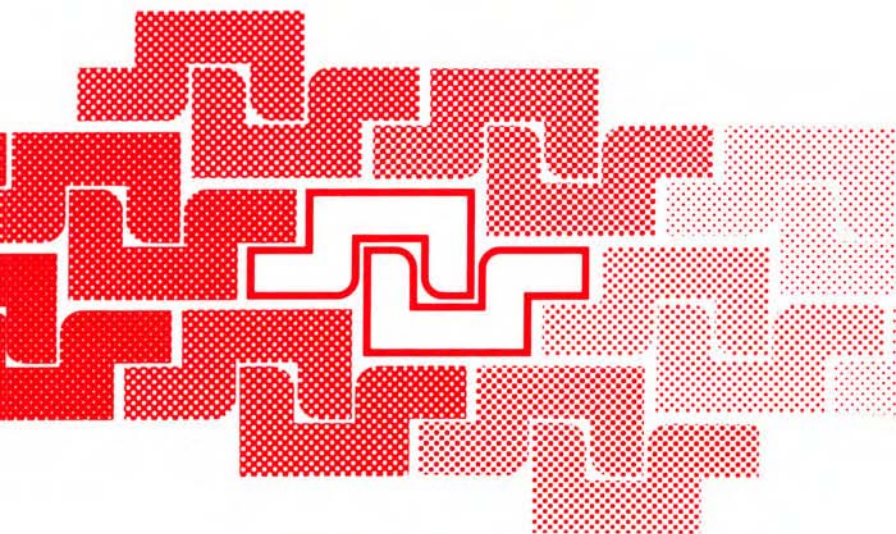




Free Radicals, Nitric Oxide, and Inflammation: Molecular, Biochemical, and Clinical Aspects

Edited by
Aldo Tomasi
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FREE RADICALS, NITRIC OXIDE, AND INFLAMMATION: MOLECULAR, BIOCHEMICAL, AND CLINICAL ASPECTS

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Foreword

Inflammation is the local response of a complex organism to an injury that serves as a mechanism initiating the elimination of noxious agents and of damaged tissues. It is now well understood that damaging mechanisms at the basis of very common human pathologies, such as atherosclerosis, neurodegenerative diseases, and cancer, i.e. the most common human pathologies, are driven by the inflammatory process.

Free radicals, and the very special free radical nitric oxide, are playing a relevant role in the pathogenesis of inflammation. The book reports topics taught and discussed during the NATO Advanced Study Institute course held in Antalya, September 23–October 4 2001.

The initial chapters introduce to the general knowledge necessary to understand the inflammatory process and the role played of free radical and oxidative stress. The interplay between inflammatory molecules and cell signalling is also dealt with in depth. A second part is dedicated to nitric oxide, redox regulation and antioxidant function in inflammation. The final chapters are devoted to diseases where inflammation plays the dominant role: septic shock, end-stage renal disease, neurodegenerative, ischemic and lung diseases.

This book, while not covering the whole gamut of the massive literature on inflammation and human diseases, gives an updated and concise view on the major issues concerning the pivotal role of inflammation in so many different human pathologies. At the same time it gives directions for future paths of research leading to a control of the pathologic process.

*Aldo Tomasi, Tomris Özben and Vladimir Skulachev,
Editors*

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Alternative Functions of Mitochondria

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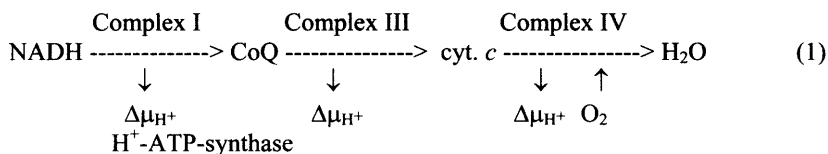
Abstract: Mitochondria are known to be multifunctional intracellular organelles. They carry out (i) energy conservation in forms of protonic potential ($\Delta\mu_{H^+}$) and ATP, (ii) thermoregulatory energy dissipation as heat, (iii) production of useful substances, (iv) decomposition of harmful substances, and (v) regulation of intracellular processes. It is suggested that mitochondria are equipped by a mechanism of self-elimination ("mitoptosis") responsible for purification of mitochondrial population from unwanted organelles (e.g., ROS-overproducing mitochondria). Massive mitoptosis is assumed to induce apoptosis due to release of the cell death proteins normally hidden in the intermembrane space of mitochondria. In this way tissues are purified from ROS-overproducing and other unwanted cells.

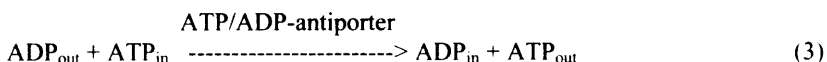
1. Energy conservation

1.1 Phosphorylating respiration

The respiration-coupled energy conservation in form of ATP is usually the most important mitochondrial function. In the aerobic cell, phosphorylating respiration is responsible, as a rule, for production of 90-95 % of the total ATP amount, the rest being synthesized by glycolytic phosphorylation. All the ATP synthesized from ADP and inorganic phosphate is hydrolyzed back to ADP and phosphate to support the energy-consuming processes in the same cell. The adult human forms and decomposes as much as about 40 kg ATP per day [1].

In mitochondria, more than 90 % of the respiratory phosphorylation is catalyzed by the H^+ -ATP-synthase, an enzyme converting the respiratory chain-produced electrochemical H^+ potential difference ($\Delta\mu_{H^+}$) into ATP [1-4]. Very small (but sometimes essential) portion of the respiratory energy is converted to GTP by succinate thiokinase [4]. Both respiratory chain enzymes (Complexes I, III and IV), catalyzing electron transfer from NAD(P)H to O_2 , and H^+ -ATP-synthase are localized in the inner mitochondrial membrane. The great majority of the formed ATP molecules is exported from mitochondria by the ATP/ADP antiporter in exchange for extramitochondrial ADP (eqs. 1-3).





1.2 Non-phosphorylating energy-conserving respiration

The respiration-produced $\Delta\mu_{\text{H}^+}$ can be utilized by mitochondria not only to form ATP but also to support some other energy-consuming processes namely reverse electron transfer in the respiratory chain and uphill transport of certain solutes from cytosol to the mitochondrial matrix.

Two reactions of the reverse electron transfer are of physiological significance. I mean (i) oxidation of succinate (redox potential, +0.03 V) by NAD^+ (redox potential, -0.32 V) and (ii) oxidation of NADH by NADPH responsible for maintenance of $[\text{NADPH}]/[\text{NADP}^+] \gg [\text{NADH}]/[\text{NAD}^+]$ in spite of the fact that redox potential of the NADPH/NADH⁺ pair is almost equal to that of NADH/NAD⁺ pair. The former process includes a reversal of NADH-CoQ reductase (Complex I of the respiratory chain). Usually it operates as a $\Delta\mu_{\text{H}^+}$ generator catalysing the downhill electron transfer from NADH to CoQ. However, when NAD^+ is reduced by succinate, the same complex acts as a $\Delta\mu_{\text{H}^+}$ -consumer carrying out the uphill transfer of electrons from CoQH_2 to NAD^+ [5].

Reduction of NADP^+ by NADH is catalysed by H^+ -transhydrogenase, a $\Delta\mu_{\text{H}^+}$ -consumer competent in the H^+ transfer between two nicotinamide adenine nucleotide in a $\Delta\mu_{\text{H}^+}$ -linked fashion. As a source of $\Delta\mu_{\text{H}^+}$, respiration or ATP hydrolysis can be used [5].

The same energy sources are employed to create gradients of solutes between cytosol and mitochondrial matrix. For instance, mitochondria accumulate Ca^{2+} by means of electrophoretic Ca^{2+} uniporter.

ATP/ADP antiporter catalyzes transmembrane exchange of ADP^{3-} for ATP^{4-} . This results in import of ADP and export of ATP at the expense of the respiration energy.

1.3 The long distance power transmission

Translated from Greek, the word “mitochondrion” means “thread-grain”. This term was introduced many years ago by cytologists who used the light microscope. The first students of mitochondria always indicated that these organelles may exist in two basic forms: (1) filamentous and (2) spherical or ellipsoid.

By applying the fluorescent cation method, it was revealed that a filamentous mitochondria may represent an electrically united system operating as intracellular electric cables. A local damage of such a filament by very narrow (0.5 μm in diameter) laser beam was shown to cause efflux of the cation and, hence, the fluorescence decreases in the entire 50 μm mitochondrial filament in a human fibroblast cell.

Later the same approach was applied to study heart muscle mitochondria that represent mainly spherical bodies. It was found that these organelles form electrically conductive intermitochondrial contacts. As a result, heart mitochondria can be united to clusters composed of tens spherical organelles (we coined them *Streptio mitochondriale*). Both mitochondrial filaments and clusters were assumed to be used by the cell to transmit $\Delta\mu_{\text{H}^+}$ inside the cell [4-6].

2. Energy dissipation

Almost all the energy conserved in form of ATP releases as heat when the ATP-dependent functions of organism are performed. Thus, then the ambient temperature lowers, a man or

a warm-blooded animal can increase their functional activity and produce in this way additional heat to keep constant the body temperature. This is the case when muscle contractions are activated by the cold (so-called shivering thermogenesis). However, such a mechanism is hardly optimal since here the main goal of thermoregulation (to make physiological functions temperature-independent) is, in fact, not realized. Moreover, shivering thermogenesis is rather complicated process requiring the H^+ -ATP-synthase-produced ATP to be transported from mitochondria to cytosol and hydrolyzed by actomyosin. Then the products (ADP and phosphate) should be transported in opposite direction i.e. from cytosol to mitochondria. It is not surprising, therefore, that during cold adaptation, the shivering thermogenesis is replaced by another mechanism which represents much simpler way from respiration to heat and does not require the main (contractile) function of muscle to be activated at cooling. The mechanism in question is thermoregulatory uncoupling of respiration and phosphorylation.

Uncoupling results in dissipation of the respiratory chain-produced $\Delta\mu_{H^+}$ due to increased H^+ conductance of the inner mitochondrial membrane. Thus energy released by respiration is immediately dissipated as heat without formation and hydrolysis of ATP. Non-esterified fatty acids proved to be compounds mediating the thermoregulatory uncoupling. They operate as protonophorous uncouplers with the help of special uncoupling proteins (UCPs) or some mitochondrial antiporters i.e. the ATP/ADP antiporter and aspartate/glutamate antiporter [1-5].

3. Synthesis of useful compounds

Both energy conservating and dissipating functions described above appear to be alternative to the functions dealing with conversion of substances rather than energy. Formally speaking, the respiration-linked substance interconversions might be carried out by the same respiratory chain which is involved in the energy-linked functions. Sometimes this really happens. However, if it were always the case, these functions would be tightly coupled to the ATP synthesis and, hence, would be dependent upon the ADP availability. Such a restriction is hardly desirable for the cell. This is why the metabolic functions of respiration are catalyzed, at least in some cases, by non-coupled respiratory enzymes that transfer electrons with no $\Delta\mu_{H^+}$ generated. For instance, some steps of the steroid hormone syntheses in adrenal cortex mitochondria are mediated by special non-coupled respiratory chain including a NADPH-oxidizing flavoprotein, the iron-sulphur protein adrenodoxin and mitochondrial cytochrome P450. All of them are localized, like the energy-coupled respiratory chain, in the inner mitochondrial membrane.

Biosyntheses of DNA, RNA and proteins in mitochondria can be another example of constructive metabolic function of these organelles. It certainly requires ATP and therefore is alternative to energy supply for extramitochondrial ATP-consuming processes [5].

4. Removal of unwanted compounds

Such a function may be exemplified by the urea synthesis from NH_3 . This ATP-consuming process is localized in matrix of liver mitochondria. Like other intramitochondrial biosyntheses, it is alternative to the ATP export from mitochondria to cytosol.

Oxidation of lactate after heavy muscle work seems to be another example of mitochondrial function dealing with removal of a harmful compound responsible for dangerous acidosis of the tissue. It was found that the ATP formation coupled to lactate

oxidation by skeletal muscle mitochondria is smaller than that coupled to oxidation of any other NAD^+ -linked substrate. This phenomenon was due to co-operation of non-coupled and coupled respiratory chains.

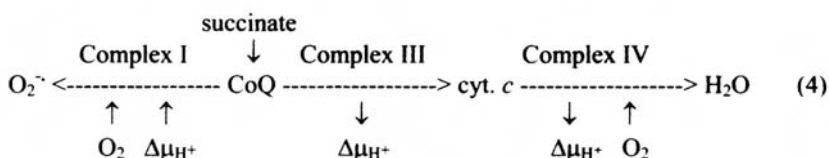
Mitochondria can take part in antioxidant defence of the cell by maintaining low intracellular oxygen concentration. In fact, this may be regarded as removal of an excess of O_2 . Under resting conditions, this process seems to be carried out by partially uncoupled or non-coupled respiration [5].

5. Mitochondria and reactive oxygen species

5.1 Mild uncoupling

Parallel with normal (enzymatic) four electron reduction of O_2 to H_2O by cytochrome oxidase, non-enzymatic one electron reduction of O_2 to superoxide (O_2^-) takes place in mitochondria. This “parasitic” chemical reaction appears to be inevitable since the initial and middle steps of the respiratory chain contain very reactive electron carriers of negative redox potential (e.g., chemically component in the one electron reduction of oxygen).

Besides non-enzymatic O_2^- generation, O_2^- can be enzymatically formed as a result of the $\Delta\mu_{\text{H}^+}$ -consuming reverse electron transfer from succinate to O_2 . In fact, standard redox potential of fumarate/succinate is slightly positive whereas that of O_2/O_2^- is negative. It was found that $\Delta\mu_{\text{H}^+}$ generated by succinate oxidation via Complexes III and IV can be used to reduce O_2 to O_2^- (eq. 4):



The process proved to be inhibited by even a small $\Delta\mu_{\text{H}^+}$ decrease (“mild uncoupling”) [5]. It was suggested that mild uncoupling is carried out by free fatty acids operating as protonophores with the help of UCPs and ATP/ADP-antiporter [5].

5.2 Cytochrome *c* as an enzyme regenerating O_2 from O_2^-

Mild uncoupling seems to be a first line of the mitochondrial antioxidant defence which prevents the O_2^- formation. If, nevertheless, some O_2^- is still formed, the next line of the defence is actuated. This role can be performed by cytochrome *c* dissolved in the solution occupying the intermembrane space of mitochondria. In fact, cytochrome *c* is competent in oxidizing O_2^- back to O_2 :



where cyt. c^{3+} and cyt. c^{2+} are for the oxidized and reduced cytochromes *c*, respectively.

Reduced cytochrome *c* formed by reaction (5) can then be oxidized by O_2 via cytochrome oxidase. In fact, the O_2^- oxidation by cytochrome c^{3+} represent the most effective way to scavenge $\Delta\mu_{\text{H}^+}$ since O_2^- formed from O_2 is converted back to O_2 . As for the other reaction product, cyt. c^{2+} , it can then be used to produce some $\Delta\mu_{\text{H}^+}$ in terminal segment of the respiratory chain. We found, however, that the only the soluble, but not the membrane-bound, cytochrome *c* is competent in superoxide oxidation. This means that desorption of cytochrome *c* from the inner mitochondrial membrane can, in principle, be

regarded, besides an apoptosis-inducing events (see below, Section 8), also as activation of an antioxidant system scavenging $O_2^{\cdot -}$.

5.3 Other ROS scavengers

Besides cytochrome *c*, there are several other compounds operating as the ROS scavengers but none of them can qualitatively convert $O_2^{\cdot -}$ back to O_2 . Some scavengers are irreversibly damaged when reacting with ROS, others can be regenerated from ROS-oxidized form back to reduced form. For the water phase of the cell, reduced glutathione and ascorbate are most important antioxidants whereas in membranes this function is inherent, first of all, in tocopherol, carotenoids and $CoQH_2$.

Important role is played by superoxide dismutase (SOD) converting the membrane-impermeable superoxide anion ($O_2^{\cdot -}$) to the membrane-permeable hydrogen peroxide (H_2O_2). The latter can escape the cell to be diluted by extracellular medium. For unicellular organisms, such a dilution is the final step of ROS detoxication. On the other hand, in higher organisms hydrogen peroxide escaping the ROS-producing cell can be used an alarm signal for its neighbours. Moreover, H_2O_2 is utilized inside the cell by glutathione peroxidase. Oxidized glutathione formed is regenerated to the reduced glutathione by glutathione reductase oxidizing NADPH. One more very important process of H_2O_2 removal is carried out by catalase which decomposes $2H_2O_2$ to O_2 and $2H_2O$ [5].

5.4 Inhibition of aconitase by superoxide

Mitochondrial aconitase, enzyme catalyzing the first steps of the citric acid cycle, is known to be reversibly inactivated by very low concentrations of $O_2^{\cdot -}$. This should result in (i) inhibition of supply of the respiratory chain by reducing equivalents and, hence, of the $O_2^{\cdot -}$ formation, and (ii) accumulation of citrate, an excellent Fe^{2+} and Fe^{3+} chelator.

Autooxidable citrate³⁻- Fe^{2+} complex immediately reacts with O_2 . As a result, Fe^{2+} is oxidized to Fe^{3+} , an effect preventing the production of $OH^{\cdot}$, the most aggressive ROS, which requires Fe^{2+} to be formed from H_2O_2 ("Fenton reaction"). The Fe^{3+} obtained remains bound to citrate since its binding to citrate is much stronger than that of Fe^{2+} [5].

Interestingly, cytosolic aconitase was recently shown to function also as an iron sensor. Earlier the cytosolic form of aconitase seemed to be an enzyme-"unemployed" since the majority of other citric acid cycle enzymes are absent from cytosol. It was found, however, that this enzyme plays a crucial role in regulating both the iron delivery to the cell and iron storage [5].

6. Mitoptosis, programmed elimination of mitochondria

There is some indications that mitochondria possess a mechanism of self-elimination. This function was ascribed to the so-called permeability transition pore (PTP). The PTP is a rather large nonspecific channel located in the inner mitochondrial membrane. The PTP is permeable for compounds of molecular mass < 1.5 kDa. The PTP is usually closed. A current point of view is that PTP opening results from some modification and conformation change of the ATP/ADP antiporter. Oxidation of Cys56 in the antiporter seems to convert it to the PTP in a way that is catalyzed by another mitochondrial protein, cyclophilin. When opened, the PTP makes impossible the performance of the main mitochondrial function, i.e., coupling of respiration with ATP synthesis. This is due to the collapse of the membrane potential and pH gradient across the inner mitochondrial membrane that mediate respiratory phosphorylation. Membrane potential is also a driving force for import of

cytoplasmic precursors of mitochondrial proteins. Moreover, it is strictly required for the proper arrangement of mitochondrially-synthesized proteins in the inner membrane of the mitochondrion. Thus, repair of the PTP-bearing mitochondrion ceases, and the organelle perishes.

It is noteworthy that the above scheme of elimination of a mitochondrion does not require any extramitochondrial proteins. It can be initiated by a signal originating from a particular mitochondrion, such as reactive oxygen species (ROS) produced by the mitochondrial respiratory chain. ROS seem to oxidize the crucial SH-group in the ATP/ADP-antiporter, thereby actuating the elimination process. This is why one can consider this effect as the programmed death of the mitochondrion (mitochondrial suicide). For this event, I coined the word *mitoptosis*, by analogy with apoptosis, the programmed death of the cell. I also suggested that the biological function of mitoptosis is the purification of the intracellular population of mitochondria from those that became dangerous for the cell because their ROS production exceeded their ROS scavenging capacity. It seems very probable that antioxidant defense is not the only function of mitoptosis. However, at least some alternative mitoptotic functions require ROS to be formed as mediators of mitoptosis (for example, disappearance of mitochondria during the maturation of the mammalian erythrocytes) [6-8].

7. Massive mitoptosis results in apoptosis

Opening of the PTP leads to an osmotic disbalance between the mitochondrial matrix and cytosol, swelling of the matrix and, consequently, the loss of integrity of the outer mitochondrial membrane, thus releasing the intermembrane proteins into the cytosol. Among them, four proteins are of interest in this context: cytochrome *c*, apoptosis-inducing factor (AIF), the second mitochondrial apoptosis-activating protein (Smac; also abbreviated DIABLO), and procaspase 9. All these proteins are somehow involved in apoptosis.

In cytosol, cytochrome *c* combines with very high affinity with a cytosolic protein called Apoptotic Protease-Activating Factor 1 (Apaf-1) and dATP. The complex, in turn, combines with an inactive protease precursor, procaspase 9, to form the "apoptosome". As a result, several procaspase 9 molecules are placed near each other, and they cleave each other to form active caspases 9. When formed, caspase 9 attacks procaspase 3 and cleaves it to form active caspase 3, a protease that hydrolyses certain enzymes occupying key positions on the metabolic map. This causes cell death.

Considering these data, the following scenario of the final steps of the defense of a tissue from mitochondrion-produced ROS seems to be most likely.

ROS induce PTP opening and, consequently, release of cytochrome *c* and other proapoptotic proteins from mitochondria to the cytosol. If this occurs in a small fraction of ROS-overproducing mitochondria, these mitochondria die. The cytosol concentrations of proapoptotic proteins released from the dying mitochondria appear to be too low to induce apoptosis. If, however, more and more mitochondria become ROS-overproducers, the concentrations in question reach a level sufficient for the induction of apoptosis. This results in purification of the tissue from the cells whose mitochondria produce too many ROS.

In 1994, I postulated a scheme in which mitoptosis is an event preceding apoptosis [9]. In the same year, Newmeyer and coauthors published the first indication of a requirement of mitochondria for apoptosis [10]. And quite recently, Tolkovsky and her coworkers presented direct proof of the mitoptosis concept [11,12]. In the first set of experiments, axotomized sympathetic neurons deprived of neuron growth factor were studied. It was found that such neurons died within a few days, showing cytochrome *c*

release and order typical features of apoptosis. However, the cells survive if a pan-caspase inhibitor Boc-Asp (O-methyl)-CH₂F (BAF) was added a day after the growth factor deprivation. The cell survival was due to that the mitochondrion-linked apoptotic cascade was interrupted downstream of the mitochondria. Electron microscopy showed that in such cells *all the mitochondria disappear within 3 days* after the BAF addition. Later, the same group reported that a similar effect could be shown using such classical experimental models of apoptosis as HeLa cells treated with staurosporin. Again, addition of BAF to the staurosporin-treated cells resulted in that (i) the cells lived longer and (ii) mitochondria disappeared in the time scale of days. This was shown to be accompanied by disappearance of mitochondrial DNA and as well as the cytochrome oxidase subunit IV encoded by nuclear DNA. On the other hand, nuclear DNA, Golgi apparatus, endoplasmic reticulum, centrioles, microtubules, and plasma membrane remained undamaged. Mitoptosis was prevented by overexpression of antiapoptotic protein Bcl-2, which is known to affect mitochondria upstream from the cytochrome *c* release.

Apparently, disappearance of mitochondria in the apoptotic cells without BAF could not be seen since the cells die too fast to reveal mitoptosis and subsequent autophagia of dead mitochondria. On the other hand, inhibition of apoptosis at a post-mitochondrial step prevented fast death of the cells so there was time for mitoptosis to be completed [6,7].

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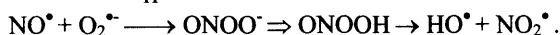
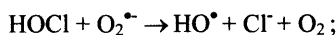
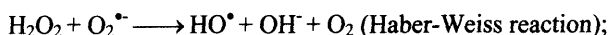
The Enzymatic Systems in the Regulation of Free Radical Lipid Peroxidation

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Abstract: Reviewing the data available in the literature and their own findings, the author consider the role of enzymatic mechanisms in the regulation of lipid peroxidation in the living cells. The paper provides a good evidence that phospholipase A₂ hydrolysis for reduction of hydroperoxy-derivatives of unsaturated phospholipids by non-selenic glutathione S-transferase is not obligatory moreover glutathione S-transferase may be inhibited by the products of phospholipase A₂ hydrolysis — by free unsaturated fatty acids. On the other hand, Se-contained glutathione peroxidase is capable of reducing unsaturated hydroperoxy-acyls of membrane phospholipids only if the phospholipids have been hydrolyzed by phospholipase A₂ and this enzyme is not inhibited in the presence of free fatty acids. It can be suggested from the results that in normal conditions glutathione S-transferase catalyzes direct reduction of oxidized membrane phospholipid acyls, but during pathological stations, when the products of phospholipase-mediated hydrolysis are accumulated (such as tissue ischaemia), the major role in lipoperoxides detoxification in the cells belongs to Se-containing glutathione peroxidase. In addition the accumulation of primary products (hydroperoxy- and hydroxy-derivatives) of polyunsaturated acyl oxidative metabolism in the phospholipid membranes induced the changes in the membrane fluidity, that were opposite to those observed upon cholesterol incorporation into membranes. It was found that antioxidative enzymes such as superoxide dismutase and glutathione peroxidase may play a leading role in the prevention of the pancreas β -cells in vivo from reactive oxygen species injury in alloxan-treated rats.

Reactive oxygen species (ROS) represent groups of oxygen-containing molecules in different states of oxido-reduction and electronic excitation, as well as compounds of oxygen with hydrogen, chlorine and nitrogen, such as superoxide anion-radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), hypochlorous acid ($HOCl$), nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^{\bullet}$) [1]. Some of ROS such as $O_2^{\bullet-}$, $HO^{\bullet}$ and $NO^{\bullet}$ are free radicals. Free radicals can be defined as any species that contain one unpaired electron (symbolized by $\bullet$) on the external orbital of molecule [1]. Free radicals are highly reactive species and can react with different organic compounds of the living cell — unsaturated lipids of biomembranes, proteins and nucleic acids and cause the oxidative damage of its molecules [1-3]. It was known to chemistry that hydroxyl radical ($HO^{\bullet}$) is the most reactive radical [1]. Endogenous prooxidants such as H_2O_2 , $HOCl$ and $ONOO^{\bullet}$ can be regarded as potentially dangerous molecules for living cells so far as they are degraded with $HO^{\bullet}$ formation:

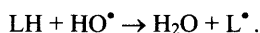


The different ROS, free radicals and endogenous inducers of free radical oxidation which are frequently found in nature are presented in Figure 1.

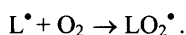
	Free radicals	Endogenous prooxidants
Reactive oxygen species	$\text{O}_2^{\bullet-}$ $\text{HO}_2^\bullet$ $\text{OH}^\bullet$ $\text{NO}^\bullet$	$\text{H}_2\text{O}_2 \rightarrow \text{OH}^\bullet$ $\text{HOCl} \rightarrow \text{OH}^\bullet$ $\text{ONOOH} \rightarrow \text{OH}^\bullet$
Lipid radicals	$\text{L}^\bullet$ $\text{LO}_2^\bullet$ $\text{LO}^\bullet$	$\text{LOOH} \rightarrow \text{LO}^\bullet$
Radicals of antioxidants	$\alpha\text{-TO}^\bullet$ $^\bullet\text{QH}$ $\text{HO-Asc-O}^\bullet$	

Figure 1. The main forms of reactive oxygen species, free radicals and endogenous inducers of free radical oxidation which are widely distributed in the living cells.

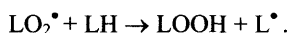
In the living cells the $\text{HO}^\bullet$ preferentially attacks polyunsaturated fatty acids (PUFA) of membrane phospholipids and it abstracts an atom of hydrogen from one of carbon atoms in the side chain PUFA and combines with it to form water [1]:



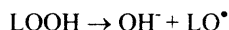
Lipid carbon-centered alkyl radical ($\text{L}^\bullet$) is to combine with molecule of oxygen with peroxy radical ($\text{LO}_2^\bullet$) formation:



Peroxy radical is reactive to attack another PUFA acyls, abstracting hydrogen. In this reaction lipid hydroperoxide (LOOH) is formed and a new lipid alkyl radical is generated [1,2]:



The LOOH is very labile and can be decomposed with formation of secondary lipid alkoxy radical which interact with PUFA and over again generate lipid carbon-centered radical:



The decomposition of LOOH can also yield a number of highly cytotoxic products, malondialdehyde and 4-hydroxynonenal are most unpleasant among them. Lipid radicals and cytotoxic aldehydes can also cause severe damage of membrane proteins, inactivating receptors and membrane-bound enzymes [1-3].

There are three initiation mechanisms for the free radical lipid peroxidation in the living cells. At the first lipoperoxidation in the body can be induced by non-enzymatic mechanism. In this processes different physical factors such as ionizing irradiation or UV radiation as well as action of some chemical toxicants including air pollutants, pesticides and herbicides from food and drinking water may act as a initiating factors.

The second initiation way for the lipoperoxidation in the organism can be defined as semi-enzymatic or quasi-enzymatic. During this mechanism the $\text{O}_2^\bullet$ radicals are generated by enzymes including NAD(P)H-dependent oxidases of mitochondrial and microsomal electron transport chains, NADPH-dependent oxidase of phagocytes, xanthine oxidase and other flavine oxidases. After the $\text{HO}^\bullet$ formation the oxidation process develops in non-enzymatic way.

Finally the lipoperoxidation process can be fully enzymatic and this is carried out by heme-containing cyclooxygenases (prostaglandin-, thromboxan- and prostacyclin-synthases) or ferrous iron-containing lipoxygenases which are oxidized arachidonic acid and another PUFA by means of free radical mechanism [4,5] as can be seen in Figure 2.

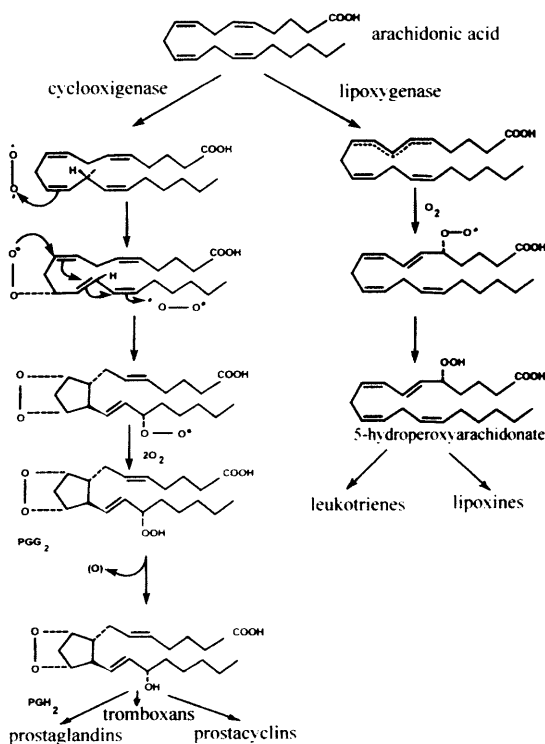


Figure 2. Free radical mechanism of enzymatic arachidonate oxidation by cyclooxygenase or lipoxygenase.

In particular the C-15 animal lipoxygenase may oxidize unsaturated acyls of membrane phospholipids [6,7] (Figure 3) and this process plays the leading role in the internal cell membranes decomposition during maturation of reticulocyte to erythrocyte [6].

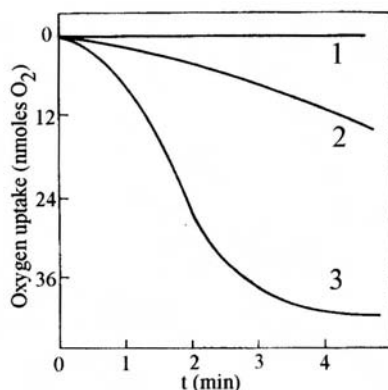


Figure 3. The oxidation of various native membrane preparations by animal (rabbit reticulocyte) C-15 lipoxygenase: (1), erythrocyte ghosts; (2), liver microsomes; (3), liver mitochondria.

In addition lipohydroperoxides formed by C-15 lipoxygenase after its homolysis can give rise to lipid alkoxyl radicals which induce cooxidation of other unsaturated lipids such as β -carotene [8] (Figure 4).

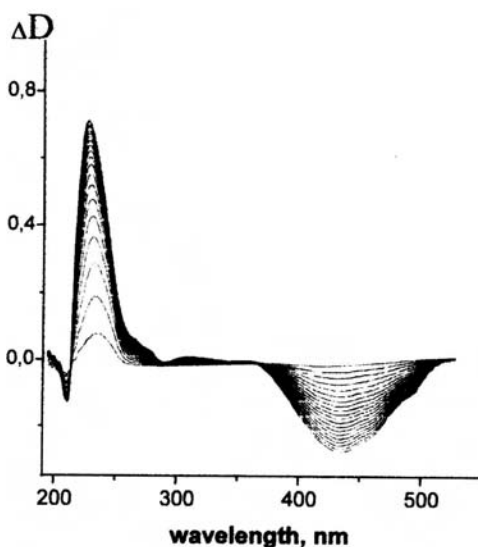
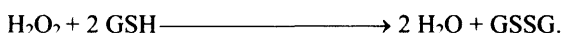
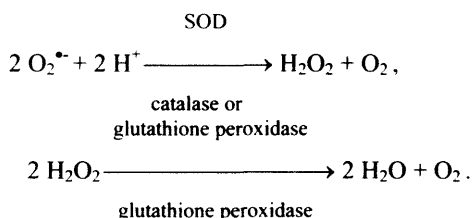


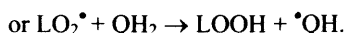
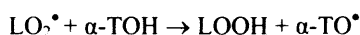
Figure 4. The cooxidation of β -carotene ($\lambda=450$ nm) by secondary lipid free radicals which formed during arachidonic acid peroxidation ($\lambda=233$ nm) by animal (rabbit reticulocyte) C-15 lipoxygenase in the water dispersions.

At present there can be no doubt that investigations into the enzymatic regulation of free radical reactions in the body is of high priority. A number of enzymes called as "antioxidative enzymes" may act as effective antioxidants *in vivo*. It is known that

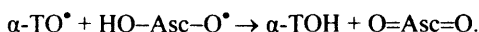
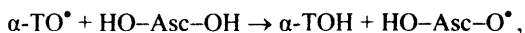
superoxide dismutase (SOD), utilizing superoxide radical and catalase or glutathione peroxidase, utilizing hydrogen peroxide, prevent accumulation of hydroxyl radicals able to initiate free radical peroxidation of lipids in the biomembranes [1,2]:



The inactivation of lipid peroxyl radicals by bioantioxidants such as α -tocopherol (α -TO $^{\bullet}$) and reduced form ubiquinol Q₁₀(Q) - ubiquinol Q₁₀(QH₂) occurs in a non-enzymatic fashion:

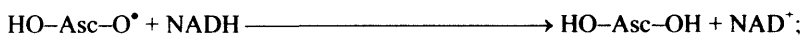
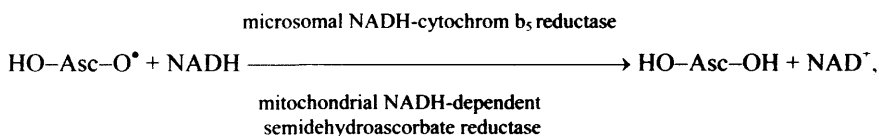


The bioregeneration of α -tocopherol phenoxyl radical which is formed in this reaction take place with vitamin C (HO-Asc-OH) participation also in a non-enzymatic fashion [9,10]:

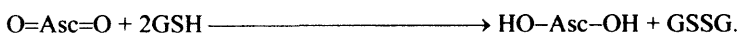
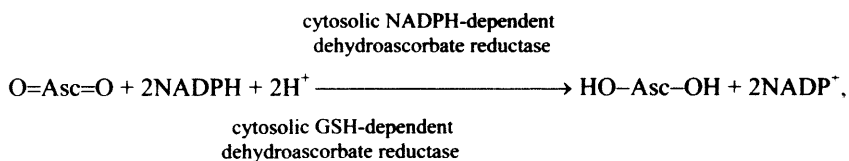


So far as radicals of natural antioxidant is reduced in non-enzymatic reactions, ascorbic acid in the same way may also reduce the free radicals of synthetic antioxidants, for example phenoxyl radical of probucol during this antioxidative drug treatment [11,12].

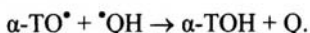
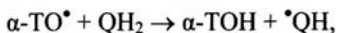
On the other hand different enzymes participate in the ascorbic acid free radical — semidehydroascorbate (HO-Asc-O $^{\bullet}$) tissues reduction [13]:



and dehydroascorbate (O=Asc=O) – oxidized form of ascorbic acid [14,15]:



The ubiquinol Q₁₀ can reduce phenoxyl radical of α -tocopherol with formation of ubisemiquinon radical ($^{\bullet}\text{QH}$) as intermediate [16]:

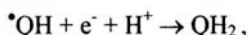


At the same time ubiquinone Q_{10} itself is the subject of reduction by enzyme NAD(P)H-dependent quinone oxidoreductase (DT-diaphorase) [17]:

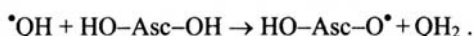
DT-diaphorase



and reduction of ubiquinon Q_{10} semiquinon radical proceeds also in mitochondrial electron transport chain [18]:



or with vitamin C using [19]:



Thus, a conclusion can be made that different enzymes involved in the natural antioxidants bioregeneration.

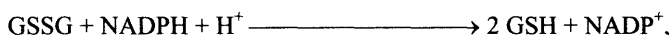
Glutathione-dependent peroxidases family includes two main enzymes — Se-contained glutathione peroxidase [20] and glutathione-S-transferase [21,22] utilizing lipohydroperoxides and preventing the production of alkoxyl radicals also play an important role in the regulation of lipid peroxidation in cells:

glutathione peroxidase or
glutathione S-transferase



The bioregeneration of oxidized glutathione (GSSG) which is formed in glutathione peroxidase reaction occurs with involving of glutathione reductase and enzymatic systems of NADP^+ reduction, in particular during process pentose phosphate pathway of glucose-6-phosphate in 6-phosphoglucono lacton oxidation [20]:

glutathione reductase



glucose-6-phosphate
dehydrogenase



The scheme in Figure 5 indicates that enzymatic regulation of lipoperoxidation is well exercised in the body and takes place in various stages of oxidation.

As shown on the scheme given in Figure 5, there are three main steps of enzymatic prevention from free radicals in the living cells. On the first step the detoxification of $\text{O}_2^\bullet$ by superoxide dismutase and H_2O_2 by catalase or Se-containing glutathione peroxidase occurs that protect from formation of reactive $\text{HO}^\bullet$. On the second step the inactivation of organic peroxy radicals by bioantioxidants such as α -tocopherol and ubiquinol Q_{10} takes place as well as reduction of potential dangerous antioxidant free radical with participation of ascorbic acid and enzymatic systems of it bioregeneration. On the last third step reduction of lipohydroperoxides by glutathione-dependent lipoperoxidases (Se-contained glutathione peroxidase and glutathione-S-transferase) and enzymatic bioregeneration of oxidized glutathione is brought about. This mechanism protects from formation of secondary alkoxyl radicals which can be formed during lipoperoxide decomposition.

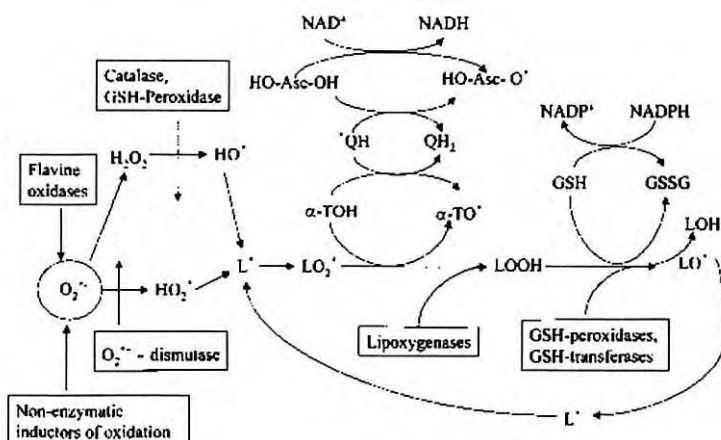
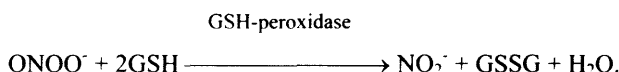


Figure 5. The enzymatic regulation of free radical lipoperoxidation in the living cells.

It is important also to note that Se-containing glutathione peroxidase may protect the cells against peroxynitrite-mediated oxidation [23]:



On the other hand some glutathione S-transferase isozymes may catalyzed the detoxification of cytotoxic unsaturated aldehyde — 4-hydroxynonenal [24], which is formed during decomposition of lipohydroperoxides, however it is important to note that 4-hydroxynonenal inhibits Se-containing glutathione peroxidase [25]. Thus glutathione-dependent lipoperoxidases may play the exceptionally role in the detoxification of not only primary but also secondary products of the lipoperoxidation and contribution of these enzymes in the regulation of free radical processes in the body are very significant.

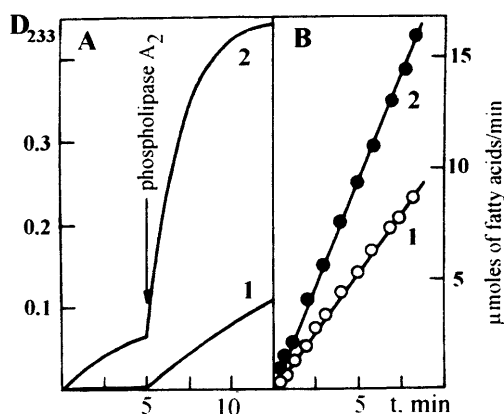


Figure 6. (A) – The oxygenation of dilinoleoylphosphatidylcholine (DLPC) liposomes by C-15 plant (from soybeans) or animal (from rabbit reticulocytes) lipoxigenase; (B) – The enzymatic hydrolysis of β -acyls of dilinoleoylphosphatidylcholine (DLPC) in the liposomal membrane by phospholipase A_2 from *Apis mellifera* venom: (1), hydrolysis rate of unoxidized DLPC liposomes; (2), hydrolysis rate of DLPC liposomes which preliminary was oxidized by C-15 rabbit reticulocyte lipoxigenase.

Both enzymes — Se-containing glutathione peroxidase and non-selenic glutathione S-transferase reduces hydroxy-derivatives of PUFA using glutathione (GSH) as a proton donor [20-22,26-28]. The “classical” Se-containing glutathione peroxidase of erythrocytes and cell cytosol is capable of reducing unsaturated hydroperoxy-acyls of phospholipids only if phospholipids have been hydrolyzed by phospholipase A₂ [20,28,29].

We found [26,27] that the enzymatic reduction of hydroperoxy-derivatives of phospholipids catalyzed by glutathione S-transferase does not require preferentially phospholipase-mediated hydrolysis of oxidized acyl of phospholipids. There is evidence that phospholipase A₂ preferentially catalyzes hydrolysis of oxidized acyl of phospholipids [30,31], which should facilitate their enzymatic reduction by Se-containing glutathione peroxidase [26-29] (Figure 6). It is interesting to note that C-15 animal lipoxigenase oxidized free PUFA with higher rate than unsaturated acyls of membrane phospholipids, at the same time C-15 plant lipoxigenase is unable to oxidize phospholipids in the liposomes and natural lipid-protein submolecular complexis [26,27] (Figure 6).

It is know that during myocardial infarction a greater extent of membrane lipids oxidation in ischemic cardiomyocytes is accompanied by the activation of phospholipase A₂ [32]. Under these conditions, there is an abrupt increase in the content of both oxidized and unoxidized free PUFA in the cells. This may have a substantial effect on the efficiency of enzymatic reduction of lipid hydroperoxides catalyzed by GSH-dependent lipoperoxidases. Since there is a close metabolic connection between “classical” Se-containing glutathione peroxidase and phospholipase A₂, we investigate the effect of the products of phospholipase-catalyzed hydrolysis (long-chain free fatty acids) on the lipoperoxidase activity of the “classical” Se-containing glutathione peroxidase from bovine erythrocytes and non-selenic glutathione S-transferase from porcine liver [33].

The results obtained in our work (Figures 7 and 8) show that free unoxidized PUFA have virtually no effect on the rate of lipoxydroperoxides reduction catalyzed by glutathione peroxidase within a broad range of PUFA concentrations in the incubation medium (up to 70-100 μ M).

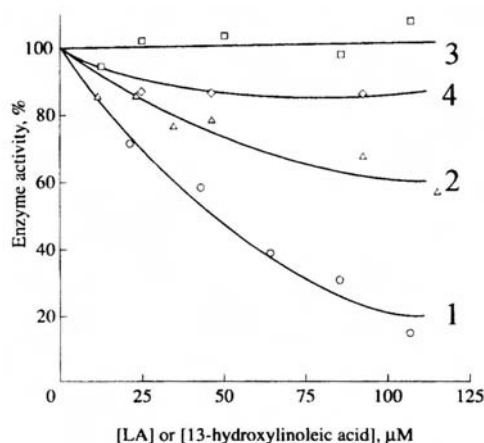


Figure 7. Effect of free linoleic acid (LA) and 13-hydroxylinoleic acid on the lipoperoxidase activity of: (1,2) non-selenic glutathione S-transferase from porcine liver and (3,4) Se-containing glutathione peroxidase from bovine erythrocytes (substrate – 25 mM 13-hydroperoxylinoleic acid). Here and in Fig.8 and 9, the enzyme activity in the absence of free fatty acids was taken as 100%: (1) and (3) in the presence of free linoleic acid; (2) and (4) in the presence of 13-hydroxylinoleic acid [33].

However, 13-hydroxylinoleic acid, a product of enzymatic reduction of 13-hydroperoxylinoleic acid, caused an insignificant inhibition of the enzymatic reduction of PUFA catalyzed by glutathione peroxidase (Figure 7). On the other hand, both unoxidized PUFA and hydroxy-derivative of PUFA had a significant inhibitory effect on the lipoperoxidase activity of glutathione S-transferase (Figures 7 and 8).

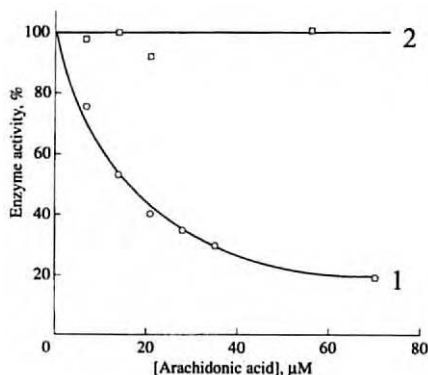


Figure 8. Effect of free arachidonic acid on the lipoperoxidase activity of: (1) non-selenic glutathione S-transferase from porcine liver and (2) Se-containing glutathione peroxidase from bovine erythrocytes (substrate – 15 mM 15-hydroperoxyarachidonic acid) [33].

Also it should be noted that saturated free fatty acids with a chain length of 14-18 carbon atoms have a significantly lower inhibitory effect on the glutathione S-transferase activity than free PUFA (Figure 9). Therefore, Se-containing glutathione peroxidase is capable of reducing hydroperoxy-derivatives of polyenoic fatty acids in the presence of unoxidized PUFA or products of their enzymatic reduction. On the other hand, free PUFA are strong inhibitors of the lipoperoxidase reaction catalyzed by glutathione S-transferase (Figures 7-9).

It is known that most polyunsaturated acyls occupy the second position among natural phospholipids [26,27]. As a result, free PUFA are the main products of phospholipid hydrolysis by phospholipase A₂. Our data showed (Figures 7-9) that free PUFA were the strongest inhibitors of non-selenic glutathione S-transferase, whereas saturated acids were the least potent inhibitors of this enzyme. It is seen from Figures 7 and

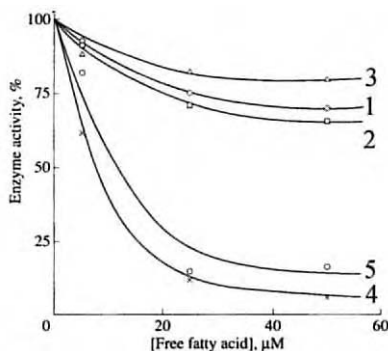


Figure 9. Effect of free long-chain saturated and unsaturated fatty acids on the total activity of non-selenic glutathione S-transferase from porcine liver (substrate – 1 mM 1-chloro-2,4-dinitrobenzene). Enzymatic activity was measured in the presence of follow free fatty acids: (1) myristic (C_{14:0})*; (2) palmitic (C_{16:0})*; (3) stearic (C_{18:0})*; (4) linoleic (C_{18:2})*; and (5) arachidonic (C_{20:4})*. (*) The first figure is the number of carbon atoms, and the second figure is the number of double bonds in the molecule of fatty acid [33].

8 that Se-containing glutathione peroxidase is absolutely insensitive to free PUFA, the strongest inhibitors of non-selenic glutathione S-transferase. It was shown in our previous studies that, in contrast to Se-containing glutathione peroxidase, non-selenic glutathione S-transferase reduces both hydroperoxy derivatives of free PUFA and hydroperoxy acyls of membrane phospholipids [26,27]. It can be suggested from the results of our work that in normal metabolic processes glutathione S-transferase catalyzes direct reduction of oxidized acyls of membrane phospholipids. In pathological conditions, when the products of phospholipase-catalyzed hydrolysis are accumulated [32], the major role in lipoperoxide detoxification in the cells belongs to Se-containing glutathione peroxidase.

The scheme in Figure 10 demonstrates the relationship between enzymatic reactions of oxidation, hydrolysis and reduction in metabolism of membrane lipoperoxides during normal state and pathological conditions.

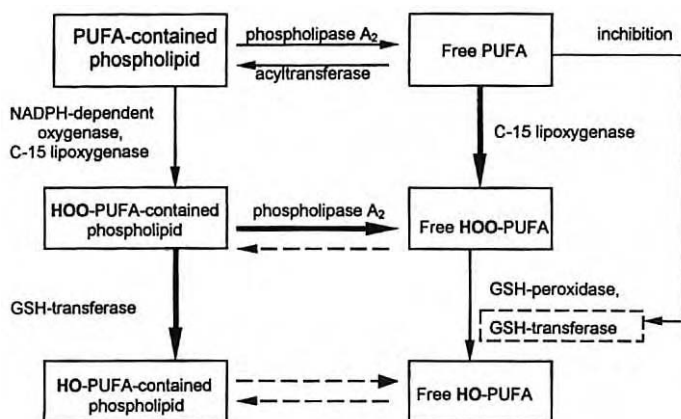


Figure 10. The enzymatic oxidation, hydrolysis and reduction in metabolism of membrane lipoperoxides during normal state and pathological conditions.

Intensification of free radical lipid peroxidation promotes oxidative stress on cell and leads to the accumulation of primary and secondary products of lipoperoxidation in biomembranes. These products induce not only chemical and structural modifications of lipid-protein supramolecular complexes such as intracellular organelles and blood plasma lipoproteins but also cause impairments in their normal functioning. The latter often contributes to the development of pathological process [1]. In particular, the oxidative modification increases the atherogeny of low density lipoproteins causing their intensive absorption by the vessel wall cells [34]. The secondary aldehyde products of the free radical lipoperoxidation (4-hydroxynonenal, malondialdehyde, etc.) can react with amino groups of proteins as well as aminophospholipids with their formation of stable complexes [1]. The effects of the secondary products of the free radical lipoperoxidation on the structural parameters of phospholipid bilayer can be opposite to those of the primary products, namely hydroperoxides [35]. Probably, this may explain that the literature contains an abundance of conflicting opinions on the effects of free radical lipoperoxidation on the membrane structure [36-38], since commonly used methods for induction of the free radical oxidation promote simultaneous accumulation not only of lipid hydroperoxides but also significant amounts of the secondary products of peroxidation [35]. Nevertheless, in native cells, the produced lipoperoxides are rapidly reduced into the corresponding alcohols by Se-containing glutathione peroxidase or non-selenic glutathione S-transferase [26,27]. It thus appears that main products of the polyunsaturated fatty acid oxidative metabolism in the cell are their more polar hydroperoxy and hydroxy derivatives

[26,27]. After enzymatic reduction of very labile lipohydroperoxides, their oxidative break-down prove to be impossible, and structure of modified biomembranes become stable [26,27]. In this connection, it is important to obtain experimental data on the changes in the conformation of the phospholipid membranes containing enzymatically produced hydroperoxy and hydroxy-derivatives of PUFA or corresponding acyl derivatives. For this goal the effects of the primary products of free radical lipoperoxidation on the membrane structure were studied by the earlier developed methods for accumulation of hydroperoxy and hydroxy derivatives of unsaturated fatty acids and phospholipids in the liposomes using C-15 lipoxigenase from rabbit reticulocyte and glutathione-S-transferase from rabbit liver [26,27].

Liposomes (200 μg of phospholipids per 1 ml) were prepared from dilinoleoyl phosphatidylcholine (DLPC) or from dipalmitoyl phosphatidylcholine (DPPC) containing 5% of DLPC (or 20% of linoleic acid). The microviscosity of the liposome membranes was determined according to the fluorescence polarization parameters of the probe 1,6-diphenyl-1,3,5-hexatrien as described in [39]. The experimental conditions were selected that after the enzymatic oxidation by C-15 reticulocytes lipoxigenase, the concentrations of the hydroperoxy derivatives were identical for the liposomes composed of 100% DLPC and those composed of DPPC containing 5% of DLPC ($2,37 \pm 0,28 \mu\text{M}$ and $2,44 \pm 0,21 \mu\text{M}$, respectively). The efficiency of the enzymatic reduction of these phospholipid hydroperoxides by glutathione-S-transferase was over 90-95% [26,27]. After consecutive enzymatic oxidations and reductions of membranes, the concentration of the linoleic acid hydroperoxy- and hydroxy-derivatives in the liposomes composed of DPPC and 20% of linoleic acid was $8,0 \pm 1,2 \mu\text{M}$. The level of secondary products of the free radical lipoperoxidation (2-thiobarbituric acid-reacting substances) in the initial liposomes was extremely low ($2,65 \pm 0,04 \text{ nmol}$ per 1 mg of phospholipid) and did not increase after incubation with C-15 reticulocyte lipoxigenase or liver glutathione-S-transferase.

Increased content of conjugated dienes in linoleate acyls in the mixed liposomes composed 95% of DPPC and 5% of DLPC caused the increase in their microviscosity (Figure 11, curve 1). The microviscosity of liposome membranes containing 100% DLPC was considerably decreased upon the enzymatic oxidation by C-15 reticulocyte lipoxigenase (Figure 11, curve 2).

The microviscosity of the liposome membranes containing saturated lecithins (95% of DPPC and 5% of DLPC) during the enzymatic reduction of the DLPC hydroperoxy derivatives in the membranes showed a sharp rise (Figure 11, curve 1) but the microviscosity of the membranes containing unsaturated lecithins (100% DLPC) during enzymatic reduction of hydroperoxy acyls on the contrary was drastically lowered (Figure 11, curve 2). It can be supposed that consecutive enzymatic oxidations and reductions of polyunsaturated acyls in the membranes is accompanied by the increase in the degree of ordered acyl organization in the membranes with high content of saturated phospholipids (Figure 11, curve 1) due to exposure of more polar hydroperoxy and hydroxy acyls into the water phase [26,40]. Decreased microviscosity during consecutive oxidations and reductions of membranes from unsaturated phospholipids (Figure 11, curve 2) may be due to increase of water content in these membranes as it was found earlier [41]. As might be expected the incorporation of non-oxidized free linoleic acid into the liposomes composed of saturated DPPC is accompanied by the rapid decrease in the initial membrane microviscosity. The enzymatic oxygenation of incorporated free linoleic acid sharply increased the microviscosity of the mixed liposome membrane containing saturated lecithins and subsequent reduction of the formed hydroperoxy linoleic acid into corresponding hydroxy acid a new increased the membrane microviscosity to the initial level (Figure 11, curve 3). It is not inconceivable that the observed changes in the membrane fluidity are due to the washing out of more hydrophilic linoleic acid derivatives

(13-hydroperoxylinoleate and 13-hydroxylinoleate) from liposomes into water medium [42]. Since the incorporation of linoleic acid into the liposome mimicked the effect of unsaturated acyls hydrolysis by phospholipase A₂, that destabilizes membrane, the subsequent enzymatic oxidative transformation of polyunsaturated fatty acids can be considered as a reparative process for maintaining the initial membrane structure (Figure 11, curve 3).

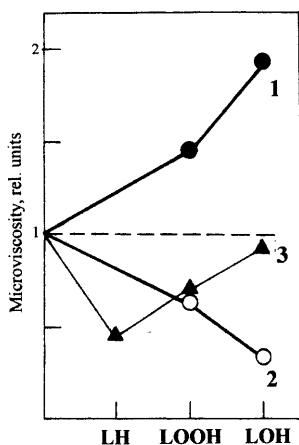


Figure 11. Effect of the hydroperoxy and hydroxy derivatives of free PUFA and phospholipids on the microviscosity of liposomes composed of saturated and unsaturated phosphatidylcholine:

(1) "saturated" liposomes composed of 95% dipalmitoyl phosphatidylcholine (DPPC) and 5% of dilinoleoyl phosphatidylcholine (DLPC); (2) "unsaturated" liposomes composed of 100% DLPC; liposomes composed of 80% DPPC and 20% of free linoleic acid.

LH – non-oxidized free PUFA; LOOH – hydroperoxy-derivatives of free PUFA and phosphatidylcholines;

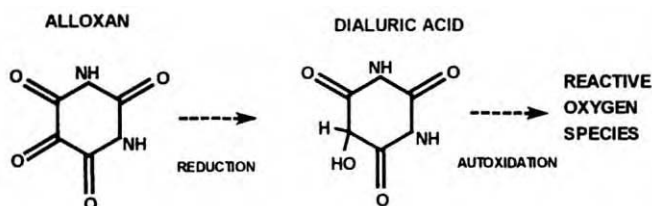
LOH – hydroxy-derivatives of free PUFA and phosphatidylcholines.

The results of two series of independent experiments (3–5 measurements for each experimental point) are given; the difference between microviscosity values of the modified and initial membranes (the initial phosphatidylcholine microviscosity was taken as 1 for every type of liposomes) was significant at $p < 0.05$ [40].

However, the accumulation of primary products of polyunsaturated acyl oxidative metabolism in the phospholipid membranes induced the changes in the membrane fluidity (Figure 11, curves 1-2), that were opposite to those observed upon cholesterol incorporation into membranes [43]. Cholesterol is known to loose an ordered acyl organization in the membranes composed of saturated phospholipids and to compact acyls in the membranes composed of unsaturated phospholipids [43]. It appears reasonable to assume that induction of free radical lipoperoxidation may be a normal physiological response of organism directed mainly to the compensation of changes in the membrane structure during pathologies accompanied by drastic increase in the levels of cholesterol (atherosclerosis, hypercholesterolemia) and free fatty acids (ischemia, activation of phospholipase A₂) in the biomembranes. Thus, the obtained results, indicating different effects of lipohydroperoxides (or corresponding hydroxy acids) and cholesterol (or free fatty acids) on the membrane conformation, can help in understanding the pathophysiological mechanism of intensification of free radical lipoperoxidation in the biomembranes during atherogenesis and myocardial ischemia [32].

Our investigation revealed that enzymatic systems of oxygen radicals and lipoperoxides detoxification may play a leading role in the inhibition of free radical

reactions in the living cells during free radical pathologies development. Alloxan rat diabetes can be regarded as a experimental model of free radical pathology. In the mammalian pancreas cells alloxan very easy reduced to dialuric acid which quickly autoxidized with superoxide radical and other ROS formation [44]:



The injury of pancreas β -cells by ROS produces the hyperglycemia and hypoinsulinemia development in alloxan-treated rats (Figure 12).

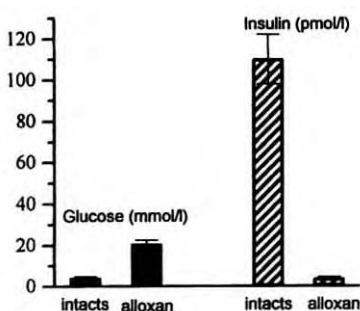


Figure 12. The level of glucose and insulin in the blood plasma of alloxan-treated rats.

In addition in the pancreas cells of alloxan-treated rats we observed the decreasing in the activity of key antioxidative enzymes namely SOD and glutathione peroxidase (Figure 13).

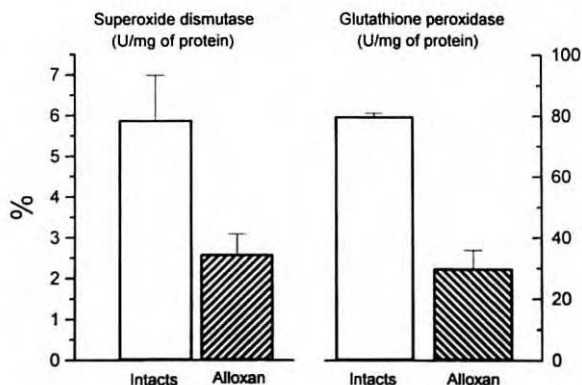


Figure 13. The activity of key antioxidative enzymes (superoxide dismutase and glutathione peroxidase) in pancreas cells of alloxan-treated rats.

We detected also that the antioxidative enzymes activity in the pancreas of rats which are susceptible to alloxan-induced diabetes is significantly lower than in pancreas cells of guinea pigs which are very resistant to diabetogenic action of alloxan (Figure 14).

It seems unavoidable to conclude that high level of antioxidative enzymes activity in pancreas cells of guinea pigs is a cause of resistance of this kind animals to diabetogenic

alloxan action [45]. As appears from the above antioxidative enzymes may act in the body as a very effective natural antioxidants and their deficiency may be the main cause of different pathologies development.

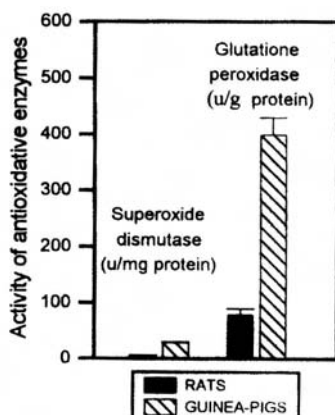


Figure 14. The activity of key antioxidative enzymes (superoxide dismutase and glutathione peroxidase) in pancreas cells of animals which are susceptible (rats) or are resistant (guinea pigs) to diabetogenic action of alloxan.

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Abstract. By means of fluorescent probes of pyrene and 4-dimethylaminochalcone an increase of polarity of the lipid phase of low density lipoproteins (LDL) during their autooxidation was discovered. The observed change of polarity is explained by the penetration of water molecules into the LDL phospholipid layer, which form hydrogen bonds with polar groups resulting from the peroxidation of lipids. Such "water corrosion" of the membranes is suggested to bring about a change of matrix properties of the phospholipid layer, particularly a change in LDL protein conformation discovered earlier during peroxidation.
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Flavanols and Procyanidins as Modulators of Oxidation *in vitro* and *in vivo*

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Abstract: Numerous epidemiological studies show an inverse association of the consumption of plant phenols with the occurrence of certain chronic diseases, including vascular disease. Reducing excessive oxidative damage is one mechanism for minimizing the cell (tissue) damage that can lead to the establishment and progression of vascular disease. The antioxidant effects of the flavonoids present in diverse plant foods represent one mechanism that could contribute to the cardiovascular protective effects of plant-rich diets. Cocoa and chocolate can represent particularly rich sources of dietary flavonoids, cocoa containing up to 10% flavonoids by weight. Flavanols and procyanidins (oligomers of flavanols) isolated from cocoa display strong antioxidant properties in a number of *in vitro* systems. In acute feeding trials with healthy adult subjects, the consumption of flavanol-rich cocoa and chocolate was associated with increases in plasma antioxidant capacity, reduction in the rate of LDL-oxidation, and reduction in platelet reactivity. Collectively, the results from several studies on flavonoid-rich foods support the concept that the consumption of these foods can be associated with improvements in oxidant defenses, and a reduced risk for certain vascular-related diseases.

1. Introduction

Recently, increasing effort has been given to studies on how diet can be used in the prevention, and treatment, of age-related diseases, such as cardiovascular disease, age-related vision loss, and osteoporosis. While the classic essential nutrients are still widely studied in this regard, there is increasing interest in the evaluation of a number of plant compounds that may have positive health effects. To a significant extent, this interest has been fueled by a number of recent epidemiological studies that suggest that diets rich in fruits and vegetables are associated with a reduced risk for several chronic diseases [1,2]. These studies have led to the development of several new diet education programs, such as the U.S. National Cancer Institutes 5-A-Day Program, that are built on the concept that increased consumption of fruits and vegetables will result in reduced risk for cancer in the general population. While these programs have laudable goals, to date, there is considerable uncertainty regarding the identity of the chemical factors that provide these protective effects. In the absence of this knowledge, it is difficult to identify specific fruits and vegetables (or other plant foods) that may be particularly rich in these factors. Thus, while education programs that emphasize the fact that a variety of plant foods should be consumed for optimal health are clearly appropriate and well grounded in science, they can lack specificity when it comes to making dietary recommendations for specific health outcomes. For example, it is unlikely that the exact same collection of plant biofactors protect against cancer and vascular disease.

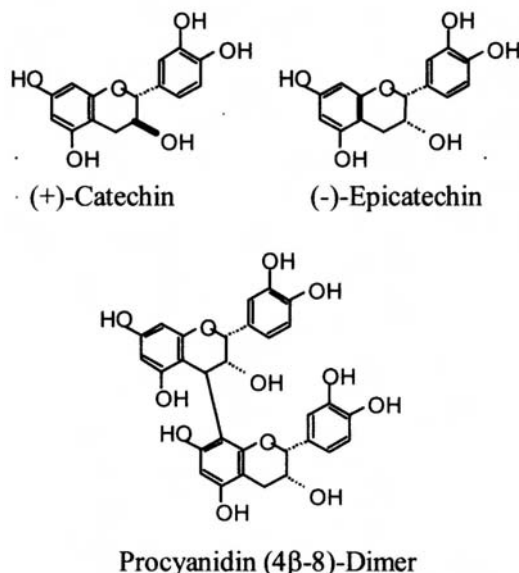


Figure 1. Chemical structures of flavanols and a cocoa (-)-epicatechin dimer. Higher molecular weight procyanidins typically oligomerize through the 4β-8 bond.

Flavonoids represent one class of bioactive compounds that may have multiple beneficial effects on several chronic diseases [3-4]. Cocoa represents an example of a potentially rich dietary source of flavonoids. High concentrations of flavonoids are present in certain cocoas, predominately as the flavanol monomers (-)-epicatechin (epicatechin) and (+)-catechin (catechin), and as oligomers of these monomeric base units which are known as the procyanidins (Figure 1) [5]. Other potential rich dietary sources of flavonoids include tea, wine, grape juice, apples, onions and certain nuts.

Cocoa is derived from the beans of *Theobroma cacao*, a tree native to South America [6]. While cocoa and chocolate are widely viewed today as confectioneries that have minimal nutritional value, historically, cacao has been thought to have strong medicinal properties, having been used for the treatment or prevention of infection, inflammation, heart palpitations and angina [6]. The rationale for the study of the potential health benefits of cacao and chocolate then, is based on cultural, epidemiological, and biochemical information [7].

The objective of this paper is to summarize some of the recent research that has been conducted on the potential nutritional value of cocoa, with a focus on its ability to serve as a rich source of flavonoid antioxidants.

2. Antioxidant action of flavanols and related oligomers on liposome and LDL-oxidation

While the positive health benefits associated with the consumption of a flavonoid-rich diet cannot be attributed to any one factor, the antioxidant properties of certain flavonoids have been the focus of considerable attention [8]. The antioxidant actions of chocolate flavonoids were first studied inhibiting LDL-oxidation [9,10]. As a consequence of the availability of purified procyanidins from cocoa (dimer-decamer), this particular family of

flavonoids has been studied in some detail. An early question that was asked concerning procyanidins involved to what extent does the oligomerization of several units of epicatechin influence the overall antioxidant capacity of these procyanidins.

In a series of experiments conducted to evaluate the inhibition of the oxidation of synthetic liposomes by flavanoids and procyanidins purified from cocoa, Lotito et al. observed that the influence of the degree of oligomerization on the antioxidant activity depended on the mechanism of oxidation [11]. In the above work, liposomes were incubated in the presence of four different oxidation systems: i) a water soluble free radical generator (2,2'-azobis-(2- amidinopropane) hydrochloride, AAPH; ii) a lipid soluble free radical generator (2,2'-azobis-(2,4-dimethylvaleronitrile), AMVN; iii) a redox active metal, ferrous iron; and iv) UV-C irradiation. In all four systems, epicatechin and its oligomers exerted antioxidant protection in a dose-dependent manner, at micromolar concentrations. When the liposomes were oxidized with AAPH (Figure 2A) or UV-C (Figure 2D), there was only a minimal effect of oligomerization on the degree of oxidant protection, suggesting that the antioxidant capacity of the molecules was related to the amount of hydroxyl groups available to react with the radicals (most likely the OH groups in the 3' position). In contrast, in the presence of AMVN (Figure 2B) or ferrous iron (Figure 2C), the degree of oligomerization significantly influenced the antioxidant capacity of the procyanidins. Thus, oligomerization had opposite effects in these two oxidation systems: increasing procyanidin chain length was associated with increased oxidative defense against AMVN-mediated liposome oxidation, while the capacity to protect against ferrous-mediated liposome oxidation was inversely associated with procyanidin chain length.

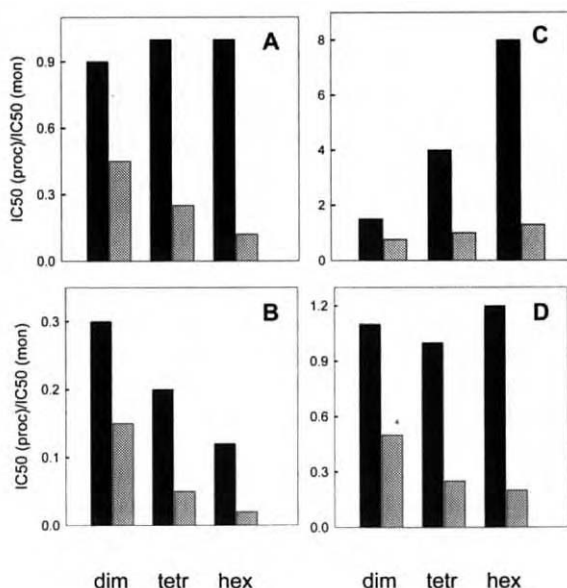


Figure 2. Relative IC₅₀ values for the antioxidant effect of dimer, tetramer, and hexamer. Antioxidant effect was evaluated in liposomes incubated at 37°C during 60 min in the presence of different oxidant systems: I) AAPH 10 mM; II) AMVN 10 mM; III) 25 μ M ferrous iron/25 μ M ascorbate; IV) UV irradiation. Black bars are relative values for IC₅₀ calculated considering the concentration of the procyanidins based on moles of monomer equivalent; and gray bars for relative IC₅₀ calculated considering the concentration of procyanidins based on mol of procyanidins.

These *in vitro* results could be attributed to either the chelating capacity of these compounds (monomers chelate better than oligomers), or the ability of the oligomers to differentially adsorb to the membrane lipids. Differences in membrane absorption could result in marked differences in membrane stabilization, reducing their susceptibility to oxidation (longer chain oligomers adsorb better than the shorter chain oligomers) (Verstraeten et al., unpublished). These observations stress the importance of the degree of oligomerization, and the origin of the oxidant insult, when one is discussing the antioxidant ability of compounds in physiological settings. While the occurrence of significant concentrations of the high molecular weight oligomers in most tissues is unlikely, their ability to bind to membranes allows them to have potential regulatory effects, even at low concentrations. Similar to their protective effects against liposome oxidation, the flavonoids isolated from cocoa have been shown to reduce LDL-oxidation *in vitro* [9-12]. Significantly, the inhibitory effects of the flavanols as well as the procyanidins can be demonstrated at a concentration of 1 μM . This is a critical point, since plasma concentrations of epicatechin can reach this level after the consumption of a flavanol-rich meal (see below). Similar to the findings with liposomes, there is structural specificity of the flavonoids with respect to their ability to inhibit LDL-oxidation [12].

3. *In vitro* interaction of flavanols with other antioxidants

While flavanols and their oligomers can have direct oxidant scavenging effects, it should be recognized that they can also have indirect effects through their interaction with other antioxidants [13-16]. The data presented in Figure 3 illustrate the above point. When plasma obtained from healthy adult humans (ascorbate 35-55 μM and α -tocopherol 24-27 μM) was incubated in the presence of 50 mM AAPH, the consumption of ascorbate followed first order kinetics ($k = 0.11/\text{min}$; $t_{0.5} = 6.0$ min). The consumption of α -tocopherol started once all the ascorbate was depleted (60 min), and also followed first order kinetics ($k = 4 \times 10^{-3}$ min; $t_{0.5} = 173$ min) (Figure 3A). When the plasma was oxidized in the presence of 100 μM epicatechin (Figure 3B) the rate of ascorbate still followed a first order kinetics, but the rate of depletion was significantly reduced ($k_{\text{epi}} = 0.07/\text{min}$; $t_{0.5} = 9.9$ min). Interestingly, when plasma was oxidized in the presence of 100 μM (+)-catechin (Figure 3C), the rate of ascorbate depletion was barely increased ($k_{\text{epi}} = 0.15/\text{min}$; $t_{0.5} = 4.6$ min). The lag times for epicatechin and catechin consumption were 75 and 45 min, respectively. The depletion of α -tocopherol was entirely prevented in the presence of catechin or epicatechin.

These experiments demonstrate that: a) as can be predicted by their reduction potentials, flavanols can have an intermediate reactivity between ascorbate and α -tocopherol; b) epicatechin seems to protect ascorbate better than catechin from oxidation. Considering that the difference between epicatechin and catechin is the orientation of the OH group in the position 3, it is clear that the differential effects are not directly related to their capacity to trap radicals.

4. Bioavailability of flavanols and related oligomers

While the flavanols and procyanidins isolated from cocoa clearly have a number of interesting properties *in vitro*, the important question from a nutrition point of view is whether the same effects can be observed *in vivo*. Spencer et al. [17] recently reported that *in vitro* there is significant decomposition of the procyanidins isolated from cocoa when they are incubated in simulated gastric juice. Given the above, it can be argued that some of

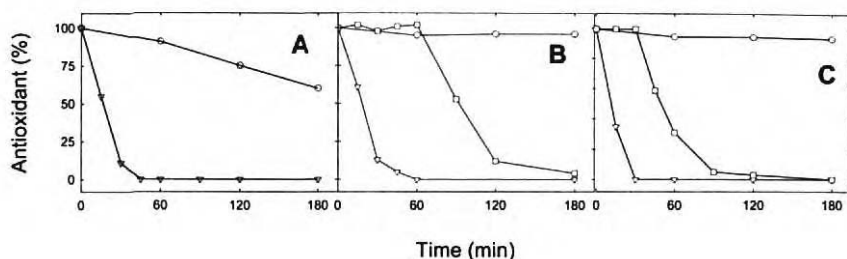


Figure 3. Kinetics of antioxidant depletion. Human plasma was incubated at 37°C with 50 mM AAPH in the absence (A) or the presence of 100 μ M epicatechin (B) or catechin (C). Flavanols (squares), ascorbate (triangles), and α -tocopherol (circles). Values are mean of at least three independent experiments. SEM were always under 15%.

the *in vitro* effects reported for these compounds may not occur *in vivo*. Critical to this issue is the extent to which flavanols and procyanidins in cocoa are absorbed, and the extent to which they are metabolized following absorption. In this regard, Richelle et al. [18] monitored the plasma kinetics of epicatechin over an 8-hour time period after volunteers consumed two different doses (40 and 80 g) of black chocolate. These investigators reported that there was a dose-responsive increase in plasma epicatechin following the consumption of the chocolate, with plasma epicatechin reaching its peak approximately 2 hours post consumption.

Similar to the above, in an acute study with healthy young adults, Wang et al. [19] reported a dose-responsive increase in plasma epicatechin concentrations after the consumption of increasing amounts (27 g, 53 g, and 80 g) of a flavanol-rich chocolate (6.9 mg of flavonoids/g). It is important to note that in both of these studies [18,19], plasma epicatechin concentrations were below detection at baseline. While the bioavailability of the flavanols epicatechin and catechin has been well documented, there is still limited information concerning procyanidin absorption. Radiolabelled techniques have indicated that the procyanidins are bioavailable, although these studies did not demonstrate whether the procyanidins were intact or depolymerized prior to absorption [20]. Recently, Holt et al. [21] reported that cocoa procyanidin dimer B2 (epicatechin-(4 β -8)-epicatechin) can be detected in the plasma of human subjects within 30 minutes of consuming a cocoa beverage, reaching a maximum concentration in the plasma approximately 2 hours after consumption. Consistent with the above, Zhu et al. [22] reported the occurrence of dimer B2 in plasma obtained from rats given a large dose of a flavanol-rich cocoa. While the above results are exciting, the physiological consequences of nanomolar concentrations of the procyanidin dimers and higher procyanidins respect to health remains to be determined. An additional observation made by Holt et al. [21] was that the plasma concentration of epicatechin could greatly exceed that of catechin, despite the fact that their concentrations were not markedly different in the consumed meals. Similar findings have been observed for rats by Zhu et al. [22] and by Baba et al. [23]. The significance of this difference in the absorption or metabolization of epicatechin and catechin is underscored by their differential ability to protect plasma vitamin C as shown in Figure 3.

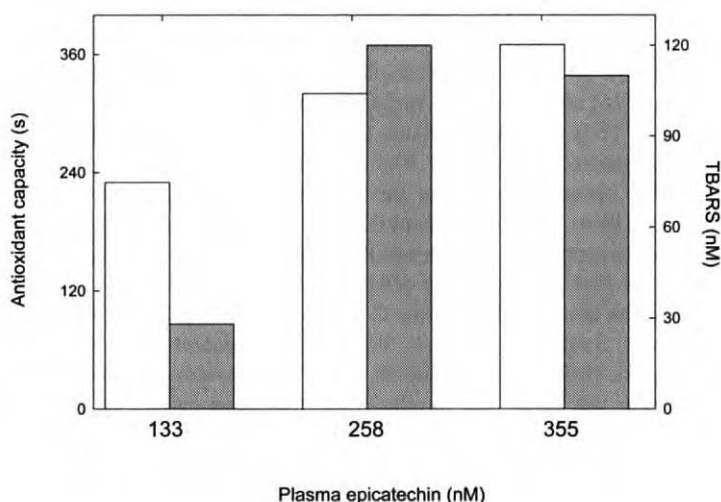


Figure 4. Antioxidant capacity and lipid oxidation in plasma of volunteers consuming different amounts of procyanidin-rich dark chocolate (6.9 mg of procyanidins per g of chocolate). Antioxidant capacity was evaluated by the ability of plasma to inhibit luminol-dependent chemiluminescence and lipid oxidation by plasma TBARS. Plasma epicatechin concentrations are the average amount of epicatechin determined two hours after chocolate consumption. Ordinate values indicate increases over basal levels of plasma antioxidant capacity (white bars), or decrease over basal values for TBARS (gray bars).

5. Presence of epicatechin and oxidative stress *in vivo*

Plasma epicatechin concentrations following the consumption of a flavonoid-rich meal are typically in the low micromolar range, thus it could be argued that the epicatechin may have only a minimal impact on plasma oxidant defense given the relative higher concentrations of vitamin C, vitamin E, and others substances with antioxidant activity. However, Wang et al. [19] reported a dose-response relationship between the levels of epicatechin in the plasma and plasma antioxidant capacity in healthy human subjects (Figure 4). In addition, there was a decrease in plasma TBARS that was inversely related to plasma epicatechin (Figure 4), suggesting that this flavanol (or one of its metabolites) is providing antioxidant protection at physiologically relevant concentrations. Zhu et al. [22] have also reported a rapid rise in plasma antioxidant capacity in rats given a single meal of a cocoa beverage.

Osakabe et al. [24] recently reported a study in which subjects consumed 36 g of cocoa, which provided 2610 mg of total polyphenols (measured using a non-specific assay) daily for 2 weeks. A control group consumed an equivalent amount of sugar as that contained in the cocoa beverage. Compared to baseline, lag time to LDL-oxidation was significantly increased in the cocoa group by 29% and 14% depending on the oxidant used (V-70 radical initiator, or Cu ion). The finding that the rate of LDL-oxidation was reduced following the consumption of flavanol-rich beverages is consistent with the *in vitro* data discussed above. It is interesting to note that Osakabe et al. [24] were unable to detect epicatechin in the plasma, although an increased urinary excretion of epicatechin was observed 1 and 2 weeks after cocoa consumption. It is likely that the timing of blood draws explains the lack of epicatechin in the plasma, as the blood samples in this study were taken following a 12-hour fast. In a similar experiment, a group of young volunteers did not show increases in plasma levels of epicatechin after the consumption of a procyanidin-rich

chocolate confectionary for 4 weeks, when blood was collected after an overnight fast (Actis-Goretta et al., unpublished). This is consistent with reports from acute feeding studies that the majority of absorbed epicatechin is cleared from the blood by 8 hours [18,19,25]. Wan et al. [26] also reported a rapid clearance of epicatechin in subjects fed 22 g of cocoa powder and 16 g of dark chocolate. Consistent with the findings by Osakabe et al. [24], these investigators observed an 8% increase in LDL-oxidation lag time after subjects consumed the chocolate products for a period of 4 weeks. The observation by Osakabe et al. [24] that there was a significant increase in lag time to LDL-oxidation after 1 and 2 weeks of cocoa consumption, independent of the concurrent presence of epicatechin in the plasma, suggests that the protective effects of flavanols on LDL-oxidation may be due to their effect on the amount of vitamins C and/or E, or other antioxidants, associated with the LDL particle. Such a mechanism would be consistent with the *in vitro* data discussed above [13-16]. However, it should be noted that flavanol-induced changes in the LDL surface that make the lipids less available for oxidation cannot be ruled out as an additional mechanism [27].

Regardless of the mechanisms involved, these results provide additional evidence for the concept that the intake of dietary flavonoids can be associated with improvements in the oxidant defense system that are physiologically relevant. This concept is further supported by the finding by Actis-Goretta et al. (unpublished), that 2 hours after the consumption of 105 g of chocolate, the depletion of α -tocopherol in plasma oxidized with AAPH was slower than in the same subjects before, and 6 hours after, chocolate consumption.

It is reasonable to speculate that flavonoid-induced reductions in the rate of LDL-oxidation may contribute to the positive vascular effects associated with these compounds, given the pivotal role that oxidized LDL is thought to play in the initiation and progression of vascular disease. It is important to note, however, that these compounds may also be providing an alternative form of antioxidant protection. For example, Zhu et al. [22] have recently reported that erythrocytes obtained from rats given a flavonoid-rich meal showed an enhanced resistance to oxidation-induced hemolysis.

6. Additional physiological properties of flavonoids

During the past five years, numerous research groups have reported on the biological effects of cocoa, and its flavanol and oligomer components. As is discussed above, it has been reported that *in vitro*, cocoa, and isolated cocoa flavanols and their oligomers, have the ability to increase the antioxidant capacity of solutions and slow the oxidation of LDL and liposomes [9-11]. While the ability of flavonoids to inhibit oxidative damage may explain some of their health benefits, it is important to recognize the fact that they can have numerous other effects. For example, in *in vitro* models, they have been shown to induce endothelium dependent vessel relaxation (EDR) [26]; reduce the production of inflammatory cytokines, while increasing the production of anti-inflammatory cytokines [29,30]; increase the synthesis of the antithrombotic lipid prostacyclin, while reducing the production of the proinflammatory cysteinyl leukotrienes [31]; protect against peroxynitrite-dependent oxidation and nitration reactions [32]; and decrease the expression of the activated conformation of glycoprotein IIb/IIIa (GPIIb/IIIa) and CD62P (P-selectin) on epinephrine activated platelets [33]. Similar to tea flavonoids, cocoa flavanols and their related oligomers can inhibit platelet activation *in vitro*, following stimulation with epinephrine [33]. Importantly, these effects have also been observed *in vivo* following the consumption of a flavanol-rich cocoa beverage. Rein and co-workers [34] determined that in subjects who consumed cocoa containing 897 mg of flavanols and related oligomers, the expression of P-selectin platelet surface receptor was lower than in control subjects.

Similarly, at 2 and 6 hours following the consumption of the cocoa beverage, GPIIb/IIIa expression on unstimulated platelets and those stimulated with epinephrine and ADP was significantly decreased. In contrast, GPIIb/IIIa expression was significantly increased following stimulation with epinephrine in subjects who consumed a caffeine-containing control beverage, and there was a trend for increased expression when platelets were stimulated with ADP. In support of the idea that these changes in platelet reactivity can be physiologically significant, coagulation as assessed by platelet function analysis was significantly decreased in subjects following the consumption of a flavonoid-rich beverage [35]. In theory, a reduction in platelet reactivity, leading to an increase in coagulation time, should reduce the risk for thrombi.

Taken together, these studies show that cocoa components can exert positive effects on the biological mechanisms underlying several inflammatory and vascular diseases.

7. Conclusion

Collectively, the data obtained over the past five years on the biological effects of flavonoid-rich cocoas and chocolate support the concept that the consumption of flavonoid-rich foods can be associated with positive health effects. It is important to note that clear epidemiological data concerning the influence of cocoa and chocolate consumption on the risk for cardiovascular disease are lacking. Unfortunately, such data will be difficult to collect, as the flavonoid content of these foods can be markedly influenced by food processing. For example, dutching, a common treatment used in the production of cocoa, results in a marked reduction in its flavonoid content. Similarly, depending on the processing employed, the flavonoid content of other beverages, as wine, tea, and grape juice, can vary considerably.

8. Summary

Even in a “balanced” diet that meets macronutrient recommendations and micronutrient requirements, there is a growing body of evidence that other compounds play an important role in optimizing health. Flavonoids, such as those occurring in cocoa, wine, tea, and purple grape juice are examples of a class of bioactive compounds that may confer beneficial effects on a number of important risk factors for cardiovascular disease. As we gain a better understanding of how bioactive compounds in various foods improve health, we will need to devise strategies for increasing their intake within the context of a healthy diet that meets energy requirements. Clearly, more research is necessary to establish: i) which compound(s) is(are) responsible for given biological effects; ii) the extent to which the bioavailability of the compounds are influenced by food processing and the food matrix; iii) the amount of the compound(s) that is needed to induce the desired biological effects; and iv) the extent to which these compounds interact with other essential nutrients, such as vitamin C and vitamin E.

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Estimation of Oxidative and Lipids Peroxidation DNA Adduct in Urine and DNA. Methodological Aspects and Application in Molecular Epidemiology

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1. Introduction

Mammalian life is based on oxygen and uses oxygen reduction for energy production and synthetic processes. By 4-electron reactions oxygen is reduced to water and the energy released is stored for controlled use. However, one electron reduction occurs in minor amounts giving rise to various reactive oxygen species (ROS) [1,2]. The reactive oxygen species potentially oxidises important macromolecules and structures in the body. Oxidation processes are prone to occur in the earth's environment, including in test tubes, refrigerators, freezers, laboratories etc. due the ubiquitous oxygen. This poses a major challenge to anybody studying these processes since artefacts can arise from oxidation during sample handling. Particularly, most methods rely on storage or prolonged preparation of samples before the initial analysis. In addition to storage, most procedures are carried out at conditions that clearly make spontaneous oxidation possible. Often it will be found that immense differences are reported between different laboratories. Consequently published data always should be scrutinised bearing this aspects in mind.

2. Analysis of oxidised DNA and excreted repair products

HPLC, high performance (or pressure) liquid chromatography, is particularly suited for small water-soluble molecules and proteins. Most used for analysis of DNA fragments is the reverse phase HPLC. Detection with electrochemical detectors are preferred. There are many different brands of ECD detectors and electrodes/cells. In our laboratory we have found that the ESA Coulochem is working excellent for our purposes and provides excellent sensitivity. Similar experience with other detectors can be found. We have found that for HPLC-ECD analysis of urine, separation is critical due to electrochemically active peaks eluting close to that of 8-oxodG. Ways to detect a false peak is given in details elsewhere [3].

The quantification also requires special attention since in HPLC it is not possible to use a true internal standard, i.e. an internal standard that behaves exactly as the substance you want to measure. An internal standard, 2,6-Diamino-8-oxopurine, has been suggested [4], but is probably only useful in controlling variations in the injection volume, and cannot

be used for other purposes that pose more severe problems like artificial oxidation, degradation of 8-oxodG on the column etc. Presently there is no experience with the use of this internal standard for urine measurement. We use external standard addition in different concentrations and evaluate the response ratios [3], and this methodology appears to function satisfactory.

Also HPLC with tandem mass spectrometric detection (HPLC-MS/MS) provides a suitable method of analysis. We have found that a single column is sufficient [3,5], however, we must emphasise that unknown substances similar in mass to 8-oxodG needs to be separated from 8-oxodG. For high sensitivity in mass spectrometry the peak height in HPLC is very important. The amount detected is proportional both to the peak height and to the area under the curve.

By derivatization it is possible to use the GC separation procedure coupled with mass spectrometry to measure oxidised DNA products. However, this method has with few exceptions not been used for urinary measurements on DNA, but has been the method used for estimation of 8-oxodG, actually the base after hydrolysis, and other DNA oxidation products in tissue DNA.

For urine measurements a semi-preparative HPLC procedure was applied, followed by hydrolysis, derivatization and GC-MS [6,7].

Gas-chromatography-mass spectrometry used for quantification of oxidative DNA products has been criticised for errors due to artificial oxidation, however, provided that sufficient precautions are taken, this can be avoided and results similar to those from HPLC-ECD can be provided regarding 8-oxodG in DNA [8]. Presumably this is also valid for other oxidative DNA products, but needs to be validated. In case of 8-oxodA the validity has been questioned [9] in an experiment with vitamin C and vitamin E intervention [10] and using HPLC-MS/MS it seems likely that the high reported 8-oxodA values relates to artefactual oxidation [5]. Many of the problems regarding artificial oxidation relates to the very high content of non-oxidised dG in DNA hydrolysates, about 1.000.000 times higher. This means that oxidation of only a very minute fraction of dG gives serious artefacts. For urine measurements the levels of oxidised and non-oxidised nucleosides are similar and would a priori not present a problem of the same magnitude.

GC-MS has been used to measure urinary DNA oxidation products, however, various clean-up or up-concentration methods are necessary. The choice for urinary measurement is therefore either HPLC-EC, which is limited mainly to 8-oxodG measurement or to HPLC-MS/MS where multiple products can be measured. Both of these methods can be set up with very little preparation of urine, just a simple centrifugation and dissolving of possible sediments.

The use of a specific antibody could be the basis for a fast and effective methodology to measure 8-oxodG. However, it has proven difficult to produce an antibody with sufficient specificity for analysis in urine. Several publications have appeared [11-14]. However, although some characterisation of the antibody and epitope is given, it appears not to be tested against the many different DNA and RNA products in urine [15]. Furthermore, testing against the present method of choice HPLC-ECD, GC-MS or HPLC-MS/MS has only been stated without data, and at present time the data have not been made available in the literature [12]. One particular problem with the immunologically based assays may relate to the high number of DNA/RNA products excreted into urine. In case of RNA products high concentrations of very similar chemical substances are excreted in to urine [15]. A similar myriad of DNA products undoubtedly is also excreted. Together this may make it very difficult to produce a specific antibody. A commercially available kit tested out against the three dimensional HPLC-ECD showed clear non-specificity [16]. Until clear demonstration of close correlation to the verified HPLC-ECD method the use of immunologically based methods for quantification of 8-oxodG in urine cannot be

recommended. Since there is a very close correlation between HPLC-ECD and HPLC-MS/MS measurements, presently these methods may be regarded as the golden standard.

The urinary excretion of 8-oxodG in pigs following i/v injection follows simple kinetics with a half-life of about 2.5 hours, a clearance of about $4 \text{ mL min}^{-1} \text{ kg}^{-1} \text{ BW}^{-1}$ and a volume of distribution close to $1 \text{ L kg}^{-1} \text{ BW}$ [17], and moreover the urinary excretion rate corresponded to the infusion rate. After liver transplantation we observed an increased urinary excretion of 8-oxodG and in a caval clamp experiment the excretion was temporarily reduced. These experiments indicate that steady state between formation and urinary excretion is obtained rapidly.

The reported values of urinary excretion of 8-oxodG in the literature are in agreement. The reported 8-oxodG urinary excretion rates measured with HPLC-ECD or GC-MS [18] vary from about 100 to 600 pmol kg $\text{BW}^{-1} \text{ 24 h}^{-1}$, excluding the measurements with immunologically based estimations that vary between 1600-4800 pmol kg $\text{BW}^{-1} \text{ 24 h}^{-1}$ most presumably for the reasons about lack of specificity given above.

Classic pharmaco-kinetic consideration gives a theoretical steady state plasma concentration equal to production (dosing rate) divided by clearance, i.e. between 0.017 and 0.100 nmol/L. The conventional HPLC-ECD and HPLC-MS/MS methods have sensitivity close to that level. Using up-concentrations and a HPLC-ECD system with a non-commercially available carbon column Bogdanov et al. [19] reported plasma values of 0.014 - 0.070 nmol/L (4- 21 pg/ml), i.e. in close agreement with the theoretical values.

Collectively, these data indicate that the 8-oxodG in the urine mainly originates from genomic DNA. However, on a more detailed level the contribution of 8-oxodG from the nucleotide pool cell turnover, cell death, and from inflammatory cells is unknown. Presently, neither direct nor indirect data from the in-vivo situation are available. Accepting that the contribution of nuclear DNA reflects the oxidation of nuclear DNA, the urinary excretion is a reflection of the average total oxidative stress to DNA of all body cells. In most experimental situations *in vivo* it is reasonable to argue that a given person is in a steady state, i.e. a constant 8-oxodG level in DNA and a constant repair. Mass conservation will be applicable and consequently the amount of excreted 8-oxodG will equal newly formed 8-oxodG. The urinary measurement is therefore equal to the rate of oxidative stress to DNA. If an experimental or other form of change happens (say smoking cessation, anti-oxidant intervention) a new steady state will soon be reached and a change in the rate of oxidation of DNA can be identified. It is important to stress that this measure is independent of DNA repair, a point often not recognised.

The concentration of say 8-oxodG in DNA reflects a balance between newly formed 8-oxodG's and removal. An increased level can consequently reflect either an increased formation (increased oxidative stress) or a decrease in repair or any combination. It is important to note that this cannot be determined from measurement of the level. A similar argumentation can be made for decreased levels.

It can further be argued that comparing two persons with different oxidative stress to DNA, i.e. different urinary excretion rates, the one with the higher stress will statistically have a higher chance for a mutation in DNA. Increased levels can not necessarily be interpreted in the same way, unless it can be established whether it originates from increased stress or decreased repair.

It should be noted that for urinary excretion studies the preferred design is to collect 24 h urine. In some special designs it can be argued that the use of spot urine samples and correction for urinary creatinine concentration may be a valid measure. A prerequisite for the spot urine - creatinine correction design is a solid argumentation that creatinine excretion is unchanged by the experimental condition or that it is not different between groups. A theoretical example is comparison of lean men versus fat females. Their cell number is comparable but muscle mass very different. Creatinine excretion is mainly

dependent on muscle mass, and there can easily be a difference in creatinine excretion of say 3 fold between the two groups. If they have the same oxidative stress to their DNA, females would appear to have 3 times higher values, simply because the male excretion is divided by a three times higher creatinine concentration. The same argumentation can be applied to comparison of catabolic patients versus normal controls, and old versus young adults. Preferentially 24 hours urine, overnight urine(s) or at least 8 hours urine on a defined period of the day should be collected and the 8-oxodG excretion given as amount per time unit and kg BW, preferentially lean body weight.

The most studied oxidative modification of DNA relates to direct oxidation of DNA, the 8-hydroxylation of guanine being the one most extensively studied, particularly regarding urinary excretion of the repair product 8-oxodG.

The excretion of the base, 8-oxoGua, is much less studied, although it is excreted in larger amounts, about 5-10 times larger than 8-oxodG [20]. There is general agreement that the modifications like 8-oxodG are the result of reactions between DNA and reactive oxygen species. However, other oxidative processes e.g. lipid peroxidation gives rise to reactive intermediates that in turn can modify DNA. Lipid peroxidation leads to formation of malondialdehyde, crotonaldehyde and acrolein that in turn lead to propano- and etheno-DNA adducts, called exocyclic adducts. These adducts are found in lower quantities than e.g. 8-oxodG and require ultra-sensitive methods. The urinary excretion of 1,N⁶-ethenodeoxyadenosine (ϵ AdA) ranges from about 0.1 to 4 fmol/micromol creatinine in human urine [21]. Human studies on the exo-cyclic adducts and their excretion into urine so far are limited indeed. A comprehensive overview is given in a recent IARC publication [22].

3. Future perspectives

The formation of DNA adducts from endogenous processes and from exogenous factors has emerged as an important factor in the pathogenesis of cancer and ageing. The development of accurate, reliable methods to determine DNA oxidation is essential for understanding the processes. Presently, there has been a fast growing knowledge about the 8-oxodG lesion, and particularly there has been improvement in the knowledge about how to avoid artefacts during the process of quantifying the damage. However, there is only limited knowledge about other lesions than 8-oxodG, particularly *in vivo* in humans. Measurement of single lesions may be misleading and just because one lesion is the most dominating it is not necessarily the most important. Free radicals generate many products at the same time [23].

Furthermore other free radical induced processes, e.g. lipid peroxidation, produce reactive intermediates that may be important. Examples of such other lesions are for example malondialdehyde induced DNA damage and exocyclic DNA adducts [22].

Development of methodologies to detect these DNA modifications are in progress. Furthermore, molecular biology methods, e.g. variants of the PCR methods, and newer mass spectrometry methods like time of flight will in the future make it possible to detail the various DNA modifications not only by reliable methods for quantification but also for position in specific genes. Increasingly, we will see animal studies using genetically modified animals, studies that will clarify specific mechanisms, including studies with DNA array techniques to quantify mRNA to give deeper insight into the cellular biology of oxidative stress.

Furthermore, the future will improve the technologies for measurement on smaller samples and for measurement of large number of samples with reasonable use of time and money. This will enable large scale epidemiological and intervention trials with reliable estimates of the precise role of these modifications in the pathogenesis of disease and ageing.

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Oxidative and Nitrosative Stress Mediated by Cyclosporine A in Endothelial Cells

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Abstract: Cyclosporine A (CsA) therapy is associated with side effects related to oxidative stress. We characterized the reactive oxygen and nitrogen species produced in the extracellular and intracellular compartments of bovine aortic endothelial cells (BAEC) exposed to CsA. CsA induced a dose-dependent increase of the intracellular oxidation of the NO-sensitive fluorescent probe DAF-2/DA. In agreement with this, CsA produced a dose-dependent accumulation of nitrites in the supernatants of BAEC. In contrast, in BAEC treated with CsA, the presence of superoxide anion could only be detected in the intracellular compartment, as measured by the oxidation of dihydroethidium. The formation of peroxynitrite was assessed in the intracellular compartment, by flow cytometry with dihydrorhodamine 123 and immuno-cytochemical nitrotyrosine formation. Tyrosine nitration of endothelial cells could represent a relevant pathophysiological mechanism underlying the vascular injury associated to the use of Cyclosporine A.

1. Introduction

Cyclosporine A (CsA) is a drug with potent immunosuppressant activity in both experimental models and in the clinical setting. However its use encompasses serious side effects such as renal toxicity and hypertension [1-3] and thrombotic events [4]. At present, cardiovascular disease is the most frequent cause of mortality in renal transplant patients. Injury to the vascular endothelium is considered to be an important component of the vascular lesion promoted by CsA. The presence of CsA considerably worsens atherosclerotic lesions within the intima while increases in the thickening of the neo-intima up to 65% have been described [5]. Both endothelial dysfunction and structural damage of endothelial cells have been described. Studies performed in animal models and in vitro suggest that CsA may affect several regulatory functions of endothelial cells, such as NO-dependent vasodilatation [6-8]. In previous work from our laboratory we could demonstrate that CsA enhances the expression of eNOS and increases its activity [9-10], at least after prolonged exposures of endothelial cells to the drug. These observations have been confirmed in rats [11] and healthy volunteers in whom infusion of CsA through the antecubital vein increased NO activity in the forearm vascular bed both basally and after receptor-mediated stimulation [12].

Incomplete reduction of molecular oxygen, potentially favored by a reducing ambient inside cells, generates reactive oxygen species (ROS). These mediate oxidative damage to membrane lipids [13], DNA [14], proteins [15] and lipoprotein peroxidation [16]. This is generally known as oxidative stress. In analogy with this concept, the term nitrosative stress has been coined, alluding to reactions mediated by NO-related species, denominated

reactive nitrogen intermediates (RNI) [17]. These reactions have functional consequences, some of which may be deleterious for the cell. Nitrosative damage may occur *in vivo* in several pathophysiological conditions [18,19-20]. The biological consequences of NO formation in cell systems are controlled by a complex balance of competing reactions between NO and molecular oxygen, ROS, transition metals and thiol groups, which is still not well clarified. These reactions lead to the synthesis of several RNI including nitrosyl-metal complexes, S-nitrosothiols, N_2O_3 and peroxynitrite [21-25]. Other potent oxidants derived from NO, such as nitryl chloride or NO_2 may arise as a product of the oxidation of nitrate through the myeloperoxidase pathway [26-27]. Many studies which examine the participation of ROS/RNI in a given effect do not identify the chemical species involved. However, precisely defining the ROS/RNI involved may help to understand more profoundly the specific pathways of regulation of a phenomenon.

2. Generation of nitric oxide by Cyclosporine A

The effect of CsA on NO synthesis has been the object of several studies, some of them offering contradictory results. It is now accepted that vasoconstriction induced by CsA does not result from a decreased activity or expression of eNOS. In order to check if the problem could be due to a disturbance in the bioavailability of NO we used fluorescent probes in combination with other techniques, in order to identify the ROS/RNI generated by endothelial cells when exposed to CsA for short periods of time.

We had previously tested that the cell-permeable fluorescent probe diaminofluorescein/diacetate (DAF-2/DA) could be used with the flow cytometry technique to detect intracellular NO [28]. A dose-dependent increase in the intracellular fluorescence of DAF-2 was detected in BAEC treated with CsA (Figure 1, right panel). When the accumulation of NO was evaluated in the supernatants of BAEC upon treatment with CsA, a dose-dependent increase in the accumulation of extracellular nitrite was detected (Figure 1, left). As shown in this figure, the concentration of CsA of 1 μM , which lies within the therapeutic range, only produced a moderate increase of NO.

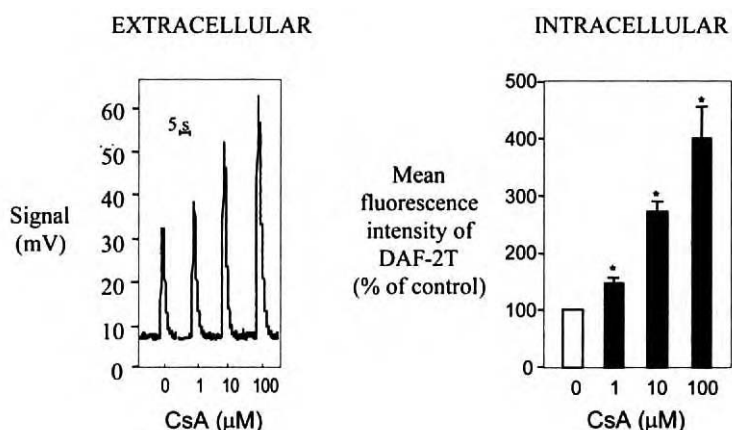


Figure 1. Detection of NO in BAEC exposed to CsA. Detection of extracellular and intracellular RNI in BAEC treated with CsA. Left: representative experiment showing the effect of the indicated doses of CsA on the 2h-accumulation of nitrites in supernatants of BAEC detected by chemiluminescence. Right: flow cytometry detection of NO with the probe DAF-2/DA (10 μM) in BAEC incubated for 2 hours with the indicated doses of CsA. Data are represented as mean intracellular fluorescence of DAF-2T (oxidized fluorescent form of DAF-2/DA) (n = 4). * p < 0.05 versus vehicle (0 μM CsA).

3. Oxidative stress associated to Cyclosporine A. Generation of superoxide anion

During the metabolism of CsA the presence of oxidative stress has been described [29-32]. However, the chemical nature of the ROS involved has not been characterized. Pro-oxidative capacity of CsA has been suggested in extravascular territories, such as isolated liver microsomes (detected as the capacity to produce malonyldialdehyde [29-32]) and in renal mesangial cells (measured as the ability to oxidize the probe dihydro-dichlorofluorescein [33-34]). The bioavailability of endothelial NO is linked to the presence of superoxide anion which significantly shortens its half-life. CsA-elicited vasoconstriction is significantly abrogated by the presence of SOD [6]. Several evidences point towards the participation of ROS in the pathophysiology of the secondary effects of CsA. Antioxidants such as vitamin E and lazaroids have been shown to reduce lipid peroxidation, renal vasoconstriction and nephrotoxicity related to the use of CsA [35-36]. Electron spin resonance using the spin trap 4-POBN made it possible to detect an increase in free radicals in the urine of CsA-treated rats [37]. The antioxidant N-acetylcysteine seems to have a protective effect against nephrotoxicity [38].

The origin of ROS does not seem to be related to the CsA molecule itself and their formation is inhibited by glycine [39]. These ROS have not been the object of a precise identification so far. We took advantage of previous studies in which we had previously validated the specificity of the fluorescent probe DHE to detect $O_2^{\cdot -}$ in our cell culture conditions [28]. Using flow cytometry, a dose-dependent intracellular increase in the 2h-accumulated fluorescence of ethidium (oxidized and fluorescent form of DHE) was detected in intact BAEC treated with CsA or DMNQ (Figure 2 right). In contrast, when the extracellular presence of superoxide anion was evaluated in the supernatant of BAEC treated with CsA, this free radical could not be detected by EPR (Figure 2 left).

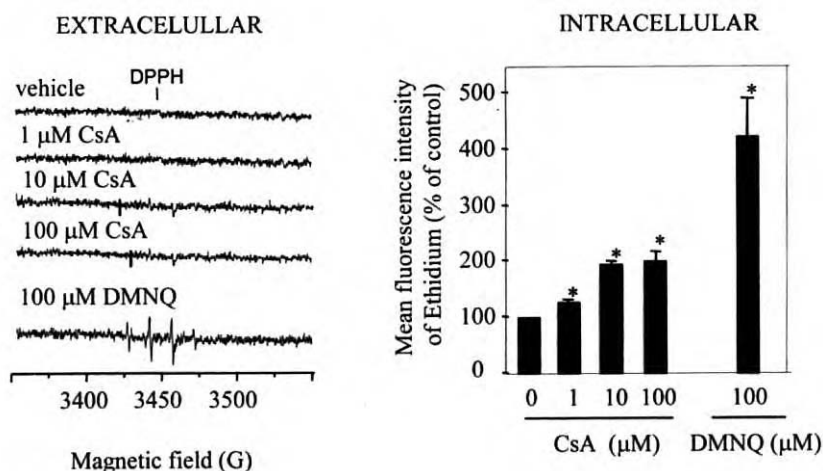


Figure 2. Detection of $O_2^{\cdot -}$ in BAEC upon treatment with CsA. Left: representative experiment of superoxide detection in supernatants of BAEC measured by electron spin resonance (ESR) using the superoxide-sensitive spin trap DMPO. The superoxide generator DMNQ was used as a positive control. Right: flow cytometry detection of $O_2^{\cdot -}$ with the probe DHE. BAEC were preincubated for 1 hour with 5 μ M DHE and then incubated for 2 hours with the indicated doses of CsA. Data are represented as mean intracellular fluorescence of ethidium (oxidized fluorescent form of DHE) (n = 7). * p < 0.05 vs CsA vehicle (0 μ M CsA). DPPH: magnetic field marker.

4. Nitrosative stress associated to CsA. Generation of peroxynitrite

Nitric oxide is not in itself very reactive or particularly toxic. However, its interaction with other oxidants may yield products whose toxicity may result of significance. Such is the case of peroxynitrite (ONOO^-) which is formed in a non-enzymatic reaction between NO and O_2^- . This reaction follows a 1:1 stoichiometry and it has a very fast reaction speed ($6.7 \times 10^9 \text{ mol/L}^{-1} \cdot \text{s}^{-1}$ [40] which is 3 times higher than the capacity of O_2^- dismutation by SOD and around 1000 times greater than the reaction of NO with iron-sulfur clusters [41].

Peroxynitrite is one of the known agents able to promote nitrosative stress. One single molecule of peroxynitrite is capable of oxidizing 2 electrons from the amino acid residue tyrosine to form 3-nitrotyrosine [42]. Formation of 3-nitrotyrosine at a physiological pH has been recently confirmed [43-44]. Several pathological conditions may represent in vivo targets for peroxynitrite-mediated tyrosine nitration. These include ageing [45-47] amyotrophic lateral sclerosis [48], Alzheimer [49] and Parkinson's [50] diseases, cancer [51] atherosclerosis [52] and myocardial contractility failure [53]. The biological relevance of this molecule as a defence mechanism has gained support after the identification of bacterial peroxynitrite reductases [54]. These enzymes would contribute to metabolize peroxynitrite, thus conferring resistance to the host immune response, as suggested for example in *Mycobacteria* [55].

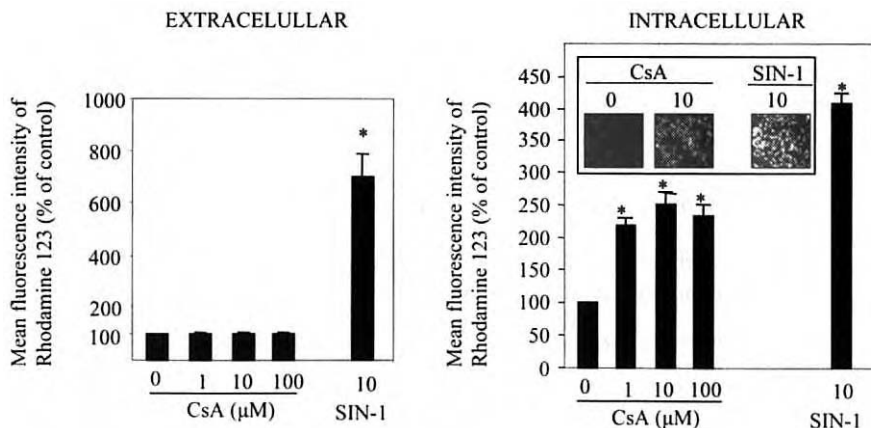


Figure 3. Detection of ONOO^- in BAEC exposed to CsA. Left: extracellular oxidation of the peroxynitrite-sensitive fluorescent probe DHR 123. BAEC were treated for 2 hours with the indicated concentrations of CsA and incubated for the last 15 minutes with DHR123 (2 mM). Triplicate samples of the supernatants were evaluated by fluorimetry (excitation wavelength of 488 nm and fluorescence emission signals were collected with a 525 nm band pass filter). SIN-1, a generator of ONOO^- , was used as a positive control. Right: the intracellular oxidation of DHR 123 (to form Rhodamine 123) by flow cytometry in BAEC upon treatment with CsA. BAEC were preincubated for 1h with DHR and incubated for an additional 2h with the indicated concentration of CsA or SIN-1. For each condition, the mean intracellular fluorescence is shown ($n = 7$). * $p < 0.05$ vs control. In the inset a representative experiment of the immunocytochemistry for the detection of nitrotyrosine in BAEC upon treatment with CsA is shown. BAEC were treated for 2h with vehicle, 10 μM CsA or 10 μM SIN-1. BAEC were examined with a CCD camera after fixation and incubation with a polyclonal rabbit anti-nitrotyrosine antibody, followed by incubation with a porcine anti-rabbit IgG fluorescein-coupled secondary antibody.

As NO and O_2^- appeared to be accumulated in endothelial cells exposed to CsA, the potential formation of peroxynitrite was evaluated. Although the cell-permeant fluorescent probe DHR is commonly used as a non-specific ROS detector, this probe has been recently suggested as a peroxynitrite sensor in cellular systems [56]. We have previously validated

the use of DHR as a peroxynitrite-sensitive sensor in endothelial cells [28]. As shown in Figure 3 (right part), the intracellular 2h-oxidation of DHR by CsA showed a dose-dependent increase, but there was no oxidation of this probe in the extracellular compartment. The intracellular formation of $O_2^{\cdot-}$ and NO, and the oxidation of the peroxynitrite-sensitive probe DHR in BAEC treated with CsA suggested that this drug could be inducing the formation of peroxynitrite. Hence, we addressed the presence of peroxynitrite-related biological markers such as the widely recognized formation of nitrotyrosine, itself a marker of endothelial damage [52]. Immunodetection with a polyclonal antinitrotyrosine antibody showed an increase of nitrotyrosine formation in BAEC treated with CsA (inset of right part of Figure 3). Further investigation led us to find that $O_2^{\cdot-}$ represents the limiting factor for ONOO⁻ formation mediated by CsA, and that this phenomenon could be also recapitulated in the context of pathophysiological conditions where superoxide generation is increased, such as hyperglycemia [57].

We propose that peroxynitrite may represent a potential mediator of endothelial cellular changes associated to CsA treatment. An eventual tyrosine nitration of specific endothelial proteins could represent an underlying mechanism for the vascular damage generated by this immunosuppressant. Further in vivo approaches will facilitate testing the potential clinical importance of this hypothesis.

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Early Signaling with Iron and Copper in Ischemic Preconditioning of the Heart

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Abstract: Iron and copper play major roles in biological systems, catalyzing free radical production and consequently causing damage. The relatively high levels of these metals, which are mobilized into the coronary flow following prolonged ischemia, have been incriminated as key players in reperfusion injury to the heart (*M. Chevion et al., Proc. Nat. Acad. Sci. USA 90:1102-6 (1993) & E. Berenshtein et al., J. Mol. Cell. Cardiol. 29:3025-34 (1997)*). In the present communication we investigated other roles of iron — providing protection to the ischemic heart via preconditioning (PC).

PC was accomplished by subjecting isolated rat hearts to three episodes of 2 min ischemia separated by 3 min of reperfusion. Prolonged ischemia followed the PC phase. PC hearts (group I) were compared to hearts subjected to normal perfusion (group II, no ischemia) and to ischemia without PC (group III). Group I showed a marked improvement in the recovery of hemodynamic function *versus* group III. Biochemical parameters further substantiated the PC protection provided to group I against prolonged ischemia. Correspondingly, group I presented markedly lower re-distribution and mobilization of iron and copper into the coronary flow, following prolonged ischemia, as evinced from the decrease in *total* levels, and in the “free” fraction (redox active levels) of either iron or copper.

During the PC phase no loss of cardiac function was observed. A small wave of re-distribution and mobilization of iron and copper (typically less than 4-8% of the value of 35 min ischemia) was recorded. The cellular content of ferritin measured in the heart was significantly higher in group I than in group III (0.90 and 0.54, respectively). Also, iron-saturation of ferritin was significantly lower for PC hearts, compared to both group II & III (0.22 *versus* 0.32 and 0.31 $\mu\text{g}/\mu\text{g}$, for 35 min ischemia, respectively). These findings are in accord with the proposal that intracellular re-distribution and mobilization of small levels of iron, during PC, cause a switch between cellular IRP-1 and aconitase, reversing the inhibitory control of translation of the ferritin message, and allowing a subsequent rapid accumulation of this iron-storage protein.

It is proposed that iron plays a dual role: (i) It serves as a signaling pathway for the accumulation of ferritin following the PC phase. This iron is not involved in cardiac injury, but rather prepares the heart against future high levels of “free” iron, and thus reduces the degree of myocardial damage after prolonged ischemia. (ii) High levels of iron (and copper) are mobilized following prolonged ischemia and cause

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tissue damage. It is tempting to speculate that by analogy to iron and ferritin, copper and its binding protein — metallothionein, are involved not only in reperfusion injury to the heart, but also in cardiac protection by PC.

Keywords: Heart, preconditioning, ischemia, iron, copper, ferritin, metal mobilization, reperfusion, isolated rat heart, free radicals

Abbreviations: IRP (iron responsive protein); ROS (reactive oxygen-derived active species); DP (developed pressure); EDP (end diastolic pressure); WI (cardiac work index); DHBA (dihydroxybenzoic acid); PC preconditioning; CFF (coronary flow fraction).

1. Introduction

Preconditioning (PC) is a process for protecting tissues against severe insult by exposing the system to minor and non-injurious stress. Heart ischemic PC is induced by a single or several repetitive *short* episodes of ischemia, separated by intermittent reperfusion. This procedure yields a heart more tolerant to subsequent prolonged ischemia. Cardiac PC has been shown to reduce infarct size, conserve high-energy phosphates, and induce improved recovery of hemodynamic function after subsequent prolonged ischemia [1,2]. It has recently been suggested that similar protection also occurs in humans during angioplasty and coronary bypass surgery [3].

The detailed mechanism of myocardial protection via PC is not fully understood yet. Many pathways have been proposed and include myocardial stunning, synthesis of heat-shock proteins, involvement of G-proteins, and nitric oxide production [3-5]. The generally accepted model is that the ischemic phase leads to enhanced catabolism of purine nucleotides, resulting in a high level of adenosine. These activate PKC and a cascade of signaling steps leading to activation of MAP, MAPK and MAPKK, culminating in a marked effect on ATP-dependent K^+ channels [3,4,6,7].

It is considered that tissue injury following ischemia and reperfusion is mediated by reactive oxygen derived-species (ROS) and pools of redox active iron and copper. The role of “free” iron and copper is visualized mainly as causing the conversion of low reactive species, such as superoxide radical anion, to the highly reactive hydroxyl radicals. The significance of iron in ischemia and reperfusion is supported by studies showing that metal chelation protected post-ischemic tissue [8], whereas the addition of iron to the perfusate increased the rate of injury [9].

We have previously shown [10,11] that following myocardial ischemia iron and copper are mobilized from the heart into the coronary flow, in an ischemia-duration-dependent manner, and that the level of the mobilized metals is well correlated with the degree of heart injury. Similar results were found by Cotin et al. [12] in patients with acute myocardial infarction treated by thrombolytic therapy.

While iron and copper ions are found in their “free state” only in minute concentrations within cells, their total levels are at the micromolar range. In general, a shift in the cellular balance between “free” and protein-bound iron (and copper) may be deleterious to cells due to their high reactivity in producing reactive free radicals. Much of the “free” iron is probably derived from an enhanced catabolism of heme-iron, non-heme-iron and iron-and-copper-containing respiratory proteins, which is a normal consequence of the break-down of the respiratory apparatus during times of famine and ischemia [13]. Under conditions prevailing in the tissue during ischemic insult, iron can also be mobilized from its major storage protein, i.e. heart ferritin [14-19].

Ferritin contains up to 4.000–4.500 iron atoms per protein molecule. During ischemia and reperfusion iron can be mobilized from ferritin by superoxide radical anion [15,20] and

other small molecules, including adrenergic agents (norepinephrine and epinephrine) [18]. The release of iron by epinephrine is significantly enhanced under anaerobic conditions that dominate the ischemic state. Iron release from ferritin can also be mediated by NADH-lipoamide dehydrogenase under aerobic conditions [21]. The destruction of the ferritin protein-shell seems to be necessary for iron release, *in vivo* [21-25].

A rapid translational response of ferritin has been reported to occur after ferritin mRNA was recruited to polysomes [14]. An increase in the cytosolic ferritin mRNA and ferritin protein following ischemia and reperfusion of the intestine was also reported [26]. Reperfusion has been shown to cause ferritin degradation, followed by activation of ferritin synthesis [27,28].

In the present paper we have analyzed the changes in iron and ferritin levels in ischemia and reperfusion of the isolated rat heart subjected to prolonged ischemia, with and without protection via PC.

2. Materials and methods

Sodium chloride was obtained from Frutarom Ltd., Israel; EDTA, salicylic acid, 2,3- and 2,5-DHBA, catechol, and ascorbic acid, ATP, ADP, AMP, GTP, GDP, adenosine, inosine-monophosphate, inosine, xanthine, hypoxanthine and uric acid were obtained from Sigma Chemical Company (St. Louis, MO); glucose, potassium chloride, magnesium sulfate, potassium dihydrogen phosphate and sodium hydrocarbonate were obtained from Merck Chemical Company (Darmstadt, Germany), and methanol hypersolv, HPLC-grade from BDH (Poole, England).

Sprague-Dawley male rats (250–350 g) were housed under standard conditions and fed a regular diet and water. The rats were injected with sodium heparin (500 units, i.p.) 30 min prior to anesthetization with sodium pentobarbital (60 mg/kg, i.p.). Hearts with part of the ascending aorta attached were rapidly excised, placed in ice-cold heparinized saline, and then perfused orthogradely in the Langendorff configuration [10,29]. The aorta was ligated with a plastic, rather than a metal, cannula, thereby minimizing both the contamination of the coronary flow with adventitious metal ions, and the background levels of iron and copper in the coronary flow fractions (CFF).

All buffers were freshly prepared on the day of the experiment, filtered prior to use, continually gassed with 95% O₂/5% CO₂, and the pH maintained at 7.4. All vessels were pre-washed with 0.1 M EDTA and Chelex-treated double-distilled water. Hearts were maintained at (37.0±0.1)°C throughout the experiment. The perfusate, modified Krebs-Henseleit (KH) buffer containing (as previously described [10] (in mM): NaCl (118), KCl (5.8), CaCl₂ (2.5), MgSO₄ (1.2), NaHCO₃ (25) and glucose (11.1) was circulated through the hearts using hydrostatic pressure achieved by a 85 cm H₂O column. The hemodynamic parameters of cardiac function were continually monitored as previously described [10].

2.1 Experimental protocol

Hearts were divided into 3 groups. Group I was the experimental group subjected to preconditioning (see details below) and prolonged ischemia (18–60 min) and reperfusion; Group II—hearts subjected to normal perfusion, without ischemia; and group III—control hearts subjected to prolonged ischemia of varying duration.

Control hearts (group III) were perfused for 25 min (stabilization period), subjected to global normothermic (37°C) ischemia for various period of time (18, 25, 35, 45, 52, 60 min) and then reperfusion for 20 min.

Experimental hearts of the PC group (group I) were equilibrated for 10 min and subsequently exposed to 2 min of global ischemia followed by 3 min reperfusion. This process was repeated 3 times. Preconditioned hearts were then subjected to global ischemia (for 18, 25, 35, 45, 52, 60 min) followed by 20 min of reperfusion.

In the normal non-ischemic group (group II), hearts were perfused for 80 min under normothermic conditions.

An additional control group was used (group IV). These hearts were subjected to stabilization and PC alone (without ischemia and reperfusion).

The left ventricular developed pressure, DP ($DP = PSP - EDP$), rates of contractility and relaxation ($+dP/dt$ and $-dP/dt$, respectively), and end diastolic pressure (EDP) were continuously monitored during the phases of the experiment (preischemia, ischemia and reperfusion).

Small fractions of coronary flow (CFF), each fraction containing only three drops (0.15 ml), were collected from the pulmonary artery prior to ischemia and during reperfusion. For control hearts, samples (3 drops each) were collected at the end of the stabilization period (preischemia), immediately after ischemia (10 consecutive samples), and at 2 min and 5 min intervals during reperfusion (4 samples). For preconditioned hearts, samples were collected in a similar manner, and also immediately after each 2 min ischemic episode (2x3 samples).

Tissue and CFF samples were kept at -80°C until analysis. Samples for HPLC analysis were prepared by extraction with perchloric acid under liquid nitrogen.

2.2 Determination of metal concentration

CFFs (0.15 ml each) were serially collected from the pulmonary artery prior to ischemia and during reperfusion. Fractions were collected in the reperfusion phase (10 first CFF samples and 5 CFF samples — after 2, 5, 10, 15 and 20 min of reperfusion, respectively). Iron and copper concentrations in the CFFs were measured according to standard procedures, using a Zeeman atomic absorption spectrometer (Varian, Spectron AA-300/400) [30]. Protein content was determined according to the Bradford method [31].

The CFF-mediated conversion of salicylate to 2,5- and 2,3-DHBA and catechol was used as a semi-quantitative assay to determine the levels of redox-active Fe/Cu, as follows: in 100 μl reaction mixture, containing 50 μl CFFs and salicylate and ascorbate (1 mM, each) in KH buffer was incubated for 1 h at 37°C . To terminate the incubation, ice-cold TCA (3% final concentration) was added, and the suspensions were centrifuged at 12,000g for 1 min. The supernatant was analyzed for 2,5- and 2,3-DHBA and catechol by HPLC coupled to an electrochemical detector (HPLC-ECD), as previously described [10].

2.3 Determination of ferritin levels

Preparation of rabbit-immune serum against rat heart ferritin. Ferritin was isolated from rat hearts as described previously [32]. The purity of the isolated protein was determined by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions. Antibodies to ferritin were raised in New Zealand rabbits (body weight 2.5 to 3.0 kg). Rabbits were injected with 1.5 to 2.0 mg of pure ferritin solubilized in complete Freund's adjuvant. After 30 days a booster containing 1.5 to 2.0 mg of ferritin in incomplete Freund's adjuvant was given, and the rabbits were bled 7 days later. The titer of the serum was tested by double immuno-diffusion in agar. The serum was then centrifuged at 10,000 g for 10 min and stored at -20°C .

ELISA for measurement of ferritin. Antibodies against rat heart ferritin were purified

from the immune serum by affinity chromatography on a column containing agarose-immobilized rat heart ferritin. The antibodies were coupled to galactosidase by the one-step glutaraldehyde method as described elsewhere [33]. This antibody-enzyme conjugate was used as a soluble phase to the immunoassay, whereas the whole rabbit immune serum was used as an immobilized phase. Optimal working concentrations for all reagents were determined by "checkerboard" titration. The assay was calibrated using pure rat heart ferritin.

Preparation of rat heart tissue extracts. Lysis buffer containing 1% deionized Triton X-100 and 0.1% sodium azide in 50 mM Tris-HCl pH 7.5 was incubated with Chelex-100, for a minimum of 24 h at room temperature prior to use. Phenyl-methyl-sulfonyl-fluoride (PMSF) (final concentration 0.25 mM) was added to the buffer immediately prior to use. Rat hearts were frozen in liquid nitrogen, and stored at -80°C until analyzed. Lysis buffer was added to the homogenized tissue, the mixture was vortexed, sonicated for 1 min and incubated on ice for 30 min, with vortexing every 5–10 min. Aliquots were taken, centrifuged at 3,000 rpm for 15 min, and the supernatant analyzed for total protein and ferritin. Total protein was determined in the extracts by the BCA method (Pierce).

Immunoprecipitation of ferritin from heart tissue extracts. The remaining suspension of homogenized heart tissue in the lysis buffer was incubated at 70°C for 10 min, cooled on ice and centrifuged at 10,000 g for 20 min. The supernatant was collected and the pellet discarded. Total amount of ferritin in each extract was calculated using the results of the ELISA. Saturating amounts of immune serum against rat heart ferritin were then added to the extracts. The mixtures were incubated at 4°C for 72 h, immunoprecipitates were washed twice in lysis buffer and stored "dry" at -80°C until analyzed for iron concentration by atomic absorbption.

Statistical analysis. All data are presented as mean $\pm$ SEM. Statistical analysis was performed using the Mann-Whitney and repeated ANOVA tests. Values corresponding to $p \leq 0.05$ were considered significant.

3. Results

3.1 Definition of the experimental groups

Rat hearts were harvested, prepared for perfusion in the Langendorff configuration as described in 'Methods' [10,11], and divided into four groups as follows:

Group No.	Stabilization (min)	Preconditioning (min)	Ischemia (min)	Reperfusion (min)	Comments
I	10	15 (3 episodes)	Variable Duration (18–60'; typically 35')	20	PC (+ischemia)
II	10+15+35+20	0	0	0	Normal perfusion
III	10+15	0	18 – 60', typically 35'	20	Ischemia no PC
III	10	15	----	----	PC alone

During the first two episodes of preconditioning (PC) no change in the hemodynamic parameters of the heart was observed. In some hearts, at the end of the third episode a drop of up to 7% (and an increase in EDP 0.4-0.7 mmHg) was observed. Nevertheless, complete recovery was recorded when the perfusion of these hearts was extended beyond 3 min. to ~10 min.

3.2 Hemodynamic parameters of hearts subjected to ischemia with and without PC

Table 1 depicts the values of several hemodynamic parameters at the end of 20 min reperfusion (as % of the pre-ischemic value), after ischemia of increasing duration (18–60 min). A time-dependent decrease in these parameters was observed for *both* group I and III (PC+ischemia and the ischemia without PC). Group III hearts, after 35 min ischemia, lost most (60–80%) of their function, while after 45 min the loss was complete.

PC hearts of group I demonstrated a marked and clear protection of their function against injury following prolonged ischemia (when compared to group III). The residual function after 35 min ischemia was 56–78%, and 27–49% after 45 min, as indicated by the various hemodynamic parameters.

Likewise, biochemical parameters of heart tissue and coronary flow (ATP, ADP, AMP, catabolites of adenine nucleotides, ascorbate and urate), following 18, 35 and 60 min ischemia, clearly indicated an analogous myocardial protection (data not shown).

Table 1. The hemodynamic parameters of hearts following ischemia and reperfusion with or without cardiac preconditioning (PC). The data represent the recovery values (% of the pre-ischemic value).

* = $p \leq 0.05$ — for the PC group as compared to the control (ischemia/reperfusion) group.

Ischemic duration	Group no.	WI (% recovery)	DP (% recovery)	EDP (mm Hg)	+dP/dt (% recovery)	-dP/dt (% recovery)
18 min	I PC+Ischemia (35')	99 ± 6*	103 ± 5*	0.0 ± 2*	96 ± 6*	95 ± 7
	III Ischemia 35' no PC	76 ± 6	76 ± 6	19 ± 4	76 ± 6	82 ± 5
25 min	I PC+Ischemia (35')	78 ± 11*	79 ± 8*	31 ± 7*	79 ± 17*	85 ± 15*
	III Ischemia 35' no PC	44 ± 5	47 ± 4	56 ± 9	42 ± 5	49 ± 6
35 min	I PC+Ischemia (35')	56 ± 9*	74 ± 4*	42 ± 5*	68 ± 4*	78 ± 9*
	III Ischemia 35' no PC	19 ± 7	21 ± 7	67 ± 5	29 ± 6	37 ± 6
45 min	I PC+Ischemia (35')	27 ± 7*	48 ± 4*	68 ± 7	39 ± 3*	49 ± 6*
	III Ischemia 35' no PC	0 ± 0	0 ± 0	75 ± 5	0 ± 0	0 ± 0
52 min	I PC+Ischemia (35')	5 ± 2*	25 ± 6*	85 ± 4	16 ± 4*	22 ± 6*
	III Ischemia 35' no PC	0 ± 0	0 ± 0	90 ± 2	0 ± 0	0 ± 0
60 min	I PC+Ischemia (35')	4 ± 1*	21 ± 3*	86 ± 5	12 ± 2*	14 ± 2*
	III Ischemia 35' no PC	0 ± 0	0 ± 0	93 ± 5	0 ± 0	0 ± 0

*: $p < 0.05$ for group I versus group III.

DP: left ventricular developed pressure (PSP-EDP).

EDP: the end diastolic pressure.

dP/dt: rate of heart contraction (+) and relaxation (-).

3.3 Analysis of metal content in coronary flow fractions

Coronary flow fractions (CFF) were collected and analyzed using previously published procedures (see 'Methods' and refs. [10,11]). The first CFFs of reperfusion contained relatively high levels of iron and copper (Table 2). The values were dependent on the duration of ischemia, while the concentrations of group I CFFs were systematically and markedly lower (by a factor of 2–5) than the corresponding values of group III. The mobilization patterns of these metals from the tissue to the CFF were similar to each other, but distinctly different for iron, copper, and protein, indicating that this mobilization does not reflect a simple necrotic process, but rather a selective one [11].

3.4 Determination of redox-active fraction of iron and copper in coronary flow fractions

The total levels of iron and copper, in each CFF, were determined by atomic absorption spectroscopy. In contrast to the total levels of iron and copper, the redox-active fractions ("free" metal fractions), that could potentially play a causative role in the injurious process

to the heart, were monitored using a biochemical assay. In this the ability of added CFF to catalyze the ascorbate-driven conversion of salicylate to its dihydroxybenzoate derivatives (DHBA) was measured. Experimentally, HPLC-ECD method [34,35] was employed [10]. Table 3 shows the production of 2,5- and 2,3-DHBA that was mediated by the first CFF, for increasing ischemic duration, with and without PC. Cathechol is an additional minor product of this reaction, and is probably produced by the decarboxylation of 2,3-DHBA. Thus, the combination of these products is also presented in Table 3. These parameters, too, were found to be dependent on the duration of ischemia; they are lower (typically by a factor of 2-3) for CFF from group I hearts than from group III.

Table 2. The levels of iron, copper and proteins in coronary flow fractions (CFF) of hearts following ischemia and reperfusion with or without PC.

* = $p \leq 0.05$ — for the PC group compared to the control group (ischemia and reperfusion without PC).

	CFF No.	18 min Ischemia		25 min Ischemia		35 min Ischemia		45 min Ischemia	
		Group I	Group III	Group I	Group III	Group I	Group III	Group I	Group III
Cu (ng/ml)	1	2.3±1.6	4.5±1.7	3.0±1.3*	9.5±1.6	7.9±1.1*	11.4±0.7	8.2±0.9*	14.6±1.1
	2	0.6±0.4	0.8±0.4	1.5±0.3	4.1±1.5	4.0±0.4	2.7±0.4	1.5±0.8	3.8±2.1
	5	0.0±0.0	0.5±0.3	0.0±0.0	1.5±0.5	0.4±0.2	1.6±0.8	0.5±0.5	1.5±0.9
Fe (ng/ml)	1	14±3	31±11	15±3*	63±2	25±6*	97±9	28±4*	104±28
	2	8.3±1.0	12.1±4.5	8.7±2.0*	20.7±4.2	9.1±0.5*	31.0±8.9	11.1±1.5*	30.4±6.2
	5	4.2±1.1	4.7±1.9	0.9±0.5*	8.3±1.7	0.6±0.4*	12.1±2.3	1.1±1.1*	7.8±2.4
Protein (µg/ml)	1	26±8	42±1	30±3*	54±2	32±5*	71±6	36±7*	83±11
	2	12±1	28±22	15±5*	38±5	17±4*	30±0.2	17±4*	34±4
	5	2.5±1.3	13.1±10.6	1.9±1.9*	12.4±1.3	4.6±1.1*	14.3±0.3	3.1±1.0*	11.0±1.8

* denotes $p < 0.05$ of group I versus group III

Table 3. Levels of dihydroxybenzoic acids (DHBA) in CFF of hearts following ischemia and reperfusion with or without PC.

* = $p < 0.05$ — for the PC group compared to the control group (ischemia and reperfusion).

	Time	18 min		35 min		60 min	
	CFF No.	PC	Control	PC	Control	PC	Control
2,5-DHBA (ng/ml)	1	9.9±5.3	17.0±8.0	15.0±5.4	42.4±11.0*	31.2±2.1	49.5±8.7
	2	11.2±6.5	9.2±3.9	8.3±2.6	9.3±6.2	13.4±1.4	15.7±9.1
2,3-DHBA (ng/ml)	1	3.9±3.9	8.6±4.4	6.9±3.3	33.5±7.1*	27.7±2.5	41.4±9.1
	2	0.6±0.6	8.5±3.7*	6.7±2.7	10.9±1.9	15.1±1.7	26.3±9.8
2,3-DHBA + Cathechol (ng/ml)	1	5.0±4.8	11.3±5.7	8.0±3.9	34.6±7.8*	27.7±2.7	47.1±10.0
	2	0.6±1.1	11.0±4.9*	7.6±4.1	13.1±3.5	16.6±2.6	27.7±10.6
SUM** (ng/ml)	1	14.9±10.1	28.3±13.7	23.0±9.4	77.0±18.8*	58.9±4.8	96.6±18.7
	2	11.8±7.6	20.2±8.8	15.9±5.7	22.4±9.7	30.0±4.0	43.4±19.7

*: $p < 0.05$ for this group versus the corresponding control group.

**: denotes the sum of 2,3-DHBA and 2,5-DHBA and Cathechol

The earlier findings, that increasing ischemic duration resulted in a parallel increase in the total and the “free” fraction of iron [10,11] were reconfirmed here. The marked suppression of iron levels in the CFF by the PC process showed that both ischemia and PC had affected, in opposite directions, the level of “free” iron in the system. Prolonged ischemia followed by reperfusion triggered a prominent increase in the level of “free” iron, whereas PC seems to have grossly inhibited this effect. The pool of “free” cytosolic iron, in mammalian cells, is controlled predominantly by ferritin. This multimeric protein incorporates and stores excessive “free” iron, functioning as an iron scavenger [36-38].

Ferritin can also release its iron causing an increase of the cellular “free” iron pool [23,24,39]. These findings indicate that ferritin may be responsible for modulations of the amounts of “free” iron in the heart, and thus, its susceptibility to injury. The ferritin contents in these hearts have therefore become of interest.

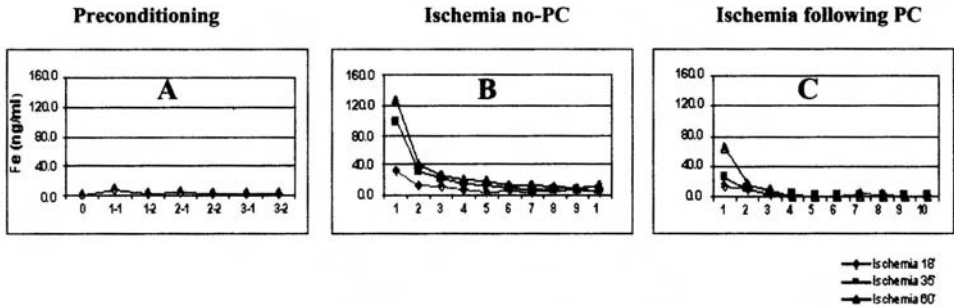


Figure 1. Levels of mobilized iron from the heart tissue to the first coronary flow fraction, following prolonged ischemia of 18, 35 and 60 min duration, under the following conditions:

Panel A: During the PC phase; Panel B: Following prolonged ischemia without PC; Panel C: Following PC and prolonged ischemia.

3.5 Analysis of Fe/Cu mobilization from the heart during the preconditioning phase

Coronary flow fractions were collected during the short reperfusion between each of the short ischemic episodes of the PC phase. The mobilization levels of iron and copper from the heart tissue were determined, and were found to be small but significant, when expressed either as concentrations or as the integrated amount in the entire volume of the short reperfusion. These amounts were fixed (for all subsequent ischemic duration).

Figure 1 shows the concentrations of iron and copper that were mobilized from the heart under different sets of experimental conditions: (i) during the ischemic-PC phase alone; (ii) following prolonged ischemia of 35 min (no PC, group III); and (iii) after PC and 35 min ischemia (group I). It is clear that the amounts mobilized following prolonged ischemia (without PC) are markedly higher (> 10-fold) than the small levels associated with the PC phase alone. While the amounts mobilized in the PC phase are not associated with heart injury, the high levels of group III hearts correlate well with the observed functional damage. The amounts of group I, following PC and ischemia of 35 min, are intermediate (2–4 fold lower than those of group III), and correlated well with an analogous attenuation of tissue damage.

3.6 Determination of ferritin contents in heart tissue and coronary flow

Ferritin content was determined in heart tissue extracts and in coronary flow, for groups I, II and III, by ELISA, using polyclonal antibodies against rat heart ferritin (Table 4). Normally perfused hearts had 0.64 $\mu\text{g}/\text{mg}$ protein. Following 35 min ischemia this amount was reduced (non-significantly) to 0.54 $\mu\text{g}/\text{mg}$ protein. Subjecting hearts to PC prior to the prolonged ischemia (35 min) yielded substantially *higher* levels of ferritin in the cardiac tissue (0.90 $\mu\text{g}/\text{mg}$ protein). The difference between ferritin contents in hearts from group I and III was statistically significant ($p < 0.05$).

Table 4. Ferritin levels in hearts and in coronary flow following prolonged ischemia of 35 min, with or without PC.* = $p < 0.05$.

	Ferritin/Protein $\mu\text{g}/\text{mg}$	Fe in Ferritin $\mu\text{g}/\mu\text{g}$	Total ferritin in Coronary flow (μg)
Group I: PC+Ischemia	$0.90 \pm 0.13^{+,*}$	$0.22 \pm 0.04^{\circ}$	$0.73 \pm 0.11^{*,\ddagger}$
Group II: Normal perfusion (80')	0.64 ± 0.10	0.32 ± 0.04	$0.19 \pm 0.05^{\ddagger}$
Group III: Ischemia without PC (80')	0.54 ± 0.07	0.31 ± 0.04	$1.30 \pm 0.14^*$
Group IV: Only PC (25')	0.44 ± 0.03	0.30 ± 0.01	---

* denotes $p < 0.05$ for this group *versus* group II.+ denotes $p=0.07$ for this group *versus* group II. $\ddagger$ denotes $p < 0.05$ for this group *versus* group III.° denotes $p < 0.05$ for this group *versus* group IV.° denotes $p < 0.06$ for this group *versus* group II.

This result was found to be in accord with the data concerning the degree of iron-saturation of ferritin. In group I, heart ferritin-saturation was $0.22 \mu\text{g Fe}/\mu\text{g Ft}$, a value lower than for groups II & III, which were 0.31 - $0.32 \mu\text{g Fe}/\mu\text{g Ft}$. These data indicate that an increase in the content of heart ferritin had occurred *only* in the PC group (group I), and not in the normally perfused hearts (group II), nor in those that were subjected to prolonged ischemia without prior PC (group III). Furthermore, the high levels of iron mobilization from the cardiac tissue to the coronary flow following prolonged ischemia coincided with the observed decrease in tissue ferritin content in group III. Also, the suppressed mobilization of "free" iron in group I, when compared to group III, was associated with the observed increase in tissue ferritin content.

The mobilization of iron from the heart to the coronary flow is in accord with excessive degradation of ferritin during prolonged ischemia, causing the release of previously ferritin-bound iron. The suppressed levels of iron mobilization, in group I hearts, are in accord with both weaker degradation *and* enhanced synthesis of ferritin.

4. Discussion

This communication has reconfirmed the marked protection against myocardial reperfusion injury afforded by ischemic PC. The novel findings in this communication are that following prolonged ischemia, PC causes a marked decrease of the levels of cellular re-distribution and extra-cellular mobilization of iron and copper. Furthermore, PC is associated with an accumulation of intracellular ferritin, and a concomitant decrease in ferritin-iron saturation. It is postulated that the low, but significant and reproducible, mobilization levels of intracellular iron, following each cycle of PC, have led to the conversion of the iron-responsive proteins, notably IRP-1, to cytosolic aconitase, and the consequent relief of the tight inhibitory control of ferritin synthesis.

We have previously demonstrated that the extent of intracellular re-distribution and mobilization of iron, copper and proteins into the coronary flow depends on ischemic duration, and could serve as predictive criteria for the degree of heart injury [10,11]. The observed increase in iron and copper levels in the CFF ("free" and "bound" states) resulted in the increase in free radicals production, which in turn could explain the post-ischemic heart damage.

It was shown that the hemodynamic performance of the heart, following prolonged ischemia, is strongly dependent on whether the heart was subjected to PC. Hearts protected by PC demonstrated a markedly better performance than those without PC. Analogous

differences in the re-distribution and mobilization of (total) iron and copper, between these groups, are seen in Figure 1 and Table 1. Table 4 demonstrates that the redox-active fractions of iron and copper, which are responsible for the conversion of salicylate to its DHBA derivatives, were smaller for the PC group, when compared to the control (ischemia and reperfusion without PC).

While PC was found to protect hearts for the various ischemic times examined, we chose 35 min ischemia as a typical duration, which was associated with severe, but reparable, damage; 70–80% loss of function that could be improved by PC, to only 30–40% loss of function.

The ability of ferritin to incorporate *and* release iron renders this protein a major active regulator and passive responder to cellular “free” iron pool, playing dual roles in oxidative stress. On one hand, scavenging of catalytic iron from the cytosol inhibiting the formation of reactive oxygen species (ROS), thus producing a protective effect [40,41]. On the other hand, releasing iron and increasing its availability for Fenton chemistry, thus producing a pro-oxidant effect [23,24,36,37]. Therefore, it is expected that the balance between its protection and pro-oxidant activity will determine the net effect of ferritin. An increase in ferritin synthesis led to a concomitant increase in iron incorporation [37,38,42–44], while degradation of ferritin allowed the release of iron [22–25,45]. The changes in ferritin levels reflected the balance between synthesis and degradation, and indicated the resultant direction of cellular-iron distribution.

Ferritin synthesis is primarily regulated at the post-transcriptional level [42–44], through a rapid translational response to influx of “free” iron [14], allowing the detection of changes in ferritin content shortly after stimulation. The changes in tissue ferritin content may therefore reflect the participation of this protein in the modulations of free iron in cardiac tissue following ischemia and reperfusion with or without PC.

It is proposed that during the PC phase small, but significant, levels of intracellular iron undergo re-distribution and mobilization. This, in turn, produces the necessary signal for enhanced translation of ferritin mRNA, increasing its level and its capacity to scavenge and store iron.

The mechanism of ferritin synthesis regulation has gained broad understanding in recent years. The efficient inhibition of translation results from the binding of iron-responsive elements (IREs) to the ferritin iron-responsive proteins (IRP). Following an initial signal of available iron the (IRP-1)-(IRE) complex is broken, yielding a translationally active ferritin message and cytoplasmic aconitase. Usually, this “free” iron signal is created through the influx of iron into the cell. In our system, this signal is being formed intracellularly during the PC phase, due to the short ischemia and reperfusion events. It is important to mention that this is a small iron signal, and the amount of iron is not sufficient to catalyze deleterious processes.

The results showed that in the CFF ferritin, levels (protein content) correlated with the levels of iron mobilization. Also, following PC, when the level of mobilization to the CFF was small, a marked increase in the tissue ferritin content was observed. No change in ferritin level was demonstrated without ischemia, when no mobilization of iron took place. Analogously, no change was noticed following prolonged ischemia (without PC), when a relatively high level of iron mobilization was recorded.

Enhanced protein catabolism by virtue of intracellular proteolysis occurs in an ischemic organ [46–49]. Ferritin degradation during prolonged ischemia, together with acidosis and reducing environment of the ischemic cell lead to the release of iron [12]. Each iron-loaded ferritin molecule (containing ~1500–2500 Fe atoms), releases many redox active iron ions that catalyze the formation of free radicals, during the reperfusion phase, and yields tissue injury. The prominent protective effect following PC is explained by *de novo* synthesis of ferritin in the preconditioned hearts, culminating in the preparation of the

heart to scavenge future release of excessive “free” iron [40,50-54].

Increasing amounts of free iron and over-production of ROS have been reported to stimulate translation of ferritin sub-units [41,43,55-58]. Other reports have shown that hypoxia suppresses the translation of the ferritin message [59]. In summary, ischemia and reperfusion activate ferritin degradation as well as its synthesis. The net changes in ferritin levels stem from the interactions among these factors, and possibly additional ones.

It seems that during and following prolonged ischemia ferritin degradation prevails over ferritin synthesis, leading to the release of iron, and thus increasing the susceptibility of cardiac tissue to oxidative damage. This is supported by the observed decrease in ferritin content (Table 4). In contradistinction, ischemic PC produces small, non-toxic, but stimulating amounts of “free” iron, which enhance ferritin production, and do not promote its degradation. The short duration of the ischemic PC periods prevents activation of cellular proteolysis. The increased amounts of intracellular ferritin sequester excessive catalytic iron and prevent oxidative damage. The increase in ferritin content provoked by PC serves as a mechanism of cardiac protection.

The typical function of IRP-1 is to inhibit the synthesis of apo-ferritin, while maintaining an adequate level of inhibitory control of the mRNA translation. Upon a small influx of iron the inhibition is relieved, ferritin synthesis is activated, protecting the cell against iron-mediated injury. Ischemic PC leads to re-distribution of small amounts of iron *within* the cell and its mobilization out of the cell. The newly released intracellular iron serves as the trigger for the relief of the inhibitory control of translation of the ferritin message through the conversion of IRP-1 to the cytosolic aconitase. The result is the production of a marked reservoir of apo-ferritin, which is ready to scavenge “free” iron, to be released only following the prolonged ischemia.

Indeed, inhibitors of protein synthesis have been used in the *in vivo* rabbit [60] and in the rat Langendorff models [61]. Cycloheximide or actinomycin D did not reverse the PC effect [60]. This is probably due to non-complete inhibition of protein synthesis. The latter study [61] showed that the PC effect was abolished only with cycloheximide, but not with actinomycin D, indicating that PC protection is regulated at the post-transcriptional level. Recent findings [62] have reconfirmed the translational control of protein synthesis in ischemic (but not pharmacological) PC. These are in accord with our findings concerning *de novo* synthesis of ferritin.

These results together with published data [63,64] render the suggestion that copper and its storage protein — metallothionein — play similar roles in PC tempting.

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Multiple Mechanisms Regulating Endothelial Nitric Oxide Synthase

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1. Regulation of NOS

Nitroglycerin has been used since the early 1870's as a vasodilator in the treatment of angina pectoris, however the cellular mechanisms involved in its actions were not elucidated until early in 1970. Murad et al. (1979) reported that nitroglycerin and other nitro-containing compounds stimulated guanylyl cyclase leading to an increase in cGMP accumulation in smooth muscle cells [1]. Later experiments by Ignarro et al. (1980) in various tissues, showed that the addition of nitroglycerin in combination with thiols resulted in the generation of unstable S-nitrothiols which degrade to form nitric oxide leading to vasorelaxation [2]. Experiments performed by Furchgott and Zawadzki in 1980 using artery rings illustrated the dependence of acetylcholine-induced relaxation on an intact endothelium [3]. Acetylcholine acts on muscarinic receptors on endothelial cells leading to an increase in an endothelium-derived relaxing factor (EDRF), which in turn diffuses to the underlying smooth muscle cells, activates soluble guanylyl cyclase, increases cGMP and mediates relaxation *via* activation of protein kinase G (PKG), ion channels and phosphodiesterases (PDE) resulting in vasorelaxation. EDRF was subsequently identified as nitric oxide (NO) by Palmer et al. (1987) on the basis that NO and EDRF possess similar features such as vasodilatory actions, inhibition of platelet aggregation, and exhibits similar half lives [4].

The free radical gas NO is produced by the enzymatic actions of nitric oxide synthases (NOS) on the cationic amino acid, L-arginine (Figure 1) [5]. Three isoforms of NOS have been identified: nNOS (neuronal), eNOS (endothelial) and iNOS (inducible) [6]. The genes encoding the 3 NOS isoforms have a similar genomic structure. All three NOS isoforms possess an oxygenase domain, which contains binding sites for haem, tetrahydrobiopterin (BH₄) and L-arginine. This oxygenase domain is linked to a C-terminal reductase domain (containing FAD, FMN and NADPH recognition sites) by a binding site for calcium-calmodulin. nNOS is a constitutively expressed calcium-calmodulin dependent NOS isoform originally identified in neuronal tissue. eNOS is also a calcium-calmodulin dependent enzyme constitutively expressed in vascular endothelial cells. The inducible iNOS, is calcium-independent and can be activated by endotoxins and cytokines. As NO plays a pivotal role in the regulation of the vasculature and in the maintenance of vascular tone, understanding the regulation of this signalling pathway is of importance.

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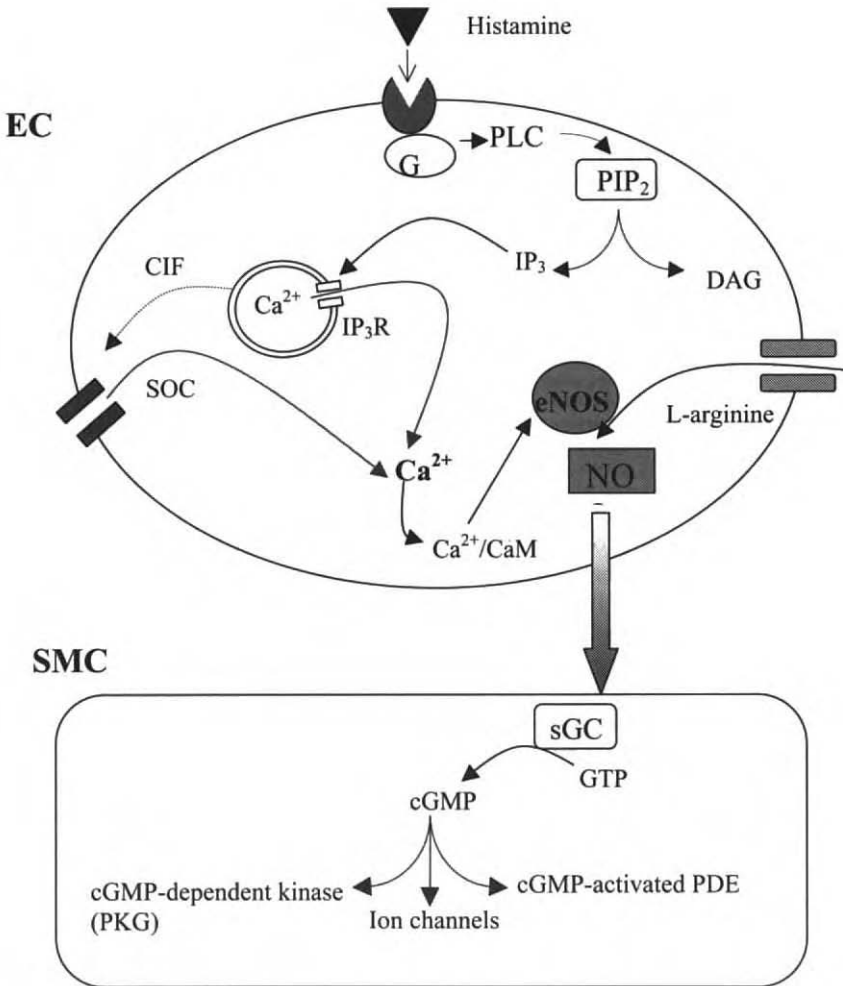


Figure 1. Calcium-dependent regulation of eNOS. Agonists such as bradykinin and histamine activate phospholipase C (PLC) in endothelial cells (EC) resulting in the generation of phosphatidylinositol 4,5 biphosphate (PIP₂) and subsequently inositol 1,4,5 triphosphate (IP₃) and diacylglycerol (DAG). IP₃ then mediates Ca²⁺ release from intracellular stores, the empty stores then release a 'Ca²⁺ influx factor' (CIF) and the increase in intracellular Ca²⁺ activates calmodulin (CaM). Ca²⁺/CaM then binds to eNOS thus activating it and L-arginine is metabolized to nitric oxide (NO) and L-citrulline. Hecker et al. (1990) have also reported that L-citrulline can be recycled to L-arginine via a transamination reaction [8]. Recycling of L-citrulline into L-arginine is dependent on availability of exogenous L-citrulline and is sensitive to inhibition by L-glutamine (K_i ~50–100 μM), with L-glutamine (L-Gln) reported to decrease L-arginine synthesis from L-citrulline by inhibiting membrane transport of L-citrulline [9–11], and L-glutamine and its metabolite glucosamine reducing intracellular NADPH levels, leading to inhibition of NO synthesis. Diffusion of NO to the underlying smooth muscle cells (SMC) activates soluble guanylyl cyclase (sGC), increasing cGMP levels which in turn modulate protein kinase G (PKG), ion channels, cGMP-activated phosphodiesterases (PDE) and vascular tone.

1.1 Calcium-dependent regulation of NO production by eNOS

1.1.1 'Classical' NO regulation

eNOS is classically activated in a calcium-calmodulin dependent manner by agonists such as histamine or bradykinin (Figure 1). Calcium is an important second messenger in cell signalling, however, at high sustained concentrations it becomes cytotoxic hence the intracellular calcium elevations are typically transient. Basal intracellular calcium is maintained at concentrations ranging between 50–100 nM *via* regulation of efflux (*via* the plasma membrane Ca^{2+} ATPase pump (PMCA)) and entry into the cell *via* store- and receptor-operated channels (SOC and ROC), respectively and release of calcium from intracellular stores (endoplasmic reticulum) [7]. Release of Ca^{2+} from intracellular stores occurs *via* ryanodine- or inositol 1,4,5 triphosphate (IP_3)- sensitive pathways. These pathways can be activated by calcium, IP_3 , ryanodine or ADP ribose.

In endothelial cells, agonists such as bradykinin and histamine activate phospholipase C (PLC) which in turn hydrolyses phosphatidylinositol 4,5 biphosphate (PIP_2) leading to subsequent production of IP_3 and diacylglycerol (DAG) (Figure 1). The newly formed IP_3 then acts on IP_3 -receptors located on intracellular stores leading to release of calcium. Consequently the emptying of intracellular stores causes the activation of store operated calcium channels (SOC) in the plasma membrane resulting in increased calcium influx. The factor(s) responsible for activation of SOC ('calcium influx factor', CIF) is unknown, however several potential mechanisms have been postulated [12]. The overall increase in cytosolic calcium in response to histamine or bradykinin activates calmodulin. The active calcium-calmodulin binds to eNOS and increases the rate of electron transfer from NADPH to the reductase flavins and transfer from the reductase domain to the haem centre [6] resulting in increase enzyme activity and an increase in NO production. eNOS, however, associates with several intracellular proteins affecting subcellular localisation and activity of the enzyme. One such protein is cavolin-1, the structural coat protein of caveolae (Figure 2).

1.1.2 Caveolae

Caveolae were originally identified by electron microscopy as small 'flask-shaped' invaginations of the plasma membrane in epithelial cells [13]. However, caveolae are not necessarily flask-shaped, they vary in morphology from single vesicles or tubules to 'grape-like' structures. They are lipid enriched and make up between 5-10% of plasma membrane proteins [13] in cells such as smooth muscle cells, endothelial cells, fibroblasts and adipocytes [14]. Caveolae are mainly composed of cholesterol and sphingolipids (sphingomyelins and glycosphingolipids). Caveolae appear to be enriched with many lipid modified signalling molecules such as eNOS, Src-kinases and Ras as well as G-proteins indicating their importance in cell signalling mechanisms within the cell.

The major protein located within the cytoplasmic membrane coat of caveolae is caveolin of which there exist three types, 1, 2 and 3 [15]. Caveolin is between 21-25 kDa in weight with three domains: the N-terminal domain, a membrane spanning domain and a C-terminal domain with both the N- and the C-terminals facing the cytosol [14]. Apart from performing a structural role in caveolae, caveolin-1 also appears to be important in regulating the cholesterol content of caveolae [16] as cholesterol-binding drugs cause invaginated caveolae to flatten and reduces the number of caveolae invaginations [16].

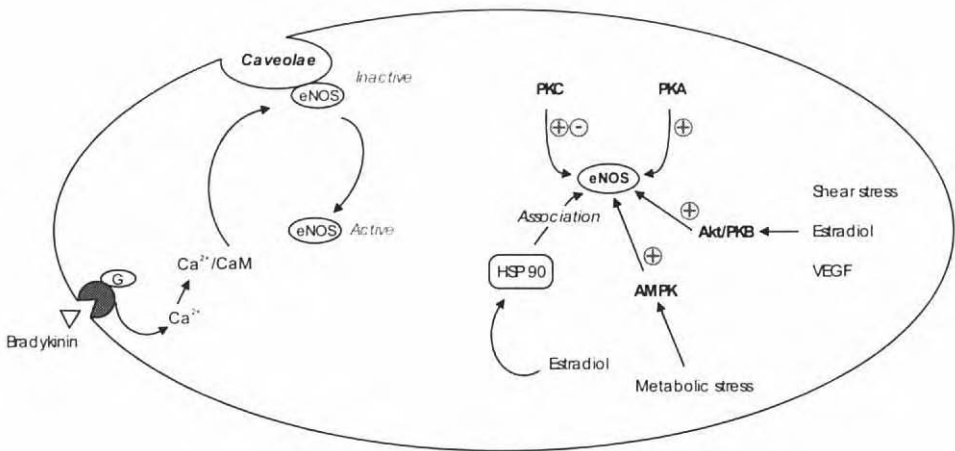


Figure 2. Regulation of eNOS. eNOS can be regulated in a number of ways one of which concerns its association with caveolin-1, the coat protein of caveolae, resulting in inhibition of NOS activity. This inhibition of eNOS is reversed by binding of calcium/calmodulin ($\text{Ca}^{2+}/\text{CaM}$) to the enzyme removing this inhibitory interaction with caveolin-1. Also eNOS can be phosphorylated on serine residues by a number of kinases such as protein kinase C (PKC), protein kinase A (PKA), protein kinase B (Akt/PKB) or AMP activated protein kinase (AMPK) resulting in activation (+) or inhibition (-) of eNOS activity. eNOS can also associate with heat shock protein 90 (Hsp90) leading to activation of the enzyme.

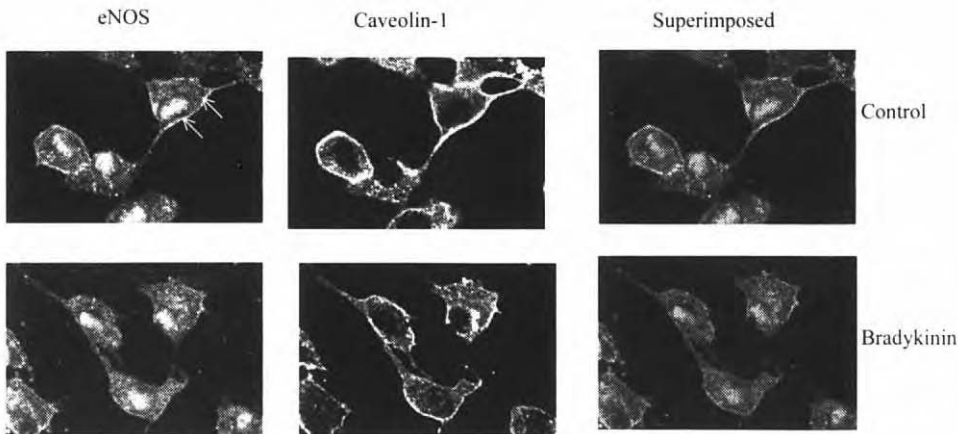


Figure 3. Co-localisation of eNOS and caveolin-1 in human umbilical vein endothelial cells. Endothelial cells were stimulated with bradykinin ($1\mu\text{M}$, 5 min), cells permeabilised and antibodies against eNOS and caveolin-1 applied. Appropriate fluorescent secondary antibodies were used and images viewed using conventional confocal microscopy. eNOS staining in control cells was predominately at the cell periphery (see arrows), whereas in bradykinin treated cells less eNOS was seen at the membrane. When eNOS and caveolin-1 images are superimposed, it is apparent that in control cells eNOS and caveolin-1 are co-localised. The co-localised staining is less apparent in cells treated with bradykinin implicating that eNOS is no longer inhibited and an increase in NO occurs. Unpublished data from Wyatt, Pedley & Mann confirming findings by Prabhakar *et al.*, (1998) in BAEC [19].

Caveolae and eNOS

Garcia-Cardena reported in 1996 that the tyrosine phosphorylation of eNOS in bovine aortic endothelial cells (BAEC) resulted in a decrease in enzyme activity whilst immunoprecipitation of caveolin-1, the predominant isoform in endothelial cells, resulted in co-immunoprecipitation of tyrosine phosphorylated eNOS [17]. Michel et al. (1997) further reported that eNOS, also in BAEC, was co-immunoprecipitated by caveolin-1 antibodies whilst in rat myocytes eNOS was associated with caveolin-3, the cardiac muscle specific type, confirming that eNOS interaction with caveolin is not tissue specific [18]. Immunofluorescence studies in our group have shown that eNOS associates with caveolin-1 in human umbilical vein endothelial cells (HUVEC), and this interaction is abolished by bradykinin (Wyatt, Pedley & Mann, 2001 unpublished data) (Figure 3).

The co-localisation of eNOS with caveolin-3 in rat cardiac myocytes at the boundaries of the cells may aid receptor stimulated eNOS activation and/or signalling. Many other signalling components required for eNOS activity, are also localised in caveolae such as IP₃ channels, Ca²⁺-ATPase and L-arginine transporters and hence targeting of eNOS to these structures may facilitate calcium-calmodulin activation of eNOS. Michel et al. (1997) demonstrated in BAEC that association of eNOS and caveolin is inhibitory on enzyme activity [18]. Caveolin-1 interacts with the eNOS protein within amino acid residue 350-358 resulting in an inhibition of electron transfer between the reductase and oxygenase subunits effectively rendering eNOS inactive [20]. This interaction can be antagonised by calcium-calmodulin, thus there is a reciprocal regulation of eNOS by calcium-calmodulin and caveolin-1. We have recently shown that caveolin-1 and eNOS association can be disrupted by agonists which do not elevate intracellular Ca²⁺ such as adenosine (unpublished data (not shown) Wyatt, Pedley & Mann, 2001).

Recently caveolin-1 deficient mice have been generated [21]. The homozygous mice were viable and fertile exhibiting no phenotypic abnormalities. However when the physiological response of aortic rings to phenylephrine was examined the caveolin deficient mice exhibited a significantly greater relaxation compared to wild type mice which was restored after application of L-NAME to the deficient mice. This report is consistent with reports by Drab et al. (2001) who also documented that caveolin-1 deficient mice demonstrated significantly greater in response to phenylephrine compared to normal mice [22]. These *in vivo* data supports the *in vitro* observations that caveolin-1 acts as a tonic inhibitor of eNOS.

1.1.3 HSP90

Aside from negative regulatory association with proteins a positive regulatory association of heat shock protein-90 (HSP90) with eNOS has been widely reported in the literature. HSP90 is a highly conserved stress protein present mainly in the cytosol of cells where it plays an important role as a chaperone ensuring appropriate folding of proteins [23]. HSP90 is not only involved in a multicomponent chaperone system but also forms an integral part of signal transduction pathways within cells.

HSP90 and eNOS

It has been reported in bovine aortic endothelial cells and human umbilical vein endothelial cells that stimuli such as bradykinin, VEGF and shear stress promote association of HSP90 with eNOS resulting in enhanced NO production [24] (see Figure 2). In this study, co-immunoprecipitation of HSP90 with eNOS was enhanced by agonist stimulation and both co-immunoprecipitation and increased NO production was abolished in the presence of geldanamycin (an antibiotic that inhibits HSP90). The mechanism by which HSP90 binds

to eNOS and leads to activation is unresolved. However, studies by Garcia-Cardena et al. (1998) have reported that HSP90 directly activates eNOS by acting as an allosteric modulator of eNOS resulting in conformational change in the enzyme [24]. Gratton et al. (2000) have implicated a reciprocal regulation of eNOS by both caveolin-1 and HSP90 [25]. In bovine lung microvascular endothelial cells these authors showed that eNOS and HSP90 are present in the caveolin-1 complex. Addition of calcium-calmodulin only slightly reduces the binding of eNOS to caveolin-1, however, in the presence of HSP90, addition of calcium-calmodulin significantly disrupts the association of eNOS with caveolin-1 [25]. Thus, the activation of eNOS by dissociation from caveolin-1 is facilitated by the presence of HSP90 [25].

1.1.4 Phosphorylation of eNOS

The regulation of eNOS activity by phosphorylation is a well documented subject as the eNOS protein possess several consensus sequences for phosphorylation by protein kinase A (PKA), protein kinase C (PKC), protein kinase B (Akt/PKB) and AMP-activated protein kinase (AMPK). The eNOS enzyme has been reported to be phosphorylated on threonine, serine and tyrosine residues in response to various agonists. Many studies have shown that phosphorylation of eNOS on serine 1177 leads to an activation of eNOS [26-29], whereas phosphorylation on threonine 495 inactivates eNOS as this site is in the Ca^{2+} /calmodulin binding domain [26]. Fleming et al. (2001) demonstrated that bradykinin (100 nM) increased eNOS activity in both porcine coronary artery endothelial cells (PCEC) and HUVEC *via* dephosphorylation of threonine 495 and phosphorylation of serine 1177 [30]. The bradykinin-induced phosphorylation of serine 1177 was abolished in the presence of a calmodulin dependent kinase II inhibitor whilst the dephosphorylation of threonine 495 was abolished by a protein phosphatase I inhibitor [30]. Harris et al. (2001) documented in BAEC that bradykinin (1 μM)-induced eNOS activity was mediated by activation of Akt/PKB [31] (see section 1.2.2) resulting in NOS phosphorylation at serine 1179 (bovine sequence) and a de-phosphorylation at threonine 497 mediated by calcineurin phosphatase. Typically, phosphorylation of eNOS at either of these sites is coordinated with dephosphorylation at the alternate site.

PKC and PKA

Protein kinase C (PKC) has been implicated in the regulation of NO production in endothelial cells. Castro et al. (1998) reported that in porcine aortic endothelial cells ATP induced increase in NO production was mediated *via* activation of PKC [32]. There are, however, reports of PKC mediated inhibition of eNOS *via* phosphorylation in BAEC treated with a phorbol ester [33] as it has been demonstrated that PKC activation inhibits eNOS activity by phosphorylating at threonine 495 and dephosphorylating at serine 1177 [34]. Unlike PKC, activation of eNOS by PKA occurs *via* phosphorylation of the enzyme at serine 1177 and de-phosphorylation at threonine 495 [34] (see Figure 2).

AMPK

AMPK is a serine/threonine kinase which belongs to a family of kinases known as 'metabolite sensing kinases' [35]. AMPK, and other members of this family, are activated by stimuli such as heat shock, vigorous exercise, hypoxia and starvation. Activation of AMPK results in the inhibition of cellular changes to maintain intracellular ATP levels e.g. inactivation of ATP consuming anabolic pathways, thus preventing ATP depletion. AMPK is co-immunoprecipitated with eNOS from various endothelial cells and following activation of AMPK (in the presence of calcium-calmodulin), eNOS is phosphorylated on serine-1177 leading to activation of eNOS [36]. However in the

absence of calcium AMPK induced phosphorylation of eNOS results in a decrease in enzyme activity (see Figure 2).

1.2 Calcium-independent regulation of NO

There are numerous reports in the literature regarding Ca^{2+} -independent regulation of eNOS in response to stimuli such as estradiol and shear stress where an increase in NO production is not accompanied by an increase in intracellular Ca^{2+} . Agonists that stimulate NO production in a Ca^{2+} -independent manner still however require a basal intracellular Ca^{2+} level as chelation of calcium abolishes eNOS activity [28].

1.2.1 HSP90

Association of eNOS with HSP90 has also been implicated in the regulation of NO production in a calcium-independent manner as well as in a calcium-dependent regulation. Estrogen stimulates NO production without an increase in intracellular calcium in endothelial cells. Russell et al. (2000) reported that estradiol increases NO production in human umbilical vein endothelial cells by enhancing the association of HSP90 and eNOS (Figure 2) [37].

1.2.2 Phosphorylation

The molecular mechanism proposed for the Ca^{2+} -independent regulation of eNOS by phosphorylation implicates a role for the autoinhibitory control element, a 45 amino acid sequence located near the calcium-calmodulin binding site of eNOS [38]. This autoinhibitory domain is believed to hold eNOS in a stable inactive conformation and binding of calcium-calmodulin displaces this element thereby gating the electron flow through eNOS [38]. The autoinhibitory domain is rich in positively charged amino acids and thus phosphorylation of serine residues (see later sections) increases the negative charge in the autoinhibitory domain enabling displacement of the domain at basal intracellular Ca^{2+} levels and thus activating eNOS [39]. A variety of protein kinases have been reported to activate eNOS *via* phosphorylation of amino acid residues as discussed below.

Akt/PKB

The discovery of cDNA encoding a novel serine/threonine kinase was reported by two independent groups in 1991, one group named the kinase c-Akt (as it was a cellular homologue of an oncogene v-Akt from a transforming retrovirus) and the other group termed the kinase protein kinase B (PKB) due to its structural identity to PKA and PKB [40]. Thus, the kinase is referred to, interchangeably, as Akt, PKB or Akt/PKB in the literature. There are three reported isoforms of Akt/PKB expressed in mammalian cells: Akt1/PKB α , Akt2/PKB β and Akt/PKB γ . Akt/PKB is normally activated by phosphorylation on serine 473 and threonine 308 in the regulatory and catalytic domains respectively. Activation of Akt/PKB occurs *via* stimulation of phosphatidylinositol 3-kinase (PI3-kinase). PI3-kinase comprises a family of lipid signalling enzymes activated by a variety of agonists [40]. Once Akt/PKB is activated it leaves the plasma membrane and exerts its effects on various intracellular proteins such as eNOS.

eNOS and Akt/PKB

Two recent reports have shown that the increase in Ca^{2+} -insensitive NOS activity occurs *via* Serine 1177 phosphorylation of eNOS by Akt/PKB [27] [28] (Figure 2). Dimmeler et

al. (1999) documented that human umbilical vein endothelial cells exposed to shear stress increases NOS activity which is unaffected by removal of extracellular calcium or addition of calmodulin antagonists [28]. Also, NO production in HUVEC exposed to shear stress was abolished by a PI3-kinase inhibitor, wortmannin, and resulted in phosphorylation of Akt/PKB. These authors concluded that Akt/PKB is activated by shear stress which in turn phosphorylates eNOS on serine 1177 resulting in maximal enzyme activity at basal intracellular concentrations of Ca^{2+} . Shear stress is not the only stimulus to modulate calcium-independent eNOS activity, activation has also been reported in response to insulin [41] and estrogen [42]. As already stated, eNOS activity can be enhanced by HSP90 association and this interaction can also be stimulated by estrogen [37]. Haynes et al. (2000) suggest that Akt/PKB activation and HSP90 association may both be necessary for NOS activity but are independent mechanisms [42].

PKG/PKA

PKG and PKA are serine/threonine kinases which become activated by agonists that evoke an increase in either cGMP levels or cAMP levels, respectively. Butt et al. (2000) recently reported that activation of PKG and PKA induced serine phosphorylation of eNOS rendering the enzyme Ca^{2+} -independent [39]. In this study eNOS was found to be phosphorylated on serine 1177, serine 633 and threonine 495 allowing for enzyme activation in the absence of calcium-calmodulin. Moreover as there are reports that PKA can activate Akt/PKB *via* a PI3-kinase dependent pathway [43], various intracellular kinase cascades may interact with one another leading to the control of eNOS activity *via* phosphorylation.

Tyrosine kinases

eNOS activity can also be regulated by phosphorylation on tyrosine residues. In bovine aortic endothelial cells, fluid shear stress has been reported to induce a 6-fold increase in NO production within 1 min which is unaffected by chelation and removal of calcium [44]. Fluid shear stress activated NO release has been reported to be prevented by tyrosine kinase inhibitors [45] in the same manner as estrogen induced NO production can be inhibited by herbimycin A [46] suggesting that tyrosine phosphorylation of eNOS or an associated protein enhances NOS activity. Venema et al. (1996) reported that bradykinin induced NO production was associated with tyrosine phosphorylation of a 90kDa protein termed eNOS associated protein-1 (ENAP-1) [47]. However, there are several reports in the literature that tyrosine phosphorylation of eNOS actually leads to enzyme inactivation. Garcia-Cardena et al. (1996) documented that tyrosine phosphorylation of eNOS in bovine aortic endothelial cells decreased enzyme activity as phosphorylation resulted in enhanced eNOS/Caveolin-1 association [17]. Thus, depending on the cell type used and the stimulus applied, tyrosine phosphorylation of eNOS may result in activation or inactivation of NOS activity.

2. Summary

From this brief overview, it is apparent that eNOS can be modulated in a variety of different ways in either a Ca^{2+} -dependent or Ca^{2+} -independent manner depending on the agonist and cell type investigated. A thorough understanding of the mechanisms involved may yield novel therapeutic targets for modulating NO production in health and disease.

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Nitric Oxide. Its Generation, Reactions and Role in Physiology

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Abstract: Nitric oxide is a pivotal reactive nitrogen species that can be generated in a variety of ways. It functions in both physiology and pathology and its reactions regulate its function.

1. Nitric oxide

Voted as molecule of the year in 1992, the gaseous molecule nitric oxide (NO) has become an area of heightened interest and of a great deal of research [1].

2. The historical background of NO and physiology

The initial interest in nitric oxide as a physiological mediator came in the mid-1970's from investigations into the role of cyclic nucleotides guanosine and adenosine mono phosphates (cGMP and cAMP) in smooth muscle relaxation. The levels of cGMP were elevated by a potent vasodilator, glyceryl tri nitrate (GTN) in arterial and other tissues [2,3]. It was hypothesised by Ignarro and co-workers that agents that activate soluble guanylate cyclase should cause smooth muscle relaxation if cGMP was involved. It was also noted that sodium nitroprusside which was unstable in aqueous solution released nitric oxide and was a potent activator of guanylate cyclase. This therefore suggested that nitric oxide should also be a vasodilator.

Using nitric oxide gas and nitrosoguanidine compounds it was demonstrated that a marked relaxation occurred in precontracted bovine coronary artery [4,5]. Other chemical agents were also shown to give similar effects including the use of inorganic sodium nitrite [6].

Furchgott and Zawadski [7] demonstrated the requirement of the endothelium for relaxation responses in vascular tissues and suggested a humoral factor, a lipoxigenase-derived or free radical species to be the endothelium derived relaxing factor (EDRF). Griffith and co-workers [8] confirmed the humoral and endothelium-dependent nature of EDRF, but showed evidence against a lipoxigenase-derived species and against EDRF as a free radical in rabbit aortic preparations.

Further to these and other experiments in 1986 both Furchgott and Ignarro independently suggested nitric oxide or a closely related species was responsible for the effects of EDRF [9,10]. The effects of EDRF and NO were compared by Palmer et al. [11] on vascular smooth muscle and platelet aggregations and observed that their actions were identical.

3. Nitric oxide synthase

Following the discovery of the endogenous nitrodilator substance as NO, the mechanism of its formation became an important aspect of research. From experiments on the activated macrophage it was noted that these cells could generate nitrite and nitrate on the addition of L-arginine [12]. Further to this, the nitrogen atoms in the nitrate and nitrite were shown to come from the terminal guanidino nitrogen atoms of L-arginine by gas chromatography and mass spectroscopy of labelled precursor. Then in 1989 Palmer and Moncada gave evidence of an enzyme-generated source of NO from L-arginine that required NADPH and calcium and generated L-citrulline as a by-product. The use of NG-monomethyl-L-arginine (L-NMMA) an analogue of L-arginine, caused a reduction in the stimulated release of NO from cultured endothelial cells [13] and caused an endothelial-dependent increase in vascular tone [14]. These data suggested that L-NMMA was an inhibitor of a nitric oxide synthase (NOS) and its effects on vascular tone were as a direct inhibition of NO generation.

4. NOS isotypes

NOS is a cytochrome P450 reductase-like haemoprotein and requires cofactors for the generation of NO. Flavin adenine dinucleotide (FAD), flavin mono nucleotide (FMN) tetrahydrobiopterin and calmodulin are all required and at least three distinct types that catalyse the production of NO have been described.

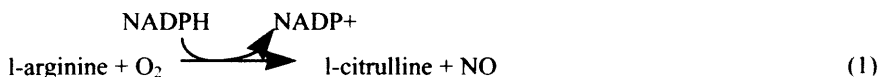
Type I NOS is a 168KDa protein found in the neurons which has been isolated and cloned [15]. This enzyme is responsible for the calcium-dependent release of NO from neurones and non-adrenergic, non-cholinergic nerves and also from skeletal muscle [16]. The activity of type I and type III NOS enzymes is regulated by oestradiol which is a phenomenon observed during pregnancy [17].

Type II NOS has been shown to respond to bacterial endotoxin or inflammatory cytokines and is an inducible, calcium-independent, 130KDa protein. The rate of NO production is apparently raised over that of the previous two types mentioned and can be localised to macrophages [18]. This isoform has associated with it a tightly bound calmodulin, which removes the requirement for calcium in stimulated NO formation. The induction of Type III NOS has been shown in vascular smooth muscle [20] and cardiac myocytes [21].

Type III NOS is endothelial cell-derived and is a 135KDa protein which is activated by the increase in intracellular calcium concentration within the physiological range [21]. This isoform is unique in that it can be localised to the cell membrane as it contains a N-myristoylation site [22].

5. NOS enzyme reactions

The evidence for NOS has steadily grown and the reaction of L-arginine to L-citrulline has been followed in a variety of tissues. The reaction is given by equation (1).



The substrates for NOS are L-arginine, oxygen and NADPH [23]. L-arginine is synthesised as a product of the urea cycle and circulates in the blood at ~ 100 μM [24]. In

endothelial cells, however, the apparent concentration has been estimated to be in the millimolar range [25]. The apparent binding constant, the K_m for L-arginine and NOS has been calculated as $\sim 5 \mu\text{M}$ [26] which suggests that the availability of this substrate is not limiting under normal physiological conditions.

However, Meyer et al. [27] and Heinzel et al. [28] have described the generation of hydrogen peroxide by purified porcine neuronal NOS at low concentrations of L-arginine. This was followed by the measurement of superoxide anion from purified rat neuronal NOS in a NADPH and calcium calmodulin dependent manner [29]. This leads to the possibility of peroxynitrite generation under certain conditions as discussed by Xia and colleagues [30]. Using a kidney cell line and transfecting with a stable rat neuronal NOS with spin-trapping techniques, this group were able to show that a reduction in L-arginine resulted in the generation of superoxide anion. The simultaneous generation of NO and superoxide leading to the formation of peroxynitrite. It was also noted that under prolonged ischaemic conditions a lack of perfusion would lead to the depletion of L-arginine [31] but the concentration of oxygen may control the rate of production for both products.

Recently the possible mechanism of superoxide generation by neuronal nitric oxide synthase has been suggested by Pou et al. [32]. It was reported that superoxide could be measured in the presence of saturating levels of L-arginine. This leads to the relative competition for available electrons that can be donated to oxygen or to L-arginine.

The catalytic mechanism of NOS involves the flavin-mediated electron transport from NADPH to the terminal haem, where oxygen is bound and incorporated into NO and citrulline [33]. The relative affinity of L-arginine ($K_m \sim 5 \mu\text{M}$) compared to oxygen may give clues as to the activity of the enzyme under ischaemic and reduced L-arginine conditions.

6. The effect of oxygen concentration on conventional NOS activity

As can be seen from reaction (1), oxygen is an integral part of the generation of NO from L-arginine. Experiments have been carried out into the effect of oxygen concentration on the rate of enzyme reaction.

In the lung of normal individuals, NO is generated and can be measured in the expired air. Consequently, it has been suggested that NO plays a central role in oxygen-induced vasodilation [34]. The lung contains all three NOS isotypes [35] and in animal studies NO was suggested as the oxygen sensitive vasodilator [36]. Kantrow et al. [37] gave evidence to suggest that hypoxia inhibited NO synthesis in the isolated rabbit lung and caused vasoconstriction which was reversed on the addition of oxygen to the perfusion system.

In human studies Dweik and colleagues [38] measured the kinetics of purified NOS enzymes and from measurement of expired NO from subjects breathing a range of oxygen concentrations. They showed that the expired NO concentrations were dependent on the inspired oxygen concentration with a K_m of $190 \mu\text{M}$ ($\sim 17\%\text{sat}$). From purified NOS II the K_{mO_2} was $135 \mu\text{M}$ ($\sim 10\%\text{sat}$) which, in the lung, covers the likely physiological range of oxygen concentrations. However, in tissues distant from the lung, the apparent oxygen concentration is reduced and this lung-derived NOS activity may therefore be limited.

The apparent activity of neuronal NOS was also studied in relation to oxygen concentration [39]. As discussed previously the NADPH-dependent reduction of bound oxygen will occur in the absence of L-arginine. The electrons donated from NADPH allow the NOS haem iron to bind oxygen which will then generate NO in the presence of L-arginine, or superoxide in its absence [40]. During NO generation, NOS apparently

binds NO to the haem iron and generates an inactive ferrous-NO complex which decomposes in the presence of oxygen to ferric-NOS allowing the return of activity [41]. In the absence of oxygen, however, the ferrous NOS is stable and activity is reduced. In the absence of L-arginine NOS catalysed the simple reduction of oxygen and gave an apparent K_mO_2 of 40 μM which saturated at 100 μM . In the presence of NO the oxygen concentration dependence showed K_mO_2 values of 400 μM which saturated at $\sim 800 \mu M$. It was therefore suggested that the concentration of oxygen controlled the inactive ferrous-NOS, which in turn controlled the rate of NO generation by altering the affinity of the haem iron binding to oxygen. This may also be a method of self-regulation for NOS-I.

Rengasamy and Johns [42] used bovine brain, cultured aortic endothelial cells and rat macrophages to generate K_mO_2 values for NOS enzymes. They found a range of apparent values of ~ 25 , 8 and 7 μM respectively and suggested that pathophysiological conditions would decrease the NO production where oxygen concentrations were limiting.

The apparent difference between the results of Dweik et al. [38] and Rengasamy and Johns [42] may reflect a tissue specific activity for NOS isoforms where adaptation to apparent oxygen concentration regulates the activity of the NOS enzymes.

7. NOS-independent NO generation

For many years the therapeutic application of nitrate drugs has given relief from angina pectoris by a mechanism which remains obscure. Formation of NO from glyceryl tri nitrate (GTN) has been demonstrated in intact bovine pulmonary and coronary artery and in cultured porcine aortic smooth muscle cells [43]. Both enzymic and non-enzymic methods have been proposed with an unidentified microsomal protein of 160-210KDa from bovine coronary artery mediating NO formation from GTN [44] and the interaction with cysteine at high concentrations of GTN [45]. The location of nitrate reduction was investigated by Feelisch et al. [46]. They showed the generation of NO from cultured endothelial cells using oxyhaemoglobin oxidation, cGMP accumulation and the inhibition of platelet aggregation; a bioassay of NO generation. The results suggested that human endothelial cells were capable of the bioactivation of organic nitrates and to some extent this was via an enzymic mechanism that had some requirement for thiols.

8. Xanthine oxidase

Recently, we were the first to detail the nitrate and nitrite reductase activity of xanthine oxidase leading to the generation of NO [47]. This activity occurs following reduction of the enzyme by a variety of substrates including, hypoxanthine, xanthine and NADH. The maximal NO generating activity occurs at low oxygen concentrations as the affinity of the enzyme for oxygen as the electron acceptor is lower than for nitrate and nitrite. The evidence for NO generation was enhanced by the observation of XO/GTN mediated inhibition of platelet aggregation [48] and a further kinetic study has been recently published showing the physiological capacity of XO mediated NO generation [49].

We were also the first to show the NO generating capacity of human milk derived xanthine oxidase [50] which has been shown to have antibacterial capacity in the presence of superoxide to form peroxynitrite. These observations have allowed us to show xanthine oxidase as a putative "peroxynitrite synthase", the first time such a single enzyme system has been suggested.

This multi-substrate and multi-product enzyme system can therefore function

throughout all the physiological and pathological conditions seen in the body concerning oxygen as this is the main regulator of activity at the substrate and molecular level.

9. Organic nitrate reduction

The organic nitrate nitroglycerin or glyceryl tri nitrate (GTN) a polyol ester of nitric acid was first synthesised in 1846 by Sobrero as an explosive and reported as a therapeutic agent in 1879 by Murrell for the relief and prophylaxis of angina pectoris. This was preceded by the use of the organic nitrite, amylnitrite, in 1857 for the treatment of angina and was the first described use of a nitrovasodilator.

Their mechanism of action is proposed to be dependent on bioactivation once in the circulation, with consequent relaxation of the vessels to reduce the pressure on the heart during an attack of angina. Early work described the effect of nitrate drugs on dog and rabbit arteries [51] and further evidence of a mechanism came with the description of the guanylate cyclase enzymes and the effects of azide and other NO donors by Kimura et al. [52].

In 1980 Ignarro and colleagues [53] published a possible mechanism requiring the reduction of nitrates intracellularly by sulphhydryl donors to form an S-nitrosothiol active intermediate that in turn directly, or by degrading to nitric oxide, activated guanylate cyclase. As discussed above, these and other experiments led to the description of the EDRF and NOS enzyme systems.

10. Glutathione S-transferase

Other methods of bioactivation have been suggested. Using a pig liver enzyme Heppel and Hilmoe [54] observed that GTN reacted with reduced glutathione to form oxidised glutathione and inorganic nitrite. This led to the suggestion by Needleman and Hunter [55] that the major route for GTN transformation appeared to be denitration in the presence or reduced glutathione.

Using liver homogenates Needleman et al. [55] gave apparent K_m values for the reduction of GTN of 1.5×10^{-5} M but the reduction of the dinitrate and mononitrates occurred at much slower rates. The final end product of GTN denitration is glycerol but this was not measurable following incubation with reduced glutathione and liver homogenates [56]. In terms of blood pressure depressants GTN was shown to be at least 10 times more potent than glyceryl di-nitrate, 40 times more potent than inorganic nitrite and inorganic nitrate had no measurable effect [57].

More recently Simon et al. [58] measured an inhibition of response of cGMP production from nitrates by the addition of the glutathione S-transferase inhibitor Basilen Blue (BB) by electroporation into porcine epithelial kidney cells. Using an alternative inhibitor of GST, ethacrynic acid, Kenkare and Benet [59] on rabbit aortic strips were able to inhibit relaxation responses to GTN treatment and also to inhibit the increase in cGMP generation. Nigam et al. [60] using the GST inhibitors Basilen Blue, bromosulphophthalein, Rose Bengal, haematin, chlorotriphenyltin and (octyloxy)benzoylvinylglutathione were able to show no inhibition of rabbit aortic strip relaxation by GTN when the strips were pre-constricted by phenylephrine. In contrast, both Basilen Blue and bromosulphophthalein significantly inhibited GTN-induced relaxation of potassium-contracted aortic strips and Basilen Blue significantly inhibited GTN biotransformation in aortic strips pre-exposed to 25 mM potassium. This was suggested to be due to a more favourable electrochemical gradient for entry of the inhibitors into membrane-depolarized tissues.

11. Cytochrome P450

Evidence for the role of NADPH-cytochrome P450 reductase (cyt P450) system in the biotransformation of organic nitrates has been obtained using hepatic microsomes [61] to generate NO in the presence of NADPH and GTN. NADPH-dependent biotransformation of GTN in rat aortic microsomes was inhibited by SKF525A, carbon monoxide and oxygen [62]. Furthermore, GTN-induced relaxation is enhanced under low oxygen conditions and in aortae from animals treated with inducers of cytochrome P450 [63]. However, using inhibitors of cyt P450 in intact blood vessels, evidence has been shown to have both a positive and negative outcome. Treatment of blood vessels with the classical cyt P450 inhibitors SKF525A, metyrapone, cymetidine or carbon monoxide did not affect the GTN-induced relaxation or GTN biotransformation. This suggests that either cyt P450 is not involved in the mechanism of GTN biotransformation, or that isoforms not sensitive to the inhibitors used are present [63,64].

Using the cyt P450 substrate, 7-ethoxyresorufin (7-ER) and the flavoprotein inhibitor diphenyleneiodonium (DPI), it was reported that a substantial inhibition of GTN-induced relaxation occurred and these compounds also reduced cGMP accumulation and inhibited transformation of GTN to 1,2-GDN [63,65].

DPI, however, is not specific for any one flavoprotein, it is also known to inhibit neutrophil NADPH oxidase [66] and the NADH oxidase activity of xanthine oxidase [67]. It has additionally been reported to affect potassium and calcium currents in isolated pulmonary smooth muscle cells [68] and its use in organ based bioassays must be treated with caution.

12. Nitrate and nitrite reduction *in vivo*

Over recent years it has become apparent that other mechanisms of NO generation exist under normal physiological and pathological conditions or through the utilisation of reducible, naturally occurring substrates. Nitrite is such a substrate that may be reduced to NO in the body.

Zweier et al. [69] pointed out the non-enzymic reduction of nitrite to NO in the ischaemic heart. It was shown that the acidotic nature of the ischaemic heart led to a reducing atmosphere that caused the reduction of nitrite to NO. This NO generation was independent of NOS and NOS inhibitors had only minimal effects on the NO generated. Using labelled nitrite ions they were able to demonstrate that nitrite can be reduced to NO in this model and was negatively correlated with intracellular pH and the production of NO with a threshold for NO detection at pH 6.

Human saliva contains both nitrate and nitrite. Dietary nitrates, which are derived from green leafy vegetables, are absorbed in the gastrointestinal tract [70]. Apparently nitrate is concentrated in the saliva up to 25% of circulating levels and is secreted during salivation [71]. The reduction of salivary nitrate to nitrite by bacteria on the tongue has been described by Duncan and colleagues [72] and this source of nitrite could be reduced to NO in the stomach following reduction in the acid conditions found in the adult [73]. Levels of nitrite in the stomach have been measured at 14ppm in the fasting subjects, which was increased to ~ 90 ppm following the ingestion of nitrate [74]. It was suggested then that the reduction of nitrate by commensal bacteria on the tongue generate nitrite which will then be reduced further to NO in the acidic environment of the stomach.

This generation of NO was also described on the skin surface. Weller et al. [75] were able to increase the NO generated from skin by the topical addition of nitrite or by acidification of the skin. This was not inhibitable by classical NOS inhibitors and was

suggested to follow a similar mechanism to the generation of NO in the stomach from nitrite utilising commensal nitrate reducing bacteria.

13. Reactions of nitric oxide

The accumulation of nitrate and nitrite in the body may be through ingestion but also from the reaction products of NO itself. Nitric oxide will react with a range of compounds, dependent on the particular environment and available reactants.

14. NO reaction with oxygen

The best known gas-phase reaction of NO is its conversion to nitrogen dioxide (NO₂) given in reaction (2).



This reaction occurs rapidly at apparently high concentrations and forms characteristic brown fumes [76]. However in the aqueous phase, the reaction of NO and molecular oxygen is slow with a half-life of several hours [77]. The suggested physiological half-life of NO, ~ 3 to 50 seconds [78] in solution, rules out its oxidation by molecular oxygen [79].

NO₂ reacts with water to give a mixture of nitrate and nitrite although it is suggested that nitrite is the major product obtained from this reaction [77]. The reaction of NO with NO₂ is supposed to occur quite readily to produce N₂O₃, an anhydride of nitrous acid (Equations (3) and (4)).



The oxidation of NO₂⁻ to NO₃⁻ occurs readily especially in the presence of oxyhaemoglobin [80].

15. With nitrosothiols

These compounds contain NO and have received attention in discussions concerning EDRF and the physiological role of NO [81]. Nitrosothiols can occur in human plasma as the nitrosothiol of human serum albumin [53] but no particular function was assigned to them in this study. The biosynthetic generation of these nitrosothiols has also come under some scrutiny. It was suggested by Ignarro and Gruetter [82] that NO reacts with thiol at ~ pH7. However more recent evidence has suggested that the required species is the nitrosonium ion (NO⁺) but this also has its problems in that it is reported as a transient species in solution at pH7. Pryor et al. [83] were able to use NO₂ as the reactant with thiol to produce nitrosothiols. However, this is also an unlikely biosynthetic mechanism due to the slow rate of NO₂ formation from NO and molecular oxygen in solution [84].

More recently the possible interaction of peroxynitrite with excess NO may cause the production of the nitrosonium ion, as peroxynitrite can cause one electron oxidations, which may then go on to form nitrosothiols by a nitrosation reaction [85]. Peroxynitrite itself is apparently not capable of this nitrosation reaction but in the presence of metal catalysts it can act as a nitrating agent [86].

16. With iron to form iron-nitrosyls

The reaction of NO with iron is possibly the most significant in terms of biological activity. It is the reaction of NO with iron in guanylate cyclase that allows its activation [87]. The related reaction is the binding of NO to haemoglobin to form nitrosylhaem and this has been extensively studied due to the electron paramagnetic spectrum that is generated [88]. As the switch for guanylate cyclase activation, NO binding to haem must be reversible and this is apparently the case [89]. This is important with respect to the active site of many haem-containing oxidase enzymes, including NOS.

The binding of NO to biological non-haem iron has also been reported. The inhibition of nitrogen fixation has been observed by the binding of NO with iron-sulphur (Fe-S) clusters of nitrogenase enzymes using electron paramagnetic resonance spectroscopy (EPR) [90,91]. The binding of NO to Fe-S to form the Fe-S-NO adduct contrasts with NO-haem binding in that it usually causes the destruction of the Fe-S cluster. This method has been suggested as the cytotoxic mechanism for NO action on key enzymes, especially the inhibition of cytoplasmic aconitase [92].

17. With superoxide to form peroxynitrite

In recent years it has become apparent that the reaction of NO with superoxide radical is possibly the most likely reaction to occur *in vivo*. Peroxynitrite is a strong oxidant and the cytotoxicity previously ascribed to NO or superoxide alone may actually be due to their reaction and degradation products. The reactions are shown below.



The reaction occurs at a rate constant near the diffusion-controlled limit [93]. The rate constant for peroxynitrite formation has been calculated by a variety of methods and the most recent figure was measured by Kissner et al. [94] using laser flash photolysis. By subjecting peroxynitrite to a burst of laser energy superoxide and nitric oxide is formed. By measuring the rate of recombination to form peroxynitrite the rate of reaction has been calculated as $1.9 \pm 0.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$. This is important when assessing the reaction characteristics of nitric oxide and superoxide when other reactions may occur. The reaction of superoxide with superoxide dismutase would reduce the production of ONOO⁻ but the rate constant for superoxide with SOD is given as $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ [93]. The rate of reaction of NO with haem compounds varies depending on the nature of the haem. For example myoglobin has a rate constant ranging from 10^3 to $10^7 \text{ M}^{-1} \text{ s}^{-1}$ depending on the source of the myoglobin. The rate constant also varies with the type of haem, i.e. ferrous or ferric in nature, with ferrous compounds having rate constants $\sim 2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The relative reactions are therefore dependent on the concentrations of NO, superoxide, SOD and haem compounds in the general milieu [94].

Peroxynitrite itself, apparently exists in equilibrium with its conjugate acid peroxynitrous acid (ONOOH) at pH 7.2. The decomposition of ONOO⁻ is complicated [95], as the anion is stable in alkaline conditions but decays rapidly to ONOOH at physiological pH with a pKa 6.8 [96,97]. Three pathways of ONOOH decomposition have been proposed. It was suggested by Beckman et al. [98] that ONOOH decomposes to form hydroxyl and NO₂ radicals based on the sensitivity of peroxynitrite induced reactions to hydroxyl radical scavengers. This was independently supported by EPR data suggesting evidence of free hydroxyl radicals on decomposition of peroxynitrite [32]. However Koppenol et al. [97] concluded from molecular dynamic calculations that homolytic cleavage of ONOOH was improbable. This led to the hypothesis by Pryor et al. [99] of a

caged radical form of ONOOH - ONOOH* which decomposes to radical species that rapidly react again due to the viscosity of the surrounding media and the diffusion limited reaction. They also suggested that ~ 99% of the caged radicals [HO] 'NO₂] would return to reform ONOOH and just ~ 1% would form nitrate, the isomer of ONOO⁻.

A third decomposition mechanism was suggested by Pfeiffer et al. [100] suggesting that the decomposition of authentic peroxynitrite prepared by two different methods produced nitrite and oxygen in a 2:1 stoichiometry at pH 7.5. It was suggested that this mechanism was due to the reaction with ONOO⁻ to form biologically active metabolites which may contribute to physiology and/or pathology of NO and superoxide.

The metabolic generation and fate of peroxynitrite remains an area of intense study with many complex reactions and interactions occurring. The physiological actions of nitric oxide and superoxide or peroxynitrite are leading to a reassessment of their individual and combined activities.

18. NO in physiology and pathology

The physiological role of NO has been studied since the mid-1970's due to its effects on guanylate cyclase and on smooth muscle relaxation. The pathological role of NO has been noted previously as a toxic gas and a constituent of smog formation from exhaust fumes and industrial pollution.

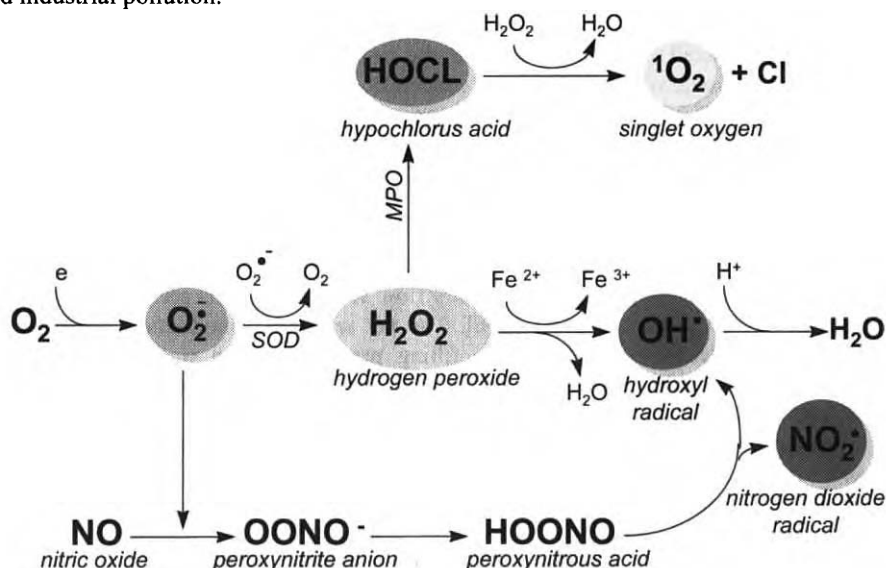


Figure 1. Summary of the possible reaction products of nitric oxide and superoxide with some of the breakdown products and metabolising enzymes.

Sir Humphry Davy in 1800 showed the toxicity of NO during experiments on the effectiveness of inhaled gases for relief of asthma in his work on nitrous oxide. The paradoxical actions of nitric oxide may come down to the particular concentration and duration of production and also on the availability of molecular targets and reactions with other available substrates [101].

The concentration of NO that causes a physiological or pathological effect *in vivo* has been difficult to elucidate. The apparent K_m for NO binding to guanylate cyclase enzyme is

in the nanomolar range and the steady state concentration of NO of $\sim 4\text{--}5\ \mu\text{M}$ can be reached in the immediate vicinity of a cell monolayer [102]. The diffusion distance of NO secreted by a single cell has been calculated to be $150\text{--}300\ \mu\text{m}$ in $4\text{--}15$ seconds but the concentration over the larger distances will be diluted [103]. The focal nature of cellular toxicity was demonstrated by Steiner et al. [103] using activated macrophages or islet endothelial cells to cause the lysis of syngeneic islet cells within 15 hours with $\sim 15\text{--}40\ \mu\text{M}$ NO over that time. It is therefore possible that the nature of NO actions is dependent on the relative concentrations of the NO produced, with higher concentrations responsible for pathological actions.

19. Regulatory role

The regulation of vascular tone has been dealt with previously in this chapter as it was the first description of NO generation in a physiological role. The generation of continuous amounts of NO from endothelial cells allows relaxation of the vessels and the control of both local and systemic blood pressure.

Penile erection is apparently regulated by the oxygen sensitivity of NOS enzyme in the corpus cavernosum. On stimulation the oxygen tension in the corpus cavernosum is increased from a near venous level to an arterial level of saturation [104]. The trabecular smooth muscle of the corpus cavernosum is stimulated by NO and relaxation of smooth muscle occurs. Endogenous NO generation is regulated by the oxygen concentration ~ 25 mm Hg in the flaccid penis compared to the relaxation induced by the addition of endogenous nitric oxide which was oxygen concentration independent ~ 100 mm Hg [104].

Previously in this chapter the use of organic nitrates was discussed in terms of their bioactivation to NO *in vivo*. These drugs are used in a clinical setting for the relief of angina pectoris. The major effect of the organic nitrates is in the modulation of blood pressure to reduce the load on the heart itself. Their action tends to be greatest in the venous circulation followed by the coronary arteries and at high concentrations they have effects in the arterial system [105]. Their combined response is to cause a reduction in venous return and an augmentation in coronary flow and these effects are most pronounced in the poststenotic collateral vessels [106]. The net result of venodilation and coronary vasodilation is a decrease in ventricular filling pressure and in wall tension and an augmentation in coronary flow. The oxygen demand is reduced and improved oxygen delivery occurs particularly in ischaemic regions [107].

The organic nitrates also appear to have antithrombotic properties. Loscalzo [109] was able to demonstrate the inhibition of platelet aggregation with GTN. GTN, or a metabolite of it, causes the activation of guanylate cyclase in the platelet and increases cyclic guanosine monophosphate (cGMP). This is accompanied by inhibition of agonist-mediated calcium flux and reduction of fibrinogen binding to the glycoprotein IIb/IIIa receptor. This system was also seen to reduce platelet adhesion to damaged intimal linings [110] and can possibly dissolve platelet aggregates [111].

20. Protective role

The antioxidant role of NO comes from its reaction with oxygen, carbon and nitrogen centred radicals and can be seen to have a scavenger role under a range of conditions [112]. This is because of the unpaired electrons of NO which react rapidly with alkoxy and alkyl hydroperoxyl radicals at near diffusion reaction rates ($2 \times 10^9\ \text{M}^{-1}\text{s}^{-1}$ [113]). It is these reactions that have been suggested to have a modulatory role in enzyme- or metal-catalysed

lipid peroxidation [113].

Peroxidation of polyunsaturated lipids is thought to be an important pathological event involved in the development of tissue damage and dysfunction. In recent studies, Rubbo and colleagues [113] demonstrated that NO inhibits lipid peroxidation and therefore may be important in the modulation of the inflammatory response by inhibiting the formation of proinflammatory lipids.

The iron or haemoprotein catalysed oxidative reactions may mediate the responses associated with acute and chronic inflammation. In the post-ischaemic, reperfused heart the role of oxidant stress has been linked with increases in leucocyte adhesion and transendothelial cell migration from oxidant production within the microcirculation [115]. This is probably caused by an increased expression in adhesion molecules or the fixation of transiently expressed adhesion molecules by the peroxidation of membrane lipids which reduces membrane fluidity [117,118]. This oxidant stress may also lead to apoptosis induction, DNA damage, inflammatory mediator synthesis and regulate gene expression [119,120,121].

NO was shown by Kanner et al. [120] to inhibit iron-catalysed oxidation reactions by binding to ferrous complexes. It was also shown that NO inhibited the superoxide driven Fenton reaction which, in the presence of iron, generates hydroxyl radical (OH) *in vitro*. By adding varying amounts of NO to a Fenton reaction process the hydroxylation of benzoic acid was reduced. This demonstrates that depending on the fluxes of the different reactive species, NO may have an antioxidant capability.

21. Deleterious role

In an almost reverse manner to those mentioned above, the reaction of NO with oxidants may lead to the generation of toxic compounds that may cause cellular damage. NO itself has been suggested as an enzyme inhibitor. This has been suggested as having a direct effect by binding to enzymes or by inhibition of enzyme assembly processes. The pig neutrophil NADPH oxidase is one such system where the assembly process is inhibited by NO. Fujii and colleagues [122] were able to demonstrate neutrophil NADPH oxidase inhibition which was not due to direct interaction of NO and enzyme nor to the reaction of peroxynitrite with the enzyme, but was greatly enhanced during the assembly process.

Direct reaction of NO with enzymes has been shown for cytochrome c oxidase (cyt c oxidase). The reaction of NO with the binuclear metal centre of cyt c oxidase apparently leads to the formation of nitrite at the active site [123] the mechanism of which was described as the opposite of nitrite reduction to NO by non-haem nitrite reductases [124]. The inhibition was caused by the binding of NO to the reduced copper centre of the enzyme rather than the expected reaction with Fe^{2+} .

Nitric oxide binding to the aconitases and specifically the four iron-four sulphur (4Fe-4S) domain of mammalian cytoplasmic and mitochondrial enzymes has been suggested to be part of pathology [125]. These are inorganic prosthetic groups whose iron atoms are co-ordinated to inorganic sulphides and usually liganded to protein by cysteine thiolates [91]. By co-culturing L10 hepatoma cells with NO producing mouse macrophages, Drapier and Hibbs [125] were able to show that there was a hierarchy in sensitivity of the mitochondrial Fe-S-containing enzymes. Aconitase was most sensitive to NO, followed by complex I and complex II, with complex III unresponsive to NO treatment. This gradation in sensitivity to NO was suggested as being caused by access to the cluster, or by the interaction of NO with the ligands that anchor the cluster to the protein.

The reaction of NO with iron, Fe-S and haem proteins leads to the possibility that there may be regulation of certain protein activities due to the binding of NO. NO

generation itself relies on the binding of oxygen and nitrogen to the haem moiety of NOS enzymes. NOS activity has been linked to the binding of NO to haem iron of neuronal NOS in both the ferrous and ferric states under anaerobic conditions to generate a stable NOS haem iron-NO complex [126]. This binding caused an inhibition in NO generation from NOS and may therefore be a method of NO regulation. In fact, the same group [40] has also suggested this in the case of oxygen availability as a regulator of NOS activity. In this case the rate of the ferrous NO complex breakdown in neuronal NOS was dependent on oxygen concentration. It is this complex breakdown that is vital for enzymic turnover with the ferrous NO complex remaining stable at low oxygen concentrations.

Over production of NO can lead to mutagenesis and cell death and has been shown to be mutagenic in a variety of systems [40]. These range from mutations in *E. coli* and human cell lines [39] to an *in vivo* mouse model [127]. Using cell lines as an *in vitro* model of NO toxicity, a range of pathways have been identified from inhibition of DNA synthesis, mitochondrial damage, apoptosis, cell cycle distribution changes and DNA strand breaks [128]. An effect on ribonuclease reductase activity has also been shown, which seemed only to be temporary in nature and the activation of poly(adenosine 5'-di-phosphoribose) synthetase has also been attributed to both NO and ONOO⁻ [129].

The formation of the anhydride (N₂O₃) from equation (3) can lead to both direct and indirect DNA damage. Direct action results from nitrosation of primary amines on DNA bases which leads to deamination and at physiological pH, N₂O₃ has been demonstrated to be the most important species [130]. Indirect actions are due to mutations that can arise from the deamination of bases where guanine deaminates to xanthine, mispairing of which can cause a G:C to A:T transition which will ultimately lead to single strand breaks [131].

The action of ONOO⁻ on DNA rather than deamination is oxidative and ONOO⁻ addition leads to more damage than treatment with an equivalent amount of NO. The spectrum of ONOO⁻ damage is also increased over NO, which is probably accounted for by their relative reactivity. The addition of ONOO⁻ to naked plasmid DNA can cause strand breaks using as little as 2–5 μM compared to no detectable strand breaks in NO treated plasmids at millimolar concentrations [132,133].

22. Conclusions

As can be seen from the review above, nitric oxide plays an important part in both physiology and pathology. From the first suggestions for its action as a mediator of muscle relaxation, to the explanation of the generating systems, the complex nature of this gaseous molecule has kept the science community intrigued by its versatile nature. Compared to other generated mediators, NO is still relatively novel and much further work is anticipated to follow the action of this enigmatic molecule.

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Redox-Regulated Glutathionylation of Transcription Factors: A Regulatory Mode for Gene Expression

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Abstract: Nitric oxide (NO) is a biological mediator involved in the regulation of a wide variety of functions, including gene expression. The interaction of NO with proteins may take place through its binding to heme groups, Fe-S or Zn-S centers and modification of cysteine (nitrosylation) and tyrosine (nitration) residues. The cellular redox status is determined by the relation between oxidized and reduced forms of intracellular redox molecules as glutathione (GSH). A decrease in the ratio GSH/GSSG to oxidative states may induce the production of posttranslational modifications that affect the protein functionality. Both, the NO and redox status changes, are powerful regulators of gene expression. This property is related with the modulation of the expression and/or activity of transcription factors like AP-1 or NF- κ B that is also governed by the cellular redox status. Our interest has been to focus on how the NO and the redox pair GSH/GSSG can regulate the activity of AP-1 or NF- κ B, exploring their effect over the recombinant proteins cJun and p50 (the AP-1 and NF- κ B subunits, respectively). We observed that c-Jun suffers a specific and reversible S-glutathionylation in the Cys 269 that inhibits its DNA binding activity. The utilization of a S-nitrosoglutathione sepharose in nuclear extracts of HeLa cells allowed the identification of diverse transcription factors that could experiment glutathionylation. A deep study about the S-glutathionylation of p50 showed us a complex scenario in the regulation of this protein in oxidative conditions that is implicated in an inhibition in its DNA binding activity. Finally, molecular modeling studies suggest the existence of specific electrostatic interactions between glutathione and c-Jun or p50 that could favour the S-glutathionylation. This modification could represent a biochemical mechanism by which oxidative and nitrosative stress signals could be reflected in gene expression changes.

1. Introduction

The regulated expression of the genome is essential for the homeostatic maintenance and correct cell differentiation and morphogenesis of an organism. Transcription is the primary regulatory step in the control of genome expression. Transcription factors play a preponderant role in this regard. In most cases, the regulation of transcription relies on co-operative effects amongst several transcription factors binding in close vicinity to each other. Far bound factors can still co-operate in the regulation of the transcription of a particular gene. This occurs through the looping out of the intervening DNA and “facilitated tracking” mechanisms, as has been proposed for the co-operative effects of enhancers upon promoters. In recent years, the role of transcriptional co-activators has been

put forward by many examples [1] and is now believed to be an essential mode of differentially regulating gene expression. Likewise, the ability of histone proteins to dynamically facilitate transcription by chromatin remodeling is another hot topic in the field of transcriptional regulation [2]. Beyond these inter-factor co-operative effects, the capability of transcription factors for regulating transcription may be further improved by mechanisms that modify their intrinsic functionality. This is essentially achieved by post-translational modifications, the best known of which is phosphorylation, which has been shown to ultimately regulate the activity of many factors [3].

The regulation of protein function by the cellular redox status has gained increasing importance in recent years. In this context it is now well known that oxidative stress is an important regulator of gene expression, both in physiological and pathophysiological conditions [4]. Transcription factors are now recognised as targets for oxidative and nitrosative stresses and more and more examples appear every day in the literature to confirm this contention. In general, activation or inactivation relies upon oxidation or nitrosylation of thiol groups located in cysteine residues which are critical for the interaction with DNA. Reactive oxygen (ROS) and nitrogen species (RNS) produced *in vivo* at levels that cannot be dealt with adequately by endogenous antioxidant systems can lead to the damage of lipids, proteins, carbohydrates and nucleic acids. Oxidative modification of these molecules by toxic levels of ROS and RNS represents an extreme event that can lead to deleterious consequences such as loss of function. By the use of a variety of cell types it has been shown that numerous cellular processes including gene expression can be regulated by subtle changes in redox balance. Examples of this include the activation of certain nuclear transcription factors. Thiols, by virtue of their ability to be reversibly oxidised to sulfenic acid or to formation of inter, intra or mixed disulfides, are recognised as key components involved in the maintenance of redox balance. Additionally, increasing evidence suggests that thiol groups located on various molecules act as redox sensitive switches thereby providing a common trigger for a variety of ROS and RNS mediated signaling events. Particular attention has been paid to the importance of thiols and thiol-containing molecules in these processes. As the regulation of transcription factor activity in these contexts has been reviewed elsewhere [4], we will focus on the potential importance of the formation of mixed disulfides between these factors and glutathione — a process known as glutathionylation — for the regulation of their activity.

2. Glutathionylation: a posttranslational modification with functional consequences

Glutathione (GSH) is a tripeptide (g-Glu-Cys-Gly) which is present in mammalian cells in concentrations ranging between 1 and 10 mM. It represents the major low-molecular-mass antioxidant, playing a key role in cellular resistance against oxidative and nitrosative damage. It acts as a scavenger of NO and oxidants by providing reducing equivalents for enzymes involved in the metabolism of ROS and RNS as well as by eliminating potentially toxic oxidation products and reducing oxidised or nitrosylated protein sulfhydryls. The availability of GSH in situations of oxidative stress is ensured by GSH-recycling and biosynthetic pathways which can be upregulated in situations of oxidative and nitrosative stress.

Apart from providing the cell with a reducing environment and maintaining proteins in a reduced state, accumulating evidence suggests that the glutathione redox couple GSH-GSSG dynamically regulates protein function by the reversible formation of mixed disulfides between protein cysteines and GSH, a process termed S-glutathionylation, and generally though less correctly, S-thiolation. Protein S-glutathionylation has been implicated in the buffering of oxidative stress, stabilisation of extracellular proteins,

protection of proteins against irreversible oxidation of critical cysteine residues as well as in the regulation of enzyme activity. Furthermore, recent evidence relates mixed disulfide formation to ROS-mediated inhibition of protein synthesis. Protein S-glutathionylation may be induced by changes in the intracellular redox potential as well as by the formation of ROS and RNS. Reversion of S-glutathionylation may occur by non-enzymatic reduction or by enzymatic cleavage of the disulfide bond involving the action of thioredoxin, glutaredoxin and/or protein disulfide isomerase. The specificity and efficacy of these enzymes is governed by the structural context of the disulfide bond. Interestingly, there is preliminary evidence that thiol transferases might eventually shift the GSH/mixed disulfide-equilibrium in both directions, i.e. also facilitating protein-S-glutathiolation [5]. It is attractive to speculate, therefore, that enzymatically controlled thiolation/dethiolation may confer specificity and regulatory potential to the post-translational control of protein function by S-glutathionylation.

The potential relevance of protein S-glutathionylation as a functional response to oxidative stress in intact cells is supported by a large number of studies performed during the past 20 years which demonstrate that oxidative challenge of intact tissue or cells in culture results in the transient accumulation of protein mixed disulfides [reviewed in 6-9]. Although in many of these studies relatively high concentrations of exogenous oxidants such as H_2O_2 , t-butyl hydroperoxide, menadione or diamide were used to simulate oxidative stress, there is evidence that mixed disulfide formation can take place in a (patho)-physiologically relevant context and is mediated by endogenous cellular oxidants. This is supported by the detection of mixed disulfide formation in response to phorbol myristate acetate stimulation of murine macrophages [10] and human neutrophils [11,12] as well as during phagocytosis-associated respiratory burst in human monocytes [13,14]. More recently, protein mixed disulfides were reported to accumulate as *in vivo* markers of oxidative tissue damage in lungs of rats exposed to cigarette smoke [15], in a cellular model of cadmium-induced oxidative stress [16] and in cataractous human eye lenses [17]. In addition, S-thiolated proteins were detected in quiescent cells probably due to basal levels of ROS formation [18].

Previous efforts to identify proteins that become S-thiolated during oxidative challenge in intact cells were based on the observation that the major part of GSH, which becomes bound to cellular proteins through oxidant-induced mixed disulfide formation, is associated with a discrete subset of a relatively small number of proteins. These studies have identified several proteins involved in metabolic and redox functions capable of suffering glutathionylation in cellular models of oxidative stress. Although these reports provide additional information about the kinetics and molecular mechanisms of redox-dependent and ROS-induced mixed disulfide information, they do not directly address the issue whether protein S-glutathionylation is in fact a prerequisite for the expression of the cellular response to oxidative stress and, if so, what are the mechanisms by which S-glutathionylation interferes with signal transduction cascades that define such functional response.

It is now well known that S-glutathionylation can be promoted by oxidative or nitrosative stress, independently. Intermediate steps may involve the activation of the protein sulfhydryl or of glutathione (Figure 1). In the next step reactions with reduced glutathione or a reduced protein sulfhydryl respectively would lead to the formation of a mixed disulfide. From a functional point of view, oxidative stress has provided examples in which S-glutathionylation may act as a defensive regulator. One of these is represented by yeast in which resistance of Tdh3 to ROS was shown to involve the redox-dependent and reversible S-glutathiolation of this GAPDH isozyme. Although there is convincing evidence that human GAPDH becomes oxidatively inactivated by S-glutathionylation both *in vitro* [5 and references cited therein] and in intact cells [13,20], an extrapolation of the

above results obtained in a yeast model to a role of GAPDH for the expression of oxidative stress resistance in mammalian cells awaits further studies.

It has been shown that the cellular phosphorylation state may be regulated by S-glutathionylation. In this regard both protein kinases and protein tyrosine phosphatases have been reported to suffer S-thiolation. Reversible regulation of protein tyrosine phosphatases (PTPs) is of pivotal importance to the dynamic regulation of the cellular protein tyrosine phosphorylation state in response to extracellular signals [21]. It has been shown that stimulation of cells with epidermal growth factor results in the ROS-mediated and reversible inactivation of the ubiquitous PTP isoform PTP-1B [22]. Recent data suggest that the redox mechanism by which ROS regulate PTP-1B activity involves oxidation of its active site cysteine 215 [23,24]. Treatment of purified PTP-1B with H_2O_2 resulted in largely irreversible inactivation of the protein whereas exposure to superoxide induced a reversible oxidation of cysteine 215 to a sulfenic acid intermediate, which was demonstrated to react with GSH to a more stable mixed disulfide [25]. Given that S-glutathionylated PTP-1B could be reactivated enzymatically by glutaredoxin [26], this may provide an efficient mechanism for the regulation of PTP-1B by superoxide. Importantly, the proposed regulatory mechanism is supported by the observation that PTP-1B becomes glutathionylated at cysteine 215 in intact cells upon stimulation with epidermal growth factor by a mechanism that involves intracellular ROS generation [25]. Finally, there is experimental evidence that PTP S-glutathiolation may not only be induced by oxygen radicals but also by changes in the redox potential, i.e. by accumulation of GSSG [26]. One of these tyrosine phosphorylation-dependent pathways that could be modulated by S-glutathionylation, is the redox-dependent recruitment of neutrophils to sites of vascular damage and inflammation. It was found that H_2O_2 and tumour necrosis factor- α promote neutrophil adhesion by redox-dependent β_2 -integrin (CD11b/Cd18 or Mac-1) activation which is suggested to involve S-thiolation of a component of this ROS-activated tyrosine kinase signalling cascade [28]. Recently, the family of protein kinase C isozymes has been shown to suffer S-glutathionylation. As a result of this process they become inactivated. This, if extended to the *in vivo* situation, could have important consequences for the regulation of cell growth and differentiation.

The ubiquitin-proteasome pathway has also been shown to be affected by S-glutathionylation. Proteins can be modified post-translationally by the attachment of ubiquitin, which targets the protein to proteolytic degradation by the proteasome. Protein ubiquitination involves the concerted action of ubiquitin-activating, ubiquitin-carrier and ubiquitin-ligating proteins which are also known as E1, E2 and E3 enzymes, respectively. It is generally accepted that ubiquitin-dependent protein degradation protects the cell against the accumulation of oxidatively damaged or aberrant proteins and regulates the levels of important key molecules involved in cytokine-induced gene expression, cell cycle progression, differentiation and cell death such as I κ B, cyclins and p53 [29]. Recent evidence suggests that the ubiquitin-proteasome pathway is subjected to redox control. It could be demonstrated that this process involves a reversible S-glutathionylation in the active sites of the E1 and E2 enzymes [30,31]. By this mechanism, S-glutathionylation might serve to protect the repair and signalling functions of ubiquitinating enzymes from permanent oxidative inactivation.

NO-induced protein S-glutathionylation was proposed for the first time in 1988 by J.W. Park as a possible mechanism for the inactivation of yeast alcohol dehydrogenase by NO [32]. However, it took almost 10 years until the possibility that NO might be able to direct the incorporation of GSH to protein sulfhydryls was reconsidered. In 1997, it could be demonstrated that micromolar concentrations of GSNO inhibit aldose reductase through site-specific mixed disulphide formation at a conserved cysteine residue in its catalytic site

[33]. One year later, a role of NO as mediator of protein thiolation in intact cells was highlighted by experiments demonstrating that endothelial cells respond to exogenous NO production with the transient S-thiolation of a number of as yet unidentified cellular proteins [34]. Similarly, S-nitrosocysteine was found to induce some S-glutathiolation in NIH-3T3 cells and rat hepatocytes. It is important to mention that other NO-derived species with a strong oxidative potential, such as peroxynitrite (ONOO-) are able to induce S-glutathionylation as is the case with the Ca-ATPase of sarcoplasmic reticulum which *in vitro* becomes inactivated [35].

Interestingly, cysteine proteases have been found to be exceptionally sensitive to NO. Such NO-mediated regulation of proteases appears to be implicated in the inhibition of bone resorption by NO synthase-expressing osteoclasts by, as yet, not fully understood mechanisms [36,37]. Recent work proposes that this may occur through the NO-induced inhibition of the collagenolytic activity of the cysteine protease cathepsin K [38]. The authors of this study demonstrate that NO donors and GSNO potently inhibit the activity of cathepsin K both *in vitro* and in intact cells through oxidation of its active site cysteine. In the absence of GSH NO donors convert this cysteine residue irreversibly into a sulfinic or sulfonic acid via intermediate oxidation to a sulfenic acid. In the presence of GSH NO generation results in the accumulation of GSNO and it has been shown that this nitrosothiol induces the reversible formation of a mixed disulfide between GSH and the active-site cysteine of cathepsin K. Similarly, another cysteine protease, caspase-3, was discussed as a target of NO-mediated S-glutathiolation. In this case, however, mixed disulfides are formed at various cysteines of both protein subunits but not at the active-site sulfhydryl which reacts to a relatively stable S-nitrosothiol [39].

An interesting link between RNS-induced protein S-glutathionylation and the adaptation of intracellular signalling to nitrosative stress is provided by the observation that GAPDH can be inhibited in a reversible manner both *in vitro* and in intact endothelial cells by GSNO-dependent S-glutathiolation [40]. As discussed above, GAPDH is one of the best-characterised targets of redox-dependent S-glutathiolation and, more importantly, has been related to oxidative stress resistance in eukaryotic cells [41]. In this context, it would be interesting to see if the selective expression of GAPDH isozymes confers cellular resistance to nitrosative stress to a comparable extent as this has been observed recently in a yeast model of GAPDH-dependent oxidative stress resistance [41].

A further potential target for NO-dependent S-glutathionylation is the human platelet L-arginine transporter which was found to be upregulated by GSNO probably through mixed disulfide formation [42]. Of note, the NO synthase substrate L-arginine may become limiting in NO producing cells and, in this case, NO formation becomes dependent on cationic amino acid transporters that shuttle L-arginine into the NO producing cell [43]. Thus, it is attractive to speculate that, by this thiolation mechanism, continuous supply of the NO synthase substrate might be coupled to the rate of NO synthesis. Finally, it was shown that NO modifies H-ras and carbonic anhydrase III in intact cells by S-glutathionylation [44] and that GSNO may thiolate alcohol dehydrogenase, glycerol-3-phosphate dehydrogenase, creatine kinase, glycogen phosphorylase b, carbonic anhydrase I, Cu-Zn superoxide dismutase, thioredoxin and glutaredoxin *in vitro* [45]. The functional implications of these findings, however, remain to be investigated.

3. The transcription factors AP-1 and NF- κ B as targets for S-glutathionylation

It is well known that the redox responsiveness in proteins may be conferred by S-NO (nitrosylation), S-OH (sulfenic acid), S-S (intramolecular disulfide), and S-SR (mixed disulfide or S-thiolation), all potential reversible modifications of reactive cysteines (Figure

1). Most redox-responsive transcriptional activators in mammalian cells are likely to be regulated by one of these modifications [45]. Among them, S-glutathionylation has emerged as a potential reaction by which the cells may transduce oxidative stress into repression of gene expression.

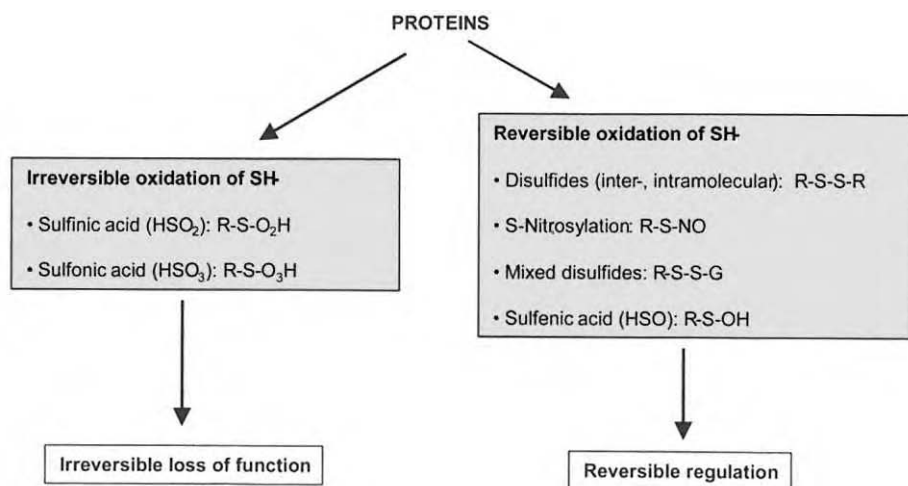


Figure 1. Oxidative modifications of protein thiols.

The first indirect evidence about the potential role of the S-glutathionylation as a regulatory mechanism for the transcription factors function was shown in a study about the dependence of the DNA binding activity of nuclear factor- κ B on the intracellular levels of the redox pair GSH/GSSG [46]. Since then, two well-defined transcription factors, nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1), have been found to be potentially regulated by S-glutathionylation *in vitro* [47,48]. Both contain highly conserved redox sensitive cysteines in their DNA binding domains which can form mixed disulfides with glutathione. This modification, which occurs with different stoichiometry in the subunit cJun of AP-1 and p50 of NF- κ B, induces an inhibition in the DNA binding activity of both proteins.

S-glutathionylation of AP-1 was the first one to be studied by our group. We demonstrated that changes in the redox status, which favor pro-oxidative conditions (represented by different ratios of GSH/GSSG ranging from 100 to 0.1) produce an inhibition in the DNA binding activity of this transcription factor. The maximal inhibition was around 98 % at GSH/GSSG values of 0.1 (Figure 2A). In these conditions, [3 H]GSH incorporation studies demonstrated that the protein c-Jun (structural component of AP-1) was able to incorporate 1 mol of glutathione per mol of protein (98 per cent of the protein is in a S-glutathionylated form, Figure 2B). The cysteine residue located in the basic region of the DNA binding domain (Cys 269) was the specific target for this S-glutathionylation, and it could be completely reverted by DTT or GSH treatment [47]. Alternatively, it has been shown that new mechanisms for the glutathione incorporation to c-Jun that do not include oxidative changes in the redox potential may exist. Accordingly, it has been demonstrated that millimolar concentrations of the nitric oxide donor DEA/NO between 0.01 and 1 can also promote S-glutathionylation of Cys 269 within c-Jun with comparable stoichiometry (1 mol per mol of protein, 87% of maximal inhibition) [49]. In this case, the reaction seems to occur by a different mechanism which does not involve previous glutathione oxidation to GSSG. Furthermore, S-nitrosoglutathione was formed during the reaction so it was

speculated that an alternative explanation for the NO-induced S-glutathionylation of c-Jun could occur through the formation of GSNO (after nitrosylation of GSH by NO) and the subsequent reaction of GSNO with the protein. The capacity of GSNO to interact with the redox sensitive thiol of c-Jun has been shown by a study of the binding of c-Jun to GSNO-Sepharose [48]. Given the complex and as yet not entirely elucidated chemistry of the GSH/GSNO system, further studies are required to establish the molecular mechanism underlying GSNO-induced mixed disulfide formation.

Minimal structural requirements for specific GSH binding have been derived from the crystal structure and NMR analysis of GSH-binding proteins [50].

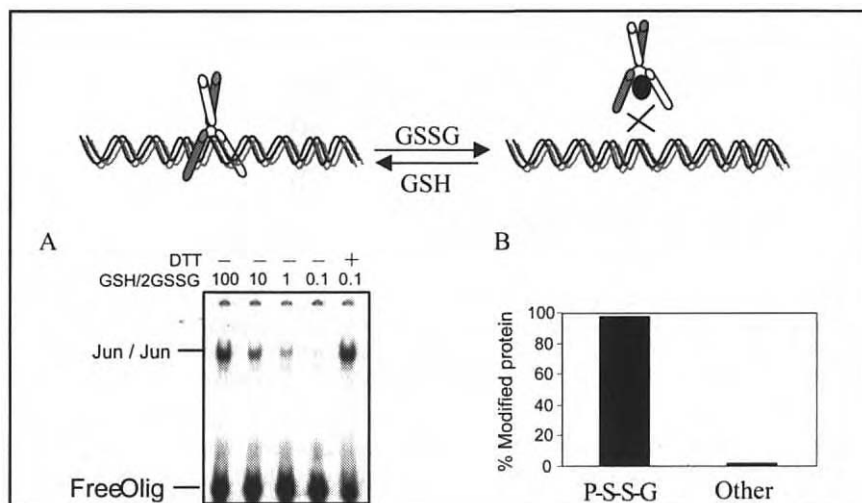


Figure 2. The incubation in the presence of GSSG induces the S-glutathionylation of c-Jun and an inhibition in its DNA binding activity. A) The DNA binding of c-Jun was subjected to EMSA experiments in the presence of different GSH/GSSG ratios or DTT. B) In the figure is represented the amount of c-Jun protein which is modified to mixed disulfide with glutathione (P-S-S-G) or to other modifications.

Another prototypic form of redox-sensitive transcription factors is NF- κ B. Its activation is modulated at multiple levels by reactive oxygen species or nitric oxide [52-55]. One of the subunits of NF- κ B, p50 contains a cysteine residue (Cys 62) in its DNA binding domain whose reduced state is essential to allow the binding to DNA [56]. In diverse oxidative stress models, different oxidants were shown to induce an inhibition in the p50 DNA binding activity by oxidation of reactive protein thiols. However, there is no firm evidence about the nature of the posttranslational modifications that may occur in these mechanisms of cellular regulation. S-nitrosylation of Cys 62 has been shown to occur [57]. In addition, the formation of an intermolecular disulfide has been proposed to mediate the observed inhibition [57]. Recently, we have shown that S-glutathionylation of Cys 62 is another candidate modification for this inhibitory effect. We observed that NF- κ B DNA binding activity was inhibited when changes in the GSH/GSSG ratio, ranging from 100 to 0.1, were applied (Figure 3A). The maximal inhibition reached (66%) was obtained with the highest pro-oxidative conditions (Ratio of 0.1 GSH/GSSG). With these, S-glutathionylation of Cys 62 was detected by mass spectrometry experiments (MALDI-TOF, nano ESI QIT). In contrast to c-Jun, [3 H]GSH incorporation assays demonstrated a maximal incorporation of 0.4 moles of glutathione per mol of protein (Figure 3B). This modification did not appear to be the only responsible for the inhibition as we were able to prove concomitant formation of sulfenic acid within the same cysteine, albeit at a much lower proportion.

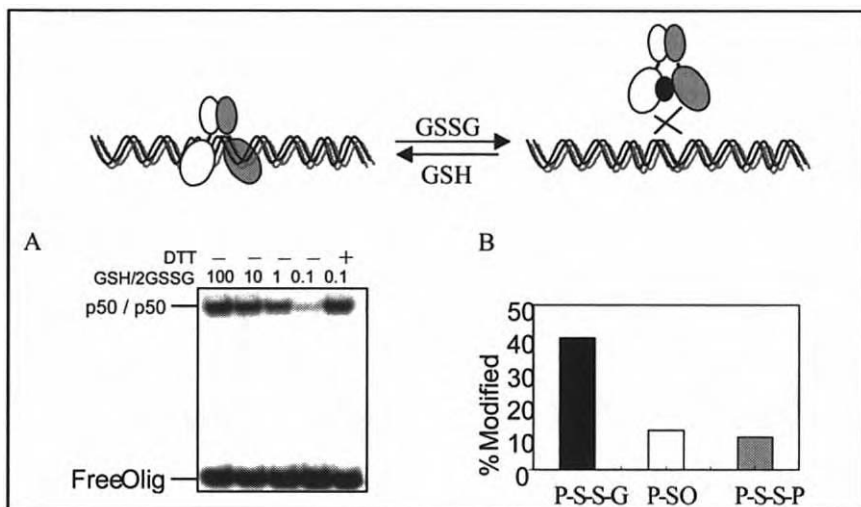


Figure 3. The treatment with GSSG produces an inhibition in the DNA binding activity of p50 by S-glutathionylation. A) The DNA binding of p50 was subjected to EMSA experiments in the presence of different GSH/GSSG ratios or DTT. B) In the figure represented the amount of protein which is modified to mixed disulfide with glutathione (P-S-S-G), sulfenic acid (P-S-OH) or intermolecular disulfide (P-S-S-P).

Potential S-glutathionylation of p50 by GSNO was also confirmed [48]. As in the case of c-Jun, p50 can be attached to a GSNO matrix apparently through a covalent interaction between a reactive thiol of the protein and the thiol group of GSNO. Nevertheless, the S-glutathionylation of p50 induced by nitric oxide or related compounds has not been demonstrated. Finally, in contrast to c-Jun, molecular modelling studies suggest that glutathione binding to the p50 molecule is not physically favored as in the case of c-Jun. This might contribute to explain the lesser degree of glutathione incorporation into p50.

Although S-glutathionylation of both transcription factors has been exhaustively studied *in vitro*, the functional relevance of this modification for the cell remains unclear. Moreover, the difficulty for the study and detection of this reaction *in vivo* have notably delayed the understanding of the molecular events in which S-glutathionylation could be involved.

4. Methods useful in the study of S-glutathionylation

In the majority of cases, the phenomenon of S-glutathionylation has been demonstrated by qualitative experiments that do not permit to conclude how susceptible a protein is to suffer this reaction. Qualitative approximations include mass spectrometry experiments, which can be performed with the intact or the trypsinized protein. The use of mass spectrometry technology (MALDI-TOF and nano ES QIT) has allowed the identification of many targets for S-thiolation and, in many cases, it has made possible to determine the specific thiol that is involved in the glutathione adduct. However, these techniques cannot be used to determine the degree of glutathione incorporation because they measure an intrinsic property of a molecule, its mass. Furthermore, it requires an exhaustive and optimal sample preparation. Other emerging techniques are focusing on the detection of mixed disulfides by the introduction of a "modified" glutathione molecule in the cell. They include glutathione esters [58], biotinylated glutathione [59] or any procedure that allows

glutathione to cross the plasmatic membrane. Alternatively, the use of radioactive glutathione incorporation experiments ($[^3\text{H}]\text{GSH}$, $[^{35}\text{S}]\text{GSH}$) has proved to be an effective method at least to detect S-glutathionylation *in vitro*. In this case, the formation of the mixed disulfide is evaluated through several procedures. Thus, it is possible to measure the radioactivity released from the protein, previously incubated with the labeled thiol, by its purification and subsequent treatment with a reducing agent [60]. In other cases, it is easier to quantify the labeled molecule that remains attached to the protein after precipitation [61]. Classic $[^{35}\text{S}]\text{GSH}$ -labelling studies have turned out to suffer from experimental artifacts during the detection of mixed disulfides in intact cells [62].

The use of isoelectric focusing methods in combination with immunodetection of the S-glutathionylated protein has been used to identify a wide variety of proteins [63]. Nevertheless, this procedure only allows a semiquantitative study of the mixed disulfide and requires additional confirmation by other methods. Moreover, the absence of reliable glutathione antibodies has hindered the detection of S-glutathionylated proteins in intact cells. The development of specific antibodies against S-glutathionylated proteins will be a crucial step in establishing analytical methods that will allow to determine whether what is currently becoming biochemically possible in terms of ROS/RNS-induced protein S-glutathionylation is of biological relevance.

All the techniques described above are represented in Table 1.

Table 1. Methods to describe S-glutathionylation *in vitro*.

Technique	Advantages	Disadvantages
Isotopic labelling	Specific quantitative	Artefacts (in vivo)
Mass spectrometry	Fast results, peptides sequencing, low amounts of sample	Qualitative, sample preparation, tedious results analysis
Immunodetection	Specific, detection in vivo	No GSH antibodies available
Isoelectric focusing	Resolution	Sample preparation, slow, semiquantitative
Glutathione labelling	Useful for in vivo studies, cross the plasmatic membrane	Qualitative and quantitative, optimization

5. Functional implications and in vivo approaches for S-glutathionylation

The intracellular GSH redox homeostasis is strictly regulated in order to govern cell metabolism and protect cells against oxidative stress. This may occur in part by the formation of mixed disulfides with protein thiols. Growing evidence has suggested that cellular oxidative processes have a fundamental role in inflammation through the activation of stress kinases and redox-sensitive transcription factors such as NF- κ B and AP-1, which differentially regulate the genes coding for proinflammatory mediators and protective antioxidant factors. It is well established that oxidative inhibition of transcription factors whose activity relies on the redox status of oxidant-sensitive cysteines in their structures such as AP-1, NF- κ B, nuclear factor-1, Sp-1, hypoxia-inducible factor-1 and p53, is one of the mechanisms by which cells may transduce oxidative stress into repression of gene expression [64]. S-glutathionylation of nuclear factor-1, p50, and c-Jun has been demonstrated *in vitro*, but the possible implications of such modification in the modulation of their activities *in vivo* require a more exhaustive study. However, the reversibility of this process lends speculation to the idea that the glutathione molecule attached to the corresponding sensitive cysteine could protect the protein from oxidative inactivation during adverse conditions. When the normal conditions were restored, the modification could be reverted and the protein could function normally. In summary, the effect that S-glutathionylation could have over the gene expression is still unknown. There are only

recent studies about the modulation of transcription factors activity by mixed disulfide formation *in vitro* or about the regulation of signalling pathways involved in the cellular response to oxidative stress. A possible explanation for this lack of knowledge may lie within limitations in the methods that allow the detection of S-glutathionylated forms in the cells.

In recent years, the development of the proteomic technology has permitted new advances in the detection of postranslational modifications [65]. By using cellular extracts from cells that have been exposed to oxidants it is possible to analyze the molecular modifications of an specific protein. These modifications produce changes in the net charge of a molecule so this property can be used to separate the different forms by isoelectrofocusing methods. Then, the use of bidimensional electrophoresis methods will allow an optimal separation of different species. The problem emerges when the amount of protein object of study is very low or the degree of modification is not very high. It might be necessary to improve sample preparation by desalting, purification or precipitation methods prior running a 2D-gel. When the gel is ready, the second step will be to analyze the obtained spots. A good detection system will be necessary according to the detection level desirable. The Coomassie or silver stain methods and the detection by the use of fluorescent or radioactive molecules are all potential options to achieve best results. Subsequently, the digestion of the sample in gel or in solution, with an appropriate protease, will yield a wide variety of peptides which can be detected by mass spectrometry. The resulting mass spectra may be analyzed more exhaustively and each peptide fragmented used to obtain the specific sequence. The comparison between a treated and a control sample will provide information about a potential modification.

The study of the S-glutathionylation of transcription factors may be focused on the detection of glutathione incorporation by these methods. These approaches will lead to a more profound understanding of the possible implications of this postranslational modification in the modulation of gene expression by transcription factors as AP-1 or NF- κ B. Knowledge of the mechanisms of redox GSH regulation and gene transcription in inflammation could lead to the development of novel therapies based on the pharmacological manipulation leading to a desired intracellular availability of this important antioxidant in situations of inflammation or vascular injury.

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Sulphur-Containing Amino Acids, Glutathione and the Modulation of Inflammation

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Abstract: The diet of industrialised countries is usually rich in amino acids, which are partly used as a source of calories. However, metabolic alterations are observed in diseased patients and a preferential retention of Sulphurated Amino Acids (SAA) occurs during the inflammatory response. It has been demonstrated in an acute sepsis phase model in rats that the metabolism of L-Cysteine (Cys) is modified. Glutathione (GSH) concentration is greater in the liver, kidneys and other organs and Cys incorporation into proteins is higher in the spleen and lungs. In the plasma Acute Phase Proteins are released while Albumin is decreased. The pro-inflammatory cytokines such as Interleukin-1, Interleukin-6 and TNF- α are the main initiators altering protein and amino acid metabolism.

L-Methionine (Met) conversion to Cys is impaired under stress, such as in premature infants or AIDS patients. Thus, the metabolic flow through the trans-sulphuration pathway may be inadequate to meet the Cys demand under critical conditions.

These altered biochemical rules during inflammation weaken the anti-oxidizing functions, while the extra-supply of SAA under inflammatory conditions may help restore homeostasis.

1. Introduction

The release of radicals and oxidants from cells is a physiological process essential to defend against infection. However, oxidative stress is defined as a consequence of the production of reactive oxidative species (ROS) at a rate higher than that of antioxidant protection: a protective and physiological function turns negative and into a damaging mechanism. The immune system reaction may turn dangerous and overpowering when the production of ROS causes tissue injury. Moreover, this article describes the fate of sulphur-containing amino acids (SAA) in a general perturbation of the metabolism of amino acids during stress. The altered biochemical rules during inflammation weaken the anti-oxidizing functions and the extra-supply of SAA under inflammatory conditions may help restore homeostasis.

2. Acute Phase Response

The Acute Phase Response (APR) is a complex phenomenon involving many biochemical and functional mechanisms [1]. A recent review published in New England Journal of Medicine [2] gave the following definition:

"The Acute-Phase Response, an important pathophysiologic phenomenon, replaces the normal homeostatic mechanisms with new set points that are presumably contributing to defensive or adaptive capabilities. The functions of these changes are highly variable and diverse: some participate in initiating or sustaining the inflammatory process, others modulate it, and still others have adaptive roles".

APR also affects many organs far removed from the site of injury, as well as causing a deep perturbation of basal metabolism.

The main metabolic changes generally include:

- Loss of muscle tone and negative nitrogen balance
- Decreased gluconeogenesis
- Increased osteoporosis
- Increased hepatic lipogenesis
- Increased lipolysis in adipic tissues
- Decreased lipoprotein lipase activity in muscles and adipic tissues

Also, the liver is strongly affected and the main hepatic changes include:

- Increased synthesis of Metallothionein, inducible nitric oxide synthase, heme oxygenase, manganese superoxide dismutase, and tissue inhibitor of metalloproteinase-1
- Increased release of Retinol and Glutathione (GSH) in plasma

APR is characterised (Figure 1) by the hepatic synthesis of a large number of factors and proteins known as Acute Phase Proteins (APP). Based on the broad spectrum of its activities, this heterogeneous group of circulating proteins assists the injured organism in restoring homeostasis by assuming a protective role.

APP achieves this by inactivating vasoactive, proteolytic and cytotoxic molecules liberated from damaged tissues, accumulating phagocytes and participating in a feedback control mechanism that prevents the organism's immune response from being overloaded.

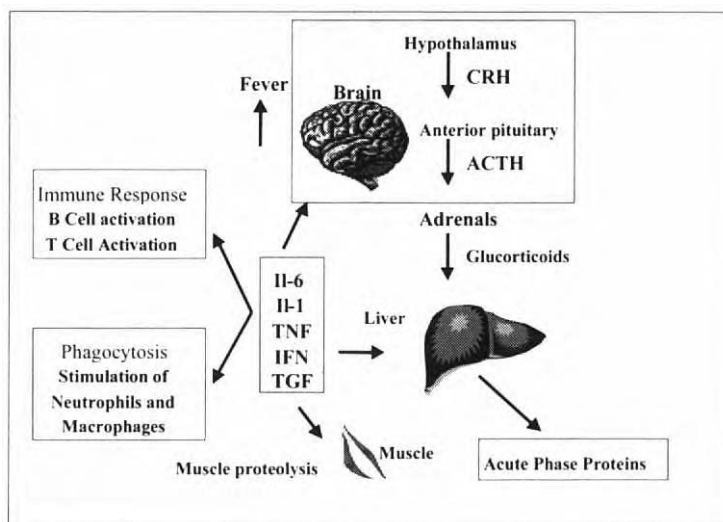


Figure 1. Acute Phase Reaction.

The stimulation of transcription of the APP genes in the liver is incorporated in a complex interchange of cytokines, growth factors and glucocorticoid hormones that are released during a systemic defence reaction.

These changes are induced by a complex intercellular signalling system, whose main constituents are inflammation-associated cytokines. Among other functions, Interleukin-1, Interleukin-6 and Tumour Necrosis Factor- α initiate the alteration of protein and amino acid metabolism designed to support the increased demand of amino acids to sustain the immune response. In particular, Interleukin-6, stimulates the production of hepatic APP. The relationship with the sulphurated amino acids (SAA) will be discussed in the following chapters.

3. The Metabolism of SAA is Modified During Stress

The diet of industrialised countries is rich in proteins and provides the physiological amount of SAA needed for the turnover and synthesis of the proteins in the organism. The absorption of L-Cysteine/L-Cystine by the intestines, originated by a normal or supplemented diet, is practically total. The excess of Cys is known to be quickly catabolized [3] (Figure 2).

The synthesis of both Piruvate and Taurine accounts for a significant fraction of the Cys catabolism in mice [4] and rats [5].

Under stable conditions, the tissue levels of free Cys and Cys equivalent are ultimately regulated and limited by the reaction rate of the Cys catabolism [6]. As an example of the great capacity to convert a high dosage of Cys, the metabolism of intravenous multi-grams infusion of N-acetylcysteine (NAC), the prototype of the Cys precursor [7–9], has been reported in patients undergoing liver transplantation [10].

Also, NAC is extensively catabolized into Sulphate and Taurine just after the implantation of a new organ, thus confirming a high hepatic capacity in metabolising a Cys excess even under such particularly severe stress conditions.

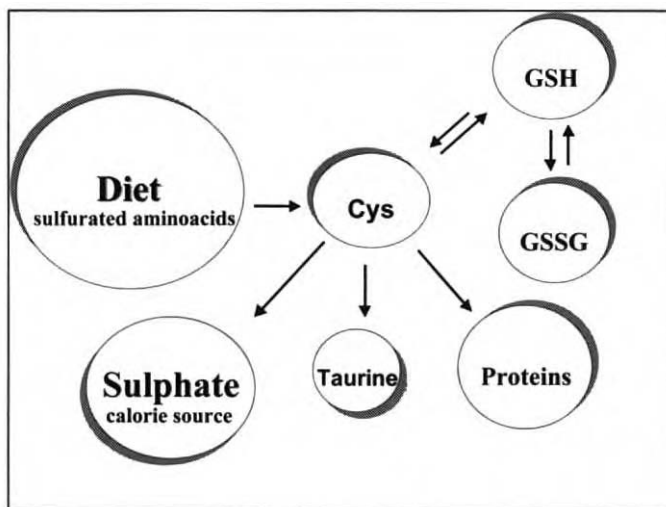


Figure 2. Cysteine is in excess in stable conditions.

However, metabolic alterations are observed under pathological conditions and a preferential retention of sulphur amino acids, evaluated as a urinary sulphur excretion, occurs during an inflammatory response [11–13]. Following a fracture or burns, urinary nitrogen excretion is enhanced to a greater extent than sulphur excretion [14]. Moreover, a

massive loss of sulphur has been shown to occur in HIV patients, thus confirming the peripheral tissue to be a site of massive Cys catabolism [15]. Indeed, the observation of such phenomena dates back to 1931 [16].

The metabolism of Cys has been studied in a model of sepsis set up to mimic the human state [17]. In rats infected with live *Escherichia Coli*, Sulphate production is significantly lower, whereas the higher production of Taurine in the liver could may play a protective role against oxidative stress [18]. GSH concentration is significantly greater in the liver, kidneys and other organs. Finally, Cys incorporation into protein is higher in the spleen and lungs, and in particular in the whole plasma proteins while the albumin level decreases. The latter effects are interpreted to be inducing APP synthesis. Thus, inflammation modifies the contribution of different organs to whole-body protein synthesis, and a protein shortage may impair the APP protein response in human and experimental animals.

Other data further support the increased requirement for Cys during infection [19,20].

Finally, the food intake is generally decreased due to the anorexia induced by the fundamental action of cytokines decreasing the supply of amino acids and other nutrients.

I am indebted in my presentation of the excellent work of Robert Grimble of the Southampton University [21–24], who studied the key role of Cys in the amino acid economy of the body under inflammatory conditions. He effectively explains the biochemistry of SAA as being linked-up with the recent findings of molecular biology on the regulation of transcription factors. Based on the proposed immunomodulatory role played by SAA, he wrote:

“... Within the liver there will be competition between acute phase protein and GSH synthesis for the cellular sulphur amino acid pool. The question therefore arises whether incorporation of Cysteine into both of these end-products, during the inflammatory response, is influenced equally by alteration in dietary sulphur amino acid intake... An insufficient intake of sulphur amino acids will thereby exert a pro-inflammatory influence...

The ability to maintain and enhance tissue GSH may be of particular importance in controlling cytokines production in response to inflammatory stimuli, because the stimulatory influence of oxidant molecules and TNF-alpha, on NFkB activity, is decreased by GSH and other sulphur-containing compounds...

The typical acute phase response included increases in C-reactive protein, fibrinogen ... amounting to a total increased in acute phase protein of 850 mg/kg body weight. To cover the requirements for all amino acids to support this increased synthesis of hepatic proteins, a breakdown of 1980 mg/kg of muscle proteins were required, because there is a mismatch between the amino acid composition of the APP and muscle proteins”.

Another aspect influencing the availability of SAA is the impairment of the Met conversion to Cys under stress.

The rate of Cys synthesis from Met (a process dependent on the Cystathionase pathway) was found to be significantly higher in isolated hepatocytes than in hepatocyte controls in rats suffering from surgical stress [25]. The same has been observed in septic rats [26].

Premature infants synthesise GSH from Met at a much lower rate than fully developed infants [27]. Most recently, the same impairment has been reported in AIDS patients [28].

It is likely that the conversion of Met to Cys is generally impaired during inflammation. Thus, the metabolic flow through the trans-sulphuration route may be inadequate to meet the GSH and Cys requirement.

Figure 3 summarises the modified SAA biochemistry during the inflammation. This confirms why Cys, a simple non-essential amino acid and present in large excess during the diet, may be considered to be a conditionally-essential agent.

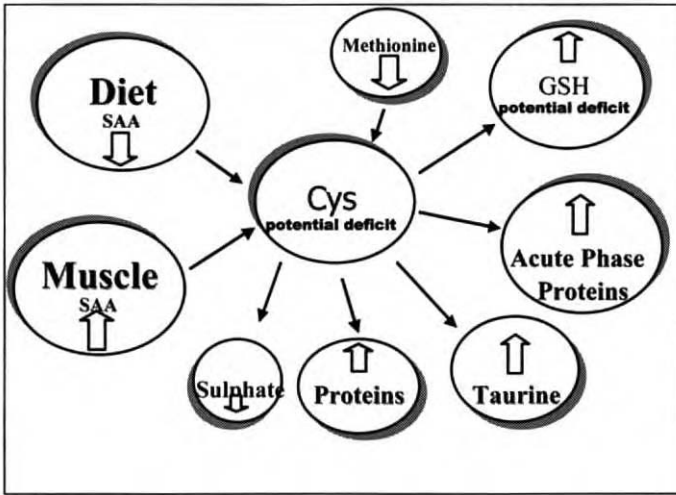


Figure 3. Metabolism of cysteine changes in inflammation.

4. The importance of Stable GSH Levels

GSH performs many physiological functions including antioxidant defence, detoxification of xenobiotics, modulation of redox regulated signal transduction, storage and transport of Cys, regulation of cell proliferation, synthesis of deoxyribo-nucleotide, regulation of immune response and regulation of leukotriene and prostaglandin metabolism [29,30].

In partnership with ascorbic acid, GSH constitutes the body's major antioxidant defence system [31]. The liver is a main source of circulating GSH. It has been estimated that approximately 45 mmol (or 14 g) of GSH are released into the circulatory system of humans during a 24 h period [32].

GSH is capable of increasing the activation of cytotoxic T-cells *in vivo* [33]. The normal functioning of T-lymphocytes depends on the cellular supplies of Cys. The cells acquire the amino acid largely as a result of an uptake of GSH by macrophages and lymphocytes. Impaired immune responses are associated with a reduction in the GSH concentration of immune tissues [34].

In rats, an infiltration of inflammatory cells into the lungs, in response to cytokines, was noted to occur in the absence of Cys and Met in a low protein diet, and was prevented by their addition to the diet [35].

In addition, in rats a non-lethal dose of TNF- α becomes lethal if the ability of the animal to increase and maintain GSH synthesis is prevented by administration of diethylmaleate [36].

The exposure to hyperoxia for several days increases epithelial lining fluid (ELF) GSH levels, and tobacco smokers also show an increase in ELF GSH at a concentration of nearly twice the normal level [37,38]. On the contrary, reduced leukocyte concentration of GSH, Met and Cys and decreased urinary excretion of inorganic Sulphate are observed in severely burned patients [39].

Critical illness is associated with alterations in muscle GSH metabolism [40–42]. Skeletal muscles are reduced and total GSH concentrations drop, thus indicating a condition of oxidative stress and an augmented release of GSH from this tissue.

Such findings are reported even under chronic conditions.

Chronic Obstructive Pulmonary Disease (COPD) is often characterised by an impaired

skeletal muscle energy metabolism associated with decreased GSH muscle levels [43].

In a large number of uremic patients affected with chronic mild to severe renal failure and a group suffering from terminal renal failure and placed on maintenance hemodialysis, the total GSH was evaluated and found to lead to a progressive decrease in the plasma levels. The GSH loss is correlated with the intensity of renal failure, culminating in dialysis. A GSH decline could therefore contribute to a progressive renal insufficiency and associated complications, such as the accelerated atherosclerosis typical of this class of patients [44]. A growing evidence in several other diseases including HIV, cancer, sepsis, trauma, and diabetes suggests that the abnormal metabolism of Cys and GSH is involved in developing a catabolic activation followed by immunological disfunctions [45,46].

In addition to GSH, a new line of research is devoted to the evaluation of the total intracellular thiol status. The defective thiols status of the peritoneal macrophages in peritoneal dialysis patients [47] and of the alveolar macrophages in COPD patients and smokers [48] has recently been reported.

GSH can exert an influence on the immune function not directly related to its role as an antioxidant.

Besides the physiological relevance of reactive oxygen species (ROS) in regulating the intracellular signalling and activating the transcription factors encoded during the synthesis of pro-inflammatory molecules e.g. cytokines and leukotrienes, oxidative stress further lowers the intracellular thiol and GSH levels related to the activated metabolism of SAA.

The ability to maintain and enhance tissue GSH may be of particular importance in controlling cytokine production in response to inflammatory stimuli, because GSH and SAA decrease the stimulatory influence of oxidizing molecules on the NF- κ B activity.

The pro-inflammatory mediators stimulate the cellular synthesis of ROS, which once more activates the cell functions [49,50].

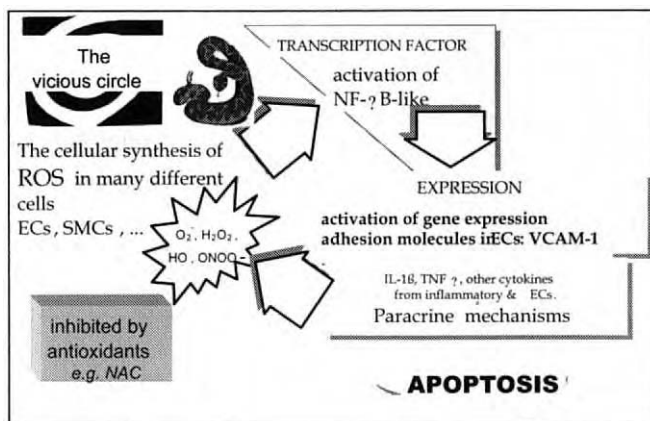


Figure 4.

A vicious cycle is thus activated (Figure 4) which amplifies the inflammation stimuli at each cycle. GSH and thiol depletion may thus exert an influence on the immune functions not directly related to their role as antioxidants but as key activators of cell functions.

The literature abounds in studies on thiol regulation of transcription factor activation. However and more relevant to physio-pathological conditions, a great body of evidence is available on supplementing NAC in several different animal models.

NAC abolishes NF- κ B activation in a model of:

- acute lung injury by LPS in rats [51]
- alloxan-induced diabetes in mice [52]
- cerulein-induced pancreatitis in rat [53]
- doxorubicin-induced cortical tubulointerstitial injury in protein-uric rats [54].

In this complex picture, the administration of a Cys precursor like NAC may elicit an anti-inflammatory effect, as it may break the vicious cycle.

5. Glutathionylation to Protect Protein Functions

Under moderate oxidative stress conditions, intracellular protein thiols can be modified by reversible S-thiolation, but the prolonged oxidative stress can cause irreversible modifications [55–57].

S-Thiolation of proteins is the formation of mixed disulphides between protein thiols and low molecular-weight thiols such as Cys and GSH (Figure 5).

S-glutathionylation is defined as a condition when the thiol is GSH. Due to the fact that GSH represents 95% of free intracellular thiols, S-glutathionylation is actually the most important reversible modification of the Cys residue of a protein structure. Such a phenomenon is observed during the respiratory burst of neutrophils and in cells exposed to oxidants.

Protein glutathionylation may play a key role in regulating protein functions and consequently in controlling the signal transduction.

In conclusion, in addition to regulating the thiol Redox State, S-glutathionylation appears to be a protective mechanism of the 3D structure in cases of oxidative stress, and is reversed when oxidative stress is decreased. On the other hand, such a phenomenon is also a means of storing GSH.

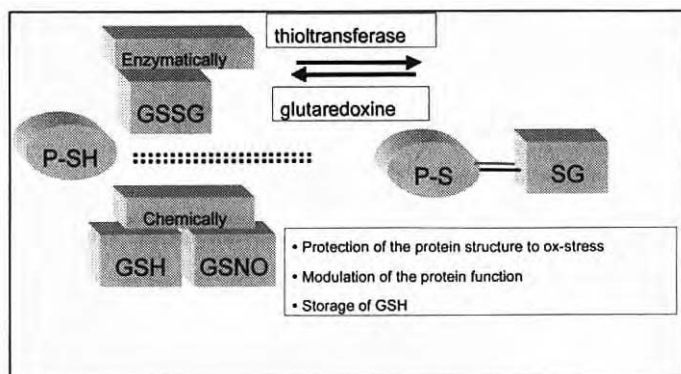


Figure 5. S-Glutathionylation of Enzymes and Transcription Factors.

6. Conclusions

- Production of cytokines, APP and GSH are strongly modified during inflammation.
 - GSH participates in many important physiological process of a cell's control of homeostasis.
 - Higher levels of Cys supply are necessary in maintaining a constant GSH level.
 - The role of GSH as a key regulator of thiol redox intracellular balance is confirmed.
- During inflammation a shortage of GSH may occur due to the oxidative stress and the

alliterated consumption of GSH in relation to the APP synthesis. The activation of an inflammatory mediator production induces an accelerated oxidative stress and perturbation of the metabolism of SAA. The increased GSH consumption reduces the protection of protein integrity by S-glutathionylation. This vicious cycle is occurring where the physiological immune response paradoxically causes a self-propagating disease (Figure 6).

It is evident that GSH plays an essential role in regulating the cell's life cycle, and that the decrease of intracellular GSH contributes to chronic inflammation.

A scientific approach capable of stopping this spiral should bring further surprises in the future.

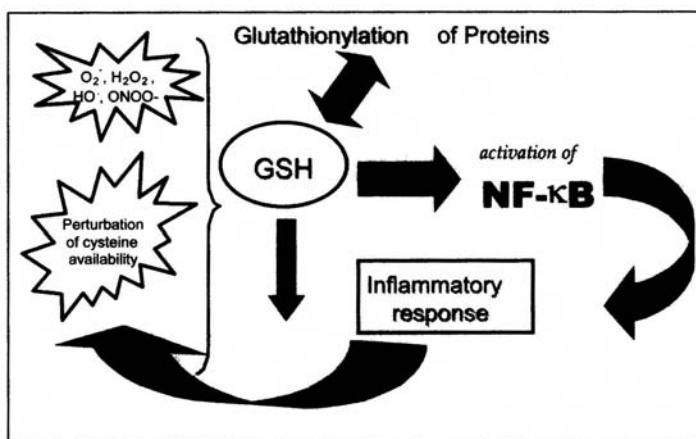


Figure 6. Central role of GSH regulation of cell life.

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Molecular Events of the Inflammation Process that are Affected by α -Tocopherol. Antioxidants and Gene Expression in the Process of Inflammation and Wound Repair

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Abstract: The properties of vitamin E and in particular of α -tocopherol that are relevant to the process of inflammation and wound repair are discussed below. They will be integrated in the description of the inflammatory process and of tissue repair.

1. Vitamin E

The term "Vitamin E" was introduced by Evans and Bishop to describe a dietary factor important for reproduction in rats [1]. Natural vitamin E includes two groups of closely related fat-soluble compounds, the tocopherols and tocotrienols, each with the four α -, β -, γ -, δ -analogs (Figure 1). The eight analogous compounds are widely distributed in nature and the richest sources are latex lipids (8% w/v), followed by edible plant oils. Sunflower seeds contain almost exclusively α -tocopherol (59.5 mg/g of oil), oil from soybeans contains the γ -, δ -, and α -tocopherol (62.4, 20.4, and 11.0 mg/g oil), while palm oil contains high concentrations of tocotrienols (17.2 mg/g oil) and α -tocopherol (18.3 mg/g oil) [2]. Although the antioxidant property of these molecules is similar, clear individual biological effects can be distinguished at a molecular level. The resulting specificity is the consequence of a selective retention of α -tocopherol in the body, and to the preferential interactions of some of the compounds with molecular components of the cells.

2. Antioxidant properties of α -tocopherol

Although it is common believe that phenolic compounds like vitamin E exert a protective role against free radical damage, antioxidant molecules can exert additional biological functions. The estrogen 17- β -estradiol, for instance, has antioxidant capacity [3] which has been proposed to protect women from coronary artery disease, but the determination of secondary sexual features is not mediated by its antioxidant activity. *All-trans*-retinol is again a potent antioxidant [4], but the main function of retinol in rhodopsin and vision is not related with this property.

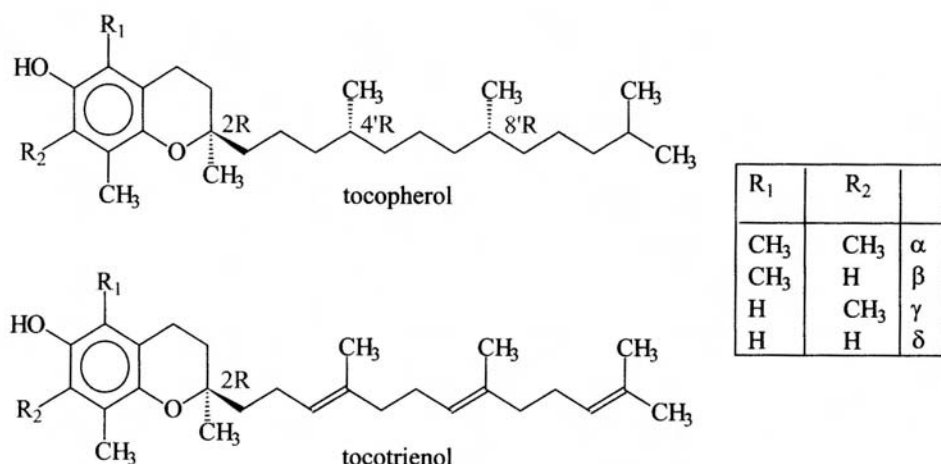


Figure 1. Molecular formulae of tocopherols and tocotrienols

Vitamin E is the major hydrophobic chain-breaking antioxidant that prevents the propagation of free radical reactions in the lipid components of membranes, vacuoles and plasma lipoproteins.

The antioxidant properties of vitamin E are well known and documented [5]. In particular, prevention by α -tocopherol of LDL oxidation has been studied [6]. Although the correlation between the level of LDL oxidation and atherosclerosis is not always evident [7], alternative studies have suggested that α -tocopherol protection against LDL oxidation may be secondary to the inhibition of protein kinase C (PKC). This enzyme seems to be responsible for the release of reactive oxygen species and lipid oxidation [8,9].

3. Pro-oxidant properties of α -tocopherol

In contrast with all the described antioxidant properties of vitamin E, it has been shown that lipid peroxidation of LDL is faster in the presence α -tocopherol, and is substantially accelerated by enrichment of the vitamin in LDL, either *in vitro* or *in vivo* [10, 11]. It was thus proposed that peroxidation is propagated within lipoprotein particles by the vitamin E radical (*i.e.* α -tocopheroxyl radical) unless it became reduced by vitamin C or ubiquinol-10 [12]. However, the importance of pro-oxidation reactions of α -tocopherol *in vivo*, under physiological conditions, appears to be questionable.

4. Antialkylating properties of α -tocopherol

Nitric oxide released by macrophages during inflammation reacts with active oxygen to form peroxynitrite. Peroxynitrite nitrates protein and peroxidizes lipids. γ -Tocopherol (the principal form of vitamin E in the United States diet) and α -tocopherol (the major form present in the European diet and in supplements), both protect against peroxynitrite-induced lipid oxidation. [13]. Christen et al. reported that lipid hydroperoxide formation in liposomes is inhibited more effectively by γ -tocopherol than α -tocopherol by a non-antioxidant mechanism [14]. However, Goss et al. [15] concluded that the presence of α -tocopherol attenuates nitration of both γ -tocopherol and tyrosine, showing that nitration of

γ -tocopherol becomes significant only after α -tocopherol depletion. This would imply that α -tocopherol alone is sufficient to remove any peroxynitrite-derived reactive nitrogen species *in vivo* [15].

5. Non-antioxidant effects of α -tocopherol

The non-antioxidant properties of tocopherol were discovered when, in several experimental models, the four tocopherol analogues had different effects, although they share a similar anti-oxidant capacity. It can be speculated that the selective uptake and transport of α -tocopherol represents the evolutionary selection of a molecule with specific functions, different from its antioxidant properties.

In the sections below, a discussion of the effect of α -tocopherol at cellular level will be carried out, particularly focusing on the non-antioxidant properties shown by the molecule (Tables 1 and 2).

Table 1. Inhibition of cell proliferation by α -tocopherol in different cell lines

Sensitive cells	Insensitive cells	Tissue and origin
A10, A7r5		Rat aorta smooth muscle
T/G		Human aorta smooth muscle
NB2A		Mouse neuroblastoma
U937		Human leukemia
C6		Glioma
DU-145, PC-3		Human Prostate Cancer
LNCaP		Human Prostate Cancer (androgen sensitive)
HPRE		Human Pigmented Retinal Epithelial Cells
Balb/3T3		Mouse fibroblast
Human fibroblast		Primary cell lines
	P388 D1	Mouse monocyte macrophage
	Saos-2	Human osteosarcoma
	HepG2	Human hepatocarcinoma

Table 2. Effects of α -tocopherol and their supposed molecular mechanisms

Reaction	Proposed mechanism ¹	Reference
Inhibition of cell proliferation	NA	[46,47]
Inhibition of platelet adhesion and aggregation	NA/ND/A	[75-77]
Inhibition of cell adhesion	NA/ND/A	[69-74]
Inhibition of ROS ² in monocytes and neutrophils	NA/A	[52,81]
Inhibition of PKC	NA/A	[46,47,56,57]
Activation of PP ₂ A	NA	[56,58]
Inhibition of 5-lipoxygenase	NA/A	[53]
Activation of diacylglycerol kinase	NA/A	[79]
Inhibition of α -tropomyosin expression	NA	[61]
Inhibition of liver collagen α 1 expression	ND	[62]
Inhibition of collagenase MMP1 expression	NA	[63]
Modulation of α -TTP expression	NA	[64]
Inhibition of scavenger receptor SR-A	NA	[65]
Inhibition of scavenger receptor CD36	NA	[66,67]
Inhibition of ICAM-1 and VCAM-1 expression	ND	[72]

¹A, antioxidant; NA, nonantioxidant; ND, not discussed

²ROS, reactive oxygen species; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule.

5.1 Effects of α -tocopherol at cellular level

In 1991 inhibition of PKC activity was found to be at the basis of the vascular smooth muscle cell growth arrest induced by α -tocopherol [16,17]. A number of reports have subsequently confirmed the involvement of PKC in the effect of α -tocopherol on different cell types, including monocytes, macrophages, neutrophils, fibroblasts and mesangial cells [8,18-20]. α -Tocopherol, but not β -tocopherol, was found to inhibit thrombin-induced PKC activation and endothelin secretion in endothelial cells [21]. α -Tocopherol, and not β -tocopherol or trolox, inhibits the activity of PKC from monocytes, followed by inhibition of phosphorylation and translocation of the cytosolic factor p47(phox) and by an impaired assembly of the NADPH-oxidase and of superoxide production [22]. α -Tocopherol has the important biological effect of inhibiting the release of the proinflammatory cytokine, IL-1 β , via inhibition of the 5-lipoxygenase pathway [23].

Inhibition of PKC by α -tocopherol in vascular smooth muscle cells is observed to occur at concentrations of α -tocopherol close to those measured in healthy adults [24]. β -Tocopherol *per se* is not very effective but prevents the inhibitory effect of α -tocopherol. The mechanism involved is not related to the radical scavenging properties of these two molecules, which are essentially equal [25]. *In vitro* studies with recombinant PKC have shown that inhibition by α -tocopherol is not caused by tocopherol-protein interaction. α -Tocopherol does not inhibit PKC expression as well. Inhibition of PKC activity by α -tocopherol occurs at a cellular level by producing dephosphorylation of the enzyme, whereby β -tocopherol is much less potent [26]. Dephosphorylation of PKC occurs *via* protein phosphatase PP_{2A}, which is activated by the treatment with α -tocopherol [26-28].

The group of King [29] has reported that prevention of glomerular dysfunction in diabetic rats can be achieved by treatment with α -tocopherol. Such a protection occurs through inhibition of PKC. In this case, however, α -tocopherol would act on the diacylglycerol pathway, by activating the enzyme diacylglycerol kinase with consequent diminution of diacylglycerol and PKC activation. In these studies, high glucose was responsible for increased diacylglycerol synthesis, which was counteracted, in the presence of α -tocopherol, by the activation of diacylglycerol kinase.

5.2 Transcriptional regulation by α -tocopherol

Recently, the possibility of gene regulation by α -tocopherol has been analyzed [30]. Upregulation of α -tropomyosin expression by α -tocopherol, and not by β -tocopherol, once more suggests a non-antioxidant mechanism [31]. Long- and short-term α -tocopherol supplementation inhibits liver collagen α 1(I) gene expression [32]. In human skin fibroblasts age-dependent increase of collagenase expression can be reduced by α -tocopherol [33].

In rats, the liver α -tocopherol transfer protein (α TTP) and its mRNA are modulated by dietary vitamin E deficiency [34]. Scavenger receptors, particularly important in the formation of atherosclerotic foam cells, are also modulated by α -tocopherol. In smooth muscle cells and monocytes/macrophages, the oxidized LDL scavenger receptors SR-A and CD36 are down regulated at transcriptional level by α -tocopherol but not by β -tocopherol [35-37]. The relevance of CD36 expression in the onset of atherosclerosis has been clarified by Febbraio and coworkers, who have shown that disruption of the CD36 gene protects against atherosclerotic lesion development in mice [38].

The following questions remain open. In some cases differential effects of α -tocopherol and β -tocopherol have been found, pointing to a non-antioxidant mechanism at the basis of gene regulation [31,36]. In other cases, however, only α -tocopherol has been tested leaving the mechanism of α -tocopherol action unclarified. Furthermore, the

involvement of PKC has not always been assessed and it remains to be established whether the transcriptional regulation of certain genes is a consequence of PKC inhibition.

5.3 Inhibition of monocyte-endothelial adhesion

α -Tocopherol enrichment of monocytes and polymorphonuclear leukocytes decreases agonist-induced and LDL-induced adhesion to human endothelial cells both *in vivo* and *in vitro* [39-41]. Monocytes as well as neutrophils diminution of adhesion induced by α -tocopherol is dependent on the inhibition of adhesion molecules expression [42-44]. These events are relevant to the onset of inflammation as well as in the early stages of atherogenesis.

5.4 Inhibition of platelet adhesion and aggregation

α -Tocopherol inhibits aggregation of human platelets by a PKC-dependent mechanism both *in vitro* and *in vivo* [19,45-47]. Another study has indicated that both α - and γ -tocopherol decrease platelet aggregation and delay intra-arterial thrombus formation [46]. The fact that γ -tocopherol was significantly more potent than α -tocopherol suggests that a simple antioxidant mechanism is not applicable to these effects.

The studies reported above are consistent with the conclusions of Iuliano et al. [48] that circulating LDL accumulates in human atherosclerotic plaques and that such accumulation by macrophages is prevented by α -tocopherol *in vivo*. The protection by α -tocopherol may not be due only to the prevention of LDL oxidation, but also to the down regulation of the scavenger receptor CD36 and to the inhibition of PKC activity.

Although not all scientific groups agree on the molecular details, PKC inhibition is accepted as a common denominator of a number of cellular events regulated by α -tocopherol: cell proliferation, cell adhesion, enhancement of immune response, free radical production and gene expression. However, the molecular mechanisms at the basis of these events are not yet fully elucidated. A number of observations, such as PP2A [16] and diacylglycerol kinase [49] activation, 5-lipoxygenase [50] and cyclooxygenase [51] inhibition, still miss a mechanistic explanation. On the other hand, the expression of several genes, such as CD36 [36], SR class A [35], collagenase [33], and ICAM-1 [42], appears to be regulated by α -tocopherol in a PKC independent way. A further understanding of the molecular events at the basis of α -tocopherol gene regulation is part of current studies.

In conclusion, a number of events are related to non-antioxidant properties of α -tocopherol (Table 2), both at transcriptional and posttranscriptional level. However, whether α -tocopherol acts by a pleiotropic mechanism, or it binds to a receptor capable of regulating different reactions, still remains unknown.

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Redox Regulation, Cytokine, and Nitric Oxide in Inflammation

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Abstract: Inflammation induces oxidative stress, lipid and protein oxidation and modifies redox equilibrium. At the whole body level, inflammation and oxidative stress contribute to the pathogenesis of many human diseases including atherosclerosis, and related cardiovascular diseases, leading cause of morbidity and mortality in Western countries.

Inflammation is evidenced by increased levels of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, tumour necrosis factor (TNF α), and C-reactive protein (CRP). The search for the link between these molecular events recorded in the inflammatory process and the derangement of redox equilibria along with the consequence on nitric oxide regulation is the aim of this chapter.

The molecular mechanism linking the inflammatory response to redox equilibria and modification of nitric oxide production will be explored in an animal model system of septic shock, a generalized inflammation induced by bacterial lipopolysaccharide (LPS). It is known that endotoxemia induces a complex interplay between the activation of nuclear transcription factors such as nuclear factor kappa B (NF κ B) and a cascade-activation of various enzymatic activities, mostly mediators of the inflammatory response with particular attention to the variation of the inducible form of nitric oxide synthase (iNOS).

The inflammatory reaction is a complex series of iterating cascades with positive and negative feedback loops that provide a vast range of responses. Messengers and effectors of the inflammatory process have been identified in a wealth of studies. Probably the best series, where detailed and up-to-date information can be found on these topics is the "Progress in Inflammation Research" series published by Birkhäuser (Basel). Of particular interest for the readers are the following books: "Free Radicals and Inflammation" (Winyard et al.) [1] and "Nitric Oxide and Inflammation" (Salvemini et al.) [2].

Since the early studies, where the involvement of the superoxide anion radical in the bactericidal action of inflammatory cells was demonstrated, free radical-dependent oxidative stress and the inflammatory response are inextricably linked [3]. Oxidative stress has traditionally been viewed as a stochastic process of cell damage resulting from aerobic metabolism, and antioxidants have been viewed as free radical scavengers. Recently it has been recognized that reactive oxygen species (ROS) are widely used as second messengers to propagate pro-inflammatory or growth-stimulatory signals [4,5], and that classic antioxidants, like α -tocopherol, plays important non-antioxidant roles [6,7].

We would like to draw here the attention to a few relevant aspects necessary to comprehend the specific theme of this chapter.

1. Inflammation and oxidative stress

ROS are known to be generated in large amount under inflammatory conditions. At low levels, ROS can function as signalling molecules, participating as signalling intermediates in regulation of fundamental cell activities, such as cell growth and cell adaptation responses, whereas at higher concentrations, ROS can cause cellular injury and death. Oxidative stress increases vascular endothelial permeability and promotes leukocyte adhesion, which are coupled with alterations in endothelial signal transduction and redox-regulated transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappa B (NFkB).

Oxidative stress constitutes a major driving force leading to the inflammatory response. It can be asserted that oxidative stress and, especially chronic inflammation are related, probably inseparable phenomena. Pharmacological strategies should aim at supplementing antioxidant defence systems while antagonizing redox-sensitive signal transduction leading to improved clinical management of the inflammatory process.

1.1 Iron and inflammation

An important contribution to the increase in oxidative stress observed in the inflammation is given by iron. During inflammation iron is delocalised from specific carriers and from intracellular binding sites. The release of iron in tissues induces free radical reactions, leading in turn to functional disorders and eventual organ failure [8,9]. Generally, intracellular iron is derived from plasma transferrin which is delivered by relatively well characterized mechanism involving receptor-mediated endocytosis [10,11]. Transferrin-transferrin receptor complex becomes trapped within endocytic vesicles, termed endosomes. The endosomes facilitate the reductive release of iron from transferrin. Reduced iron is transported across the endosomal membrane into the cytosol and becomes included in different intracellular iron pools. The main part of intracellular iron is bound by the iron storing protein ferritin; another part is used for synthesis of iron-containing enzymes; a small part, the so called 'free' iron or transit iron pool [12], is not bound by these proteins and occurs mostly as low molecular complexes, with a low stability constant. These iron ions are available for a number of chelators, like desferrioxamine and phenanthroline. Iron pool associated with ferritin has no catalytic activity, in contrast to free iron pool which is able to catalyse free radical reactions like lipid peroxidation, oxidative degradation of proteins and DNA [13,14].

There are major analytical difficulties in determining free iron concentration; in relation to the relevance of free iron in modulating inflammation. Our laboratory has developed a technique to determine free iron using Electron Spin Resonance (ESR) spectroscopy. Two tests, measuring the nitric oxide (NO)-available and the desferal-chelatable iron, have been developed and applied to the measurement of 'free' iron *in vivo*, giving comparable results [9,15]. The assays are based on the formation of relatively stable paramagnetic compounds. In the first case the reaction of free iron with NO, produced by the reduction of sodium nitrite, lead to the formation of a dinitrosyl-iron complex, characterized by an ESR absorption at $g = 2.03$ [16]. In the second case, the test measures the desferal available iron, which also give rise to a broad ESR spectrum, absorbing at $g = 4.3$.

There are some experimental evidences that the control of iron ameliorates the outcome of a grave inflammatory process. For instance, in a model system of sepsis induced in mice by the injection of lethal doses of lipopolysaccharide (LPS), the pre-treatment with desferal caused the reduction of tumour necrosis factor alpha (TNF- α) serum levels and an increase in the rate of survival [17]. Nonetheless there are very few studies in this area.

1.2 Lipid peroxidation

There is increasing evidence that the lipid peroxidation process is involved in many of the pathological expressions associated with inflammation, particularly in diseases characterised by chronic inflammation lesions. The most widely spread disease and major cause of death in the western hemisphere, is atherosclerosis. In the pathogenesis of atherosclerosis enhanced oxidative stress, along with a consequent alteration of the redox state, modulates a set of pro-inflammatory genes regulated directly, or indirectly, by ROS. Inflammatory phenomena at the site of the atherosclerotic plaque are the major determinants of the progression and clinical outcome of the disease. The LDL oxidation theory is one important consequence of a generalized metabolic abnormality of the arterial wall in atherosclerosis, rather than being the core pathophysiological feature. The fact that hypercholesterolemia, hypertension, and diabetes mellitus all activate similar redox-sensitive pro-inflammatory genes associated with the pathogenesis of atherosclerosis provides the potential for the development of unifying concepts concerning the etiology of the disease.

The recent finding that lower, rather than higher, cholesterol levels are associated with poor clinical outcome in patients with chronic heart failure has lead to hypothesise an important role of endotoxins, and the ensuing inflammation induced by it, in the atherosclerotic lesion progression. The ability of lipoproteins to bind endotoxins and to serve as natural buffer substances may explain the relationship between lower lipoprotein levels, higher cytokine concentrations and impaired prognosis [18].

Among the products which originate from the peroxidation of cellular membrane lipids, 4-hydroxy-2-nonenal (HNE) is believed to play a major role in causing the cytopathological effects observed during oxidative stress *in vivo* [19]. However a definite role for this widely studied compound is still under much debate. Aldehydes are mitogenic to vascular smooth muscle cells and sustain a vascular inflammation, hence are thought to favour atherosclerosis. However it has been reported that HNE down-regulates the LPS-induced activation of the transcription factor NF- κ B [20], followed by the inhibition of the expression of adhesion molecules induced by inflammatory stimuli in human aortic endothelial cells [21]: hence an anti-inflammatory role. Nonetheless, this inhibitory effect may well lead to a low levels chronic inflammation and may also be involved in other inflammatory/degenerative diseases.

1.3 Glutathione

Glutathione (GSH) has a vital role as an antioxidant, a regulator of inflammation, immune response, and cell viability, controlling the redox status in the human body. Its intracellular concentration, especially in inflammatory cells, is particularly high and new findings are beginning to reveal the role that the GSH pool plays in controlling inflammation.

As mentioned, ROS generated by cells recruited to the site of inflammation, are a major cause of oxidative stress and ensuing cell damage. When ROS production increases, the redox balance alters, being GSH, along with the regulatory machinery, the major intracellular antioxidant.

GSH is synthesized from its constituent amino acids by the sequential action of gamma-glutamylcysteine synthetase (γ -GCS) and GSH synthetase. The rate-limiting enzyme in GSH synthesis is γ -GCS. Interestingly γ -GCS expression is also modulated by intracellular redox state in a delicate balance among oxidants, antioxidants, inflammatory and anti-inflammatory agents [22].

GSH plays a relevant role in detoxification processes, such as those linked to aldehyde metabolism, source of highly reactive and oxidative stress inducing compounds,

derived from the oxidation of low-density lipoproteins. From LDL oxidation high concentrations of unsaturated aldehydes, such as HNE are generated. Aldehydes are mitogenic to vascular smooth muscle cells and sustain vascular inflammation. Major metabolic pathways and products of HNE are linked to GSH: glutathionyl-1,4 dihydroxynonene and 4-hydroxynonanoic acid have been identified as the major metabolites of HNE [23].

Moreover GSH concentration indirectly controls a host of redox-sensitive transcription factors such as NF- κ B and AP-1, modulates the genes for pro-inflammatory mediators as well as protective antioxidant genes such as γ -GCS, Mn-superoxide dismutase, and heme oxygenase-1. Also TNF- α , p38 MAP kinase activation and p38 MAP kinase-mediated RANTES (regulated upon activation, normal T-cells expressed and secreted) production is redox regulated [24]. The role of RANTES in the inflammatory and allergic response has been recently elucidated [25], indicating a role of intracellular GSH also in this particular field of inflammation.

In summary, GSH regulates the critical balance between the induction of pro-inflammatory mediators and antioxidant genes, hence it can be considered as a very important modulator of the inflammatory process. Despite this extremely important function, the regulation of the levels of GSH in response to free radical production and oxidative stress at the site of inflammation is poorly known. Knowledge of the mechanisms of redox GSH regulation and gene transcription in inflammation could lead to the development of novel therapies based on the pharmacological manipulation of the production of this important antioxidant in inflammation and injury.

1.4 Markers

There is a great need for non-invasive tests of several parameters of oxidative stress and redox homeostasis during inflammation. Many tests are available, a few entered the clinical practice, most still do not reach to the clinics.

More and more markers have been proposed for a concomitant determination of inflammation, oxidative stress and redox status markers. Inflammation is readily identified by a long list of "classic" markers such as white blood cell count (WBC) cytokines and chemokines, acute-phase proteins (C-reactive protein, fibrinogen and alpha1-antitrypsin), determination of erythrocyte sedimentation rate, rheumatoid factor (RF), serum iron levels, total iron-binding capacity (TIBC) and serum ferritin levels [26]. A wide list of "novel" markers also exists, including NF- κ B, AP-1, soluble ICAM-1 and soluble thrombomodulin, prothrombin fragment, fibrin and fibrinogen degradation products, eosinophil cationic protein (ECP), soluble receptor of interleukin-2 and 4, soluble intercellular adhesion molecule-1, granulocytic proteins myeloperoxidase and lactoferrin, LPS-binding protein [27]. A marker strongly associated to inflammation and of routine clinical use is homocysteine. Hyperhomocysteinemia has been related to cardiovascular diseases and inflammation [28,29]. It is now widely accepted that inflammation is accompanied by hyperhomocysteinemia, and is associated with cardiovascular risk in the general population. While there are substantial epidemiological data confirming that this risk factors is associated with cardiovascular risk, a causal relationship has not been established. Homocysteine regulates NF- κ B controlled gene transcription, posing itself at a crossroad as a marker of redox status and inflammation, opening a new perspective for a pathway by which homocysteine might enhance chronic inflammation of the endothelium, contributing to the development of atherosclerosis [30].

Various markers, as stressed above, often indicates not only the presence of an inflammatory process, but also give hints to the determination of redox status and oxidative stress. Unfortunately there are no reliable and clinically useful parameters for the specific

determination of oxidative stress and redox status.

The assays most widely employed are the measurement of thiobarbituric acid-reactive species (TBARS) and the formation of conjugated dienes, markers of lipid peroxidation [31-33]; the determination of advanced oxidation protein products (AOPP), a marker of protein oxidation, and of advanced glycation end-products (AGE) [34-37]; the measurement of erythrocyte antioxidant potential [38]. Of particular importance is the isoprostanes determination, since these compounds are formed by the free radical catalysed peroxidation of arachidonic acid, which is independent of the cyclooxygenase enzyme, giving rise to stable compounds, measurable in urine [39]. As recently assessed in a Workshop on markers of oxidative damage and antioxidant protection [40], currently available methods for the determination of antioxidant and redox status are not yet generally suitable for routine clinical applications, essentially for the lack of standardized tests.

2. Redox-regulated transcription factors and inflammation

Of the burgeoning number of regulatory factors, those who appear to be sensitive to a modification of the redox equilibrium are NF- κ B and AP-1 [41,42].

NF- κ B resides in the cytosol of many cell types involved directly and indirectly in the inflammatory process coupled to its inhibitor I κ B α . The degradation of I κ B α permits the migration to the nucleus of the active molecule, where it regulates a battery of inflammatory genes. NF- κ B induces gene programs leading to transcription of factors such as leukocyte adhesion molecules, cytokines, chemokines [43,44], heme oxygenase (HO)-1, cyclooxygenase-2 (COX-2). Of particular interest is the specific induction of gene expression of HO-1, COX-2, and inducible nitric oxide synthase (iNOS). The transcriptional activation of the RANTES promoter, which plays an important role in the production of allergic inflammation of the airway, is also NF- κ B-dependent. Cellular redox changes are involved in the activation of p38 mitogen-activated protein (MAP) kinase, leading to the induction of cytokine expression [45,46]. Recently it has been demonstrated that monocyte chemotactic protein-1 receptor CCR2 (CC chemokine receptor-2) is redox regulated. Antioxidant such as pyrrolidine dithiocarbamate (PDTC), N-acetyl cysteine (NAC) and 2-mercaptoethanol all strongly inhibits chemokine receptors 2 and 4, clearly indicating that the redox status of cells is a crucial determinant in the regulation of the chemokine system.

In vitro and animal studies clearly point to a central role of several distinct but interconnected redox-sensitive pathways in the pathogenesis of inflammation [47]. It has been hypothesized that oxidative stress occurs as a result of the depletion of the cellular content of reduced glutathione. A sublethal oxidative stress can activate redox-sensitive kinase cascades and transcription factors, NF- κ B and AP-1, with resulting increases in the expression of factors associated with an inflammatory response and cellular proliferation. Evidence has been collected suggesting that oxidative stress and the ensuing modification of the cellular redox status may be associated with the induction of cell death either via stimulation of apoptosis and/or necrosis. It is well known that cell death is a common feature of the inflammatory process [48].

Redox status can be restored by the use of SH group donor, such as NAC, which is an antioxidant and SH group donor, and is also cell membrane permeable. It attenuates cytokine production in various experimental model systems both *in vitro* and *in vivo* [24, 49]. The search for molecules with good bioavailability and able to modulate redox status is very active with the target of therapies that not only protect against the injurious effects of oxidants, but also may fundamentally alter the inflammatory event.

At the present, however, clinical evidence that the modulation of the redox state can

be used therapeutically to modify the inflammatory response is not convincing [48]; introduction of redox-modulating therapies into mainstream medicine is possible and promising, but will require significant advances.

3. Nitric oxide (NO) and Inducible NO synthase in inflammation

3.1 NO and inflammation

The molecule appears to be a two-edged sword since it may have both physiological and pathological roles. NO has relevant physiological functions as paracrine messenger, bronchodilator, blood pressure regulator, neuronal messenger. Moreover NO and its derivatives are important weapons against invading pathogens.

As far as the inflammatory process concerns, the bulk of the data indicates that NO is pro-inflammatory. However, the conflicting notion that NO may be protective during an inflammatory insult also exists. For instance, leukocyte adhesion and infiltration, characteristic of the initial steps of inflammation, depends on the interaction of the leukocytes with the endothelial cell surface via glycoproteins (endothelial cell adhesion molecules, ECAM). NO modulates cytokine-induced ECAM expression in cultured endothelial cells *in vitro* by regulating the activation of NF- κ B. Hence NO activity may result in this case as anti-inflammatory [50].

Various studies by a number of different laboratories have implicated NO as an important modulator of a variety of acute and chronic inflammatory disorders [50-52].

The molecular mechanism of the NO pro-inflammatory activity is also multifaceted: NO regulates the inflammatory responses by cell-specific inhibition of the transcription factor NF- κ B, IL-1 β , interferon- γ (IFN γ). At sites of inflammation, increased free radical activity is associated with the activation of the neutrophil NADPH-oxidase and/or the uncoupling of a variety of redox systems, leading to a substantial increase in ROS. Free radicals thus produced, have the capacity to mediate tissue destruction, either alone or in concert with proteases [53].

In this scenario, NO reacts with superoxide, an inflammatory mediator, forming peroxynitrite. Peroxynitrite is a highly reactive compound with harmful effects on cells, and also an important microbicidal compound. The high reactivity of NO with superoxide implies that, in the presence of high concentration of superoxide, NO will be effectively wiped out, interfering with NO signalling mechanisms [54]. NO autoxidation leads to the formation of N₂O₃, which is a nitrosating species. Nitrosation of amine, thiol, and hydroxyl residues can modulate critical cell functions [55].

To further emphasize the double face (Janus face) of the molecule, it has been recently demonstrated that the transfer of a NO group to cysteine sulfhydryls on proteins, known as S-nitrosylation, is a ubiquitous regulatory reaction. It represents a form of redox modulation in diverse tissues. Nitrosylated proteins are relevant in many processes ranging from signal transduction, DNA repair, host defence, and blood pressure control to ion channel regulation and neurotransmission [56].

Finally, a new aspect of NO activity is related to its reported carcinogenicity observed during chronic inflammation. The mediators linking chronic inflammation to carcinogenesis are numerous, though it appears now clearly that NO contributes to carcinogenesis during chronic inflammation [57,58].

3.2 Inducible NO synthase and inflammation

Nitric oxide synthase (NOS) isoenzymes generate NO; a recent paper summarizes and

reviews all the recent data accumulated on this matter [59]. High NO levels are produced by an inducible NOS (iNOS), particularly in response to pro-inflammatory agents. Agents such as LPS, and the pro-inflammatory cytokines IL-1, TNF α , and IFN γ are the most potent inducers of iNOS. As mentioned above, the redox-sensitive transcription factors NF- κ B and AP-1 are the final intermediates in the amplificatory chain stimulating the gene expression of iNOS. Hence the regulation of iNOS is exquisitely redox-sensitive [42].

An interesting link between inflammation, atherosclerosis and NOS has been recently described [60]. In this study, the stimulation of iNOS through endothelin resulted in an increased production of NO, along with a concurrent suppression of the expression of vascular cell adhesion molecule-1 (VCAM-1). It is well known that a hallmark of inflammation is the adhesion of leukocytes to post-capillary venular endothelium and the consequent infiltration of leukocytes into the tissue interstitium. NO, by modulating cytokine-induced ECAM expression through the regulatory factor κ B, may here act as anti-inflammatory, keeping under control the very basic mechanisms of the atherosclerotic lesion.

In general, it can be said that the increase in iNOS and the consequent high output of NO translates into tissue damage. Low level NO produced by constitutive NOS, (nNOS and eNOS) generally results in an anti-inflammatory activity.

Recent studies on this anti-inflammatory role of constitutive NOS (cNOS) has further strengthened the above statement by demonstrating a protective effect mediated by low-level constitutive NOS-derived NO on preservation injury observed in experimental liver transplantation [61]. On the same line, it has been demonstrated that pneumococcal meningitis occurs in graver form in eNOS deficient mice [62].

Concluding this section, it appears clear that the full comprehension of the role of NOS in inflammation is necessary for giving a clear direction to the research for new anti-inflammatory drugs. Drugs able to modulate NOS expression, by inhibiting iNOS expression and/or activity and preserving nNOS and eNOS activity may well represent an important therapeutic goal that can be reached in the near future.

4. Septic shock

Severe sepsis and septic shock are common and are associated with a mortality rate which is still around the 50% mark. There are an estimated 751,000 cases of sepsis or septic shock in the United States each year, and they are responsible for as many deaths as acute myocardial infarction [63]. The transition from a systemic inflammatory response syndrome, typical of the initial onset of a septic shock, to severe sepsis, multi-organ failure, and irreversible shock, involves a multitude of pathogenic changes.

It has been recognised that sepsis is characterised by a dysregulated host response to microbial components, such as LPS, from gram-negative bacteria, and peptidoglycan or extracellular toxins, from gram-positive bacteria. Neutrophils and monocyte exposed to microbial components are activated and release proinflammatory cytokines.

Shock-dependent initialising mechanisms cause the induction of iNOS, COX-2, and CD14. The early response genes, iNOS and COX-2, promote the inflammatory response by the rapid and excessive production of NO and prostaglandins. The transcription factor hypoxia-inducible factor-1 (HIF-1) may also regulate the induction of iNOS during the ischemic phase of shock, contributing to the excess NO formation.

4.1 NO role in septic shock

The role of NO in septic shock has been extensively studied and, not surprisingly, a Janus-type behaviour has been ascribed to it.

Plasma concentrations of NO metabolites were found to be markedly increased in septic shock shortly after the discovery that NO is a potent endogenous vasodilator. NO production is increased as a result of increased expression of the iNOS [64]. The vasodilating action of NO in shock is mediated by the activation of myosin light-chain phosphatase [65] and by the activation of potassium channels in the plasma membrane of vascular smooth-muscle cells [66]. Nitric oxide can activate KCa channels by two mechanisms: direct nitrosylation of the channel and activation of a cGMP-dependent protein kinase [67,68].

Beneficial and detrimental effects of NO in septic shock have been described by Squadrito and coworkers [69]. A protective effect ascribed to NO has been described in the lungs [70], and in the liver [71]. NO in the advanced phases of septic shock is mainly produced by iNOS, and iNOS mRNA increases *in vivo* in various organs during septic shock in rats [72,73], but does not increase in LPS-treated dogs [74]. Szabo hypothesises that during the initial period of shock, NO is mainly produced by cNOS and has a beneficial effect; in an advanced phase, a massive amount of NO is produced by iNOS, leading to toxicity [75,76].

A selective inhibition of the iNOS, but not of the cNOS have been found beneficial in experimental shock treatment [77-79]. The research of iNOS inhibitors which could be useful in septic shock treatment is now very active [80]. Following this line, various research groups tried to modify redox equilibria administering NAC, a well-known thiol-containing antioxidant and radical scavenger. NAC is known to inhibit TNF α secretion and TNF- α mRNA expression *in vitro* in human macrophages [81], in glial cells and primary astrocytes [82] exposed to LPS. The rationale of investigating the effect of NAC stays in the reported redox sensitivity of inflammatory amplifying factors, in particular NF- κ B. Also in this case, an apparently conflicting picture has been reported. Van Dervort et al. [83] demonstrated that NAC substantially increased the production of TNF α in human neutrophils exposed to LPS and IFN γ incubated with an exogenous source of NO. NAC-dependent elevated iNOS expression was also reported by Duval [84].

NAC administration *in vivo* during endotoxic shock gives also rise to conflicting results. NAC inhibits TNF α *in vivo* in LPS treated mice [85] and in dogs [86]. No significant effect on plasma TNF α in human and rat septic shock was seen [87]. NAC depresses cardiac performance [88] increases cardiac index and rate, and causes a decrease in systemic vascular resistance in human septic shock [89]. It is known that LPS-induced expression of iNOS is dependent on the activation of tyrosine kinase and on the binding of the transcription factor NF- κ B heterodimers p50/c-rel and p50/RelA [90]. It has been suggested that the thiol donor activity of NAC improves the cellular antioxidant network and down regulates the redox sensitive mechanisms of NF- κ B activation. This effect can be achieved either by providing the fuel for the ex-novo GSH synthesis, or more in general by increasing the thiol intracellular levels improving antioxidant defences [91].

5. Conclusions

The complex mechanisms underlying inflammation control and its implication in various pathologies have been delineated in this overview. The full comprehension of these mechanisms is necessary to indicate a path for experimenting new treatments for the control of inflammation. The challenge being that of controlling chronic inflammatory processes, at the basis of diseases such as atherosclerosis, neurodegenerative and autoimmune diseases.

Data discussed here demonstrate how deeply the current research has delved into the

understanding of NO and cytokines generating pathways and has indicated some of the regulatory mechanisms. It is likely that an eventual important biochemical therapeutic goal will involve re-establishing cellular redox homeostasis not only to ensure cellular structural integrity, but also to re-establish normal secondary cellular signal transduction mechanisms.

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Non-Traditional Cardiovascular Disease Risk Factors and Arterial Inflammatory Response in End-Stage Renal Disease

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Abstract: Patients with end-stage renal disease (ESRD) have a reduced life expectancy due to an accelerated rate of cardiovascular disease (CVD). In patients with chronic renal failure, cardiovascular morbidity and mortality are higher than in non-uremic controls. The overall risk of cardiac death in the uremic population is increased by a factor of 5 to 20. Traditional risk factors, such as hypertension, dyslipidemia, smoking, advanced age, gender, high-fat diet, physical inactivity, left ventricular hypertrophy, an early CVD-related death in a close relative, hyperparathyroidism, hyperfibrinogenemia and diabetes mellitus may account for the increased cardiovascular mortality rate in these patients. Non-traditional risk factors might also contribute to the high cardiovascular mortality rate in dialysis patients. Prominent among these are oxidative and carbonyl stress, oxidized low density lipoprotein (ox-LDL), advanced glycation end-products (AGEs), accumulation of asymmetrical dimethyl arginine (ADMA) and hyperhomo-cysteinemia. Atherosclerosis is accepted as a common mechanism underlying all CVDs. It is a disease of large and medium sized arteries, which in its more advanced stages affects all three coats of the arterial wall. This artery wall disease is progressive and multifactorial. There is increasing evidence that atherosclerosis should be considered as an inflammatory disease. Chronic inflammation, as evidenced by increased levels of pro-inflammatory cytokines and C-reactive protein (CRP), is a common feature in dialysis patients. Endothelial cell activation which is induced by a number of mediators including, tumor necrosis factor- α (TNF- α), interleukin 1 β (IL-1 β), thrombin, and ox-LDL preludes migration of monocytes to the subendothelial space. The adhesion of leukocytes are mediated by adhesion molecules on the endothelial cell membrane that mainly belong to two protein families: the selectins and adhesion molecules of the immunoglobulin superfamily. Expression of adhesion molecules has been demonstrated in various cell types forming the atherosclerotic plaque. Increased ROS generation and decreased antioxidant defenses have been implicated in the pathogenesis of oxidative stress in uremia. Oxidized LDL (ox-LDL) is an important component of the atherogenic cascade. Oxidized LDLs activate endothelial cells to express surface adhesion molecules for circulating monocytes and lymphocytes, are chemotactic for the same cell types, and are more avidly taken up by macrophages in the subendothelial space to form foam cells. Advanced glycation renders tissues, cells and lipoproteins more susceptible to atherogenesis. AGEs generate reactive oxygen intermediates and can play a role in atherogenesis through oxidant stress. In chronic renal insufficiency NO production is reduced due to accumulation of ADMA, an endogenous competitive inhibitor of NO synthase and decreased L-arginine synthase activity. Accumulation of ADMA may be an important pathogenic factor for atherosclerosis in chronic renal failure and ADMA may be a new uremic toxin. Homocysteine accumulates in chronic renal patients due to both decreased clearance and impairment of renal metabolic function. The pathogenesis of homocysteine-induced vascular damage is not fully

understood. The vascular changes in hyperhomocysteinemia are rather multifactorial. Hyperhomo-cysteinemia may contribute to the pathogenesis of atherosclerosis by injuring the endothelium, damaging endothelial cells and their functions, increasing platelet adhesiveness, enhanced LDL deposition in the arterial wall and direct activation of the coagulation cascade and promoting coagulation. An increase in total plasma homocysteine also reduces the endothelial production of thrombomodulin thus impairing the activation of the anticoagulant, protein C. Auto-oxidation of homocysteine generates ROS. Homocysteine promotes oxidation of LDL, inhibits glutathione peroxidase in vitro and decreases endothelial cell mRNA expression of the enzyme. Therefore, hyperhomocysteinemia attenuates the antioxidant properties of glutathione and thereby potentiates peroxide-mediated cell injury.

1. Atherogenesis in end-stage renal disease

Patients with end-stage renal disease (ESRD) have a reduced life expectancy due to an accelerated rate of cardiovascular disease (CVD) [1-6]. In patients with chronic renal failure, cardiovascular morbidity and mortality are higher than in non-uremic controls [2]. For a dialysis population, the probability of cardiovascular death is reported 50% after ten years [2]. The overall risk of cardiac death in the uremic population is increased by a factor of 5 to 20 [2]. The increased risk of CVD is evident also in predialysis patients (before the onset of ESRD) [4]. CVD is a general expression used to designate a wide range of diseases. According to the World Health Organization (WHO) CVD classification, they are divided into 69 groups, such as ischemic heart disease (IHD), transient cerebral ischemic attack, stroke, occlusive arterial disease of the lower extremities, and acute myocardial infarction (AMI) [3].

Despite the improvements in dialysis technology, the cardiovascular mortality rate is still unacceptably high among dialysis patients. It is obvious that traditional well characterized risk factors, such as hypertension, dyslipidemia, smoking, advanced age, gender, high-fat diet, physical inactivity, left ventricular hypertrophy, an early CVD-related death in a close relative, hyperparathyroidism, hyperfibrinogenemia and diabetes mellitus may account for the increased cardiovascular mortality rate in these patients [1,3-6]. Other, non-traditional risk factors might also contribute to the high cardiovascular mortality rate in dialysis patients. Prominent among these are oxidative and carbonyl stress, oxidized low density lipoprotein (ox-LDL), advanced glycation end products (AGEs), accumulation of asymmetrical dimethyl arginine (ADMA) and hyperhomocysteinemia [1]. Figure 1 shows a number of factors and the interactions between them leading to accelerated atherogenesis in end-stage renal disease.

2. Main events in atherosclerosis

Atherosclerosis is accepted as a common mechanism underlying all CVDs [2]. Atherosclerosis is a disease of large and medium-sized arteries. It affects all three coats of the arterial wall in its more advanced stages. The arterial wall consists of three layers: intima, media, and adventitia. The most inner luminal part of the intima is a monolayer of endothelial cells lining the whole wall. The intact endothelial layer is a selective barrier for plasma lipids and also has antithrombotic properties [3,7]. The pathogenesis of atherosclerosis can be divided into three main stages.

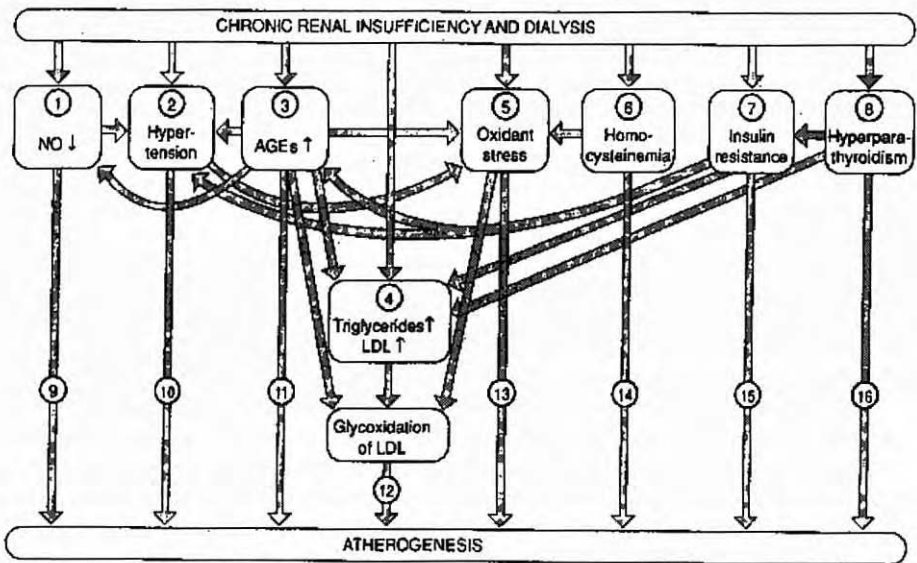


Figure 1. Influence of renal failure and dialysis on atherogenesis. From Huysmans et al. [2] by copyright permission of the Italian Society of Nephrology.

Stage 1. In the initial stage, endothelial dysfunction results in increased endothelial permeability, adhesion of activated leukocytes, and migration of leukocytes into subendothelial tissue [8]. It leads to formation of fatty streak in which subendothelial lipid-filled macrophage-derived foam cells are the dominant feature [2]. These lesions are considered to be reversible or precursors of the more advanced fibrous plaques. Factors involved are: intimal influx of plasma lipoproteins such as LDL and Lp(a); monocyte recruitment and activation; intimal foam cell formation by macrophage scavenger receptor pathways and non-receptor-mediated uptake of oxidatively modified lipoproteins [2,9]. Lipid-laden monocytes and foam cells secrete growth factors and mediate T-cell activation, smooth muscle migration, and remodeling of vascular tissue [8].

Stage 2. Progression to the fibrous plaque occurs during this stage. The main cells involved are phenotypically modified smooth muscle cells, monocyte-derived macrophages and T lymphocytes. The latter two are chemo-attracted into the vessel wall by substances such as oxidized lipoprotein. Smooth muscle cells are phenotypically altered and smooth muscle cell migration, proliferation and connective tissue synthesis are seen [2,9]. A central core of cell debris and cholesterol are formed from necrotic lipid-filled macrophages and smooth muscle cells [2]. Transformation into advanced lesions with a fibrous cap comprises accumulation of lipids and formation of a necrotic core, which is covered toward the lumen by a dense fibrous tissue. Excessive matrix deposition is triggered by platelet-derived growth factor (PDGF) and/or transforming growth factor- β (TGF- β) [8].

Stage 3. In the final stage, an advanced or complex lesion is formed with plaque vascularisation, surface ulceration, chronic inflammation and calcification [9]. Complications occur such as plaque fissure or intramural hemorrhage [2]. Destabilization and rupture of the plaque occur as a result of proteolytic degradation of the fibrous cap [8]. Ulceration and splitting of the fibrous cap expose the highly thrombogenic necrotic core to the flowing blood, resulting in thrombus formation and occlusion of the vessel [2,8].

3. Arterial inflammatory response in end-stage renal disease

Early atherosclerosis shows a massive accumulation of monocytes and cholesterol-containing lipid-loaded foam cells in the subendothelial space. These foam cells are derived from migration of monocytes from circulation into the subendothelial space, differentiation to macrophage, and ultimately conversion to foam cells. There is increasing evidence that atherosclerosis should be considered as an inflammatory disease [3,5]. Chronic inflammation, as evidenced by increased levels of pro-inflammatory cytokines such as interleukin-1, -6 (IL-1, IL-6) and tumour necrosis factor (TNF), and C-reactive protein (CRP), is a common feature in dialysis patients and is associated with an increased cardiovascular morbidity and mortality [3,5,6]. Endothelial cell activation which is induced by a number of mediators including, tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), thrombin, and ox-LDL preludes migration of monocytes into the subendothelial space [3]. The adhesion of leukocytes on endothelial cells and their transendothelial migration are mediated by adhesion molecules on the endothelial cell membrane that mainly belong to two protein families: the selectins and adhesion molecules of the immunoglobulin superfamily [10,11]. The integrins cluster designation 11 (CD11), and the very late activation antigen-4 (VLA-4) belong to the latter group [3]. The direct migration of monocytes into the subendothelial space is mediated by chemoattractants, including monocyte chemotactic protein-1 (MCP-1), ox-LDL, and possibly, collagen and elastin peptides and C5a [3,12]. For two members of the first group (E-selectin and P-selectin) and two members of the latter group (ICAM-1 and VCAM-1), expression has been demonstrated in various cell types forming the atherosclerotic plaque, for example, endothelial cells, vascular smooth muscle cells, and macrophages [10]. Especially in intimal neovasculture, the expression of VCAM-1, ICAM-1, and E-selectin is upregulated [10,13]. Circulating, shedded forms of adhesion molecules have been described that are probably generated by cleavage at a site close to the membrane insertion [10,14]. The amount of soluble ICAM-1 and E-selectin released has been demonstrated to be directly correlated with the surface expression of ICAM-1 and E-selectin in endothelial cells in culture [10,15]. Furthermore, a correlation of circulating VCAM-1 with VCAM-1 mRNA expression in human atherosclerotic aorta and plaques has been reported [10,16]. Elevated circulating VCAM-1 levels have been reported in patients with an atherosclerotic aorta compared with asymptomatic control subjects [10,16]. Furthermore, patients with dyslipidemia demonstrated elevated levels of circulating adhesion molecules [10,17]. Karlheinz et al. found that the serum concentration of circulating VCAM-1 strongly correlates with the extent of human atherosclerosis and can be used to grade atherosclerosis. They suggest an important role of circulating VCAM-1 as a potential serum marker for atherosclerosis [10].

Risk and protective factors of atherosclerosis influence VCAM-1 expression [10,19]. A possible relation between VCAM expression and oxidized LDL was established when an important component of this modified lipoprotein, lysophosphatidylcholine, was shown to stimulate VCAM expression and increase adhesion of monocytes on endothelium in cell cultures [10,18,19]. Modified LDL and its constituents augment cytokine-activated VCAM-1 expression in human vascular endothelial cells [10,20]. In contrast, HDL inhibits cytokine-induced expression of endothelial cell adhesion molecules [10,21]. ω -3 Fatty acids have been found to decrease mRNA levels and surface expression of VCAM-1 in endothelial cells [10,22]. Aspirin inhibits induction of mRNA and cell surface expression of VCAM-1 by TNF- α and thereby inhibits monocyte adhesion on stimulated endothelial cells [10,23]. In contrast to ICAM-1, E-selectin, and P-selectin, endothelial VCAM-1 can mediate leukocyte adhesion via its sole interaction with the integrins $\alpha_4\beta_1$ or $\alpha_4\beta_7$ [10].

Hyperlipidemia and the recruitment of inflammatory cells into the atherosclerotic arterial wall are suggested to cause excessive production of oxygen free radicals [19]. Ohara et al. found that aortas from rabbits that had been fed a high cholesterol diet for several weeks produced several fold more oxygen free radicals than did control aortas [19,24]. Furthermore, removal of the endothelium resulted in reduction of free radical production, suggesting that the endothelium is a major source of the reactive oxygen species in this model. It was hypothesized that the abnormal redox state in the arterial wall may be a fundamental metabolic feature of atherosclerosis [19].

Marui et al. clarified the relation between oxidative stress in the arterial wall (in particular, the endothelium) and the development of the inflammatory response [19,25]. Expression of VCAM-1 by human endothelial cells stimulated by cytokines such as interleukin-1 (IL-1) is mediated by redox-sensitive control mechanisms [25]. The redox-sensitive nature of this gene regulation was determined by the use of antioxidants that are active intracellularly. It was reported that the antioxidant pyrrolidine dithiocarbamate (PDTC) inhibited the IL-1-induced endothelial expression of mRNA for VCAM-1 and was as effective as a monoclonal antibody against the VCAM-1 counterligand very late antigen-4 (VLA-4) in inhibiting binding of Molt-4 cells, which express VLA-4 [25].

The inhibition of VCAM-1 expression by PDTC was suggested to occur at the level of gene transcription [19,25]. Constructs of the VCAM-1 gene promoter were connected to the gene encoding for the enzyme chloramphenicol acetyltransferase (CAT), which provides a readout of promoter activation. These constructs were transfected into endothelial cells for assessment of transcriptional control mechanisms. CAT activity was found to be stimulated by IL-1 and inhibited by PDTC, suggesting that a redox-sensitive mechanism controls transcription of the VCAM-1 gene. A clue to the potential identity of the protein factors controlling transcription was the presence in the VCAM-1 gene promoters of consensus binding sites for the protein nuclear factor kappa B (NF- κ B). NF- κ B represents a family of so-called transcription factors that are present in the cytoplasm and translocate to the nucleus and bind to gene promoters when activated [19,26]. NF- κ B proteins were found to be present in the nuclei of IL-1-stimulated endothelial cells, as reflected in their binding to NF- κ B sequences from the VCAM-1 promoter [19,25]. This translocation to the nucleus was found to be inhibited by PDTC. The redox-sensitive control mechanism was specific for VCAM-1. Other endothelial leukocyte adhesion molecules (eg, E-selectin and ICAM-1) were not regulated in this manner [19,25]. The current hypothesis suggests that hyperlipidemia induces an oxidative stress on the endothelium that leads to the production of oxygen radical species that stimulate VCAM-1 expression, contributing to monocyte and lymphocyte recruitment [19,27]. This suggests that the pathogenesis of atherosclerosis reflects in part the stimulation in the endothelial cell of a set of redox-sensitive genes by oxygen free radicals. The free radical production also accounts for the defective endothelium-dependent vasodilation characteristic of atherosclerotic vessels [19,27].

4. Oxidative Stress in Uremia

Reactive oxygen species are implicated in cell signaling, gene transcription, mitosis, apoptosis and vasoconstriction [1]. They may mediate vascular remodeling and hypertrophy [1]. Oxidant stress can induce proto-oncogene expression that appears to regulate smooth muscle cell migration and proliferation [2]. Lipid and protein oxidation products contribute to atherosclerosis. The nonenzymatic reaction of O_2^- with arachidonate yields a family of isoprostanes that promote mitosis, vasoconstriction and platelet aggregation. O_2^- inactivates nitric oxide which has been implicated in the

pathogenesis of hypertension and renal damage resulting from hypertension [1]. Oxidative stress occurs when the generation of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defense systems [1,28]. Increased ROS generation and decreased antioxidant defenses have been implicated in the pathogenesis of oxidative stress in uremia [1,29,30]. Lipid peroxidation products were reported to be increased whereas plasma vitamin C levels decreased in uremic patients [1,29,30]. Most studies report a progressive increase in oxidative stress markers with declining renal function. An increase in low molecular weight dialyzable oxidizing species has been detected in the plasma of uremic individuals [1,31]. Increased basal $O_2^{\cdot-}$ generation by leukocytes was found in uremic patients due to chronic, low grade inflammation [1,30]. Hemodialysis is accompanied by a measurable oxidative stress and plasma lipid peroxidation [2]. The high level of anti-oxidized-LDL antibodies indicates oxidant stress in chronic renal failure [2,32,33]. This increased oxidant stress can be explained by the following mechanisms. First, hemodialysis itself can generate ROS from leukocytes during exposure to bio-incompatible membranes [1,30]. During bio(in-)compatible hemodialysis, granulocytes aggregate and adhere to the pulmonary vasculature. This causes production of oxygen free radicals, hydrogen peroxide and other reactive molecules [2]. Both the use of biocompatible membranes and the reuse of dialyzers reduce ROS generation [1]. Bicarbonate and erythropoietin were reported to enhance oxidative stress [1,34-36]. Free Fe^{+2} generates highly toxic $OH^{\cdot}$ [1,28,37]. Activation of the renin-angiotensin system and hypertension can increase $O_2^{\cdot-}$ generation via vascular nicotine adenine diphosphate (NADPH) oxidase [1,38]. Second, advanced glycation end-products (AGEs), which are increased in chronic renal failure, can generate reactive oxygen intermediates [1,2]. Third, the anti-oxidative capacity of uremic patients is lower than normal subjects [1,2]. A decrease in antioxidants in uremic individuals may reflect enhanced ROS generation which has overwhelmed the defense mechanisms. In addition, water-soluble vitamins are removed by dialysis [1,29,30]. The plasma and vessels from patients with ESRD contain markedly elevated concentrations of oxidatively modified proteins [1]. An enhanced myeloperoxidase activity has been detected in the blood of uremic patients [1,39].

5. Oxidation of LDL

Oxidized LDL (ox-LDL) is an important component of the atherogenic cascade [3]. LDL is accumulated in the subendothelial space due to increased endothelial cell permeability [3]. In the subendothelial space, LDL undergoes oxidative modifications by endothelial cells, smooth muscle cells, and resident macrophages [3,40-42]. Macrophages derive their lipids mainly from plasma LDL-cholesterol to form foam cells. Macrophages have a higher affinity for oxidized LDL [1-3]. Total and mild LDL oxidation occur within the subendothelial space [3]. Mild LDL oxidation causes formation of LDL minus (LDL-) which is minimally modified LDL [3,43]. LDL oxidation occurs both in the subendothelial space and in the circulation. The enzymes responsible for LDL oxidation are 15-lipoxygenase, myeloperoxidase, NADPH oxidase, and nitric oxide synthase in the artery wall [3,44]. Fully oxidized LDL is not found in blood. On the other hand, LDL- is found both in the subendothelial space and blood. LDL-has some atherogenic properties, some of them shared with ox-LDL. Oxidized LDLs (Ox-LDLs) are pathogenic derivatives of native LDL [1]. They stimulate the chemokine, monocyte chemoattractant protein-1 (MCP-1) and the production of monocyte colony stimulating factor (mCSF) [19]. Oxidized LDLs activate endothelial cells to express surface adhesion molecules for circulating monocytes and lymphocytes, are chemotactic for the same cell types, and are

more avidly taken up by macrophages in the subendothelial space to form foam cells [20,32]. The molecular mechanisms responsible for LDL oxidation *in vivo* and the nature of the initiating stimuli are not clarified yet [11]. *In vitro* models of LDL oxidation developed may not reflect *in vivo* oxidation [32,45]. In the presence of hydrogen peroxide, several peroxidases, including myeloperoxidase, has been shown to promote the peroxidation of polyunsaturated fatty acids in LDL, and the derivatization of amino acid residues in ApoB100 by reactive aldehydic products of lipid peroxidation, such as MDA and 4-OH nonenal [32,45]. LDL can also be directly oxidized by hypochlorous acid or advanced glycation end-products (AGEs) [1,46]. Oxidative modifications generate molecular epitopes in LDL that exhibit peculiar biological activities [32]. Oxidatively modified LDLs are antigenic and elicit an immune response with the generation of circulating autoantibodies often detected in plasma and within plaques of atherosclerotic patients [32,47]. Although Cu^{2+} -oxidized LDL and malondialdehyde (MDA)-modified LDL are usually used as antigens in immunoassays, other, still unrecognized epitopes may be formed *in vivo* during LDL oxidation and may induce antibody production. Seccia et al detected antibodies recognizing LDL oxidatively modified by Cu^{2+} , 2,2'-azobis-(2-amidino propane)hydrochloride (AAPH), and the combination of horseradish peroxidase and H_2O_2 (HRP) in serum of a group of 90 unselected patients [32]. HRP-oxidized LDL was the antigen that revealed the highest IgG titers, although the extent of LDL oxidation (evaluated as conjugated diene formation, loss of tryptophan fluorescence, production of fluorescent aldehydic adducts, and change in electrophoretic mobility) was comparable to that obtained with Cu^{2+} and AAPH. They found a significant correlation between the IgG titers detected using Cu^{2+} - and AAPH-oxidized LDLs as antigens, but no correlation was found between the IgG titers revealed by HRP and Cu^{2+} or AAPH. In addition, the antibody titers against MDA-modified LDL exhibited a significant correlation with those against Cu^{2+} - or AAPH-oxidized LDL, but did not correlate with those against HRP-oxidized LDL. Immunocompetition experiments revealed that IgG recognizing HRP-oxidized LDL did not cross-react with Cu^{2+} -oxidized LDL and vice versa. These findings indicate that peroxidase(s)-dependent mechanisms can trigger peculiar lipid peroxidation-independent modifications of LDL *in vivo* [32].

6. Mechanism of LDL oxidation

The exact mechanism involved in LDL oxidation is not clear. This is the result of the complexity and heterogeneity of human LDL composition both among individuals, and in response to dietary variations within individuals. The density range of human LDL is 1.019 and 1.063 g/mL. It is a spherical particle with a diameter ranging from 19 to 25 nm [3]. The typical LDL particle consists of a central lipophilic core containing approximately 1600 molecules of cholesteryl ester and 170 molecules of triacylglyceride [3,48]. A monolayer of approximately 600 free cholesterol molecules and 700 of phosphatidylcholine surrounds this lipid core. The protein portion of the LDL particle embraces its entire surface and consists of apolipoprotein-B (apoB). Apo B is a glycosylated protein containing approximately 4500 aminoacid residues [3]. LDL particle contains 2700 molecules of fatty acids [3,48]. Almost 50% of these fatty acids are polyunsaturated (PUFAs), mainly linoleic acid (18:2) and arachidonic acid (20:4). PUFA content of LDL is highly variable. Depending on this, individual LDL samples differ in relation to their susceptibility to oxidative modification [3].

The antioxidants associated with LDL determine LDL PUFAs' resistance to oxidation (Table 1). The lipid-soluble antioxidant in the particle are alpha-tocopherol.

gamma-tocopherol, ubiquinol-10, beta-carotene, lycopene, cryptoxanthine and alpha-carotene. The content of lipid-soluble antioxidants in LDL varies considerably among individuals as a function of dietary fat intake and rate of fat absorption.

Table 1. Antioxidant content of human LDL.

Antioxidant	LDL protein (mean $\pm$ SD, $\mu\text{mol}/\text{mg}$)	LDL (mean, mol/mol)
Vitamin E (alpha+gamma tocopherol)	15.5 $\pm$ 2.9	7.95
Ubiquinol-10	0.65 $\pm$ 0.28	0.33
Beta-carotene	0.53 $\pm$ 0.47	0.27
Lycopene	0.41 $\pm$ 0.20	0.21
Cryptoxanthine	0.25 $\pm$ 0.23	0.13
alpha-carotene	0.22 $\pm$ 0.25	0.11

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Oxidation of LDL can be divided into different stages: i) initiation of lipid peroxidation; ii) propagation of PUFA-mediated lipid peroxidation; iii) decomposition of lipid hydroperoxides into reactive aldehydes and ketones, and iv) modification of apo B, leading to recognition of LDL by the macrophage scavenger receptor.

7. Antioxidants and LDL oxidation

The lipid-soluble antioxidants present in the LDL particle are responsible for the LDL particle resistance to oxidation [3]. LDL copper-mediated oxidability in vitro, has been used by several researchers to evaluate oxidation resistance of LDL. LDL oxidation is evaluated by following in vitro copper-mediated oxidation of LDL [3,49]. Duration of the lag phase determines the resistance of LDL to oxidation and depends on the content of antioxidants in the LDL molecule. During the lag period, the alpha-tocopherol and other antioxidants are lost from LDLs. The length of the lag phase reflects the protective effects of chain-breaking antioxidants, especially alpha-tocopherol. When LDL particles, isolated from subjects who have consumed vitamin E supplements, or are enriched with vitamin E, the length of lag period is significantly increased [3].

Table 2. Threshold plasma levels of antioxidants considered as optimal levels regarding risk cardiovascular disease.

	Mean $\pm$ SD
Ascorbate (μM)	≥ 50
Alpha-tocopherol (μM)	≥ 30 (lipid standardized)
Beta-carotene (μM)	> 0.4
Retinol (μM)	> 2.2
Lycopene	> 0.4 to 0.5
Total carotenoids	> 3.2
Alpha-tocopherol/cholesterol ratio ($\mu\text{M}/\mu\text{M}$)	> 5.1 to 5.2

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There are many discrepancies in the literature regarding the amount of antioxidants in healthy subjects and the effects of antioxidants either in preventing LDL oxidation and cardiovascular diseases. Different results are obtained when different methods, with different sensibility and accuracy, are used for measurement of plasma antioxidants, and

lipoproteins. Correction of lipid-soluble antioxidant concentration by total plasma lipid content, is not always done [3] and the results were not always obtained in a healthy and genetically comparable population. The threshold levels of plasma antioxidants that can be considered as optimal levels in relation to risk of CVD are shown in Table 2 [3,50].

There is indirect evidence for excessive oxidation of LDL in patients with ESRD [1]. There are several reports that patients with renal failure have an increase in plasma lipid peroxidation markers such as malondialdehyde in lipoprotein particles and adipose tissue [1,51,52]. Susceptibility of LDL to undergo oxidation in vitro in plasma from patients with ESRD has yielded conflicted results [1,32,53]. Nguyen-Khoa et al demonstrated recently that the high plasma levels of uric acid, triglycerides, and advanced oxidation protein products in the plasma of ESRD patients, although not efficient antioxidants in vivo, can scavenge the peroxy radicals involved in the assay for total peroxy radical-trapping antioxidant potential when tested in vitro [1,54]. Patients with chronic renal failure have diminished capacity against oxidation of LDL particles [1]. Antioxidant protection against lipid peroxidation involves high-density lipoprotein enzymes, such as paraoxanase, and platelet activating factor acetylhydrolase that hydrolyzes lipid peroxides. Paraoxanase activity has been found to decrease, whereas platelet activating factor acetylhydrolase activity did not change or was increased in patients with ESRD [1,55,56].

8. Advanced Glycation End-Products (AGE)

Glycation is the non-enzymatic reaction of glucose with amino groups in proteins. Glucose and other saccharides are important glycation agents, but the most reactive glycation agents are oxoaldehydes, glyoxal, methylglyoxal, and 3-deoxyglucosone (3-DG). Early glycation adducts are Schiff's base and fructosamine. Advanced glycation adducts are carboxymethyllysine, pentosidine and imidazolone. The initial reaction is reversible (clinically used by the Hb A1c laboratory test) but the advanced glycation end products or "AGEs" are not reversible any more [2,57,58]. AGEs are implicated in aging, diabetic microvascular complications, atherosclerosis, and dialysis-associated amyloidosis [1,59].

Pathways for AGEs generation are shown in Figure 2. In the classical pathway, AGEs are formed from the condensation of reducing sugars with the amino group of lysine residues in proteins. The resulting Schiff base undergoes rearrangement to form relatively stable Amadori products. These compounds undergo a slow and irreversible chemical transformation into a range of AGEs, some of which form protein-protein cross-links [1]. In the oxidative pathway AGEs are generated from carbonyl intermediates by glycoxidation of free carbohydrates such as pentoses and ascorbate or by lipoxidation to form malondialdehyde-lysine [1,59]. 3-deoxyglucosone (3-DG), a very reactive carbonyl compound, was originally detected as a reactive intermediate of the Maillard reaction in vitro. However, recent studies have demonstrated that 3-DG is synthesized in human bodies via the Maillard reaction and the polyol pathway. 3-DG rapidly reacts with protein amino groups to form advanced glycation end products (AGEs) such as imidazolone, pentosidine, pyrraline, and N(epsilon)-carboxymethyllysine (CML), among which imidazolone is the AGE that is most specific for 3-DG [60]. CML is an AGE formed on protein by combined nonenzymatic glycation and oxidation (glycoxidation) reactions, but is also formed during metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein. Both glycoxidation and lipid peroxidation are important sources of CML in tissue proteins in vivo. Pentosidine is also a glycoxidation product. Pyrraline is an AGE formed by a nonenzymatic reaction initiated by glucose with lysine residues on proteins and this reaction involves 3-DG as an intermediate [60]. 3-deoxyglucosone (3-DG) is accumulated not only in uremic serum, but also in uremic erythrocytes. 3-DG has some toxic effects on

cells and enzymes. 3-DG shows cytotoxicity by inducing intracellular oxidant stress. In contrast, oxidant stress was demonstrated to cause accumulation of intracellular 3-DG. It was demonstrated that the intracellularly accumulated 3-DG inactivates antioxidant enzymes such as glutathione peroxidase, thereby enhancing the oxidative stress [60]. These studies have emphasized an important role of 3-DG and AGEs in the development of uremic complications.

Oxidant stress decreases concentrations of GSH and NADPH. GSH is a cofactor for the detoxification of glyoxal and methyl glyoxal by the glyoxalase system while NADPH for 3-DG by 3-DG reductase. Hence, glyoxal, methylglyoxal and 3-DG accumulate in oxidative stress, thereby AGE formation is increased. High glucose in dialysis fluid also increase glycation.

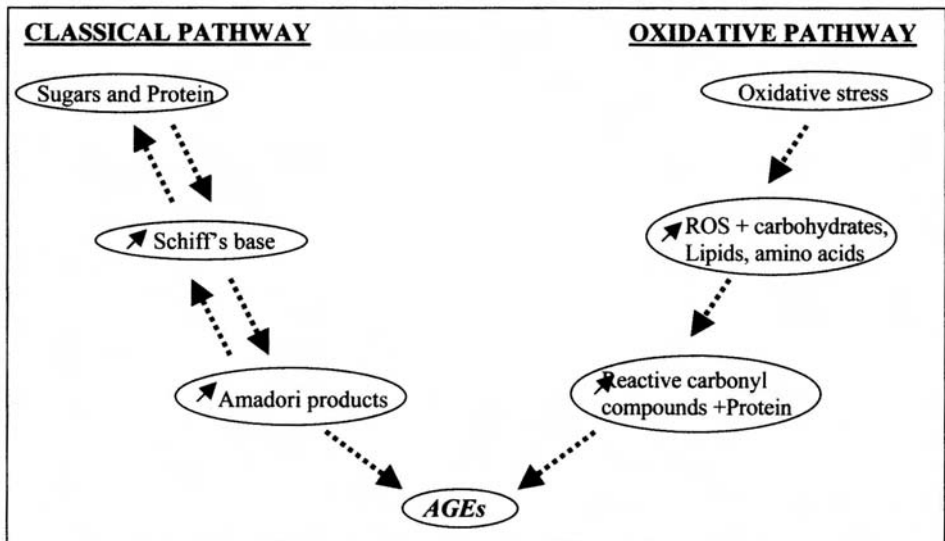


Figure 2. Pathways for AGEs formation.

Advanced glycation renders tissues, cells and lipoproteins more susceptible to atherogenesis. AGEs cross-link with subendothelial collagen tissue. After binding with their receptor on endothelial cells, AGEs undergo endocytosis, transcytosis and finally cross-linking with the subendothelial matrix [2,61]. Activation of receptors for AGE (including RAGE) generates cytokines that lead to inflammatory and proliferative responses [1]. Normal endothelial cells do not constitutively express a receptor for AGEs (RAGE), but in chronic renal insufficiency, RAGE is expressed by arterial and capillary endothelial cells [2,62]. AGEs binding with endothelial cell receptors leads to the activation of a nuclear factor $\text{NF-}\kappa\text{B}$ with transcriptional activation of cell adherence molecules, such as the vascular cell adhesion molecule-1 (VCAM-1) [2,61,63]. This results in increased adhesivity of monocytes and contributes to the accelerated atherogenesis [2,61]. AGEs also crosslink with proteins and thereby derange structural and functional integrity [1,59]. AGE-modified collagen tissue is chemotactic for macrophages [2]. These macrophages are then immobilized and stimulated to release TNF, IL-1, and PDGF. This causes recruitment of connective tissue cells and smooth muscle cells, cellular proliferation and production of extracellular matrix [2,57,58,61].

AGEs have an effect on apo-B-containing lipoproteins VLDL, IDL and LDL. LDL

normally transfers endogenously synthesized lipids to peripheral tissues. Glycation of LDL decreases its systemic clearance by the LDL receptor but enhances its uptake by the macrophage scavenger receptor and may thereby increase its atherogenicity [1,2,57,58]. AGEs can crosslink LDL to matrix protein and impair LDL removal [1,59]. Glycated LDL is suggested to be cytotoxic causing endothelial injury [2,64]. Glycated LDL induces foam cell formation, forms atherogenic LDL-immune complexes and has procoagulant effects [2,57,65]. AGEs generate reactive oxygen intermediates and can play a role in atherogenesis through oxidant stress [2,61,66].

Subendothelial accumulated AGEs were reported to inactivate NO and thus reduce its anti-atherogenic effects [2,58,67]. AGEs alter endothelial cell function, and cause increased vascular permeability, decreased expression of the anticoagulant thrombomodulin and increased synthesis of a procoagulant tissue factor [2,57,58,61]. AGEs binding with smooth muscle cells may promote cytokine and growth factor release and matrix protein synthesis. Evidence for the roles of ROS and AGEs in the pathogenesis of atherosclerosis in vivo derives from the identification of lipid peroxidation products, AGEs, and modified LDL in atherosclerotic plaques from animal and human studies [1,29,30,59]. It was demonstrated that antioxidants, or therapies that decrease AGEs, can decrease atherosclerosis in experimental models and improve endothelial function in human individuals [1,29,59,68].

Serum AGEs and tissue-derived AGE degradation products, low molecular weight AGEs (LMW AGEs), are elevated in diabetics and in non-diabetic patients with renal insufficiency because AGEs are primarily removed from the circulation by renal clearance [2,58]. It was suggested that AGEs are not removed as efficiently as other toxic agents by current dialysis treatments. However, plasma concentrations are higher in low flux hemodialysis than in peritoneal dialysis and are lowest in high flux hemodialysis [2,69]. Serum levels of both pentosidine and N-carboxymethyl-lysine are increased over 20-fold in uremic individuals, even in those without diabetes [1,59]. The plasma levels of pentosidine are slightly lower in patients dialyzed with high flux membranes or those on chronic ambulatory peritoneal dialysis [1,59]. In uremia, 3-deoxyglucosone (3-DG) was shown to be accumulated in serum and erythrocytes [60]. The loss of 3-DG reductase activity in the end-stage kidneys may lead to a high plasma 3-DG level. The elevated 3-DG levels in uremic patients may promote the formation of AGEs such as imidazolone in erythrocytes, aortas, and dialysis-related amyloid deposits. It was reported that treatment with an aldose reductase inhibitor reduced the erythrocyte levels of 3-DG and AGEs such as imidazolone in diabetic uremic patients. The increase in carbonyl stress end products is likely to be a consequence of increased generation as a result of oxidative stress and decreased detoxification and clearance due to renal failure. It was suggested that 3-DG may play a principal role in the development of uremic complications, such as dialysis-related amyloidosis, atherosclerosis, and enhanced oxidative stress [60].

9. Nitric oxide (NO) and asymmetric dimethylarginine (ADMA)

NO is synthesized by stereospecific oxidation of the terminal guanidino nitrogen of L-arginine by the action of a family of NO synthases (NOS) with endothelial, neuronal and macrophage isoforms [2,70]. L-arginine is synthesized from citrulline in the kidney by L-arginine synthase [2,71]. Endothelium-derived NO is continuously released and causes vasodilation, inhibition of smooth muscle cell proliferation, decreased platelet aggregation and leukocyte adhesion to the endothelium and decreased macrophage toxicity [2,72]. NO has been shown to modulate the behavior of circulating blood elements. In vivo, NO inhibits leukocyte adherence in the early stages of

hypercholesterolemia in the rat [73,74]. Furthermore, endothelium-derived NO is able to increase cGMP and reduce the ability of platelets to aggregate [73,75]. These observations suggest that endothelium-derived NO plays an important role as a modulator of leukocyte and platelet function [73]. These effects of endothelium-derived NO may play a role in preventing atherosclerosis [2,71].

The synthesis of NO can be selectively inhibited by guanidino-substituted analogs of L-arginine like N-monomethyl-L-arginine, which acts as competitive antagonists at the active site of the enzyme [70]. Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of NOS [70,73,76-78]. It is thought to be derived from proteins that have post-translationally methylated arginine residues and subsequently hydrolysed to release ADMA [70,73,76,78]. ADMA is produced by endothelial cells in culture and blood cells and is present in plasma and urine of human subjects. ADMA is an endogenous inhibitor of NOS in vivo [72,78-82]. It was found that plasma ADMA levels and adhesiveness of mononuclear cells (specifically, monocytes and T lymphocytes) were elevated in hypercholesterolemic patients. Adhesiveness was inversely correlated with the plasma L-arginine/ADMA ratio. It was also shown that oral administration of L-arginine normalized plasma L-arginine/ADMA ratios and attenuated monocyte and T-lymphocyte adhesiveness. ADMA had no direct effect on the adhesiveness of mononuclear cells. However, monocytes became hyperadhesive when cocultured with ADMA-exposed endothelial cells. In human hypercholesterolemia, the plasma L-arginine/ADMA ratio was shown to be inversely correlated with mononuclear cell adhesiveness. Restoration of the L-arginine/ADMA ratio to control levels normalized mononuclear cell adhesiveness [73]. Insulin release by high doses of L-arginine has been implicated as a mechanism by which L-arginine might stimulate vasodilation in vivo independent of its serving as a substrate for NOS. Endogenously released insulin may contribute to the vasodilation and inhibition of platelet aggregation that is observed during intravenous L-arginine administration in healthy volunteers. However, this endocrine effect requires large doses of intravenous L-arginine [73].

ADMA is degraded by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which hydrolyzes ADMA to L-citrulline and dimethylamine [70,83]. Two isoforms of this enzyme have been characterized and cloned to date. DDAH I predominates in tissues that express neuronal NOS and DDAH II predominates in tissues expressing endothelial NOS [70,84]. Activity of DDAH has been shown to be decreased by oxidized low density lipoprotein (LDL) or tumor necrosis factor- α (TNF- α) in vitro yielding increased levels of ADMA. Plasma levels of ADMA were found elevated in hyperhomocysteinemia, hypercholesterolemia and in hypertensive patients on a high salt diet [70,72,73].

In chronic renal insufficiency reduced NO production is explained in different ways [2]. First, in the diseased kidney, L-arginine synthase activity is reduced. Second, there is accumulation of asymmetric dimethyl-L-arginine (ADMA), an endogenous competitive inhibitor of NO synthase because it is excreted via the kidneys [2,71]. More efficient dialysis, causing better clearance of ADMA, increases NO production. The accumulation of ADMA in the interdialytic period, leading to diminished NO synthesis and diminished vasodilation, can also explain why even in the absence of volume expansion, blood pressure may increase. In chronic renal failure, ADMA accumulates in the presence of reduced L-arginine synthase activity [2,71]. Therefore the dietary contribution of L-arginine becomes more necessary. This could explain why poor survival and cardiac death are related to low cholesterol, low plasma urea, low body mass index and low serum albumin, all indicative of a poor nutritional status [2,71]. It was suggested that accumulation of ADMA may be an important pathogenic factor for atherosclerosis in

chronic renal failure and ADMA may be a new uremic toxin [70,84,85]. Accumulation of ADMA in end-stage renal disease are shown in Figure 3.

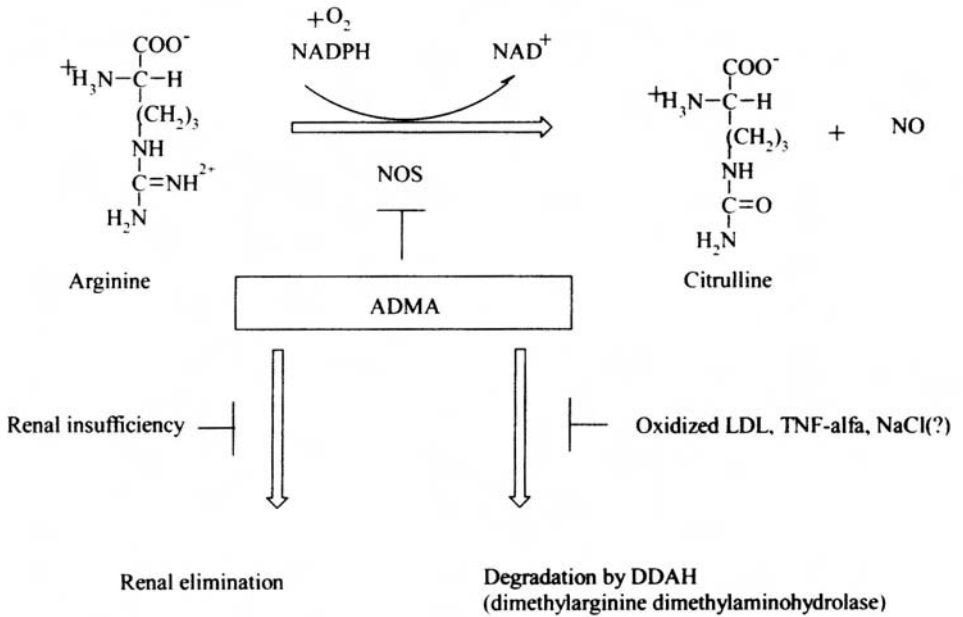


Figure 3. Accumulation of ADMA in end-stage renal disease (ESRD). Adapted from Kielstein et al. [70] by copyright permission of the International Society of Nephrology.

10. Homocysteine

The increased risk for mortality from cardiovascular disease cannot be fully explained by traditional risk factors. Hyperhomocysteinemia is being recognized as a serious, and independent risk factor for the development of atherosclerosis [1-3,87]. No association was found between homocysteine and any of the conventional risk factors for coronary artery disease [2,88]. Homocysteine is generated by metabolism of methionine [1-3]. This redox compound can be readily oxidized to disulphides [1]. Plasma homocysteine represent the sum of concentrations of free reduced homocysteine (2-3%), protein-bound homocysteine (70%), the oxidized dimeric form of homocysteine and cystein-homocysteine dimers (~30%) [1,3,89,90]. Homocysteine is an intermediate of methionine metabolism, which is closely related to the metabolism of thiol-containing compounds (cysteine, glutathione, some proteins), and to several one-carbon transfer reactions (methylations, formylations, carboxylations) [3,91]. This thiol-containing aminoacid is metabolized by remethylation to methionine or by transsulfuration to cysteine [1-3,91]. There are two remethylation pathways. One occurs in all mammalian tissue. It requires N⁵-methyl-tetra-hydrofolate as the methyl donor and reduced cobalamin (vitamin B₁₂) as a cofactor. The other involves betaine and occurs in the liver and the kidney [1]. Methylation of homocysteine is catalyzed by 5-methyltetrahydrofolate-homocysteine methyltransferase, which transfers a one-carbon unit from 5-methyltetrahydrofolate to homocysteine, or by phosphatidyletanolamine methyltransferase, which uses betaine (trimethylglycine) as the one-carbon unit donor to homocysteine, releasing dimethylglycine. Betaine is formed from the polar head of

phosphatidylcholine, a common phospholipid in cell membranes [1,3,91].

Methylation of homocysteine by 5-methyltetrahydrofolate-homocysteine methyl reductase depends on an adequate supply of 5-methyltetrahydrofolate. The unmethylated folate is recycled in a cobalamin-dependent pathway, by remethylation to 5,10-methylene-tetrahydrofolate, and subsequent reduction to 5-methyltetrahydrofolate. The transferase enzyme, also named 5,10-methyltetrahydrofolate reductase catalyzes the whole cycle [3,91]. S-adenosylmethionine and 5-methyltetrahydrofolate are the most important methyl unit donors in biological system. S-adenosylmethionine is reported to regulate methylation and transsulfuration pathways in the homocysteine metabolism [3,91].

Homocysteine is metabolized in the liver, kidney, small intestine and pancreas also by the transsulfuration pathway [1,3,89]. It is condensed with serine to form cystathione in an irreversible reaction catalyzed by a vitamin B₆-dependent enzyme, cystathionine-synthase. Cystathione is hydrolyzed to cysteine that can be incorporated into glutathione or further metabolized to sulfate and taurine [1,3,89]. The transsulfuration pathway enzymes are pyridoxal-5-phosphate dependent [3,91]. This co-enzyme is the active form of pyridoxine. So, either folates, cobalamin, and pyridoxine are essential to keep normal homocysteine metabolism. The former two are coenzymes for the methylation pathway, the last one is coenzyme for the transsulfuration pathway [1,3,89,91].

The reference values for human adults are in the range of 6 to 12 mM. Values exceeding 16 μ M characterize hyperhomocysteinemia. Hyperhomocysteinemia can be further subclassified as mild, intermediate, and severe [3,92]. Elevated homocysteine levels are found in 1-2% in the general population. Higher prevalence is associated with vascular diseases [3,93]. It increases with age. The vitamin deficiency, frequent in the elderly, contributes for the elevation of homocysteine levels [3,94]. Homocysteine levels increase in post-menopausal women, and are attenuated by hormone replacement therapy [3,94]. Hyperhomocysteinemia may result from [2,3,88,93]:

- pyridoxine, cobalamin or folate deficiency;
- inherited or acquired enzyme deficiencies or inherited thermolability of enzymes,
- decreased urinary excretion or
- excessive protein dietary intake.

Inherited cystathionine-beta-synthase deficient activity causes severe hyperhomocysteinemia. Deficiencies in two other enzymes, namely, 5,10-methyltetrahydrofolate reductase and methionine synthase activity may also occur. Homocysteine levels are usually lower in the methylation pathway disorders than in cystathionine-beta-synthase deficiency. Homozygotes for cystathionine-beta-synthase deficiency develop premature and severe vascular disease and present high incidence of thromboembolic events in young adulthood or even in childhood [3,93]. In chronic renal failure homocysteine levels are significantly elevated at a relatively early stage, and are correlated with the serum creatinine concentration [2]. Homocysteine accumulates in chronic renal patients due to both decreased clearance and impairment of renal metabolic function [3]. Marked hyperhomo-cysteinemia is frequently observed in end-stage renal disease. Decreased catabolism of homocysteine in the proximal tubular cells due to reduced filtration and to tubular dysfunction, and decreased extrarenal homocysteine metabolism due to uremic toxins may contribute [1-3]. High dose multiple vitamin supplementation was shown to lower homocysteine levels in dialysis patients [3,95]. The plasma total homocysteine concentration correlates inversely with the glomerular filtration rate [1,96]. Van Guldener et al. found a significant decrease in homocysteine remethylation, but not in transsulfuration pathway in the homocysteine metabolism in hemodialysis patients [1,97]. They could not detect a compensatory increase in the transsulfuration of homocysteine in response to the elevated total levels [1,97]. Henning et al. reported that some, although not

all, of the metabolites of the transsulfuration pathway were elevated in patients with chronic renal failure [1,98]. These studies prove that the transsulfuration pathway was generally intact in patients with renal dysfunction [1,97,98]. In contrast to these findings, Suliman et al. reported that a defect exists in the transsulfuration pathway at the site of the decarboxylation of cysteinesulfinic acid in hemodialysis patients causing elevations in the levels of the transsulfuration pathway metabolites, cysteine and cysteinesulfinic acid and a decrease the plasma levels of taurine, an end-product of the transsulfuration pathway [1,99,100].

The pathogenesis of homocysteine-induced vascular damage is not fully understood [2]. The vascular changes in hyperhomocysteinemia are rather multifactorial [3]. Hyperhomocysteinemia may contribute to the pathogenesis of atherosclerosis by injuring the endothelium, damaging endothelial cells and their functions, increasing platelet adhesiveness, enhanced LDL deposition in the arterial wall and direct activation of the coagulation cascade and promoting coagulation [1-3,87,93]. The cystathionine-beta-synthase deficiency heterozygotes are very susceptible to homocysteine mediated endothelial injury [3,102]. Auto-oxidation of homocysteine generates ROS, including superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) [1-3,88,101,102]. Homocysteine may act as a pro-oxidant factor. This process has been shown to promote oxidation of LDL [2,103]. The generation of superoxide anion and hydrogen peroxide through autooxidation of thiol compounds may contribute to LDL oxidation and atherogenesis in hyperhomocysteinemic patients [2,104]. The thiolactone form of free homocysteine readily reacts with primary amine groups of lipoproteins, by nucleophilic addition [3,105]. The homocystamide-LDL adduct, an acylation product of the reaction between homocysteine thiolactone and the ϵ -aminogroups of Apo-B lysyl residues, has been reported to be cytotoxic to endothelial cells and to increase atherogenicity of LDL in vitro [3,106]. It was observed that the whole vascular cell turnover is affected during hyperhomocysteinemia. A higher DNA synthesis in smooth muscle cells is coupled to a lower DNA synthesis in endothelial cells, suggesting a growth promoting effect in the vascular muscle cells along with an inhibitory effect on endothelial cell growth, a pro-atherosclerotic combination [3,107].

Normal endothelial cells modulate the effects of homocysteine by facilitating the S-nitrosilation of homocysteine by nitric oxide. The formed S-nitrosothiol adduct is a potent vasorelaxing substance [2,108]. So, when high levels of homocysteine occur, they may overcome or impair the endothelial capacity for NO synthesis. Endothelial cell damage may result from increased production of reactive oxygen species or from impaired production of nitric oxide [3,102]. In endothelial cells, total homocysteine reduces the levels of tetrahydrobiopterin (BH_4), relative to dihydrobiopterin (BH_2), thereby creating a dysfunctional eNOS causing a reduced amount of nitric oxide [1,101].

It was reported that increased plasma homocysteine inhibits glutathione peroxidase in vitro and decreases endothelial cell mRNA expression of the enzyme. In these conditions, glutathione is oxidized thus decreasing substrate for glutathione peroxidase. Therefore, hyperhomocysteinemia attenuates the antioxidant properties of glutathione and thereby potentiates peroxide-mediated cell injury [1,101]. Endothelial dependent coagulation and fibrinolysis are modified by homocystein [3]. Homocysteine may induce a pro-coagulatory state [2,88]. High concentrations of homocysteine inhibit thrombomodulin. Thrombomodulin physiologically binds thrombin so enhancing anticoagulant protein C activation and thrombin cleavage of fibrinogen. An increase in total plasma homocysteine also reduces the endothelial production of thrombomodulin thus impairing the activation of the anticoagulant, protein C [1,3,102,109].

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Significance of Reactive Oxygen Species for Neuronal Function

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Abstract: Metabolism of reactive oxygen species (ROS) in living cells is analyzed. Short life-span and high reactivity of these compounds are in agreement with the presence of specific antioxidant system in tissues which controls level of ROS in the cells. Excess of ROS production accompanies the oxidative stress appearance and opportunity to damage cellular proteins, lipid and nucleic acids. However, ROS play positive role in signal transduction mechanisms, blood pressure and other metabolic events. Thus balance between damaging and functional features of ROS should be constantly equilibrated. When the balance is destroyed the signal to cellular death is formed and ROS are involved in the selection of kind of cellular death, which can be apoptotic or necrotic one. Change in metabolic mechanisms regulating cellular death can result in chronic inflammatory reaction.

*Stress is a period, which is characterized
by a selection of strategy for adaptation of organism
in response to un-favorable environmental factors.*

Hans Selye

1. Oxygen radicals in living cells

Molecular oxygen is widely spread oxidant in living systems. Its function in a cell is based on some features of its molecular structure. Whereas electrons in molecules of stable organic compounds are coupled in pairs with anti-parallel spins, oxygen molecule has two non-paired electrons with parallel spins. Correspondingly, the transfer of electrons from an oxidized molecule to oxygen has to induce inversion of electron spin because two electrons can not occupy the only orbital until their spins will be antiparallel (Figure 1). This inversion takes more time than that of existence of complex of oxygen with an oxidized molecule. That is why, during interaction of oxygen with several organic compounds unstable complexes appear.

For total utilization of oxidant ability of molecular oxygen resulting in the formation of water molecule four electrons should be accepted by the molecule. During step-by-step acceptance of electrons, superoxide anion radical (SAR, I), hydroperoxide (II), and hydroxyl radical (III) are formed in series (Figure 2).

As it is seen from Figure 2, SAR can play the role of both oxidant and reducer. Its oxidative potential is relatively small but within cells SAR can oxidize catecholamines, low molecular weight thiols, ascorbate, tetrahydropterins. At alkaline pH SAR forms hydroperoxyl radical HO_2^- (pKa 4.8) being better oxidizer but its amount is no more than 1% of the total SAR pool.

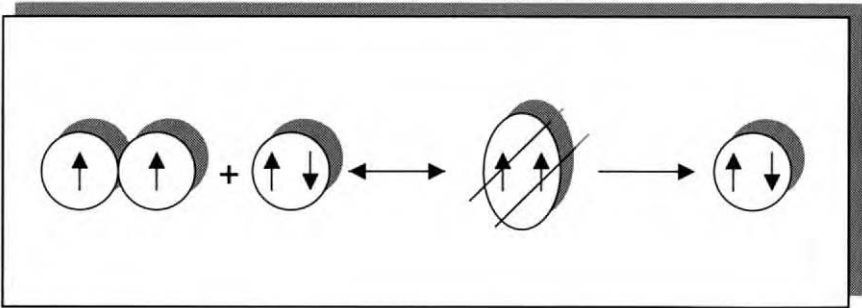


Figure 1. Inversion of electronic spin state in molecular oxygen induced by interaction with nuclear spin.

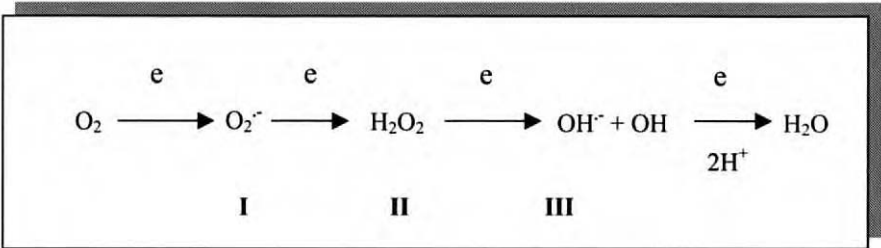


Figure 2. Step-by-step (one-electron) reduction of molecular oxygen.

Table 1. Characteristics of several reactive oxygen species.

Compound	Chemical symbol	Life-span at 37 °C, s	Properties
Superoxide anion radical	$O_2^{\bullet -}$	10^{-6}	Good reductant, poor oxidant
Hydroxyl radical	OH	10^{-9}	Very active in reactions of acceptance, donation and transfer of electrons. Diffusion distance is short
Hydroperoxyl radical	$HO_2^{\bullet}$	10^{-8}	Stronger oxidizer and more hydrophobic then superoxide anion radical. Can initiate lipid peroxidation in membrane lipids
Peroxyl radical	ROO	10^{-2}	Low oxidizable ability compared to OH, but higher rate of diffusion
Alcoxyl radical	RO	10^{-6}	Possessing efficiency of interaction with lipids being intermediate between ROO and OH
Hydrogen peroxide	H_2O_2	10-100	Oxidant with low rate of interaction with organic substrate; possesses high ability to penetrate through cellular membrane
Singlet oxygen	1O_2	10^{-6}	Strong oxidant
Molecular oxygen	O_2	$>10^2$	Weak oxidant

Fe/S-containing dehydratases (aconitase, fumarase, 6-phosphogluconate dehydratase) are easily oxidized by superoxide, their Fe/S-cluster being unstable and loses ferrous ions. Simultaneously hydroxyl radical is formed, which is one of the most active oxidizer. It is able to attack nuclei acids, proteins and phospholipids. Contrary to hydroxyl radical, hydroperoxide, H_2O_2 , forming after SAR reduction, is an inert hydrophobic molecule, which

readily avoids the cell. The difference in properties between SAR and H_2O_2 explains biological importance of superoxide dismutase (SOD), which converts former into latter in biological tissues.

All products of molecular oxygen reduction forming in cellular metabolic reactions listed above are the most widely spread reactive oxygen species (ROS). In Table 1, characteristics of these and several related compounds discovered in living systems are present.

One more oxidant is formed after interaction of SAR with NO-radical formed by NO synthase and possessing a number of useful biological functions as neuromediator, second messenger or neuromodulator. Two important properties of NO should be stressed: i) relaxation of smooth muscle cells of vascular walls resulting in vasodilatation (increase in peripheral blood circulation) and ii) ability to react with SAR to form peroxynitrite ONOO^- . The rate constant for such interaction is about $10^{10} \text{ M}^{-1}\text{s}^{-1}$. Pair “superoxide anion - nitric oxide” is considered to be universal regulator of vascular tone: NO induces vasodilatation, while SAR works as NO neutralizer providing vasoconstriction. Peroxynitrite possesses powerful oxidizing ability, which belongs to both molecule itself and its product, hydroxyl radical, which can be easily formed from ONOOH in the presence of ferrous ions. Because of this, metabolism of NO in a cell is usually analyzed together with typical ROS and with hypochlorous anion, OCl^- produced by myeloperoxidase from H_2O_2 . Hypochlorous anion, one of the strongest oxidants in living systems, is also able to form hydroxyl radical. While OCl^- is rather active form of chlorine than that of oxygen, all these radicals are combined in a unique metabolic pathway. In Figure 3 relationships between these radicals and their effects on cellular functions are schematically present.

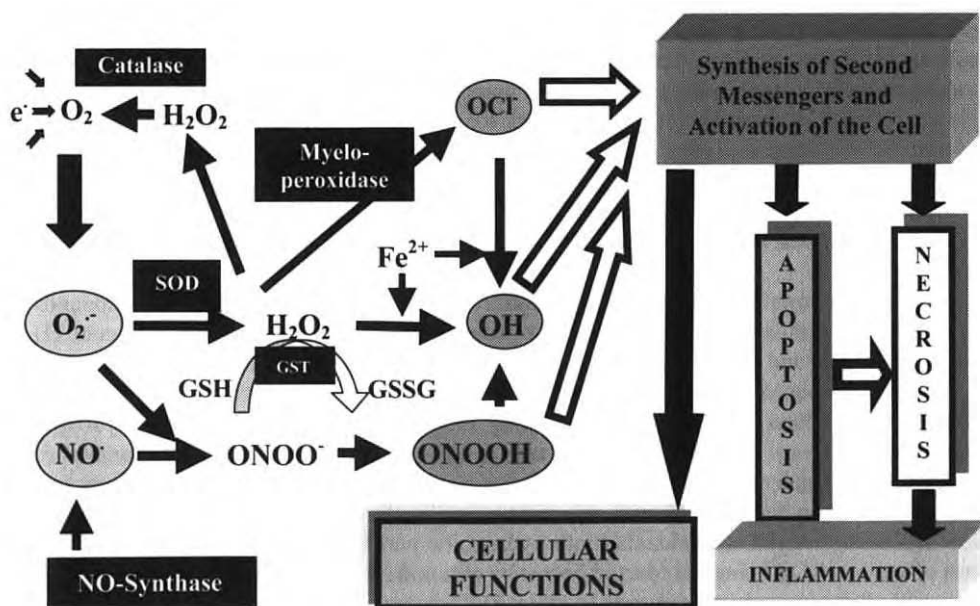


Figure 3. Relationship between several radicals in tissues and their effects on cell functions.

All the oxidants mentioned above possess a damaging effect on the cells. During oxidation of membrane lipids (especially those containing unsaturated fatty acid tails) chain reactions easily appeared, which result in irreversible violation of membrane integrity being inconsistent with viability of the cell. Protein and nucleic acids can be oxidized even earlier

than lipids, their damage is, as a rule, more serious for cellular function. Actually, when ROS levels are increased, protein and nucleic acid start to oxidize before pronounced oxidation of membrane lipids [1-3].

In oxidation modification of proteins several amino acid residues are involved. Amino and SH-groups are especially fast oxidized by ROS and OCl^- . These modifications are reversible in their nature but the restoration depends on energetic potential of the cell and the presence of reduced forms of glutathione, cysteine and thioredoxine. Besides cysteine, in protein molecule lysine, tyrosine and free carboxyl groups of dicarbonic amino acids are accessible for oxidation. Because of such transformations o- and m-tyrosine, methionine sulfoxide and various protein carbonyls are accumulated. Carbonyl groups easily interact with aldoses resulting in protein glycation. The latter process can be performed as a direct interaction of free sugars with ϵ -amino groups of lysine. Schiff bases formed in both cases are transformed into stable Amadori products. Together with products of lipid peroxidation they are accumulated in lipofuscin complexes forming specific hypo-epidermal "senile spots".

Oxidation, as a rule, results not in proteins inhibition but in their modification. For example, oxidative modification of Na/K-ATPase is accompanied by loss of enzyme sensitivity to regulating effect of ATP, while formation of S-S bonds in xanthine dehydrogenase modulates its properties and character of the reaction: dehydrogenase transforms into oxidase and oxidation of hypoxanthine (xanthine) results in the generation of SAR as a second product. For brain tissue containing high level of xanthine dehydrogenase this transformation can be especially dangerous. Oxidation of SH-groups of glutamate receptors of NMDA subtype results in modification of their affinity to ligands [4].

Radical attack of nucleic bases in DNA and RNA results in their hydroxylation, disorders their regular package and decreases stability of the macromolecules with subsequent fragmentation. Actually, in cells with marked oxygen metabolism oxidative modification of nucleic acids takes place in amount exceeding ten thousand hits a day [5]. However, most of them have no after-effects for cell viability demonstrating the presence of specific cellular antioxidant defense repair system.

2. Antioxidant defense system

This system consists of both enzymes and various low molecular weight compounds preventing accumulation or scavenging free radicals (Table 2). Their coordination controls both generation and metabolic transformation of ROS in cells and tissues. In agreement with noted properties different antioxidants play specific roles in different tissues. SOD follows the cellular level of SAR providing dismutation of its excess into hydrogen peroxide. That one, if not leaving the cell is neutralized by catalase or a number of glutathione dependent enzymes. Proteins chelating the metals of transient valency prevents electron donation for one-electron reduction of molecular oxygen by these ions. Finally, low molecular weight antioxidants (both hydrophilic and hydrophobic) are picking up the rest of various ROS being not reacted before by antioxidant enzymes.

Of all the antioxidant enzymes in brain, SOD is one of the most important. Eucariotes possess several isoforms among those are mitochondrial Mn-SOD, cytosolic Cu/Zn-SOD and another Cu/Zn-SOD found in extracellular fluids. Plant species contain additionally Fe-SOD. For procariotes, various combinations of above isoforms including the recently described Ni-SOD (in *Streptomyces*) are characteristic.

Many pathologies of human beings accompanied and/or induced by ROS accumulation such as lateral amyotrophic sclerosis, Alzheimer disease, parkinsonism, brain stroke, etc. develop under decreased activity or genetically induced deficit of SOD [6].

Table 2. Antioxidants in living systems.

Antioxidants	Location	Function
Enzymes and proteins		
Cu/Zn-SOD	Erythrocytes, cytoplasm	Quenching of $O^{\cdot-}$
Mn-SOD	Mitochondria	Quenching of $O^{\cdot-}$
Extracellular SOD	Blood plasma, vascular system	Quenching of $O^{\cdot-}$
Catalase	Peroxisomes	Quenching of H_2O_2
Glutathione peroxidase	Cytoplasm, Mitochondria	Degradation of H_2O_2 and lipoperoxides
Glutathione transferase	Cytoplasm, outer mitochondrial membrane, endoplasmic reticulum	Degradation of H_2O_2 and lipoperoxides
Ferritins	Cytoplasm	Chelation of Fe ions
Transferrin	Extracellular medium	Chelation of Fe ions
Lactoferrin	Extracellular medium	Chelation of Fe ions
Ceruloplasmine	Extracellular medium	Chelation of Cu ions, oxidation of Fe ions, quenching of SAR
Albumin	Extracellular medium	Chelation of Cu ions, quencher of $OH\cdot$, $LOO\cdot$, $HOCl$
Low molecular weight compounds		
Vitamin E	Cell membranes	Quenching of $OH\cdot$, $LOO\cdot$, $HOCl$, etc.
Ubiquinol	Mitochondrial membranes	Quenching of $OH\cdot$, $LOO\cdot$, $HOCl$, etc.
Carotinoids	Cell membranes	Quenching of $OH\cdot$, $LOO\cdot$, $HOCl$, 1O_2
Vitamin C	Cytoplasm	Quenching of $OH\cdot$, $O_2^{\cdot-}$
Carnosine	Cytoplasm	Quenching of various ROS
N-acetylcysteine	Cytoplasm	Quenching of $OH\cdot$, $LOO\cdot$, $HOCl$, etc.
Taurine	Cytoplasm	Hypochlorite neutralization
Glutathione	Cytoplasm, mitochondria	Quenching of $OH\cdot$, $O_2^{\cdot-}$
Uric acid	Blood	Prevention of lipid peroxidation
Bilirubin	Blood	Prevention of lipid peroxidation

One of specific features of SOD is an ability to form SAR in an excess of H_2O_2 , which, in turn, can destabilize Cu in active center of the enzyme and to be transformed into hydroxyl radical [7]. Under such conditions, free radical attack of the protein molecule may result in its fragmentation and loss of the activity. Thus one of the conditions of successful operation of SOD in tissues is simultaneous presence of low molecular weight antioxidants. This example demonstrates the importance of concerted interaction of several components of cellular antioxidant defense.

For antioxidant defense of brain cells SOD is the most important enzyme. Firstly, brain tissues consume the largest amount of oxygen comparing to other tissues (if only 0.1% of the total oxygen consumed will be transformed into SAR it will be toxic for brain survival). Secondly, the activity of catalase in brain is very low. Thirdly, neuronal membranes contain enormous amount of arachidonic and other highly unsaturated fatty acids, which are easily oxidized. Finally, disordering of oxygen metabolism induces acidosis and subsequent release of ferrous ions from its complexes with Fe-transporting proteins. All these events make ROS generation in brain cells very likely.

Glutathione dependent enzymes (glutathione peroxidase, glutathione transferase and glutathione reductase) play limited roles in brain antioxidant defense. Nevertheless, glutathione peroxidases are of great interest by the broad number of reductants, which are used by this enzyme to transform hydrogen peroxide into water molecule. Besides glutathione, ascorbate, ferrocytochrome *c*, thioredoxine, NADH or even chlorine and iodine ions can take part. Peroxidases may serve for another goal, for example, to catalyze oxidation of polyunsaturated lipids into subsequent alcohols.

Low molecular weight antioxidants react with ROS in cell compartments which for some reasons are lack of antioxidant enzymes. Thus, suppression of bifurcate chain reactions of lipid peroxidation in hydrophobic core of cell membrane is mostly effectively performed by vitamin E (α -tocopherol). Interaction of lipid molecules with hydroxyl radical in the absence of vitamin E results in bifurcation of oxidative processes and formation of peroxy and alkoxy radicals. They are quickly accumulated in the restricted volume of the membrane and reaction began to be uncontrolled. α -Tocopherol interacts with peroxy radicals with high affinity, reduces them and is then oxidized itself into relatively non-active phenoxyl radical [8]. The latter can be accumulated within the bilayer until it will be returned to initial state by reduction by ascorbate [9]. Pair "Vitamin E - Vitamin C" is a good example of a mutual interaction between hydrophobic and hydrophilic low molecular weight antioxidants. Recently, tight relations were demonstrated for several natural antioxidants which interaction balances the red/ox state of the cell [3,5,10-12]. Figure 4 demonstrates such interaction between some of them.

All natural antioxidants under special conditions possess pro-oxidant activity. It was primarily noted for ascorbic acid by describing the initiation of lipid peroxidation by pair "Fe - ascorbic acid" because vitamin C easily reduces Fe^{3+} into Fe^{2+} supporting its ability to regenerate SAR from molecular oxygen. Ascorbate itself is transformed into oxidized state:

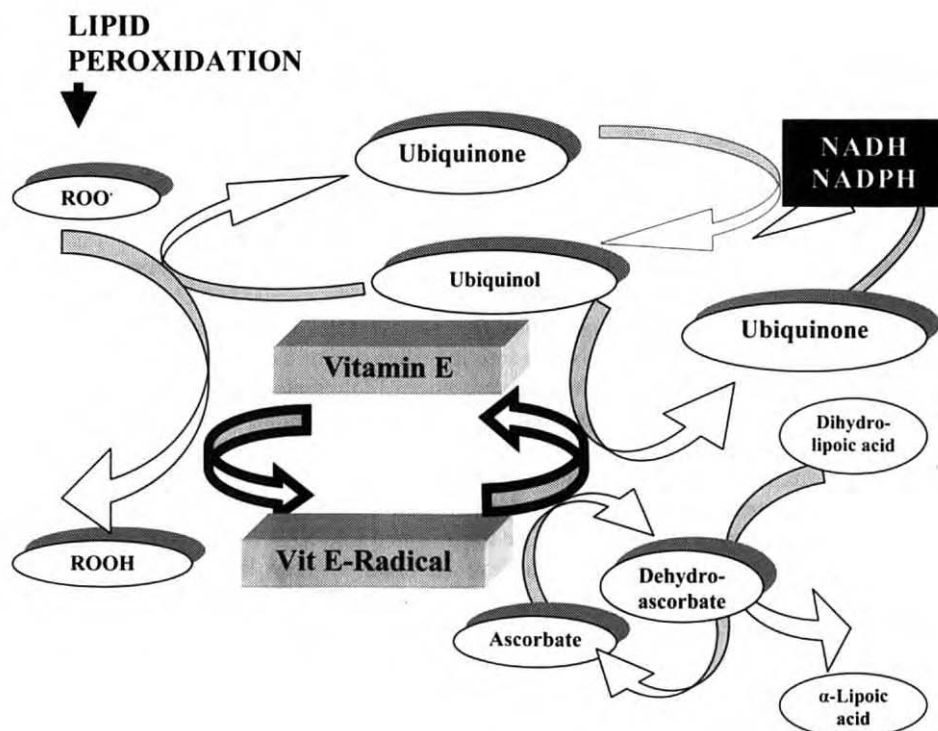


Figure 4. Regeneration of vitamin E from the radical form by a number of natural cell reducers (from [5] and [10] with modifications).

The above reaction is reversible; there are many candidates to return oxidized ascorbate into reduced form [5]. The pro-oxidant effect of ascorbate was recently demonstrated within the living cells [13]. The reversibility of such ascorbate transformation

makes it an important neuroprotector in human beings (as well as in other mammals) while this compound is not synthesized in their tissues [14]. Glutathione, α -lipoic acid, carnosine can also play a dual role in ROS metabolism [5,12,15]. Such feature of natural antioxidants can be one of the reasons of only partial (restricted) success in antioxidant therapy of the diseases accompanied by the rise in ROS production [16,17]. The pro-oxidant ability of α -tocopherol was also demonstrated. Moreover, it was found that vitamin E does not protect against some damaging effects of ROS [18]; post-ischemic reparation of brain tissues are not accompanied by vitamin E utilization [19]; some protecting action of vitamin E can be related to its direct effect on the level of ionized calcium [20,21] or on the membrane bilayer structure [8]. This evidence can open absolutely new direction in the study of regulation of cellular function. Package and lipid asymmetry of the membrane bilayer are important parameters regulating transfer of information from outer medium onto the cell (Signal Transduction Mechanisms). Variation of membrane lipid microviscosity will affect interactions of membrane bound proteins with each other and with regulatory proteins as well. A number of receptors like insulin receptor, may be dimerized within the bilayer, which results in their mutual phosphorylation and involves cytoskeletal actin into association with membrane proteins thus simplifying signal transfer to cytoplasmic protein kinases [22]. Many membrane bound receptors are associated with G-proteins and their association is under the control of membrane microviscosity [23]. Thus, effect of α -tocopherol and other antioxidants on cell membrane properties will result in modification of modes of regulation mentioned above. One of the demonstrations of such effects has been recently announced by A. Azzi, describing inhibiting effect of α -tocopherol on cytoplasmic protein kinase C occurring via specific tocopherol binding proteins while γ -tocopherol, inspite of the same antioxidant ability did not demonstrate such effect. Moreover, it competed with α -tocopherol for binding with its cytoplasmic target [24]. Therefore, low molecular weight antioxidants work as ROS buffers rather than ROS scavengers and simultaneously demonstrate besides of antioxidative effects the diverse regulatory functions.

3. Metabolic sources of reactive oxygen species

There are two sub-types of glutamate receptors in glutamatergic structures in brain: ionotropic and metabotropic ones, which differ in both localization (the former — predominantly on the postsynaptic membrane and the latter — on both post- and presynaptic membranes) and their functional role. Activation of ionotropic receptors provides electric activity of neurons (generation of action potential) whereas that of metabotropic receptors modifies metabolism of the neuronal cell and, in particular, conformational state and properties of ionotropic receptors; this modifying effect is performed by second messengers which formation involves activation of subsequent G-proteins.

Glutamate can provide diverse response of a neuronal cell depending on which kind of receptors is included in realization of a pre-synaptic signal. Such phenomenon is explained by different sensitivity of receptors to the neurotransmitter as well as mutual influence of several sub-types of receptors on each other. In whole, activation of ionotropic receptors results in excitation and that of metabotropic receptors — in regulation of its amplitude and duration.

Activation of glutamate receptors of each sub-type leads to increase in intracellular ROS level [12,25,26] — mainly, SAR and hydroxyl radical, as well as Ca-dependent stimulation of NO-synthase, which results in appearance of NO-radical [27]. When arginine is in deficit, NO-synthase may generate SAR [28]. Under conditions of overproduction of

both these radicals interaction of SAR and NO can result in appearance of peroxynitrite, which can provoke massive damage of cellular structure. On this reason, stable excitation of postsynaptic membrane results in toxic injury (that is why glutamate belongs to excitotoxic neuromediators).

In neuronal function ROS play a role of metabolites immediately participating in the excitation process. In the intracellular space there are both enzymic (cyclooxygenases, monoamine oxidases) and non-enzymic (spontaneous oxidation of biogenic amines) reactions where they are formed. Mitochondrial respiratory chain also provides ROS production in a cell under conditions of changeable oxygen pressure [29,30].

Flow cytometry approach to study neuronal suspensions [31] allows to measure directly an increase in ROS level when the cells are activated by glutamate or its agonists [25,31,32]. Activation of glutamate receptors of several kinds was found to result in activation of different metabolic processes. As it is seen from Table 3, ROS signal is suppressed to different extent by different metabolic inhibitors depending on which ligand stimulates the neurons.

Both NMDA, and kainite generate ROS signal, which is not sensitive to rotenone. One can suggest that only cytoplasmic sources of ROS are displayed, whereas mitochondrial ROS are quenched by Mn-SOD. Nialamide inhibits ROS signal by about 40% in the case of kainite and only by 16% in the case of NMDA. When other inhibitors are used, the inhibitory level is also different depending on the kind of receptors activated. Thus, kainite receptors can be concluded to activate more easily monoamine oxidases while NMDA — cyclooxygenases. At the same time, phorbol myristate acetate (PMA), membrane penetretable activator of proteine kinase C, results in ROS generation in the other reactions, insensitive to the inhibitors used (Table 3).

All these data allow us to demonstrate red/ox regulation of ionotropic receptors [33], which suggests that antioxidant/prooxidant balance in the cell manages the neurocomputing and learning process. Actually, NMDA receptors are mainly responsible for toxic effect of glutamate when brain blood supply is damaged and overproduction of SAR and NO takes place [34,35].

Table 3. Effects of rotenone (20 μ M), indomethacine (100 μ M), 4-methylpyrasole (4-MP, 50 μ M) and nialamide (100 μ M) on ROS level measured in rat cerebellum granule cells using flow cytometry [12].

Conditions	Inhibition of ROS level, %	Immediate target
Kainate (0,25 mM)		Kainate receptors
+ rotenone	0	Respiratory chain
+ indametacine	46,0 $\pm$ 13,1	Cyclooxygenases
+ 4-MP	42,7 $\pm$ 14,3	Cytochrome P ₄₅₀
+ nialamide	40,0 $\pm$ 7,2	Monoamine oxidases
NMDA (0,25 mM)	-	NMDA receptors
+ rotenone	0	Respiratory chain
+ indometacine	35,3 $\pm$ 4,5	Cyclooxygenases
+ 4-MP	15,5 $\pm$ 4,9	Cytochrome P ₄₅₀
+ nialamide	16,0 $\pm$ 7,1	Monoamine oxidases
PMA (1 μ M)	-	Protein kinase C
+ rotenone	-7,0 $\pm$ 5,0	Respiratory chain
+ indometacine	-15,0 $\pm$ 14,0	Cyclooxygenases
+ nialamide	-16,0 $\pm$ 16,0	Monoamine oxidases

Antioxidants might be expected to be a useful tool to protect brain from toxic effects of glutamate. It was found, however, that while NMDA-antagonist orphenadrine partially protects neurons from glutamate induced toxicity both in vitro, and in vivo [36], the agonists of metabotropic receptors, ACPD and L-AP4 [35] or lazardoid U-83836E.

possessing no antioxidant capacity but activating protein kinase C [32] are much more effective. Neuropeptide carnosine, which efficiently protects neurons against damaging action of ROS [12] can be also considered as rather protein kinase C activator than ROS quencher [37]. All these facts point out the protecting role of metabotropic receptors in function of excitotoxic mediators.

Further study on free radical signaling of neurons using flow cytometry elucidated the ability of cells to generate ROS under activation of metabotropic receptors [12,38]. Compared response of the ROS signal was nearly two times higher in the case of ACPD (metabotropic agonist) than in the case of kainate or NMDA (ionotropic agonists). Moreover, simultaneous presence of ACPD and NMDA results in summation of ROS level, while ACPD and kainate — in its decrease. Thus, metabotropic receptors serve as natural regulators of activity of ionotropic receptors [38-41]. One can suggest that ROS provide the intrinsic signal, which is used for such regulation.

What is especially demonstrative is a sharp rise of ROS within the neuronal cells when modest hypoxia is substituted by reoxygenation. As V. Skulachev suggested [42], under oxygen deficit some special conditions can appear when reducibility of intermediate components in mitochondrial respiratory chain will be high enough to provide the interaction of ubiquinol with O_2 to form SAR even under low molecular oxygen pressure. We have examined this suggestion using experimental ischemic model of Mongolian gerbils' brain [30]. It was found that an increased ROS level is measured even under hypoxic conditions, as it is evidenced by accumulation of hydroperoxides and other toxic compounds resulting in delayed death of the neurons.

Thus, overproduction of ROS can be registered in brain after overloading the receptors with excitotoxic neuromediators and brain blood disordering as well as a result of genetically determined defects. If antioxidant defense system is not powerful enough to neutralize excess of ROS the products of their interaction with cellular components are accumulated. This is a sign to stimulate a cell repairing mechanism including methionine sulfoxide reductase (reverses methionine oxidation), proteases (disrupt irreversibly oxidized proteins favoring their substitution by novel molecules), several phospholipases (remove oxidized fatty acid tails from phospholipids) and acyl-CoA transferases, repairing the membrane structure. Endonucleases exclude oxidized nucleic bases and stimulate reparation of nucleic acids. How effectively this repairing system will work is preferentially determined by balancing between generation and neutralization of ROS.

4. Dual role of free radicals in neuronal life

There is no uniform opinion in the modern literature concerning the role of ROS in cell metabolism. Usually, useful role of ROS was restricted by their bactericidal function belonging to SAR produced by NADPH oxidase of cell plasmatic membrane. Recently, ROS has been evidenced to be able to affect a number of metabolic processes including protein synthesis and cell differentiation [1,43-45]. There are direct data published that ROS may be involved in the control of gene expression [45].

Selected information is present in Table 4 illustrating a dual role of ROS in cell function. For excitable cells, ROS are playing a very special role. They are involved in normal metabolic pathway, which is demonstrated by increasing level of ROS within the cells shortly exposed to ligands of glutamate receptors. Functional role of such signal is still obscure, however, it has no relation to the signal for cell death and its height is proportional to neuron activation [12,25,26]. Novel data evidences that free radicals take part in cell-to-cell cross talk [46] as well as are involved in a long-term memory formation [47,48].

Table 4. Role of ROS in cell metabolism.

Source of ROS	Role in metabolic pathway
SAR generation by NADPH oxidase on outer plasma membrane of phagocytes	Protecting (antiseptic) function
Formation of SAR in mitochondrial respiratory chain as a result of interaction of ubiquinol with molecular oxygen	Attack of non-histone-protected nucleic acids in mitochondria, induction of apoptosis
Spontaneous or enzyme dependent oxidation of biogenic amines resulting in formation of SAR, H ₂ O ₂ and toxic aldehydes	Oxidative damage of macromolecules (modification of NH ₂ - and SH-groups of proteins, breaking of DNA chains, lipid peroxidation)
Formation of hydrogen peroxide and hydroxyl radical from excess of SAR being not neutralized by SOD	Activation of translational factors (NF- κ B, AP-1, etc.) and red/ox regulation of gene expression
Activation of NO-synthase and formation of NO and then peroxynitrite (in the presence of excess of SAR)	Activation of guanylate cyclase and intracellular effects of cGMP (on vasodilatation, of peroxynitrite on nitration of tyrosine, oxidation of SH-groups)
Activation of NMDA receptors in hippocampal area	Long-term potentiation, effects on learning processes
Generation of ROS during activation of glutamate receptors in brain neurons	Signal mechanism involving mutual regulation of glutamate receptors of different sub-types

With modest increase in intracellular ROS levels, activation of NF- κ B takes place, which protects the cell against oxidative stress [45]. Direct root of ROS participation in signal transduction from cell membrane to intracellular metabolic reactions were recently described. Among them – activation of potential-dependent K-channels and variation of membrane potential, inhibition of cellular protein phosphatases and restriction of activity of MAP-kinase [49]. Such view on intracellular role of ROS consider them as second messengers, which together with cyclic nucleotides, calcium ions, and other biologically active compounds provides adequate cell response to the outer signals.

It is hardly possible to pass over in silence the dual role of NO-radicals in neuron stabilization against oxidative damage. Two properties of nitric oxide noted above — the ability to activate cGMP formation by binding to guanylate cyclase haem and the ability to react with SAR, makes it simultaneously a useful factor in the hypoxic period (improvement of peripheral blood stream) and a damaging factor — at the re-oxygenation step (peroxynitrite formation). Moreover, NO is known to activate a number of ROS producing enzymes, particularly, cyclooxygenase, increasing stationary level of ROS. One more property of NO is its extremely fast interaction with tyrosine residues in protein molecules resulting in nitro-tyrosine formation and, thus, avoidance of sensitivity of some proteins to tyrosine kinases. All these features may explain a diversity of NO effects described by a number of investigators (see [50]).

5. Oxidative stress and brain

A non-compensated increase in intracellular ROS level evidences on exhaustion of antioxidant defense system and provides the cells with danger of mutagenic defects. Such unfavorable situation is characterized by accumulation of modified lipid molecules (hydroperoxides of fatty acids, malonic dialdehyde, MDA, etc.) and proteins (containing S-S bounds, carbonyl groups, and other modified residues) as well as products of degradation of nucleic acids (mainly — 8-hydroxy-2'-deoxyguanosine). All metabolites are accumulated in different tissues including blood and urine, which evidences on the misbalance of oxygen metabolism named as "oxidative stress".

The common point of view has been widely accepted that oxidative stress results in multiple defects in cellular structure and, thus, is damaging for cells, tissues and the body. Nowadays accumulated information allows to accept another concept (which, by the way, is close to that of Hans Selye who introduced this term to modern biology) — that oxidative stress is a way to mobilize adaptive and protective mechanisms of the organism to survive under extreme conditions.

In the case of brain, however, the simplicity to transform the exciting effect of neuromediators into the excitotoxic one can be very dangerous. Uncontrolled rise in intracellular ROS level may result in undesirable consequences — oxidative modification of Na/K-ATPase, which is one of the first target of oxidative stress [51], will be carried out with the Na-pump damage disordering asymmetric distribution of sodium and potassium ions and glutamate re-uptake will be suppressed. Hypoxia induced acidosis will stimulate ferrous ion release from the protein-transporters (transferrin, lactoferrin, etc.). This, in turn, will induce oxidation of brain membrane lipids. Disruption of cell membrane will result in massive influx of calcium ions into the cytoplasm and activation of the enzymes damaging for the cell integrity (calpains, phospholipases, etc.).

In the literature dangerous for brain neurons effect of ROS was traditionally underestimated — one can believe that multi-step antioxidant system should provide reliable protection. Actually, antioxidants are able to remove toxic component of glutamate overloading and keep neurons viable. However, this point of view is under serious doubt because of both a multiple role of antioxidants in brain metabolism [1,38,52] and apparent absence of therapeutic effect of antioxidant protection [42].

Thus, normal function of glutamatergic neurons would be hardly possible if special protective system against glutamate excitotoxicity did not exist in brain. As we noted earlier, such protection is provided by metabotropic receptors. Their effect on ionotropic receptors is realized via ROS production [39-41] and results in varying of duration and intensity of ionic fluxes through the membrane, thus supplying long-term potentiation or long-term depression of the electric activity. All these data illustrate participation of ROS in Signal Transduction Mechanisms.

How serious are the anxieties that free radical damage of brain under disordering of brain blood supply can take place? Proper function of above described antioxidant defense system makes hardly possible an appearance of any traces of ROS in the cells... As a matter of fact, various radicals are constantly formed in brain neurons and their content is high enough to ascribe them definite functional significance. Disordering of metabolism and increase in ROS level being characteristic of model experiments or during senile or neurodegenerative processes was not defined in brain under ischemic conditions for a long period of time. Modern neurochemical methods allow to demonstrate an increase in a number of ROS when oxidative stress is developed in injured brain. SAR and NO-radicals are increased in amount to 100 pM and 100 nM respectively (10-fold increase compared to the normal level); hydroxyl radical and peroxynitrite (which are not determined under normal conditions) are amounted to 150 nM and 120 μ M [50]. Serious potential damage by these ROS is no doubt because massive ROS attack to biomacromolecules brings signal of cellular death, which can be developed via immediate type (necrosis) or delayed process (apoptosis). Thus, one more signal function of ROS is a sign of cellular death [52-54].

6. Cell response to unfavorable factors — selection between apoptosis and necrosis

Long term activation of glutamate receptors taking place during disordering of neuronal function is a factor, which can result in cell death. Excitotoxic mechanisms of cell death are

the leading ones during ageing, a number of neurodegenerations like Alzheimer disease, parkinsonism, as well as in the case of acute disordering of brain blood supply.

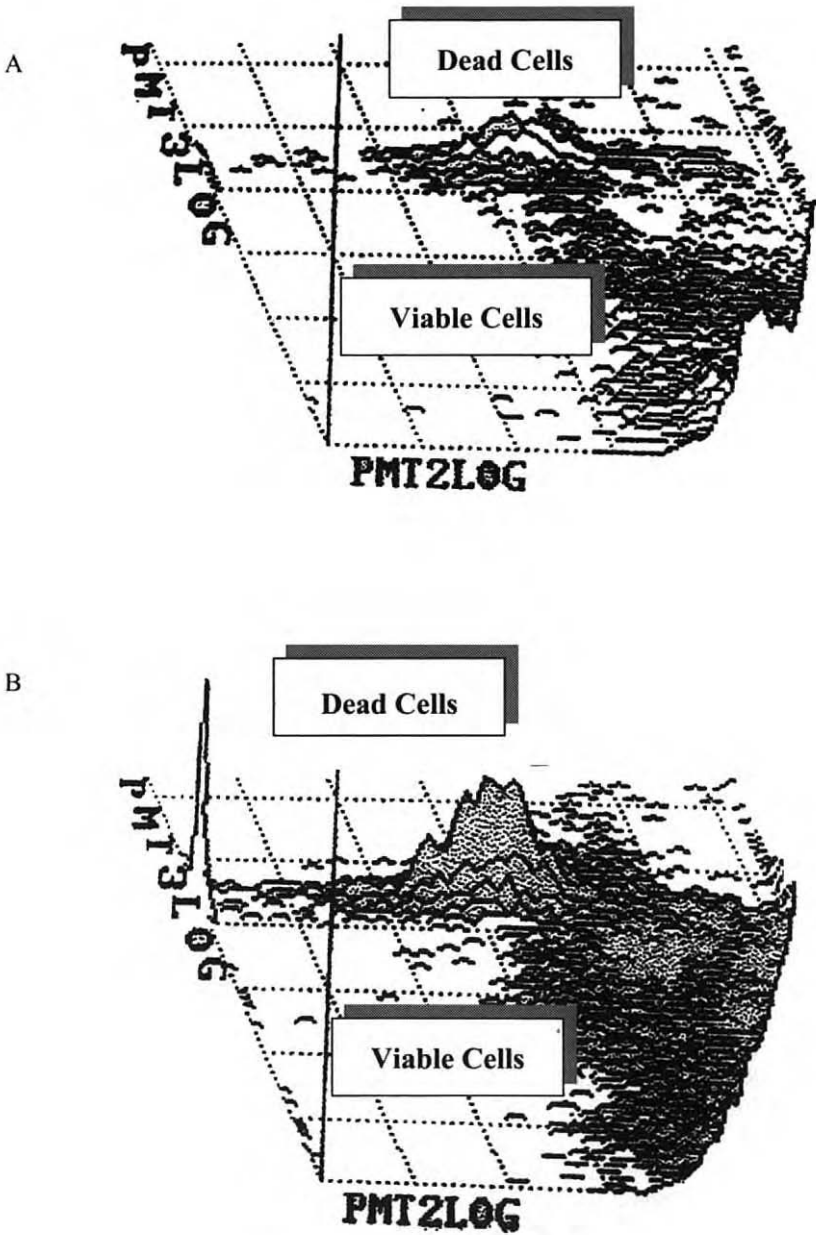


Figure 5. Distribution of neurons between viable and dead populations before (A) and after (B) ischemic injury in vitro [12].

Massive cell death in the experiments in vitro can be induced by high (0.5-3.0 mM) concentrations of kainate or NMDA. After exposure of neurons to such drugs the amount of cells being stained with propidium iodide, PI (so being dead) is proportional to the ROS

level [12,31]. Neuronal death induced by disordering of brain blood supply can be imitated when the suspension of neurons is placed into short-term hypoxia burdened by hypoglycaemia with subsequent reoxygenation. Data on viability of the population of neurons under such conditions are illustrated by Figure 5. Sharp increase in a portion of cells sensitive to PI staining (in other words, being dead) can be induced by combination of ischemic factors with exposition of cells to excitotoxic compounds [26].

One important question is which kind of cellular death takes place after brain injury. It is well known that time course of necrosis is differed from that of apoptosis. The latter is suppressed by protein synthesis inhibitors and need in cellular pool of ATP. This coincides with activation of specific genes and synthesis of a number of proteins regulating delayed cell death. Thus, programmed cell death depends on appearance of novel proteins, information about which is potted in cellular genome. In agreement with this paradigm, apoptosis is genetically programmed cellular death participating in ontogenic development (like degeneration of tail in tadpole or reorganization of up to 70% of body in insects during their transformation) or as a response to unfavorable environmental factors (neurodegenerative diseases).

Initiation of apoptosis includes several alternative (or, at least, independent) mechanisms. The cell makes a conclusion about "preferable" mode of cellular death choosing between variety of factors, the level of ROS being only one of them. In fact, not the ROS level itself but an inability of cellular defense system to resist their unfavorable effects results in decision to dye.

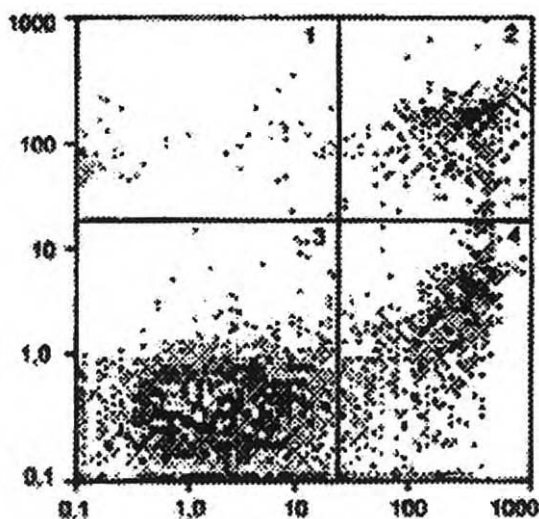


Figure 6. Discrimination between sub-populations of viable (3), necrotic (1+2) and apoptotic (4) neurons using double staining during flow cytometry approach (see text for explanation).

One of the earliest events of apoptosis is oxidative damage of the contacts between cytoskeleton and membrane bilayer. Among cytoskeletal proteins annexins is the family of proteins responsible for such contacts, while phosphatidylserine is involved from membrane bilayer site. After interruption of these contacts phosphatidylserine is disengaged and migrates from the inner (normal location) to the outer side of the bilayer (initiation of apoptosis). Thus, appearance of phosphatidylserine on the outer side of the membrane is a sign that apoptosis begins. Addition of fluorescent labeled annexin V (usually, FITS labeled annexin V) allows to mark the cells with phosphatidylserine located

on the outer side of the membrane, i.e. apoptotic ones. Such approach can be used for early recognition of cells with apoptosis initiated [31]. Fig. 6 demonstrates an example of how to discriminate the neurons between three populations — viable, necrotic and apoptotic when double staining of cells with PI and FITS-annexin V was used. It is seen that annexin V positive cells are concentrated in the fourth quadrant, PI-positive cells — in the first quadrant, and annexin and PI negative cells — in the third quadrant. A part of the cells is labeled both with annexin and PI — the second quadrant contains cells with seriously damaged membranes, which are labeled with both dyes. The cells in this quadrant are corresponded to “heavy” necrosis while those in the first quadrant — to “light” necrosis [12,31].

Figure 7 demonstrates excitotoxic effect of NMDA on neuron suspension. Exposure of the cells to 2 mM NMDA for 3 hrs results in cellular death by both necrosis and apoptosis. It is also seen that light necrosis in a control sample (Figure 7A) is substituted by heavy necrosis (Figure 7B). Lower concentration of this and other glutamate agonists exposed to the cells for a shorter time results in apoptosis not affecting amount of necrotic cells; the earliest features of apoptosis appeared 1-3 hrs after beginning of the experiment.

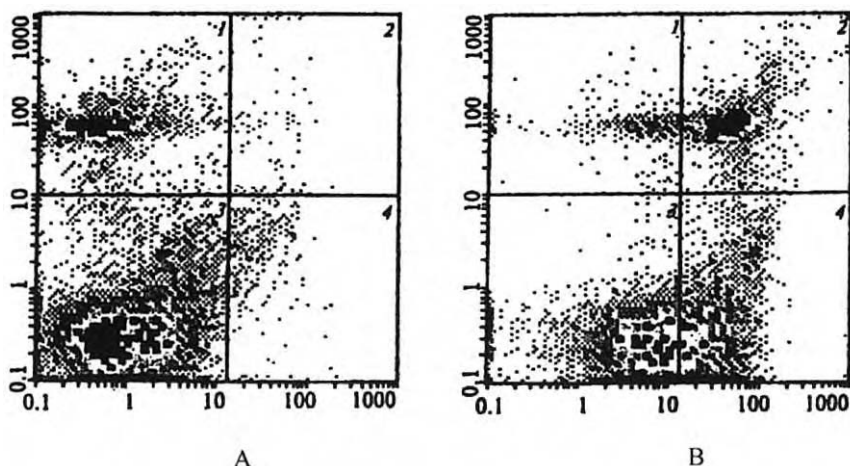


Figure 7. Induction of neuronal death by NMDA (A – control, B – after 3 hrs exposure to 2 mM NMDA).

Thus, under disordering of metabolism of excitotoxic mediators (glutamate, for example) neurons begin to be stressed by a non-compensated effect of ROS, which directly or via H_2O_2 work as other cytokines (TNF, several ILs, lipopolysaccharides, oxidized LDL) activating transcriptional factor NF- κ B being one of the key factor of expression of a number of cellular proteins. One of such proteins is inducible NO-synthase. One can think that its activation reflects an attempt to use NO in protection against disordering in brain blood supply. A good example demonstrating usefulness of NO in such protection is that transgenic knockout mice lacking nNOS are more vulnerable in relation to hypoxia [3].

Among NF- κ B activators there are free fatty acids, especially linoleic acid (C 18:2), and partial inhibitors — vitamin E. When inactive NF- κ B is present in cytosol it is associated with the inhibiting molecule, I κ B. Cytokines activate NF- κ B via cascade of kinases resulting in dissociation of I κ B and its proteolytic degradation [45]. It is important that ROS having signaling function in this process are usually formed by NADPH-oxidase located in plasma membrane of other than leucocytes (fagocytes) cells [45,55]. In the activated state, however, NF- κ B must go through red/ox regulation which is occurred by

thioredoxine. This is cysteine containing low molecular weight (12 kDa) protein controlling reducibility of many cellular proteins and NF- κ B is one of its important targets (reduction or oxidation of thioredoxine itself occurs in the cytosol because of the intracellular reductants or ROS). The effect of thioredoxine is directed on Cys62 in p50 subunit of NF- κ B, which is recognized by specific binding loupe of DNA only if Cys62 is reduced. Thus, the oxidation of Cys62 by ROS and its reduction by thioredoxine is another example of multi-step participation of ROS in survival of neurons under oxidative stress.

Relatively mobile (low molecular) cytosolic components taking part in red/ox regulation of metabolism (glutathione, thioredoxine) serve as rather regulators of key proteins like Na/K-ATPase, NF- κ B, than ROS scavengers or antioxidants. In response to any metabolic discomfort the cell increases the stationary level of ROS, which works as a signal for mobilization of metabolism to adapt to new conditions. Inability of red/ox system of the cell to quench the excess of ROS results in activation of a cascade of reactions inducing cellular death either chaotic (necrosis) or programmed (apoptosis). Efficiency of apoptosis depends very strongly on energetic level of the cell (red/ox state), that's why disordering of energetic metabolism and exhaustion of cellular ATP during apoptotic program realization may result in the change of the program and substitute the apoptosis by necrosis. In such a case, broadening of necrotic area and infiltration of phagocytes to the defect zone (as well as release of a number of cytokine including H_2O_2 and OCI^-) necrotic response generates inflammation area, in which abnormal components of tissue will be destroyed (Figure 3). Thus, we can see that intracellular rise in the same compounds — ROS — can provide signal either to activate metabolism and successfully neutralize ROS, or to induce apoptotic or even necrotic cell death — depending on the presence of a number of metabolic regulators and red/ox state of the cell.

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Protein Aggregates and the Development of Neurodegenerative Diseases

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Abstract: Protein aggregation seems to be a common feature of several neurodegenerative diseases and to some extent of physiological brain aging. We focus in this short review on the occurrence of protein aggregates in the most eminent neurodegenerative diseases. The current knowledge about the protein aggregates and the ways of their formation will be highlighted as well as the main proteolytic system counteracting the process of aggregation inside the cells — the proteasome. The different ways in which protein aggregates and the proteasome interact will be highlighted and the factors inhibiting the proteasome are discussed. Finally, the question whether protein aggregates are cause or consequence in the neurodegenerative process will be dealt with. In the light of the previously discussed information different therapeutic approaches are briefly consulted at the end of this short review.

1. Introduction

A variety of diseases and physiological processes is characterized by the occurrence of intra- or extracellular accumulation of proteins. When it comes to the terminology of these often cross-linked accumulations of protein, terms and definitions are not yet commonly agreed upon. Among others, the terms ‘protein aggregates’, ‘plaques’, ‘inclusion bodies’ or ‘aggresomes’ are used. Johnston et al. defines aggresome as “a pericentriolar, membrane-free, cytoplasmic inclusion containing misfolded, ubiquitinated proteins ensheated in a cage of intermediate filaments formed specifically at the microtubuli organization center (MTOC)” [1]. This seems to be the most narrow definition of all. Kopito uses the term ‘inclusion body’ as a somewhat broader definition, that does not include the microtubule dependence [1].

The term ‘protein aggregate’ appears to have a rather wide specificity, requiring mainly the existence of aggregations of misfolded protein. For extracellular protein aggregates the term ‘plaque’ is more common.

In this context we will use the broad definition of ‘protein aggregate’ referring to all aggregations of misfolded and accumulated ubiquitinated protein no matter whether extra- or intracellular, cytoplasmic or nuclear and whether or not associated with microtubuli.

Protein aggregation seems to be a common feature of many, albeit diverse neurodegenerative diseases and to a lesser extent, of physiological brain aging. At the moment for this class of diseases several names are common up, like ‘conformational diseases’, ‘protein deposition diseases’ or ‘gain of function diseases’. We shall focus on the occurrence of protein aggregates in the most eminent neurodegenerative diseases. To get an overview of the different diseases with their in part very divert pathologies we first shall give a short description focusing on the occurrence of protein aggregates. We will then turn to the aggregates themselves, analyzing the knowledge about their structural makeup and

discuss the different ways in which aggregation is thought to occur. The cell has evolved mechanisms to counter aggregation. From these proteolytic devices we have chosen to focus on the proteasome-ubiquitin system. The different ways in which protein aggregates and the proteasome interact will be highlighted and the factors inhibiting the proteasome are shown. Among these, biological aging stands out as the most prominent. How does the protein aggregation encountered in neurodegenerative diseases relate to the protein aggregation in aging? Summarily, the question whether protein aggregates are cause or consequence and various therapeutic approaches are briefly discussed.

2. Neurodegenerative diseases

2.1 The pathology of different diseases

Neurodegenerative diseases of the human brain are characterized by the death of selected populations of neurons and the occurrence of protein aggregates in the brain. Table 1 shows the mutated proteins found in some of these diseases, the form of the protein aggregates and the place of occurrence.

2.1.1 Neuronal Ceroid Lipofuscinosis (NCL)

NCL constitutes a group of neurodegenerative storage diseases, showing an accumulation of autofluorescent material in lysosome-derived organelles. Neurons of the central nervous system appear to be selectively affected and undergo progressive death. One form, the congenital ovine NCL shows a mutation in the cathepsin D gene leading to production of an enzymatically inactive but stable protein [2]. Cathepsin D is one of the major lysosomal enzymes responsible for protein degradation. In the late-infantile NCL another lysosomal protease is mutated, the pepstatin-insensitive proteinase (CLN2P) [3].

2.1.2 Morbus Parkinson (PD)

Parkinson disease is an age-related neurodegenerative disorder that affects in the US approximately 1×10^6 persons [4]. Pathological features include degeneration of dopaminergic neurons in the substantia nigra coupled with intracytoplasmic aggregates known as Lewy bodies. Neurodegeneration and Lewy bodies can also be found in the locus ceruleus, nucleus basalis, hypothalamus, cortex, motor nuclei. Risk factors are toxins e.g. MPP⁺, which causes a defect of the mitochondrial complex I [4].

In PD a mutation in the gene for a protein called synuclein was found. The function of this protein is unknown. Oxidation of normal synuclein could trigger aggregation. Synuclein was detected in many types of neurodegenerative diseases and it could be that this protein acts as a seeding factor in initiating aggregation formation.

2.1.3 Familial Amyotrophic Lateral Sclerosis (fALS)

Amyotrophic lateral sclerosis is an adult onset neurodegenerative disease. The motoneurons in the brainstem and in the spinal cord are selectively damaged. 15-20% of the patients have a mutation in the gene for the cytosolic superoxide dismutase (SOD I). It is thought that superoxide is not detoxified, side reactions of this enzyme form oxidants including peroxynitrite and the formation of nitrated proteins, is one of the reasons for cellular death [5].

It has been suggested that perhaps one of these reactions are up-regulated in animals with mutated SOD. But tests with low or high SOD expression levels did not show

differences in the development of the ALS syndromes [6].

An other explanation would be that the aggregation of SOD proteins, which had been found in astrocytes and in the neurons of ALS mice could kill the cells [7]. Injection of mutated or native SOD proteins into motoneurons of mice show that only the mutant form could kill the cells [8].

Wild-type SOD molecules that are damaged by oxidation were shown to aggregate spontaneously in vitro [9].

2.1.4 Huntington's Disease (HD)

HD is a progressive neurodegenerative disease with neuronal loss in the striatum and cortex. A mutation in a gene coding for a protein called Huntingtin is responsible for the symptoms, which are personality changes, motor impairment and subcortical dementia. Protein aggregates from Huntingtin with a fibrillar morphology are found in neurons [10,11]. The function of the wild-type protein is unknown [10,12]. The mutation is a CAG repeat expansion translated into a polyglutamine stretch. The disease breaks out if the critical length of 37 residues is reached. There is also a strong correlation between the length of the repeat and the age of onset and severity of symptoms.

Similar neuronal inclusions with aggregated polyglutamine containing protein were found in SCA-1 [13], SCA-3 [14], SCA-7 [15], and DRPLA [16].

2.1.5 Alzheimer Disease (AD)

AD is characterized by the presence of two types of senile plaques, dense core plaques and diffuse plaques mainly in the enterorinal cortex. The major component of the dense plaques is the fibrillar β -amyloid peptide. Around these plaques activated microglia could be found [17]. The accumulation and formation of β -sheet amyloid plaques outside the cells, the accumulation of diffuse amyloid aggregates around dead neurons and the formation of intracellular neurofibrillar tangles build up from tau proteins, are hallmarks of the disease. β -Amyloid peptides have been implicated as a causative agents in the brain of Alzheimer patients [18]. The β -amyloid forms fibrils and although these eventually aggregates as amyloid plaques, it is the fibrils themselves that are believed to be neurotoxic [19]. However several authors link also the accumulation of intracellular hyperphosphorylated tau proteins into neurofibrillar tangles to the progression of the disease [20].

2.1.6 Prion diseases

This group of diseases is characterized by the accumulation of a specific isoform of the normal prion protein (PrP), termed PrP^{Sc}, which seems to be the main infectious agent. PrP^{Sc} is derived by a conformational change from the isoform of the prion protein PrP^C. PrP^{Sc} is a copper-binding glycoprotein attached to the cell membrane of neuronal cells [21]. The PrP^{Sc} form is protease resistant, has a beta-sheet structure and possesses a tendency to polymerization [22].

The pattern of these prion diseases differs related to the pattern of PrP^{Sc} depositions, based on the occurring genotype of the prion protein and to the related different pathological conformer's of the PrP^{Sc} [21].

Situations which can change the conformation of prion proteins are related to the activation of microglial cells, releasing proinflammatory cytokines and reactive oxygen species. This elevated oxidative stress may somehow alter the conformation of the protein. Experiments with synthetic human prion peptides (PrP 106-126, PrP 127-147) revealed that the peptide structure is the relevant toxic factor for neuronal cells. PC 12 cells in vitro died

after addition of these peptides, but peptide solutions lacking stable beta-sheet structures or amyloid structures were nontoxic [22].

2.2 Occurrence of protein aggregates in neurodegenerative diseases

At the beginning there seemed to be no common feature to all these diseases, but since about 1963 the fact that protein aggregates could be found in a wide variety of neurodegenerative diseases gained recognition, when Orgel presented the thesis, that accumulated proteins lead to senescence of cells through toxic accumulation. [4,23].

Even in diseases where seemingly no protein inclusions could be found and no mutated protein acting as aggregator nucleus, recent use of immunochemical techniques has identified protein aggregates [24].

In each familiar form of a given disease, a mutation in the gene for a specific aggregated protein [25] was found. Thus, each aggregated protein is intimately connected to a different disease.

Table 1. Protein aggregation in neurodegenerative disease.

Name of disease	Protein aggregated	Localisation	Aggregate/ structure
Huntington	Huntingtin	nuclear	β -sheet
Parkinson	synuclein, tau	cytoplasmic	β -sheet
Alzheimer	tau, amyloid	extracellular/ intracellular	amyloid/ β -sheet
Creutzfeld-Jakob	prion protein	extracellular	amyloid/ β -sheet
Amyotrophic lateral sclerosis	SOD1/synuclein	cytoplasmic	β -sheet
Multiple system atrophy	synuclein	cytoplasmic	β -sheet
SCA-1 and SCA-3	Ataxin 1 and 3	nuclear	β -sheet
DRPLA	Atrophin 1	cytoplasmic	β -sheet
SBMA	Androgene receptor	cytoplasmic	β -sheet
Cerebellar ataxia	synuclein	cytoplasmic	β -sheet
Pick's disease	tau	cytoplasmic	β -sheet
Neuronal ceroid lipofuscinosis	lipofuscin	cytoplasmic	amyloid
Friedreich's	frataxin	nuclear	β -sheet
Alexander's	GFAP, tau-2	cytoplasmic	amyloid
Hallervorden-Spatz	synuclein, tau	cytoplasmic	β -sheet

(SCA: Spinocerebellar ataxia; DRPLA: dentatorubal pallidolusian atrophy; SBMA: spinal bulbar muscular atrophy)

Friedreich's disease: Chamberlain S., et al., Mapping of mutation causing Friedreich's ataxia to human chromosome 9. *Nature*, 1988, **334**, 248-250.

Alexander's disease: Towfighi J., et al., 1983, Alexander's disease: further light, and electron microscopic observations. *Acta Neuropathol* (Berlin), **61**, 36-42.

Brenner M., et al., 2001 Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander's disease, *Nature Gen*, **27**, 117-121.

Hallervorden-Spatz disease: Wakabayashi K., et al., juvenile-onset generalized neuroaxonal dystrophy (Hallervorden-Spatz Disease) with diffuse neurofibrillary and lewy body pathology, *Acta Neuropathol*, **99**, 331-336.

3. Protein aggregates

3.1 What characterizes a protein aggregate?

Protein aggregates are oligomeric complexes of normally not interacting molecules, that can be either structured or amorphous. Both types are insoluble and metabolically stable under normal physiological conditions [1]. Amorphous aggregates have been found in Alzheimer's and prion disease (see Table 1).

Aggregates are often accompanied by displaced and abnormally phosphorylated intermediates filaments, e.g. in Picks disease (GFAP), Parkinsons disease (Lewy bodies),

neurodegeneration type 4 (intermediate filaments) and amyotrophic lateral sclerosis (hyaline inclusion bodies) [26].

The aggregate is unrelated to the original function of the protein, but introduces a new element into the cell which might be toxic.

3.2 *Why and how does aggregation occur?*

Regular proteins will or will not aggregate depending on its function. Aggregations of the kind discussed here occur if the protein involved is misfolded.

It is estimated that about 30% of the newly synthesized proteins are misfolded [27]. Protein misfoldings also occur due to partial unfolding during thermal or oxidative stress and also because of alterations in primary structure caused by mutation [28,29].

The misfolded proteins connected to neurodegenerative diseases have a strong molecular propensity to aggregate [25].

A single event on the molecular level of a protein may theoretically trigger a chain reaction leading to the formation of an aggregate. When a soluble β -amyloid protein (in Alzheimer's disease) or prion protein (in prion disease) interacts with the seeding molecule, a change in its three-dimensional rather than in its covalent structure [30] takes place, rendering it prone to aggregation.

The process of aggregation is slow due to the complex process of specific intermolecular interactions required. The higher the concentration level of the protein, the higher the rate of aggregation. In so-called 'ordered aggregation' [31], polymerization will not be significant until a certain critical concentration is reached. In a saturated solution, aggregation can be triggered by a seed, similar to the way seeding works in crystallization [14].

One might speculate that seeds arise from mutant forms of protein. For some of the discussed diseases this could be proven in cell culture models. Proteins like Huntingtin, amyloid- β and alpha-synuclein aggregate in vitro and in transfected COS cells. Models of SCA-3 show nuclear inclusions with wildtype and mutated protein [14]. Another example for this situation is found in AD plaques, where a peptide with a partial alpha-synuclein sequences seeds β -amyloid polymerization in vitro [32].

Interestingly the aggregating disease-specific protein, is often not restricted to the disease specific brain areas, e.g. polyglutamine containing proteins — aggregations of which are typical for the trinucleotide repeat order diseases — are also expressed in non-affected brain regions and even outside of the brain. The specificity of the neurodegenerative processes for a definite brain area, therefore may be determined by associated binding proteins, accelerating or even starting the formation of aggregates [32].

Kopito suggests the process of aggregation to be a controlled process [24]. In animal cells this requires an active, retrograde transport of misfolded proteins on microtubuli [33,34].

3.3 *How does proteolysis work?*

Cells have evolved mechanisms to prevent aggregation. Molecular chaperons like HSP 90 or HSP 70 bind non-native protein conformations or aggregation-prone folded intermediates from the cytosol, thereby reducing the likelihood of aggregate formation [35].

On the other hand misfolded intracellular proteins are efficiently degraded by the proteasome. The proteasome is one of the major proteolytic systems in the cytosol of eukaryotic cells [36]. Misfolded cytoplasmic or nuclear [37,38] proteins are the target for destruction by the proteasome. Many misfolded proteins are recognized directly by their changed conformation [39] or targeted by covalent attachment of multiubiquitin chains for

degradation by the proteasome [40]. The ubiquitin-proteasome system is a quality control system for the prevention of protein aggregations. The occurrence of 20 S and 26 S proteasomes has been reported in aggregates of various cell lines including neuronal cells [41-44,35]. In SCA-1 neurons the 20 S proteasome was found near the nuclear inclusion of proteins. Furthermore, in some neurodegenerative diseases colocalization of proteasome subunits and aggregated proteins could be proven [45,46].

As mentioned above, Johnston et al. [1] suggests that there is an active transport by microtubuli that brings aggregates to the microtubuli organization center 'MTOC', where he identifies a 'proteolytic center'. Lysosomes and endosomes are also delivered by microtubuli to the same region of the cell [47]. This centralization would not only increase the efficiency of the proteasome-ubiquitin system and facilitate the disposal by an autophagic route but also avoid an interfering of aggregated material with proteins from the cytosol.

3.4 Factors inhibiting proteolysis

There is a constant competition between proteasomal degradation and aggregation of misfolded proteins in the cell. In normal cells improperly folded proteins are selectively degraded before they can aggregate [48,49]. However, the effectiveness of proteolysis may be hampered by a number of occurrences.

The most obvious errors may occur when the proteolytic system is overcharged by the mass of degradable material. This could happen if an elevation of oxidative stress in the brain leads to an increase in the production of misfolded proteins thus raising the probability and occurrence of aggregation. Another variant would be a change in the gene code which produces a protein that aggregates more easily [50].

A further set of factors can be seen in those errors and irregularities that occur in one facet or the whole proteolytic process and affect the efficiency of proteolysis. At first, a gene which participates in proteolysis could be hampered or damaged. The ubiquitination machinery could be damaged, resulting in incorrect tagging of misfolded protein.

The relationship between proteasome inhibition and aggregates was focused on by a number of studies: tests with inhibited proteasome in HeLa cells transfected with mutant ataxin-1 [51] showed increased accumulation of subnuclear aggregates. These aggregates alter the staining pattern of the 20 S proteasome, suggesting that it is targeting the aggregated proteins [52]. The abnormal distribution after overexpression of mutated ataxin suggests that it is targeting the inclusion bodies in an attempt to degrade them. Tests with ataxin-2 and ataxin-3 did show that an inhibition of proteasomal breakdown leads to increased accumulation of these proteins [49].

Proteins with an expanded polyglutamine repeat are more resistant to proteasomal degradation [53]. Inhibition of the proteasome also induced accumulation of aggregated forms of a viral nucleoprotein reported by Anton et al. [54]. Recently was found an inhibition of the proteasome in a transfected cell line with a protein FLAG-F508 and with the Huntingtin protein [55].

Figueiredo-Pereira et al. discovered that proteasome inhibition leads to accumulation of ubiquitinated proteins [56]. Proteasome inhibition is also connected with the activation of the cellular stress response including an increased expression of HSP 70 and activation of the inflammatory pathway shown by the increase of COX-2 expression and the production of the pro-inflammatory prostaglandin PGE2. An interaction of these two pathways may happen in the cascade of events leading to neurodegeneration [56].

To degrade proteins they must be unfolded before to be able to enter the 20S proteolytic core particle. Generally speaking, oxidized, therefore partially unfolded proteins, are better substrates for the proteasome, in comparison to native, normal folded

proteins [36]. However, overly oxidized and aggregated proteins no longer ‘fit’ the proteolytic ‘grip’ of the proteasome. The changed conformation of these proteins may result in restricted entry into the core particle of the proteasome or incomplete degradation.

According to the ‘bite and chew model’ proposed by Kisselev et al. 1999 [57], the proteasome loses its proteolytic power if it is clogged up by non-degradable material. Results from our group demonstrate, that non-degradable protein aggregates may inhibit the proteasome [58]. Tests with non-dividing and postmitotic fibroblasts reveal that the proteasome is gradually inhibited through aggregates, which bind to the proteasome, but can not be degraded. It is suggested that a constant minor accumulation of aggregates occur with time, because some misfolded proteins will always ‘escape’ the proteasome. After adding artificially cross-linked proteins the proteasome displays a decrease in activity in fibroblasts [59]. After adding artificially cross-linked β -amyloid peptide or cross-linked albumin, these aggregates inhibited the proteasome in vitro [58].

It can be summarized, that errors in proteolysis — especially proteasome inhibition — and protein aggregation are processes that promote each other.

Table 2. Age-related changes of proteasome-activity or subunit-composition of the proteasome [63-66,82-86].

	Human	Mouse	Rat
20S Proteasome subunits:	alpha 2 (HC3, P25787) alpha 7 (HC8, P25788)	LMP7 (AAA75033) LMP2	alpha 2 (P17220) alpha 3 (P21670) beta 6 (P18421)
19 S cap subunits:	p42 (ATPase, Q92524) MSS1 (ATPase, P35998) p55 (NP_002807) p44.5 (NP_002806)	TBP1 (ATPase, AA145829)	ATPase (BAA09341)
Proteasome activator PA28:		alpha subunit (U60328)	
Chymotrypsin-like activity	decreased in lens, t-lymphocytes and fibroblasts		tested in heart, lung, kidney, liver, decreased by 3vs 28 months
trypsin-like activity	decreased in fibroblasts		tested in muscle decreased 4 vs 34 months
PGPH-like activity	different results		tested in muscle decreased 4 vs 34 months

(In brackets: other name, sequence number (NCBI data base) or correct enzyme number.)

3.5 Aging

Focusing on the proteasome, a general decline of the proteasomal system was found in aged tissue cultures. This includes the decreased activity of the proteasome towards artificial peptide substrates as well as the ability to degrade oxidized model proteins [59-61].

It can be observed that with time the proteasome can be damaged or inactivated by oxidants [62]. Furthermore, the subunit composition of the proteasome changes with age. Alterations for LMP2, LMP7, subunit Z, Ub Thiolesterase and 26 S components have been found [63-66]. Mitochondrial mutations accumulating with aging [67] often decrease ATP production. This also affects the ATP-dependent proteolysis process.

If one understands the process of aging as an accumulation of damages with time [67] such a process is especially critical within postmitotic cells such as neurons. During aging of these cells, the process of seeded polymerisation (see above) could play an important role. In these cells the required concentration will be reached sometimes during aging, initiating a rapid degeneration once a certain level has been reached.

3.6 What are the results of aggregation?

The question arises, whether protein aggregates are cause or consequence of neurodegeneration. The mentioned findings of aggregates in disease afflicted regions of the brain could suggest a detrimental effect of the aggregates on the cell.

One possible way in which an aggregate could bring about neurotoxicity would be through mediation of radical formation. In PD elevated membrane peroxidation products, increased 8-hydroxy-2-deoxyguanosine, lower content of polyunsaturated fatty acids and higher carbonyl-content of proteins, indicate an increased ROS-level [68].

One of the best studied proteins is the amyloid peptide, which seems capable of inducing free radical production in Alzheimer disease [69]. Amyloid peptide can bind metals and these can produce radicals through the Fenton reaction [70,71]. Since aggregation of proteins is also promoted through metals, a spiral of aggregation steps starts [72].

A second way to produce elevated oxidative stress in the brain, could be the radical mediated uptake of aggregates: if microglial cells encounter extracellular β -amyloid deposits, the aggregates bind to the RAGE or to a type 2 scavenger receptor. This activates the microglia. Activation of microglia, produces large amounts of free radicals by oxidases [73] that contribute to the burden of oxidative stress in the affected brain. In the case of AD this was tested in a clinical study. Microglial cells are triggering additive oxidative stress in PD through release of high amounts of γ -IFN, TNF-alpha and interleukin 1 β . The level of all of these factors are increased by 7-15fold [74] This additive oxidative stress supports the progression of the disease. Patients who were treated with an anti-inflammatory medicament had reduced prevalence of AD [71].

It could be proven with oxidative markers like the carbonyl content, nuclear 8-oxoguanosine and the 3-nitrotyrosine level, that patients with ALS show elevated levels of oxidative stress [75-77]. As already mentioned some families with familiar ALS have mutations in the SOD I gene, so it is easy to suggest that oxidative stress may play an important role in the pathogenesis of ALS. Animals transfected with a truncated form of the enzyme show an ALS phenotype [76].

The conclusion could be drawn that oxidative stress acts as a promoting factor of neuronal cell death. Oxidative stress seems to alter the onset and the development of the disease, but is not necessarily causal for the spreading of neuronal death. However if a particular protein does not cause a detrimental effect itself, the protein-aggregates cause secondary changes which may be harmful. Protein aggregates are ubiquitinated and accumulation of intracellular ubiquitin conjugates leads to cell cycle arrest [77]. Furthermore while the proteasome is inhibited by aggregates transcription factors cannot be degraded in time and initiate the apoptosis-pathway [78]. Therefore, the disturbance of the normal level of proteins, can cause the induction of apoptosis. Studies show, that protein aggregates are present in the brain before patients show clinical symptoms [4].

All these findings suggest that the above question could be answered in the following fashion: protein aggregates are more than just a consequential side effect in neurodegenerative diseases. While the initial onset of the degeneration process might be disease-induced, protein aggregates are an enhancing factor that drives neurodegeneration.

Summarizing, no matter what the initial onset — whether a major error affecting the proteolytic system or the slow accumulation of additive errors during the aging process, the effects of the presence of protein aggregates are very similar: once aggregation has occurred, the aggregated protein hampers the activity of the proteasome. The more the proteolytic systems loses activity the more misfolded or oxidized proteins are accumulating. Abundance of cellular waste eventually proves toxic to the cell.

4. Therapeutic approaches against protein aggregates

One therapeutic approach could be the stimulation of the proteasomal activity and perhaps specificity. On the other hand it seems to be possible to prevent the formation of aggregates through induction of chaperone genes, which help to reestablish the normal conformation of proteins. Furthermore the induction of other small molecules inhibiting the formation and the seeding of aggregates at an early stage, capable of crossing the blood-brain barrier, could be a strategy against neurodegenerative diseases. Some tests with HSP 70 and HSP 40 have shown to inhibit the self-association of polyglutamine proteins [79]. By in vitro experiments these heat shock proteins shift the self-association from fibrillar β -sheet formation to amorphous aggregates, which are then amenable to be degraded by the proteasome system [24].

Another possibility is the reduction of the concentration of the disease-related protein in neurons by antisense strategies. That should slow down the disease progression, although it is not clear whether here not a serious malfunction due to the missing of the protein might take place. Heiser et al. [80] demonstrated that a monoclonal antibody 1C2, recognizing elongated polyglutamine tracts, Congo red and thioflavine slows down Huntingtin exon 1 aggregation in vitro and in cell culture.

It should even be possible to break-up already existing aggregates. Melatonin has the capacity to intercalate into β -pleated sheet structures and break them up [25].

If the production of free radicals is involved in the aggregation processes in neurodegenerative diseases, then inhibiting free radical production might be an important pharmaceutical strategy.

Another mechanism preventing aggregation is the immunization against aggregated protein structures. A dramatic success had been archived using this method by Schenk et al. 1999 in the case of amyloid β [81]. He was able to prevent further amyloid deposition, in older mice that already had neuritic senile plaques due to a overexpression of a mutant form of the Alzheimer precursor protein (APP) that generates high level of amyloid β .

As manifold as the triggering events for aggregate induced neuronal degeneration are, as numerous are the approaches for intercepting, stopping and maybe eventually curing the neurodegenerative diseases.

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Inflammatory Response of the Brain Following Cerebral Ischemia

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Abstract: Brain injury following cerebral ischemia develops from a complex series of pathophysiological events. Ischemia due to middle cerebral artery occlusion encompasses a densely ischemic focus and a less densely ischemic penumbral zone. Cells in the penumbra may be salvaged by reperfusion or by drugs that prevent an extension of the infarction into the penumbral zone. Factors responsible for such an extension include acidosis, edema formation, acute local inflammation, dissipative ion fluxes, calcium overload, glutamate excitotoxicity, free radical formation, nitric oxide overproduction and programmed cell death. There is increasing evidence that ischemic brain injury secondary to arterial occlusion is characterized by acute local inflammation, which involves accumulation of polymorphonuclear neutrophils (PMN). Factors that influence the recruitment of PMN could represent new therapeutic targets in acute stroke. Overexpression of inflammatory mediators such as cytokines, chemokines and adhesion molecules promotes recruitment of leukocytes in the ischemic area. Leukocytes have deleterious effects in brain ischemia and play key role in the progression from ischemia to irreversible injury. An inflammatory reaction is a common response of the brain parenchyma to various forms of insult. It is characterized by the infiltration of leukocytes, which are mainly polymorphonuclear leukocytes and monocytes/macrophages. Human data regarding inflammation in stroke are scarce. The inflammatory response of ischemic brain parenchyma has been more thoroughly explored in animal models. In animal models of cerebral ischemia, accumulation of PMN has been detected within the first 12 hours after induction of ischemia. During reperfusion after acute ischemia, polymorphonuclear neutrophils (PMN) are believed to exacerbate tissue damage by both physical obstruction of vessels and release of oxygen radicals, proinflammatory cytokines, and cytolytic enzymes. There are a number of mechanisms by which leukocytes may produce deleterious effects on ischemic parenchyma. It has been proposed that leukocytes obstruct the microvessels and contribute toward the so-called "no-reflow" phenomenon. This indicates the lack of complete recovery of cerebral blood flow in the ischemic area after reperfusion. Other detrimental effects of leukocytes during ischemia may be due to the release of vasoconstrictive mediators, such as thromboxane A_2 , endothelin-1, and prostaglandin H_2 ; an alteration in cerebral artery vasoreactivity; the release of cytotoxic enzymes; free oxygen radicals; NO; and products of the phospholipid cascade. It is believed that the release of proteolytic enzymes such as elastase might damage endothelial cell membranes and the basal lamina, alter the blood-brain barrier, and contribute to the formation of postischemic edema. In addition, loss of the integrity of the endothelial cell-basal lamina lining might facilitate the escape of red blood cells and the hemorrhagic transformation of a brain infarct. An understanding of the role of leukocytes and the mediators of inflammation in cerebral ischemia may have a very great impact on therapy. An increasing number of molecules are currently being investigated in animals for their possible effectiveness in human acute stroke. One group of studies has focused on therapeutic strategies related to the role of proinflammatory cytokines. A second group of studies has concentrated on molecules that are capable of blocking the adhesion between endothelial cells and leukocytes. Another group of studies consist of antiinflammatory agents such as cyclooxygenase inhibitors. Broad spectrum antibiotics with antiinflammatory effects

have been investigated in focal and global ischemia. Drugs capable of interfering with inflammation-related mechanisms have given encouraging results in experimental stroke models in animals. Possible future pharmacological treatments could be based on the inhibition of proinflammatory mediators, prevention of adhesion between the leukocytes and endothelial cells, controlling the specific transduction pathway signals following cytokine production and promoting neovascularization.

1. Cerebral Ischemia

Cerebral ischemia is a common and devastating neurological disorder which is the third leading cause of death in major industrialized countries and also a major cause of long-lasting disability [1-8]. Cerebral ischemia is always vascular origin and can be divided into haemorrhagic and thromboembolic, with the latter accounting for approximately 90% of strokes and results from embolic or thrombotic occlusion of the major cerebral arteries, most often the middle cerebral artery [1].

Cerebral ischemia can be classed by topography as global or focal and by chronology as reversible and irreversible. Focal hypoxia-ischemia also occurs in such contexts as traumatic insults or cerebral hemorrhages, while global hypoxia-ischemia occurs in cardiac arrest, near drowning and carbon monoxide poisoning [9].

Occlusion of middle cerebral artery (MCA) develop an infarct area in the MCA territory. Ischemia due to middle cerebral artery occlusion encompasses a densely ischemic focus and a less densely ischemic penumbral zone. A rim of moderate ischemia surrounds the severely ischaemic area. This is called penumbra which lies between normally perfused brain and infarct area. Penumbra doesn't exist in global ischemia. The penumbra defines regions with blood flow below that needed to sustain electrical activity, but above that required to maintain cellular ionic gradients, and that lead in time to infarction [10]. In penumbra, pathophysiological mechanisms are dynamic, cell death occurs last and pharmacological intervention has been most successful. Penumbra may extend outside [11]. Cells in the focus are usually doomed unless reperfusion is quickly instituted. In contrast, although the penumbra contains cells "at risk", these may remain viable for at least 4 to 8 hours. Cells in the penumbra may be salvaged by reperfusion or by drugs that prevent an extension of the infarction into the penumbral zone [12].

2. Mechanisms involved in ischemic brain damage

Many events occurring during and after cerebral ischemia are well known, but they are not known enough to fully elucidate the mechanisms of brain damage. Factors responsible for the extension of infarction into the penumbral zone include acidosis, edema formation, acute local inflammation, dissipative ion fluxes, calcium overload, glutamate excitotoxicity, free radical formation, nitric oxide overproduction and programmed cell death [2,13-16].

Central to any discussion of the pathophysiology of ischemic lesions is energy depletion. Energy failure alone cannot explain the functional damage occurring during the reperfusion phase. The brain is critically dependent on its blood flow for a continuous supply of oxygen and glucose. Within minutes of the onset of ischemia, energy demands exceed the brain's capacity to synthesize ATP anaerobically. Energy depletion has fundamental importance in the genesis of subsequent injurious events. Lactate and unbuffered hydrogen ions accumulate in tissue in proportion to the carbohydrate stores present at the onset of ischemia. In addition to the rapid change in tissue acid-base status, failure of all energy dependent mechanisms, including ion pumps, leads to the deterioration

of membrane ion gradients, opening of selective and unselective ion channels and equilibration of most intracellular and extracellular ions (anoxic depolarization). As a consequence of anoxic depolarization, potassium ions leave the cell, sodium, chloride and calcium ions enter. Cellular accumulation of ions cause formation of cytotoxic edema. Brain edema is one of the major determinants of the survival of stroke patients. Since most mechanisms that maintain membrane calcium gradient are either directly or indirectly energy dependent, loss of ATP rapidly leads to a massive calcium influx and release of calcium from intracellular compartments. Extracellular concentrations of glutamate are markedly elevated in ischemic brain tissue as a consequence of both enhanced release of the amino acid from neurons and its impaired uptake into glia and neurons. Glutamate released from depolarized presynaptic endings activates several postsynaptic receptor/channel complexes which are named according to the preferred agonist (the quisqualate, kainate and NMDA-preferring receptor). Of these, the N-methyl-D-aspartate (NMDA) receptor/channel complex is permeable to calcium ions. Calcium ions are among the most powerful intracellular messengers, able to give rise to a great variety of events. Intracellular Ca^{2+} overload during ischemia is thought to have several deleterious consequences including: a) beginning of the metabolic cascades, which include activation of phospholipase A_2 , attacking cellular membranes, liberating fatty acids (mainly arachidonic acid) and altering membrane permeability and cell function; b) mitochondria accumulates calcium, which uncouples oxidative phosphorylation at a time when ATP production is already reduced; c) alteration of receptor function; d) toxic excitatory amino acid (EAA) release, precipitating neurons in a state of hyperexcitability [15,16].

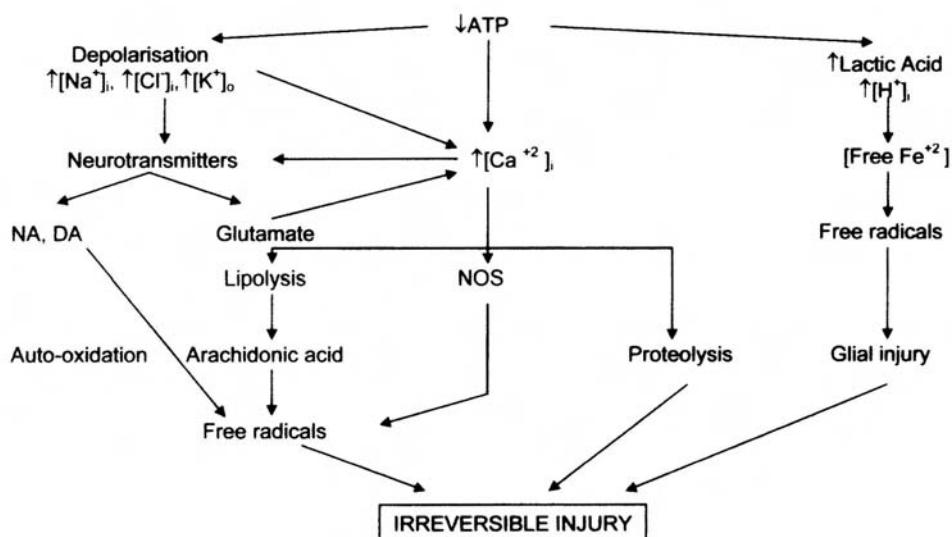


Figure 1. Potential mechanisms of ischemic brain damage. From T. Özben [15] by copyright permission of Plenum Press, New York and London.

Intracellular Ca^{2+} overload can also set off a cascade of events which may lead to the formation of reactive oxygen species, promoting arachidonic acid metabolism and converting xanthine dehydrogenase into xanthine oxidase. Injury to brain cells may release iron ions that can stimulate free radical reactions. In addition, there is a high concentration of ascorbic acid in the gray and white matter of the CNS. Ascorbate/iron and

ascorbate/copper mixtures generate free radicals. One more source of oxygen free radicals is the intramitochondrial electron transfer chain. Free radicals produced in mitochondria may cause point mutations, DNA cross link and DNA strand breaks in mitochondrial genes. Damage to the mitochondrial genome results in impaired respiration, further increasing the possibility of oxygen radical production. In addition, the brain is poor in catalase activity and has only moderate amounts of superoxide dismutase and glutathione peroxidase [15,16].

Nitrogen monoxide (NO) has recently emerged as an important mediator of cellular and molecular events which impacts the pathophysiology of cerebral ischemia. An increase in intracellular Ca^{2+} resulting from the activation of voltage-gated Ca^{2+} channels or ligand-gated Ca^{2+} channels or from the mobilization of intracellular Ca^{2+} stores could activate the enzyme NO synthase (NOS; EC 1,14,13,39) which catalyzes the synthesis of NO from the guanido nitrogen of L-arginine and molecular oxygen. NO is produced in neurons, glia cells and vascular endothelium in central nervous system (CNS). Depending on its origin, its effects are varied. NO is a mediator having both neurotoxic and neuromodulator effects. Neuronal NO is proposed as the neurotoxic agent mediating NMDA toxicity and increasing acute ischemic damage. It causes cytotoxicity through formation of iron-NO complexes with several enzymes including mitochondrial electron transport chain, oxidation of protein sulfhydryls and DNA nitration. NO may mediate cell death also through formation of the potent oxidant peroxynitrite (ONOO^-). ONOO^- decomposes to the hydroxyl radical ($\text{OH}^\bullet$) and nitrogen dioxide radical ($\text{NO}_2^\bullet$) which is a potent activator of lipid peroxidation. On the other hand, vascular NO as a potent vasodilator and an inhibitor of platelet aggregation, may be beneficial in the early stages of focal cerebral ischemia. It may facilitate collateral blood flow to the ischemic territory [15,16]. A third isoform, iNOS, is normally not present in most cells, but its expression is induced in pathological states associated with inflammation. iNOS generates toxic levels of NO, and may contribute to the cytotoxicity induced by inflammation [13]. In the brain, iNOS is induced by postischemic inflammation. After transient or permanent middle cerebral artery (MCA) occlusion in rodents, iNOS messenger RNA has been reported to be upregulated and peaks at 12-48 h after ischemia [13]. iNOS is induced in neutrophils infiltrating the injured brain and in cerebral blood vessels in the ischemic territory. It has been also reported that postischemic NO production continues during the recovery phase of ischemic stroke. The data with iNOS inhibitors along with the data from studies in iNOS-null mice, suggest that NO produced by iNOS is an important factor in ischemic damage [13].

Recent studies have provided evidence that expression of the inflammation-related enzymes, nitric oxide synthase (iNOS) and cyclo-oxygenase (COX)-2 are critical mechanisms by which inflammatory cells influence the progression of cerebral ischemic damage [13]. COX is a rate limiting enzyme in the synthesis of prostaglandins and thromboxanes. Two isoforms have been described: COX-1 and COX-2. COX-1 is involved in normal cellular function. COX-2 is normally expressed at low levels in neurons. COX-2 is upregulated in response to mitogens, inflammatory mediators and hormones. In inflammation, it contributes to tissue damage through the production of reactive oxygen species and toxic prostanoids. Superoxide produced by COX-2 reacts with NO to form the powerful oxidant peroxynitrite [13]. There is evidence that COX-2 participates in cerebral ischemia [13]. It was shown that COX-2 messenger RNA and protein expression are upregulated 12-24 h after cerebral ischemia in rodents. COX-2 expression in rodents has been observed in neurons at the periphery of the infarct, in vascular cells, and in microglia [3,13]. It was reported that administration of a selective COX-2 inhibitor, 6 h after ischemia reduced infarct volume in a model of focal cerebral ischemia in rats [3,13].

There is increasing evidence that ischemic brain injury secondary to arterial occlusion is characterized by acute local inflammation, which involves accumulation of poly-

morphonuclear neutrophils (PMN). An inflammatory reaction is a common response of the brain parenchyma to various forms of insult. It is characterized by the infiltration of leukocytes, which are mainly polymorphonuclear leukocytes and monocytes/macrophages [5,7,17]. Overexpression of inflammatory mediators such as cytokines, chemokines and adhesion molecules promotes recruitment of leukocytes in the ischemic area. Leukocytes have deleterious effects in brain ischemia and play key role in the progression from ischemia to irreversible injury. Human data regarding inflammation in stroke are scarce. The inflammatory response of ischemic brain parenchyma has been more thoroughly explored in animal models. In animal models of cerebral ischemia, accumulation of PMN has been detected within the first 12 hours after induction of ischemia [5,7,18]. During reperfusion after acute ischemia, polymorphonuclear neutrophils (PMN) are believed to exacerbate tissue damage by both physical obstruction of vessels and release of oxygen radicals, proinflammatory cytokines, and cytolytic enzymes [5,19]. There are a number of mechanisms by which leukocytes may produce deleterious effects on ischemic parenchyma [5,7,19]. It has been proposed that leukocytes obstruct the microvessels and contribute toward the so-called "no-reflow" phenomenon [7,20]. This indicates the lack of complete recovery of cerebral blood flow in the ischemic area after reperfusion [7,21-23]. Other detrimental effects of leukocytes during ischemia may be due to the release of vasoconstrictive mediators, such as thromboxane A₂, endothelin-1, and prostaglandin H₂; an alteration in cerebral artery vasoreactivity; the release of cytotoxic enzymes; free oxygen radicals; NO; and products of the phospholipid cascade [5,7,19,24]. It is believed that the release of proteolytic enzymes such as elastase might damage endothelial cell membranes and the basal lamina, alter the blood-brain barrier, and contribute to the formation of post-ischemic edema. In addition, loss of the integrity of the endothelial cell-basal lamina lining might facilitate the escape of red blood cells and the hemorrhagic transformation of a brain infarct [7,25-27].

3. Cytokines and cerebral ischemia

It is believed that cytokines play a key role in the entry of leukocytes into the ischemic area [2-8]. Cytokines are low-molecular-weight glycoproteins that act as intercellular messengers and mediate and regulate immune and inflammatory responses [5,7]. They are produced by activated macrophages, monocytes, lymphocytes, endothelial cells, fibroblasts, platelets, and many other cell types [7,28]. In vitro, cytokines can be produced by astrocytes, neurons, endothelial and microglia cells [7]. Cytokines act at very low concentrations on specific target-cell receptors, whose expression is modulated by the cytokines themselves. Binding of cytokines to receptors activates intracellular second messenger systems and several protein kinases and phosphatases. These enzymes trigger the expression of a number of proinflammatory genes by inducing the synthesis of transcription factors including nuclear factor- κ B, hypoxia inducible factor-1, interferon regulatory factor-1 and Stat3 [2,29-32]. For example, cytokines such as TNF- α activates transcriptional factor nuclear factor κ B (NF κ B), which is an important event in the signal transduction cascade that leads to the transcription of the genes. In resting cells, NF κ B is located in the cytosol in an inactive form due to its binding of the NF κ B inhibitor, I κ B. If upstream signals of the signal transduction cascade induce a conformational change of I κ B, NF κ B is released. NF κ B translocates to the nucleus where it binds to NF κ B regulatory elements in the promoter regions of the adhesion molecules such as VCAM-1, ICAM-1 and E-selectin genes. Cytokines are considered to be the principal mediators of immunologic and inflammatory responses [7]. During cerebral ischemia cytokines can attract leukocytes and stimulate the synthesis of adhesion molecules

in leukocytes, endothelial cells, and other cells, thus promoting the inflammatory response of damaged cerebral tissue. They can facilitate thrombogenesis by increasing levels of plasminogen-activating inhibitor-1, tissue factor, and platelet-activating factor and by inhibiting tissue plasminogen activator and protein S [7,33-35].

Increased production of several cytokines, including interleukin IL-1 β , IL-6, IL-8, IL-10, TNF- α , interferon- γ (IFN- γ) and granulocyte-macrophage colony-stimulating factor, has been demonstrated intrathecally in patients with acute ischemic stroke [5,36-38]. Increased synthesis of cytokines in acute stroke is, however, not restricted to the central nervous system (CNS) but can also be detected systemically [5,8,39,40].

3.1 IL-1

It was observed that rats with a transient MCA occlusion have a larger brain infarction when recombinant human IL-1 β is injected into the lateral ventricle immediately after reperfusion [7,41]. Similar results have been obtained in rats with a permanent MCA occlusion [7,42]. The intraventricular injection of recombinant human IL-1 β also enhances the formation of brain edema and increases both the number of neutrophils in ischemic areas and neutrophil-endothelial cell adhesion. The most widely recognized functions of IL-1 appear to be the induction of endothelial cell adhesion molecule expression and the promotion of neutrophil tissue infiltration [7,41]. These observations suggest that IL-1 may play a deleterious role in cerebral ischemia. Studies showing a reduction in infarct size after the administration of IL-1 antagonists or inhibitors provide further evidence of the importance of IL-1 in cerebral ischemia [41,43-49]. The possible harmful mechanisms induced or activated by IL-1 include fever, increased heart rate and arterial blood pressure, enhancement of N-methyl-D-aspartate-mediated injury, proliferation of microglia, release of arachidonic acid, and stimulation of NO synthesis [7,50].

IL-1 exists in two separate forms (α and β), which have only one-third sequence homology [7]. IL-1 is overexpressed during brain ischemia, as documented by the induction of IL-1 β mRNA synthesis in rats with permanent MCA occlusion, transient global forebrain ischemia, or a ligated carotid artery associated with hypoxia [7,51-57]. The complex functions of IL-1 are mediated by specific cell-surface receptors and regulated by the IL-1 receptor antagonist [7,58]. Two main receptors for IL-1 have been identified; type I is present in many cell types and binds IL-1 α and IL-1 β with similar affinity [7,59]. Type II is found on the surface of B cells, neutrophils, and macrophages and shows higher affinity for IL-1 β [7,59]. Types I and II are regulated differently in brain ischemia and may thus play separate roles. In spontaneously hypertensive rats, the mRNA for the type I IL-1 receptor was found to be relatively highly expressed in the normal cortex, with a marked increase 5 days after cerebral ischemia [7,58]. Type II mRNA has low basal expression and a peak 12 hours after the onset of ischemia [7,58]. The possible mechanisms of intracellular signal transduction for IL-1 on peripheral immune cells include effects on cAMP, protein kinase C, and protein phosphorylation. These effects remain to be proved in cerebral ischemia [7,59]. The IL-1-receptor interaction is quickly followed by the induction of immediate-early genes such as *c-jun* and *c-fos* [7,60].

3.2 IL-6

IL-6 plays a central role in host defense and in acute and chronic inflammatory activities. It is expressed in response to various forms of cerebral injury [7,61,62]. It was found that in the rat, IL-6 mRNA is overexpressed 3 hours after permanent MCA occlusion and reaches a peak at 12 hours; its expression remains high for at least 24 hours [7,63]. Higher IL-6 levels have been detected in the peripheral blood of patients with acute cerebral ischemia

than in control subjects [7,36]. It has not yet been completely clarified whether IL-6 exerts anti-inflammatory or pro-inflammatory effects or both [7]. Its anti-inflammatory effect depends on the inhibition of IL-1 and TNF- α production via a negative-feedback mechanism and stimulation of the production of their circulating antagonists which are soluble TNF- α receptor and IL-1 receptor antagonists [7,61,64]. IL-6 induces the release of corticotropin and cortisol and promotes the expression of acute-phase proteins that has anti-proteinase and oxygen scavenger functions [7,62,65-68]. It has been shown that IL-6 induces phospholipase A₂ gene expression and as a consequence, stimulates the production of leukotrienes, prostaglandins, and platelet activating factor, all of which are involved in ischemic brain damage [7,69,70].

3.3 IL-17

IL-17 induces the production of IL-8 in endothelial and parenchymal cells, indicating an indirect role in PMN recruitment. IL-8 is a potent chemoattractant for polymorphonuclear neutrophils (PMN) and can also stimulate the release of neutrophil granules and the respiratory burst of these cells [5,71-78]. In their study, Kostulas et al. found that the proinflammatory cytokine IL-17 was elevated systemically after ischemic stroke. IL-17 induces the secretion of cytokines, including IL-8, and enhances the expression of ICAM-1 in cultures of stromal cells and human fibroblasts [5,77,79].

3.4 TNF- α

TNF- α induces the expression of adhesion molecules by glial and endothelial cells, facilitates neutrophil adherence and accumulation in microvessels [7,80,81]. TNF- α is suggested to be involved in blood-brain barrier alterations, a proadhesive-procoagulant transformation of endothelial cell surfaces, and glial cell activation [7,80]. Its role in cerebral ischemia has not been clarified in detail. TNF- α gene upregulation has been demonstrated in transient and prolonged cerebral ischemia by the increased synthesis of TNF- α mRNA in the parenchyma [7,57,81,82]. TNF- α plasma levels have also been found to be higher in acute stroke patients than in healthy control subjects [7,83]. Barone et al. showed that administration of TNF- α exacerbated the ischemic injury provoked by MCA occlusion in spontaneously hypertensive rats, and also demonstrated that anti-TNF- α antibodies have a neuroprotective effect [84]. It was also demonstrated that the inhibition of TNF- α in mice with permanent MCA occlusion caused a smaller infarct volume [85]. Conflicting results also exist in the literature [7]. It was reported that when TNF- α was administered to mice 48 hours before MCA occlusion, it induced a protective effect [86]. Mice lacking TNF- α receptors showed a larger infarct area after MCA occlusion than do normal controls [87]. These findings suggest that TNF- α may be beneficial in the poststroke recovery phase. TNF- α was found to be expressed also in the contralateral, nonischemic hemisphere after cerebral ischemia [88].

There are specific receptors for TNF- α . They mediate the effects of TNF- α . The most well known of which are two proteins called p75 and p55 named according to their molecular weight [7,89]. The binding of TNF- α to its receptor is followed by the activation of a variety of proteins, such as protein kinase C, tyrosine kinase, mitogen-activated protein kinase, phospholipase A₂, and phosphatidylcholine-specific phospholipase C [7,90]. Among these, the mitogen-activated protein kinases are the most extensively studied. They are divided into three main families: (1) the extracellular signal-regulated kinases, which are activated by growth factors; (2) the c-Jun NH₂-terminal kinases; and (3) the p38 kinases. The last two groups are activated by pro-inflammatory cytokines [91,92]. The second step in the TNF- α signal transduction pathway, as for other cytokines, is

intranuclear, with the activation of several transcription factors. One of these, nuclear factor- κ B, translocates from the cytoplasm to the nucleus, where it activates the promoter of the genes for adhesion molecules and other cytokines [7,93].

3.5 TGF- β

TGF- β regulates and stimulates cell proliferation and differentiation and plays a central role in tissue repair mechanisms [7,94]. It was reported that TGF- β reduced neutrophil adherence to endothelial cells; suppressed the release of potentially harmful oxygen- and nitrogen-derived products by macrophages; promoted angiogenesis in the penumbra area; and reduced the expression and efficacy of other cytokines, such as TNF- α , possibly by blocking p38 kinase and the consequent inhibition of the TNF- α transduction mechanism [7,95-99]. All of these results indicate the beneficial effects of TGF- β in cerebral ischemia. In rats exposed to 10 minutes of global cerebral ischemia, increased expression of TGF- β mRNA was observed after 6 hours throughout the brain; this expression increased further at day 2 and subsided afterwards [100]. TGF- β mRNA overexpression was found in ischemic tissue in comparison with samples taken from the contralateral, non-ischemic side in an human autopsy study [97]. The highest expression of TGF- β mRNA was detected in the penumbra [97].

3.6 IFN- γ

IFN- γ is produced by activated CD4⁺ and CD8⁺ T cells and, to a lesser degree, by natural killer cells [7,101]. IFN- γ is not produced by central nervous system cells. Following cerebral ischemia, damage of the blood-brain barrier allows infiltration of lymphocytes into the brain parenchyma and release of IFN- γ . IFN- γ induces the expression of a variety of cytokines by stimulating p38 kinase and class II major histocompatibility complex [7,99]. Major histocompatibility complex is essential for macrophages to recognize antigen. It was postulated that IFN- γ plays a crucial role in the development of brain necrosis after an ischemic insult. Another possible role of IFN- γ in cerebral ischemia is the production of NO, with a consequent cytotoxic effect on brain cells. It was shown that IFN- γ stimulates in vitro the production of interferon regulatory factor-1, which induces NO synthase mRNA expression [7,102].

4. Chemokines in cerebral ischemia

Inflammatory cells infiltrating postischemic tissue are considered to contribute to disability after cerebral ischemia [5,8,17]. Identification of factors involved in the selective recruitment and accumulation of inflammatory cells into ischemic brain tissue and the mechanisms behind the entry of leukocytes through the blood-brain barrier into sites of ischemia are not completely understood [5,8]. Locally produced proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6 initiate the inflammatory process. TNF- α and IL-1 β mRNA elevate in the brain after experimental middle cerebral artery occlusion [5,51,81]. While, IL-1 β and TNF- α play a major role in promoting adhesion between endothelial cells and leukocytes, they are poor attractants for polymorphonuclear leukocytes and monocytes [7]. Astrocytes and endothelial cells can respond in vitro to such proinflammatory cytokines with enhanced expression of chemokines, which results in the influx of leukocytes to areas of inflammation [5,8,103].

Chemokines constitute a subgroup of the cytokine family, which may play a pivotal role in the attraction and accumulation of leukocytes through the parenchyma and toward

the ischemic area [5,7,104]. Chemokines are low-molecular-weight molecules with chemotactic activities on selective leukocyte subpopulations [7,8]. The number of discovered chemokines is continuously growing, and so far, more than 20 members of this cytokine family have been identified [7]. They are characterized by the presence, as a common structural pattern, of four cysteine residues. They are divided into two main subfamilies (α - or C-X-C subfamily and β - or C-C subfamily) according to the presence or absence of an amino acid between the residues of the two most amino-proximal cysteines [7,105]. Members of the IL-8 family belong to α -chemokines, while monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1 α , and MIP-1 β belong to the C-C chemokines. The structural distinction is reflected *in vitro* by a peculiar effect on different cell types: C-X-C chemokines tend to attract polymorphonuclear leukocytes (PMNL), primarily neutrophils, whereas C-C chemokines preferentially act on monocytes/macrophages [7,8]. Each chemokine family binds to specific receptors formed by seven transmembrane domains that activate G proteins and subsequently an intracellular kinase cascade [7,106]. Chemokine production and secretion are stimulated by a number of compounds, such as bacterial lipopolysaccharide, IL-1 α and IL-1 β , and TNF- α [7,105].

Few chemokines have so far been studied in detail in experimental cerebral ischemia. Cytokine-induced neutrophil chemoattractant (CINC) is a member of the interleukin-8 family. Significant expression of CINC mRNA has been detected in the ischemic areas of spontaneously hypertensive rats 6 hours after permanent MCA occlusion [5,7,107]. This expression reaches its peak at 12 hours and rapidly decreases at 24 hours. CINC has been shown to be homologous to three "gro" human proteins and "KC" in the mouse, and all of them are molecules acting predominantly as neutrophil chemoattractants [7,108]. In a model of rat transient focal cerebral ischemia, the first detectable level of CINC in the brain was observed 3 hours after reperfusion and preceded leukocyte infiltration [5,7,109]. In the sera of the same animals, a high concentration of CINC was found 60 minutes after MCA occlusion, with the peak concentration being reached 3 hours after reperfusion; a reduction in levels was observed between 6 and 48 hours [5,7,109]. Kostulas et al demonstrated elevated levels of IL-8 expressing peripheral blood mononuclear cells (PBMC) in patients with acute ischemic stroke [5,40]. They found that the upregulation of IL-8 mRNA expression occurred within the first few days after onset of symptoms, and remained elevated during the observation time of up to 1 month [5]. Numbers of IL-8 expressing PBMC correlated with the severity of the ischemic event. It was suggested that enhanced production of IL-8 locally in the ischemic brain could lead to a concentration gradient of IL-8 over the blood-brain barrier, which partly can be detected systemically [8]. In chemotaxis, the migration of cells in the direction along the concentration gradient results in a rapid influx of neutrophils to the brain parenchyma, thereby leading to a local inflammation [8]. It was reported that the administration of a neutralizing antibody against IL-8 prevented cell infiltration and tissue damage [8]. Compounds neutralizing IL-8 may be used to prevent postischemic brain injury mediated by PMNL. In a rabbit model of cerebral reperfusion injury, systemic administration of monoclonal antibodies against IL-8 at the initiation of reperfusion prevented PMN infiltration and reduced the size of brain edema at 6 hours and infarction at 12 hours after reperfusion [5,76]. Another potential agent of interest in this context is related to IL-1, a proinflammatory cytokine with the ability to induce IL-8 production [8,78]. A human IL-1 receptor antagonist has been shown to influence the postischemic injury in murine studies by reducing the number of necrotic neurons, decreasing the number of leukocytes in the ischemic brain, and causing a significant decrease in the pallor area [8,47].

The expression of monocyte chemoattractant protein-1 (MCP-1) in brain ischemia follows that of CINC [7]. High levels of MCP-1 mRNA have been found in the brains of rats with transient and permanent MCA occlusion at 6 hours, with the highest expression

occurring between 12 hours and 2 days [7,110,111]. Increased MCP-1 expression was still detectable 5 days after permanent MCA occlusion [110]. It was reported that IL-1 β and TNF- α induce MCP-1 expression in cerebral ischemia [7,51,81,112]. Gourmala et al demonstrated that 6 hours to 2 days after MCA occlusion, MCP-1 mRNA is present in rat astrocytes surrounding the ischemic tissue [113]. After 4 days, MCP-1 mRNA was found in macrophages and microglial cells in the infarcted tissue [113]. On the other hand, Kostulas et al couldn't detect any elevation in the numbers of mononuclear cells (MNC) expressing MCP-1 in patients at the early stages of ischemic stroke [8]. They reported that only sporadic stroke patients had transcripts detectable in their blood MNC, with no difference between patients and healthy controls [8].

Macrophage inflammatory protein-1 α (MIP-1 α) attracts monocytes and macrophages and modulates their activity in tissues undergoing an inflammatory process [7]. The mRNA of MIP-1 α , has been found to be overexpressed in cerebral ischemic areas [7,111]. It was found that the temporal expression of MIP-1 α parallels that of MCP-1 and that the distribution of MIP-1 α -positive cells was similar to that of activated astrocytes [7,111]. Kostulas et al assessed mRNA expression for the β -chemokines, MIP-1 α and MIP-1 β . They found no difference in numbers of MIP-1 α mRNA expressing blood MNC in patients with ischemic stroke in comparison to the healthy control subjects [5,8]. There was a tendency for increasing numbers of MIP-1 α mRNA expressing PBMC during follow-up after ischemic stroke, but the differences were small and did not reach statistical significance [5].

Interferon (IFN)-inducible protein-10 is a chemoattractant for macrophages and activated T lymphocytes [7,114]. It was demonstrated that in vitro, smooth muscle cells stimulated by IFN and IL-1 β or TNF- α produce IFN-inducible protein-10 [114]. IFN-inducible protein-10 mRNA expression has been demonstrated in the rat after endothelial damage by balloon angioplasty. It seems likely that this molecule could also play a role in cerebral ischemia because endothelial damage occurs during brain ischemia [7,114].

5. Cell adhesion molecules in cerebral ischemia

The inflammatory process initiated by locally produced proinflammatory cytokines induce or enhance the expression of several adhesion molecules [7,8,39,103]. The adhesion of leukocytes to the endothelial surface and their subsequent migration from the microvessels into the brain parenchyma are mediated by a variety of molecules located on the surface of both leukocytes and endothelial cells [7]. Adhesion molecules are divided into four main families: integrins, the immunoglobulin superfamily, cadherins, and selectins [115]. Under normal conditions, there is little or no cell-surface expression of adhesion molecules [116]. Inflammatory processes, such as cerebral ischemia induce their expression with the upregulation mediated by cytokines [7]. They are glycoprotein in nature and are the anchors that mediate the attachment of leukocytes [117].

Intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) belong to the immunoglobulin superfamily. They are single-chain glycoproteins with a polypeptide core containing two (intercellular adhesion molecule-2, or ICAM-2 for short), five (ICAM-1) and six (vascular cell adhesion molecule-1, or VCAM-1) extracellular immunoglobulin-like domains, followed by a transmembrane domain and a short cytosolic tail [117]. ICAM-1 is expressed on endothelial cells and other cell types, including lymphocytes and monocytes. In addition to these cells, ICAM-2 is expressed on granulocytes. VCAM-1 is expressed predominantly by the endothelial cells present in the postcapillary venules and by the endothelial cells covering atherosclerotic lesions [117].

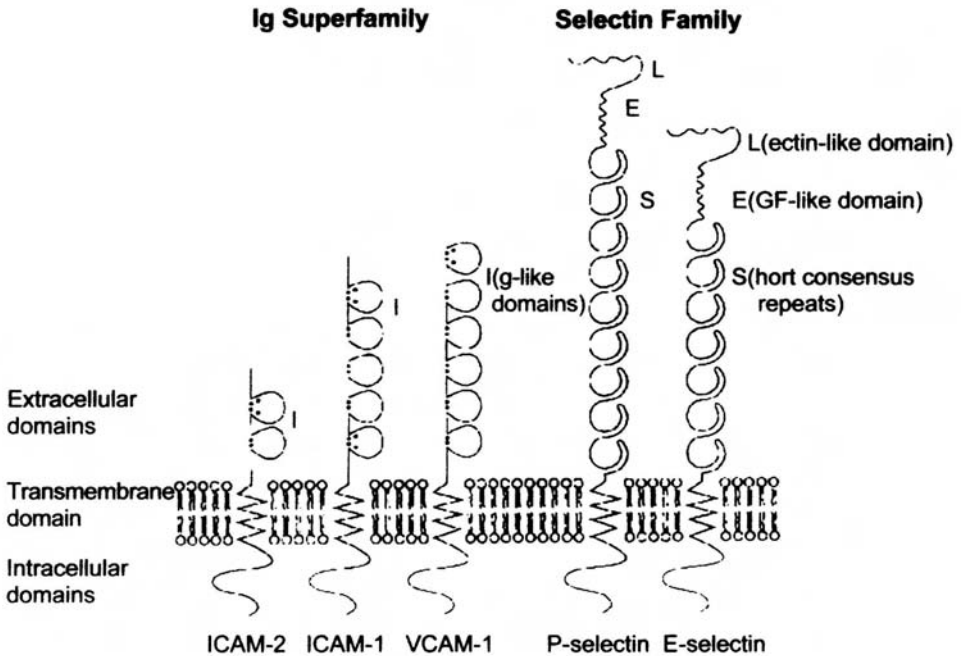


Figure 2. The basic molecular structure of the members of the Ig superfamily and the selectin family of leukocyte adhesion molecules expressed by the vascular endothelium. Black dots represent disulfide bridges within the molecule. From W. Sluiter et al [117] by copyright permission of Plenum Press, New York and London.

Selectins have an N-terminal lectin followed by an epidermal growth factor (EGF) motif, six (endothelial leukocyte adhesion molecule-1, ELAM or E-selectin) or nine (granular membrane protein-140, GMP-140 or P-selectin) short consensus repeat motifs, a transmembrane domain and a short cytosolic peptide tail [117]. Only endothelial cells express E-selectin which can bind lymphocytes, granulocytes and monocytes. The ligand for E-selectin is not well characterized, but the lectin domain of E-selectin recognizes sialated forms of the Lewis X and A glycans (SLe-X and SLe-A) of unidentified glycolipids and glycoproteins. A possible candidate is E-selectin (ELAM-1), a highly glycosylated selectin family member, which is expressed by all leukocytes. P-selectin is found in the Weibel-Palade bodies of the endothelium [117]. Figure 2 shows the basic molecular structures of the members of the Ig superfamily and the selectin family. Table 1 summarizes the properties of adhesion molecules related to Ig superfamily and the selectin family. It should be realized that there is a time-dependent difference in the expression of the adhesion molecules. P-selectin is translocated in the first minutes of stimulation and its expression lasts about 5-30 minutes. ICAM-1 is constitutively expressed but can be upregulated by stimulation with tumor necrosis factor (TNF)-alpha with a maximum at 4 to 6 hours of stimulation. VCAM-1 is normally absent, but its expression reaches a maximum at 4 to 6 hours of stimulation. After 24 hours only ICAM-1 and VCAM remain demonstrable at high levels [117]. Among the most studied adhesion molecules are ICAM-1, which is located on the endothelial surface, and its leukocyte counterpart, integrin CD11/CD18 [7]. According to the results of the current research selectins, such as endothelial-leukocyte adhesion molecule-1 (ELAM-1, E-selectin), mediate the initial low-affinity interaction between leukocytes and endothelial cells and promote the margination

and rolling of leukocytes in the blood stream. Vascular cell adhesion molecule-1 (immunoglobulin superfamily) causes the tight leukocyte-endothelial attachment and transendothelial migration [7]. Intensive research both in humans and experimental animals is ongoing to clarify the roles of adhesion molecules in inflammation following cerebral ischemia. Focal cerebral ischemia in rodents and in nonhuman primates caused upregulation of endothelial-leukocyte adhesion molecule-1 (E-selectin), ICAM-1, and P-selectin [7,118-121]. Cytokines induce in vitro expression of adhesion molecules in astrocytes, oligodendrocytes, and microglia. It is postulated that the presence of adhesion molecules on the surface of glial cells may facilitate the postischemic migration of leukocytes through the brain parenchyma [7,80]. It was demonstrated that mice belonging to an ICAM-1-deficient strain had a marked reduction in cerebral infarction size after transient MCA occlusion [122]. This indicates role of adhesion molecules in the pathogenesis of ischemic brain damage.

Table 1. Adhesion molecules on endothelial cells involved in leukocyte adhesion. From W. Sluiter et al [117] by copyright permission of Plenum Press, New York and London.

Molecule	Family	Basal expression	Stimulators of expression	Minimal time for maximal expression	Ligands on leukocytes
P-selectin (GMP-140)	selectin	absent	histamine, thrombine, ODFR	5- 30 min	SLe ^a -sugars SLe ^x -sugars
E-selectin (ELAM-1)	selectin	absent	IL-1, TNF-alpha ODFR (?)	2- 6 hr	SLe ^a -sugars SLe ^x -sugars
L-selectin	immunoglobulin	low	IL-1, TNF-alpha	4- 6 hr	LFA-1,CR3
ICAM-1			IFN-gamma		(CD 11a/CD18, CD11b/CD18)
ICAM-2	immunoglobulin	moderate	none, refractory to stimulation	constitutive expression	L FA-1 (CD11a /CD18)
VCAM-1	immunoglobulin	very low	IL-1, TNF-alpha	4-6 hr	VLA-4 (CD 49d/CD29)

GMP, granular membrane protein; ODFR, oxygen-derived free radicals; ELAM, endothelial leukocyte adhesion molecule; IL-1, interleukin-1; TNF-alpha, tumor necrosis factor-alpha; ICAM, intercellular adhesion molecule; IFN-gamma, interferon -gamma; VCAM, vascular cell adhesion molecule; LFA, lymphocyte-associated antigen; CR, complement receptor.

Upregulation of adhesion molecules has been documented in human stroke patients [7]. It was demonstrated that leukocytes from patients suffering an ischemic stroke or transient ischemic attack showed increased CD11a expression within 72 hours of the onset of symptoms [123]. Increased ICAM-1 expression on the surface of vessels from cerebral cortical infarcts was detected in four patients [124]. In some studies, soluble isoforms of adhesion molecules shed from the surfaces of activated cells were quantified in serum. Serum endothelial-leukocyte adhesion molecule-1 (ELAM-1, E-selectin) levels increased up to 24 hours after stroke. Similar increases were observed in serum vascular cell adhesion molecule-1 (VCAM-1) levels and these increases were sustained up to 5 days [125]. In contrast, serum ICAM-1 levels in acute ischemic stroke patients have been found to be lower than or the same as those of asymptomatic control subjects matched for age, sex, and vascular risk factors [125,126]. The reason not to detect an increase in serum levels of adhesion molecules might be due to the late enrolling of patients. Once adhesion molecules bind to leukocytes and endothelial cells, they can no longer be detected in serum [7].

Integrins are adhesion molecules involved in cell-matrix interaction. Integrins are heterodimeric membrane glycoproteins formed by the combination of an α - and β -subunit with an intracellular and an extracellular domain [115]. In the brain, endothelial cells, astrocytes, and the basal membrane such as laminin and collagen contribute to form the

blood-brain barrier, and their interconnection is mediated by integrins. Damage to these molecules may therefore lead to severe damage of the blood-brain barrier. The integrin $\alpha_6\beta_4$ was demonstrated to mediate the interaction between astrocytes and extracellular matrix under normal conditions. It was found to be rapidly damaged during focal cerebral ischemia/reperfusion [127]. Other integrins play an important role in inflammatory neoangiogenesis, wound repair, and ontogenesis and may therefore be important for tissue repair after an ischemic insult [7].

6. Therapeutic Interventions

Heavy studies are being performed to develop drugs that will prevent neurodegeneration following acute ischaemic stroke [15,16,128-133]. For this purpose, animal models have been produced that mimic the neuropathological consequences of stroke. During the past 10-15 years of stroke research, reproducible techniques for the induction of focal and global ischaemia have been developed. These models have several advantages and disadvantages. Reversible or irreversible focal ischaemia models like stroke in humans are useful for investigations of molecular mechanisms of stroke and also for the development of neuroprotective drugs. The advantages of using rats for stroke study include the similarity of their intracranial circulation to that of man and the relatively low animal cost which is important for large scale studies for statistical analysis. They provide the exact time of the onset of ischemia and the possibility to test new drugs.

Even though a large number of different compounds have been proven to reduce the size of brain infarct in animal studies, these drugs caused disappointing results in stroke patients. The reasons for the unsuccessful clinical trials have been either the toxic side effects, which have overridden the neuroprotective potential of the compounds demonstrated in animals, or a limited time window for human therapy. Compounds with no or tolerable side effects combined with a protective potential when administered several hours after ischemic insult are under heavy research [2,6,7].

Currently, the only treatment of patients with acute ischemic stroke is thrombolysis and restoration of blood flow [3,6,7]. Only a fraction of stroke patients benefits from this therapy [3,6,7]. Therapeutic recanalization of an occluded cerebral artery is a risky option that can be applied only in the case of selected patients. The main limitation of cerebral thrombolysis is the narrow, 3-hour therapeutic "window" during which the thrombolytic agent has to be administered to be effective. Beyond this time limit, its effectiveness is neutralized by the high risk of cerebral hemorrhage [7]. In acute stroke, only a small fraction of patients benefit from intravenous administration of recombinant tissue plasminogen activator, which is the only drug with proven effectiveness in reducing the size of infarct in humans [6].

The rationale for the use of a number of other pharmacological treatments for acute stroke is based on recent advances in the pathophysiology of brain ischemia [2,4,6,7]. One of the most rapidly expanding and promising areas of which is the role of inflammation in stroke [2,4,6,7]. Factors that influence the recruitment of PMN could represent new therapeutic targets in acute stroke [5]. Drugs capable of interfering with inflammation related mechanisms have given encouraging results in experimental stroke models in animals.

An understanding of the role of leukocytes and the mediators of inflammation in cerebral ischemia may have a very great impact on therapy. An increasing number of molecules are currently being investigated in animals for their possible effectiveness in human acute stroke. Treatments to induce neutropenia, to reduce infarct volume and improve functional outcome are under heavy study. One group of studies has focused on

therapeutic strategies related to the role of proinflammatory cytokines. The effects of IL-1 receptor antagonist on infarct volume in rats caused by MCA occlusion or the ligation of one carotid artery associated with hypoxia were investigated by several researchers [42,44,46,47,49]. Other IL-1 inhibitors such as anti-IL-1 β neutralizing antibody, zinc protoporphyrin, and fragments of lipocortin-1 were applied to animals in different models of cerebral ischemia [41,43,45,47,48]. A second group of studies has concentrated on molecules that are capable of blocking the adhesion between endothelial cells and leukocytes such as anti-CD11b/CD18 monoclonal antibodies, anti-ICAM-1 antibodies [134-141]. Mice genetically lacking adhesion molecules, such as ICAM-1 and P-selectin have been shown to be less susceptible to ischemic damage. Another group of studies consist of anti-inflammatory agents such as cyclooxygenase inhibitors. Broad spectrum antibiotics with anti-inflammatory effects such as tetracycline have been investigated in focal and global ischemia [3,4,6].

Possible future pharmacological treatments could be based on the inhibition of proinflammatory mediators, prevention of adhesion between the leukocytes and endothelial cells, controlling the specific transduction pathway signals following cytokine production and promoting neovascularization. Strategies that block the activity of inflammation-induced enzymes, such as iNOS and COX-2 should also be investigated.

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Carnosine as Natural Antioxidant and Neuroprotector: Biological Functions and Possible Clinical Use

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Abstract: Distribution and quantification of carnosine and related compounds in excitable tissues of vertebrates are analyzed. Description of various biological effects of carnosine, such as protons and transition metals buffering, antioxidant activity, and anti-glycating ability, resulted in suggestion on important functional role of carnosine and related compounds in nerve and muscle cells. Biological function of carnosine is protection of tissues from oxidative damage under conditions of oxidative stress resulting in increase of the longevity of life of the organisms. In conclusion, relation of carnosine and homocarnosine to brain and muscle pathologies is discussed.

1. Classification, distribution and quantification of carnosine and other histidine containing dipeptides

Among nitrogenous compounds contained in muscle extracts one group is remarkable by its extremely high concentration: it is a family of histidine containing dipeptides. The most widely distributed is carnosine (β -alanyl-L-histidine), primarily described in 1900 [1] within the meat extract, which explains the given name of the compound (from Latin: *caro*, *carnis* – meat). In 1929 the methyl derivative of carnosine was found in goose muscles and was named «anserine» (goose – *Anser anser*) [2,3]. Later on homocarnosine was described in bovine brain [4]. By the present time, the family of carnosine and related compounds (CRC) includes more than 10 natural derivatives (see Table 1). They are accumulated in vertebrate muscles in amount 5-10 times exceeding that for essential amino acids and ATP (for example, concentration of phenylalanine is 0.3-0.5 mM and ATP – 2.5-5 mM).

In muscle and nervous tissue some other low molecular weight peptides are also present: β -alanyl-tirosine and β -alanyl-lysine, tripeptide consisting of aspartate, glutamate and γ -aminobutyric acid, tetrapeptide containing histidine, glutamate, aspartate, α -alanine and/or glycine. However, their content is low and relation to functional activity is obscure.

Carnosine and related compounds in skeletal muscles. Systematic study on distribution of carnosine and anserine in different tissues has been carried out by Sergey Severin and his co-workers [6]. It was found that these compounds are present in metabolically sufficient amounts only in skeletal muscles of vertebrates. The more efficient contractile ability was noted the higher level of these compound was found. For example, high content of the dipeptides is characteristic for flight muscles of birds, muscles of racing horses; opposite examples are tonic and smooth (slowly contracting) muscles. However, some exclusions are also known – anserine is present in turtle muscles. In heart muscles the

dipeptides are predominantly acetylated at free β -amino group [7]. In other tissues, like spleen, liver, kidney, they are found only in trace amounts. In blood stream they appear after food digestion (short-term increase in their concentration may be observed after meat uptake). In invertebrate tissues CRC are mainly not presented with a few exceptions — carcine in crab muscle and carnosine (or homocarnosine) in blowfly [8].

Table 1. Classification and distribution of carnosine related compounds in vertebrates (placed according to date of discovery) [5].

Trivial name	Rational name	Distribution in tissues	Date of discovery
Carnosine	β -alanyl-L-histidine	Vertebrate skeletal muscles, olfactory epithelium, amphibian and snake skin	1900
Anserine	β -alanyl-N ¹ -methyl-histidine	Vertebrate skeletal muscles, heart, brain	1929
Ophidine	β -alanyl-N ³ -methyl-histidine	Snake, dolphin and whale musculature	1939
Homocarnosine	γ -aminobutyryl-histidine	Animal and human brain	1961
Neurosine	N-acetyl-histidine	Central nervous system and eyes	1964
Homoanserine	γ -aminobutyryl-N ¹ -methyl-histidine	Cardiac muscle and brain	1969
Carcine	β -alanyl-histamine	Central nervous system and heart	1975
N-Acetyl-carnosine	N-acetyl- β -alanyl-L-histidine	Brain and heart of vertebrates	1975
N-Acetyl-homocarnosine	N-acetyl- γ -aminobutyryl-histidine	Brain tissue	1975
N-acetyl-methyl-histidine	N-acetyl-N ¹ -methyl-histidine	Cardiac muscle and brain	1988
N-Acetyl-anserine	N-acetyl- β -alanyl- N ¹ -methyl-histidine	Heart muscle	1988

Table 2. Content of carnosine and CRC in muscle tissue of some animals (mg/100 g wet weight).

Source	β -Alanine	Histidine	Carnosine	Anserine	Ophidine
Actinia	-	10	-	-	-
Crab	-	7-15	-	-	-
Giant oyster	150	7	-	-	-
Octopus	345	2	-	-	-
Squid	5	65	-	-	-
Skate	175	-	-	-	-
Lamprey	65	25	80	120	-
Pelamyd	-	1600	-	-	-
Sturgeon	140	-	250	-	-
Siberian salmon	-	15	-	1200	-
Frog	-	-	220	-	-
Snake	-	-	-	-	560
Chicken	-	1	280	980	-
Rook	-	-	-	350	-
Ox	-	-	150	25	-
Cat	7	4	150	200	-
Dolphin	-	-	200	-	480
Whale	-	1	10	-	1080
Man	-	-	150-200	-	-

The content of carnosine and related compounds in muscles of several animals is shown in Table 2. One can see that in many cases CRC are present in extremely high

amount and evolutionary complication (with minor exceptions) correlates well with substitution of histidine by carnosine and then — by anserine or ophidine. Similar situation was described in the study of appearance of these compounds in ontogeny. The substitution of histidine by carnosine (at the formation of neuromuscular junctions of duck) and carnosine — by anserine (when rooks start to flight) in ontogenic development of birds was found to take place in rabbit muscles. In this latter case sufficient part of carnosine is bound with muscle proteins.

Enzymes regulating carnosine level in tissues. These data evidence that CRC are active metabolites, which accumulation in muscles is supported by specific enzyme system and results in more efficient muscle performance. Actually, two enzymes are known: cytosolyc (EC 3.4.13.3) and serum (EC 3.4.13.20) dipeptidases, which demonstrate specificity in relation to carnosine, thus being carnosinases. A non-specific dipeptidase (EC 3.4.13.18) also demonstrates partial ability to hydrolyze carnosine (Table 3). All these enzymes hydrolyses CRC with the following rank of efficiency: carnosine > anserine = ophidine >>> homocarnosine being not effective with respect to carinine and N-acetylcarnosine [9].

Table 3. Characteristics of tissue dipeptidases.

Characteristics	Cytosolyc carnosinase, EC 3.4.13.3	Serum carnosinase, EC 3.4.13.20	Cytosolyc dipeptidase, EC 3.4.13.18
Source	Brain (including olfactory mucosa), placenta, ovary, testes, uterus, pancreas, adrenal gland	Serum, plasma, brain	Liver, kidney, brain, intestinal mucosa, milk, T-lymphocytes
Organisms	Rat, sheep, pig, mouse, rabbit, dog, cat, human	Higher primates, human	Mouse, pig, rat, monkey
Natural substrates	Carnosine > anserine > homocarnosine	Carnosine > anserine = homocarnosine	Several dipeptides including carnosine (low rate) excepting anserine and homocarnosine

Localization of the carnosinases is one of the reasons of specific spreading of carnosine and its derivatives in different tissues. Another reason is localization of carnosine synthase (EC 3.2.11). This enzyme is found in skeletal muscle, brain and heart. It was purified from the latter source in 1981 [10]. Subsequent gene expression resulted in accumulation of carnosine in tissue. Thus, distribution and activity of carnosine synthase and carnosinases define tissue specificity and content of CRC in the body.

Carnosine synthase, as a rule, is not found in tissues where active carnosinase is present. The only organ, containing both enzymes simultaneously is brain but even in this case, carnosine synthase is located in glial cells, while carnosinase — in the intercellular space [11]. Food carnosine does affect sufficiently the tissue level of CRC only under systemic use. In spite of carnosinase presented in the intestine about 70% of carnosine can penetrate through the brush border into blood stream via Na-dependent carnosine transporter [12]. Appearing in blood carnosine exists there not longer than 30-60 min (depending on its amount in food received) because of digestion by carnosinase if it is not accumulated in tissues. Active transport of carnosine is demonstrated for several tissues. In brain astrocytes it occurs using dipeptide transporter of PepT2 type [13]. At the same time, native outer membrane of muscle is not permeable for the accumulated dipeptides, which may not leave the cells while they are alive.

Carnosine and related compounds in non-muscular cells. CRC are found not only in skeletal and heart muscles but also in nervous tissue, their content in several regions of brain being close to that in muscle or even higher. Thus, in different areas of human brain homocarnosine content is equal to (mM): frontal cortex – 0.29, cerebellum – 0.63.

substantia nigra – 0.88, nucleus dentatus – 1.55 [14]. In rodent brain, average content of carnosine and related compounds is 1.2-1.5 mM [15], but in special regions of brain its level can be sufficiently higher (in olfactory bulb - 10 mM or more) [16].

2. Tissue specific metabolic transformation of carnosine

From all known CRC, carnosine first appeared in muscle maturation. Its transformation into N-acetylcarnosine (heart), anserine or ophidine (skeletal muscles) depends on specific enzymes, thus the amount, to which these compounds are accumulated within the muscle, depends on functional activity of muscle [17]. In brain, besides carnosine, homocarnosine is also present, which is synthesized by the same enzyme carnosine synthase having similar affinity to both β -alanine and γ -aminobutyric acid. Thus, the ratio between carnosine and its homolog in different regions of brain is defined by the accessibility of substrates for dipeptide synthesis. In whole, tissue specificity in distribution and accumulation of different CRC allows to suggest correlation between the biological features of CRC and functional specificity of different excitable tissues [18].

3. Carnosinase paradox

Alternative distribution of carnosine synthase and carnosinases in tissues supports an idea of definite physiological meaning of CRC accumulation. Moreover, the presence of specific carnosine degrading enzyme during the evolution is some kind of paradox because carnosine is one of the less toxic nitrogen-containing compound (from animal experiments, LD₅₀ for carnosine exceeds 20 g/kg body mass). Another paradox is that carnosine itself is not accessible to regular (di)peptidases owing to the presence of free amino group in β -position. One can suggest that it can serve as a special pool for biologically important histidine and β -alanine [19] but this suggestion is not substantial because cellular carnosine usually is not accessible to carnosinase. Another suggestion is that there can be some unknown conditions when carnosine is harmful for tissues and has to be eliminated [20]. It could be a serious argument as several diseases related to brain dementia may accompany deficiency of serum carnosinase and increased level of carnosine in blood and urea [21]. How serious these suggestions are, can be concluded after final elucidation of functional properties of carnosine and related compounds.

4. Biological activity of carnosine and related compounds

Carnosine is a polifunctional molecule and different researches paid their attention to its different properties, which as they considered were most important for its functional activity. Present day information on CRC functions allow to estimate a contribution of these properties in functional activity of carnosine comparing change of these properties with modification of structure of the molecule.

pH-Buffering capacity. Because of the presence of several ionogenic groups carnosine can serve as a good buffer for protons in the neutral area of pH. It was noted as early as in 1938 [22] and according to recent calculations, in skeletal muscles carnosine together with anserine can provide for about 60% of proton buffer capacity whereas soluble muscle proteins — only from 9% to 38% [23-25]. Highly hydrophilic small molecule may perform pH buffering function in cell more efficiently than, for example, large protein molecules. Thus Skulachev [26,27] was the first who has used for carnosine the term

“mobile proton buffer”.

Moreover, Skulachev turned his attention to the change in the meaning of pKa for different CRC, which are changed in a profitable way when histidine is substituted by carnosine and later on — by anserine in muscle (see Table 4). These results in a shift into basic area of the pH being characteristic of the highest proton capacity and in all the cases (excepting histidine) this point lies in a more acidic area compared to normal pH value (7.4-7.6). Hence biological importance of CRC as pH buffers results from high proton capacity exactly in the physiologically important area.

Various muscles differ sufficiently in their ability to neutralize protons (the muscles with different functional activities were compared including sprinters, as well as humans) and in all cases this ability correlates well with functional activity of a muscle. Especially high proton buffer capacity is in fast contracting muscles, which totally fit to anaerobic metabolism [25].

Table 4. pKa for carnosine and several related compounds at 25°C [25].

Compound	pKa
Free imidazole	6.23
Imidazole group in protein	6.50
The same close to acidic group	7-8
The same close to basic group	5-6
L-Histidine	6.21
N ¹ -Methyl-histidine	6.62
N ³ -Methyl-histidine	5.98
Histamine	5.00
L- Carnosine	7.01
Homocarnosine	7.10
Anserine	7.15
Ophidine	6.93
Carcinine	> 8
Phosphate inorganic	6.88

Some CRC, however, don't differ sufficiently from carnosine by their proton buffering capacity (acetylated dipeptides) or have pKa in an alkaline area (carcinine). Their formation in tissues has to be induced by other metabolic needs.

Transient metal complexes. Carnosine and anserine are able to form complexes with transient metal ions Cu, Zn, Co, Va, Mn, Ni and Fe. Cu(II) complexes are better studied. Cu(II) and carnosine form both monodentate and bidentate complexes being in equilibrium with each other and interconverting depending on surrounding conditions; under physiological conditions only monodentate complexes exist [28]. Stability constants, pKa at room temperature are 13.3 for Cu-Carn-H complex and 8.47 for Cu-Carn complex. Complex between Cu(I) and carnosine is also formed [29], which is characteristic of unpredictable low ability to interact with molecular oxygen contrary with that of similar complexes of histidine and histamine.

Zn(II)-carnosine complexes were only shown for narrow pH area (6-7.5), they are inclined to form polidentates [29]. Carnosine complexes with Zn(II) are less stable than that with Cu(II). Carnosine complexes with Co, Mn, Ni and Fe are monodentate ones and characterized by progressively decreased stability.

It is noteworthy that addition of another ligand (which can be histidine or cysteine) to carnosine complexes cause increased stability [30]. Addition of albumin leads to the similar effect [28], thus carnosine can effectively compete with albumin for copper or zinc ions [29].

It is little known about complex forming ability of other CRC. As N³-nitrogen of

imidazole ring, deprotonated nitrogen of peptide bond and nitrogen of free amino group participate in complex forming N-acetylated derivatives as well as anserine and ophidine can be concluded to possess less ability to form metal complexes. Homocarnosine is known to be weak copper chelator [28].

Biological meaning of these metal complexes was considered mainly for most spread CRC — carnosine and anserine. Superoxide dismutase like activity was described for copper-carnosine complexes; similar ability was established for zinc-carnosine [31-33]. Cobalt-carnosine complexes are able to catalyze formation of superoxide anion from molecular oxygen thus demonstrating pro-oxidant properties [34]. Ni-carnosine complexes possess modest superoxide dismutase and catalase activity which under special conditions can result in ROS generation; chelating copper or ferrous ions allows to suppress their activity in the presence of carnosine. For example, carnosine inhibits Cu-induced HADH oxidation by hydrogen peroxide and direct oxidation of ascorbic acid (see: [29]). Zn-carnosine complex possess pronounced ability to neutralize hydroxyl radical thus working as an antioxidant [35]. Ability to chelate Fe ions (with pKa of about 3.2 mM) allows to demonstrate anti-oxidant ability of carnosine under conditions when Fe(II) initiates superoxide anion formation [36].

Antioxidant activity. First publication dealing with ability of carnosine to prevent accumulation of products of membrane lipid peroxidation and thus preserve activity of sarcoplasmic reticulum Ca-pump appeared in 1984 [37]. These data were confirmed later in a number of laboratories [35,38-42]. Carnosine was also shown to be able to quench singlet oxygen [33,43,44]. Moreover, it could interact with intermediates of lipid peroxidation to decrease hydroperoxide formation [45], and to form anion charge transfer complex with superoxide decreasing its activity [46,47]. Exactly because of this ability carnosine suppresses “respiratory burst” of lymphocytes after their activation [48]. Carnosine can also effectively neutralize hydroxyl radical [35,41], which results in protection of membrane lipids and proteins during oxidative stress [49]. Finally, carnosine was also able to neutralize another active oxidant, hypochlorite anion as it was found during chemiluminescence analysis of myeloperoxidase reaction [50].

Antioxidant activity of carnosine can be explained partially by its ability to form complexes with copper [31,32] or ferrous ions [36], however even in the absence of transient ions carnosine demonstrates pronounced antioxidant ability [50,51].

Antioxidant abilities of several CRC were compared in several articles. All CRC tested containing imidazole ring were roughly equally effective as singlet oxygen quenchers. The constant for interaction of ligands with the singlet molecule was about the same as for that of imidazole — $(2-4) \cdot 10^{-7} \text{ M}^{-1} \cdot \text{sec}^{-1}$ [44]. Carnosine, anserine and homocarnosine ($K_{0.5} = 1.5-2 \text{ mM}$) demonstrated closely similar ability to neutralize hydroxyl radical, while relative activity slightly decreased in above line. N-acetylated carnosine is practically inactive [17,50]. At the same time, anserine inhibits accumulation of the end-product of (Fe+ascorbate)-induced lipid peroxidation, malonic dialdehyde (MDA), whereas histidine demonstrated rather pro-oxidant properties [52]. In these experiments, homocarnosine demonstrated weaker antioxidant effect than carnosine did — lag period of oxidation became longer and rate of accumulation of MDA decreased while the stationary level of MDA accumulation was similar to the control. In the presence of anserine or carnosine MDA accumulated in the medium to 10-50% less level (depending on dipeptide concentration).

Chemiluminescent analysis of accumulation of lipid hydroperoxides during Fe-induced oxidation of human plasma lipoproteins showed that different CRC possess non-equal protecting activity. The following rank of increased protection was found (at 5 mM concentration of each CRC): N-acetylcarnosine (13%) < N-acetylanserine (29%) < homocarnosine (60%) < ophidine (62%) < carnosine (74%) < anserine (97%). Thus, change

in the structure of carnosine sufficiently modulates its anti-oxidant activity [53]. Methylation of N³-nitrogen of imidazole ring of carnosine (resulting to ophidine) or substitution of β -alanine by γ -aminobutyric acid (conversion of carnosine to homocarnosine) both slightly decrease its protecting ability, whereas methylation of the ring in N¹ position (anserine) sufficiently increases its efficiency and on the contrary acetylation of the molecule at free β -amino group makes molecule practically inactive.

Effective concentrations of carnosine vary from 0.5 to 50 mM depending on the model used but in all cases are within the range of its physiological concentrations. In protection of blood plasma lipoproteins K_{0.5} for suppression of their oxidation was 3.5 mM, K_{0.5} for neutralization of hydroxyl radicals was about 1.5 mM, and for protection of muscle membrane lipids against peroxidation — 20 mM [52]. It was important to compare efficiency of carnosine with that of other natural antioxidants (see Table 5).

Affinity of carnosine to superoxide anion is two-fold higher than that of ascorbic acid or α -tocopherol, while it is much less efficient than SOD. However, it is necessary to take into consideration that carnosine content in muscle tissues is much higher than content of vitamins C and E, which makes carnosine much more important protector of muscle against ROS. It is also important that affinity of carnosine to hydroxyl radical is higher than to superoxide anion [41,46].

Table 5. Relative ability of antioxidants to neutralize superoxide anion [47].

Compound	K _{0.5} (M)	Quenching constant, K (M ⁻¹ ·sec ⁻¹)
Carnosine	$(7.1 \pm 0.2) \cdot 10^{-5}$	$(0.83 \pm 0.05) \cdot 10^5$
Ascorbic acid	$4 \cdot 10^{-5}$	$2.7 \cdot 10^5$
α -Tocopherol	$5 \cdot 10^{-5}$	$2.0 \cdot 10^5$
N-Acetyl-cysteine	Does not interact with O ₂ ⁻	-
Superoxide dismutase	$(1.1 \pm 0.1) \cdot 10^{-9}$	$(5.35 \pm 0.07) \cdot 10^9$

Finally, one can note that interaction of carnosine with superoxide does not result to such potentially toxic compound as H₂O₂, which takes place in the case of SOD. Thus, carnosine can serve as effective hydrophylic anti-oxidant in excitable tissues in which relative deficit of such anti-oxidants as vitamin E (skeletal muscles) or catalase (brain) takes place.

Effects on antioxidant enzymes. Along with anti-radical effects carnosine can influence enzymes which activity is connected directly or indirectly to free radical metabolism. Carnosine and other CRC may work as NO synthase inhibitors in muscles [54]. Low concentrations of carnosine (until 2.5 mM) can activate rabbit platelets 5'-lipoxygenase, whereas higher concentrations inhibit it [55]. Carnosine, homocarnosine or anserine (15-25 μ M) inhibit rat brain tyrosine hydroxylase by 50% at very low concentrations [56]. It was demonstrated recently that carnosine may protect SOD from ROS attack under *in vitro* [57], and *in vivo* (our unpublished data) oxidative stress.

Anti-glycating effects. It was shown in 1990 that carnosine (50-100 mg/kg body weight) increases survival of rodents when it was administered to animals before sub-lethal dose of γ -irradiation [5]. Kurella et al. [58] have found that under these conditions viability of haemopoietic stem cells is significantly increased and their colony forming activity is activated as well. These phenomena can be addressed to anti-radical protection of biomacromolecules by carnosine, however carnosine was additionally found to protect nuclear DNA from oxidative modification induced by hyperoxia, to preserve its native structure and to synchronize cell cycle *in vitro* [59]. Its addition to the medium where fibroblasts were cultivated increased the longevity of cell life and reversed the senescence features of the cells [60]. Moreover, carnosine was demonstrated to increase stability of

lymphocytes [61] and fibroblasts [62] against osmotic shock.

These data suggested that carnosine can be drawn into the protein synthesis providing specific regulatory role directed to support of essential genes in active state and to increase cell viability. In agreement with such suggestion, it was found that addition of carnosine to cell cultures promotes their viability and stimulates synthesis of a number of proteins, particularly, vimentin, which takes part in interaction of cytoskeleton with membrane lipid bilayer [63].

These effects could not be restricted by the only anti-radical activity. It is very likely that ability of carnosine to prevent non-enzymic glycosylation (so called glycation) of cell proteins involves, the process, which usually attacks ϵ -amino group of lysine and is activated by a neighbor proline residue. The structure of lysyl-proline is close to that of β -alanyl-histidine, by virtue of this fact free amino group of carnosine fits very well for binding of glycosyl radicals; thus carnosine can protect proteins from glycation. As glycation induces cross-linking between protein molecules [64] in the same manner as protein carboxyl groups are oxidized by ROS (which results in protein carbonyls are formed) anti-glycating effect of carnosine could get an additional protection stabilizing protein structure under unfavorable conditions (oxidative stress, etc.). There is strong evidence obtained in model experiments in vitro that carnosine can interact with low molecular weight aldehydes and ketones [65-67]. It was found that carnosine protects skeletal muscle actin from glycation [68] and prevents aldehyde modification of α -crystallins [20]. This ability is much lower in the case of homocarnosine and practically absent for N-acetylcarnosine, thus, confirming direct participation of free β -amino group in this process [52]. Recently some evidence was obtained in favor of such mechanism in the organism [20].

Such protective action could be useful under several diseases like diabetes mellitus when hydrocarbon metabolism is disordered and stationary level of blood sugar is increased. As a rule, this disease is accompanied by increased ROS production, which activates glycation efficiency of monosaccharides. Carnosine can serve as a useful protector from both ROS and glycation agents. Thus, in the case of carnosine, anti-radical and anti-glycation activity are combined in one molecule. Both protection of cells against physiological senescence and anti-glycation effect became apparent at high enough concentrations of carnosine (10-50 mM), which is in agreement with non-enzymic nature of this process.

Does carnosine receptor exist? All effects of carnosine mentioned above are performed with no participation of specific receptor proteins. In some cases, however, specific receptors can be involved. In brain tissue carnosine is released together with glutamate in response to electric stimulation [11]. It can modulate the affinity of glutamate receptors to the neuromediator controlling concentration of free zinc ions [69]. Modulation of H_1 and H_2 histamine receptors in heart (the former stimulate and the latter prevent mobilization of carnosine into histamine, thus regulating inflammation development) by carnosine is apparently carried out with participation of zinc ions [70]. Similar interrelations were found between carnosine and histamine receptors in blood vessel muscles responsible for the tone of blood vessels [15]; this is closely related to hypotensive response appeared after intravenous administration of carnosine noted as early as in 1936 [71].

The ability of carnosine to modulate Ca-induced contraction of chemically skinned muscle fibrils is also well known [72], which takes along this process muscle ryanodine receptors [73]. In most cases (excluding the latter one) carnosine acts at very low concentrations (10^{-7} - 10^{-5} M), which suggests participation specific protein structures in its modulating effects. However, no data were found related to proteins possessing specific ability to bind carnosine molecule. This means that receptor mediated effects of carnosine are realized by means of modulation of functions of several known receptors resulting in formation of adequate reaction on changed environmental conditions.

5. Functional role of CRC

Properties of carnosine and related compounds discussed above provide diverse manifestations of their functional activity.

Activation of muscle working capacity. First demonstration of functional importance of the dipeptides was presented in 1953 [71], when the phenomenon was described later on named as «Severin's phenomenon». Concerning author's description, addition of carnosine or anserine (10 mM) to the medium surrounding rhythmically contracting muscle cause fast and long-lasting increase in muscle working capacity. The stimulation was more pronounced the more exhausted by the previous performance the muscle was. Such mobilization of muscle contractility was accompanied by over-production of lactic acid and decrease of ATP content. However, the muscle continued to contract in spite of these factors and by the end of the experiment performed several fold larger work than the control one.

The preliminary suggestion that dipeptide effect is directed toward synaptic junction was not irrefragable one, because they also stimulate working capacity at direct electrical stimulation of muscle [75]. The contractile system was not also involved in "Severin's phenomenon" whereas protection effect on muscle membranes and activity of membrane bound enzymes was extremely pronounced [5,6]. Carnosine effect can be partially mimicked by several pH-buffers confirming importance of proton buffering capacity of the molecule [27].

It was important to note the sufficient decrease (to 25-30%) of carnosine amount after exhaustive muscular work [76]. This was not accompanied by appearance of histidine or β -alanine, which was in agreement with the absence of carnosinase in skeletal muscle. This fact illustrated that carnosine is involved in metabolic processes when supporting functional activity of skeletal muscles. These processes can include interaction with ROS and neutralization of glycosyl radicals.

Effects on muscle enzymes. In addition to mentioned above 5'-lipoxygenase, tyrosine hydroxylase and NO-synthase, some other enzymes are known to be affected by carnosine. Carnosine (as well as histidine) protects 3-phosphoglycerate dehydrogenase from heavy metals [6] and activates phosphorylase *a* and *b* (the former — in the acidic pH areas) [77]. Stimulation of the over-all glycolytic process by the dipeptides is usually explained by their pH-buffering capacity and protection of individual glycolytic enzymes from contaminations of heavy metals [78].

Carnosine (as well as anserine) exerts positive effect on oxidative phosphorylation in mitochondria and maintains P/O ratio in the in vitro experiments at higher level than that in control samples having no dipeptides added [79]. The same effect of the dipeptides was noted in terms of ionic pumps of skeletal muscles, such as Ca-pump and Na/K-pump, supporting their activity under unfavorable conditions [6,45].

Protection of cellular structure. In several cases favorable effect of carnosine and related compounds on enzyme function are explained by preservation of native structure of proteins and their environment. Because of anti-oxidant and anti-glycating mechanisms carnosine prevents disordering of natural genome structure [59], glycation of contractile (actin) and structural (α -crystallins) proteins [20,68], suppresses oxidative modification of membrane lipids [36,39] and proteins [80,81]. Pronounced membrane protecting action was demonstrated for carnosine and N-acetylcarnosine (but not histidine, anserine or homocarnosine) in the model of ischemic heart in vitro [82]. Carnosine and its acetylated derivative restored force of myocyte beating and prevented myoglobin and nucleosides release from heart damaged by oxidative stress, N-acetylcarnosine being sufficiently more effective than carnosine. On the contrary, anserine and homocarnosine did not demonstrate protecting effect and even slightly stimulated myoglobin and nucleosides release. The

effect of N-acetylcarnosine was fully prevented by chelerithrine, evidencing protein kinase C being involved in such protection [83].

Immunomodulating and wound healing effects. Wound healing effect of carnosine was first described by Frolov et al. as early as in 1936 [84]; the authors have noted accelerated epithelization of gastric mucosa during treatment of gastro-duodenal ulcer. Later on, similar effects were found during treatment of trophic ulcers [85], surgical damage of muscles [86], lungs [87], and liver [88]. Systematic treatment of eye tissues by carnosine solutions results in normalization of internal pressure, prevents glaucoma and cataract development in a dog [38,89] and a man [90].

Wound healing effect of carnosine is potentiated by zinc [35,91]. Its mechanism probably related to ability of carnosine to support functional activity of lymphocytes [61], to reveal the immunomodulating activity depending on age of animals and concentration of carnosine [86,92], to neutralize ROS formed in the inflammatory area. In some cases, increased formation of granulating tissues was noted which stimulated healing of damaged surface because of protein synthesis activation [86,88].

Apoptotic/necrotic transformation of excitable cells. Effects of dipeptides directed to support stability of cellular structures increase the reliability of cellular functions under normal conditions and especially during oxidative stress, which accompanies effect of several extreme factors. It was found in experiments on individual neurons that carnosine prevents cell death induced by excitotoxic compounds, N-methyl-D-aspartic acid (NMDA) or kainate [93-95] or experimental hypoxia/reoxygenation [96]. Apoptosis induced by exposure of cerebellum neurons to kainic acid (see Table 6), was arrested if the cells were pre-incubated with carnosine or anserine and simultaneously heavy necrotic processes were substitute by light (reversible) necrosis. At the same time, N-acetylcarnosine or homocarnosine did not reveal protecting action [94,95].

Table 6. Effect of carnosine and related compounds (10 mM) on death of cerebellum neurons induced by excitotoxic action of kainate (500 μ M for 3 hrs). Data are presented in %% to total amount of cells in the sample [95].

Conditions	Viable cells	Apoptotic cells	Light necrosis	Heavy necrosis
Control	79 $\pm$ 5	2 $\pm$ 3	6 $\pm$ 4	9 $\pm$ 3
Kainate	40 $\pm$ 7	37 $\pm$ 3	3 $\pm$ 2	20 $\pm$ 3
+ carnosine	67 $\pm$ 4	3 $\pm$ 2	23 $\pm$ 7	7 $\pm$ 8
+ anserine	67 $\pm$ 4	2 $\pm$ 1	21 $\pm$ 4	1 $\pm$ 1
+ N-acetylcarnosine	36 $\pm$ 8	38 $\pm$ 8	3 $\pm$ 3	23 $\pm$ 3
+ homocarnosine	37 $\pm$ 4	40 $\pm$ 5	4 $\pm$ 3	20 $\pm$ 6

Effect of carnosine on the longevity of life (from cells to whole organism). Increase in cellular stability toward unfavorable factors by carnosine, which was noted during culturing cells of different types [60,62,63] or under conditions of experimental ischemia [80,97], suggesting possible protective action of carnosine on the level of whole organism. Actually, different kinds of experimental brain injury in rodents (rats, mice, mongolian gerbils) are manifested with lighter "neurological symptomatic" and resulted in lower mortality if carnosine was preliminary administered in a dose of 100-200 mg/kg body weight [80,97,98]. Simultaneously, higher survival rate of the animals corresponds with positive effect on their learning capacity, as a rule disordered by experimental brain ischemia [97]. Similar protecting effect of carnosine was found in the case of semi-lethal dose of γ -irradiation of mice – the survival rate decreased slower and to less extent when animals were treated with carnosine (50 mg/kg daily); moreover, the amount of animals survived to the 30th day after irradiation was much higher (70% toward 20%) [5,58].

Systemic use of carnosine as a food additive has been studied in Senescence

Accelerated Mice (SAM) line being characterized by accelerated rate of accumulation of senescence features and essential (approximately two-fold) shortening of lifespan in relation to the animals of the control line [99]. SAM mice are characterized by multiple defects in the system of anti-oxidant defense and increased level of chromosomal aberrations in the stem cells [99,100]. Every day use of carnosine by the animals of this line (100 mg/kg body weight) results in deceleration (or reversion) of senescence, increase in average lifespan (approximately by 20%) as well as favored the exterior of the animals [101-103].

6. Relation to pathologies

Age changes appeared in muscle tissue results in decreased stability to un-favorable factors, for example, to oxidative stress. The intensity of protein synthesis falls and muscle mass decreases (approximately by 25% to age of 70 compared to the age of 20), and efficiency of contractile response decreases as well. Muscle anti-oxidant system deficiency flings into a deficit and CRC content in heart and skeletal muscles is decreased [104,105]. While distinct correlation between age changes and muscle pathologies has not been found, in whole aging is a strong prognostic factor for several muscle myasthenia and other age disfunctions.

Progressive muscle dystrophy. As Stepanova and Grinio demonstrated [106] this disease in children is accompanied with decrease in muscle carnosine content progressively and amount of carnosine falls proportionally to depth of muscle atrophy. Other diseases accompanied with muscle atrophy (like spastic tetraparesis) are also characterized by progressive decrease in carnosine content. As authors suggested, possible explanation of this phenomenon is a deficiency of system of carnosine synthesis [106].

Huntington disease. This progressive inherited disease is accompanied by locomotive and mental disordering. Brain of such patients is characterized by sufficient (nearly two-fold) decrease of GABA and homocarnosine content, followed by decreased activity of glutamate decarboxylase. Treatment of patients with isoniazide (inhibitor of GABA transaminase) results in partial restoration of homocarnosine in brain, which, however, does not correlate with improvements of neurological and psychological defects [107].

Serum carnosinase deficiency. This relatively rare disease described in children becomes apparent as carnosinemia and carnosinurea (increased carnosine level in blood and urea). This is caused by very low activity of serum carnosinase which in half of the cases analyzed was characterized by sufficiently elevated K_m for substrates [108]. From 23 sick children (belonging to different families) 14 were found to have neurological lesions, myoclonal convulsions, mental retardation; at the same time, the correlation between intensity of neurological disorders and residual activity of carnosinase was not found [108].

Homocarnosinosis. This disease is accompanied with pronounced increase of homocarnosine level in brain and cerebrospinal liquid as well as carnosine in urea with simultaneous disability to metabolize anserine into N¹-methyl-histidine (see: [107]). Normal level of carnosine and homocarnosine is exceeded 20 and more times and this is accompanied with apparent neurological deficit. At the same time, relatives of the patients can get similar shifts in dipeptides metabolism with no neurological symptomatic. The cause of such disorder of metabolism in patients with homocarnosinosis is that carnosine splitting enzyme, carnosinase is mainly present in the inactive form [109,110].

Thus, above-mentioned pathologies are not restricted by disordering of CRC metabolism. Nevertheless, these compounds are involved in disease development. It is seen that both deficit and uncontrolled excess of CRC are both undesirable. It is quite questionable that neurological disorders accompanied by carnosinemia are the result of

toxic action of the dipeptides themselves. These facts rather illustrate the necessity for normal metabolic pathway not only dipeptides but also products of their hydrolysis by carnosinase.

7. Conclusion

Carnosine is a very simple molecule, which, however, possesses a diversity of properties being extremely useful for the metabolism of excitable cells. Being the proton buffer, chelator of a number of transient metal ions, quencher of free radicals and active sugars carnosine serves as a poli-functional protector of cells and tissues under different extreme conditions like oxidative stress, excitotoxic disordering of neurons, healing defects, different immunological deficits, etc. Such a view on biological role of carnosine is very likely but one question is out of answer: why carnosine and CRC are accumulated in high quantity only in excitable tissues and what is the cause to transform carnosine into CRC providing tissue specific distribution of these compounds in different excitable tissues?

Absence (or very low concentration) of CRC in liver, kidney, and other organs where detoxication of xenobiotics takes place is easily to explain by possible interference of carnosine as hydrophylic antioxidant with a system of microsomal oxidation. For the same reason, its presence can be unadvisable in the cellular immune system using ROS as a "bactericidal remedy". On the contrary, carnosine is very pertinent component of muscles and neuronal tissues where ROS are used as signal transducing molecules and are potentially damaging for many reasons. However, why carnosine is transformed into different CRC in such a way that the molecule becomes not accessible not only to regular peptidases but to carnosinases as well [9]. Another words, one can suggest that anserine is preferable in skeletal muscle, N-acetylated derivatives of the dipeptides — in heart, and carnosine homolog, homocarnosine — in the brain.

To answer this question needs more knowledge, which level is not enough at present and only preliminary assumptions can be discussed. This appeared from comparison of properties of the compounds under analysis and specificity of ROS turnover in excitable cells [18]. There is no doubt that to perform large and changeable amount of work skeletal muscle should be protected against free radicals and glyating agents as well as against over-loading with acidic products (lactate) accumulating during performance of anaerobic muscle fibrils. In terms of specificity of blood supply in the heart, these factors are not critical or solved by other means but necessity exists to support functional activity of myocytes involving protein kinases (as it was demonstrated in the experiments with chelerithrine) in order to increase stability toward ischemic damage [83]. At the same time, for neuronal cells in which apoptosis is involved both in the brain formation (during ontogeny) and in removal of defect cells being victimized by excitotoxic attack (oxidative stress), the couple carnosine — homocarnosine, in addition to other useful properties can affect switching on/off mechanisms of cellular death.

In spite of the absence of clarity of real biological importance of CRC in excitable cells, our knowledge in this field strongly evidences that these compounds play a crucial role in protection of muscle and neuronal cells of vertebrates against oxidative stress and toxic environmental factors and thereby are involved in evolutionary perfection of cellular functions.

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Atherosclerosis as a Free Radical Pathology and Antioxidative Therapy of this Disease

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Abstract: Reviewing the data available in the literature and their own findings, the authors consider the role of lipid peroxidation in the etiology and pathogenesis of atherosclerosis. The paper provides a good evidence for intensified peroxidation of hepatic microsomal phospholipids and liver-secreted atherogenic lipoproteins in atherogenesis, which is followed by blood lipid peroxides accumulation in hypercholesterolemia and atherosclerosis. The excess of aortic lipid peroxides occurs concurrently with a sharp decrease in the activity of the enzymatic systems which utilizing lipid peroxides. Accumulation of lipid peroxides in the aorta during atherosclerosis may be a factor that induced the inhibition of prostacyclin biosynthesis and may be a direct cause of thrombosis that are the most frequent and severe event in this disease. The products of cholesterol oxidation were also reported to exert an atherogenic action. It was found that cholesterol-lowering drugs from HMG-CoA-reductase inhibitors family (statins) may induced free radical lipoperoxidation of low density lipoproteins (LDL) in vivo so far as this drugs may depress not only cholesterol but also ubiquinon Q₁₀ biosynthesis reduced form of those act as a main antioxidant in human LDL particles. Treatment of patients with ubiquinon Q₁₀ preparation or synthetic antioxidant probucol not produces the increase of the liperoxide level in the LDL during 6 months observation.

Atherosclerosis and coronary heart disease continue to be leading cause of mortality in industrial countries and cause of many deaths in developing countries. The main risk factors of atherosclerosis include smoking, obesity, high blood pressure and diabetes [1]. High blood level of cholesterol, especially low density lipoprotein (LDL) cholesterol, also have been associated with an increased risk of atherosclerosis development [2]. Data of literature [3-5] and our findings [6-10] confirm that the intensification of free radical lipid peroxidation play the important role in the atherogenesis. In particular, there is evidence that oxidative modification of LDL leads to enhanced and unregulated LDL cholesterol uptake by arterial macrophages that leads to foam-cell formation on the early stages of atherosclerotic injury of vascular wall [3-5].

In our experiments [7] the feeding of cholesterol to rabbits and mini-pigs is followed by the sufficiently decrease in the activity of key cytosolic antioxidative enzymes of hepatocytes - superoxide dismutase and glutathione peroxidase (Figure 1).

At the same time increasing in the level of hydroperoxides in the phospholipids of liver microsomal membranes was observed (Figure 2).

It is well known for a long time that activity of key enzyme of cholesterol catabolism in the liver — microsomal 7 α -cholesterol hydroxylase is inhibitable by lipoperoxides in vitro [11,12]. Indeed, we observed [12] decreasing in the 7 α -cholesterol hydroxylase activity in vivo during lipoperoxidation of liver microsomes induced by cholesterol feeding to animals (Figure 2). Since synthesis of components and the assemblage of atherogenic

very low density lipoproteins occurs in the hepatocytes, the intensification of lipoperoxidation processes in this cells may be accompanied by increased the secretion of oxygenated lipoproteins in the blood flow [13].

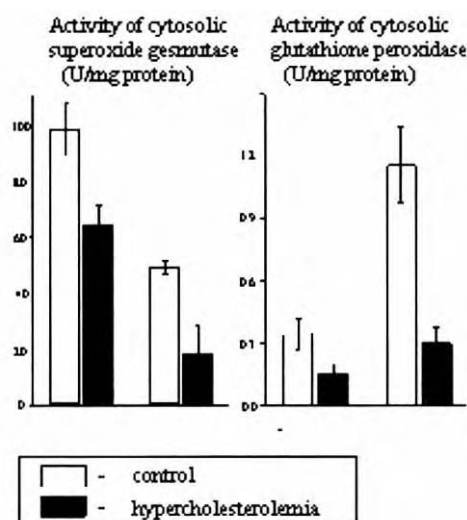


Figure 1. The activity of antioxidative enzymes — superoxide dismutase and glutathione peroxidase in the liver of rabbits and mini-pigs with experimental hypercholesterolemia.

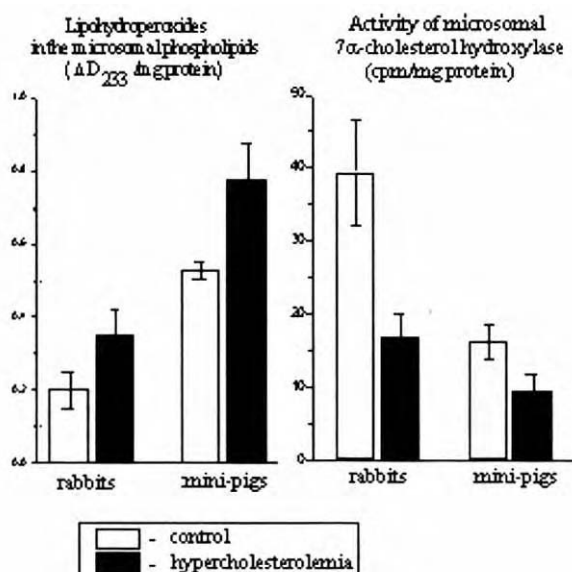


Figure 2. The level of lipoperoxides in the liver microsomes and 7α-cholesterol hydroxylase activity in the same membranes of rabbits and mini-pigs with experimental hypercholesterolemia.

In patients with postinfarct cardiosclerosis we found [14] a sharp increase in the blood plasma levels of primary and secondary products of free radical lipoperoxidation (Figure 3).

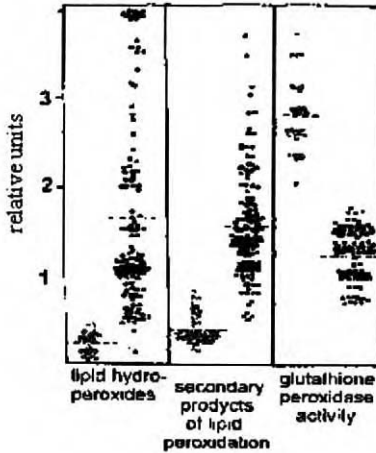


Figure 3. The content of primary and secondary products of free radical lipoperoxidation in plasma and activity of erythrocyte glutathione peroxidase in the blood of patients with atherosclerosis.

With this the activity of glutathione peroxidase in red blood cells, an enzyme utilizing lipohydroperoxides drastically decreased (Figure 3). Thus, the intensification of blood lipoperoxidation in atherosclerosis must favour the higher penetration of oxidized atherogenic lipoproteins, namely LDL, into the wall of a vessel.

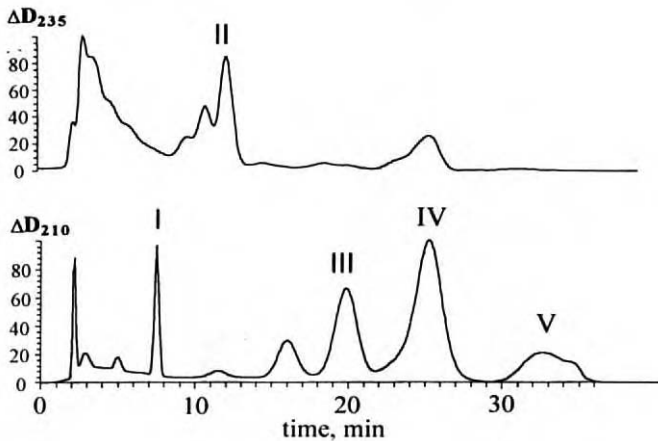


Figure 4. Normal phase high-pressure liquid chromatography of cholesterol esters isolated from atherosclerotic lesions of human aorta: (I), free cholesterol; (II), oxygenated cholesterol esters; (III), cholesteryl arachidonate; (IV), cholesteryl linoleate; (V), cholesteryl oleate and cholesteryl palmitate.

A number of authors have repeatedly reported that in experimental [7] and human atherosclerosis [15] lipoperoxides and secondary products of lipoperoxidation accumulate

in the aorta. In our study, that was carried out in collaboration with our colleagues from Biochemistry Institute of Humboldt's University in Germany [16], we investigated by HPLC-method the lipid composition of human aortas with atherosclerotic lesions, obtained at autopsy made in one to four hours after sudden death. The detection of oxygenated cholesterol esters in the lipofibrous plaques of human aorta confirms earlier findings by Harland et al. [17] who isolated hydroperoxy-derivatives of cholesteryl linoleate from the lipids of atherosclerotic plaques. The major component of oxygenated cholesterol esters in the regions of atherosclerotic plaques in aorta was hydroperoxy-derivative of cholesteryl linoleate, the major lipid component in this specimens [17-19]. (Figure 4).

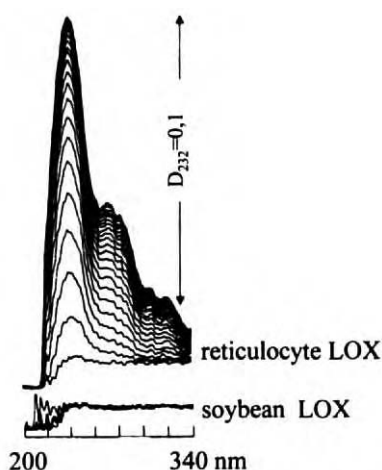


Figure 5. The oxygenation of cholesteryl arachidonate by animal or plant C-15 lipoxygenases: (1), oxidation by rabbit reticulocyte lipoxygenase; (2), oxidation by soybean lipoxygenase.

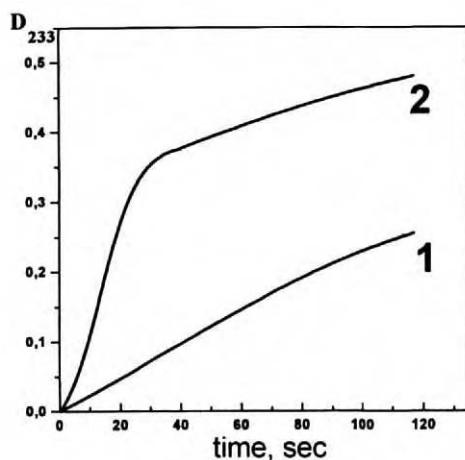


Figure 6. Stimulation by human LDL of the arachidonic acid oxidation catalyzed by C-15 animal lipoxygenase (reticulocyte lipoxygenase): (1), in the absence of LDL; (2), in the presence of LDL.

Since unsaturated cholesterol esters are one of the main class of unsaturated lipids in atherosclerotic human aorta they might serve as a substrate for the vascular wall

lipoygenase. We found that unsaturated cholesterol esters are oxygenated with high rate during incubation of lipid dispersions with animal C-15 lipoygenase (reticulocyte lipoygenase) in vitro [20] (Figure 5). In opposition, plant C-15 lipoygenase (soybean lipoygenase) is unable to oxygenate the unsaturated cholesterol esters [20] (Figure 5).

Therefore, the nature of the oxygenated cholesterol products in atherosclerotic human aorta does not exclude the fact, that they have been formed during lipoygenase catalysis. Really, our results demonstrated that the activity of C-15 animal lipoygenase may be greatly stimulated by addition of the atherogenic LDL to the incubation media [21,22] (Figure 6).

Figure 7 shows that in the human aortas we observed a significant decrease in the antioxidative enzymes activity such as superoxide dismutase and glutathione peroxidase, in the areas of atherosclerotic lesions [23]. A significant drop in the activity of both key antioxidative enzymes was seen in the intimal specimens taken from the area of fatty streaks, yet a severe decrease in the activity of these enzymes was found in those taken from the area of fibrous plaques (Figure 7).

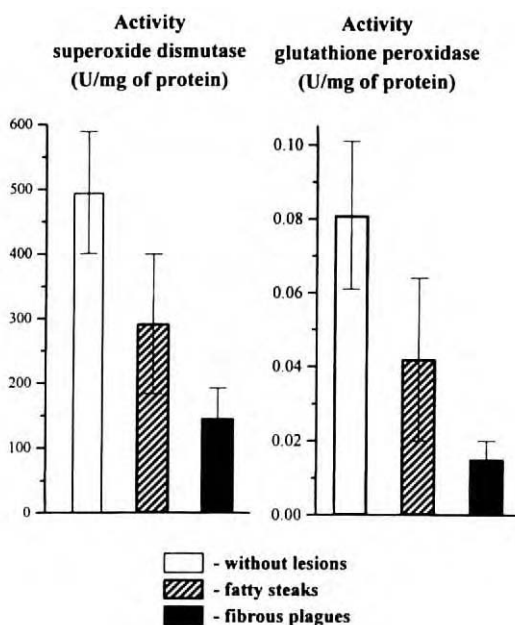


Figure 7. The activity of superoxide dismutase and glutathione peroxidase in the specimens from intima of human aorta with atherosclerotic lesions obtained at autopsy made in the 1-4 hours after sudden death.

Consequently, in the atherosclerotic aorta accumulation of oxidized LDL and activation of LDL oxidation by C-15 lipoygenase in the vascular wall in situ may be enhanced, and rate of enzymatic detoxification of reactive oxygen species and lipohydroperoxides in the areas of atherosclerotic lesions may be drastically decreased. All these mechanisms induced the accumulation of lipohydroperoxides in the regions of lipid streaks and fibrous plaques of atherosclerotic aorta [7,8,16] (Figure 8).

The data available from the literature [24,25] and our findings [16] enable us to explain the known fact of diminished prostacyclin production in the atherosclerotic aorta by accumulating lipoperoxides inhibiting prostacyclin synthetase. Thus, intensified lipoperoxidation in the atherosclerosis may be a direct cause of thrombosis which is the most frequent and severe event in this condition.

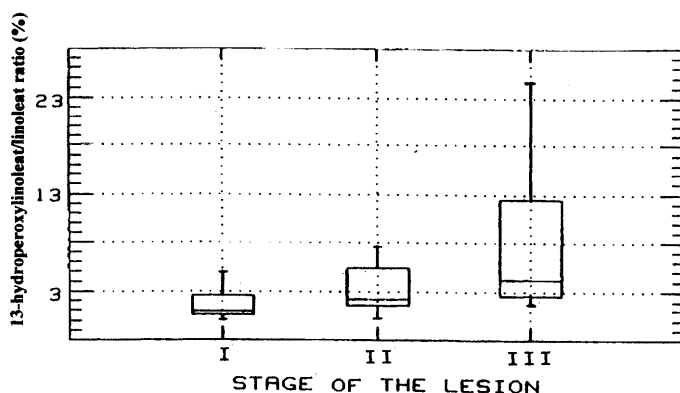


Figure 8. Dependence of the oxygenation degree of tissue lipids (hydroxy linoleate/linoleate ratio) on the stage of atherosclerotic lesion of human aorta (multiple box plot). (I), without lesions; (II), fatty streaks; (III), fibrous plaques.

There is one more aspect of the problem under consideration. Cholesterol autoxidized on the air with formation epoxides, ketones, hydroperoxy- and hydroxy-derivatives [26] (Figure 9).

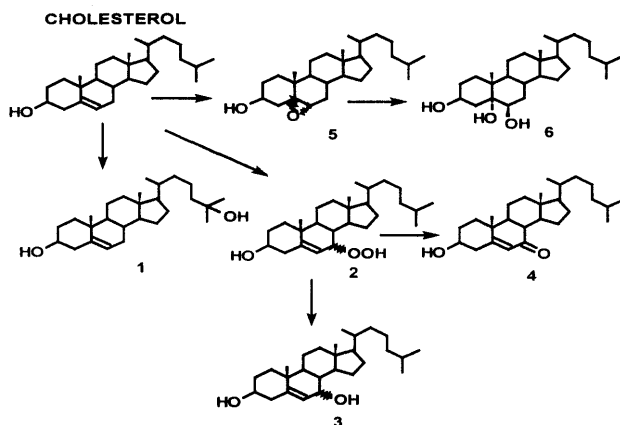


Figure 9. The products of free radical autoxidation of cholesterol: (1), 25-hydroxycholesterol; (2), 7 α -hydroperoxycholesterol; (3), 7 α -hydroxycholesterol; (4), 7 α -ketocholesterol; (5), 5 α ,6 α -epoxycholesterol; (6), cholestane-3 β ,5 α ,6 β -triol.

These oxysterols may be formed during *in vivo* cooxidation of cholesterol with unsaturated phospholipids of biomembrane and LDL as well as may be absorbed from food [26]. We investigated the influence of feeding of rabbits with oxidized (commercial cholesterol preparation including about 5% of cholesterol autoxidation products, mainly 7 α -hydroxycholesterol and cholestane-3 β ,5 α ,6 β -triol) or purified cholesterol (non-contained of any cholesterol oxidation products) on the total plasma cholesterol level and aorta lipoidosis after 12 weeks experiment starting [27-29]. We observed the increasing of the hypercholesterolemia rate during feeding oxidized cholesterol to animals [27,28] (Figure 10).

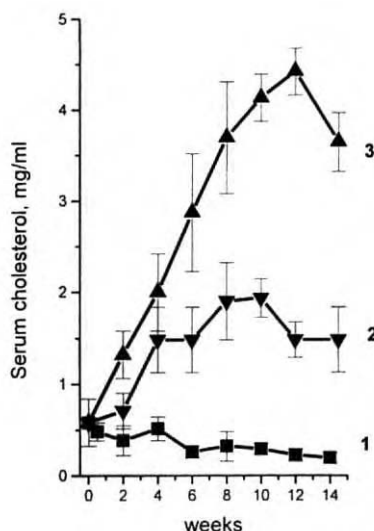


Figure 10. The total cholesterol level in blood plasma of rabbits fed with oxidized or non-oxidized cholesterol: (1), intact animals; (2), animals fed with purified cholesterol; (3), animals fed with cholesterol preparation which contain about 5% oxysterols.

Cholesterol ester content of the hepatocytes from oxysterol-fed rabbits was significantly higher then in control and purified cholesterol-fed rabbits [28] (Figure 11).

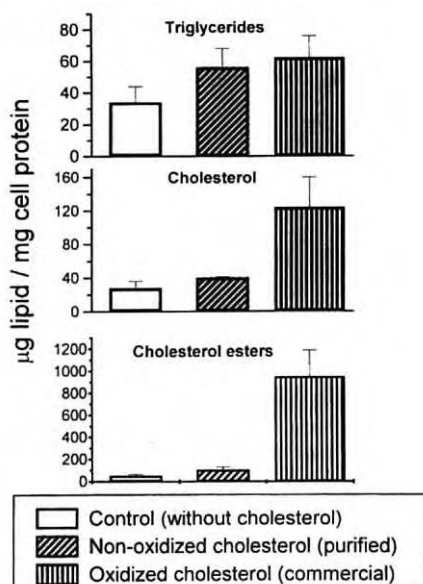


Figure 11. The cholesterol level in isolated rabbit hepatocytes after 6 weeks oral administration diet without cholesterol, with purified (non-oxidized) cholesterol and with commercial cholesterol preparation which contain about 5% oxysterols (oxidized cholesterol).

In addition the rate of NADPH-dependent microsomal lipoperoxidation was higher in the hepatocytes of rabbits which were fed with oxidized cholesterol (Figure 12).

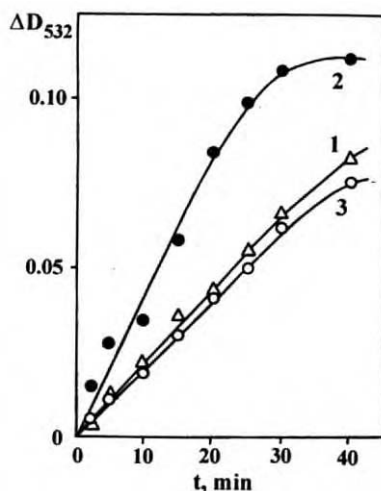


Figure 12. The NADPH-dependent microsomal lipoperoxidation in the isolated rabbit hepatocytes from animals which fed during 6 weeks with diet without cholesterol (1), and with cholesterol preparation which contain about 5% oxysterols (2) and with purified cholesterol (3) .

As it was found in our experiments the aorta lipoidosis was dramatically profound in the group of rabbits fed with oxysterol-rich diet in comparison with animals which were on purified cholesterol administration [27,29] (Figure 13).

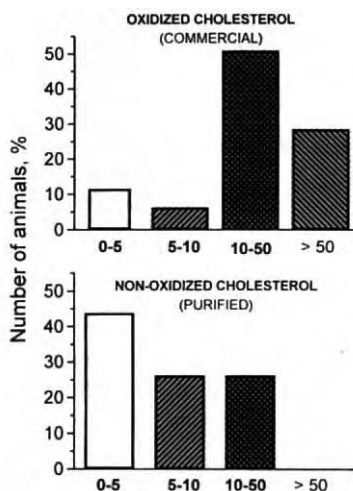


Figure 13. The aorta lipoidosis degree (%) in the rabbits fed during 3 months with purified cholesterol or with commercial cholesterol preparation which contain about 5% oxysterols.

We treated rabbits with very high content of oxysterols (about 20%) for 2 weeks. After it we observed by scanning electron microscopy the different oxysterol-induced endothelium injuries such as microthrombosis, cell protrusions and peeling of aorta endotheliocytes [29].

Therefore, intensified lipid peroxidation may promote enhanced thrombogenesis and

other vascular wall injuries by accumulating cytotoxic and angiotoxic products of cholesterol oxidation. The findings also support our assumption that not only cholesterol itself but its oxy-derivatives formed in the tissues in situ or given with food may substantially contribute to atherogenesis. It should be stressed that antioxidative enzymes may act in the body as a very effective natural antioxidants and their deficiency may be the main cause of free radical lipoperoxidation in the liver, blood and aorta during atherosclerosis. These findings also support that atherosclerosis can be considered as a classical example of free radical pathology [8,9].

Interventions that block oxidative modification of LDL are currently under intensive study [1,3-5,10]. If oxidative modification of LDL results in enhanced uptake by macrophages, use of an appropriate antioxidant should protect LDL from oxidation, decrease the rate of LDL uptake by macrophage foam cells and slow the development of fatty streaks in the arterial wall. The role of antioxidants in preventing oxidative modification of LDL has been evaluated in a number of studies [1,5,8,10]. In our investigation we studied the influence of the vitamin E rich diet on the copper-mediated oxidizability of plasma LDL from patients with atherosclerosis. So far as LDL is the main transport form of natural antioxidant α -tocopherol we were surprised to find that during 3-months vitamin E supplementation in the daily dose 400 mg the oxidation resistance of LDL did not increase (Figure 14).

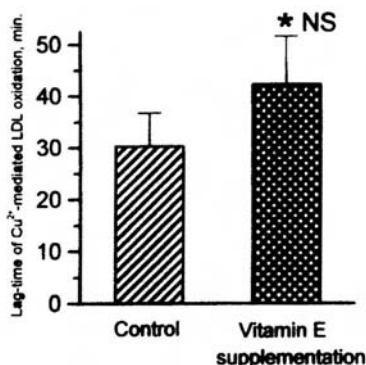


Figure 14. The susceptibility of human LDL to oxidation after 3-months treatment of patients with vitamin E in daily dose 400 mg.

These observations are consistent with the view that the most potent natural antioxidant of LDL may be not α -tocopherol but reduced form of ubiquinon Q₁₀ — ubiquinol Q₁₀ [30]. As we found the treatment of patients with the synthetic antioxidant probucol in the daily dose 1000 mg in opposition to vitamin E sharply increase the lag time of LDL oxidation in vitro [31]. It is known that probucol in daily dose 1000 mg act as cholesterol-lowering agent but this drug reduces of LDL cholesterol level very slowly and in addition induces different negative clinical effects such as increased of Q-T interval on the cardiogram. Used in quarter of the usual dose (250 mg per day) probucol demonstrated the same inhibition of lipohydroperoxide LDL accumulation in patients with cardiosclerosis as with high probucol dose — 1000 mg per day [31].

We isolated the LDL fraction from plasma of patients with atherosclerosis who had been on probucol (daily dose 250 mg) for 3 months and oxidized this probucol-contained LDL by C-15 animal lipoxygenase in vitro [31]. After decomposition of enzymatically accumulated acyl-lipohydroperoxides in LDL phospholipids by hemin with corresponding

alkoxyl radicals formation we identified in these particles the electron spin resonance signal of probucol phenoxyl radical (Figure 15). These findings suggest the possibility of LDL-associated probucol interaction with lipid radicals *in vivo*.

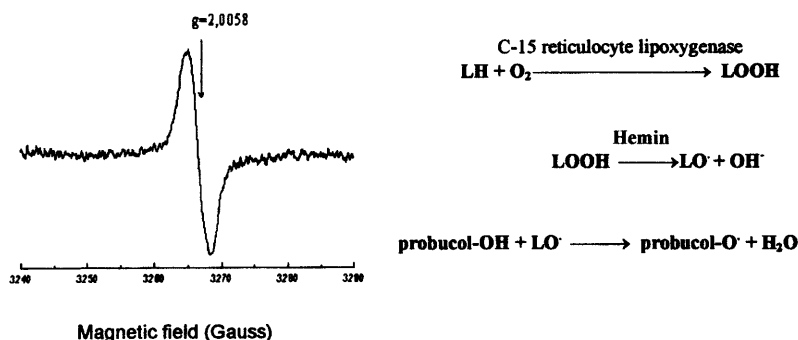


Figure 15. Electron spin resonans signal of phenoxyl probucol radical in LDL of patients with atherosclerosis, which were treated with 250 mg probucol daily during 3 months, after oxidation of those LDL by animal (reticulocyte) C-15 lipoxygenase and decomposition of LDL lipohydroperoxyde by hemin.

Several studies have demonstrated that intensive lowering of serum cholesterol or LDL cholesterol may retard progression of coronary atherosclerosis [32]. At present the inhibitors of key enzyme of cholesterol biosynthesis (HMG-CoA-reductase inhibitors) used in the clinical conditions as one of the most effective lipid-lowering drugs [32]. Note should be taken that HMG-CoA-reductase inhibitors may depress not only cholesterol but also ubiquinon Q_{10} biosynthesis so far as biosynthesis of both this substances involved a common precursor [33] (Figure 16).

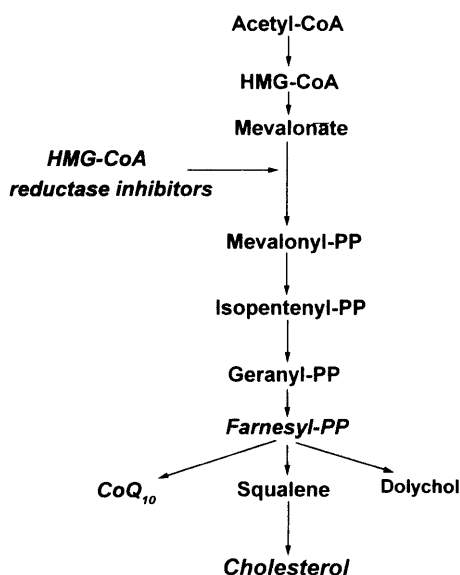


Figure 16. Scheme of cholesterol and ubiquinon Q_{10} biosynthesis depression by HMG-CoA reductase inhibitors.

Really the data available from the literature indicates decreased levels of ubiquinon Q_{10} in the LDL of patients with hypercholesterolemia during treatment with HMG-CoA-reductase inhibitors such as lovastatin, pravastatin and other drugs from this family [34,35]. There are also some experiments suggesting that ubiquinon Q_{10} in reduced form is an important antioxidant in human LDL [30].

Hence we study the level of LDL lipoperoxides in patients with atherosclerosis who had been for a long-time treated with HMG-CoA-reductase inhibitors in monotherapy as well as in combination with natural or synthetic antioxidants such as ubiquinon Q_{10} and probucol in double-blind placebo controlled trials [36,37]. The treatment of patients with inhibitor of cholesterol and ubiquinon Q_{10} biosynthesis pravastatine alone in daily dose 40 mg during 6 months was followed by accumulation of LDL lipohydroperoxides in the blood plasma [36] (Figure 17). On the other hand the 6 months administration of the same dose of pravastatine in combination with natural antioxidant ubiquinon Q_{10} in daily dose 60 mg sharply decreased even initial LDL lipoperoxides level in the plasma of patients [36] (Figure 17).

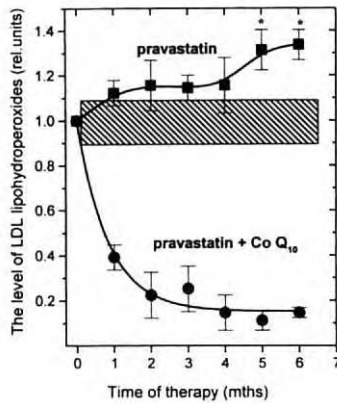


Figure 17. The LDL lipoperoxide levels in the blood of patients with atherosclerosis after 6 months treatment with HMG-CoA-reductase inhibitor – pravastatin (40 mg daily) or pravastatin in combination with natural antioxidant ubiquinon Q_{10} (60 mg daily).

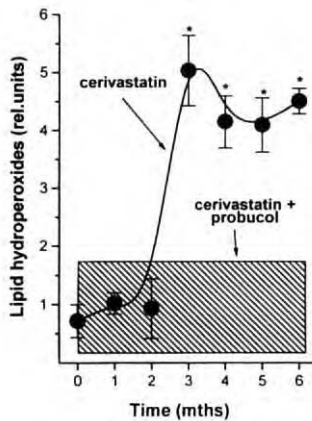


Figure 18. The LDL lipoperoxide levels in the blood of patients with atherosclerosis after 6 months treatment with HMG-CoA-reductase inhibitor — cerivastatin (0,4 mg daily) or cerivastatin in combination with synthetic antioxidant probucol (250 mg daily).

In one of our other studies [37], the 6 months therapy with 0,4 mg daily of other HMG-CoA-reductase inhibitor cerivastatin, which is more effective than pravastatin as cholesterol-lowering drug, sharply increased the level of LDL lipohydroperoxides in the blood plasma of patients [37] (Figure 18). At the same time administration of cerivastatin in combination with synthetic antioxidant probucol in daily dose 250 mg did not produce the increase of the LDL lipohydroperoxide level in the plasma during all time of the observation [37] (Figure 18).

As appears from the above, for prevention of atherogenic oxidative modification of LDL in the blood of patients with atherosclerosis HMG-CoA-reductase inhibitors must be used in combination with antioxidants. The most attractive conclusion is that synthetic antioxidant probucol may act in the LDL as a trap for lipid free radicals and may be effective in the prevention of LDL peroxidation in atherogenesis and during cholesterol-lowering therapy.

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Abstract. The significance of free radical oxidation of phospholipids in tissues of animals with experimental atherosclerosis was investigated. By using modern physico-chemical methods an elevated content of polyunsaturated fatty acids and other lipids peroxides was discovered in the blood and the aorta of rabbits with experimental atheromatosis. The human blood demonstrated a low level of protective enzymatic systems and a high content of products secondary to peroxidal oxidation of the lipids. The mechanism accounting for the action of lipids peroxides on the vascular wall resulting in the formation of atheromatous plaques is considered.
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H_2O_2 Sensors of Lungs and Blood Vessels and their Role in the Antioxidant Defense of the Body

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Abstract: This paper considers the composition and function of sensory systems monitoring H_2O_2 level by the lung neuroepithelial and carotid body. These systems are localized in the plasma membrane of cells of the corresponding organs and are composed of (i) O_2^- -generating NADPH-oxidase and (ii) an H_2O_2 -activated K^+ channel. This complex structure of the H_2O_2 sensors is probably due to their function in the antioxidant defense. By means of these sensors, an increase in the H_2O_2 level in lung or blood results in a decrease in lung ventilation and constriction of blood vessels. This action lowers the O_2 flux to the tissues and, hence, intracellular $[\text{O}_2]$. The $[\text{O}_2]$ decrease, in turn, inhibits intracellular generation of reactive oxygen species. The possible roles of such systems under normal conditions (e.g., the effect of O_2^- in air) and in some pathologies (e.g., pneumonia) is discussed.

It is obvious that oxygen simultaneously performs several functions essential for aerobic life. It plays the role of terminal electron acceptor for the respiratory chain, which is the major energy-conserving mechanism for respiring cells. Moreover, O_2 is a substrate of oxygenases as well as of oxidases alternative to cytochrome oxidase of the respiratory chain. Some of these oxidases produce reactive oxygen species (ROS), namely O_2^- and H_2O_2 , instead of the inert H_2O which is formed by cytochrome oxidase. ROS can be used by the organism as a tool to attack pathogens or as a signal. In the majority of cases, this is the signal for self-elimination of organelles, cells, organs, or even the entire organism. ROS are also formed nonenzymatically as a result of "parasitic" chemical reactions of one-electron reduction of O_2 by the respiratory chain electron carriers and some other natural reductants [1-3].

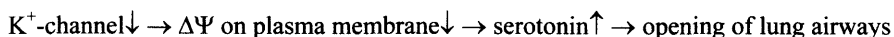
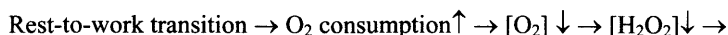
It is noteworthy that H_2O_2 can be reduced by Fe^{2+} and Cu^+ ions to extremely dangerous hydroxyl radical ($\text{OH}^\cdot$), which is able to oxidize almost all cellular compounds including DNA. The high toxicity of ROS is due first of all to $\text{OH}^\cdot$.

Higher organisms possess a multilevel system of anti-ROS defense. The first line of this system is to decrease the intracellular $[\text{O}_2]$ to a level still saturating cytochrome oxidase but insufficient for non-enzymatic ROS formation. One of the great achievements of evolution of aerobic life was the invention of cytochrome oxidase, an enzyme able to reduce O_2 at a high rate at O_2 levels even 100-fold lower than that in water under normal atmospheric pressure. As to ROS, their nonenzymatic formation parallels the decrease in $[\text{O}_2]$ according to the mass action law. This is why a decrease in intracellular $[\text{O}_2]$ over wide limits does not affect the cytochrome oxidase reaction but strongly inhibits nonenzymatic one-electron reduction of oxygen [1-5].

The strategy of higher organisms is that the rate of O₂ delivery to a tissue, being high in the state of active work, decreases dramatically during rest. This effect is achieved first of all by means of a decrease in lung ventilation and constriction of blood vessels at the work-to-rest transition. It should be emphasized that reactive oxygen species, rather than O₂ per se, are dangerous. Therefore, it would be desirable for organisms to have a sensor monitoring the level of OH[•], the most aggressive ROS. However, this is hardly possible since OH[•], in fact, is too aggressive. On the way to the active site of the sensor, it would be discharged, spoiling thereby any cellular component including the hypothetical OH[•] sensor itself. On the other hand, it would be much easier to monitor the level of H₂O₂, a direct precursor of OH[•] in the chain of ROS interconversion reactions.

There are some indications that mammals possess at least two H₂O₂ sensors. One is located in cells of the lung neuroepithelial bodies, being responsible for constriction of the lung airways when the H₂O₂ level rises [6,7]. The other performs the same function in the blood vessels, being found in cells of the carotid body [8-10].

The two H₂O₂ sensors have very similar mechanisms as shown in Fig. 1. They are composed of two independent protein systems, one H₂O₂-forming and another responding to H₂O₂. H₂O₂ is formed by an NADPH-oxidase which is of the same type as that found in the plasma membrane of phagocytes, where this enzyme forms O₂^{•-} to suppress pathogens (for review, see [11]). The enzyme oxidizes intracellular NADPH, transporting electrons through the membrane to its outer surface (FAD and special two-heme cytochrome *b* are involved). Here one-electron reduction of O₂ to O₂^{•-} occurs. Two O₂^{•-} molecules dismutate to form O₂ and H₂O₂. The latter interacts with the outer part of a K⁺ channel protein located in the plasma membrane. As a result, the channel is stabilized in its open conformation. If the O₂ concentration drops, the rate of the NADPH-oxidase reaction decreases, [H₂O₂] decreases, the K⁺ channel closes, the membrane potential on plasma membrane decreases, and the cell is excited. The excitation gives rise to release of intracellular serotonin to the extracellular medium. Serotonin operates as a mediator of opening of the lung airways (it is significant that the neuroepithelial cells are located in places of branching of these airways). This situation is typical for periods of active work when mitochondrial cytochrome oxidase consumes large amounts of oxygen:



The work-to-rest transition results in a decrease of the O₂ consumption in cells, a rise in the blood O₂ concentration, and consequent lowering of O₂ diffusion from the lungs to the blood. As a result, [O₂] outside the neuroepithelial cells rises, NADPH oxidase is activated, [H₂O₂] increases, K⁺ channel opens, and serotonin is not released. The final event will be a decrease in the O₂ supply to the body due to a constriction of airways [8].

Similar events occur in the carotid cells. The only difference is that they release catecholamines instead of serotonin, causing dilatation of the blood vessels.

It is generally assumed that the lung neuroepithelial cells as well as carotid cells are O₂ sensors [8]. From this point of view, however, it is difficult to understand why the O₂ sensors of animals are organized in such a complex manner. It is known that the O₂ sensors are already inherent in bacteria, where they are much simpler than in animals and are competent in [O₂] monitoring by means of a direct O₂ binding¹.

¹ One of them was quite recently described by Alam and coworkers in *Halobacterium salinarum* and *Bacillus subtilis*. This is a single protein composed of two domains. The first (175 amino acid residues) is homologous to the animal myoglobin, whereas the second (amino acids 222-489) is very similar to the bacterial methyl-accepting proteins taking part in chemotaxis. They assume that the O₂ binding by the first domain results in a conformational change transmitted to the second domain participating in transduction of

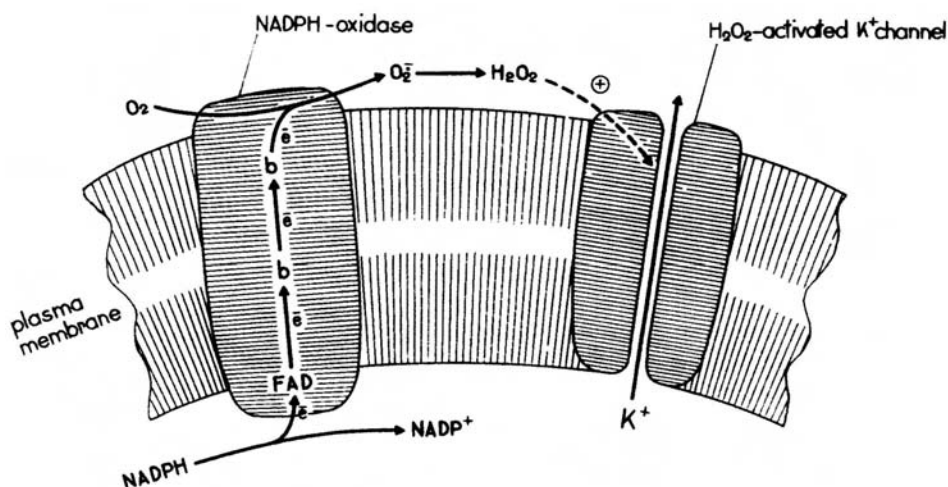


Figure 1. H₂O₂ sensor in the plasma membrane of the lung neuroepithelial and carotid cells.

These relationships might be explained suggesting that the major function of the sensors in neuroepithelial and carotid cells is that of the antioxidant defense of the organism. The very fact that it is [H₂O₂] rather than [O₂] that is monitored by these sensors allows the organism to effectively perform such a function. The described organization of the sensors causes constriction of the airways and the blood vessels due to an increase in [H₂O₂] independently of the reasons causing this increase. The reasons may be not only elevation of [O₂] because of a decrease in O₂ consumption in the tissues, but also activation of H₂O₂ production or inhibition of the H₂O₂ decomposition. This means that any damage to the antioxidant system of the body will actuate such an effective defense mechanism as a decrease in the O₂ supply to tissues and cells. Such a response would be impossible if in the above-mentioned sensory cells a simple bacterial-type O₂ sensor would be employed.

Quite recently, Weintraub and coworkers [16] reported that H₂O₂ activates an O₂⁻-generating NAD(P)H oxidase in a non-phagocytic cell type of vascular origin (smooth muscle cells and fibroblasts). This means that production of H₂O₂ by, say, carotid cells can initiate a feed-forward mechanism amplifying the H₂O₂ signal. It is quite obvious that such a cascade may strongly reinforce the ability of ROS to down-regulate the O₂ delivery to the tissues.

The concept described above can explain a number of physiological and pathological phenomena. For example, bronchospasms in the case of pneumonia may be a consequence of an increased O₂⁻ production by the phagocyte NADPH oxidase in the inflamed regions [11], an event erroneously interpreted by the organism as a signal of oxygen danger. The same situation may take place as a result of a viral infection in lungs due to activation of xanthine oxidase. As reported by Maeda and coworkers [17-19], the influenza virus causes strong (by 2-3 orders of magnitude) activation in lungs of xanthine oxidase, an enzyme forming O₂⁻ and H₂O₂ from O₂ (concerning the possible significance of this effect for suppression of the viral infection, see [20]).

It seems possible that O₂⁻ in the air (so-called negative aeroions) may regulate the work of the lungs. An increase in [O₂⁻] in the consumed air may be interpreted as a signal

the signal to the bacterial flagellum [12]. There are some reasons to suggest that heme-containing O₂ sensors that bind O₂ without its subsequent reduction operate also in animals, but their role consists in regulation of some events at the level of the cell rather than the organism (for review, see [13-15]).

to decrease lung ventilation. Respectively, a decrease in $[O_2^{\cdot-}]$ will lead to hyperventilation, tissue $[O_2]$ increase, stimulation of ROS production, and, as a consequence, acceleration of ageing [2].

In this context, it should be noted that one of the most aggressive types of cancer, small cell lung carcinoma, represents, in fact, a result of malignant transformation of the lung neuroepithelial cells. These tumor cells still produce the same neuromediators [21, 22] and contain both NADPH oxidase and the H_2O_2 -stimulated K^+ channels [8]. Even more, malignant transformation was found to be oxygen-dependent [23].

It seems possible that the favorable effects of the $O_2^{\cdot-}$ -generating devices (see, e.g. [24,25]) is also mediated by some H_2O_2 sensor(s). As shown by Goldstein and coworker [24], mice and rats die when kept under $O_2^{\cdot-}$ -free conditions for 16 and 23 days, respectively. Most probably, the death is a consequence of deregulation of some functions of vital importance occurring due to the absence of signals from $O_2^{\cdot-}$ - and/or H_2O_2 sensors normally reporting about the level of these reactive oxygen species in airways. These signals might be produced by either lung neuroepithelial bodies (see above) or the so-called vomeronasal organ [26,27].

There are indications that in large cities the air $[O_2^{\cdot-}]$ is strongly reduced due to antioxidant actions of products of decomposition of rubber and some other polymers [28,29]. The air $O_2^{\cdot-}$ deficiency could be compensated by artificial $O_2^{\cdot-}$ -generators. However, here we should be very careful since hyperproduction of $O_2^{\cdot-}$ can lead to catastrophic consequences due to constriction of the lung airways. This is why the use of $O_2^{\cdot-}$ generators should be considered only after detailed investigation of their effects on lung function. In any case, it is very probable that air $O_2^{\cdot-}$ monitoring should be useful to improve conditions of existence of humans in the modern world.

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Oxidative Lung Injury

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Abstract: Considerable interest has developed in the idea that oxidative stress is instrumental in the aetiology of a range of respiratory diseases. Recent findings of oxidant/antioxidant imbalances in respiratory disorders such as cystic fibrosis, adult and neonatal respiratory distress syndromes and asthma has brought the lung to the forefront of such work. In each of these conditions it is a combination of events that give rise to the oxidative stress. Understanding the interrelationship of these events is key to a better understanding of the respective pathologies. Work is now ongoing in a number of groups to improve our understanding of the response of the lung to oxidant injury. Such findings will be important in designing strategies that result in more appropriate remodelling of the airways. These strategies are likely to include provision of specific components of the antioxidant system or substances that stimulate the increased production of endogenous antioxidant defences.

1. Introduction

All tissues are vulnerable to oxidant damage but by virtue of its location, anatomy and function, the epithelial surface of the lung is one of the most vulnerable targets in the body. The surface of the lung is enormous (equivalent to a couple of tennis courts) and it is continually exposed to gases, vapour and particulate matter present in the atmosphere. Many of these, such as ozone, nitrogen dioxide and tobacco smoke are powerful oxidants (due to direct and indirect free radical activity) and if left unopposed lead to the oxidation of lipid, protein and nucleic acids in the respiratory epithelium. In addition, a number of respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) involve pulmonary inflammation. In these conditions reactive oxygen species (ROS) are produced by activated alveolar macrophages, and invading neutrophils and eosinophils.

A third and often-overlooked source of oxidative stress in the lung is the generation of ROS from the utilisation of oxygen as the terminal electron acceptor in the mitochondrial respiratory chain. Under normal oxygen concentrations the generations of intermediate ROS is minimal and not a significant burden for the cell. However, when the partial pressure of oxygen is increased, for example to compensate for a under performing lung as in a patient with respiratory distress, the increased generation of ROS may unbalance the delicate redox balance of the cell. Under such circumstances a range of responses will ensue which may lead to the ultimate death of the cell. If cell loss becomes excessive then the respiratory distress will increase and extensive lung injury can occur.

None of the above mentioned forms of oxidative stress are mutually exclusive as pulmonary inflammation usually follows cell injury occurring both as a result of respiratory support or exposure of the airways to ambient pollutants. In the following review, each of these three forms of oxidative stress will be considered in more depth and their inter-relationships will be explored using a range of respiratory diseases.

2. Environmental oxidative challenges

Ambient air contains a range of oxidant pollutants, the exact combination of which varies from one microenvironment to the next. One of the most important of these is ozone, a highly reactive gas that is a major constituent of photochemical smog [1]. Both controlled human exposure studies and field studies have revealed that frequently encountered ambient concentrations of ozone induce transient functional and inflammatory changes in the lung. Interestingly, marked responses are seen in only about 10-20% of the healthy population. Moreover, those with conditions such as asthma and COPD generally experience increased symptoms. Together, these observations suggest that certain individuals are particularly susceptible to this oxidant gas.

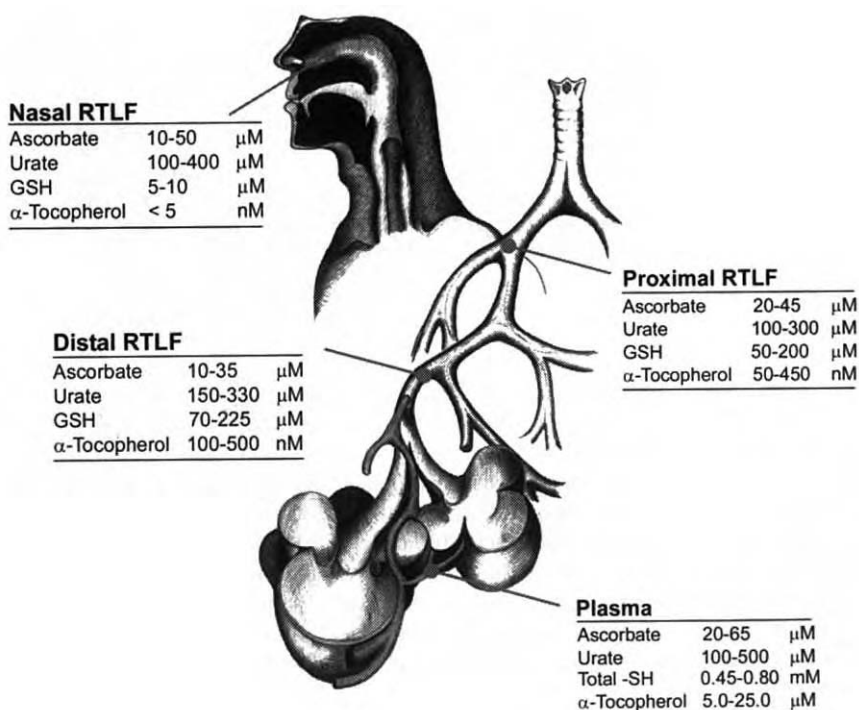


Figure 1. Distribution and concentration of lung lining fluid antioxidants within the respiratory tract.

Values are based on lavage values corrected for the lung lining fluid dilution. Corresponding plasma concentrations are provided for comparison. Plasma reduced glutathione is not given as concentrations are very low, typically less than 5 μM.

Ozone is a highly reactive gas that is consumed by reactive processes on reaching the first interface in the lung, the respiratory tract lining fluid (RTLF) compartment. Reactions between ozone and antioxidants tend to dominate in this compartment and these are generally thought of as beneficial, or protective, interactions. In those instances when ozone reacts with other substrates in RTLF such as protein or lipid, secondary oxidation products arise which transmit the toxic signals to the underlying pulmonary epithelium. The rules that govern the balance between beneficial and detrimental interactions in the RTLF compartment are not well established but these may contribute, in part, to sensitivity.

Under normal circumstances, oxidative injury of the respiratory epithelium is

minimised by a thin layer of fluid rich in antioxidant defences, the RTLf, that buffers the extracellular surface of the respiratory epithelium [2]. The SOL phase of RTLf contains enzymes such as superoxide dismutase and catalase, macromolecules such as caeruloplasmin and transferrin and an array of small molecules including glutathione, uric acid, cysteine, methionine, vitamin C (ascorbic acid), and vitamin E (α -tocopherol) (Fig. 1). The overlying GEL phase of RTLf, which is rich in mucoglycoproteins, probably also has antioxidant activity but this is as yet undefined. It is likely that the quantity and quality of this airways antioxidant network is an important determinant of the susceptibility of the underlying respiratory epithelium to resist oxidative stress [1].

The uptake of ozone relates directly to its reactions with substrates present in the lung lining fluid, a mechanism referred to by Postlewaite as 'reactive absorption' [3]. The uptake of ozone is thus related not only to its concentration but also availability of substrates within the RTLf [4]. As these are numerous, ozone does not actually transit RTLf and hence cannot interact directly with the pulmonary epithelium. Rather it is consumed during reactions with compounds in this compartment (Fig. 2). Therefore, cellular responses to ozone are not a result of the direct reaction of ozone with cell surface component/receptors but rather are mediated through a cascade of secondary, free radical derived, ozonation products [2,4].

In addition to having to breathe ozone-laden air, modern living results in the exposure of the respiratory tract to other oxidant gases such as nitrogen dioxide and particulate pollution such as that arising from diesel exhaust emissions. For many of us, urban living ensures daily contact with these oxidant challenges. Another recurrent, but avoidable, oxidant challenge is cigarette smoking. Cigarette smoke contains a range of oxidant gases and a high number of particulates and much work has been undertaken to understand its impact on lung biology [5].

3. Pulmonary inflammation

A feature common to many respiratory diseases is the influx of activated inflammatory cells, such as neutrophils to the lung. The generation of oxygen free radicals by activated inflammatory cells is likely to be involved in the pathogenesis of these conditions. Neutrophils, eosinophils and macrophages possess a membrane bound flavoprotein cytochrome b245 NADPH oxidase that is induced during cell activation. Using molecular oxygen, the NADPH oxidase produces superoxide anions, which if removed by superoxide dismutase, result in H_2O_2 generation. Nathan and Root (1977) estimated that activated macrophages produce H_2O_2 at a rate of 2.5×10^{14} mol/hr/cell. Due to the relatively low reactivity of H_2O_2 it can easily pass across cell membranes, where it may activate intracellular signalling pathways, or lead to the generation of other reactive oxygen species. For example, in the presence of transition metals H_2O_2 leads to the production of the more toxic, hydroxyl radical.

Nitric oxide ($\bullet NO$) also contributes to the alveolar epithelium's oxidant burden, primarily as a result of the formation of reactive oxygen or nitrogen species. $\bullet NO$, one of the smallest and most distinctive biological mediators, is generated by nitric oxide synthase (NOS) which has three isoforms; neuronal (nNOS, isoform I), inducible (iNOS, isoform II) and endothelial (eNOS, isoform III). nNOS and eNOS are constitutively expressed in cells and generate $\bullet NO$ in small quantities for brief periods of time in response to increased intracellular Ca^{2+} concentrations. It is currently unclear whether the level of expression or the enzymatic activity of either eNOS or nNOS is modulated by pathogens or inflammatory stimuli.

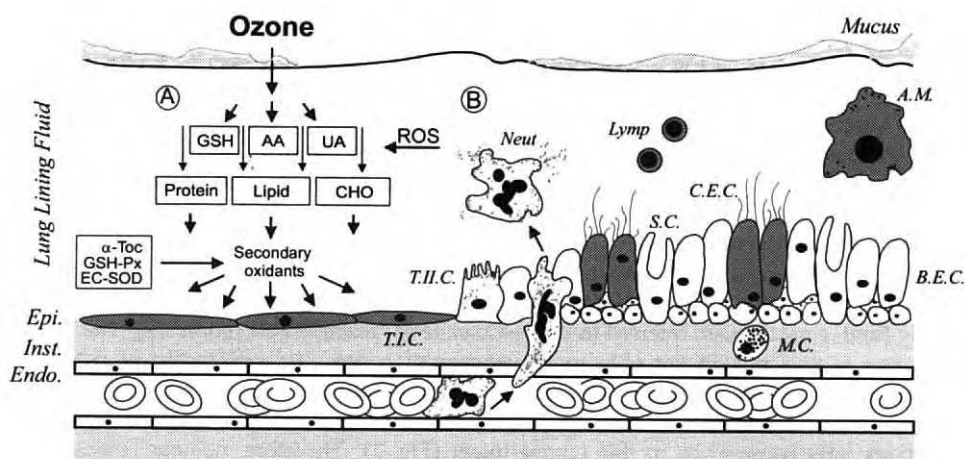


Figure 2. Proposed mechanism of ozone toxicity in the lung.

Ozone is both highly reactive to a broad spectrum of substrates and relatively water insoluble. As a result of these two physical characteristics its uptake into the LLF is intrinsically coupled to its reaction with moieties within this compartment, such that little or no ozone is thought to react directly with the underlying cells, except in areas where the lung lining fluid is denuded. Ozone is very highly reactive toward the water soluble antioxidants, ascorbate (AA), urate (UA), and reduced glutathione (GSH), which are present at high abundance in the lung lining fluid. It is thought that these antioxidants provide protection against damaging oxidation reactions in the extracellular pool by preferentially reacting with ozone to prevent its direct reaction with the macromolecular components of this compartment, 'A'. Reactions with proteins may lead to the inactivation of enzymes, whilst ozonation of unsaturated lipids is known to give rise to cytotoxic and pro-inflammatory lipid oxidation species, which may react with the underlying cells to transmit the toxicity of ozone from the extra-cellular compartment to the cell. Additional antioxidant moieties within this compartment, alpha-tocopherol (α -Toc) and glutathione peroxidase (GSH-Px) may act to prevent the propagation of lipid auto-oxidation reactions, whilst extra-cellular SOD (EC-SOD) may be important in removing superoxide formed as a by-product of the oxidation reactions occurring. Ozone is also known to be a strong promoter of neutrophil migration to the surface of the lung 'B'. If these cells are activated on entering the lung they will create a second wave of oxidative stress within this compartment which will further deplete the endogenous antioxidants and cause cellular injury. In addition to neutrophils a number of cell types have been shown to be influenced by ozone exposure, either because they are especially sensitive to acute injury (usually reflecting a large surface area), or because post exposure increases have been observed. Type I cells (T.I.C.), alveolar macrophages (A.M.) and ciliated epithelial cells (C.E.C) appear to be especially prone to injury. Lymphocytes and mast cells populations have been shown to be increased post-ozone. Other abbreviations: Epi.: epithelium, Inst.: interstitium, Endo.: endothelium, TIIC: type II cell, BEC: bronchial epithelial cell.

In contrast to nNOS and eNOS, iNOS protein is generally not constitutively expressed. Rather, transcription of iNOS in alveolar macrophages and probably neutrophils is triggered by pro-inflammatory stimuli including cytokines such as IFN- α/β , IFN- γ , TNF- α and IL- β . Many anti-inflammatory agents, including glucocorticoids, cytokines (IL-4, -8 and -10) and growth factors (TGF- β) inhibit iNOS expression. Provided that substrate and cofactors are available, iNOS can generate large amounts of $^{\bullet}\text{NO}$ for an extended period of time. It is important to note that, although iNOS and the NADPH-oxidase system are differentially regulated they are both induced by similar pro-inflammatory stimuli and therefore likely to be simultaneously active and generating reactive species during an inflammatory response.

Potential sources of $^{\bullet}\text{NO}$ in the lungs include activated alveolar macrophages, neutrophils; alveolar type II cells endothelial cells and airway cells. nNOS is localized to nonadrenergic/noncholinergic nerve terminals and is present in human airway epithelial cells. eNOS is localized to human pulmonary epithelium and bronchial epithelium. Studies have suggested that iNOS is constitutively expressed in human upper airway epithelium

and occasional alveolar macrophages, but this may be as a result of chronic exposure of these cells to inhaled pollutants and microbes. Expression of iNOS in other regions of the normal lung is believed to be minimal. However, iNOS has been immunolocalised to airway cells or human lung tissue obtained from patients with conditions as diverse as bacterial pneumonia, lung cancer, pulmonary sarcoidosis, idiopathic pulmonary fibrosis, asthma and the adult respiratory distress syndrome.

Because cytotoxic effects of $\text{}^{\bullet}\text{NO}$ are non-specific, they are not limited to invading pathogens but can also damage the cells and tissues that produce it. Moreover, $\text{}^{\bullet}\text{NO}$ may contribute to the systemic morbidity of pathological processes through its proposed activity as a peripheral vasodilator and because $\text{}^{\bullet}\text{NO}$ has an unpaired electron, it can readily react with other free radicals. In pathological states, most of the toxic effects of $\text{}^{\bullet}\text{NO}$ have been attributed to its rapid reaction with $\text{O}_2^{\bullet-}$ to form peroxynitrite ($\text{ONOO}^{\bullet}$) which is a potent oxidising and nitrating agent as well as a vasodilator in its own right.

4. Excess oxygen and lung injury

Pure oxygen breathing is lethal to mammals within days. Death is due to the extensive lung damage that occurs and the consequent demise of gaseous exchange. The early signs of injury are seen in endothelial cells, although bronchial and alveolar lining cells are also damaged [6,7]. Following damage of the capillary endothelium oedema develops and the efficiency of gaseous is reduced. As oxidative damage proceeds the alveolar Type I cells also show alterations and subsequently with sustained insult Type II cells are also injured.

Present understanding of these events began with the astute observations of Gerschman and colleagues in 1954, when they noticed that the lung injury seen following hyperoxic exposure was similar to that seen following exposure to ionising radiation [8]. This led them to suggest that hyperoxic-induced lung injury, like radiation injury, was due to excessive free radical production. This fundamental observation initiated a whole new era of investigations in free radical biology and led to the present situation where free radicals are implicated in the pathology of numerous diseases.

The area next moved forward in 1969 following the discovery of superoxide dismutase (SOD) [9]. With the advent of methods to measure SOD activity and superoxide production great interest was shown in examining the relationship between pulmonary antioxidant defences, oxygen free radicals and tissue injury.

At this time the evidence that hyperoxic-induced lung injury was due to excessive oxygen free radical production was still largely indirect. The next major event which specifically addressed this question was the important experiments of Crapo and colleagues in the early 1980's [10]. These studies conducted with lung tissue were similar to those performed earlier in liver [11] and heart [12]. Crapo and co-workers found that superoxide production, measured as CN-insensitive respiration, was increased in lung slices when exposed to 100% O_2 rather than air [10]. This work was extended to the measurement of O_2 production in submitochondrial particles [13]. Clearly then exposure of tissues to elevated concentrations of O_2 leads to ROS production, the extent of which, in association with local antioxidant defences, will determine the redox balance of the tissue.

5. Respiratory diseases that involve oxidative stress

As stated previously oxidant stress in the lung does not arise as the result of only one of the three pathways described above. Rather, direct oxidant challenge of the lung by ambient

oxidant gases or increased intracellular ROS generation due to increased mitochondrial respiratory chain activity is usually accompanied by pulmonary inflammation. Invading inflammatory cells then contribute to the increased oxidant load in their own right. Such interactions will now be considered in a range of respiratory diseases.

6. Chronic lung disease of prematurity

Chronic lung disease (CLD) or Bronchopulmonary Dysplasia was first described by Northway and colleagues in 1967 [14]. It was seen to develop in a number of infants treated for severe hyaline membrane disease (HMD) with high oxygen concentrations. The chest radiograph of HMD progressed over 3 to 4 weeks to emphysema with uneven aeration and prominent stringy radiodensities. Hence the definition emerged that an infant had CLD if it showed these characteristic chest radiographic changes and was still oxygen dependent after 28 days. As the survival of extremely premature infants has increased in the intervening period the number of infants developing CLD has increased. It has been estimated that between 20 and 45% of infants born prior to 32 week develop this condition [15]. Reported incidences of occurrence, however, depend critically on the criteria used and population studied.

Whilst both high concentration oxygen therapy [16], the pulmonary prematurity itself [17] and positive pressure ventilation [18], have all been incriminated in the pathogenesis of CLD, little is known about the cellular basis of the disorder. It is highly likely that chronic lung disease, complicating RDS in prematurity is multifactorial and may differ from case to case. Some of the factors that have been suggested as contributing causes are listed in Table 1. Nevertheless, out of a confusing literature emerges a view that “oxygen toxicity” may be responsible in large part for the clinical events associated with the subsequent development of CLD. Indeed, Northway et al. [17] drew attention to an animal model that demonstrated the histopathological similarities between hyperoxic injury to the lung and CLD. This and other experimental observations have led to the postulate that administration of high concentrations of oxygen to premature infants results in damage to the bronchial epithelium and increased airways resistance. This acute response of the lung necessitates an increase in oxygen tension of the inspired gas and increased ventilatory pressure to maintain adequate arterial oxygen saturation. Thus, on the basis of the resuscitative measures surrounding prematurity and the development of CLD, attention has been focused on the role of oxygen toxicity to the bronchial epithelium.

Table 1. Risk factors for Chronic Lung Disease.

Pulmonary parenchymal immaturity
Surfactant deficiency
Antioxidant deficiencies
Hyperoxic exposure
Barotrauma
Patent ductus arteriosus
Pulmonary oedema
Protease/anti-protease imbalance

At this same time there was also considerable interest in examining the antioxidant defence resources of the immature lung. Attention was focused on this question, as it was appreciated that birth itself represented a period of oxidative stress, as at birth the foetus moves from a relatively anoxic environment to an oxidative one. The 5-fold increase in oxygen tension would presumably lead to increased oxygen free radical production and if

lung damage were to be avoided then antioxidant defences would have to increase at this time. Such a theory had support from the rapid development of a parallel biochemical system prior to birth, the surfactant system.

Early studies designed to address this question were mostly animal based as suitable foetal lung tissue was difficult to obtain. A study by Author and colleagues [19] in a small number of infants, did however fuel speculation, when they concluded that premature birth would indeed result in infants with lungs deficient in superoxide dismutase. Numerous studies examining the ontogeny of pulmonary antioxidant development in mammalian lungs soon appeared to support this finding [20-26]. It rapidly became widely accepted that birth prior to full gestation would result in the use of lungs with immature antioxidant defence levels.

This concept, which rapidly became ingrained in researchers minds, led rapidly to the concept that many of the disorders of prematurity such as retinopathy of prematurity, intraventricular haemorrhage and chronic lung disease, could be tackled therapeutically by antioxidant supplementation. Of course this was not a totally new concept, as it had been recognised for years, that preterm infants had low circulating vitamin E levels and vitamin E supplementation had been used, mostly unsuccessfully, to try and alleviate or prevent these conditions.

The concept that the preterm infants lung is deficient in antioxidant enzymes, based mainly on these animal studies remained largely unchallenged for a decade. In the mid 1980's however, Strange and co-workers and Kelly et al., began to examine in detail antioxidant enzyme development in the human lung during gestation.

The clear conclusion of these studies was that human lung antioxidant enzyme development was not a 'late gestational' process as had been cited for a number of laboratory mammals, but rather Cu/Zn-SOD, Mn-SOD and GSH-Px are expressed constitutively throughout gestation and early neonatal life [27-30]. The exception to this rule was the developmental pattern of catalase in the lung, which was found to increase markedly throughout gestation. While the functional significance of the increase in catalase activity in the absence of coordinated changes in SOD and GSH-Px activities is not presently understood, indirect evidence suggests that there may be a link with the maturation of the surfactant system. Studies in liver have located catalase activity to peroxisomes, where it serves to reduce H_2O_2 generated by a variety of oxidases [31]. The function of peroxisomes in the developing lung has not been studied, but histological studies have shown their numbers to increase in concert with the acquisition of cytosolic lamellar body stores of surfactant in the maturing alveolar epithelium [32]. In our study we found a strong temporal relationship between pulmonary DPPC fractional content and catalase activity during gestation [27]. Hence, the preterm infant born with an immature lung in respect of morphological and biochemical development is not, with the exception of catalase, deficient in antioxidant enzymes. Notwithstanding, these babies often require treatment with supplemental oxygen, and although this has not been possible to show directly, indirect evidence would suggest that hyperoxic exposure will lead to increased ROS production. Therefore there still remains a strong case to investigate the benefit of exogenous antioxidant supplementation in such circumstances.

7. Asthma

Asthma is a chronic relapsing inflammatory disorder that can lead to tissue destruction and airway remodeling characterised by epithelial disruption with smooth muscle and microvascular proliferation. Oxidative stress appears to play a central role in these changes as both increased ROS generation and decreased antioxidant defenses have been identified

in asthmatic patients. Moreover ROS can reproduce many of the pathophysiological features of asthma including enhanced arachidonic acid release, airway smooth muscle contraction, increased airway reactivity and secretions, increased vascular permeability, increased synthesis of chemoattractants and impaired β -adrenergic responsiveness [33].

Evidence for the involvement of ROS in asthma include reduced SOD activity in both bronchial cells and RTLF in asthmatics [34,35], increased basal and stimulated superoxide production from both eosinophils and neutrophils [36,37] and a correlation between such increased production and leakage of albumin [36]. Mice over expressing the cytosolic Cu/Zn-SOD were resistant to changes in bronchial neural control following ovalbumin challenge [38]. There are also reports of increased nitrotyrosine concentrations in asthmatic lungs, presumably arising from peroxynitrite, the reaction product of NO and superoxide [39,40].

RTLF antioxidant status in patients with mild asthma has been found to differ from healthy control subjects [41]. Whereas nasal lavage and RTLF reduced glutathione (GSH) content was similar in asthmatic and control subjects, ascorbate, urate and α -tocopherol concentrations were all modified (Fig. 3). RTLF ascorbate levels were particularly low or undetectable in asthmatics even though their plasma ascorbate levels were similar to control subjects. Likewise, α -tocopherol concentrations were significantly lower in RTLF from asthmatic patients, even though they had higher plasma levels of this primary lipid soluble antioxidant. Conversely urate concentrations, although lower in plasma and NL fractions in asthma patients, were higher in RTLF, perhaps in part compensation for the low ascorbate and α -tocopherol concentrations. Also, RTLF oxidised glutathione (GSSG) content was higher in asthma subjects compared to control subjects indicating the presence of oxidative stress in their airways.

An association between asthma and dietary antioxidant intake, in particular vitamin C, has been recognised for some time. Both blood leukocyte (35%) and plasma (50%) vitamin C levels have previously been reported low in asthmatics [42]. Although the asthmatic patients in the study of Kelly et al. [41] did not have low plasma vitamin C levels, their RTLF levels were low or non-existent. This important finding indicates that reliance on plasma measurements alone is not a sufficient indicator of airways antioxidant status. Moreover, it highlights the fact that the nature of the relationship between plasma and RTLF antioxidant pools is unknown. The occurrence of low ascorbate and α -tocopherol concentrations in the airways of asthmatic patients is particularly worrying. It has already been noted that consumption of fresh fruit and vegetables has decreased in recent decades [43] and this may explain, in part, the lower RTLF antioxidant defences in this group. If there is a direct relationship between dietary antioxidant intake and RTLF levels then presumably the healthy control subjects also have lower RTLF antioxidant defences than previous generations. In this case, the addition of the oxidative burden of inflammation, as in asthma, is sufficient to overwhelm this important defence screen as seen by the presence of oxidised glutathione in asthmatic airways. These findings provide a basis for understanding why antioxidant intake may play an important role in the aetiology and severity of asthma and why these patients are more susceptible to inhaled irritants and allergens.

8. Cystic fibrosis

Cystic fibrosis (CF) is a genetic condition that mainly affects the lungs and gastrointestinal tract. Great advances have been made in understanding the underlying genetic defect in CF. The cause of the severe lung damage that arises, and the subsequent pulmonary fibrosis that develops, however remain unclear. Irregularities in ion transport leading to inspissation of

mucous secretions, along with increased adherence of bacteria to epithelia and reduced muco-ciliary clearance are all thought to contribute to the recurrent, progressive, pulmonary infections characteristic of the disease [44].

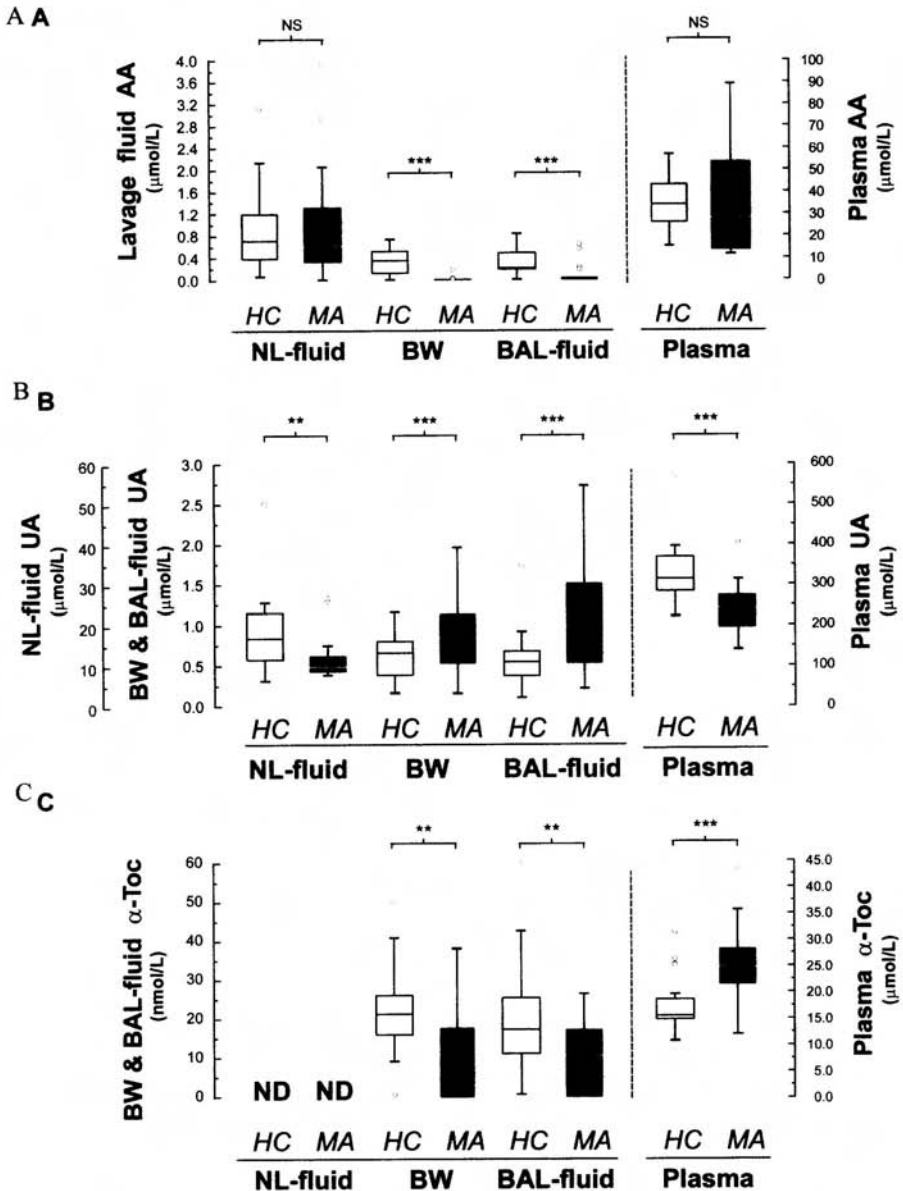


Figure 3. Comparison of the antioxidant concentrations (AA: ascorbate, UA: urate and α -Toc: alpha-tocopherol) in differential lavage fluid obtained from mild asthmatic and healthy control subjects. HC=healthy controls (n = 20), MA=mild asthmatics (n = 20), NL: nasal lavage, BW: bronchial wash, BAL: bronchoalveolar lavage. Data represented as medians, with interquartile and full ranges. Comparison of concentrations was performed using Wilcoxon's-Signed-Rank-Test. *p < 0.05, **p < 0.01, ***p < 0.005. Plasma concentrations are illustrated for comparison. Based on data published by Kelly et al., 1999 [41].

Patients with CF are particular at risk from oxidative lung injury. They have the combined problem of acquiring sufficient fat-soluble dietary antioxidants such as vitamin E as well as experiencing regular respiratory infections. Infection of the lungs by bacteria such as *P. aeruginosa*, causes an extremely vigorous inflammatory response with massive neutrophil infiltration of the airways [45]. Unfortunately, not only does the inflammatory response often fail to eradicate the inciting organism [46] but, as a growing body of evidence suggests, it also contributes to a local defect in the host defence that interferes with eradication of the infection [47-49]. Thus, a vicious cycle of infection and inflammation is established. Normal homeostatic regulatory mechanisms fail to break the cycle and sustained production of inflammatory mediators continues the recruitment of additional inflammatory cells whose products cause bronchospasm, increased secretions and other changes that exacerbate the underlying pulmonary abnormalities and lead to further deterioration in lung function [50].

In addition to the increased oxidative burden arising from the immune response to pulmonary infection in cystic fibrosis patients, there may also be an intracellular source of heightened free radical generation: namely increased leakage from the electron transport chain in mitochondria (Table 2). Feigl and Shapiro [51] have shown that Ca^{2+} uptake and oxygen consumption is increased in cystic fibrosis fibroblasts. They also showed that oxygen uptake was completely inhibited by cyanide, indicating that it was mitochondrial based. They used this data to hypothesise that the electron transport system in cystic fibrosis patients was more active than in controls. Von Ruecker et al. [52] expanded on this work and showed that the specific electron transfer activities of various enzymes of redox components of the respiratory chain, reduced nicotinamide adenine dinucleotide (NADH) oxidase, NADH cytochrome c reductase, and succinate cytochrome c reductase, were significantly elevated in cystic fibrosis.

Table 2. Strategies for elevating the antioxidant status of the lung.

Aim	Method of achievement
Elevated SOD activity	Administer free enzyme Administer enzyme-PEG conjugate Administer in liposomes Induce by drug treatment
Elevated CAT activity	Administer free enzyme Administer enzyme-PEG conjugate Administer in liposomes Induce by drug treatment
Elevated GSH concentration	Administer GSH Administer GSH ester Administer N-acetylcysteine Administer cystearine

The implication of these findings is that various components of the electron transport chain can "leak" electrons and act as sources of partially reduced oxygen intermediates [53]. Thus, increased oxygen consumption by cells from cystic fibrosis patients and the consequent increased activity of the electron transport chain will be potentially damaging through an increase in the intracellular generation of oxygen free radicals. For example, significantly elevated ($p < 0.01$) concentrations of 8-hydroxydeoxyguanosine are present in the urine of cystic fibrosis patients compared with age-matched controls [54]. The presence of this marker substantiate data that indicate that intracellular oxidative metabolism is increased in tissues of cystic fibrosis patients.

A further source of increased intracellular free radical generation in cystic fibrosis patients may be heightened P_{450} activity (Table 2). A study of the metabolism of

theophylline [55] in cystic fibrosis patients concluded that heightened free radical production could be a reflection of enhanced P_{450} activity, since oxygen free radicals play an important role in oxidative detoxification reactions. These reports receive further support from a study by Matkovics et al. [56] showing that the activities of intracellular antioxidant enzymes in plasma erythrocytes were elevated above control values, possibly due to "priming" by increased exposure to reactive oxygen species.

Numerous reports of elevated concentrations of lipid [57,58], protein [57,58] and DNA [54] oxidation products in CF patients have now been published. Importantly, oxidative stress is not present in all CF patients at all times. Oxidative stress, like the recurring infections, is probably cyclic. Importantly, antioxidant status tends to decrease with age in CF [58], hence older CF patients are particularly susceptible to renewed cycles of pulmonary inflammation. It is tempting to speculate that it is this oxidant/antioxidant imbalance that is responsible, in part, for their decline in lung function with advancing age. The reason for the fall in antioxidant status in CF is not clear, however decreased compliance in taking vitamin supplements may play a role. Alternatively, it is conceivable those repeated cycles of pulmonary inflammation, and associated oxidative stress, also contributes to the decline in antioxidant status. Whatever the exact cause, it is probable that the worsening antioxidant status of the CF adolescent contributes to their deteriorating clinical circumstances.

9. The common cold

The common cold is the most frequent cause for people having to visit the doctor. Adults succumb to 2-4 colds per year while children can develop as many as 8 colds each year. As a consequence of the large numbers of cold episodes, time lost from the workplace and/or school each year is enormous. Obviously this has considerable implications for the individual, but the employer and the national economy is also affected. Despite considerable research into the problem, there is currently no treatment to prevent, or cure, the common cold.

Colds are due primarily to viral infection of the upper respiratory tract. Rhinoviruses are by far the most prevalent type of viruses involved in colds. One of the most distinguishing features of human rhinoviruses is that there are over 100 variations. This large number of different rhinoviruses is partly responsible for individuals being susceptible to several colds each year. Neutrophil recruitment in particular is a major feature in the pathogenesis of the common cold [59]. Indeed, there is a significant correlation between the number of neutrophils recovered by nasal lavage and the severity of symptoms [60]. This host response tries to clear the rhinovirus infection through neutrophil-generated ROS production. ROS have the ability to oxidise many of the rhinovirus's key proteins and lipids constituents. The mechanism, although not instantaneous is, because of its powerful localised approach, usually effective.

During the past 30 years numerous studies have assessed the potential role of vitamin C in the treatment or prevention of the common cold. During this time, articles have been published which both support [61-64], and refute [65-68] any positive benefit for vitamin C in the common cold. These differences arise mainly from the fact that investigators have employed different study protocols, which makes inter-study comparisons difficult. Moreover, the use of vitamin C had become an emotive issue and this has led to the publication of a number of articles with considerable bias (both for and against) its use as a treatment for the common cold. However, careful retrospective analysis of these data indicates that supplementation with gram amounts of vitamin C may indeed decrease the severity of cold symptoms, although the incidence of infection appears to be changed little.

Given that most individuals receive a reasonable amount of vitamin C from their diet, it is likely that the greatest benefit of vitamin C supplementation for treatment of the common cold will be seen in specific groups such as those with low dietary intakes of vitamin C. This will include children, young men, the elderly and in those undertaking heavy physical exercise.

10. Strategies for therapeutic intervention

In its simplest form, oxidative stress is a state of excess oxidants and/or deficient antioxidant defenses. Strategies for decreasing the oxidative burden depend largely on the source of the oxidant stress. In the case of the oxidant burden arising from an external source (such as ozone, or cigarette smoke) then strategies include reducing the source at origin (expensive in the case of air pollution) or reducing the inspiration of the oxidant gas (stop smoking!). If the oxidant burden is due more to secondary sources such as activated inflammatory cells, therapeutic strategies include pharmacological approaches to reduce inflammatory cell recruitment and/or cell activation.

Strategies to improve antioxidant defenses first require information regarding which particular antioxidant(s) are lacking or present in insufficient quantities. For example, deficiency of glutathione in the lower respiratory tract as has been reported for HIV and CF may be approached by treated with N-acetylcysteine (NAC). NAC reacts rapidly with hydroxyl radicals and hypochlorous acid and has been demonstrated to protect against breathing pure oxygen [69]. Others have demonstrated that Ebselen, which contains selenium, is beneficial in experimental alveolitis and broncholitis [70]. In addition to these a range of synthetic antioxidants are being reviewed for therapeutic potential. Many of these mimic natural antioxidants for example by scavenging superoxide or hydrogen peroxide. Several pharmaceutical companies are now engaged in antioxidant trials and the results are eagerly awaited. Given the clear evidence of ROS involvement in a range of respiratory disorders and the accessibility of the lung it seems likely that antioxidant therapy will be part of a range of future treatments.

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Proper Design of Human Intervention Studies, Power Calculations

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1. Introduction

Several elements are mandatory for a trial, particularly a trial on humans, to adhere to modern scientific demands. The basic elements are an a priori defined primary hypothesis and definition of primary and secondary endpoint and in some cases also tertiary endpoints. Once this is defined the design of the trial, the statistical analysis and the control group can be defined.

2. Designs

Only two major design types will be dealt with here, cross-over and parallel groups designs, also called paired and un-paired designs.

2.1 Historical controls

A large number of studies use comparison of people before and after, say e.g. antioxidant intervention. Such a design is considered based on historical controls and should not be performed.

2.2 Cross over designs

Rather, the persons should be randomised to two different treatments, placebo and active treatment one after the other, with a wash-out period between, the randomisation gives the random order of the treatments. By such a design effects e.g. due to season is randomly allocated to the groups. The advantage of the paired design is that each person serves as his own control, and the number of subjects in the trial is reduced compared with the un-paired trial. Among the disadvantages are that every time a person drops out the first measurement he/she cannot be included in the analysis. Furthermore, if the variation within individuals is comparable with that between individuals, extra power is not obtained.

2.3 Parallel groups

The parallel group is a more simple design. A group of people is randomised to two treatments, e.g. active treatment and placebo or two different active treatments, and the primary variable is then compared between the groups.

More complicated designs can be used but is not mentioned here. Most important is to stress the proper use of randomisation and controls.

2.4 Power analysis

In the planning of a trial it is necessary to calculate the number of persons needed to be able to detect a predefined difference. In many countries, e.g. in Denmark, ethical approval is not given if a proper statistical power analysis is not given.

The power analysis is a calculation of the number of people to enter the trial, provided there is knowledge about the defined type I error risk (significance level), the type II error risk (power), the defined difference the trial is supposed to detect (Δ) and the variation of the measurement in the trial. A simple mathematical relationship between these factors exists. For details readers should look in statistical textbooks. Also electronic books are available on the net, e.g. http://www.graphpad.com/articles/interpret/principles/stat_principles.htm.

The calculation of number of persons needed in a trial can be done by several statistical programs, such as nQuery® and Statistica®. Also there are websites where calculations can be made. For simple designs it is very easy to make this calculation by hand or a simple electronic calculator. For the most common design two parallel groups, e.g. one active treatment and one placebo group, and assuming that both group are of equal size the number in each group is calculated as:

$$N1 = N2 = 2(t_{2\alpha,df} + t_{\beta,df})^2 \times (SD^2 / \text{MIREDF}^2)$$

where the t-values can be obtained from a statistical t-table, SD is the standard variation of the measurement measuring on e.g. a control population, and MIREDF is the Minimum RElevant DIFference.

If the $N1 = N2$ is large i.e. about 200 the t-values are about 2 and 1.7. This simplifies the equation to

$$N1 = N2 = 2(2+1.7)^2 \times (SD^2 / \text{MIREDF}^2) = 27.4 \times (SD^2 / \text{MIREDF}^2) \cong 30 \times (SD^2 / \text{MIREDF}^2)$$

As an example you want to find a change of 8% in the excretion of 8-oxodG in a group given antioxidants compared with a placebo group and you know that the SD of the urinary 8-oxodG is 32%. The number needed in each group is therefore about $30 \times (32/8)^2 = 30 \times 4^2 = 30 \times 16 = 480$ (note that SD and MIREDF are given in percent). It is quite straight forward that the most important factor in the power calculation is the SD of the measurement, and that the best way to reduce the number needed for the trial is to reduce SD.

2.5 Conclusion

Power calculation is thus a simple task to perform and should be included in the planning of all biological experiments.

References

Readers are referred to standard textbook in Statistics or similar items available on the net:

- [1] <http://www.ebook.stat.ucla.edu/calculators/powercalc/>
- [2] <http://www.davidmlane.com/hyperstat/power.html/>

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