Part I

Basic aspects of neurodegeneration

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Endogenous free radicals and antioxidants in the brain

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The role of oxygen free radicals and antioxidants in Central Nervous System (CNS) pathology is of major interest for many reasons. While accounting for only 2% of the body weight, the brain uses 20% of the oxygen consumed by the resting body. Polyunsaturated lipids and catecholamines prone to autoxidize are abundant, and since the neurons are postmitotic, efficient protection against oxidants is vital. Accordingly, changes in most antioxidant and prooxidant factors result in phenotypes in the brain, which is exemplified in this chapter. Evidence for attack by oxygen free radicals on lipids, proteins and DNA has also been found in virtually every type of brain disease and in aging. The oxidants also exert signalling effects, but that aspect will not be covered in this chapter.

Formation of oxygen free radicals

Molecular oxygen is chemically a biradical with two unpaired electrons with equal spins in two antibonding Π*2p orbitals. This has the consequence that oxygen primarily is reduced one step at a time, since reactions with paired electrons are "forbidden." Reduction of oxygen to water may, in principle, be described in steps:

$$O_2 + e^- \rightarrow O_2\cdot^-$$
$$O_2\cdot^- + e^- + 2H^+ \rightarrow H_2O_2$$
$$H_2O_2 + e^- + H^+ \rightarrow OH\cdot + H_2O$$
$$OH\cdot + e^- + H^+ \rightarrow H_2O$$

The results are the intermediates: the superoxide anion radical, hydrogen peroxide and the hydroxyl radical. Hydrogen peroxide is not a free radical but is still reactive, and the intermediates are together often termed reactive oxygen species (ROS). Sometimes peroxyl (LOO·) and alkoxyl (LO·) radicals, e.g. formed in lipid peroxidation (below) are included among the ROS. The term is commonly used, because often the actual damaging species in the biological system under study is not well defined.

The bulk of oxygen in the body is reduced in four steps to water in the mitochondrial respiratory chain, without release of intermediates. A few percent of the oxygen consumed is estimated to be reduced stepwise under formation of ROS. With the exception of a few oxidase-catalyzed reactions, the reduction primarily proceeds via formation of superoxide radical.

Superoxide anion radical

Mitochondria

The major source of superoxide radicals in the body is the reaction of oxygen with electrons leaking from the mitochondrial respiratory chain. The major sites of superoxide formation are apparently FeS clusters or semiquinones in complex I and ubisemiquinone in complex III. Studies with depletion of Mn-SOD suggests that complex III superoxide formation may be directed vs. both the inner matrix and the intermembrane space (Raha *et al.*, 2000). From the intermembrane space, the radicals may then enter the cytosol via the voltage-dependent anion channels (VDAC) (Han *et al.*, 2003). Increased mitochondrial superoxide production apparently plays important roles in a variety of pathologies including glutamate-induced excitotoxicity (Nicholls *et al.*, 1999), hyperglycemia (Du *et al.*, 2000), TNF action (Hennet, Richter & Peterhans, 1993), some mitochondrial diseases (McEachern *et al.*, 2000), and ischemia-reperfusion (Li & Jackson, 2002). (See also Chapter 3, Greenamyre and Chapter 62, Shapira.)

The NADPH oxidases

These are membrane-bound complexes that are major sources of superoxide radical in the body. Normally, the activity has useful purposes: in phagocytes for the defense against infections and in other cell types probably for signalling. However, activation of the oxidases may also exert toxic collateral effects. The catalytic subunit gp91phox and p22phox are membrane bound, while there are several cytosolic components that assemble with the membrane-bound components upon activation (Babior, Lambeth & Nauseef, 2002). There are at least five gp91phox homologs (NOX) (Bokoch & Knaus, 2003; Cheng *et al.*, 2001). The most extensively studied is the NOX2 of neutrophils, which also exists in macrophages/microglia. Oxidant production by activated microglia has been implicated in virtually every neurodegenerative disease, e.g. Gao *et al.* (2002). (See also Chapter 14, Pasinetti.) NOX isoforms are also expressed in vascular endothelial and smooth muscle cells (Sorescu *et al.*, 2002), and may be sources of superoxide in pathologies with vascular components, e.g. as induced by peptides derived from APP (Iadecola *et al.*, 1999). Furthermore, components of the NADPH oxidase complex have been demonstrated in cultured cortical neurons and astrocytes, and have been implicated in oxidant production induced by Zn (Noh & Koh, 2000), and neuronal apoptosis following NGF deprivation (Tammariello, Quinn & Estus, 2000).

Autoxidations

Autoxidations generally result in one-step reduction of oxygen to the superoxide radical. Multiple endogenous and exogenous drugs and toxins in the CNS are prone to autoxidation including dopamine, L-DOPA, adrenalin, noradrenalin, 6-OH-dopamine. Most reactions are relatively slow, but are promoted by transition metal ions. Such reactions are suspected to contribute to neurodegeneration, e.g. in Parkinson's disease (Jenner & Olanow, 1996). (See also Chapter 6, Przedborski.)

Xanthine oxidase

This is the final enzyme in the catabolism of purines in humans and catalyzes the stepwise formation of xanthine and urate from hypoxanthine under formation of both superoxide and hydrogen peroxide. Xanthine oxidase is proteolysed from its NADH-accepting form, e.g. following ischemia/reperfusion, which also will lead to increased formation of hypoxanthine. The enzyme has been demonstrated in the brain of some rodents (Patt *et al.*, 1988), but not with certainty in humans. The inhibitor allopurinol (Palmer *et al.*, 1993) or inhibition by pretreatment with tungsten (Patt *et al.*, 1988) has shown protective effects in some brain i/r models, for example, but many studies have failed to show effects (Nakashima *et al.*, 1999).

Nitric oxide synthase

Under suboptimal concentrations of arginine all nitric oxide synthase isoenzymes generate $O_2\cdot^-$ under enzymic cycling (Pou *et al.*, 1992). This side reaction is also influenced by the cofactor tetrahydrobiopterin, which increases the rate of oxygen reduction, but also leads to some direct formation of hydrogen peroxide (Rosen *et al.*, 2002).

Prostanoid metabolism

Both PGH synthase and lipoxygenases release in the presence of NAD(P)H superoxide radical as a byproduct (Kukreja *et al.*, 1986). Such formation has been shown to contribute to microvascular abnormalities in the brain, e.g. induced by hypertension (Kontos *et al.*, 1981).

CYP450

The drug-metabolizing cytochrome P450s leak electrons to oxygen to form the superoxide radical. The 2E1 isoform is particularly prone to form superoxide and occurs in some parts of the brain (Tindberg & Ingelman-Sundberg, 1996). It is induced by ethanol and some xenobiotics, IL-1β, and ischemia/reperfusion (Tindberg *et al.*, 1996). Whether the activities in the brain are sufficient to exert toxic effects is not known, but 2E1 is strongly implicated in, e.g. liver pathology (Cederbaum *et al.*, 2001).

RAGE

Advanced glycation end products react with a cell surface receptor (RAGE) that transduces proinflammatory signals including formation of oxygen radicals, possibly via activation of NADPH oxidases (Wautier *et al.*, 2001). The receptor exists on microglia, astrocytes and hippocampal neurons and has been implicated in cellular activation, e.g. by Aβ peptides in Alzheimer's disease (Lue *et al.*, 2001; Sasaki *et al.*, 2001).

Reactions of the superoxide radical

The superoxide radical reacts very rapidly with NO to form peroxynitrite. This reaction is of great importance for the physiology and pathology of both molecules. (See Chapter 2, Crow and Chapter 12, Dawson.) There is also a rapid reaction with ascorbate (Nishikimi, 1975) and a slower complex reaction and interaction with reduced glutathione (Winterbourn & Metodiewa, 1994). The superoxide radical has, however, a low reactivity with most other biomolecules. The most important cellular targets are

proteins with FeS clusters (Gardner & Fridovich, 1991), which occur particularly in mitochondria. The inactivation of the cytosolic and mitochondrial aconitases, which both contain FeS clusters, are useful markers for increased superoxide radical formation (Gardner & Fridovich, 1991; Huang *et al.*, 2002). Participation of superoxide radical in Haber Weiss chemistry may also be of importance, see below. The corresponding acid form, HOO·, is more reactive and may, e.g. initiate lipid peroxidation (Bielski, Arudi & Sutherland, 1983). The acid–base couple has a pK_a of 4.75, which is why such reactions will increase in importance with decreasing pH. It has been suggested that the superoxide radical can terminate lipid peroxidation chains and there is evidence for the existence of optimal superoxide concentrations in vivo (Nelson, Bose & McCord, 1994). The superoxide radical inhibits glutathione peroxidase (Blum & Fridovich, 1985) and catalase (Kono & Fridovich, 1982). On the other hand, the radical reactivates catalase inhibited by NO (Kim & Han, 2000). There are other in vivo effects of superoxide radical with more undefined mechanisms, but with a neurobiological interest. Thus superoxide activates mitochondrial uncoupling proteins (Echtay *et al.*, 2002), potentiates hippocampal synaptic transmission (Knapp & Klann, 2002) and inactivates a plasma membrane surface glutamate receptor-like complex (Agbas *et al.*, 2002).

Hydrogen peroxide

The major part of the hydrogen peroxide in the body is formed by superoxide dismutase-catalyzed or spontaneous dismutation of the superoxide radical. Some hydrogen peroxide is, however, formed directly by oxidases such as glycollate oxidase, urate oxidase (not in humans) and D-amino acid oxidase, which are localized to peroxisomes. Of particular importance in the CNS are the monoamine oxidases (MAO), situated in the outer membrane of mitochondria. MAO A primarily degrades serotonin and norepinephrine, and MAO B degrades phenylethylamine. Dopamine is metabolized by both (Shih, Chen & Ridd, 1999). Toxic effects may be caused by both the hydrogen peroxide formed upon the oxidative deamination of catecholamines as well as by some of the resulting metabolites. MAO effects have been suggested to contribute to, e.g. the loss of dopaminergic neurons in Parkinson's disease (Jenner & Olanow, 1996), where impairment of mitochondria may contribute (Gluck *et al.*, 2002).

Hydrogen peroxide generally has a low reactivity, but may oxidize some cysteines. Cysteine thiolate anions are readily oxidized to cysteine sulfenic acid, whereas (base) thiols are very resistant. The thiolate anions exist at physiological pH in cysteines with low pK_as caused by adjacent stabilizing positively charged amino acid residues. Protein tyrosine phosphatases can be inactivated by such cysteine oxidation, and this reaction is involved in some of the signaling effects of hydrogen peroxide (Meng, Fukada & Tonks, 2002). Another susceptible target is the active cysteine in glyceraldehyde-3-phosphate dehydrogenase (Brodie & Reed, 1987). This thiol is very susceptible overall for reactions with ROS. Most toxicity of hydrogen peroxide is caused apparently by the interaction with transition metal ions (below).

Hydroxyl radical

The hydroxyl radical reacts at close to diffusion limited rates with most biomolecules and is by far the most reactive of the ROS. It is mainly formed in vivo in the reaction of hydrogen peroxide with reduced forms of transition metal ions:

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

The most important participating metals in vivo are Cu^+ and Fe^{2+}. The reaction with iron is called the Fenton reaction from its first description (Winterbourn, 1995). For the reactions to occur, the metal ions have to be reduced, e.g. by superoxide:

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

The sum of the two reactions is called the Haber–Weiss reaction (Halliwell, 1978; McCord & Day, Jr., 1978). The transition metal ion reduction can, however, be accomplished by many other reductants, e.g. ascorbate. Fenton/Haber–Weiss chemistry probably underlies a major part of the toxicity of both hydrogen peroxide and the transition metal ions.

Hydroxyl radical may also be formed from peroxynitrite (Halliwell, Zhao & Whiteman, 1999) and by the reaction of superoxide with hypochlorite (Candeias *et al.*, 1993). HOCl can, for example, be formed from hydrogen peroxide and Cl^- catalysed by myeloperoxidase in neutrophil leukocytes.

$$HOCl + O_2\cdot^- \rightarrow OH\cdot + Cl^- + O_2$$

Singlet oxygen

An input of energy can transform ground state (triplet) molecular oxygen to singlet oxygen. The physiologically relevant form is called $^1\Delta_g$ singlet oxygen, which has the two antibonding electrons with opposite spins in one of the Π*2p orbitals. Thereby, spin restrictions for reactions

are removed and singlet oxygen reacts avidly with a wide variety of biomolecules, e.g. those containing double bonds. The reaction with unsaturated fatty acids results in the formation of lipid hydroperoxides, cf. below. Among amino acids residues, methionine and histidine are particularly susceptible, and in DNA the reaction primarily occurs with guanine residues (Sies & Menck, 1992). Singlet oxygen has a very short half-life in water, 3.8 μs. Protection can be achieved by quenching reactions where the energy is transferred to another molecule, which in turn dissipates it as heat. β-carotene and other carotenoids are efficient singlet oxygen quenchers.

Singlet oxygen can be formed by type II photosensitizing reactions, where molecules excited by light transfer the energy to (triplet) molecular oxygen. Porphyrins and many drugs, e.g. tetracyclins are efficient sensitizers, and singlet oxygen is a major cause of the light-induced skin reactions caused by these compounds. Singlet oxygen is also formed in the reaction between two lipid peroxyl radicals, cf. below, and slowly in a reaction between hydrogen peroxide and hypochlorite. Except for photosensitized reactions in the retina, singlet oxygen is probably not a major player in pathology of the nervous system.

Lipid peroxidation and vitamin E

The basis for lipid peroxidation is the labile *bis*-allylic hydrogens (LH below) bound to the methylene carbons between the double bonds in polyunsaturated fatty acids. The process can be initiated, for example, with the reaction of the hydrogen with a hydroxyl radical. Molecular oxygen rapidly adds to the resulting carbon-centred free radical, forming a lipid peroxyl radical. This, in turn, abstracts a *bis*-allylic hydrogen from a neighbor fatty acid forming a lipid hydroperoxide and again a carbon-centred free radical. Molecular oxygen adds to this radical resulting in a propagated chain reaction called lipid peroxidation.

$$LH + OH\cdot \rightarrow L\cdot + H_2O$$

$$L\cdot + O_2 \rightarrow LOO\cdot$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot$$

The reaction chain can be terminated by the radicals reacting with each other, e.g. the so-called Russel mechanism:

$$>LOO\cdot + >LOO\cdot \rightarrow >CHOH + >C=O + (\text{singlet})O_2$$

The "phenolic" hydrogen (Toc-OH) on the chroman ring of the lipid-soluble vitamin E (α-tocopherol) efficiently competes with the *bis*-allylic hydrogens for donation to lipid peroxyl radicals.

$$LOO\cdot + \text{Toc-OH} \rightarrow LOOH + \text{Toc-O}\cdot$$

The resulting radical in vitamin E is delocalized and generally does not participate in propagation of lipid peroxidation. The result is termination of the lipid peroxidation chain. Following absorption, vitamin E is transported to the organs of the body via lipoproteins. Vitamin E enters the brain slowly, although this organ seems to be prioritized in vitamin E deficiency (Vatassery *et al.*, 1988). In a-β-lipoproteinemia there is a deficient absorption and transport of vitamin E, which results in neurological deficits that are ameliorated by vitamin E therapy. The secretion of vitamin E from the liver via VLDL is dependent on the α-tocopherol transfer protein. Loss of function mutations in this protein lead to very low plasma vitamin E levels and progressive spinocerebellar ataxia (Gotoda *et al.*, 1995) and retinitis pigmentosa (Yokota *et al.*, 1997).

The in vivo initiation mechanisms are not well understood, but commonly may involve reactions of transition metal ions with lipid hydroperoxides, which in turn can be preformed by several mechanisms in vivo:

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

$$LOOH + Fe^{3+} \rightarrow LOO\cdot + H^+ + Fe^{2+}$$

The resulting lipid alkoxyl and peroxyl radicals then abstract *bis*-allylic hydrogens initiating lipid peroxidation chains.

The lipid peroxidation results in detrimental structural changes of membranes and lipoproteins. The peroxidation-modified lipids degrade further to form a wide variety of toxic reactive compounds such as 4-OH-nonenal and malondialdehyde.

Transition metal ions

Transition metal ions are toxic, e.g. by participating in the Haber–Weiss reaction and in initiation of lipid peroxidation, and therefore have to be tightly controlled in vivo. In plasma, binding of Fe to transferrin prevents the reactivity, and normally less than 50% of the capacity is occupied. In the cytosol, storage in ferritin is protective. The form and mechanism of transit of Fe between intracellular pools and proteins, however, is not well defined, but there may be a 0.7–0.9 μM "labile" iron pool (Konijn *et al.*, 1999). Increased amounts of reactive iron may occur following release from ferritin by the superoxide radical (Biemond *et al.*, 1988) and some redox-cycling toxins (Winterbourn, Vile & Monteiro, 1991), disruption of FeS clusters, e.g. by superoxide radicals (Gardner & Fridovich, 1991), and degradation of heme from denatured hemoproteins. Heme itself is also prooxidant (Gutteridge & Smith, 1988).

The intracellular transit of Cu is better understood and involves different "Cu-chaperones" for different Cu protein targets (Huffman & O'Halloran, 2001). SOD1 is, for example, charged by a Cu-chaperone for SOD (Wong *et al.*, 2000). The intracellular concentration of "free" Cu has been estimated to be less than 1 molecule per cell (Rae *et al.*, 1999). Excess Cu (and Zn) in cells may be scavenged by metallothioneins (Hidalgo *et al.*, 2001). (See also Chapter 13, Bush, Chapter 60, Gitlin, and Chapter 61, Lewitt.)

Hemoprotein peroxidases and oxidants

Mammals contain at least four peroxidases with heme prosthetic groups; myeloperoxidase, eosinophil peroxidase, lactoperoxidase and thyroid peroxidase. Like the ubiquitous plant hemoperoxidases, they have the ability to catalyze the oxidation of a wide variety of phenolic and other substrates by hydrogen peroxide. In vivo, however, the mammalian peroxidases primarily oxidize halides.

Myeloperoxidase exists in granules of neutrophils, monocytes and can also be induced in tissue macrophages/microglia (Sugiyama *et al.*, 2001). Upon activation these cells produce hydrogen peroxide via NADPH oxidase, which myeloperoxidase uses to oxidize Cl^- to the strong oxidant hypochlorite.

$$H_2O_2 + Cl^- \rightarrow OCl^- + H_2O$$

The hypochlorite is not essential for, but contributes to, the killing of bacteria by the neutrophils (Hampton, Kettle & Winterbourn, 1996). Myeloperoxidase apparently can also catalyze the hydrogen peroxide-dependent oxidation nitrite to a nitrating species (Pfeiffer *et al.*, 2001). This leads to the formation protein-bound nitrotyrosine, previously thought to be a specific marker for the reaction product between superoxide radical and NO, peroxynitrite.

The peroxidase of eosinophil leukocytes can also produce hypochlorite, but seems primarily to oxidize bromide ions to hypobromite (Weiss *et al.*, 1986). The lactoperoxidase of tears, saliva and milk oxidizes thiocyanate to the bacteriostatic hypothiocyanate. The thyroid peroxidase iodinates and condensates tyrosine residues in thyroglobulin to form the thyroid hormones.

Myeloperoxidase has been demonstrated in microglia close to plaques in Alzheimer's disease and also in the β-amyloid, suggesting a potential involvement in the disease (Reynolds *et al.*, 1999). The enzyme may, however, also have a modulating effect on inflammatory reactions, since mice lacking myeloperoxidase are more susceptible to experimental autoimmune encephalitis (Brennan *et al.*, 2001a) and atherosclerosis (Brennan *et al.*, 2001b).

Low-molecular weight scavengers

Endogenous

GSH

The tripeptide glutathione, L-γ-glutamyl-L-cysteinyl-glycine (GSH) reaches cellular concentrations of several mmol/l, and is generally the most abundant low-molecular weight antioxidant in the body. GSH aids in metabolizing hydroperoxides by being the reducing substrate of the glutathione peroxidases (below), and of some of the peroxyredoxins (below). GSH can also directly reduce some free radical targets in biomolecules (Biaglow *et al.*, 1989). The reactions mostly result in formation of the disulfide oxidized glutathione (GSSG), which is in turn reduced back to GSH by glutathione reductase. The enzyme uses NADPH, derived from the glucose-6-phosphate dehydrogenase, the cytosolic (Jo *et al.*, 2002) and the mitochondrial (Jo *et al.*, 2001) NADP-dependent isocitrate dehydrogenase isoenzymes. These dehydrogenases thus form important parts of the antioxidant defence. GSH can also react with oxidized cysteines in proteins forming mixed disulfides. Increased concentrations of these are found in oxidant stress (Cotgreave & Gerdes, 1998). GSH is also used for conjugation of xenobiotics and other molecules catalyzed by glutathione transferases, and direct reaction with free radical intermediates, e.g. from dopamine and L-DOPA can also occur (Spencer *et al.*, 1998). These reactions can, however, also lead to depletion of GSH, causing toxic effects on tissues.

GSH is formed from its constituent amino acids in two steps synthesized by γ-glutamyl-cysteine-synthase and glutathione synthase. Cysteine may be limiting for the synthesis, and can experimentally and therapeutically be supplied by *N*-acetylcysteine or by 2-oxothiazolidine-4-carboxylate (Gwilt *et al.*, 1998). GSH can be exported over the plasma membrane and will then be hydrolyzed to γ-glutamine and cysteinyl-glycine by the ectoenzyme γ-glutamyltranspeptidase. The dipeptide is then hydrolyzed by dipeptidases. The constituent amino acids can then be taken up intracellularly for GSH resynthesis.

In the brain, the concentration of GSH appears to be higher in astrocytes (4 mmol/l) than in neurons (2.5 mmol/l) and other glial cells. Astrocytes can apparently protect neurons by degrading the diffusible hydrogen

peroxide, and also supply neurons with the substrates for GSH synthesis by export of GSH (Dringen, Gutterer & Hirrlinger, 2000).

Bilirubin and heme oxygenases

Heme is degraded to biliverdin and the transmitter substance CO by the heme oxygenases (HO), the inducible HO-1 and the constitutive HO-2. HO-2 is the most abundant in the CNS. Biliverdin is then reduced by biliverdin reductase and NADPH to bilirubin. Since heme, that for example, can be released from denatured hemoproteins, is prooxidant (Gutteridge & Smith, 1988), the heme oxygenases may exert antioxidant roles, provided that the released iron is efficiently sequestered. Bilirubin, in addition, efficiently scavenges some reactive oxygen species such as singlet oxygen and peroxyl radicals, forming biliverdin again. Bilirubin has shown in vitro neuroprotective action (Dore *et al.*, 1999), and a protective cycle involving biliverdin reductase can be envisioned. The β-amyloid precursor protein inhibits HO, and Alzheimer-linked mutant protein causes stronger inhibition. This effect has been suggested to contribute to the pathology in Alzheimer's disease (Takahashi *et al.*, 2000).

Urate

This is the end product of purine catabolism in humans, who have an inactive urate oxidase. In most other species allantoin is formed by urate oxidase. The concentration of urate in humans furthermore is kept high by tubular reabsorption in the kidney. Urate can scavenge a variety of oxidants such as ROO·, $ONOO^-$, NO_2 and O_3 (Becker *et al.*, 1991) to form allantoin, parabanic acid and some other compounds. Urate at concentrations found in humans furthermore has been shown to protect the Cu-containing SOD1 and SOD3 in vivo against inactivation by (presumably) hydrogen peroxide (Hink *et al.*, 2002). Administration of urate protects, for example, against experimental autoimmune encephalitis (Kastenbauer *et al.*, 2001), and increased levels of the oxidized metabolites in CSF have, for example, been observed in humans with meningitis (Kastenbauer *et al.*, 2002), suggesting a protective role of the compound in the CNS.

Exogenous

Ascorbate

This and vitamin E (see above) are the major dietary antioxidants in humans. Other mammals, with the exception of guinea pigs, synthesize ascorbate in the liver. Ascorbate occurs in 10–100-fold higher concentration in cells compared with blood plasma. In addition to participation in multiple metabolic reactions, ascorbate is an important antioxidant that reacts rapidly with superoxide radical, other free radicals and peroxynitrite and can also regenerate oxidized vitamin E (Chan, 1993). Ascorbate on the other hand can also reduce ferric iron and other oxidized transition metal ions and thereby promote Fenton-type reactions. Ascorbate easily autoxidizes, catalyzed by transition metal ions under oxygen radical formation (Buettner & Jurkiewicz, 1996). The importance of these prooxidant reactions may, however, be small in vivo where transition metal ions are mostly tightly controlled.

The reaction of ascorbate with oxygen free radicals primarily results in formation of semidehydroascorbate that disproportionates rapidly to dehydroascorbate and ascorbate. Semidehydro- and dehydroascorbate are reduced back rapidly to ascorbate by multiple mechanisms including GSH, thioredoxin reductase (Arrigoni & De Tullio, 2002; Nordberg & Arner, 2001), and a GSH-dependent dehydroascorbate reductase localized to the grey matter (Fornai *et al.*, 1999).

Ascorbate reaches the brain primarily via the CSF. It is actively transported from blood plasma in the choroid plexus into the CSF via a Na-coupled transporter, SVCT2, to result in high extracellular concentrations in the brain, around 200–400 μmol/l (Rice, 2000). This concentration apparently is controlled, suggesting an extracellular antioxidant role. Neurons take up ascorbate via SVCT2 and contain around 10 mmol/l. Astrocytes contain much less, around 2 mmol/l. The mechanism of loss of ascorbate from neurons is not fully understood, but may include a heteroexchange mechanism with glutamate, and an export of dehydroascorbate via the glucose transporter Glut 3. It is suggested that astrocytes acquire dehydroascorbate by Glut 1, reduce it and secrete ascorbate again. This may serve as a supply mechanism for neurons (Rice, 2000). The presence in all cells of glucose transporters (Glut) that also allow transport of dehydroascorbate, coupled with efficient intracellular reduction, seems to account for a significant part of the cellular ascorbate uptake. Such uptake is enhanced by extracellular ascorbate oxidation via, for example, superoxide radicals (Nualart *et al.*, 2003).

Flavonoids and other plant antioxidants

Dietary and intervention studies have indicated that fruit and vegetable consumption is important for neuronal health (Galli *et al.*, 2002). Plants contain multiple potential active components, and mostly the actual active compounds are not identified with certainty. Many beneficial effects are presumed to be derived from a family of polyphenolic compounds called flavonoids, which occur

abundantly in plants and are major constituents of many formulations, for example, from ginseng and *Ginkgo biloba*. Owing to their polyunsaturated substituted cyclic structures, the compounds function as reducing antioxidants, and have been shown to exert cytoprotective actions in a variety of experimental systems. The compounds may also reduce oxygen (autoxidize) under formation of oxygen radicals and thereby function as prooxidants. Many of the compounds show poor absorption and the extent of passage through the blood–brain barrier is little known. Their importance for the basal CNS integrity in humans is therefore not well established (Youdim *et al.*, 2002). Carotenoids, many of which are precursors of vitamin A, are efficient scavengers of singlet oxygen. Garlic extracts containing *S*-allycystein, *S*-allymercaptocysteine, allicin and diallosulfides have been shown to delay cognitive impairment in a senescent accelerated mouse model (Youdim & Joseph, 2001).

Antioxidant proteins and enzymes

Superoxide dismutases (SOD)
These dismute the superoxide radical under formation of molecular oxygen and hydrogen peroxide.

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

Since this reaction occurs rapidly and spontaneously, the SODs generally do not alter the hydrogen peroxide formation, but rather reduce the steady-state concentration of the superoxide radical. When there are major competing reactions that reduce the radical (e.g. with ascorbate) SOD will decrease hydrogen peroxide formation and, if competing reactions oxidize the radical, SOD will tend to increase the hydrogen peroxide formation. If SOD competes with NO for reaction with the superoxide radical, hydrogen peroxide formation will also be increased by the enzyme (Gardner, Salvador & Moradas-Ferreira, 2002).

There are three different SODs with different locations and different roles in the body. The phenotypes of knockouts are very different (Carlsson *et al.*, 1995; Li *et al.*, 1995; Reaume *et al.*, 1996), and so far there is no evidence that the isoenzymes can complement each other (Copin, Gasche & Chan, 2000). One reason apparently is the poor penetration of the superoxide radical through biological membranes (Winterbourn & Stern, 1987).

The first discovered (McCord & Fridovich, 1969) and most extensively studied is CuZn–SOD (SOD1). It is a homodimeric enzyme with each 15 kDa subunit containing one Cu and one Zn atom. The Cu is liganded by 4 His and the Zn by 3 His and one Asp residue (Tainer *et al.*, 1982). SOD1 physically is a very stable protein. SOD1 is charged with Cu by the Cu-chaperone for superoxide dismutase (CCS) (Huffman & O'Halloran, 2001). Whereas CCS is essential in yeast, some residual SOD1 activity exists in mice lacking CCS (Wong *et al.*, 2000), suggesting additional ways of charging the enzyme with Cu in mammals. The major location of SOD1 is the cytosol, but the enzyme also occurs in the nucleus (Chang *et al.*, 1988) and in the intermembrane space of mitochondria (Weisiger & Fridovich, 1973). A location for peroxisomes has also been discussed (Keller *et al.*, 1991; Liou *et al.*, 1993). Whereas the other SOD isoenzymes are widely regulated, SOD1 is essentially a constitutive enzyme with tissue-specific differences in concentration (Marklund, 1984). All the cultured or isolated cell types studied so far have contained the enzyme and generally there are rather small differences between various cell types (Marklund, 1984, 1990). Overall the brain contains intermediate levels of SOD1 compared with other tissues, with slightly more in grey matter that in white (Marklund, 1984). SOD1 is an abundantly expressed enzyme and the level in grey matter corresponds to 0.3% of the soluble protein (Marklund, 1984). By immunohistochemistry, spinal motor neurons appear to contain more SOD1 than most other cells in the CNS (Shaw *et al.*, 1997), but a likely proposition is that all other cell types in the CNS also contain sizeable levels of SOD1. More than 100 different mutations in the SOD1 gene have been linked to heritable amyotrophic lateral sclerosis (ALS) (Andersen *et al.*, 2003; Rosen *et al.*, 1993). ALS is caused by the gain of a toxic property of mutant SOD1, and mice lacking SOD1 do not develop motor neuron degeneration or show any other obvious phenotype (Reaume *et al.*, 1996). Closer inspection, however, reveals axonopathy (Shefner *et al.*, 1999), and the mutant mice also show increased susceptibility to, e.g. the neurotoxin MPTP (Zhang *et al.*, 2000), and to ischemia/reperfusion of the CNS (Kondo *et al.*, 1997).

The mammalian Mn-SOD (SOD2) is homotetrameric, with each subunit liganding one Mn atom (Borgstahl *et al.*, 1992). The subunits are synthesized in the cytosol with a signal peptide with direct import into the mitochondrial matrix where the enzyme is matured by proteolysis and equipped with Mn atoms. The latter reaction may be accomplished by a specific Mn-transfer protein (Luk *et al.*, 2003). The severe phenotype of mice lacking SOD2 suggests that the enzyme is essential for mitochondrial integrity (Lebovitz *et al.*, 1996; Li *et al.*, 1995). Mitochondrial factors containing superoxide-susceptible FeS clusters are apparently major targets in SOD2 null mice. Brain grey matter contains high levels of Mn-SOD, whereas the level in white matter is threefold lower (Marklund, 1984).

Apparently, the high levels in the brain are important since CNS injury is a prominent feature of Mn-SOD null mice (Lebovitz *et al.*, 1996). Mice heterozygous for the SOD2 null allele show increased susceptibility to the neurotoxic mitochondrial toxins, malonate, 3-nitropropionic acid and MPTP (Andreassen *et al.*, 2001), and are more susceptible to ALS-linked mutant SOD1 (Andreassen *et al.*, 2000). SOD2 is widely regulated by a variety of inflammatory cytokines (Marklund, 1992; Wong & Goeddel, 1988) and somewhat less so by oxidant stress (Stralin & Marklund, 1994).

Extracellular-SOD (EC-SOD, SOD3) is a homotetrameric secreted glycoprotein (Marklund, 1982). The subunits are synthesized with a signal peptide, which is cleaved upon maturation. They ligand Cu and Zn and the sequence is similar to that of SOD1 in the part that defines the active site (Hjalmarsson *et al.*, 1987). SOD3 shows high affinity for heparin and heparan sulfate, conferred by the highly positively charged C-terminal end of the subunits. In vivo the enzyme is anchored primarily to heparan sulfate proteoglycans in the interstitial matrix of tissues (Karlsson *et al.*, 1994). In the vasculature, SOD3 forms an equilibrium between the plasma phase and the endothelial cell surfaces. The brain contains little SOD3, both in humans (Marklund, 1984) and in mice (Carlsson *et al.*, 1995). However, in the brain the enzyme is localized to neurons in a few discrete areas, and in addition is apparently mainly intracellular (Oury, Card & Klann, 1999). This intracellular location has also been observed in some other tissues and suggests that the enzyme may have an intracellular role or is stored for secretion under some circumstances. The enzyme may thus exert some specific roles in the CNS. SOD3 null mice do not show any spontaneous CNS or other phenotypes, but the lungs are highly susceptible to high oxygen tension (Carlsson, Jonsson, Edlund & Marklund, 1995). Impaired memory (Levin *et al.*, 1998), and enhanced injury in stroke models (Sheng *et al.*, 1999) has also been observed in SOD3 null mice. SOD3 is a highly regulated enzyme and the synthesis is influenced by inflammatory cytokines (Marklund, 1992), vasoactive factors and some sulphated glycosaminoglycans (Stralin & Marklund, 2001), but hardly by oxidant stress (Stralin & Marklund, 1994).

Catalase

The homotetrameric hemoprotein catalase catalyzes the dismutation of hydrogen peroxide.

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

The catalysed reaction is first order in hydrogen peroxide at all physiological concentrations, and unlike glutathione peroxidase catalase is equally efficient at all hydrogen peroxide concentrations. Except for erythrocytes, the enzyme is intracellularly localized to peroxisomes. Overall, the brain contains little catalase activity. Both grey and white matter of the human brain contains so little catalase that it is difficult to measure reliably due to contamination of the catalase-rich erythrocytes (Marklund *et al.*, 1982). In the rat brain catalase shows a widespread distribution, both to neurons and to glia (Moreno, Mugnaini & Ceru, 1995). Since hydrogen peroxide easily penetrates membranes (Winterbourn & Stern, 1987), the catalase-rich erythrocytes in the vasculature may degrade some of the hydrogen peroxide formed in the brain. In rats, an age-dependent decline in the catalase activity in various parts of the brain has been observed (Ciriolo *et al.*, 1997).

Glutathione peroxidases (GPX)

There are four GPX isoenzymes, all with selenocysteine in the active sites (Brigelius-Flohe, 1999). The highest activity is generally shown by the cytosolic GPX, which probably is expressed by all cells in the body. This cGPX also occurs in the mitochondria (Esworthy, Ho & Chu, 1997). The phospholipid hydroperoxide (PH) GPX also shows a wide distribution. There is an extracellular plasma GPX that is mainly secreted by the kidney, but which also is expressed in the CNS (Maser, Magenheimer & Calvet, 1994). The fourth isoenzyme is the gastrointestinal (GI) GPX. All GPXs reduce H_2O_2 and some organic hydroperoxides at the expense of reduced glutathione:

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$$

In addition, PHGPX reduces a variety of complex lipidhydroperoxides and hydroperoxy groups on other biological compounds. Plasma GPX can also use thioredoxin as a reductant.

The human brain contains relatively low levels of GPX activity compared with other tissues (Marklund *et al.*, 1982), which, together with the low catalase activity, suggests that the organ may be vulnerable to increased formation of hydrogen peroxide. Note that the cGPX activity in the brain is, unlike in other organs, spared in selenium deficiency (Brigelius-Flohe, 1999). The enzyme appears to be mainly expressed in glial cells (Damier *et al.*, 1993). CGPX null mice do not show any spontaneous phenotype, but display an increased infarct size in a brain ischemia-reperfusion model (Crack *et al.*, 2001). They also show increased sensitivity to the neurotoxins: malonate, 3-nitropropionic acid and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (Klivenyi *et al.*, 2000; Zhang *et al.*, 2000).

Thioredoxin, glutaredoxin, and thioredoxin reductase

The major cytosolic thioredoxin (Trx-1) is a 12 kDA protein with a –Cys–Gly–Pro–Cys– active site motif, which is easily oxidized to a disulfide. Trx possesses potent protein disulfide oxidoreductase activity, and is involved in protein folding (Nordberg & Arner, 2001). Mitochondria contain another isoform, Trx-2. Reduced Trxs also serve as a substrate for the peroxyredoxin isoenzymes, see below. Trx-1 is widely expressed in neurons in the brain (Lippoldt *et al.*, 1995), and overexpression has been shown to protect against, for example, brain ischemia-reperfusion damage (Takagi *et al.*, 1999). Likewise, Trx-2 shows a wide distribution in the brain, apparently primarily to neurons (Rybnikova *et al.*, 2000).

Glutaredoxin (thioltransferase) shows functions overlapping with the Trxs. Unlike these, glutaredoxin can be reduced by glutathione, and it can also reduce protein-GSH-mixed disulfides (Nordberg & Arner, 2001). This effect is, for example important in recovery of mitochondrial function following excitotoxic insult (Kenchappa *et al.*, 2002).

Oxidized Trxs are reduced by thioredoxin reductases (TrxR) using NADPH as reductant (Nordberg & Arner, 2001). The TrxRs are flavoproteins containing active site selenocysteines, which occur in different isoforms in the cytosol and mitochondria (Nordberg & Arner, 2001). The TrxRs show wide substrate specificity and can, in addition to Trx, reduce GSH, dehydroascorbate and semidehydroascorbate, hydroperoxides including lipid hydroperoxides and protein disulfide isomerases (Nordberg & Arner, 2001). The main function of protein disulfide isomerases is to oxidize cysteines to form disulfide bonds during protein folding.

Methionine is easily oxidized by various oxidants to the sulfoxide form and is reduced back by methionine sulfoxide reductase. This enzyme uses the NADPH/Trx/TrxR system for reduction of its active site (Weissbach *et al.*, 2002). Overexpression of methionine sulfoxide reductase predominantly in the brain of *Drosophila* prolongs the lifespan (Ruan *et al.*, 2002). This suggests a role of methionine oxidation in aging.

Peroxyredoxins

In mammals the peroxyredoxins (Prx) comprise a family of six isoenzymes with structural and some functional similarities. The Prx isoenzymes are localized to different compartments with Prx1 and Prx2 in the cytosol, Prx3 in the mitochondria, Prx4 in the plasma membrane, Prx5 in mitochondria, peroxisomes and cytosol, while Prx6 has an extracellular location (Fujii & Ikeda, 2002). The Prx isoenzymes catalyze the reduction of hydrogen peroxide and organic hydroperoxides with all, except Prx6, using thioredoxin as the reductant. Prx1, 4 and 6 can also use GSH (Fujii & Ikeda, 2002). Notably, a bacterial peroxyredoxin has shown peroxynitrite reductase activity, which might be shared by the mammalian isoforms (Bryk, Griffin & Nathan, 2000). Unlike other enzymes that degrade hydroperoxides, the activities of Prx1 and 2 can be regulated (by phosphorylation), suggesting active involvement in cellular signaling by hydrogen peroxide (Chang *et al.*, 2002). The Prx isoenzymes are generally highly expressed and show wide tissue distributions with comparatively high expression throughout the brain (Rhee *et al.*, 2001). Given the low catalase and glutathione peroxidase activities, the Prx isoenzymes may conceivably exert particularly important roles in the CNS.

Markers of free radical damage

Chemical analysis

There are numerous relatively stable small molecules and protein and DNA modifications formed in reactions with oxygen free radicals, which can be used as markers for oxidant injury.

Peroxidative attack on polyunsaturated fatty acids results in the formation of various classes of isoprostanes, which are regarded as the most reliable markers of lipid peroxidation. The most widely studied is the F2 isoprostane 8-isoPGF2α, which derives from arachidonic acid. The isoprostanes are stable and have been determined, e.g. in brain extracts, cerebrospinal fluid, blood plasma and urine, and appear to parallel the peroxidation intensity (Greco, Minghetti & Levi, 2000; Janssen, 2001).

Malondialdehyde is a late breakdown product from peroxidized lipids and is generally detected as a reaction product with thiobarbituric acid (TBARS). The TBARS can be analysed by spectrophotometry, fluorimetry and by HPLC with increasing specificity (Moore & Roberts, 1998). A variety of other aldehydes and molecules can react with TBA, however, and MDA is also formed in prostanoid synthesis. The TBARS reaction therefore, has a very limited specificity and utility in complex matrices such as tissue extracts, plasma and CSF.

A major cytotoxic degradation product from peroxidized lipids is 4-hydroxynonenal. It easily forms thioether adducts and adducts with free amino groups in proteins. It is much less often analyzed than MDA (Moore & Roberts, 1998), but has a greater utility as a marker for immunohistochemical analysis.

Numerous oxidant-induced protein modifications are used as markers. The most commonly analyzed