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Oxidative stress in neurodegeneration and available means of protection

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1. ABSTRACT

Substantial pieces of direct and indirect evidence have mounted over the years linking the induction of oxidative stress to a plethora of disease conditions, not least those associated with the death of neurons. The causal relationship between oxidative damage and neurodegeneration is, however, not yet clear and still a subject of intense investigation. Nevertheless, the phenomenon of oxidative neuronal death has received considerable attention in a frantic search for efficacious therapies for the management of neurological and neurodegenerative conditions. The redox-active nature of reactive oxygen species (ROS), which in their excessive levels induce oxidative stress, the prevalence of ROS production in biological systems, the complexity of interrelationships among these species, and the context-dependent adequacy and resilience of the antioxidant defense systems are some of the challenges that basic research has to grapple with to advance successfully to the translational stage. Much still has to be understood for research efforts in this field to yield enduring therapies. In this review, we examine the nature (chemistry) of ROS, the relationships between them, their physiological functions, the roles of oxidative stress in neurodegeneration, the mechanisms of cell death induced by oxidant species, and the available means of protecting neurons against oxidative damage.

2. REACTIVE OXYGEN SPECIES IN BIOLOGICAL SYSTEMS

2.1. Nature and reactivity

Although oxygen (O_2) is essential to life, it is an interesting fact that toxicity can result from its excessive levels (1), and it can also play a role in molecular damage through its radical and non-radical derivatives named reactive oxygen species (ROS), which are molecules chemically reactive to different degrees (2-5). ROS are usually low-molecular-weight intracellular oxygen free radicals with an unpaired electron and the terms ROS and oxygen free radicals are sometimes considered equivalent and used interchangeably (6), though erroneously, as some ROS, such as hydrogen peroxide (H_2O_2), are not free radicals. A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons (7). Because free radicals are partially reduced and contain an orbital with an unpaired electron, they are chemically reactive, and in order to gain stability, they "steal" electrons (hence the term "electron lovers") or hydrogen atoms from their neighbors, turning these molecules also to radicals (8,9), and thereby instigating a continuous chain of reactions. Reactive oxygen species include superoxide anion radical ($O_2^{\cdot-}$), H_2O_2 and the hydroxyl radical ($^{\cdot}OH$). The one-, two- and three-electron reductions of molecular O_2 generate, respectively, superoxide, H_2O_2 and hydroxyl radical (10), but the

reductions of H_2O_2 are generally slower than those of superoxide (11). The superoxide anion, obtained by the univalent reduction (addition of an electron) of triplet-state molecular oxygen ($^3O_2$) (12), is not highly reactive, even though it is a free radical. This is because it lacks the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it is produced (6). However, it can generate more reactive radical species (11). For example, it could react rapidly with nitric oxide (NO) to produce the highly toxic radical, peroxynitrite ($ONOO^-$) (12), and can also be converted to H_2O_2 by the process of dismutation (two molecules of superoxide dismutate to H_2O_2 and molecular oxygen), catalyzed by the enzyme superoxide dismutase (SOD) (11), of which there are three isoforms (SOD1, SOD2, SOD3) (13), or by metal complexes, especially of copper (11). The peroxynitrite generated by the reaction between superoxide and NO has the activity of the hydroxyl radical and nitrogen dioxide radical, although it does not readily decompose into these entities (14), and can also nitrate and hydroxylate directly aromatic rings on amino acid residues as well as react readily as a potent oxidant with sulfhydryls and zinc-thiolate (14,15). Peroxynitrite can protonate rapidly at physiological pH to the hydroxyl radical-generating peroxynitrous acid (ONOOH) (16,17) and is also capable of reacting with carbon dioxide to form nitrogen dioxide and carbonate radical (15,16,18).

Transition metals such as iron and copper can catalyze the formation of oxyradicals, as autoxidation of metal complexes can generate superoxide radical (11). Hydrogen peroxide is not a free radical, and is often considered-like superoxide- only mildly reactive, but because it is stable (with a long half-life), produced by almost all tissue types where its concentrations can rise, and has the ability to penetrate biological membranes by diffusing within and across cells, it is highly important (10). In fact, a decrease in intracellular superoxide or an increase in H_2O_2 could lead to cytosolic acidification by shifting the cytosolic pH to a significantly acidic level (19). The toxicity of H_2O_2 is usually as a result of its profound ability to traverse cellular membranes -unlike superoxide- and the production of the extremely toxic hydroxyl radical through its reaction with transition metals, since in the presence of such transition metal ions as iron or copper, which are often bound in complex with proteins or other molecules, H_2O_2 can undergo a one-electron reduction in the Fenton reaction (Reaction 1), leading to the production of the hydroxyl radical (6,20) (which is the most reactive and most toxic of the ROS), although it can also react with organic peroxides to form alkoxyl or hydroxyl radicals (21).

$$H_2O_2 + Cu^+/Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Cu^{2+}/Fe^{3+} \quad \text{(Reaction 1)}$$

The metal ions can be recycled by superoxide as shown in Reaction 2

$$Cu^{2+}/Fe^{3+} + O_2^{\cdot-} \rightarrow Cu^+/Fe^{2+} + O_2 \quad \text{(Reaction 2)}$$

A combination of Reactions 1 and 2 produces the Haber-Weiss reaction. Hydrogen peroxide could also serve as an intermediate in the production of the more reactive hypochlorous acid (HOCl) through the action of myeloperoxidase present in the phagosomes of neutrophils (22).

2.2. Sources in biological systems

ROS can be generated in biological systems through cellular respiration, several enzyme systems, endogenous metabolism and receptor-mediated events. In the process of generating energy during aerobic metabolism, cells reduce oxygen to water and there is transfer of electrons, the result of which is the leakage of high-energy electrons resulting in the formation of ROS. It is estimated that 2-4% of the oxygen consumed during oxidative phosphorylation in the mitochondria is converted to ROS. Superoxide is the major oxygen free radical produced, generated by up to 1% of the mitochondrial electron flow (13), and its formation could occur spontaneously in the electron-rich aerobic environment in the neighbourhood of the inner mitochondrial membrane (6). The part of the electron transport chain that actually uses oxygen is the terminal oxidase enzyme, cytochrome oxidase (23). In addition to the mitochondrial generation, superoxide radical could also be produced through the activity of the electron transport chain in the endoplasmic reticulum (24), but apart from these sources, NADPH oxidase also generates a significant amount of ROS in the cell (25). It is a multi-component, membrane-associated enzyme that catalyzes the one-electron reduction of oxygen to superoxide using reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor. Both superoxide anion and H_2O_2 are established products of the "respiratory burst" when the plasma membrane NADPH oxidase of neutrophils and macrophages is activated (26). Either of them is also produced endogenously by flavoenzymes such as xanthine oxidase (27-29). When accumulated hypoxanthine and xanthine are converted by xanthine oxidase to uric acid, superoxide anion is produced. This xanthine oxidase can derive from xanthine dehydrogenase through a calcium-dependent conversion (involving calcium-activated peptidases, such as calpain I) under conditions of energy failure and elevated intracellular calcium levels (30). Other enzymes capable of producing superoxide are lipoxygenases and cyclooxygenases (31,32).

Calcium-dependent activation of nitric oxide synthase (NOS) can produce the gaseous free radical, nitric oxide. Several enzymes in the brain, including monoamine oxidase (MAO), tyrosine hydroxylase and L-amino oxidase generate H_2O_2 as a normal by-product of their activity, while auto-oxidation of endogenous substances such as ascorbic acid and catecholamines also yields H_2O_2. Phospholipase A_2 (PLA_2), when activated in a Ca^{2+}-dependent process, can generate arachidonic acid from membrane phospholipids and arachidonic acid could in turn yield superoxide anion when subsequently metabolized through the cyclooxygenase or lipoxygenase pathway that leads to the production of eicosanoids.

There is also evidence for receptor-mediated generation of ROS. Glutamate receptor activation is now known to be a major source of ROS inducing oxidative stress in the brain, where excessive stimulation of glutamate receptors results in excitotoxicity (30). Overstimulation of the population of glutamate receptors sensitive to *N*-methyl-D-aspartate (NMDA) leads to significant elevation of intracellular calcium concentration, which in turn generates ROS (superoxide and H_2O_2) through the activation of several calcium-dependent enzymes and processes (33). By damaging neurons, oxidative stress generated by ROS could itself promote the release of excitatory amino acids (34); the ROS could also reduce glutamate uptake by glial cells, prevent glutamate conversion to glutamine by inactivating glutamine synthetase (35), or damage glutamate transporters (36). Some neurons and glia contain transient receptor potential melastatin-related (TRPM)2 cation channels that rapidly permit entry of calcium ions in the presence of ROS such as H_2O_2 (37). A major endogenous route for the generation of NMDA receptor ligands and some cytotoxic compounds is the kynurenine pathway of metabolism of the amino acid tryptophan. The kynurenine pathway metabolizes over 90% of dietary tryptophan that is not used in protein synthesis to generate the essential co-factors nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Products of this pathway include quinolinic acid, an endogenous agonist ligand able to activate selectively the NMDA receptors (38,39), and kynurenic acid, a broad-spectrum glutamate receptor antagonist that acts at the strychnine-resistant, glycine-sensitive site on the NMDA receptor (39,40). The kynurenine pathway products known to generate substantial levels of ROS include quinolinic acid, 3-hydroxyanthranilic acid and 3-hydroxykynurenine. 3-hydroxykynurenine has been reported to be the most toxic of all kynurenines or tryptophan metabolites, as it generates substantial levels of H_2O_2 (41,42) and is relevant to the pathophysiology of a number of neurodegenerative conditions such as Huntington's disease (43).

2.3. Physiological roles in cell signaling

Although not the focus of this review, it is essential to first of all highlight the physiological relevance of ROS (superoxide and H_2O_2 in this context) before focusing on their pathological roles, as this enhances a better appreciation of their concentration- and context-dependent differential effects. ROS are produced and degraded by all aerobic organisms, and this leads to either physiological concentrations required for normal cell function, or excessive quantities, a state referred to as oxidative stress, as discussed later in this review (6). It is generally believed that low levels of ROS, especially H_2O_2 and superoxide, are involved in cellular signaling and may thus be beneficial and necessary for the maintenance of organism's adequate physiological functioning, while high concentrations are damaging, leading to cell death (26,44). ROS can induce increases in cytosolic calcium (33) and their signal cascades are involved in cell growth, cell death, mitogenesis, angiogenesis and carcinogenesis (25). Growth responses have been shown to be elicited by H_2O_2 in mouse osteoblastic cells (MC3T3) (44,45). Besides, exogenously added superoxide and H_2O_2 as active oxygen species can stimulate growth and growth responses in several mammalian cell types when added to the growth medium, with the effects of exogenous superoxide on cells being extremely rapid (44). Interestingly, it has also been reported that a number of polyunsaturated fatty acids (PUFAs) can promote the growth of many cell lines when added to cultures at low concentrations, whereas high concentrations are inhibitory to growth (44). In addition, superoxide and H_2O_2 could stimulate the expression of early growth-regulated genes such as c-*myc*, c-*fos,* c-*jun, erg-1, KC* and *JE*. Hydrogen peroxide can cause protein phosphorylation (46), activation of protein kinases (e.g., protein kinase C), and activation of serum-response elements in the promoters of early growth-response genes (45). ROS can also cause oxidative inactivation of phosphatases and activation of transcription factors (47). For example, they stimulate tyrosine kinase activity (48), regulate mitogen activated protein kinases (MAPKs) (JNK, p38MAPK and ERK) and activate transcription factors Nuclear Factor-kappa B (NF-kappaB) and Activator Protein-1 (AP-1) (12). In neuronal cells, a role has been demonstrated for ROS in nerve growth factor (NGF) signaling (12). Such findings have led to suggestions that these ROS might function as mitogenic stimuli through biochemical processes common to natural growth factors (44). Again, the observation that superoxide or its dismutation product, H_2O_2, is released by cells, either constitutively in the case of tumor cells, or following cytokine stimulation, has provoked the speculation that they might serve as a sort of "autocrine" growth stimulation system or a means of "intercellular communication" (26). It appears that the growth responses induced in normal cells by exogenous ROS require the additional presence of some serum components, in which case the ROS are thought to be augmenting the effects of natural growth factors (44).

Both superoxide and H_2O_2 have also been found to be involved in cellular defense system, as they could be produced *in vivo* through inflammatory cells in the vicinity of a tumor, or at an inflammation site, and are products of the "respiratory burst" when the NADPH oxidase of neutrophils and macrophages is activated (26,49). They are therefore relevant to the ability of the cells to fight infection or invading organisms. Superoxide can be released by both phagocytic (50) and non-phagocytic cells (51), by primary fibroblasts (52), and by endothelial (53) and epithelial cells (54). In a number of these cases, the release becomes significantly enhanced by cytokines such as interferon and interleukin-1, and there is involvement of protein kinase C (55). The production of ROS in non-phagocytic cells involves the activation of several signaling pathways such as cytokine receptors, G-protein-coupled receptors and receptor tyrosine and serine/threonine kinases (47).

In relation to growth factors, an example of a defined role for ROS in the context of angiogenesis is the involvement of NADPH oxidase-derived ROS in vascular endothelial growth factor receptor 2 (VEGFR2)-mediated signaling linked to endothelial cell migration and proliferation, VEGF being a potent angiogenesis factor (56). In another capacity, ROS have been shown to be important in controlling the transcriptional activity of the

hypoxia-inducible factor-1 (HIF-1), which is believed to be the master regulator of the hypoxia response and is known to regulate dozens of genes involved in the control of metabolism, angiogenesis and metastasis (57-59). The angiogenic potency of bone marrow cells, the implantation of which induces therapeutic angiogenesis, was shown to be enhanced by their short-term pre-treatment with low-dose H_2O_2 (60). In relation to oncogenesis, it was recently reported that the effect of intracellular ROS is dependent on the ratio of intracellular superoxide to H_2O_2: while a predominant increase in superoxide supports cell survival and promotes oncogenesis, a preponderance of H_2O_2 prevents carcinogenesis by activating cell death signaling (19). ROS are now known to act at different stages of carcinogenesis, thus playing multiple roles in oncogenesis, including mutagenicity and effects on tumor initiation, promotion and progression (61).

2.4. Induction of oxidative stress

While low levels of ROS have a number of useful physiological roles and are involved in cell signaling, their excessive levels result in a state commonly referred to as oxidative stress, defined as an imbalance between free radical-generating and free-radical scavenging systems, in favor of the former, a situation that arises when the amount of free radicals is increased or when the levels of antioxidant enzymes are decreased (62-65), leading to oxidative damage. Because of their high reactivity, ROS are prone to causing damage and are thereby potentially toxic, mutagenic, or carcinogenic (6), their intracellular production threatening the integrity of various biomolecules including proteins (66), lipids, and nucleic acids such as DNA (67,68), as they oxidize such molecules (68), although it has to be appreciated that there is always a basal level of oxidative damage to these biomolecules (7, 69). Cellular damage resulting from oxidative stress has been linked to the aging process (70) and a wide variety of pathological conditions including atherosclerosis (71), carcinogenesis (72), osteoporosis (73) and neurodegenerative disorders (30). Damage can be caused through a single reaction, a chain reaction, or a branching mechanism (11). A single reaction may not cause pronounced damage. A chain reaction results when a radical such as the hydroxyl radical reacts with a biomolecule, creating another radical, and only when a radical reacts with another radical or with a transition-metal ion would this vicious trend stop. The process of branching results in extensive damage (11). Chain reactions when initiated generate numerous toxic reactants that rigidify membranes by cross-linking (30). The oxygen radicals can occur as alkyl or peroxyl radicals in lipids (74) and notably, a major consequence of oxidative stress is lipid peroxidation, which results from an interaction of ROS with polyunsaturated lipids in cell membranes (75). Subsequently, changes occur in structure, function and permeability of the membrane, leading ultimately to cell death. Lipid peroxidation can decrease membrane fluidity, increase membrane leakiness, damage membrane proteins and inactivate receptors, enzymes and ion channels (7). Oxidative stress can increase Ca^{2+}, thus activating PLA_2, which can liberate arachidonic acid from membrane phospholipids (17). This arachidonic acid can undergo lipid peroxidation (76) and also act as a substrate for eicosanoid synthesis. Low levels of H_2O_2 can accelerate cyclooxygenase action on PUFAs, leading to prostaglandin synthesis (77). End-products of lipid peroxidation can also have direct damaging effects: for example, the increased formation of isoprostanes (78), biochemical markers of oxidative stress (73), has been observed in many human diseases (79).

Although superoxide and H_2O_2 at high concentrations could induce oxidative stress resulting in oxidative damage, the hydroxyl radical is possibly capable of doing more damage to biological systems than any other ROS, owing to its strong reactivity with biomolecules (6). It has been theorized that in excess of 50% of the free radical-mediated molecular destruction of cells is a direct consequence of the hydroxyl radical. Nevertheless, its absolute significance to cellular malfunction, diseases and aging has been difficult to define unequivocally (10). The hydroxyl radical is not generated directly by any known enzymatic reaction, but is produced by H_2O_2 through slow decomposition in the presence of Fe^{2+} by the Fenton reaction (30).

2.5. Relationship with nitrosative stress

Reactive nitrogen species (RNS), some of which are also free radicals, are equally a significant source of cellular damage when present in excessive amounts, as they induce nitrosative stress. Because they share a number of commonalities with ROS, we would consider them in some detail. Nitric oxide (NO), formerly identified as Endothelial-Derived Relaxing Factor (EDRF), is rather an odd member of the free radical family with a multiplicity of roles in the CNS and the periphery, including neurotransmitter functions and mediation of both proliferation and death, depending on tissue types and hence has been referred to as a Janus molecule. Studies of NO now mainly span the cardiovascular, nervous and immune systems (80). It is produced through the metabolic action of nitric oxide synthase (NOS) on L-arginine (oxidation of its guanidine group) with a stoichiometric formation of citrulline, though direct reduction of nitrite to NO has also been reported in the ischemic heart (81,82). Nitric oxide is known to be the mediator of tumoricidal and bactericidal actions of macrophages and is a likely transmitter of nonadrenergic, noncholinergic neurons (82). There are three isoforms of NOS: the calcium/calmodulin-dependent neuronal NOS (nNOS or NOS1), the endothelial NOS (eNOS or NOS3), and the calcium-independent, inducible NOS (iNOS or NOS2). Neuronal NOS (nNOS) and eNOS are the constitutive nitric oxide synthases. The inducible form (iNOS) is now known to bind, S-nitrosylate and activate cyclooxygenase-2 (83). Physiologically, NO activates soluble guanylate cyclase (sGC), leading to increase in cyclic guanosine monophosphate (cGMP), although it is now also known to inhibit mitochondrial cytochrome c oxidase. Nitric oxide is similar to superoxide in many aspects in that it does not readily react with most biomolecules, despite its having an unpaired electron, but easily reacts with other free radicals (e.g., peroxyl and alkyl radicals), generating mainly less reactive molecules and thus functioning somewhat as a free radical scavenger (6).

In fact, it has been shown that nitric oxide inhibits peroxide-mediated endothelial toxicity (84). On the other hand, as mentioned earlier, it readily reacts with the superoxide radical, forming the extremely toxic peroxynitrite. The rate of this reaction is about three times faster than the rate at which superoxide dismutase (SOD) catalyzes the dismutation of superoxide radical to H_2O_2. Nitric oxide can also be converted to other RNS such as nitrosonium cation (NO^+) or nitroxyl anion (NO^-), depending on the microenvironment (85). The production of peroxynitrite provides a case for the existence of a co-operative relationship between ROS and RNS. It is interesting to note that overstimulation of glutamate receptors causes the production of NO, superoxide and H_2O_2, and these species are now believed to be major mediators of the demise of neurons following excitotoxicity (86).

2.6. Antioxidant defense systems

Defense mechanisms to prevent or limit the damage caused by ROS are present in the body under normal circumstances (87). However, these defenses are complex, and tissues are equipped with different patterns of antioxidant defense, based on cell type and function, as well as on physiological states (88). Halliwell and Gutteridge (23) defined an antioxidant as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate," where the term "oxidizable substrate" includes every type of molecule found *in vivo*. The cellular antioxidant defense systems are classified into two groups, enzymatic and non-enzymatic, of which there are indirect- and direct-acting agents (68). The antioxidant enzyme systems include superoxide dismutases (SOD), superoxide reductases (SOR), catalases, peroxiredoxins (Prx), glutathione peroxidases (GPx) and other glutathione-related enzymes. The low-molecular-weight antioxidant compounds include vitamin C (a hydrophilic antioxidant), vitamin E or alpha-tocopherol (a chain-breaking hydrophobic antioxidant), different selenium compounds, lipoic acid (thioctic acid) and ubiquinones, all of which interact with the mammalian thioredoxin system, a ubiquitous oxidoreductase system with antioxidant and redox regulatory roles (6,30). There are also low-efficiency ROS scavengers like free amino acids, peptides and proteins (89). In addition, there are free radical-induced cytoprotective genes, such as the antioxidant-like stress protein heme oxygenase-1 (HO-1), which catabolizes the pro-oxidant heme to generate biliverdin, iron, and the vasodilator carbon monoxide (CO). Elevated HO-1 activity has been shown to be protective against several pathological conditions (90). Biliverdin is subsequently converted by biliverdin reductase to bilirubin, an antioxidant that can scavenge lipid peroxyl radicals (91), while CO has both anti-apoptotic and anti-inflammatory properties (92).

Superoxide dismutase (SOD) catalyzes the formation of H_2O_2 from superoxide radical. There are three forms of SOD expressed in the eukaryotic cells, encoded by three separate genes: the copper-zinc SOD (CuZnSOD) or SOD 1 found in the cytosol, the manganese-containing SOD (MnSOD) or SOD 2, localized to the mitochondrial matrix, and the extracellular form of CuZnSOD, which is expressed at low levels in plasma and extracellular fluids, where it partially protects NO by reducing the concentration of superoxide radical (93,94). When the activity of SOD is chronically elevated above normal, as occurs in Down syndrome subjects, it can be pro-oxidative, which is why the disease is believed to be, at least in part, a consequence of excessive free radical generation (10,95). Catalase (a hemoprotein with four heme groups) and glutathione peroxidase (GPx) (containing selenium as a prosthetic group) catalyze the breakdown of H_2O_2 to water and oxygen, with glutathione peroxidase being more important in neural tissue (87), perhaps partly because, relative to GPx which is found in high concentrations in the brain, there is little catalase in both grey and white matter (30). In addition, aside from its ability to eliminate H_2O_2, GPx is also involved in the detoxification of lipid peroxyl radicals (30) or lipid hydroperoxides (96). These lipid hydroperoxides can decompose to alkoxy radicals and aldehydes in the presence of Fe^{2+} (30). Glutathione peroxidase makes use of reduced glutathione (GSH), a tripeptide synthesized intracellularly (30), as a substrate that donates hydrogen and thus becomes converted to oxidized glutathione or glutathione disulphide (GSSG). Glutathione disulphide can be converted back to glutathione by glutathione reductase in an NADPH-consuming process (12). Another cytosolic enzyme, quinone reductase, first noted for its protection against carcinogens, also catalyzes a two-electron reduction of quinones to hydroquinones, which are more stable and less reactive (97).

Unlike superoxide anion and H_2O_2 whose cellular levels are regulated by antioxidant enzymes as mentioned, there are no analogous enzymes for regulating the hydroxyl radical, which may further explain the extreme reactivity and toxicity of the oxyradical. Its management therefore depends on the endogenous antioxidants ascorbate and reduced glutathione (GSH) (98,99). Interestingly, cells have also developed complex metal-transport systems that deliver copper and iron to metallo-enzyme and proteins, thus preventing the presence of unbound copper in the intracellular environment (33), a mechanism that may limit the Fenton reaction that generates the hydroxyl radical.

3. OXIDATIVE DAMAGE IN NEURODEGENERATION

Oxidative stress represents an important pathway leading to neuronal degeneration and is implicated in many neurodegenerative diseases (which could be familial or sporadic) including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) (Lou Gehrig's disease) and Friedreich's ataxia (FA) (33,100). Similarly, there is evidence of oxidative stress in conditions involving acute damage to the brain, such as trauma, stroke, hypoxic-reperfusion and epilepsy (13,30,101). However, it is still far from clear whether oxidative damage is a cause or consequence of neurodegeneration. Although the aetiology, symptomatology and disease locus are not the same for all

neurodegenerative diseases, oxidative damage represents one of the identified rallying point for most of them (17). It is known that increasing age represents a major risk factor in neurodegenerative conditions, and normal aging is associated with a rise in the levels of copper and iron in the brain tissue (33). Consequently, the appealing nature of the oxidative stress hypothesis is related to its ability to explain the cumulative damage associated with the delayed onset and progressive nature of neurodegenerative conditions, and if the relationship between oxidative stress and glutamate neurotransmission is well dissected, it may lead to effective therapies for the management of the conditions without jeopardizing normal excitatory neurotransmission (30). The mitochondria play a pivotal role in the intricate relationship between oxidative stress and neurodegeneration, and a dysfunction of the cytochrome oxidase and the mitochondria is a common theme in the pathophysiology of neurodegenerative diseases (102,103).

At this point, a brief overview of the major neurodegenerative conditions and their link to oxidative stress is useful. In AD, the major histopathological features are the deposition of senile plaques, accumulation of intracellular neurofibrillary tangles of hyperphosphorylated tau filaments, and oxidative damage (37,104-106). The senile plaques contain mainly amyloid beta (Abeta)-peptide derived from the proteolytic processing of the amyloid precursor protein (APP). The Abeta peptide is responsible for the development of AD, inducing neuronal injury through oxidative stress (107,108), although both APP and Abeta have been suggested to have a role in metal homeostasis and the latter could have an important physiological role as an antioxidant that is impaired by the aging process (33). Mutations have been identified in the *APP* gene, the gene encoding tau protein, the presenilin genes (*PSEN1* and *PSEN2*) and the apolipoprotein E gene (*APOE*) (APOE4 protein significantly associated with sporadic AD) in AD (104-106). The presenilins seem to be responsible for the catalytic activity of the gamma-secretase complex, the enzyme which processes APP to form Abeta (104). The site for Abeta deposition seems to be the striatum (109,110), a part of the brain where oxidative stress plays a predominant role in cell death (111) and which is highly susceptible to injury from ischemia and reperfusion (112). There is evidence for a role of Zn^{2+} in amyloid plaque formation, but the major sources of oxidative stress and free-radical production in AD are copper and iron when bound to Abeta, and the various forms of Abeta in the AD brain are commonly found to be oxidatively modified (33). The toxicity of synthetic Abeta in the presence of Cu^{2+} is inhibited by catalase, thus implicating H_2O_2 in the pathway (113).

Idiopathic PD is a chronic, progressive disorder characterized by rigidity, tremor at rest, and bradykinesia, resulting from selective degeneration of neuromelanin-containing neurons, most notably the nigral dopaminergic neurons projecting to the caudate-putamen (30). There is also the deposition of intracellular inclusion bodies which contain mainly the protein alpha-synuclein that is ubiquitously expressed in the brain (33), mutations of which result in the familial form of PD (114). The accumulation of neuromelanin is age-dependent and this pigment contains essentially products of dopamine redox chemistry. The catechol dopamine can generate H_2O_2 and the oxidative stress in PD could come from a failure to regulate dopamine-iron biochemistry (33). Interestingly, alpha-synuclein is known to modulate dopamine activity (33). Other mutations that have been identified in PD are in the genes encoding the proteins parkin, DJ-1, PINK-1 and LRRK2 (115).

Huntington's disease is a hereditary, autosomal dominant disorder with features such as disturbances in movement, psychiatric symptoms, and progressive dementia (30). The underlying mutation is an expanded CAG repeat in exon 1 of the coding region of the *HD* gene (unstable trinucleotide repeat on chromosome 4). The CAG triplet encodes the amino acid glutamine in the gene product huntingtin (116). The elongated polyglutamine (polyQ) stretch of mutant huntingtin therefore affects its interaction with huntingtin-binding proteins and increases their susceptibility to aggregation (117). In this condition, the medium spiny neurons in the striatum are lost (118). The pathology of HD has been linked to mitochondrial function and oxidative stress (30,116).

Amyotrophic lateral sclerosis occurs in mid-life, due to a selective and progressive degeneration of the lower motor neurons in the spinal cord and the upper motor neurons in the cerebral cortex (30,33). It is characterized by the deposition in neural tissue of a misfolded protein, CuZnSOD (SOD1). The mutations lead to a toxic gain of function by the cupro-enzyme SOD, the nature of which is either due to misfolded aggregated forms of SOD or a pro-oxidant activity of SOD generating ROS (33,119,120).

Friedreich's ataxia is due to an abnormal GAA trinucleotide expansion within the first intron of the gene encoding the mitochondrial protein frataxin, causing frataxin deficiency. Iron therefore accumulates in the mitochondria, precipitating oxidative stress that leads to cardiomyopathy and neurodegeneration (33).

In all of these and other CNS disorders, the brain is at risk from oxidative damage due to high oxygen consumption (20% of the total basal O_2 consumption of the body), critically high levels of both iron and ascorbate, relatively low levels of antioxidants (e.g., catalase levels are generally low in most brain regions), a tendency to accumulate metals and low regenerative capacity (17,33,121). In addition, microglia, the resident immune cells of the brain, produce superoxide and H_2O_2 upon activation; they also produce cytokines which can enhance more production of ROS and NO (17). Astrocytes equally produce cytokines through which they can be activated to generate NO from iNOS (17). The microglia and astrocytes are therefore major mediators of inflammatory processes in the brain (122). Some cytochromes P450 (CYPs) are also a source of ROS in certain brain regions (123).

It is known that neurodegeneration increases with aging, particularly of the CNS, and this is related to damage inflicted by free radicals, as there are regions of increased

iron (which catalyzes free radical formation) within the brain and neural tissue which are rich in PUFAs and therefore susceptible to attack by free radicals (87). Also, a decrease in defense mechanisms with age causes increased oxidative damage and free radical generation, and in certain disease states such as ischemia, radicals can be produced at elevated rates (124). Similarly, damage to mitochondria can lead to the production of more ROS, causing further damage, and in AD, interference with mitochondrial ATP supply through mitochondrial damage could cause cells to increase Abeta production (125). It has been proposed that oxidative stress is involved in the aging process both by inducing damage to mitochondrial DNA and by other mechanisms (126,127), and the superoxide anion can inactivate enzymes involved in energy production and amino acid metabolism (128,129). In general, the hallmarks of oxidative damage resulting from toxic oxidative stress include alterations in mitochondrial lipids (e.g., cardiolipin) and mitochondrial proteins (e.g., aconitase and uncoupling protein 2), and increases in DNA base oxidation products, oxidative protein damage, and lipid peroxidation end-products (130-133). Lipid peroxidation is one important cause of neuronal damage in neurodegenerative diseases and the accumulation of lipid peroxidation products like malondialdehyde (MDA) and 4-hydroxy-2,3-nonenal (HNE) has been demonstrated in affected regions in brains of AD patients (134). Other breakdown products linked to AD, PD and ALS include acrolein, F_2-isoprostanes, and thiobarbituric acid-reactive substances (TBARS), elevated levels of which have been found in brain tissue, cerebrospinal fluid (CSF), or plasma. These may serve as markers of oxidative stress, as could changes in the endogenous defense systems (33). Oxidized and nitrated proteins can also accumulate, producing oxidative stress, especially when there is impairment of the ubiquitin-proteasome system that is responsible for removing them (17). Increased levels of oxidative damage to DNA, lipids and proteins have been detected in post-mortem tissues from patients with PD, AD and ALS, and some of the observed changes may occur early in disease progression (130). Hydrogen peroxide is a mediator of damage in neuropathological conditions and has been shown to induce lipid peroxidation in rat brain homogenates (135). Its increased production in the CNS has been implicated in the pathogenesis of PD, AD, ischemic reperfusion and stroke (136). In PD, H_2O_2 generated from presynaptic Lewy body alpha-synuclein may be associated with neurodegeneration of nigral cell bodies in the substantia nigra and destruction to the nigrostriatal tract (137), while in AD, Abeta plaque builds up in the brain and causes intracellular accumulation of H_2O_2 (138). The use of H_2O_2 as an inducer of damage is, therefore, a potentially clinically important model of oxidative stress, although a number of reports have indicated that the modulatory and pathological consequences of H_2O_2 are often mediated by the hydroxyl radical and not by H_2O_2 per se (139,140). It should be pointed out here that H_2O_2 on its own is capable of causing damage in a number of ways, especially when it builds up in high concentrations. For example, as reported in drug-induced apoptosis, it could inhibit the ATP-dependent Na^+/H^+ antiporter which regulates cytosolic pH, thus leading to profound acidification of the intracellular milieu (19).

Relating to the roles of metal in neurodegeneration, it is agreed that iron is known to be critically vital to biological reactions in living cells and in the brain is required for sustenance of brain's high respiratory activity, myelinogenesis, and for the production of many neurotransmitters including dopamine, noradrenaline, and serotonin and the generation of GABAergic activity (141), but the divalent state of iron makes it very reactive and therefore extremely toxic if its intracellular concentrations are not tightly regulated (142). The iron content of the brain is known to increase in early life to reach a maximum at about 30 years of age (17). Iron overload in the early stages of life has been reported to induce cognitive impairment, possibly by inducing oxidative damage in the brain (143). Iron, copper and other metals promote aggregation of proteins such as alpha-synuclein and Abeta (17,33). This is why proteins such as ferritin and transferrin, which sequester transition metal ions, have neuroprotective properties. It is known that superoxide can release iron from ferritins (144), while peroxynitrite can displace iron from iron-sulfur proteins and copper from copper-containing proteins such as ceruloplasmin (145). Iron has been clearly identified with the pathology of PD, and iron, copper and zinc have all been associated with the progression of AD (121), although it is thought that iron deposition may be a late stage in tissue injury in PD or ALS (17). The proteins implicated in age-dependent neurodegenerative diseases (such as Abeta in AD, alpha-synuclein in PD, SOD1 in ALS and frataxin in FA) may bring about inappropriate reactions of Cu^{2+} or Fe^{3+} with oxygen (33).

4. MECHANISMS OF OXIDATIVE NEURONAL DAMAGE AND DEATH

Agents such as ROS which induce cell death in biological tissues bring about demise by either apoptosis or necrosis, which are the two classical extreme pathways of cellular death, each with its distinct features. This cell death pathway classification has been primarily based on morphological criteria (146). However, there is growing evidence that a number of death processes may simultaneously activate the two pathways, thus resulting in a form of "hybrid" death that is neither entirely apoptotic nor entirely necrotic (147-150). It is important to note that neuronal cell death induced by oxidative damage shares similarities with cell death in other tissues. Therefore, most of the pathways discussed here are also applicable to other tissues and even to neuronal injury resulting from non-oxidative toxic stimuli.

4.1. Apoptotic neuronal death

Apoptosis is the basis of programmed cell death (PCD), a delayed form of cell death from less severe insults that is energy-dependent and associated with activation of a genetic program (151). It is an important mechanism for the selective elimination of mammalian cells distinct from the process of cell death by necrosis (152). Recent widespread interest in cell death processes has generated controversy

over definitions that distinguish apoptosis from other forms of cell death (153). For example, apoptosis was earlier defined as the process of cell death associated with caspase activation or caspase-mediated cell death, a view that presumes that caspases represent its final common mechanistic pathway (154), but this definition now needs to be expanded, as a number of caspase-independent apoptotic mechanisms have been recently reported, mainly involving the apoptosis-inducing factor (AIF). It seems there is no consensus yet on the classification of the different forms of PCD. In their own review, Krantic and colleagues highlighted a more-encompassing classification that is based on nuclear morphology, dividing PCD into classical apoptosis, apoptosis-like PCD, and necrosis-like PCD, respectively characterized by nuclear condensation that is 'crescent-like,' partial or peripheral, or absent (101). Classical apoptosis is the best-known phenotypic expression of PCD, resulting in caspase activation. Apoptosis-like PCD is broader and includes caspase-independent mitochondrial pathways. With regard to necrosis-like PCD, the cell death program is triggered by organelles other than mitochondria, such as lysosomes, endoplasmic reticulum (ER) and the nucleus, and by proteases other than caspases, such as cathepsins and calpains originating from lysosomes and the ER, respectively (101). Generally speaking, hallmarks of apoptosis include shrinkage of the cytoplasm, phosphatidylserine translocation and condensation of nuclear material into "clumps" (155,156). Subsequently, the nucleus undergoes fragmentation and the ER fuses with the plasma membrane, forming vesicles and convoluting its surface. The final stages of apoptosis witness cellular fragmentation forming membrane-bound apoptotic bodies that contain intact cytoplasmic organelles and nuclear fragments (157).

There are two primary modes of apoptotic induction. One is through the death receptors in the plasma membrane, called the extrinsic pathway, and the other, the intrinsic pathway, is via mitochondrial dysfunction (158). The extrinsic pathway involves cell surface death receptors which are members of the nerve growth factor/TNF superfamily of receptors and include Fas (CD95/APO-1), tumor necrosis factor (TNF) receptor 1 (TNFR1), as well as death receptor (DR)-3, DR-4, and DR-5 (159-162). They can be activated by both cell surface-bound and soluble ligands such as FasL (CD95L), tumor necrosis factor-alpha (TNF-alpha), lymphotoxin-alpha (LT-alpha) and TNF-related apoptosis-inducing ligand (TRAIL) (159,160). These receptors are widely distributed in the body and are often found in cells from the immune system and in many somatic tissues (161). Tumor necrosis factor type 1 receptor (TNFR1) was shown to be required for Abeta protein-induced neuronal death (163). It is also becoming increasingly recognized from a molecular perspective that neuronal PCD consistently shows a unique property of pathological re-initiation of the cell cycle, as examined later in this review (101).

4.1.1. Caspase-dependent mechanisms

It is well recognized that in apoptosis, the effector molecules are the cysteine-dependent, aspartate-directed proteases called caspases (cysteine-aspartate proteases), even though BAD (Bcl-X_L/Bcl-2-associated death promoter) can initiate apoptosis and a reciprocal regulation of Bcl-2 and Bax expression seems to occur in glutamate-induced excitotoxicity (164). Activation of caspase-zymogens is an early event in the process of apoptosis. Once cytochrome c (a water-soluble, basic, heme-containing protein that binds to the anionic phospholipid cardiolipin, located exclusively on the inner mitochondrial membrane of eukaryotic cells) (165) is released from the mitochondria, it combines with apoptotic protease-activating factor-1 (Apaf-1) and the duo recruits and activates pro-caspase 9 to form the apoptosome by means of which cleavage and activation of pro-caspase-3 into caspase-3 occur. There are upstream initiator caspases that begin the proteolytic cascade in apoptosis (caspases 8 and 9), and downstream effector caspases that cleave cellular proteins (caspases 3, 6, and 7) (166). For the extrinsic pathway, the binding of members of the DR family (e.g. Fas–TNFR-1–TRAIL-R1) and their cognate ligands (167) results in an oligomerization of receptors and a subsequent activation of procaspase-8 and, depending on the cell type, active caspase-8 either cleaves and activates procaspase-3 directly or it cleaves the proapoptotic Bcl-2 protein Bid to tBid, which then recruits the mitochondrial apoptotic pathway, resulting in the activation of procaspase-3 and other effector caspases (168). It therefore means that the two pathways of apoptosis converge on caspase-3 induction. Both pathways are associated with activation of caspase-activated DNase (CAD) and also with typical internucleosomal DNA fragmentation (169). There is, however, growing evidence that caspases and other apoptosis regulators participate, not only in cell death, but also in the control of cell cycle (166,170).

4.1.2. Caspase-independent mechanisms

It is now clear that a number of apoptotic events occur independently of caspase activation, and energy depletion and the generation of free radicals have been shown to contribute to caspase-independent neuronal death (171). The best example of an effector of caspase-independent cell death is the AIF, which is normally localized to the inter-membrane mitochondrial space (172,173). AIF is a 67-kDa flavoprotein that is similar to bacterial oxidoreductases (172) and is evolutionarily conserved. It displays NADPH oxidase and monodehydroascorbate reductase activities (174). Upon mitochondrial outer membrane permeabilization (following cytotoxic insults such as oxidative stress from ROS), it translocates to the nucleus where it induces peripheral chromatin condensation and large (high-molecular-weight) DNA fragmentation. This translocation of AIF to the nucleus appears to be a general feature of apoptosis in mammalian cells (14,175). However, because AIF lacks any intrinsic endonuclease activity, once in the nucleus, it recruits a number of downstream nucleases including cyclophilin A and endonuclease G (176,177). Although its physiological role is not clear, it has been suggested to participate in scavenging ROS (178). DNA binding by AIF may be required for its apoptogenic function in the nuclear compartment (14).

4.1.3. Membrane Permeability Transition (MPT)

This is the phenomenon whereby there is an opening of an unspecific pore, permeability transition pore (PTP), on the mitochondrial inner membrane, allowing the flow of solutes < 1.5 kDa out of the mitochondrial matrix (179). This leads to the collapse of the mitochondrial membrane potential, ψ_m. Cytochrome c leaks through the multiprotein complex (containing hexokinase, porin, and adenine nucleotide translocator (ANT)) (180) into the cytosol, where it combines with Apaf-1 to activate procaspase-9 to caspase-9, hence forming the apoptosome, which in turn activates caspase-3, the terminal caspase that executes the apoptotic command. Agents that block the PTP are therefore able to protect against apoptotic death. Such agents include cyclosporin A, which also blocks calcineurin (protein phosphatase 2B). The opening of the pore may be involved in cellular apoptosis, as AIF is released from the mitochondrial intermembrane space as a result of the destruction of the mitochondrial outer membrane after excessive mitochondrial matrix swelling (181). Permeability transition pore formation is enhanced by increased production of ROS (179).

4.2. Necrotic neuronal death

ROS at very high concentrations are able to induce neuronal death through necrosis, which, generally speaking, results from severe insults and is associated with changes in calcium and sodium ion homeostasis. It is generally believed that low concentrations of toxic stimuli induce apoptosis while high concentrations precipitate necrosis (182). For example, stimulation of cortical neurons with high NMDA concentrations causes necrosis, while low concentrations result in apoptosis (183). The duration and extent of calcium influx could determine the fate of neurons: survival, death by apoptosis, or necrotic lyses (183,184). Major morphological hallmarks of necrotic cell death include the swelling of cells and of organelles (mainly the mitochondria), followed by disruption of the organelles. There is early compromise of the integrity of the cell membrane, and the plasma membrane ruptures, permitting the leakage of cellular contents into the extracellular compartment. Random DNA degradation also occurs following histone proteolysis. Necrotic cell death is distinct from apoptotic cell death in a number of ways (151). Necrosis is largely energy (ATP)-independent, unlike apoptosis which requires energy to proceed. In fact, the ability or otherwise of the mitochondria to produce enough ATP may switch neurons towards one or the other of the two cell death types (182,185), thus establishing further the critical role of the mitochondria in the execution of cell death. Furthermore, in necrosis, large groups of adjacent cells are usually affected -as opposed to individual cells in apoptosis- and there is a promotion of an inflammatory reaction, unlike in apoptosis where no inflammation is evident. Overall, necrosis is usually a pathological event while apoptosis could either be physiological or pathological (151,186).

4.2.1. Role of poly (ADP-ribose) polymerase (PARP)

In the process of inducing neuronal cell death, ROS are able to activate poly (ADP-ribose) polymerase-1 (PARP-1), which is the best known and most important of a family of abundant chromatin-bound nuclear proteins responsible for the repair of DNA strand nicks and breaks and important for the maintenance of genomic stability and nuclear homeostasis (14,187,188). PARP-1 generates nicotinamide and long-chained, branched polymers of ADP-ribose (PAR) from oxidized nicotinamide adenine dinucleotide (NAD^+), attaching these polymers (50-200 residues) to nuclear proteins including histones, topoisomerases I and II, DNA polymerases, DNA ligase-2, high-mobility group proteins, transcription factors, and itself (14,189,190). Poly (ADP-ribosyl)ation is a unique biochemical pathway, with PAR synthesis and degradation known to be present in all mitotic and post-mitotic cells with few exceptions in mammalian organisms (191). In response to DNA damage induced by substantial levels of ROS, PARP-1 activity becomes rapidly upregulated 500-fold upon binding to DNA strands and breaks (14). This overactivation of PARP-1 leads to cell death by a mechanism that involves metabolic derangement resulting from the depletion of NAD^+ and ATP, as the synthesis of every molecule of NAD^+ requires four molecules of ATP. The continuous depletion of NAD^+ and ATP over time brings about irreversible cellular energy failure and the demise of the cell in a characteristically necrotic manner (192,193), although PARP is also relevant to apoptotic cell death (194). Loss of energy-dependent cellular function also occurs through impairment of the oxidoreduction capacity of NAD^+, which is required in the mitochondrial electron transport chain to maintain its proton gradient and thereby generate ATP (195). These observations underlie the suicide hypothesis. It is now recognized that caspase-3 could inactivate PARP in order to turn off an energetically expensive DNA repair pathway and therefore maintain ATP levels required for the execution of apoptosis (196). The extent of oxidant-induced ATP depletion and cell fate could be modified by PARP inhibition (197).

Recently, a number of other mechanisms have been mooted to explain the basis for cellular death from PARP-1 overactivation. The most popular at the moment is the now established link between overactivation of PARP-1 and apoptosis, specifically in relation to the induction of AIF, as it is known that free radical/oxidant attacks (e.g., induced by H_2O_2) and a variety of environmental and chemical stimuli (e.g., the DNA-alkylating agent *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine or NMDA) can trigger the overactivation of PARP-1 in response to DNA damage (14,188). This process in turn stimulates the translocation of AIF from the mitochondrial intermembrane space to the nucleus, triggering chromatin condensation, massive DNA fragmentation and nuclear shrinkage (14,188,198). Following this, phosphatidylserine becomes exposed, cytochrome c is released at a later time point and caspase-3 is activated. However, AIF could mediate both caspase-dependent and caspase-independent cell death, although the cross-talk between AIF and the caspase pathway is complex (195). Pharmacological inhibition of PARP-1 or genetic knockout of PARP-1 has been shown to be therapeutically efficacious in experimental models of disorders characterized by DNA damage, such as ROS-induced injury, ischemia, ischemia-reperfusion injury, diabetes, shock, inflammation, cancer, excitotoxic neuronal

cell death (14,199,200), 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced Parkinsonism and traumatic spinal cord injury (200), most of which are associated with oxidative damage. The termination of the toxic action of PARP-generated poly (ADP-ribose) polymer (PAR) occurs through the rapid action of another enzyme, poly (ADP-ribose) glycohydrolase (PARG), which catalyzes the hydrolysis of PAR into free ADP-ribose (201,202). In the CNS, PARP and PARG are present throughout the brain and spinal cord (195).

4.3. "Hybrid" or "intermediate" cell death

It is now recognized that in most cases, neuronal death occurring following damage by ROS and other neuronal insults (e.g., excessive levels of glutamate leading to excitotoxicity) shows features of both apoptosis and necrosis, an observation that challenges seriously the binary classification of cell death. This emerging pattern now constitutes what is now referred to as "hybrid cell death" or "intermediate" type of cell death, which has recently become a subject of intense investigation (147,148,186,203). There is currently a plethora of terms used to describe distinct forms of cell death (e.g., anoikis and necroptosis). In many neuropathological conditions, apoptosis and necrosis occur simultaneously (204,205). In experimental models of stroke, neuronal death in the ischemic core is necrotic, but in the less severely affected penumbra or border regions, it is delayed and apoptotic (206-209). The resonating message from these findings is that it may take some time before we attain a reasonably clear understanding of cell death processes.

4.4. Activation of signaling cascades

Reactive oxygen species have ability to regulate several signaling cascades involving tyrosine phosphatases, tyrosine kinases, protein kinases (e.g., PKC), MAPKs (ERK1/2, JNK, p38), and transcription factors such as NF-kappaB, hypoxia-inducible factor-1alpha (HIF-1alpha), AP-1 and NF-E2-related factor-2 (Nrf-2) (6,210,211). ROS such as H_2O_2 are able to inhibit protein tyrosine phosphatases, although the high concentrations required for such oxidative inhibition call to question the physiological relevance of the inactivation. In these conditions, the activities of many protein tyrosine kinases (e.g., Lck, Fyn, Syk, ZAP70) were also found to be increased. The MAPK species JNK and p38 have been shown in several studies to be strongly activated by ROS, and H_2O_2 in particular is a strong messenger for NF-kappaB activation (211,212). Again, there is a link between oxidative stress, the phosphoinositide-3 kinase (PI3K)/AKT pathway and the MAPK pathway (213). The transcription factor Nrf-2 influences cytoplasmic responses to oxidative stress by transcriptional activation of genes involved in GSH synthesis, including xCT, gamma-GCLC, gamma-GCLM, and GPx (214-216). Nrf-2 is a basic leucine zipper transcription factor that binds to antioxidant response element (ARE) sequences in the promoter regions of specific genes. Its inactive form is bound to the Kelch-like ECH-associating protein 1 (Keap1) in the cytoplasm, under physiological conditions. However, following oxidative stress, Nrf-2 is released from Keap1, and is translocated to the nucleus (216,217), where it interacts with the small Maf proteins FosB, C-Jun, JunD, ATF2, or ATF4. The complexes (heterodimers) then interact with ARE promoter elements to induce gene expression (216,218). With regard to protein kinases, the serine/threonine kinase protein kinase C (PKC)-alpha and some other PKC isoforms can be activated by H_2O_2 in a phospholipid-independent process involving tyrosine phosphorylation in the catalytic domain, while ROS can also activate cRaf. The oxidative activation of PKC-alpha can be enhanced in the presence of vitamin A (211). It should be noted that there are a number of other signaling pathways associated with ROS that are not discussed here.

4.5. Aberrant cell cycle re-entry

It has now been recognized that one of the possible mechanisms by which oxidative stress induced by ROS leads to neuronal death is forced re-entry of such neurons into the cell cycle, and the concept has developed into an active line of research. There is growing evidence that the death of terminally differentiated neurons is intimately linked to aberrant re-entry into the cell cycle, a phenomenon that had been reported in AD patients, Down syndrome patients and in many neurodegenerative models (13). Evidence for this association is based on observations that tumors arising from terminally differentiated neurons are very rare, and forced expression of oncogenes in cells that are terminally differentiated causes cell death instead of cell proliferation (219). Many animal models have also lent support to this hypothesis. Neurotoxic insults such as kainic acid and Abeta peptides have been shown to induce unscheduled cell cycle re-entry, as indicated by increased neuronal expression of cell cycle proteins such as cyclin-dependent kinase 2 (CDK2), cyclin E, cyclin A and E2F-1, increased phosphorylation of the retinoblastoma (Rb) protein, and the replication of DNA prior to apoptosis (220-222). However, despite the link between oxidative stress and aberrant cell cycle abnormalities, the mechanisms involved are still far from clear, although the possible involvement of the AIF has been shown (178). Besides, there is another interesting but alternative concept that espouses cell cycle re-entry in neurons as a prerequisite for DNA repair (223). A better understanding of these intriguing phenomena is therefore highly important and desirable, as both oxidative stress and cell cycle re-entry have been implicated in the onset of later-onset neurodegenerative diseases (13,101).

5. PROTECTION AGAINST OXIDATIVE DAMAGE AND DEATH: EXISTING AND EMERGING MECHANISMS AND CURRENT AND FUTURE CHALLENGES

In order to protect against oxidative damage and death of neurons, a number of strategies could be adopted which either limit the levels of ROS in the brain or reduce the damage caused by oxidative stress. The protective mechanisms include the regulation of oxygen, boosting antioxidant levels, (receptor-mediated) lowering of ROS production, repairing oxidative damage, and eliminating unwanted (damaged) proteins and lipids.

5.1. Regulating oxygen, boosting antioxidant levels and lowering ROS production

The brain requires an abundant supply of oxygen. However, if the brain oxygen levels are not allowed to go beyond what is required for normal physiological functioning, it is possible to reduce neuronal damage due to oxidative stress, since excessive ROS production would be avoided (17,224,225).

Measures to boost antioxidant levels or lower ROS production could also prove significantly neuroprotective. It was reported that overexpression of antioxidant enzymes protected cultured hippocampal and cortical neurons from necrotic insults (226). Both superoxide and H_2O_2 are involved in reactions that produce more toxic ROS. Therefore, a promising neuroprotective strategy would be to reduce or remove the superoxide and H_2O_2 produced in the cell. As mentioned earlier, SOD converts superoxide to H_2O_2, thus removing superoxide; but what happens to the H_2O_2 produced? Catalase could destroy the generated H_2O_2, but it is not abundant in the brain or present in the mitochondria where much of the superoxide is produced (17,227). Fortunately, however, there are other enzyme systems which remove H_2O_2-glutathione peroxidases (GPx) and peroxiredoxins. Glutathione peroxidases are selenium-containing enzymes that reduce H_2O_2 through the oxidation of reduced glutathione (GSH). Glutathione reductases would then convert the oxidized glutathione (GSSG) back to GSH. They have an additional ability to act on other peroxides (228). Astrocytes may assist neurons to boost GSH levels by releasing a GSH precursor, cysteinyl-glycine (17,229). It appears that the peroxiredoxins are the most important removal systems for H_2O_2 in animals and they can also reduce organic peroxides (230). Although they may remove H_2O_2 more slowly than GPx, the peroxiredoxins are present in large amounts in subcellular organelles and cytosol (230) and are effective at relatively low concentrations (low K_m). However, when levels of H_2O_2 are too high, peroxiredoxins can be inactivated, thus causing neuronal damage (130).

Apart from the antioxidant enzymes, administration of antioxidant vitamins, molecules, or supplements, such as ascorbate (vitamin C), alpha-tocopherol (Vitamin E), melatonin, uric acid, lipoic acid, creatine, coenzyme Q/Q10 (ubiquinone), curcumin, carotenoids and flavonoids, is a promising strategy in protecting against oxidative neuronal damage. Growth factors such as neurotrophins and steroid hormones also have the capacity to prevent or mitigate damage due to oxidative stress. It should be emphasized that the mechanisms by which these different substances exert protection against oxidative damage are diverse. Cerebrospinal fluid (CSF) levels of ascorbate are high and neurons concentrate and take it up readily. On the other hand, while astrocytes also concentrate ascorbate, they take up dehydroascorbate for conversion to ascorbate intracellularly (99). Ascorbate is important for CNS function (231) and is generally anti-oxidant in its action (232,233) as well as having other cellular functions (234) leading to neuroprotection and improvement of cognitive function (235). However, the outcome following its administration could also be pro-oxidant, especially when iron or copper are present, which can be reduced to form hydroxyl radicals from H_2O_2 and can also decompose lipid peroxides (236). With regard to the transition metals iron and copper, a number of proteins could prevent their oxidation and thus avoid the Fenton chemistry that leads to toxic ROS formation. Examples of such proteins are ceruloplasmin (237), haptoglobin, (238), metallothioneins (239), histidine-containing dipeptides (e.g., carnosine) (240) and heme oxygenase (HO), which degrades heme, producing carbon monoxide (CO) and biliverdin. Biliverdin or bilirubin (produced from biliverdin) also has some antioxidant properties (241), although excessive bilirubin causes neurotoxicity (242). Both the inducible HO-1 and constitutive HO-2 forms are present in the brain, where the levels of the former are up-regulated following bleeding and ischemia-reperfusion and in some neurodegenerative diseases including AD (243,244). Metal chelators such as desferrioxamine could also offer protection against oxidative damage by binding iron, thus preventing its availability for neurotoxic transformation (245).

Neurotrophins promote the growth and survival of neurons and also have protective effects against oxidative stress. They include brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) (33,246,247). Gonadal steroid hormones such as estradiol and progesterone possess antioxidant properties too. Estrogens are known to have antioxidant and neuroprotective effects, as they function as radical scavengers and can inhibit lipid peroxidation both *in vitro* and *in vivo* (248-250). 17-Beta estradiol has been shown to reduce lipid peroxidation induced by quinolinic acid in brain homogenates (251), while estrogen, progesterone, testosterone and luteinizing hormone all have neuroprotective properties and can influence oxidative stress and Abeta metabolism in AD (252).

Some of the antioxidant molecules such as alpha-tocopherol (vitamin E) are tightly regulated (17,253). This compound acted synergistically with coenzyme Q to improve learning ability in old mice (254), the latter having been shown to offer protection against striatal lesions and MPTP toxicity and in Huntington's disease (255) through its electron transport action in mitochondria and/or the antioxidant property of ubiquinol (17). Vitamin E, through a phenolic hydroxy (OH) group, scavenges peroxyl radicals thereby inhibiting lipid peroxidation (256). It has been reported to reduce lipid peroxidation induced by nitric oxide in rat brain homogenates (257).

Another endowment from nature is a wide range of polyphenolic compounds, mostly flavonoids, found in a variety of natural diets including fruits, vegetables, grains, nuts, tea and wine (258), which have been shown to have a diverse range of biochemical and pharmacological activities (259), including antioxidant, anti-inflammatory, anti-carcinogenic, anti-lipidemic, anti-infective and anti-apoptotic properties (260,261). The mechanisms by which they protect cells against oxidative damage are not well known, although they may interact with mitochondria and

signaling cascades (e.g., MAPKs) (262). The *Ginkgo biloba* extract (rich in flavonoids) orally administered has been suggested to protect against the development of dementia (17). The flavonoids belong to several chemical classes. Examples of flavonoids are epigallocatechin gallate, quercetin, luteolin, hesperitin, naringin, naringenin, kaempferol, rutin, neohesperidin and resveratrol. A current challenge in antioxidant therapy is to develop molecules capable of rapidly penetrating the blood-brain barrier (BBB). Although natural antioxidants such as carotenoids and flavonoids do not enter the brain readily in the adult (130), some are able to cross the BBB (263-265). It has been suggested that many of the other actions of flavonoids unrelated to antioxidant effects may be responsible for some or all of their neuroprotective actions (265). The complexity of natural dietary products containing several flavonoids makes interpretation of their apparent antioxidant effects difficult. Again, there is a possibility that some of the biological actions of these compounds are mediated *in vivo* by their active metabolites. The biological actions of flavonoids involve complex mechanisms and are the subject of intense research. In fact, some have been shown to produce ROS (e.g., quercetin, resveratrol), thus contributing to cell death. In a study of its protective effect against H_2O_2, the same concentration of kaempferol that protected cells against loss of viability caused significant DNA damage and apoptosis when applied on its own (266). Flavonoids are also capable of inducing oxidative stress by impairing antioxidant defense systems (266). Currently, they are marketed as components of functional food and as supplements, not as drugs (266). Overall, traditional remedies may constitute lead compounds in the search for potential therapeutics for arresting neurodegeneration or improving brain function.

Another promising antioxidant strategy exploits pharmacological blockade of glutamate receptors, especially the NMDA receptor which mediates excitotoxicity. Since overactivation of the receptor causes intracellular calcium overload and the production of ROS and RNS (86), ligands that are effective in blocking it should be able to lower pathological elevations of ROS and RNS. The challenge, however, is the need for such antagonists at the NMDA receptor to spare glutamatergic neurotransmission that is essential for normal physiological functioning.

5.2. Repairing oxidative damage and eliminating unwanted proteins and lipids

In addition to the neuroprotective strategies already mentioned, measures could be developed to boost the reparative capacity of the brain. There are defenses in the brain for repairing oxidative damage, as neuronal nuclei and mitochondria are endowed with enzymes that repair oxidatively damaged DNA (267), lipids (268) and proteins (17). Phospholipase A_2 cleaves and destroys damaged lipids (268), while methionine sulfoxide reductase enzymes convert methionine sulfoxide in oxidized proteins back to methionine. There are mechanisms for marking damaged proteins for proteolytic removal in order to prevent cell death from their accumulation (269-271), although aggregation and precipitation occasionally lowers toxicity owing to sequestration (269,272). Lysosomes contain hydrolytic enzymes that degrade unwanted proteins and organelles (271), while Lon-proteinase degrades aconitase and other mitochondrial proteins that have been oxidized (273). Apart from these, the ubiquitin-proteasome system in eukaryotic cells is a major remover of unwanted proteins (274). Proteins are first marked for degradation by the process of ubiquitination, which is ATP-dependent and occurs in steps through the attachment of the heat shock protein ubiquitin, thus allowing the 26S proteasome to recognize its targets for degradation. However, proteasome activity seems to decrease with age (275) and some oxidatively damaged proteins may paradoxically inhibit proteasome function (276).

5.3. Antioxidant therapeutics: From bench to bedside and back

There is active research interest in the development of antioxidant therapeutics for the management of neurodegenerative diseases. However, to date, there is still a huge challenge in the clinical exploitation of the beneficial effects of antioxidants observed in experimental models. One major requirement for drugs that would be effective in treating neurodegeneration is the ability to penetrate the BBB (33). Some of the compounds that showed great promise in animal models of disease (e.g., alpha-tocopherol in neurodegeneration and atherosclerosis and several antioxidants in ALS) have had comparatively less beneficial effects in patients (7,130,277). However, as reviewed by Halliwell (17,130), a number of antioxidants have been shown to reduce neuronal damage in human disorders or animal models, e.g., idebenone (278) and a mixture of coenzyme Q and alpha-tocopherol in Friedreich's ataxia (279); Ebselen in subarachnoid hemorrhage and stroke (280); the modified spin trap NXY-059 in stroke (281-283); catechol-*O*-methyltransferase inhibitors tolcapone and nitecapone in PD (17); selegiline (deprenyl) and its metabolites, pergolide and ropinirole (7,284), in PD; and apomorphine (130) in PD. Some of these exert their actions in ways additional to or different from their antioxidant effects (285). Idebenone is a free radical scavenger analogue of coenzyme Q10 which has been shown to lower oxidative damage (it lowered elevated urinary excretion of 8-hydroxy-2'-deoxyguanosine) and rescue respiratory chain function (286,287). The iron chelator desferrioxamine was found to be protective in a cell-culture model of Friedreich's ataxia (288), while copper chelators do inhibit the course of ALS in cell culture and mouse models (33). Apart from chelation therapy, molecules are now being designed to inhibit aberrant metal interactions by competing with the target protein for metal ions; they are referred to as metal-protein attenuating compounds (MPAC) (33). An example is clioquinol (CQ, 5-chloro-7-iodo-8-hydroxy-quinoline), which crosses the BBB and has proven successful in animal models of AD and PD and in clinical trials for AD (33). Deprenyl is an irreversible monoamine-oxidase B inhibitor which exerts anti-oxidant, anti-apoptotic and neuroprotective effects (250). Apomorphine (Apo) is a dopamine D1/D2 receptor agonist used in the clinical treatment of PD, as it has a potent radical-scavenging property and has recently been

shown to stimulate the translocation of the transcription factor Nrf-2 (involved in the expression of numerous detoxifying and antioxidant genes) into the nucleus and the transactivation of the antioxidant response element (ARE) (289). There are now also lazaroid antioxidants, which are potent membrane-based lipid peroxidation inhibitors (e.g., U-74500A, U-74389G, U-83836E) (290). In addition to all of these, drugs that inhibit glutamate release or block the NMDA receptor are also in use for the management of neurodegenerative conditions, since pathological stimulation of the receptor enhances the production of ROS. Examples include riluzole (which inhibits glutamate release), memantine (an NMDA receptor antagonist) and amantadine (a partial NMDA receptor antagonist). Riluzole is used in the treatment of ALS, memantine in AD and amantadine in PD (33). The challenge in this arena is the development of agents that can prevent pathological stimulation of the NMDA receptor without affecting normal glutamatergic neurotransmission.

Having listed current pharmacological recipes for combating neurodegeneration, it should be added that effective lifestyle management is now seen as very important and desirable in order to lower the risk of developing neurodegenerative conditions such as AD (17). It is becoming increasingly recognized that the risk of developing AD can be significantly reduced by taking diets rich in fruits and vegetables and low in fat, and by regular physical exercise and mental activity (291-293). Interestingly, neurotrophic factors such as the BDNF protein are increased following exercise in an NMDA receptor-dependent manner (294,295).

In summary, there are now promising advances in the antioxidative approach to the management of neurodegenerative conditions, both at the basic research level and in clinical settings, although much still has to be understood before we are able to develop "ideal" therapies.

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Abbreviations: Abeta: amyloid beta; AD: Alzheimer's disease; AIF: apoptosis-inducing factor; ALS: amyotrophic lateral sclerosis; AP-1: activator protein-1; Apaf-1: apoptotic protease-activating factor-1; APP: amyloid precursor protein; ARE: antioxidant response element; BBB: blood-brain barrier; BDNF: brain-derived neurotrophic factor; cGMP: cyclic guanosine monophosphate; CO: carbon monoxide; DR: death receptor; EDRF: endothelial-derived relaxing factor; ER: endoplasmic reticulum; FA: Friedreich's ataxia; GDNF: glial cell line-derived neurotrophic factor; GPx: glutathione peroxidase; GSH: reduced glutathione; GSSG: oxidized glutathione; HD: Huntington's disease; HO-1: heme oxygenase-1; HO-2: heme oxygenase-2; HIF-1: hypoxia-inducible factor-1; HNE: 4-hydroxy-2,3-nonenal; H_2O_2: hydrogen peroxide; LT: lymphotoxin; MAO: monoamine oxidase; MDA: malondialdehyde; MPTP: 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; NGF: nerve growth factor; NO: nitric oxide; NOS: nitric oxide synthase; NAD: nicotinamide adenine dinucleotide; NADPH: reduced nicotinamide adenine dinucleotide phosphate; NF-kappaB: nuclear factor-kappa B; NMDA: *N*-methyl-D-aspartate; Nrf-2: NF-E2-related factor-2; MAPKs: mitogen activated protein kinases; PAR: poly (ADP-ribose) polymer; PARG: poly (ADP-ribose) glycohydrolase; PARP: poly (ADP-ribose) polymerase; PD: Parkinson's disease; PLA_2: phospholipase A_2; PCD: programmed cell death; PKC: protein kinase C; PTP: permeability transition pore; PUFAs: polyunsaturated fatty acids; Prx: peroxiredoxins; RNS: reactive nitrogen species; ROS: reactive oxygen species; sGC: soluble guanylate cyclase; SOD: superoxide dismutase; SOR: superoxide reductase; TBARS: thiobarbituric acid-reactive substances; TNF: tumor necrosis factor; TRAIL: TNF-related apoptosis-inducing ligand; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor

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