ACUTE CEREBRAL ISCHEMIA AND OXIDATIVE STRESS

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ABSTRACT

Oxidative stress has been involved in the pathogenesis of several neurological diseases including acute stroke. The paper describes the main features of the oxygen and nitrogen reactive species, their role in damage caused by stroke, and briefly indicates the effects of antioxidants given in acute brain lesions.
Key words: free radicals, acute stroke, antioxidants

Selected abbreviations: ATP – adenosine triphosphate; BH_4 – tetrahydrobiopterin; cAMP – cyclic adenosine monophosphate; cGMP – cyclic guanosine monophosphate; CSF – cerebrospinal fluid; GPx – glutathione peroxidases; GSH – glutathione; GSSG – glutathione disulfide; HDL – high density lipoproteins; LA – lipoic acid; LDL – low density lipoproteins; MAPK – mitogen activated proteinkinase; MDA – malondialdehyde; NADH – reduced nicotinamide adenine dinucleotide; NADPH – reduced nicotinamide adenine dinucleotide phosphate; NF-$_k$B – nuclear transcription factor; NOS – nitric oxide synthase; ROS – reactive oxygen species; RNS – reactive nitrogen species; SO – superoxide; SOD – superoxide dismutase; VLDL – very low density lipoproteins;

1. INTRODUCTION

The presence of free radicals in biological materials has been demonstrated for over 50 years ago (1). Initially they were viewed as noxious evils, that may account for gross cellular damage, mutagenesis, cancer, and the degenerative process of biological aging, but as the research of free radicals progressed their physiological roles in the biological systems were pointed out one by one (2). Now there is a large body of evidence showing that „living organisms have not only adapted to an unfriendly coexistence with free radicals but have, in fact, developed mechanisms for the advantageous use of free radicals" (1).

On the other hand, researches provided suggestive evidence for oxidative stress being implicated in initiating apoptosis and accelerating the tissue damage in several neurodegenerative diseases as well as in ischemic stroke (3).

A free radical is any chemical compound (atom, molecule, or group of molecules) that contains one or more unpaired electrons in its outer orbits. The magnetic field generated by the spin of the unpaired electron(s) not being compensated by the opposite spin of paired electron(s) makes the radical more reactive than the corresponding nonradical because it acts as an electron acceptor and essentially „steals" electrons from other molecules (4).

The free radicals produced by the human body are mainly reactive oxygen species and reactive nitrogen species.

2. REACTIVE OXYGEN SPECIES

The oxygen molecule is essential to aerobiosis being the final electron acceptor in the mitochondrial respiratory chain and in the synthesis of ATP through oxidative phosphorylation. However, a strong case can be made for molecular oxygen in the air we breathe being the most dangerous toxin and carcinogen in the environment because of its propensity to generate free radicals (5). It has been demonstrated that approximately 2% to 5 % of the electron flow in isolated brain mitochondria produces superoxide anion radicals and hydrogen peroxide (4, 6).

Molecular oxygen has an unusual bonding arrangement, with two unpaired electrons occupying separate orbitals, which implies that it preferentially exists as two free radicals rather than having all of the electrons paired. As a consequence, oxygen can accept only one electron sequentially from biological molecules at a time. This prevents oxygen from rapidly oxidizing the biological

materials because these have filled orbitals containing 2 electrons of opposite spin and giving up only one electron is energetically highly unfavorable (5).

Mitochondrial oxidative phosphorylation (ox-phos) is the major ATP synthetic pathway in eukaryotes. In this process, electrons liberated from reducing substrates are delivered to O_2 via a chain of respiratory H^+ pumps. These pumps (complexes I-IV) establish a H^+ gradient across the inner mitochondrial membrane, and the electrochemical energy of this gradient is then used to drive ATP synthesis by complex V (ATP synthase). Chemically, the stepwise reduction of O_2 proceeds via several reactive oxygen species (ROS). These ROS can damage cellular components such as proteins, lipids, and DNA, but recent evidence also highlights a specific role in redox cell signaling for mitochondrial ROS. In the fine balancing act of aerobic metabolism, mitochondrial ox-phos accomplishes the reduction of O_2 to H_2O while maximizing ATP synthesis and maintaining ROS production to only the amounts required for microdomain cell signaling.

2.1. Dysfunctional mitochondria exposed to oxygen in the penumbral area of the infarct generate higher amounts of ROS than can be scavenged by the antioxidant systems, leading to oxidative stress. The main source of **superoxide ($O_2^{-\cdot}$)** in mitochondria is the ubisemiquinone radical intermediate ($QH\cdot$), formed during the Q cycle at the Q_O site of complex III. Complex I is also a source of ROS, although the mechanism of generation is less clear than for complex III. In vitro, electrons entering at complex II (succinate dehydrogenase) can flow backward through complex I to make ROS. In vivo, this would be prevented by forward electron flow through complex I from NADH, except under pathological conditions in which NADH is depleted. Furthermore, it has been shown that ROS can stimulate mitochondrial uncoupling and that the processes of uncoupling and ROS generation exist in a feedback loop.

Alternatively, superoxide generation may be facilitated by (1, 8):

- the breakdown of adenine nucleotides during ischemia, leading to accumulation of hypoxanthine, which is then metabolized by xanthine oxidase (XO)
- oxidative metabolism of accumulated arachidonic acid via the ciclooxygenase pathway or via the lipooxygenase pathway
- reduction of oxygen, mediated by a variety of enxymes in the body, including reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and xanthine oxidases
- accumulation and autooxidation of mono-amines
- autooxidation of hemoglobin and myoglobin

Superoxide traverses the cell membrane via the anion channel and exits into the extracellular space to exert remote effects (8). It was believed initially that the toxicity ascribed to the superoxide radical was caused by superoxide's direct interaction with biological targets. However, at neutral pH superoxide is not a strong oxidant because it is negatively charged. Since oxidation would require withdrawing an electron from another molecule, the overall reaction would involve transferring a negative charge to a small molecule that is already negatively charged.

2.2. Superoxide has moderate oxidizing effects on biological molecules, but undergoes a spontaneous reaction with itself, a dismutation reaction that yields one molecule each of $H_2 O_2$ (hydrogen peroxide) and oxygen in a relatively slow reaction:

$$\mathbf{O_2^{-\cdot} + H^+ \rightarrow HO_2^{-\cdot}}$$

$$\mathbf{HO_2^{-\cdot} + O_2^{-\cdot} + H^+ \rightarrow H_2O_2 + O_2}$$

$$\mathbf{= 2\ O_2^{-\cdot} + 2\ H^+ \rightarrow H_2O_2 + O_2}$$

At physiological concentrations of superoxide, the self-reaction slows down considerably and the lifetime of the free radical extends to many seconds. Consequently, nature has evolved a class of superoxide dismutase enzymes to remove this deleterious byproduct of oxygen metabolism.

Other sources of hydrogen peroxide are:

- Oxidative deamination, mediated by the L- and D-aminoacid oxidases of the neutrophiles
- Beta-oxidation of fatty acids
- Oxidation of ascorbic acid to dehydro-ascorbic acid, a reaction catalyzed by ascorbic oxidase
- Glycolytic reactions, catalyzed by NADH oxidase

Hydrogen peroxide is a lipid-soluble molecule which diffuses easily across cell membranes and exerts remote effects (8): peroxidation of the membrane cell lipids, induction of DNA damage, oxidation of cellular enzymes. Defense mechanisms against the deleterious effects of hydrogen peroxide include the activities of catalase (CAT) and myeoperoxidases.

2.3. In the presence of trace amounts of metal ions (ferrous or cuprous ions), hydrogen peroxide

generates **hydroxyl radicals** ($OH^{\cdot}$) in the Fenton reaction, which takes place in the absence of superoxide, or in the Haber-Weiss reaction, in which superoxide serves to reduce the transition metal:

Fenton reaction:

$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$

Haber – Weiss reaction:

$O_2^{-\cdot} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$

$H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^- + Fe^{3+}$

$= O_2^{-\cdot} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^-$

Alternatively, hydroxyl radicals can be generated from superoxide reacting with nitric oxide to form peroxynitrite ($ONOO^-$), which, in turn, will produce peroxynitrous acid (ONOOH) which will decompose spontaneously to produce $OH^{\cdot}$.

Of the ROS, hydroxyl radicals are the most reactive ones in spite of their very short lifetime (10^{-9} s) and are capable of damaging lipids, DNA, and oxidize proteins (9). Being extremely reactive, hydroxyl radicals do not survive beyond a few molecular diameters from their site of production, therefore their actions are likely limited to the site of production, but they may be able to initiate chain reactions that would extend their range of action beyond the narrow limits predicted from its short life span (8).

The defense mechanisms against the damaging actions of hydroxyl radicals include (4):

- Ceruloplasmin chelates iron molecules and transforms Fe^{2+} into Fe^{3+}, thereby diminishing the rate of $HO^{\cdot}$ formation in the Haber-Weiss reaction
- Metalothioneines are cysteine-rich proteins that fixate trace metals (Co, Zn, Cd, Cu, Fe), thereby impending the hydroxyl radical-producing reactions; unfortunately, their tissue and blood concentrations are very low
- Plasma albumin
- Plasma glucose
- Ascorbic acid, its action being potentiated by flavonoids

2.4. Other ROS are the perhydroxyl radical ($HO_2^{\cdot}$), hypochlorous acid (HOCl), singlet oxygen, as well as the peroxyl ($RO_2^{\cdot}$) and alcoxyl ($RO^{\cdot}$) radicals.

From the viewpoint of patients recovering from stroke, oxidative stress is generally bad because it amplifies tissue injury. However, there has been strong evolutionary pressure for cells to produce oxygen radicals as antimicrobial defenses, even at the expense of suffering greater collateral damage in neurological diseases occurring well past the age of reproduction (5).

3. REACTIVE NITROGEN SPECIES

The air we breathe contains approximately 78% nitrogen. (4). The reactive nitrogen species yet described are radical (nitric oxide – $NO^{\cdot}$, the nitronium ion – NO_2^-), or nonradical molecules (nitrous acid – HNO_2, peroxynitrous acid – ONOOH, nitrite – NO_2^-, or nitrate – NO_3^- anions).

3.1. Nitric oxide (NO) was initially described by Furchgott and Zawadzki (cited by 4) in 1980 as an endothelium-derived vascular relaxing factor. It is a biologically active molecule, interfering with vascular tone regulation, immunity, and neuronal signal transduction. Nitric oxide is synthesized from L-arginine by a family of nitric oxide synthases (NOS) in endothelial cells, neutrophils, macrophages, fibroblasts, thrombocytes, and neurons. Several NOS isoenzymes have been described: nNOS, the neuronal constitutive enzyme, the endothelial isoenzyme (eNOS), and the inducible one (iNOS). NO may react with other molecules, like superoxide or oxygen, leading to the generation of peroxynitrite and other nitrogen radical species

($NO^{\cdot} + O_2^{-\cdot} \rightarrow ONOO^-$;

$4\ NO + O_2 + 2\ H_2O \rightarrow 4\ NO_2^- + 4\ H^+$).

3.2. Peroxynitrite anion $ONOO^-$, generated from the reaction of nitric oxide with superoxide, is a far stronger oxidant and much more toxic than either nitric oxide or superoxide acting separately. One can consider peroxynitrite to be a binary weapon assembled from two less reactive intermediates. It catalyzes the peroxidation of cell membrane lipids, the oxidation of DNA bases, may react with metals or metalloproteins (like SOD), nitrates biological molecules, and exhibits bactericidal properties. At neutral pH a substantial fraction is protonated, the resulting peroxynitrous acid being even more reactive, with 30 % decomposing to form hydroxyl radical and nitrogen dioxide ($NO_2^{\cdot}$) (5).

3.3. Peroxynitrous acid ONOOH is a strong cytotoxic agent formed extracellulary from the following reactions:

$NO^{\cdot} + O_2^{-\cdot} \rightarrow ONOO^-$

$ONOO^- + H^+ \rightarrow ONOOH$

As already mentioned, it may lead to the generation of $OH\cdot$ and $NO_2\cdot$ in tissues.

4. THE PHYSIOLOGICAL FUNCTIONS OF FREE RADICALS

Nitric oxide ($NO\cdot$) and superoxide ($O_2^{-}\cdot$) have important roles in the physiological control of biological functions and the relatively large number of NAD(P)H oxidase isoforms and NOS indicates that nature has „learned" to use free radicals to her advantage in several biological processes (1).

ROS regulate oxygen homeostasis and ventilation as well as erythropoietin production (1). The adherence of leukocytes to endothelial cells is also induced by ROS, an effect abolished by catalase but not by superoxide dismutase, suggesting that hydrogen peroxide and not superoxide is the effective agent (1, 10). Moreover, ROS activate the T lymphocytes and modulate the functions of macrophages, thereby contributing to the amplification of immune responses (1). A particular situation is the regulation of vascular tone by cGMP, because guanilate cyclase is activated both by NO and by superoxide or hydrogen peroxide (1, 2, 11). ROS also have well-described roles in receptor-mediated signaling pathways, like the nerve growth factor (NGF) signaling in neuronal cells. Stimulation by this growth factor results in a transient increase in intracellular ROS through the signaling protein Rac 1. Elimination of hydrogen peroxide by catalase was shown to inhibit NGF-induced tyrosine phosphorylation of the growth factor receptor itself (1). Moreover, ROS cause an oxidative inhibition of protein tyrosine phosphatases and increase the phosphorylation of receptor tyrosine kinases (12), regulate the insulin receptor kinase activity (1), activate the cytoplasmic protein kinases (13), the MAPK cascades (14, 15), as well as several transcription factors (16, 17), and contribute to the increase in citosolic calcium (1).

Many of the biological actions of nitric oxide are mediated through the guanyl cyclase/cyclic GMP (cGMP) system, although some actions are cGMP-independent (18). $NO\cdot$ is a small lipophilic molecule that diffuses to adjacent cells. At the low concentrations of nitric oxide produced for signal transduction, the molecule is rapidly removed by diffusing into blood vessels where it reacts with hemoglobin to form methemoglobin plus nitrates (5). It can readily enter the cytosol, where it activates soluble guanyl cyclase by binding to the iron on its heme component, thereby moving the iron out of the plane of the porphyrin ring. Increased levels of cGMP trigger a reduction of calcium concentration by enhancing calcium extrusion and its sequestration into intracellular stores. The decrease in intracellular calcium concentration is responsible for the NO-mediated relaxation of smooth muscle cells, inhibition of platelet adherence and aggregation, and inhibition of neutrophil chemotaxis (1, 18, 19). Also, cGMP modulates the function of protein kinases, phosphodiesterases, and ion channels (20), as well as the neuronal transmission (5). RNS are involved also in the iron homeostasis by inhibition of aconitase (1), and in the activation of MAPK cascades (21).

5. THE ANTIOXIDANT SYSTEMS

Living cells and tissues have several mechanisms for reestablishing the original redox state after a temporary exposure to increased ROS or RNS concentrations. The production of NO, for example, is subject to direct feed back inhibition of NOS by NO. Similarly, elevated ROS concentrations induce in many cells the expression of genes whose products exhibit antioxidative activity (1). Halliwell and Gutteridge (cited by 1) have defined antioxidants as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates.

Antioxidants are exogenous (natural or synthetic) or endogenous compounds acting in several ways including scavenging reactive oxygen species or their precursors, inhibiting ROS formation and binding to metal ions needed for catalysis of ROS generation (22). The natural antioxidant system can be classified into two major groups (22): enzymes and low molecular weight antioxidants.

5.1. Antioxidant enzymes

A. The **superoxide dismutases (SODs)** were the first antioxidant enzymes discovered (23). They dismutate superoxide to generate hydrogen peroxide, a more stable and less toxic compound. There are three major endogenous superoxide dismutases:

– SOD 1, or Cu/Zn-SOD, is principally found in the cytosolic fractions in concentrations up to 10 μM (0.5 % of the total neuronal and hepatocytic proteins), but also in lysosomal fractions as well as in the mitochondrial intermembranar space (5, 9, 24, 25). It is a 32,500 Da homodimer coded by a

gene on chromosome 21, with each subunit containing one atom of copper which contributes to the catalytic process, and one atom of zinc, which confers stability to the molecule. The catalytic mechanism of scavenging of superoxide by Cu/Zn SOD is remarkably simple: Copper normally exists in its oxidized or cupric state (Cu^{2+}). Superoxide is attracted to the active site of SOD containing the copper, and an electron is rapidly transferred from SO to copper. This generates oxygen and leaves copper in its cuprous (Cu^{1+}) state. When a second superoxide encounters the enzyme, the electron is transferred from Cu^{1+} to the superoxide to produce hydrogen peroxide and regenerates the SOD enzyme in the cupric state. The zinc atom plays an important role in the second half of the dismutation cycle. In the oxidized state, the copper is bound to a total of four histidines, including a bridging histidine that spans the copper and the zinc. When copper becomes reduced, the bridging ligand bound to the zinc detaches from the copper and becomes protonated. The strong positive charge density on the zinc atom makes this a strong Lewis acid. This helps facilitate the second step of the reaction, where an electron needs to be transferred onto a negatively charged superoxide. The proton can be transferred to the superoxide to form its conjugated acid, $HOO\cdot$. This intermediate is a powerful oxidant and will rapidly oxidize Cu^{1+} to regenerate Cu^{2+} while producing hydrogen peroxide. The loss of zinc greatly changes the properties of SOD and makes it toxic to neurons (5).

– SOD 2, or MnSod, is found in the mitochondrial matrix. It is a 80,000 Da homotetramer, coded by the SOD-2 gene on chromosome 4, which contains manganes in its active site (9, 26), and represents the first-line defense against intramitochondrially produced ROS (27, 28). MnSOD expression can be induced by $O_2^{\cdot -}$, H_2O_2, TNF-α, IL-1, or γ- interferon (29).

– SOD 3, or EC-SOD, is a tetrameric protein secreted into the extracellular compartment, where it dismutates superoxide anions produced by membrane NAD(P)H oxidase or secreted by inflammatory cells (24, 30).

The reaction catalyzed by SOD is very rapid and efficient, at rates exceeding 10^9, dismutating the radical faster than superoxide can react with other potential biological targets (4, 18).

B. Catalase (CAT) is a tetrameric enzyme, each unit containing a hem and a NADPH molecule, localized mainly in peroxysomes, where it prevents hydroxyl radical generation by dismutating hydrogen peroxide. It is especially abundant in hepatocytes and erythrocytes, being less well represented in the central nervous system (4, 24).

C. The **glutathione peroxidases (GPx)** are a group of cytosolic enzymes that reduce hydrogen peroxide by using glutathione (31). To date, 5 isoenzymes have been identified: four of them contain seleno-cysteines, and one is selenium-independent (32). In the human brain, GPx localize mainly in glial cells. Cerebral glutathione peroxidase activity is sevenfold greater than that of catalase, indicating the importance of the enzyme in the antioxidant defense of the brain (24).

D. Other antioxidant systems that use glutathione are the **glutathione transferases**, which exhibit peroxidase-like activity for the organic peroxides but not for hydrogen peroxide itself (33), and **glutathione reductase**, an enzyme that reduces oxidized glutathione. Glutathione is a tripeptid with important antioxidant functions. Oxidation of the cysteine sulfhydryl groups joins two glutathione molecules (GSH) with a disulfide bridge to form glutathione disulfide (GSSG). NADPH-dependent glutathione reductase catalyzes recovery of glutathione. Normally, the brain maintains a high ratio of GSH/GSSG for antioxidant defense (24).

E. The **thioredoxines** are a group of proteins able to reduce several organic molecules.

5.2. Non-enzimatic antioxidants

Non-enzimatic antioxidants can be found in the cell membranes (liposoluble molecules, like vitamin E, or the carotenoids) or in the cytoplasm (water-soluble molecules, like vitamin C, or glutathione).

5.2.1. The **antioxidant vitamins** are exogenous substances, derived mainly from dietary sources (22).

a) Vitamin E consists of a group of fat-soluble tocopherols (α, β, δ si γ) and tocotrienols (34) with an aliphatic chain that facilitates the incorporation of the molecule in the cell membranes. The most abundant and potent isomer is a-tocopherol. The plasma concentration of vitamin E is between 8 and 13 μg/mL, but the vitamin is unevenly distributed in the plasma lipoproteins: 65 % in LDL, 24 % in HDL and 8 % in VLDL lipoproteins (35). Vitamin E breaks the propagation of the free radical chain reaction in the lipids of biological membranes and

scavenges the alcoxyl- and peroxyl- radicals. In this process, it may be oxidized to form tocopheryl radicals, which may act as toxic prooxidants in some circumstances, and from which vitamin E will be regenerated under the influence of ascorbic acid. (35). Vitamin E deficiency also influences the activities of SOD, catalase, and glutathione peroxidase (22).

b) Carotenoids are also natural lipid-soluble antioxidants. Some carotenoids, like beta-carotene, serve as precursors of vitamin A (retinol), while others, like lycopen, zeaxanthine, or luteine, can amplify the immune response and exhibit mainly antitumoral effects (36). Because of the long polyunsaturated chain, they efficiently scavenge the peroxyl radicals and singlet oxygen (36, 37).

c) Vitamin C, or ascorbic acid, is an exclusively exogenous molecule derived from dietary sources. Thereby its plasmatic concentration varies widely, between 30 and 100 μM (or 5.45 mg/L and 18.18 mg/L), depending on the subject's diet as well as on the season (38). It inhibits peroxidation of membrane phosphorlipids and acts as a scavenger of free radicals. In vitro it has been shown to prevent the oxidation of low LDL lipoproteins; in vivo this effect is more difficult to demonstrate because vitamin C is inactivated while isolating the plasma LDLs (38). Still, ascorbic acid and ascorbate anion are considered to be the major plasma antioxidants which protect the cell membranes from oxidation because of the capacity of vitamin C to promote regeneration of vitamin E (22,39). Brain concentration of vitamin C is 10-fold higher than its plasma levels (40). This may indicate its potential role as a cerebroprotective agent (22), although in the presence of transition metals it may be oxidized to ascorbyl radicals which can exhibit prooxidant properties (38).

5.2.2. Coenzyme Q_{10}, or ubiquinone, is a lipid-soluble compound within the hydrophobic core of the phospholipids bilayer of the inner membrane of the mitochondria. It is an essential cofactor in the electron transport chain, where it accepts electrons from complexes 1 and 2 (41), but also serves as an important antioxidant in lipid membranes either directly or by regenerating vitamin E (22).

5.2.3. Glutathione, as already mentioned, plays a major role in the defense against lipi, protein, or nucleic acid oxidation. It has milimolar intracellular and micromolar plasma concentration (42). GSH has major intracellular antioxidant activity, mainly due to the thiol group within the molecule. It is an electron donor in the reaction catalyzed by glutathione peroxidase, but can also react non-enzymatically with free radicals like superoxide, nitric oxide, or hydroxyl radicals (32). Oxidation of the glutathione joins two molecules with a disulfide bridge to form glutathione disulfide (GSSG), while glutathione reductase catalyzes recovery of glutathione through a NADPH-consuming reaction. Since the pentose phosphate pathway appears to be the predominant source for regeneration of NADPH, ischemia, with its oxygen and glucose deprivation, interferes with the regeneration of glutathione (32).

5.2.4. Melatonin is an indoleamid secreted by the pineal gland, which has structural similarities to serotonin. It is highly lipophylic and can readily cross the blood-brain barrier and gain access to neurons and glial cells (22). There is experimental evidence that melatonin influences aging and age-related processes and disease states, a role which is related to its potency as a free radical scavenger (43). Although the neuroprotective effects of melatonin as an antioxidant in cerebral ischemia have been experimentally demonstrated (44), translating these results into clinical practice may be problematic due to its various physiological roles and multiple undesirable effects (22).

5.2.5. Alpha-lipoic acid (LA) is absorbed from the diet and is a sulphur-containing compound, similar to GSH, GSSG, S-methyl-glutathione, and dihydrolipoic acid (45). LA efficiently scavenges hydroxyl radicals, singlet oxygen, nitric oxide, chelates a number of transition metals, recycles other antioxidants (such as vitamin C and E), raises intracellular levels of glutathione, and modulates transcription factors activities, especially that of NF-$_k$B (22). Some researches show that LA is a very potent protector against ONOOH-mediated damage (45). The available experimental results strongly recommend alpha-lipoic acid to be investigated as an antioxidant in clinical ischemic stroke trials (46).

5.2.6. Uric acid is a waste product of the living cell, produced by xanthine oxidase, which contributes up to 60 % of the total plasma antioxidant activity in healthy subjects due to its wide distribution and relatively high concentration. It interacts with 10-15 % of the hydroxyl radicals produced daily and scavenges also both peroxyl

radicals and singlet oxygen (22). Moreover, it chelates iron ions (47) and stabilizes plasma ascorbate (48). In high concentrations, it may act as a pro-oxidant and hyperuricemia has been suggested to be a risk factor for oxidative stress-associated disorders (22).

5.2.7. Creatine is a tri-peptide produced from glycine, methionine, and arginine. Experimental findings have demonstrated that creatine provides significant neuroprotection against ischemic and oxidative insults, seemingly in relation to creatine-induced maintenance of mitochondrial bio-energetics (49). Mitochondrial membrane potential was significantly increased, intramitocondrial levels of ROS and calcium were significantly decreased, and ATP levels were maintained after creatine administration (22).

5.2.8. In addition, there are **other compounds** that have a relatively low specific antioxidant activity, but, when present at high concentrations, can contribute significantly to the overall ROS scavenging activity. The most prominent examples are free amino acids, peptides, and proteins. Of the amino acids, some (tryptophan, tyrosine, histidine, and cysteine) are particularly sensitive to ROS (1). Intact proteins have been shown to be less sensitive to oxidation than misfolded proteins (50). Protein oxidation and enhanced proteolytic degradation cause, therefore, an increase in the ROS scavenging capacity (1).

Free radicals and reactive nonradical species derived from radicals exist in biological cells and tissues at low but measurable concentrations, determined by the balance between their rates of production and their rates of clearing by various antioxidant compounds and enzymes (1). Oxidative stress has been defined as a disturbance in the pro-oxidant – antioxidant balance in favour of the former and has been implicated in the pathogenesis of neuronal damage (22, 26).

The nervous system is particularly vulnerable to oxidative stress and ROS mediated injury for a number of biochemical, physiological, and anatomical reasons (6, 32, 51):

- a high rate of oxidative metabolic activity;
- high concentration of oxidisable substrate, in particular membrane lipid polyunsaturated fatty acids
- large area of cell membranes compared to the cytoplasmic volume
- endogenous generation of ROS by specific neurochemical reactions, for example, dopamine oxidation
- low level of antioxidant enzymes, mainly catalase and glutathione peroxidase
- high levels of iron and ascorbate, and low concentrations of transferrin and ceruloplasmine

6. OXIDATIVE STRESS IN ACUTE CEREBRAL ISCHEMIA

Evidence has accumulated over the past two decades showing that reactive oxygen species are involved in brain injuries caused by cerebral ischemia. Reoxygenation during spontaneous or thrombolytic reperfusion provides oxygen as a substrate for numerous enzymatic reactions in the cytosolic compartments or subcellular organelles and mitochondria that will further increase oxidative stress and add to the cellular damage.

6.1. Potential mechanisms for radical production

6.1.1. Mitochondrial dysfunction

In normally functioning mitochondria, cytochrome *c* is involved in a four-electron transfer to reduce oxygen to water without production of oxygen radicals. During electron transport, the presence of oxygen at the terminus of the chain favors the maintenance of the members of the carrier system in an oxidized state. During ischemia, when oxygen supply is limited, the electron transport chain of the inner mitochondrial transport chain becomes highly reduced, a situation that favors oxygen radical production (52). It has been demonstrated that approximately 2 % to 5 % of the electron flow in isolated brain mitochondria produces superoxide anion radicals and hydrogen peroxide (9). Studies implicate the ubiquinone-cytochrome b region as the major site of oxygen radical production when mitochondria are in a maximally reduced state (52).

6.1.2. Free fatty acid metabolites

One of the postulated consequences of calcium influx during ischemia is in initiating pathways involved in breakdown of lipid membrane constituents and accumulation of free fatty acids. In an ATP-poor environment, calcium influx activates phospholipase C. Alternatively, ischemia, through the release of potassium, adenosine, and catecholamines, increases the concentration of cAMP, which, in turn, activates phospholipase A (53, 54). These enzymes catalyze the breakdown of lipid membranes, which leads to an increase in free fatty acids, mainly arachidonic acid, deleterious through several mechanisms:

– arachidonic acid may cause cerebral edema through direct inhibition of Na^+-K^+-adenosinetriphosphatase
– arachidonic acid may cause release of neuroransmitters from neurons and reduce their reuptake mechanisms (55).
– it readily intercalates into membranes and produces changes in packing of lipid molecules (56).

During recirculation, there is rapid utilization of free fatty acids, in particular arachidonic acid, which is metabolized via the lipooxygenase and cyclooxygenase pathways to produce prostaglandins, thromboxanes, and superoxide. The prostaglandins may be responsible for vascular sludging and altered vascular reactivity, while oxygen radicals may cause brain injury directly by lipid peroxidation and indirectly by vascular paralysis (57).

6.1.3. Purine metabolites

With the onset of ischemia, brain adenine nucleotides are metabolized to nucleosides and purine bases (51), so ischemia is associated with a rapid rise in interstitial concentration of adenosine and hypoxanthine. The increase in brain adenosine makes it available for further metabolism to inosine, hypoxanthine, and as a substrate for the xanthine oxidase pathway. Under normal circumstances, the whole brain has a low xanthine oxidase activity as compared to endothelial cells, where the enzymes activity is 20-25-fold higher (58). However, with ischemia and reperfusion there is an increase in the conversion of xanthine dehydrogenase to xanthine oxidase by calcium-activated proteases. Thus, the xanthine oxidase pathway may a principal source of free radicals mainly in the endothelial cells, in ischemic conditions and especially during reperfusion (51, 59).

6.1.4. Uncoupling of NOS

Under normal conditions, the NOS isoenzymes transfer electrons from a heme group in the oxygenase domain to the substrate L-arginine to form L-citrulline and nitric oxide; 5,6,7,8-tetrahydrobiopterin (BH_4) serves as a cofactor in this process (60). If the availability of either BH_4 or L-arginine decreases, eNOS switches from a coupled state, in which it generates NO, to an uncoupled state in which the electrons from the heme reduce oxygen to form superoxide (61). The superoxide anion reacts with nitric oxide to produce peroxynitrite, which impairs endothelium-dependent vascular relaxation (by reducing NO), and may also oxidize BH_4. This causes a deficiency of BH_4 and will further promote uncoupling of NOS (62). Furthermore, ischemia induces iNOS, an enzyme responsible for producing NOS in nanomolar concentration (as compared to the physiological picomolar ones). These high concentrations of NOS are cytostatic and cytotoxic for fungi, bacteria, protozoa, or tumoral cells, but are also cytotoxic for the neurons because they inhibit key mitochondrial enzymes like NADH-ubiquinon oxydoreductase, NADH-succinate oxydoreductase, and aconitase (18).

6.1.5. Polymorphonuclear leukocytes

Ischemia leads to activation of leukocytes trapped in the cerebral vasculature, which may result in release of chemotactic factors (e.g., leukotrienes) during reperfusion. Activated leukocytes may cause mechanical obstruction of capillaries and impair microvascular circulation, although the leukocyte accumulation is not necessarily the major cause of injury from ischemia and reperfusion (53). Likewise, leukocytes may interact with platelets to metabolize arachidonic acid and produce lipooxygenase and cyclooxygenase byproducts, including oxygen radicals (63).

6.1.6. The role of calcium in the production of free radicals

In cerebral (and more generally speaking, in any tissue) ischemia there is an increase in intracellular calcium that activates phospholipases and liberates free fatty acids, mainly arachidonic acid. During reperfusion, arachidonic acid can be metabolized by the lipooxygenase and cyclooxygenase pathways with the production of oxygen radicals and vasoconstricting prostanoids. Calcium may also play a central role in activation of the protease calpain, which cleaves a peptide bond in xanthine dehydrogenase to form xanthine oxidase, thereby contributing to the increased formation of free radicals (53).

6.2. Potential sites of radical-induced injury

Both parenchyma and vascular endothelium have potential pathways for radical production, and it is yet unclear which of these sites the main source of radical production is during ischemia and reperfusion (53). Experimentally, free radicals were produced in cultured endothelial cells mainly by the xanthine oxidase pathway and by the metabolism of prostanoids (64), while studies on brain

homogenates and mitochondrial preparations demonstrated the parenchymal radical production (52). However, accumulated evidence shows that in ischemia/reperfusion the endogenous antioxidative defenses are perturbed as a result of overproduction of free radicals by cytosolic prooxidant enzymes and mitochondria, inactivation of detoxification systems, consumption of antioxidants, and failure to adequately replenish antioxidants in ischemic brain tissue.

Within the brain, oxygen radicals impair capillary endothelial cell mechanisms that help maintain homeostasis of water and electrolytes, alter membrane fluidity characteristics and contribute to synaptic damage (53). The accumulation of conjugated dienes and thiobarbiturate-reactive material after cerebral ischemia followed by reperfusion suggests that once peroxydative reactions have begun, they are chain propagating in the presence of sufficient concentrations of oxygen. Lactic acidosis presumably increases the production of oxygen radicals because of its ability to dissociate iron-bound iron.

Of particular importance is the reaction of superoxide with nitric oxide to produce peroxynitrite. Both molecules have unpaired electrons and are highly reactive. Essentially, every collision between nitric oxide and superoxide results in the formation of peroxynitrite. Because small molecules can diffuse much more rapidly than large proteins, the reaction of nitric oxide is several times faster with superoxide than the scavenging of superoxide by SOD. However, the high intracellular concentrations of SOD greatly reduce the formation of $ONOO^-$ in vivo. Peroxynitrite is a far stronger oxidant and much more toxic than either nitric oxide or superoxide acting separately, and is also remarkably stable. It can react with metal ions bound to proteins, as well as carbon dioxide that catalyzes the addition of nitro (NO_2) groups to nitrate biological molecules like MnSOD, prostacycline synthase, or tyrosine (5, 18).

Free radical-mediated reactions can also cause structural alterations in DNA by oxidation, methyltion, depurination, and deamination reactions. The chemistry of DNA damage by several ROS has been well characterized in vitro, suggesting that different ROS affect DNA in different ways: hydrogen peroxide does not react with DNA bases at all, the hydroxyl radical generates a multiplicity of products from all four DNA bases, while superoxide selectively attacks guanine (18).

6.3. Evidence for the role of free radicals in the pathophysiology of acute cerebral ischemic lesions

A vast amount of circumstantial evidence implicates oxygen-derived free radicals and high-energy oxidants (such as peroxynitrite) as mediators of ischemic brain damage.

Human studies on stroke and oxidative stress in the brain are still scarce, mainly because of the methodological difficulties in measuring free radical production in the cerebral tissue. However, researches aimed at evaluating antioxidants in blood, urine, or cerebrospinal fluid revealed lower plasma vitamin C, E, vitamin A, and uric acid (65, 66), a decreased total plasma antioxidant capacity (67), as well as an increase in plasma concentrations of lipid peroxides, homocysteine, nitric oxide, and of malondialdehyde and other TBA-reactive molecules (66, 68, 69) at the onset of stroke. Although the pre-stroke antioxidant status of these patients is unknown, the significant inverse correlation of the plasma antioxidant capacity with the infarction volume and the degree of neurological impairment (67) suggests an excessive consumption of the antioxidants by the free radicals produced during the ischemic insult. The evaluation of oxidative damage and antioxidant status in time yielded divergent results. Some studies showed persistently elevated malondialdehyde concentrations 6 months after strokes caused by large vessel disease and microangiopathy, with no significant differences between the two stroke subtypes and regardless of the coexistence of risk factors like hypertension, diabetes mellitus, or dislipidemia (70). Others reported either no significant alterations of these parameters (68), or a progressive increase to normal levels of plasma antioxidants except for vitamin C, which remains at lower concentrations (65). Evaluation of the plasma enzymatic antioxidants showed also conflicting results: either no significant alterations of plasma SOD activity in stroke patients (66, 71, 72), or elevated SOD activity in plasma and CSF at stroke onset (73), with a gradual decline over 7 days. Elevations of glutathione and glutathione peroxidase were also demonstrated in acute stroke patients (71).

Evidence from genetic manipulation in rodents also demonstrates that transgenic mice overexpressing Cu/ZnSOD, MnSOD or ECSOD have a 35 % reduction in lesion size after focal ischemia (9, 24, 26-28, 74, 75), while SOD-knockout mice have increased vulnerability to ischemic brain lesions (9).

Similarly, glutathione peroxidase overexpression was proven to be neuroprotective (9, 76). As for NOS, the results differ depending on the NOS isoform manipulated. A 40 % reduction in lesion size was observed after permanent focal ischemia in nNOS knockout mice with even greater neuroprotection after transient ischemia, as opposed to a 20% increase in lesion volume after cerebral ischemia in eNOS knockout animals (9, 77) when compared to lesions caused by similar types of ischemia in wild-type animals.

6.4. Antioxidants in the treatment of acute cerebral ischemia

The ultimate goal for understanding the mechanism of oxidative stress in brain ischemia is to develop therapeutic interventions. To this end, many pharmacological antioxidants have been evaluated, but their clinical use is limited by bioavailability and undefined secondary effects (24). Promising results have been achieved with the following agents:

1. Ebselen is a seleno-organic compound that exhibits antioxidant activity through a glutathione peroxidase-like activity. It inhibits proteinkinase C, 5-lipooxygenase, cyclooxygenase, and NADPH-oxidase (22, 78). Administration of 10-30 mg/kg body weight was shown to reduce infarct size in a rodent model of focal cerebral ischemia, while 50 mg/kg ameliorated delayed vasospasm in a canine model of subarachnoid hemorrhage (22, 79, 80). Based on these encouraging results, ebselen entered the phase of clinical trials, and preliminary results indicate an improvement of ischemic stroke outcome in patients receiving 150-300 mg/day within 6 to 48 hours of stroke onset and continued for two weeks (81-83). Phase III clinical trials are ongoing (84).

2. Spin-trap scavenging agents are molecules (usually with a nitrone moiety) that have been used in electron paramagnetic studies for trapping highly reactive, unstable radicals (22). These compounds have been shown to protect experimental animals from ischemia-reperfusion injuries, physical trauma, oe even aging (85, 86). The first agent to enter trials was PBN (phenyl-alpha-tert-butyl-nitrone), a synthetic antioxidant capable of scavenging oxygen free radicals and that significantly reduced the magnitude of ischemia-reperfusion injuries in gerbils if given before inducing ischemia (87). Its disulfonyl derivative entered clinical trials as NXY-059, or Cerovive, and although it proved neuroprotective in a therapeutic 6 hour-window in the SAINT I trial (88), the results of the SAINT II trial reported in 2007 were rather disappointing (89).

3. Citicoline, by stabilizing the cell membrane and diminishing the range of the arachidonic acid cascade, diminishes oxidative stress. Administered within 24 hours of stroke onset, it has been shown to reduce the size of ischemic injuries by 33 % and improve recovery, being still investigated in phase III clinical trials (84, 90, 91).

Finally, it should be mentioned that some antioxidant properties have been described for drugs already recommended by guidelines to be used in the treatment of acute cerebral ischemia. These "vascular protective"drugs are the HMG-CoA reductase inhibitors, which activate the phosphatidyl-inositol-3-kinase pathway, potentiate eNOS activity, inhibit the NAD(P)H oxidases and superoxide generation, and diminish the production of free radicals (92), as well as the angiotensin converting enzyme inhibitors, which inhibit NAD (P)H oxidase (93) and increase the endothelial nitric oxide production (94).

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