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Review Article

Free Radicals: Key to Brain Aging and Heme Oxygenase as a Cellular Response to Oxidative Stress

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Aging is one of the unique features in all organisms. The impaired functional capacity of many systems characterizes aging. When such impairments occur in the brain, the susceptibility to neurodegenerative diseases amplifies considerably. The free radical theory of aging posits that the functional impairments in brains are due to the attack on critical cellular components by free radicals, reactive oxygen species, and reactive nitrogen species produced during normal metabolism. In this review, we examine this concept based on the parameters of oxidative stress in correlation to aging. The parameters for lipid peroxidation are phospholipid composition, reactive aldehydes, and isoprostanes. The parameters for protein oxidation are protein carbonyl levels, protein 3-nitrotyrosine levels, electron paramagnetic resonance, and oxidative stress-sensitive enzyme activities. We conclude that free radicals are, at least partially, responsible for the functional impairment in aged brains. The aging brain, under oxidative stress, responds by induction of various protective genes, among which is heme oxygenase. The products of the reaction catalyzed by heme oxygenase, carbon monoxide, iron, and biliverdin (later to bilirubin) each have profound effects on neurons. Although there may be other factors contributing to brain aging, free radicals are involved in the damaging processes associated with brain aging, and cellular stress response genes are induced under free radical oxidative stress. Therefore, this review supports the proposition that free radicals are, indeed, a key to brain aging.

ONE of the unique features of life in organisms is the aging process. Aging is defined as the gradual alterations in structure and function that occur over time, eventually leading to an increased probability of death not associated with disease or trauma (1). The functional capacity of many physiological systems undergoes impairment, which characterize the aging process. When such impairments occur in the brain, the physiological, or even psychological, changes can be dreadful. The impairment of functional capacity of the brain is associated with dementia, and even smaller impairments increase the susceptibility for devastating neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS). Since aging is the common risk factor among the neurodegenerative diseases and death (2), scientists have advanced numerous theories to explain the phenomenon of aging. A credent theory of aging should be able to: (a) explain how the loss of homeostasis occurs to individual organisms in the latter part of life; (b) account for the life span variation among cohort genetic strains and species; (c) identify the crucial factor(s) responsible for life-span extension by genetic mutation or experimental regimens such as caloric restriction (CR); and (d) demonstrate that variation of senescent factors can manipulate the rate of aging (3). While attempting to explain the mechanism of aging, the free radical theory of aging is able to address the above criteria and has gained relatively strong support.

The free radical theory of aging is based on the reactive free radical reactions and their ubiquitous prominent presence in living systems. Since all aerobic organisms utilize O_2 for energy, generation of free radicals by enzymatic or nonenzymatic reaction as well as by "leakage" from mitochondria is inevitable. When oxygen is partially reduced, less-toxic species with longer half-lives are formed, and these species can attack cell components distant from where those free radicals were produced. These less-toxic species and free radicals are collectively called reactive oxygen species (ROS) and reactive nitrogen species (RNS). Reactive oxygen species refer to molecules that contain oxygen with higher reactivity than the ground-state O_2. Reactive oxygen species include, among others, superoxide ($O_2\cdot$), hydroxyl radical ($HO\cdot$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). Reactive nitrogen species refer to nitric oxide (NO) and molecules derived from NO, such as peroxynitrite (-OONO), nitroyl ($\cdot NO$), and nitrogen dioxide (NO_2). Reactive oxygen species and RNS have the capacity to modify proteins, lipids, and nucleic acids to develop or enhance age-related manifestations.

Fortunately, living organisms evolved several mechanisms to protect themselves from the attack of ROS and RNS. These antioxidant defense mechanisms include: (a) scavenging ROS/RNS and their precursors; (b) binding catalytic metal ions needed for ROS formation; and (c) generating and up-regulating endogenous antioxidant defenses. Two major groups of antioxidant defense systems are

low molecular weight antioxidant compounds, e.g., vitamins C and E, lipoic acid, and ubiquinones, and antioxidant enzymes, e.g., superoxide dismutases (SOD), superoxide reductases (SOR), catalase, and glutathione peroxidases (GPx), among others (4). When the production of ROS and RNS exceed the capacity of the antioxidant defenses systems, the new state is termed oxidative stress.

The brain is more susceptible to oxidative stress for the following reasons: (a) high content of peroxidizable unsaturated fatty acids; (b) high oxygen consumption per unit weight; (c) high content of lipid peroxidation key ingredients (iron and ascorbate); and (d) the scarcity of antioxidant defenses systems (5). It is widely held that free radical-induced oxidative stress increases in brain aging. We review this concept based on the parameters of oxidative stress in the brain. This review provides a critical evaluation on whether free radicals are the key to brain aging.

Lipid Peroxidation

Background

The mechanism of lipid peroxidation can be divided into 3 stages: initiation, propagation, and termination. In initiation, since OH· is a highly reactive ROS (6), it attacks the hydrogens from nearly any C–H bond to form H_2O. The electron-rich hydrogens at the CH_2 group in the system -CH=CH-CH_2-CH=CH- of polyunsaturated fatty acids (PUFA) are even more vulnerable to the OH· attack due to the electron-donating double bonds (7). The OH·, along with other radicals (8), generate racemic peroxyl radicals that attack the hydrogens from other PUFAs and begin a chain reaction (7). These chain reactions are collectively called propagation. The peroxyl radicals then undergo the Esterbauer dioxetane mechanism to form multiple aldehydes with varying lengths of carbons, such as 4-hydroxyl-2-nonenal (HNE) (9), and terminate the chain reaction, thus the termination stage. The end products of lipid peroxidation are usually more stable than the free radicals, serving as excellent markers for free radical reactions. Although these aldehydes have lower activity than free radicals, their longer half-life potentially allows them to be more neurotoxic.

Phospholipid Composition

One way to assess lipid peroxidation is to use the compositional alterations of brain phospholipids. PUFAs, such as arachidonic (AA, 20:4n-6), docosatetraenoic acid (DCS, 22:4n-6), and decosohexaenic acid (DCH, 22:6n-6), are abundant in brains and highly susceptible to free radical attack by the mechanism mentioned above. When PUFAs act as substrates of lipid peroxidation and become oxidized, their lipid peroxidation end-product fatty acids, e.g., oleic acid (18:1n-9), godonic acid (20:1n-9), and arachidonic acid, process reduced number of double bonds as well as different chemical and physical properties. Therefore, the quantity of lipid peroxidation can be estimated by measuring the depletion of PUFAs or the ratio change between PUFAs and saturated/monounsaturated fatty acids. These measurements can be performed by simple extraction (10) and separation of the fatty acids by thin layer chromatography (TLC) (11,12), gas chromatography (GC) (13), or high-performance liquid chromatography (HPLC) (14) followed by ultraviolet (UV) spectrophotometry, gas chromatography, or mass spectrometry for identification of the fatty acids.

Brain aging is accompanied by changes in the overall membrane lipid composition (15,16). The most important changes involve a decreased level in PUFA (20:4n-6, 22:4n-6, 22:6n-3) and an increase in monounsaturated fatty acids (18:1n-9 and 20:1n-9) in cerebral cortex and cerebellum of aged rats (17). Moreover, arachidonic acid, along with n-6 and n-3 fatty acids, were also reported to decrease in the brains of aged rats with cognitive deficit (18). This study suggested that low levels of AA might be related to the cognitive deficit in rats, as membrane AA concentration were shown to be correlated with the long-term potentiation (LTP). Long-term potentiation is a form of synaptic plasticity characterized by persistent synaptic efficacious increases, which follow tetanic stimulation from an afferent pathway to one of the hippocampal subfields (19). The ability to sustain LTP is usually used as a parameter of cognitive function. The changes in phospholipid composition provide evidence that free radical-mediated lipid peroxidation occurs in brains, and such changes might also be responsible for the cognitive deficit observed in aged animals and humans.

Reactive Aldehydes

HNE is an end product of AA or linoic acid (mainly, but not exclusively) peroxidation (9). Because of its extensive half-life, HNE is able to diffuse to sites that are distant from that of its formation and cause further damage (20). HNE causes neurotoxicity and apoptosis in many ways. This major product of lipid peroxidation can inhibit proliferation and cell cycle progression by the down-regulation of D1, D2, and A cyclin expression (21). It can also react with some bases of DNA and lipid amino groups by Michael addition (22,23) to alter the proper function of these biological molecules. The most damaging effect of HNE comes from its ability to form covalent adducts of histidine, lysine, and cysteine residues in proteins to modify their activity (20). HNE deactivates aldose reductase and glutathione peroxidase, which detoxify HNE (24) and catalyze the reduction of H_2O_2 by glutathione (25), respectively. HNE reduces Na^+/K^+–ATPase activity (26). Reduction of these critical ion pumps causes neurotoxicity in rat hippocampal neurons by disrupting the Ca^{2+} homeostasis (27) and impairing glucose transport (28).

Acrolein (2-propen-1-al) is the most reactive of the α,β-unsaturated aldehydes formed by lipid peroxidation of fatty acid sources that are not fully characterized as yet (29). Sharing the chemical properties of HNE, acrolein induces toxicity to primary hippocampal culture (30). This toxicity can result from the distortion of the transmembrane and cytoskeletal proteins structures (31) as well as the inhibition of the glucose and glutamate uptake (32).

Since acrolein and HNE adducts are more stable than most free radicals, acrolein- and HNE adducts, in particular acrolein-modified and HNE-modified proteins, provide an effective marker for free radical-mediated lipid peroxidation. It was also suggested that the protein-bound acrolein is

a potential marker of oxidative stress in aging (29). Studies have shown that the amount of HNE-modified protein staining increased logarithmically with age in human oculomotor neurons but not melanized neurons (33). Additionally, the HNE-modified proteins, along with neurofibrillary tangles, are also observed in the senile plaques in aged dogs (34). Both HNE and acrolein are elevated in the AD brain (30,35,36).

Malondialdehyde (MDA) is the most abundant toxic aldehyde formed when AA acts as the PUFA source for the lipid peroxidation (37–39). Lysine or arginine can catalytically hydrolyze MDA to formic acid or acetaldehydes (40). Similar to HNE, MDA can react with amino acids in proteins to for MDA adducts. Lysine, tryptophan, and histidine react with MDA to form N-β-lysyl-amino-acrolein (β-LAA) (41,42), whereas arginine reacts with MDA to form N-(2-pyrimidyl)-l-ornithine (NPO) (43,44). β-LAA can then further react with another MDA molecule to form N-lysyl-4-methyl-2,6-dihydropyridine-3,5-dicarbaldehyde (NLMDD) (40,45). This latter molecule contains an aldehyde side-chain on the dihydropyridine ring that is responsible for reactions with lysine, to form a reversible cross-link (42). Although there is no evidence that such a reaction occurs physiologically, it is possible that HNE, arolein, and so forth can also react, in place of the second MDA, with NLMDD to form a reversible cross-link reaction. (46). MDA is also a highly reactive genotoxic compound that induces DNA damage by reacting with nucleic acids to form adducts, thus disrupting base pairing (47). One of these adducts, deoxyguanosine-MDA (dG-MDA), was shown to induce cell cycle arrests in human cell cultures (48).

MDA reacts with thiobarbituric acid (TBA) to produce fluorescent thiobarbituric acid-reacting substances (TBARS), commonly used to measure MDA levels (49,50). However, TBA also reacts with other carbonyl-containing compounds. Therefore, TBARS are generally considered a nonspecific measurement of MDA. Such a problem can be corrected through HPLC coupling with postcolumn TBA derivatization. This column can specifically identify TBA–MDA complexes, thereby providing a relevant assay in biological systems (51–53). The cellular distribution of MDA can also be assessed immunohistochemically (46).

In aged human brain, it was shown that the MDA was increased in the inferior temporal corticies (54) and in the cytoplasm of neurons and astrocytes (55) compared with young controls. Through electron microscopic immunohistochemistry, MDA was shown to form linear deposits. These deposits are cap-like when associating with lipofuscin in neurons and vacuole encircling when found in the glia in the CA4 region of the human hippocampus (55). In a human aging canine model, which naturally develops extensive diffused deposits of human-type amyloid β-peptide (Aβ), MDA was increased in the prefrontal cortex of aged brains (56). Although there is no significant increase of MDA-modified protein observed in aged rat brains (57), it was shown that the basal MDA level was significantly elevated (19%) in the hippocampi of old rats (58). Additionally, 5 dG-MDA adducts were identified as the indigenous DNA (iDNA) adducts, which is considered a biomarker of the aging brain (59) due to the increase of iDNA with age (60–63). The above studies suggest that the presence of free radical-mediated MDA production could induce further damage of the proteins or DNAs in the brain. In the Mooradian and colleagues study (57), the increased MDA-modified proteins were not observed. This could be due to the fact that MDA-modified protein reacts with lipid peroxidation products to cross-link with each other. Therefore, MDA-modified proteins could not be detected immunochemically.

Isoprostanes

Isoprostanes are another group of compounds that result from lipid peroxidation. Two groups of isoprostanes were commonly used to index lipid peroxidation: isoprostane (IP) and neuroprostanes (NP). IP is a prostaglandin F_2-like compound formed by the nonenzymatic peroxidation of AA (64). In the initiation stage when $O_2\cdot$ attacks AA, 3 arachidonyl radicals are formed to yield 4 prostaglandin H_2-like bicyclic endoperoxide intermediate regioisomers. These regioisomers are then reduced to F-ring regioisomers to form F_2-IP in different series, according to the carbon number on the hydroxyl side of the chain. However, during the reduction, rearrangement could occur to create E-, D-, throboxin-, A-, and J-ring IP as well as highly active acyclic γ-ketoaldehydes, namely isoketals (65–67). All of these isomers are termed as IP (68). F_2-IP and E_2- IP are involved in mediation of receptor action, such as vasoconstriction, due to their inherent chemical reactivity. A_2-IPs and J_2-IP, as well as isoketals, are involved in adduct formation due to their reactivity (65,69–71). F_2-IPs are an ideal index for lipid peroxidation because of their stability and specificity (72,73). F_2-IPs are initially found in esterified form. They are then released in free form by a phospholipase(s) because most AAs are esterified in phospholipids (74). Similar to assessments of PUFAs level, the assessments of free and esterified F_2-IPs can be accomplished immunochemically (75) or by TLC, HPLC (73), or GC (76) coupled with MS (77).

Neuroprostanes are a set of IPs uniquely formed from the free radical-mediated peroxidation of DCH in brains (78,79). Although DCH is highly enriched in neurons, those neurons are not able to elongate and desaturate essential fatty acids to form DCH. DCH is synthesized and secreted by astrocytes and then captured by neurons (80). Similar to the mechanisms of IP formation, $O_2\cdot$ attacks DCH in the initiation stage of NP formation. However, 8 peroxyl radicals are formed, rather than 4 in AA, since DCH is 2 carbons longer than AA and able to form different regioisomers. These peroxyl radicals then undergo a similar process as in the AA reaction to develop F_4-IP or NP (79). The rearrangement during the reduction step can also result in reactive γ-ketoaldehydes, named neuroketals (81). Both neuroketals and isoketals demonstrate the inhibition of proteasome function (82). Due to the similar chemical properties of F_4-IPs, NP can be detected by the same methodologies described above.

Although elevated free and esterified F_2-IPs in plasma were reported in aged animals (72), interestingly, the study of isoprostane levels in the brain found that F_2-NP and NP

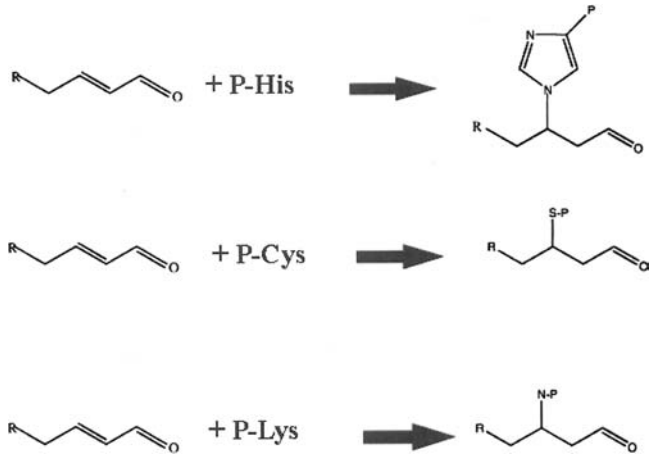

Figure 1. The carbonyl group was introduced to protein lysine (P-Lys), histidine (P-His), or cysteine (P-Cys) residues by Michael addition (233).

showed no significant differences among the brains of young, adult, and old rats (83). This finding is rather controversial to the other findings of lipid peroxidation during aging (15–18,29,33,34,54–56,58,60–62). However, such concealed differences of the IP and NP levels in aged brains could be due to the reduced glutathione (GSH) level generally in aged brains (84,85). Since GSH is an important effector for the reduction of endoperoxide intermediates to F-ring IPs (86), reduced GSH levels may not effectively reduce all the peroxyl radicals to F_2-IPs. Since GSH levels are age dependent (87) and abnormal in AD brains (88,89), the increased levels of F_2-isoprostanes were observed in the cerebrospinal fluid (CSF) of AD patients (90), as well as in the brains of AD models, e.g., mice with apolipoprotein E (ApoE) deficiency (91) and AD amyloidosis (92). Therefore, lack of differences observed in the IPs and NPs level in aged brains could be due to the reduced GSH level.

Lipid Peroxidation Summary

In short, the results above suggest that free radicals are important in lipid peroxidation in the brain. Although others might argue that lipid hydroperoxides can also be formed enzymatically by lipoxygenases (93), most lipoxygenases require as substrates free PUFAs, which are only present in trace amounts in healthy tissue (94). Free PUFAs are generated by the activation of membrane-bound phospholipase A_2 (95,96). Oxidative stress-associated activation and enhanced function of cytosolic phospholipase A_2 have been proposed to be critical factors in the accumulation of PUFAs (97). These alterations of cytosolic phospholipase A_2 are due to the increase in H_2O_2-induced intracellular Ca^{2+} concentration (98). Therefore, it seems that free radicals play a key role in lipid peroxidation, whether that be in enzymatically or nonenzymatically driven reactions.

Protein Oxidation

Introduction

Protein oxidation is an exothermic event where peptides react with free radicals. This reaction is mainly, though not exclusively, mediated by the OH; which is mostly produced by the Fenton reaction through the decomposition of H_2O_2 in the presence of redox metals (Cu^+ and Fe^{2+}) (20). Free radicals can attack peptides at two locations: back bone and side chain. In backbone modification, a free radical attacks the hydrogen on the α-carbon to form a carbon-centered radical. In the presence of oxygen, this radical then further converts into a peroxyl radical (20), which can attack other hydrogens of the same or differing peptides to propagate the free radical oxidation in a similar fashion as the initial and propagation stage of free radical-mediated lipid peroxidation. Such oxidation can lead to protein cross-linking and/or peptide bond cleavage. Four types of cleavage are reported: (a) α-amidation, which yields RCOCOOH and NH_3; (b) diamide, which yields RCOOH, CO_2, and NH_3; (c) glutamate oxidation, which yields $CH_3COCOOH$, HOOC-COOH, and NH_3; and (d) proline oxidation, which yields $H_2NCH_2(CH_2)_2COOH$ and CO_2 (99). In side-chain modification, the free radicals attack the amino acid side chains of a peptide. Although most amino acid side chains are vulnerable to oxidative modification, only some of these modifications are fully characterized: (a) histidine modified to aspartic acid, aspargine, or oxo-histidine; (b) tyrosine modified to 3,4-dihydroxyphenylalanine and Tyr–Tyr cross-links; (c) phenylalanine modified to 2-, 3-, 4-hydroxyphenylalanine; (d) tryptophan modified to N-formylkynureine, kynurenine, 2-, 4-, 5-, and 6-hydroxyvaline; (e) valine modified to 3-hydroxyvaline; (f) leucine modified to 3- and 4-hydroxyleucine; (g) lysine modified to 2-aminoadipic semialdehyde; (h) arginine modified to glutamic semialdehyde; (i) proline modified to glutamic semialdehyde, pyroglutamic acid, 2-pyroglutamic acid, 2-pyrrolidone, and 4-hydroxyproline; (j) glutamic acid modified to 4-hydroxyglutamic acid, and pyruvate α-ketoglutaric acid; (k) threonine modified to 2-amino-3-ketobutyric acid; (l) methionine modified to methionine sulfoxide and methionine sulfone; and (m) cysteine modified to disulfides such as Cys-S-S-Cys and Cys-S-S-R (100). These oxidative modifications generally cause the loss of catalytic or structural function in the affected protein and contribute serious deleterious effects on cellular and organ functions (99).

Protein Carbonyl Levels

Carbonyl level is probably the most commonly used method of assessing the oxidative modification of proteins (20). Besides introducing carbonyls to proteins directly as previously described, free radicals can introduce carbonyls to protein through covalent reaction with lipid peroxidation end products such as HNE, acrolein, and MDA (20). Due to the resonance effect between the double bonds, the α-carbon of these reactive aldehydes are highly electrophilic and able to covalently bind to the electron-rich site of Cys, His, and Lys by Michael addition (Figure 1). Therefore, the increased carbonyl content is introduced to the proteins, as these aldehydes process at least one carbonyl group. Carbonyl content of proteins are generally indexed by its 2,4-dinitrophenylhydrazone (DNP-) adduct formed by reacting the proteins with 2,4-dinitrophenylhydrazine (DNPH) (20). The DNP adduct can be detected by UV-Vis, fluorescence spectroscopy, or immunochemically. It should be noticed

that the quantity of the protein carbonyl level depends not only on its formation, but also the degradation of the oxidized protein. Therefore, we can only conclude that increased carbonyl levels are due to increased free radical attack if the protein degradation system is normal.

It was found that protein carbonyl levels were increased in the frontal and occipital cortex of aged humans (101), in the cortex of Brown-Norway rats (102) and Mongolian gerbils (103), in the forebrain of Wistar rats (104), and in brain homogenates of aged canine models (56). The carbonyl level increase of proteins at 65 kDa was also reported to be in the hippocampus of aged spatial learning-impaired rodents (105), consistent with the notion that free radical-induced protein carbonyls are involved in the dementia observed in some elderly persons. Since the alkaline protease activity in the rat brains did not decline with age (106), the carbonyl level increase reported by these studies are most likely due to the increased free radical assault rather than the decreased oxidative-modified protein degradation. In accelerated aging (SAMP8 mice), brain protein oxidation is observed, and brain-accessible antioxidants modulate these effects (107,108).

Protein 3-Nitrotyrosine (3-NT) Level

3-Nitrotyrosines result from the nitration of the *ortho* position of the aromatic ring on tyrosine residues of proteins (20,109). Such reaction is a form of protein oxidation by definition (27). 3-Nitrotyrosines can make the aromatic moiety more hydrophilic, thereby altering the secondary and tertiary structures of a protein. It can also sterically or electronically hinder the phosphorylation or dephosphorylation on the *para*-OH group of Tyr, thus interfering with the cell signaling (110). Protein 3-NT levels can be detected immunochemically or by HPLC coupled with MS/MS to avoid the electrochemical potential complications of 3-NT (111).

Although protein 3-NT levels of brain homogenate were found to decrease in aged Wistar rats (104), it was increased in the hippocampus and the cerebral cortex of aged rats (112), the CSF of aged human (113), and the subcortical white matter of aged monkeys (114). Moreover, the most prominent labeling of 3-NT was discovered in Prukije cell layers and molecular layers of the cerebellar cortex, as well as in the surroundings of neuropil in the cerebellar nuclei of aged rats (115). These findings suggest that increases in protein 3-NT levels are in a regionally specific fashion in aging. Such specificity could be attributed to the level of nitric oxide synthase, which produces NO to activate tyrosine nitration, in cells (113); in contrast, 3-NT occurs in a wide-spread manner in the AD brain (116,117). Recently, proteomics analysis identified that specific proteins were increased in 3-NT in the AD brain (118).

Electron Paramagnetic Resonance

Free radical-mediated protein oxidation can also be indirectly assessed by EPR spin labels. A 2,2,6,6-tetramethyl-4-maleimidopiperdin-oxyl (MAL-6) spin label (119–125) can covalently bond to SH groups on Cys. The ratio of the EPR signal intensity of the low-field weakly immobilized resonance line (W) to that of the strongly immobilized line (S) (the W/S ratio) is a parameter that reflects motion of the spin label bound to proteins (119–125). The amplitude of the low-field weakly immobilized line indicates the relatively fast MAL-6 motion, as this protein-specific spin label binds to SH groups in which the MAL-6 motion is only weakly immobilized. In contrast, the amplitude of the low-field strongly immobilized line indicates the relatively slow MAL-6 motion as this spin label binds to SH groups in which the MAL-6 motion is strongly immobilized. Therefore, the W/S ratio is lowered when protein cross-linkings increase, protein–protein interactions increase, and segmental motion of spin-labeled proteins decrease. Since all of these protein alterations occur when free radicals induce protein oxidation, a change in the W/S ratio indexes the protein conformation change due to free radical modification (119–125).

It was found that the W/S ratio is extremely precise when assessing the oxidative conformational changes of proteins (119–125). It was also found that the W/S ratio decreases when OH• induce oxidation to rodents' synaptosomes in vivo and in vitro (123,124). Such a W/S ratio decrease is also observed in the cortical synaptosomes and erythrocyte membranes of hyperoxia-exposed rodents (125) and the synaptosomes of senescence-accelerated mice (SAM) (108). This suggests that free radical-induced protein oxidations, and thus conformational alterations, are significant events that occur during brain aging.

Oxidative Stress-Sensitive Enzyme Activity

Oxidative deactivation of enzymes is another index of free radical oxidative damage to proteins. As described above, the free radical-induced oxidative modification of proteins usually results in structural and functional change in these proteins (119–125). Decreased activities of glutamine synthetase (GS), creatine kinase (CK), and tyrosine hydroxylase (126–129) by free radical-induced modification were observed. Since the activities of these enzymes, particularly CK and GS, can be easily determined, their activities serve as efficient indices for oxidative modification of proteins.

Studies shows that the activities of CK and GS were decreased in aged brown Norway rats brains (102), in aged gerbil brains (130), and in aged human frontal and occipital lobes (101). Since CK and GS activity is very sensitive to free radical-induced oxidative modification, the declined activity of CK and GS in aged animals demonstrates direct evidence that free radical-induced oxidative modification of proteins are essential during the aging process. That diminution of CK activity occurs by free radical-induced reactions was confirmed by the observation that vitamin E protected against the loss of CK activity by Aβ (131). It should be noticed that the activities of many enzymes are also altered during aging. These proteins include: (a) lipid metabolic enzymes phospholipase D, phosphatase phosphohydrolase, diacylglycerol lipase, phosphatidylserine synthase, and phosphatidylserine decarboxylase (17); (b) metabolic enzyme, L α-ketoglutarate dehydrogenase complex (132), pyruvate kinase, NAD-isocitrate dehydrogenase, NAD-malate dehydrogenase (133), enolase (134), and

lactate dehydrogenase (135–138); (c) mitochondrial enzymes, citrate synthetase, and electron transport complex I-IV (139); (d) ionic homeostatic and signaling proteins: Na^+, K^+-ATPase (140), and acetylcholine esterase (141); and (e) antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase, γ-glutamylcysteine synthetase, glutathione reductase, glutathione-S-transferase, and γ-glutamyl transpeptidase (142–144). Although the alteration of these enzymes are most likely due to oxidative modification of the proteins, no direct correlation has been established between the oxidative modification of the proteins and their activity decline until recently, In the AD brain, we found direct correlation of loss of CK and GS activities (125) with specific protein oxidation using proteomics (145,146). This suggests that oxidative modification of enzymes leads to their dysfunction. Further studies of this notion are required.

Protein Oxidation Summary

The studies mentioned show that free radical modification of proteins may be responsible for the gradual alteration of physiological function and accumulation of altered enzymes in aged brain. In a recent review (99), evidence supporting this view is classified into 7 major areas: (a) in vitro free radical alteration of catalytic activities, thermal stability, and sensitivity to photolytic degradation is similar to what is observed in aging; (b) inducing free radicals in young animals in vivo can change the enzymes to aged-like forms; (c) increasing the life span of animals by factors or physiological conditions can lead to decreases of protein carbonyl levels, and vice versa; (d) increased levels of oxidized protein in the frontal pole and occipital pole concur with the age-related loss of cognitive function; (e) proteins from aged animals are more sensitive to oxidative damage, compared with the proteins from young animals; (f) protein oxidation levels are correlated to increased surface hydrophobicity of protein in aged animals; and (g) protein carbonyl levels increase exponentially with age in different animal species and tissues. The review further proposed that free radical-induced protein oxidation might be the cause of changes in aging. In short, free radicals and free radical-mediated protein modification is essential to the brain aging process.

Recently, proteomics studies enabled the identification of specific proteins that undergo oxidative modification in AD patients (118,145,146). Applying the proteomics technology to brain aging potentially can increase understanding of the mechanisms of brain aging associated with protein oxidation. However, such studies conceivably could encounter problems such as the difficulty of removing lipids from transmembrane proteins, and unknown mass spectrum of proteins. Another potential major problem is that a large amount of oxidized proteins may be present in the brain due to nonspecific protein oxidation in normal aging brains. However, proteomic technology is advancing rapidly. Large-scale proteomics will eventually develop and, therefore, the complete oxidative proteome will be obtained in normal aging brains. This database, with the help of bioinformatics, will provide very valuable information and a foundation about protein oxidation in aging and in many age-related neurodegenerative diseases and dementia (147).

The Heat Shock Pathway of Cell Stress Tolerance

Background

It is well known that living cells are continually challenged by conditions that cause acute or chronic stress. To adapt to environmental changes and survive different types of injuries, eukaryotic cells have evolved networks of different responses that detect and control diverse forms of stress. One of these responses, known as the heat shock response, has attracted a great deal of attention as a universal fundamental mechanism necessary for cell survival under a wide variety of toxic conditions. In mammalian cells, heat shock protein (HSP) synthesis is induced not only after hyperthermia but also following alterations in the intracellular redox environment, exposure to heavy metals, amino acid analogs, or cytotoxic drugs. While prolonged exposure to conditions of extreme stress is harmful and can lead to cell death, induction of HSP synthesis can result in stress tolerance and cytoprotection against stress-induced molecular damage. Furthermore, transient exposure to elevated temperatures has a cross-protective effect against sustained, normally lethal exposures to other pathogenic stimuli. Hence, the heat shock response contributes to establish a cytoprotective state in a variety of metabolic disturbances and injuries, including stroke, epilepsy, cell and tissue trauma, neurodegenerative disease, and aging (148,149).

These considerations have provided new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear as possible candidates for novel cytoprotective strategies (150–156). In mammalian cells, the induction of the heat shock response requires the activation and translocation to the nucleus of one or more heat shock transcription factors that control the expression of a specific set of genes encoding cytoprotective heat shock proteins. Some of the known HSPs include ubiquitin, HSP10, HSP27, HSP32 (or HO-1), HSP47, HSP60, HSC70, HSP70 (or HSP72), HSP90, and HSP100/105. Most of the proteins are named according to their molecular weight.

HSP70

The 70 kDa family of stress proteins is one of the most extensively studied. Included in this family are HSC70 (heat shock cognate, the constitutive form), HSP70 (the inducible form, also referred to as HSP72), and GRP75 (a constitutively expressed glucose-regulated protein found in the endoplasmic reticulum). After a variety of central nervous system (CNS) insults, HSP70 is synthesized at high levels and is present in the cytosol, nucleus, and endoplasmic reticulum). Denaturated proteins are thought to serve as stimulus for induction. These denaturated proteins activate heat shock factors (HSFs) within the cytosol by dissociating other HSPs that are normally bound to HSF (157). Free HSF is phosphorylated and forms trimers, which enter the nucleus and bind to heat shock elements (HSE) within the promoters of different heat shock genes leading to transcription and synthesis of HSPs. After heat shock, for instance, the synthesis of HSP70 increases to a point to where it becomes the most abundant single protein in a cell. Once synthesized, HSP70 binds to denaturated proteins in

an ATP-dependent manner. The N-terminal end contains an ATP-binding domain, whereas the C-terminal region contains a substrate-binding domain. Heat shock proteins serve as chaperones that bind to other proteins and regulate their conformation, regulate the protein movement across membranes or through organelles, or regulate the availability of a receptor or activity of an enzyme.

In the nervous system, HSPs are induced in a variety of pathological conditions, including cerebral ischemia, neurodegenerative disorders, epilepsy, and trauma (157). Expression of the gene-encoding HSPs has been found in various cell populations within the nervous system, including neurons, glia, and endothelial cells (158). HSPs consist of both stress-inducible and constitutive family members. Whether stress proteins are neuroprotective has been the subject of much debate, as it has been speculated that these proteins might be merely an ephiphenomenon unrelated to cell survival. Only recently, however, with the availability of transgenic animals and gene transfer, has it become possible to overexpress the gene encoding HSP70 to test directly the hypothesis that stress proteins protect cells from injury, and it has been demonstrated that overproduction of HSP70 leads to protection in several different models of nervous system injury (159,160). Following focal cerebral ischemia, mRNA-encoding HSP70 is synthesized in most ischemic cells except in an area of very low blood flow because of limited ATP levels. HSP70 proteins are produced mainly in endothelial cells, in the core of infarcts in the cells that are most resistant to ischemia, in glial cells at the edges of infarcts, and in neurons outside the areas of infarction. It has been suggested that this neuronal expression of HSP70 outside an infarct can be used to define the ischemic penumbras (161). A number of in vitro studies show that both heat shock and HSP overproduction protect CNS cells against both necrosis and apoptosis. Mild heat shock protects neurons against glutamate-mediated toxicity and protects astrocytes against injury produced by lethal acidosis (162). Transfection of cultured astrocytes with HSP70 protects them from ischemia or glucose deprivation (163). HSP70 has been demonstrated to inhibit caspase-3 activation caused by ceramide and also affect JUN kinase and p38-kinase activation (164). In addition, HSP70 binds to and modulates the function of BAG-1, the bcl-2 binding protein (165), thus modulating some type of apoptosis-related cell death.

A large body of evidence now suggests a correlation between mechanisms of oxidative and/or nitrosative stress and HSP induction. Current opinion also holds the possibility that the heat shock response can exert its protective effects through inhibition of nuclear factor kappa B (NFkB)-signaling pathway (149). We have demonstrated in astroglial cell cultures that cytokine-induced nitrosative stress is associated with an increased synthesis of HSP70 stress proteins. An increase in HSP70 protein expression was also found after treatment of cells with the NO-generating compound sodium nitroprusside (SNP), thus suggesting a role for NO in inducing HSP70 proteins (167). The molecular mechanisms regulating the NO-induced activation of the heat shock signal seems to involve cellular oxidant/antioxidant balance, mainly represented by the glutathione status and the antioxidant enzymes (165–167).

Ubiquitin

Ubiquitin is one of the smallest HSPs and is expressed throughout the brain in response to ischemia. It is involved in the targeting and chaperoning of proteins degraded in proteasomes, which include NFkB, cyclins, HSFs, hypoxia-inducible factor, some apoptosis-related proteins, tumor necrosis factor (TNF), and erythropoietin receptors (168).

HSP27

HSP27 is synthesized mainly in astrocytes in response to ischemic situations or to kainic acid administration. It chaperones cytoskeletal proteins, such as intermediate filaments, actin, or glial fibrillary acidic protein following stress in astrocytes. It also protects against Fas-Apo-1, staurosporine, TNF, and etoposside-induced apoptotic cell death as well as H_2O_2-induced necrosis (169).

HSP47

HSP47 is synthesized mainly in microglia following cerebral ischemia and subarachnoid hemorrhage (170).

HSP60, Glucose-Regulated Protein 75 (GRP75), and HSP10 Chaperone Proteins

HSP60, glucose-regulated protein 75 (GRP75), and HSP10 chaperone proteins are within mitochondria. GRP75 and GRP78, also called oxygen-regulated proteins (ORPs), are produced by low levels of oxygen and glucose. These protect brain cells against ischemia and seizures in vivo, after viral-induced over-expression (171).

HSP32

HSP32, or heme oxygenase, is the rate-limiting enzyme in the production of bilirubin. There are 3 isoforms of heme oxygenase: HO-1 or inducible isoform, HO-2 or constitutive isoform, and the recently discovered HO-3 (172–174).

HO for Neuroprotective Strategies in Age-Related Neurodegenerative Disease Pathology

Background

In the last decade, the heme oxygenase (HO) system has been strongly highlighted for its potential significance in maintaining cellular homeostasis. It is found in the endoplasmic reticulum in a complex with NADPH cytochrome c P450 reductase. It catalyzes the degradation of heme in a multistep, energy-requiring system. The reaction catalyzed by HO is the α-specific oxidative cleavage of the heme molecule to form equimolar amounts of biliverdin and carbon monoxide (CO). Iron is reduced to its ferrous state through the action of NADPH cytochrome c P450 reductase. Carbon monoxide is released by elimination of the α-methylene bridge of the porphyrin ring. Further degradation of biliverdin to bilirubin occurs through the action of a cytosolic enzyme, biliverdin reductase. Biliverdin complexes with iron until its final release.

Heme Oxygenase Isoforms

HO is present in various tissues with the highest activity in the brain, liver, spleen, and testes. There are 3 isoforms of

heme oxygenase: HO-1 or inducible isoform (175), HO-2 or constitutive isoform (176), and HO-3, cloned only in rats to date (177). They are all products of different genes and, unlike HO-3, which is a poor heme catalyst, both HO-1 and HO-2 catalyze the same reaction (i.e., degradation of heme) but differ in many respects and are regulated under separate mechanisms. The most relevant similarity between HO-1 and HO-2 consists in a common 24 amino acid (aa) domain (differing in just one residue) called the ''HO signature,'' which renders both proteins extremely active in their ability to catabolize heme (178). These isoforms have different localization, similar substrate, and cofactor requirements, while presenting a different molecular weight. They also display different antigenicity, electrophoretic mobility, and inducibility as well as susceptibility to degradation. The proteins for HO-1 and HO-2 are immunologically distinct and, in humans, the two genes are located on different chromosomes, i.e., 22q12 for HO-1 and 16q13.3 for HO-2, respectively (179).

Various tissues have different amounts of HO-1 and HO-2. The brain and testes have a predominance of HO-2, whereas HO-1 predominates in the spleen. In the lung not subjected to oxidative stress, more than 70% of HO activity is accounted for by HO-2, whereas in the testes, the pattern of HO isoenzyme expression differs according to the cell type, although HO-1 expression predominates after heat shock. This also occurs in brain tissue, where HO isoforms appear to be distributed in a cell-specific manner and HO-1 distribution is widely apparent after heat shock or oxidative stress. Although previous reports from our group and other groups have not found detectable levels of HO-1 protein in the normal brain (180,181), we have recently demonstrated that HO-1 mRNA expression is physiologically detectable in the brain and shows a characteristic regional distribution, with a high level of expression in the hippocampus and the cerebellum (182,183). This evidence may suggest the possible existence of a cellular reserve of HO-1 transcript quickly available for protein synthesis and a posttranscriptional regulation of its expression.

HO isoenzymes are also seen to colocalize with different enzymes depending on the cell type. In the kidney, HO-1 colocalizes with erythropoietin, whereas in smooth muscle cells, HO-1 colocalizes with nitric oxide synthase. In neurons, HO-2 colocalizes with NOS, whereas the endothelium exhibits the same isoform to colocalize with NOS III. The cellular specificity of this pattern of colocalization lends further support to the concept that CO may serve a function similar to that of NO. Furthermore, the brain expression pattern shown by HO-2 protein and HO-1 mRNA overlaps with distribution of guanylate cyclase (184), the main CO functional target.

HO-3, the third isoform of heme oxygenase, shares a high homology with HO-2, both at the nucleotide (88%) and protein (81%) levels. Both HO-2 and HO-3, but not HO-1, are endowed with 2 Cys-Pro residues considered the core of the heme-responsive motif (HRM), a domain critical for heme binding but not for its catalysis (185,186). Although the biological properties of this isoenzyme still remain to be elucidated, the presence of 2 HRM motifs in its amino acidic sequence might suggest a role in cellular heme regulation (187). Studying the HO-3 mRNA sequence (GenBank accession n.: AF058787) shows that a 5′ portion corresponds to the sequence of a L-1 retrotransposon, a member of a family of retrotransposons recently involved in evolutionary mechanisms (188,189). Based on the close similarity to a paralogous gene (HO-2) and the preliminary data from our group demonstrating no introns in the HO-3 gene (182), it is possible that HO-3 could have originated from the retrotransposition of the HO-2 gene. In addition, this genetic mutation in the rat may represent a *specie*-specific event since no other sequence in the public databases matches the rat HO-3.

Regulation of HO Genes

The HO-2 gene consists of 5 exons and 4 introns. HO-2 has a molecular weight of 34 kDa and exhibits 40% homology in an amino acid sequence with HO-1. It is generally considered a constitutive isoenzyme, however, in situ hybridization studies have shown increases in HO-2 mRNA synthesis associated with increased HO-2 protein and enzyme activity in neonatal rat brain after treatment with corticosterone (190). The organization of the HO-2 gene needs to be fully elucidated, although a consensus sequence of the glucocorticoid response element (GRE) has been demonstrated in the promoter region of the HO-2 gene (191). In addition, endothelial cells treated with the NOS inhibitor L-NAME and HO inhibitor zinc mesophorphyrin exhibited a significant up-regulation of HO-2 mRNA.

The regulation of the HO-1 gene as well as its promoter has received more elucidation that constitutive HO-2. The HO-1 gene is induced by a variety of factors, including metallophorphyrins and hemin, as well as ultraviolet A (UVA) irradiation, hydrogen peroxide, prooxidant states, or inflammation (192,193). This characteristic inducibility of the HO-1 gene strictly relies on its configuration: the 6.8-kilobase gene is organized into 4 introns and 5 exons. A promoter sequence is located approximately 28 base pairs upstream from the transcriptional site of initiation. In addition, different transcriptional enhancer elements, such as the heat shock element and metal regulatory element, reside in the flanking 5′ region. Also, inducer-responsive sequences have been identified in the proximal enhancer located upstream from the promoter and, more distally, in 2 enhancers located 4 kb and 10 kb upstream from the initiation site (194).

The transcriptional activation of HO-1 in response to hyperoxia requires the cooperation between the promoter and an enhancer element located 4 kb upstream from the transcription site (195). This response is also mediated by increased binding activity of AP-1. The HO-1 promoter contains an antioxidant responsive element with a consensus sequence (GCnnnGTA) similar to that of other antioxidant enzymes (196,197). Heme treatment results in increased binding activity of NFkB and AP-2 transcription factors at the proximal part of the promoter, but also of AP-1. The promoter region also contains 2 metal-responsive elements, similar to those found in the metallothionein-1 gene, which respond to heavy metals (cadmium and zinc) only after recruitment of another fragment located upstream, between –3.5 and 12 kbp (CdRE). In addition, a 163-bp fragment

containing 2 binding sites for HSF-1, which mediates the HO-1 transcription, are located 9.5 kb upstream of the initiation site (197). The distal enhancer regions are important in regulating HO-1 in inflammation, since it has been demonstrated that these enhancer regions are responsive to endotoxin. In the promoter region also resides a 56 bp fragment that responds to the STAT-3 acute-phase response factor involved in the down-regulation of the HO-1 gene induced by 1 glucocorticoid (198).

Heme Oxygenase in Brain Function and Dysfunction

In the brain, the HO system has been reported to be very active and its modulation seems to play a crucial role in the pathogenesis of neurodegenerative disorders. The heme oxygenase pathway, in fact, has been shown to act as a fundamental defensive mechanism for neurons exposed to an oxidant challenge (199–201). Induction of HO occurs together with the induction of other HSPs in the brain during various experimental conditions including ischemia (202). Injection of blood or hemoglobin results in increased expression of the gene encoding HO-1, which has been shown to occur mainly in microglia throughout the brain (202). This suggests that microglia take up extracellular heme protein following cell lysis or hemorrhage. Once in the microglia, heme induces the transcription of HO-1. In human brains following traumatic brain injury, accumulation of HO-1 plus microglia/macrophages at the hemorrhagic lesion were detected as early as 6 hours post trauma and was still pronounced after 6 months (203).

Since the expression of HSPs is closely related to that of the amyloid precursor protein (APP), HSPs have been studied in the brain of patients with AD. Significant increases in the levels of HO-1 have been observed in AD brains in association with neurofibrillary tangles (204,205), and also HO-1 mRNA was found to be increased in AD neocortex and cerebral vessels (206). An HO-1 increase was not only in association with neurofibrillary tangles, but also colocalized with senile plaques and glial fibrillary acidic protein-positive astrocytes in AD brains (206). It is conceivable that the dramatic increase in HO-1 in AD may be a direct response to increased free heme associated with neurodegeneration and an attempt to convert the highly damaging heme into the antioxidants biliverdin and bilirubin (207).

Up-regulation of HO-1 in the substantia nigra of Parkinson's disease patients has been demonstrated. In these patients, nigral neurons containing cytoplasmic Lewy bodies exhibited in their proximity maximum HO-1 immunoreactivity (208). New evidences showed a specific up-regulation of HO-1 in the nigral dopaminergic neurons by oxidative stress (153,209).

It has been recently demonstrated that hemin, an inducer of HO-1, inhibited effectively experimental autoimmune encephalomyelitis (EAE), an animal model of the human disease multiple sclerosis (MS) (210). In contrast, tin mesoporphyrin, an inhibitor of HO-1, markedly exacerbated EAE. These results suggest that endogenous HO-1 plays an important protective role in EAE and MS.

All of these findings have opened up new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear to be possible candidates for novel therapeutic neuroprotective strategies (210) (see below).

Carbon Monoxide Effects on Brain Function in Health and Disease

Increasing evidence implicates CO as an emerging chemical messenger molecule that can influence physiological and pathological processes in both the central and peripheral nervous systems. This gaseous molecule is now considered a putative neurotransmitter, owing to its capability to diffuse freely from one cell to another, thereby influencing intracellular signal transduction mechanisms. However, unlike a conventional neurotransmitter, carbon monoxide is not stored in synaptic vesicles and is not released by membrane depolarization and exocitosis. It seems likely that CO is involved in the mechanism of cell injury (211,212). This is evidenced by the fact that CO binds to the heme moiety in guanylate cyclase to activate cGMP (178,184). It has been found that CO is responsible for maintaining endogenous levels of cGMP. This effect is blocked by potent HO inhibitors but not by NO inhibitors (213,214).

Based on the endogenous distribution of HO in the CNS, it has been suggested that CO can influence neurotransmission like NO (184). CO appears to be involved as a retrograde messenger in LTP and also is involved in mediating glutamate action at metabotropic receptors (213). This is evident from the observation that metabotropic receptor activation in the brain regulates the conductance of specific ion channels via a cGMP-dependent mechanism that is blocked by HO inhibitors (214). Experimental evidence suggests that CO plays a similar role as NO in the signal transduction mechanism for the regulation of cell function and cell-to-cell communication (212).

HO resembles NOS in that the electrons for CO synthesis are donated by cytochrome P450 reductase, which is 60% homologous at the amino acid level to the carboxyl terminal half of NOS (215). CO, like NO, binds to iron in the heme moiety of guanylate cyclase. However there are some differences in function between CO and NO. Thus, NO mainly mediates glutamate effects at NMDA receptors, while CO is primarily responsible for glutamate action at metabotropic receptors.

Taken together, it appears that CO and NO play an important role in the regulation of CNS function; thus, impairment of CO and NO metabolism results in abnormal brain function (207,212). A number of reports suggest a possible role of CO in regulating the transmission of nitrogen compounds. Endogenous CO has been suggested to control constitutive NOS activity. Moreover, CO may interfere with NO binding to guanylate cyclase. This is in addition to the important role of HO in regulating NO generation, owing to its function in the control of heme intracellular levels, as part of normal protein turnover (216). This hypothesis is sustained by recent findings showing that HO inhibition increases NO production in mouse macrophages exposed to endotoxin (217).

CO may also act as a signaling effector molecule by interacting with targets different from guanylate cyclase. Notably, it has been recently demonstrated that K_{Ca}

channels are activated by CO in a GMPc-independent manner (218), and also that CO-induced vascular relaxation results from the inhibition of the synthesis of the vasoconstrictor endothelin-1 (218). However, little is known about how CO is sensed on a biological ground. Interestingly, the photosynthetic bacterium *Rhodospirillum rubrum* has the ability to respond to CO through the heme protein CooA, which, upon exposure to CO, acquires DNA-binding transcriptional activity for the CO dehydrogenase gene. This property hereby encodes for CO dehydrogenase, which is the key enzyme involved in the oxidative conversion of CO to CO_2. Remarkably, heart cytochrome c oxidase possesses CO oxygenase activity, thus metabolizing CO to CO_2 (219). Whether this occurs also in brain mitochondria remains to be elucidated.

Bilirubin: An Endogenous Antioxidant Derived From Heme Oxygenase

Supraphysiological levels (>300 μM) of nonconjugated bilirubin, as in the case of neonatal jaundice, are associated with severe brain damage. This is a plausible reason whereby bilirubin has generally been recognized as a cytotoxic waste product. However, only in recent years, its emerging role as a powerful antioxidant has received wide interest. The specific role of endogenously derived bilirubin as a potent antioxidant has been demonstrated in hippocampal and cortical neurons, in which accumulation of this metabolite protected against H_2O_2-induced cytotoxicity (220,221). Moreover, nanomolar concentrations of bilirubin resulted in a significant protection against hydrogen peroxide-induced toxicity in cultured neurons as well as in glial cells following experimental subarachnoid hemorrhage. In addition, neuronal damage following middle cerebral artery occlusion was substantially worsened in HO-2 knock-out mice (222). Bilirubin can become particularly important as a cytoprotective agent for tissues with relatively weak endogenous antioxidant defences, such as the central nervous system and the myocardium. Interestingly, increased levels of bilirubin have been found in the CSF of AD patients, which may reflect the increase of degraded bilirubin metabolites in the AD brain derived from the scavenging reaction against chronic oxidative stress (223). Similarly, a decreased risk for coronary artery disease is associated with mildly elevated serum bilirubin, with a protective effect comparable to that of high-density lipoprotein cholesterol (221). The most likely explanation for the potent neuroprotective effect of bilirubin is that a redox cycle exists between bilirubin and biliverdin, the major oxidation product of bilirubin. In mediating the antioxidant actions, bilirubin would be transformed in biliverdin, then rapidly converted back to bilirubin by biliverdine reductase, which in the brain is present in large functional excess. Remarkably, the rapid activation of HO-2 by protein kinase C (PKC) phosphorylation parallels the disposition of nNOS. Both are constitutive enzymes localized to neurons, and nNOS is activated by calcium entry into cells binding to calmodulin on nNOS. Similarly, PKC phosphorylation of HO-2 and the transient increase in intracellular bilirubin would provide a way for a rapid response to calcium entry, a major activator of PKC. Whether the antioxidant bilirubin possess antinitrosative properties still remains an open question, although this possibility could partially account for the effect of NO and peroxynitrite in up-regulating HO-1 expression (193).

Conclusion

The studies discussed here provide evidence for free radical/ROS/RNS involvement in brain aging that is direct as well as correlative. Recent literature provide increasing evidence of accumulation of the oxidatively modified proteins, DNAs, and lipids in the aged brain. Numerous marker compounds of lipid peroxidation were detected, such as MDA, HNE, and acrolein. These products react with DNA and proteins to produce further damage in aged brains (20,233). It was reported that 10,000 oxidative interactions occur between DNA and endogenously generated free radicals per human cell per day (224). Additionally, at least 1 of every 3 proteins in the cells of older animals is dysfunctional as enzyme or structural protein due to oxidative modification (99). Although much evidence shows the important role of free radicals in brain aging, some argue otherwise. Some contend that damage produced by endogenously generated oxygen free radicals by mitochondria resulting in distinctive biochemical profiles only occurs under exceptional conditions (225). Others claim that elongated life span from caloric restriction is not accredited to reducing free radical production, but by metabolic-shift activation of the SIR2 protein, which slices chromatin by deacetylating the histones in targeted regions of the genome, including the rDNA (226). It was also reported that inactive SIR2 mutants shorten the life span while over-expression of SIR2 extends it (227). However, these experiments did not eliminate the likely involvement of free radicals during the aging process. As matter of fact, many studies showed a connection between other theories of aging and free radicals. The ''wear and tear'' theory stated that the cells and tissues in the system, such as neuroendocrine system (228), were damaged by toxin accumulation from diet and environment, such as consumption of excessive fat, alcohol, UV rays from the sun, or metal ions from the environment, and so forth. However, this toxin accumulation, such as UV and estrogens, could affect ROS production and indicate the involvement of free radicals in aging (229). Also, the programmed death theory stated that the unique genetic code and genetic inheritance within DNA decide the longevity of the individual. DNA can be compared to a biological clock set to go off at a particular time (230). Telomeres are the tails of DNA that shorten every time cells divide. Once the telomeres become too short, cell division slows and finally ceases, causing the cell to die (231). It was reported that the telomere repairing enzyme telomerase is activated by free iron, and iron is responsible for the production of $O_2\cdot$ by Fenton reaction and activating xanthine oxidase (232). This suggests that the loss of telomerase activity is possibly due to insufficient free iron in cells caused by free radicals.

HO provides free iron as well as CO and the antioxidant bilirubin (via biliverdin). The HSP response to oxidative stress in aging and age-related neurodegenerative diseases is primal, powerful, and pervasive (149). Therapeutic strate-

gies aimed at inducing HO-1 may be a promising approach to the oxidative stress characteristic of brain aging and age-related neurodegenerative disorders (154,159,194,196,201).

All of the studies described do not imply that free radicals are the only direct cause of brain aging. However, while other factors may be involved in the cascade of damaging effects in the brain, the significant key role of free radicals in this process cannot be underestimated. Therefore, this review supports the proposition that free radicals are, indeed, the key to brain aging.

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