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Forum Review

Redox Regulation of Heat Shock Protein Expression by Signaling Involving Nitric Oxide and Carbon Monoxide: Relevance to Brain Aging, Neurodegenerative Disorders, and Longevity

VITTORIO CALABRESE,[1] D. ALLAN BUTTERFIELD,[2] GIOVANNI SCAPAGNINI,[1,4] A.M. GIUFFRIDA STELLA,[1] and MAHIN D. MAINES[3]

ABSTRACT

Increased free radical generation and decreased efficiency of the reparative/degradative mechanisms both primarily contribute to age-related elevation in the level of oxidative stress and brain damage. Excess formation of reactive oxygen and nitrogen species can cause proteasomal dysfunction and protein overloading. The major neurodegenerative diseases are all associated with the presence of abnormal proteins. Different integrated responses exist in the brain to detect oxidative stress which is controlled by several genes termed *vitagenes*, including the heat shock protein (HSP) system. Of the various HSPs, heme oxygenase-I (HO-1), by generating the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin, could represent a protective system potentially active against brain oxidative injury. The HO-1 gene is redox regulated and its expression is modulated by redox active compounds, including nutritional antioxidants. Given the broad cytoprotective properties of the heat shock response, there is now strong interest in discovering and developing pharmacological agents capable of inducing the heat shock response. These findings have opened up new neuroprotective strategies, as molecules inducing this defense mechanism can be a therapeutic target to minimize the deleterious consequences associated with accumulation of conformationally aberrant proteins to oxidative stress, such as in neurodegenerative disorders and brain aging, with resulting prolongation of a healthy life span. *Antioxid. Redox Signal.* 8, 444–477.

INTRODUCTION

ALTHOUGH THE TERM "aging" is generally understood in broad terms, the aging process is extremely complex and multifaceted (140). Increasing evidence supports the notion that reduction of cellular expression and activity of antioxidant proteins and the resulting increase of oxidative stress are fundamental causes in the aging processes and neurodegenerative diseases (195). Within the frame of the free-radical hypothesis of aging several lines of evidence suggest that accumulation of oxidative molecular damage is a causal factor in senescence. It is also increasingly evident that the mitochondrial genome may play a key role in aging and neurodegenerative diseases. Mitochondrial dysfunction is characteristic of several neurodegenerative disorders, and evidence for mitochondria being a site of damage in neurodegenerative disorders is partially based on decreases in respiratory chain complex activities in Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD) (42). Such defects in respiratory complex activities, possibly associated with oxidant/antioxidant balance perturbation, are thought to underlie defects in energy metabolism and induce cellular degeneration (212). Efficient functioning of maintenance and repair process seems to be crucial for both survival and physical quality of life. This is accomplished by a complex network of the so-called longevity as-

[1]Section of Biochemistry and Molecular Biology, Department of Chemistry, Faculty of Medicine, University of Catania, Catania, Italy.
[2]Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, Kentucky.
[3]Department of Biochemistry, University of Rochester, Rochester, New York.
[4]Institute of Neurological Sciences, National Research Council (CNR), Catania, Italy.

surance processes, which are composed of several genes termed *vitagenes* (52). Among these, chaperones are highly conserved proteins responsible for the preservation and repair of the correct conformation of cellular macromolecules, such as proteins, RNAs, and DNA. Chaperone-buffered silent mutations may be activated during the aging process and lead to the phenotypic exposure of previously hidden features and contribute to the onset of polygenic diseases, such as age-related disorders, atherosclerosis, and cancer (46, 230). Recently, the involvement of the heme oxygenase (HO) pathway in antidegenerative mechanisms operating in AD has received considerable attention, as it has been demonstrated that the expression of HO is closely related to that of amyloid precursor protein (APP) (50, 79, 80, 192). HO induction, which occurs together with the induction of other HSPs during various physiopathological conditions, by generating the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin, represents a protective system potentially active against brain oxidative injury (142, 156–159). HO-1 gene is redox regulated and this is supported by the fact that HO-1 gene has a heat shock consensus sequence as well as AP1, AP2 and NFκB binding sites in its promoter region. In addition, heme oxygenase-1 is rapidly upregulated by oxidative and nitrosative stresses, as well as by glutathione depletion (2, 53, 179). Given the broad cytoprotective properties of the heat shock response there is now strong interest in discovering and developing pharmacological agents capable of inducing the heat shock response (55, 216, 218, 238). In the present review we discuss the role of free radicals and mitochondrial energy thresholds in brain aging and neurodegenerative disorders, then review the role of NO and CO gases in brain stress tolerance and their relevance to mechanisms of longevity.

THE "OXIDATIVE STRESS HYPOTHESIS" OF AGING

One of the most prominent current theories of aging is the free radical theory. According to this theory, free radicals generated through mitochondrial metabolism can act as causative factor of abnormal function and cell death. Various toxins in the environment can injure mitochondrial enzymes, leading to increased generation of free radicals that over the lifespan would eventually play a major role in aging (4, 195).

During the last few years, cellular oxidant/antioxidant balance has become the subject of intense study, particularly by those interested in brain aging and in neurodegenerative mechanisms (39). Several lines of evidence suggest that accumulation of oxidative molecular damage is a causal factor in senescence. The direct evidence for this hypothesis is that overexpression of antioxidative genes for Cu, Zn-superoxide dismutase and catalase in transgenic *Drosophila melanogaster* prolongs the lifespan, retarding the age-associated accumulation of oxidative damage (18). Among the correlative evidence supporting the involvement of oxidative stress are the following: (a) oxidative molecular damage to DNA and proteins increases exponentially with age, and concomitantly, the rates of mitochondrial $O_2^{-\cdot}$ and H_2O_2 generation as well as the susceptibility of tissues to experimentally induced oxidative stress are increased; (b) experimental regimens that extend the lifespan, such as caloric restriction in mammals and reduction of metabolic rate in insects, decrease the accumulation rates of oxidative damage; (c) mitochondria make two rather contradictory contributions to cell survival. The classically recognized function is the synthesis of ATP for energizing endergonic reactions, the other is generation of reactive oxygen species which may compromise the long-term survival of cells and constitute a major underlying cause of the aging process. Indeed, these two rather conflicting functions are part of the same process, namely mitochondrial respiration (42).

More than 95% of the O_2 taken up by the human body is used by mitochondrial cytochrome oxidase which adds four electrons to oxygen to generate a molecule of water. Cytochrome oxidase normally does not release reactive oxygen species into its surroundings. However, a number of investigations have indicated that brain mitochondria undergo oxidative stress damage and a decrease of cytochrome c oxidase activity during aging (117). It has been postulated that this complex may act as a bottleneck, creating a situation of "electron traffic jam" upstream, which would alter the redox state of oxidoreductases in the electron transfer chain, and increase their autoxidizability and rate of superoxide generation. A finding that lends credibility to this hypothesis is that cytochrome c oxidase activity is directly correlated with the average life-span in different species (225).

Oxidative damage to key intracellular targets such as DNA or proteins is an important feature of the normal cellular aging process in the brain, and several studies have shown that oxidative damage to DNA or protein extracted from brain tissue increases with age (130). Oxidative damage to DNA has been shown to be extensive and could be a major cause of the degenerative diseases related to aging such as cancer (204). With respect to this, it has been proposed that DNA damage is a major factor underlying neuronal degeneration in normal aging and that accelerated damage to DNA may be the basis of the neurodegenerative conditions such as Alzheimer's disease (AD), where it has been demonstrated that DNA damage distribution in the human brain, as shown by *in situ* end labeling, shows area-specific differences in aging and in Alzheimer's disease (173). Levels of the oxidized nucleotide 8-hydroxy-deoxyguanosine (8-OH-dG) a biomarker of DNA damage, also accumulate with aging. In several tissues, including brain and muscle, levels of 8-OH-dG in mtDNA exceed that of nuclear DNA (nDNA) some 16-fold, although as yet there have been no studies performed using absolutely pure mtDNA (15, 111). It has been demonstrated that 8-OH-dG most frequently base pairs with cytosine, but also mispairs with adenine approximately 1% of the time, causing misreading of adjacent residues. Mecocci *et al.* found that 8-OH-dG significantly correlates with increases in levels of a 7.4-kb deletion in human brain (172).

The major DNA product formed by methylating agents *in vitro* and *in vivo* is 7-methylguanine, and in nuclear DNA of normal mouse brain, steady-state levels of 7-methylguanine increased approximately twofold between 11- and 28-months of age and that following treatment *in vivo* with methylnitrosourea, a fraction of DNA damage in brain tissue was refractory to repair and was lost from DNA much more

slowly (101). This repair-resistant fraction of damage was greater in DNA from the old tissues and it was suggested that that although DNA repair enzymes are present and active in senescent postmitotic tissues such as brain, changes in the structure and function of "old" chromatin somehow decreases the capacity of the DNA repair enzymes present in the nucleus to repair oxidatively damaged DNA (102). In addition, single-strand and double-strand breaks in DNA accumulate in the brain with age, and on exposure of neurons isolated from young and aged rats to an excitotoxic insult, more extensive DNA breaks neurons were measured in neurons isolated from older rats (160). During the course of normal metabolism in the brain there is production of reactive oxygen species such as superoxide and hydroxyl radicals, as well as the production of reactive nitrogen species such as nitric oxide and peroxynitrite. Therefore the ability of DNA repair mechanisms within the nuclei of brain cells to repair damage caused by a diverse range of oxidizing species is central to the maintenance of normal brain function. Several enzymes systems have been found to repair damage to DNA caused by oxidizing species (76). These include endonucleases, exonucleases, thymine glycol glycolases, and DNA polymerases. DNA polymerases thus far detected in mammalian brain (alpha, beta, delta, and epsilon), undergo age-dependent changes in activity, but it is not known which cell types contain which polymerases, and the ability of nuclei from different brain regions to repair specific types of oxidative DNA damage is unknown. In addition, recent evidence indicates that genetic instability, such as telomere loss, somatic and mitochondrial DNA mutations, increases with age (6). Levels of oxidative damage in mitochondrial DNA isolated from various brain regions appear to be at least ten-fold higher than those of nuclear DNA (19), although due to technical difficulties there has as yet been no definitive study of oxidative damage to mitochondrial DNA (16). This increase correlates with the 17-fold higher evolutionary mutation rate in mtDNA compared with nuclear DNA. These higher levels of oxidative damage and mutations in mtDNA recognize different causal factors, including location of the DNA near the inner mitochondrial membrane sites (where oxidants are generated), lack of protective histones, mitochondrial polymerase errors, and activation of genes involved in error-prone DNA repair (220). The age-associated accumulation of oxidative damage to mtDNA correlates with the level of mtDNA deletions found in various tissues composed of postmitotic cells (89). It is possible that this damage leads to mutations that result in mitochondrial dysfunction, which has been suggested to be involved in the pathogenesis of neurodegenerative disorders (54, 189, 190, 196).

An increase in protein oxidative damage, as indicated by the loss of protein sulfhydryl groups and by a decline in the activity of enzymes, such as glutamine synthetase and glucose-6-phosphate dehydrogenase, has been documented to occur in brain during aging (197, 233). A number of experimental evidence indicate that increased rate of free radical generation and decreased efficiency of the reparative/degradative mechanisms, such as proteolysis, both are factors which primarily contribute to age-related elevation in the level of oxidative stress and brain damage (197, 198). With respect to this, it has been suggested that decreases in levels of enzymes which ordinarily protect neuronal cells against oxidative stress with age may be responsible for increased levels of free-radical damage in the brain, or that these enzymes themselves are susceptible to inactivation by free radical molecules which increase with age in the brain (189, 190). During aging a number of enzymes accumulate as catalytically inactive or less active forms. The age-related changes in catalytic activity are due in part to reactions of the protein with oxygen and/or nitrogen free radical species produced during exposure to ionizing radiation or to metal ion catalyzed oxidation systems. The levels of oxidized proteins in brain extracts of rats of different ages increase progressively with age, and in old rats can represent 30–50% of the total cellular protein (42, 51). The age-related increase in oxidized protein is accompanied by a loss of glutamine synthetase (GS) and glucose-6-P dehydrogenase (G-6-PDH) activities, and to a decrease in the level of cytosolic neutral protease activity which is responsible for the degradation of oxidized (denatured) protein. Of particular significance are the results of experiments showing that similar age-related changes occur in the gerbil brain and that these changes are accompanied by a loss of short-term memory. Chronic treatment of old animals with the free radical spin-trap reagent, *N-tert*-butyl-alpha-phenylnitrone (PBN) resulted in normalization of the several biochemical parameters to those characteristic of the young animals (26); coincidentally, the short-term memory index was restored to the young animal values (57). These results provide strong evidence that there is a linkage between the age-dependent accumulation of oxidized proteins and the loss in brain physiological functions. It has recently proposed that a primary mechanism leading to neuronal cell death in ageing and common neurodegenerative disorders is interference with proteasome function (126).

Proteasomal dysfunction can involve genetic defects, direct inactivation of proteasome by reactive oxygen and nitrogen species, or overloading with proteins. The latter can be caused by excessive production of normal proteins or by the formation of poorly degradable proteins as a result of genetic mutations, faulty posttranslational modification, or protein modification by free radical damage. Protein conformational diseases (PCD) is an emerging aspect of major neurodegenerative diseases: Alzheimer disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and Friedreich's ataxia (FA) are all associated with the presence of abnormal proteins (195–198, 125). The origin of HD and FA involve specific genetic defects that lead to production of abnormal proteins, whereas AD, ALS, and PD have been described mostly as sporadic, although familial types of AD, ALS and PD are recognized. Examples are the rare mutations in synuclein or parkin that cause familial PD, and the 2% of ALS cases associated with mutation in the gene encoding copper-zinc containing superoxide dismutase (CuZnSOD) (113, 190). Even in the more common sporadic versions of PD, ALS, and AD, abnormal proteins are present to a significant extent. Thus the senile plaques typical of AD contain not only β-amyloid but a wide range of other proteins. Most of them are oxidized and nitrated. Similar damage has been described for proteins in the Lewy bodies in sporadic PD. Oxidative as well as nitrative protein damage are also elevated in ALS. In nondividing cells, such as the great majority of neurons in the adult brain, the protein content of

cells is approximately constant. Since protein synthesis is continuous, there must be an equilibrium between synthesis and degradation. Cellular protein can be degraded by the lysosomal system, but a system of equal or greater importance is the proteasome. The 20S (according to its sedimentation coefficient) is a cylindrical structure comprised of multiple protein subunits and containing a narrow channel where proteolysis occurs. The 20S proteasome can degrade a wide range of proteins, including oxidatively damaged proteins, but most or all of the 20S proteasome in the cell is associated with a 19S "cap complex" that binds in an ATP-dependent manner and confers specificity for the degradation of polyubiquinated proteins (112). Several other proteins are known that can associate with proteasomes and increase (or in some case decrease) rates of protein clearance. Although it is usually assumed that increased levels of oxidative damage are due to the increased generation of free radical species, however increased levels of oxidative damage can equally ensure a decreased clearance of oxidatively-modified biomolecules. Oxidized protein levels in the CNS tend to increase with age (97, 170) consistently with several reports that proteasome activity decreases with age and in neurodegenerative disorders (170).

The age-dependent accumulation of oxidized dysfunctional proteins with reactive carbonyl groups, leads to inter- and intramolecular cross-links with protein amino groups, thus altering the efficiency of the electron transport. Imbalances in the stoichiometry of functional electron transport proteins is proposed to lead to a leakage in the flow of electrons to the terminal electron acceptor, cytochrome oxidase (183), and increased likelihood of superoxide generation. Studies on isoprostanes, end product of lipid peroxidation that can be measured in CSF and urine in various neurodegenerative disorders, suggest that lipid peroxidation is an early stage in these disease processes. Similarly, another end product of lipid peroxidation, the aldehyde 4-hydroxy-*trans*-nonenal (HNE), which is highly neurotoxic, avidly binds to proteins, and HNE-protein adducts are demonstrable in senile plaques and tangles in AD, tissues from ALS patients, and Lewy bodies in PD. Protein carbonyls can be generated by direct oxidative damage to proteins, by the binding to proteins of cytotoxic aldehyde such as HNE, and by glycosidation of proteins (81, 82). The content of protein carbonyls in Alzheimer's brain samples is greater than in age-matched controls (32–35), and this provides the clearest indication of greater accumulation of oxidized proteins in this disease. Brain regions show specific changes in this regard, and carbonyl levels correlate well with tangles (26–29). Importantly, the accumulation of oxidation products in particular regions of the brain seems to be related to specific cognitive defects (93, 30, 31). In support of this, administration of the spin-trap PBN, or nutritional antioxidants retards both protein oxidation and such neurological defects (26–28, 57).

THE MITOCHONDRIAL THEORY OF AGING

Harman in 1972 first proposed that mitochondria may have a central role in the process of aging (115, 116). According to this theory, free radicals generated through mitochondrial metabolism can act as causative factor of abnormal function and cell death. Mitochondria are the cell's most significant source of oxidants and *in vitro s*tudies have indicated that approximately 1–2% of electron flow through the ETC results in the univalent generation of superoxide (42, 52, 195). Moreover, various toxins in the environment can injure mitochondrial enzymes, leading to increased generation of free radical that over the life span would eventually play a major role in aging (48, 50, 53). Ultrastructural changes have been also reported to occur in mitochondria with age. They become larger and less numerous with vacuolization, cristae rupture, and accumulation of paracrystalline inclusions. Cardiolipin, an acidic phospholipid that occurs only in mitochondria, decreases with age (188). This inner membrane lipid is known to have optimal electrical insulating properties, thereby contributing significantly to the transmembrane potential that drives the formation of ATP via ATP synthase. Indeed, a decrease in membrane potential in mitochondria from older animals has been demonstrated (52, 53).

It has been proposed that accumulation of mtDNA during life is a major cause of age-related disease and this is because of its high mutagenic propensity. The lack of introns and protective histones, limited nucleotide excision, and recombination DNA repair mechanisms, location in proximity of the inner mitochondrial membrane which expose it to an enriched free radical milieu, are all factors contributing to a ten-fold higher mutation rate occurring in the mtDNA than in the nDNA. A large body of evidence indicates that mtDNA mutations increase as a function of age reaching the highest levels in brain and muscle. More than twenty different types of deletions have been documented to accumulate in aging human tissues. The first report on an age-related increase in an mtDNA deletion was called the "common deletion" and was found in elderly brain and in Parkinson's disease (39). This deletion has been described to occur between 13-bp sequence repeats beginning at nucleotides 8470 and 13447, removing almost a 5-kb region of mtDNA between ATPase 8 and the ND5 genes (Fig. 1). The deletion is thought to occur during replication of the mtDNA, the absent sequence encoding for six essential polypeptides of the respiratory chain and 5 tRNAs. It has been associated with several clinical diseases, such as chronic progressive external ophthalmoplegia and Kearns Sayre syndrome. Several age-related disorders are linked to higher levels of mtDNA mutations than age-matched controls. In the CNS, Ikebe *et al.* showed 17 times higher levels of the common deletion in the striatum of patients with Parkinson's disease compared to age-matched controls (195). Evidence also exists indicating higher levels of this deletion in patients with Alzheimer's disease which parallel increased levels in the oxidized nucleotide 8-OH-dG (19). A major feature of mtDNA disease in humans is the presence of cells with low cytochrome *c* oxidase activity, which indicates that the mechanism for these changes is likely to be clonal expansion of individual mtDNA deletions within single cells (220). Complex IV deficient cells, which occurred only sporadically earlier than the sixth decade of life, were present regularly after this age, with the loss of enzyme activity being always confined to single randomly distributed cells. Similarly, cytochrome *c* oxidase-negative neu-

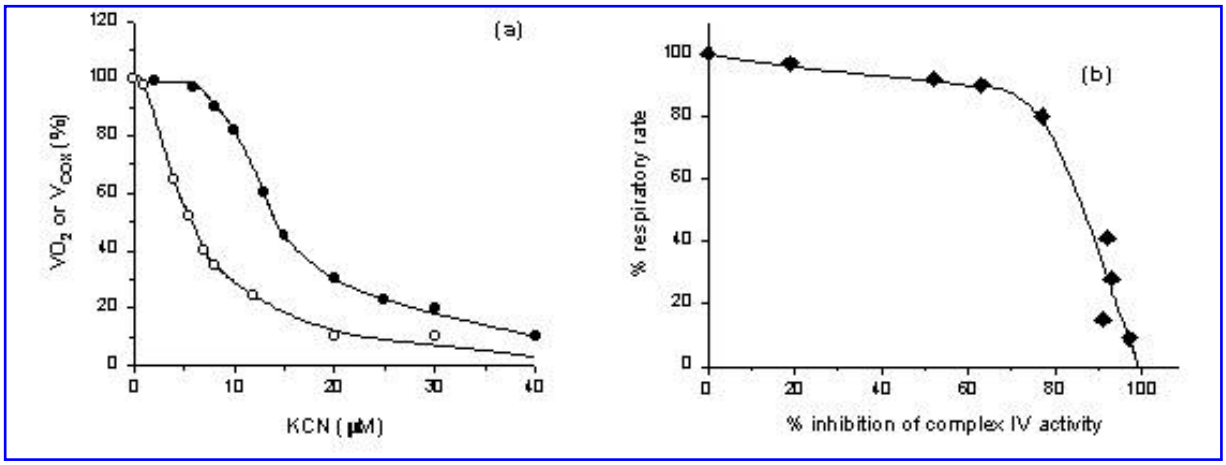

FIG. 1. Energy thresholds in brain mitochondria. (**a**): KCN titration of respiration (●) and complex IV activity (○); (**b**): Respiratory rate as a function of complex IV inhibition. The data points from (**a**) are used to plot rates of respiration against the percent inhibition of complex IV activity.

rons have been demonstrated to exist in abundance in the CNS of patients with a mitochondrial disorder (70). These findings establish the relationship between age-associated accumulation of mtDNA mutations and bioenergy degradation as a key feature of the aging process, at least in tissues predominantly composed by postmitotic cells, such as CNS and skeletal muscle. Relevant to mitochondrial bioenergetics, in fact, is the finding of a significant decrease in state 3/state 4 ratio, which has been observed to occur in brain as function of age (42). Since this ratio relates to the coupling efficiency between electron flux through the electron transport chain and ATP production, an increase in state 4 would result in a more reductive state of mitochondrial complexes and, consequently, to an increase in free radical species production. A decrease in state 3/state 4 respiration during aging is associated with a significant decrease in cardiolipin content in brain mitochondria (69). This loss could play a critically important role in the age-related decrements in mitochondrial function, and appears to be associated with both quantitative and qualitative region-specific protein changes that are parallel to structural changes, such as decrease of the inner membrane surface, smaller as well as sparser cristae, decreased fluidity, and increased fragility. Modifications in cardiolipin composition are recognized to accompany functional changes in brain mitochondria, which include all proteins of the inner mitochondrial membrane that generally require interaction with cardiolipin for optimal catalytic activity (199, 202). Acetylcarnitine fed to old rats increased cardiolipin levels to that of young rats and also restored protein synthesis in the inner mitochondrial membrane, as well as cellular oxidant/antioxidant balance (47–50, 187) suggesting that administration of this compounds may improve cellular bioenergetics in aged rats (109, 110). Interestingly, caloric restriction, a dietary regimen that extends lifespan in rodents, maintains the levels of 18:2 acyl side chains and inhibits the cardiolipin composition changes (223). In addition, caloric restriction retards the aging-associated changes in oxidative damage, mitochondrial oxidant generation, and antioxidant defenses observed during aging (164–166, 197).

ENERGY THRESHOLDS IN BRAIN MITOCHONDRIA: IMPLICATION FOR NEURODEGENERATIVE DISORDERS

Human cells contain from a few hundred to more than a thousand mitochondria; each mitochondrion in turn has 2–10 copies of mtDNA, thus, several thousands copies of the mitochondrial genome can be present within a single cell. Importantly, unique to mtDNA, is that it is inherited exclusively through the mother, and may exist in many different copies in the oocyte cytoplasm. This implies that no mtDNA recombination occurs at fertilization and only a sequential accumulation of mutations from the maternal lineage account for mtDNA variations. Moreover, mtDNA is particularly prone to mutation, being estimated as 10 times greater then nuclear DNA (54), owing to the absence of protective proteins (such as histones) and of a high-efficiency repair system. Thus, mutant and wild-type (normal) mtDNA can coexist within a cell in any proportion and this situation is termed heteroplasmy. It is becoming increasingly clear that the mitochondrial genome may play an essential role in the pathogenesis of neurodegenerative diseases, and evidence for mitochondria being a site of damage in neurodegenerative disorders is based in part on observed decreases in the respiratory chain complex activities in Parkinson's, Alzheimer's, and Huntington's diseases. Such defects in respiratory complex activities, possibly associated with oxidant/antioxidant imbalance, are thought to underlie defects in energy metabolism and induce cellular degeneration. The first hint that mitochondria play a role in human disease did not emerge until 1958, when a Swedish patient was identified who had symptoms of severe perspiration, polydipsia, polyphagia, weight loss, and weakness (155). Laboratory studies showed a basal metabolic rate 200% above normal, very low weight (37 kg), and basal temperature reaching 38°C. Biochemical studies revealed that she had a partially uncoupled respiration which accounted for her generation of excessive heat and high calorie consumption. Although the primary etiologic event in Luft's disease

remains to be identified, the disease is associated with release of mitochondrial calcium stores, abnormal calcium cycling, and sustained stimulation of loosely coupled respiration. The discovery of Luft's disease cleared the path for fertile investigations and since the 1960s, over 120 human mitochondrial diseases have been discovered, many of which involve selected populations in the central nervous system consisting of postmitotic, highly energy-dependent cells. Many of these diseases have been associated with specific inherited mitochondrial DNA mutations and respiratory chain deficiencies. Point mutations may involve either the RNA or protein-encoding genes, and rearrangements may take the form of deletions or duplications. In the presence of heteroplasmy there is a critical ratio of mutant to wild-type mitochondrial genomes that is necessary before the disease becomes both biochemically and clinically apparent. As might be expected, mtDNA disorders are phenotypically diverse given the ubiquitous presence of mitochondria and the variation in the levels of heteroplasmy in the body. Yet many have predominant neurologic and muscular symptoms, including dementia, seizures, ataxic syndromes, peripheral neuropathies, and progressive myopathy.

Postmitotic tissues typically show increased levels of mutant mitochondria due to the inability of these tissues to select against cells containing mutant mtDNA genomes. CNS imaging of patients with mtDNA disorders often reveals moderate degrees of cerebral or cerebellar atrophy that are consistent with neurodegeneration, often comparable with the pathologies associated with the brain in senescence or in dementia (69).

Several mechanisms have been proposed to explain the variability of the phenotypic expression of an mtDNA mutation, such as sporadic mutation or mitotic segregation. However, all these hypotheses incorporate a unique feature of mitochondrial genetics and pathologies; that of the heteroplasmic concept of mtDNA mutations. Levels of mutation can vary considerably between mitochondria, cells, and even tissues within the same individual. Consequently, the expression of a mutation in the mtDNA can be thought of as a function of the degree of the heteroplasmy. In general, whether a metabolic defect expresses itself as a recognizable clinical disease will depend upon the extent to which it affects the metabolic pathway in question and this can lead to a threshold expression of the disease state. One of the most important features recognized in mitochondrial diseases is the existence of a threshold in the degree of a mitochondrial deficit for the expression of the disease, and these were shown by Wallace (252) to be related to the balance between normal and mutant mtDNA. Accordingly, it has been demonstrated by Attardi (62) that only 10% of wild type DNA is enough to maintain a normal respiratory rate and this has also been confirmed by other authors who have demonstrated that 80–90% deleted mtDNA must be achieved before complex IV activity is compromised (42). All this represents compelling evidence that there is a threshold in the heteroplasmy of the mutation around 90% before a pathological consequence become manifest. Below this threshold the flux of respiration and of ATP synthesis are at a level which does not compromise normal metabolism. As consequence, there are at least four levels at which threshold effects occur in mitochondrial metabolism, with respect to their possible involvement in the pathogenesis of neurodegeneration. The first is the expression of the heteroplasmy of mtDNA at the level of a given enzymatic step, whereby mitochondria from patients might exhibit a particular ratio of defective DNA compared to normal DNA. The second is the threshold effect observed in the mitochondrial metabolism as a result of a decrease in a given mitochondrial activity. The third may occur in the expression of defective mitochondria with respect to the whole cellular metabolism. The fourth is the fact that the control coefficient of a given step may vary depending on different types of mitochondria, which leads to the observation that the threshold value for a given complex of the electron transport chain can vary according to the threshold in the energy demand of different tissues. At each level the threshold effect will reinforce the others, as interpreted by the *double threshold hypothesis* (169), whose predictions are now beginning to be documented. Data from studies of rat brain mitochondria of nonsynaptic origin have shown that thresholds exist whereby complex activities need to be reduced by at least 60% before major changes in ATP synthesis and oxygen consumption occur. Interestingly, in synaptic mitochondria, titration of various complexes with specific inhibitors generated threshold curves showing that complex I, III, and IV activities had to be decreased 25, 80, and 70%, respectively, before major changes in rates of oxygen consumption and ATP synthesis were observed (147). These results suggest that in mitochondria of synaptic origin complex I activity has a major control of oxidative phosphorylation, such that when a threshold of 25% inhibition is exceeded, energy metabolism is compromised, and reduction in ATP synthesis ensues. Moreover, the same study demonstrated that depletion of glutathione, which has been reported to be a primary event in idiopathic Parkinson's disease, abolished the threshold for complex I, providing experimental evidence that antioxidant status is critically involved in maintaining energy thresholds in mitochondria (42, 73).

Other data are also consistent with these findings, as it has been shown both in a patient with cytochrome c oxidase deficiency and in an animal model of copper deficiency that more than a 50% deficit in complex IV activity did not affect the respiratory flux (210). It is possible to explain these findings within the framework of the *metabolic control theory* (209). According to this theory, which investigates the effects of infinitesimally small parameter perturbations on the variables of metabolic systems, a crucial stage in the expression of a threshold for a clinical disease is, at molecular level, the impact that a localized defect in a given step has on the global flux of a metabolic network (209). In this theory an important parameter is the *control coefficient* which quantitatively expresses the fractional change in pathway flux of a metabolic network, under steady-state conditions, induced by a fractional change in the individual step under consideration (42, 73). For the oxidative flux (respiration) in mitochondria it can be determined according to Eq.1:

$$C = (dJO_2/d(\text{Inhibitor}))/(dVc/d(\text{Inhibitor})) \quad \text{(Eq.1)}$$

where C is the flux control coefficient of the mitochondrial complex under investigation, dVc/d(Inhibitor) is the rate of change of complex activity (individual step) and dJO_2/d(Inhibitor) is the rate of change of respiration (global flux), at

low concentrations of the complex inhibitor. In determining the control coefficients of the various steps of oxidative phosphorylation on respiratory flux with the inhibitory titration method, two very differently shaped curves are observed, for the isolated step and the whole flux. Figure 1a shows the effect of KCN titration of respiration and complex IV activity. It can be seen that even at 50% cytochrome c oxidase inhibition, there is only 20% inhibition of the whole flux, and 90% of inhibition of the isolated step is required to achieve a significant reduction of the respiration, corresponding to global flux. This is apparent from Figure 1b, obtained by plotting the inhibition of the respiratory flux as a function of the complex IV activity, given the same KCN concentration. Generation of a threshold curve is evident, and the complex activity must be decreased by 70% before a rapid decline in the rate of respiration occurs. This pattern is a direct consequence of the summation theorem of metabolic control theory (210), which states that the sum of control coefficients of a defined metabolic pathway is equal to 1 with the result that most of the control coefficients are low. Consistently, a control coefficient of 0.1 for a given complex, implies a 10% perturbation in the activity of this complex and can result in an inhibition of respiratory rate by as little as 1%. This implies that in the case of oxidative phosphorylation each single control coefficient is close to zero, producing at the beginning a quasi horizontal slope; at very low activity of the step both curves must meet again, due to the fact that the flux becomes zero and the step is completely inactivated (209).

NEUROGASOBIOLOGY: ROLES OF NO AND CO IN BRAIN PHYSIOPATHOLOGY

Carbon monoxide (CO) is the second gas discovered in the last 25 years to have salutary effects, the first being NO (108). Certain findings raise the conceivable possibility that HO-1 and/or CO and NOS2 and/or NO are functionally interrelated in mediating their protective effects. In some situations, CO can activate the expression of NOS2 and, in others, inhibits expression of NOS2 and consequently NO (179). NO upregulates HO-1 with production of CO (156–158). We have recently found evidence for a functional relationship between CO and NO. In endotoxic shock, the salutary action of CO in rat brain appears to depend sequentially on the activation of NFkB, which triggers transcription of NOS2 with production of NO, and subsequently in the upregulation of HO-1. In the absence of any of these steps, the beneficial effect of CO is lost (214–216). This has been also demonstrated in mice in the treatment of hepatitis induced by TNFα and D-galactosamine (185). To what extent CO and NO act interdependently in other physiopathological conditions that are responsive to CO and/or NO is unknown.

Nitric oxide synthase (NOS) and its isoforms in the CNS

The enzyme responsible for NO synthesis is the nitric oxide synthase (NOS) family of enzymes, which catalyze the conversion of arginine to citrulline and NO•. NOS, localized in the CNS and in the periphery (39), is present in three well-characterized isoforms (a) neuronal NOS (nNOS, type I) (b) endothelial NOS (eNOS, type III), and (c) inducible NOS (iNOS, type II). Activation of different isoforms of NOS requires various factors and co-factors. In addition to a supply of arginine and oxygen, an increase in intracellular calcium leads to activation of eNOS and nNOS, and formation of calcium/calmodulin complexes is a prerequisite before the functional active dimer exhibits NOS activity, which depends also on cofactors such as tetrahydrobiopterin (BH_4), FAD, FMN, and NADPH (85). nNOS has a predominant cytosolic localization whereas the eNOS is bound to the plasma membrane by N-terminal myristylation (39, 52). In contrast to nNOS and eNOS, iNOS can bind to calmodulin even at very low concentration of intracellular calcium, thus iNOS can exert its activity in a calcium-independent manner. iNOS, usually present only in the cytosol, also requires NADPH, FAD, FMN, and BH_4 for full activity. eNOS expressed in cerebral endothelial cells critically regulates cerebral blood flow. However, a small population of neurons in the pyramidal cells of CA1, CA2, and CA3 subfields of the hippocampus and granule cells of the dentate gyrus express eNOS. nNOS, which is expressed in neurons, is critically involved in synaptic plasticity, neuronal signaling, and neurotoxicity. Activation of nNOS forms part of the cascade pathway triggered by glutamate-receptor activation that leads to intracellular cyclic GMP elevation. The levels of iNOS in the CNS are generally fairly low. However, an increased expression of iNOS in astrocytes and microglia occurs following viral infection and trauma (22). Activation of iNOS requires gene transcription, and the induction can be influenced by endotoxin and cytokines (Interleukin-1, interleukin-2, lipopolysaccharide, interferon-γ, tumor necrosis factor). This activation can be blocked by antiinflammatory drugs (dexamethasone), inhibitory cytokines (interleukin-4, interleukin-10), prostaglandins (PGA_2), tissue growth factors or inhibitors of protein synthesis (e.g., cycloheximide) (42, 48).

Nitric oxide as a neurotransmitter

The discovery of the role of NO as a messenger molecule has revolutionized the concept of neuronal communication in the CNS. NO is a gas freely permeable to the plasma membrane. Thus, NO does not need a biological receptor to influence the intracellular communication or signaling transduction mechanisms (235). Once generated, the cell can not regulate the local concentration of NO, therefore the other way to influence NO activity is to control its synthesis. The activity of NO also terminates when it chemically reacts with a target substrate. Nitric oxide when produced in small quantities can regulate cerebral blood flow and local brain metabolism (42), neurotransmitter release, and gene expression, and play a key role in morphogenesis and synaptic plasticity. It is also generally accepted that NO is a major component in signaling transduction pathways controlling smooth muscle tone, platelet aggregation, host response to infection, and a wide array of other physiological and pathophysiological processes. Under conditions of excessive formation, NO is emerging as an important mediator of neurotoxicity in a variety of disorders of the nervous system (72, 119).

Redox activities elicited by NO

In the last several years a number of studies have shown a protective effect of nitric oxide in a variety of paradigms of cell injury and cell death. These include: (a) direct scavenging of free radicals, such superoxide with effects on intracellular iron metabolism, including interaction with iron to prevent, through formation of nitrosyl-iron complexes, release of iron from ferritin (224); (b) interaction of $NO^{\bullet}$; (through its congener NO^{+}) with thiol group on the NMDA receptor with consequent downregulation and inhibition of calcium influx (127); (c) inactivation of caspases (224); (d) activation of a cyclic GMP-dependent survival pathway, as demonstrated in PC12 cells (179); and (e) inducing expression of cytoprotective proteins, such as heat shock proteins (40, 43, 45, 48); inhibition of nuclear factor-κB activation or GADPH, whose activity appears to be required in one paradigm of neuronal apoptosis (193). In general, the current opinion holds that the intracellular redox state is the critical factor determining whether in brain cells NO is toxic or protective (207). In addition, it has been proposed that NO might inhibit T-cell activation and cell trafficking across the blood-brain barrier and hence limiting the setting of the autoimmune cascade associated with degenerative damage (74, 75). The difficulty in delineating a mechanistic involvement of NO as pro-inflammatory or antiinflammatory agent and the controversy arising on whether excessive NO elicits cytoprotective or cytotoxic actions are better appreciated by recognizing the complexity of NO chemistry when applied to biological systems (177–179). As minutely detailed by Stamler and colleagues, the reactivity of the NO groups is dictated by the oxidation state of the nitrogen atom, which enables the molecule to exist in different redox-activated forms (235). In contrast to NO, which contains one unpaired electron in the outer orbital, nitrosonium cation (NO^{+}) and nitroxyl anion (NO^{-}) are charged molecules being, respectively, the one-electron oxidation and reduction products of NO. While NO^{+} can be transferred reversibly between cysteine residues (transnitrosation), NO^{-} can be formed by hemoglobin, neuronal NOS, and *S*-nitrosothiols (RSNO). A fundamental aspect of NO biochemistry is the attachment of NO groups to sulfhydryl centers to form *S*-nitrosyl derivatives or RSNO (127). This chemical process, known as *S*-nitrosation, has been suggested to represent a refined endogenous tool to stabilize and preserve NO biological activity (180, 181, 207). It has been speculated that low-molecular weight RSNO, such as *S*-nitrosoglutathione or nitrosocysteine may also represent a mechanism for storage *in vivo* of NO (207, 234). In this regard, glutathione becomes an important determinant of the reactivity and fate of NO because this cysteine-containing tripeptide is very abundant in most tissue and biological fluids. In addition, *S*-nitrosation is also an important process in modulating the activity and function of several enzymes and proteins. However, deleterious and oxidative modification in protein structure and function may occur when reactive nitrogen species (RNS) reach a critical threshold and hence nitrosative stress may ensue (101). At the cellular level, nitrosative stress has been linked to inhibition of cell growth and apoptosis, and implicated in NO pathogenesis (224). The intriguing aspect in the parallellism between the effects mediated by increased RNS and ROS is the ability of cells to respond to these two types of stress and, depending on the severity of the nitrosative/oxidative insult, this response may result in both adaptation and resistance to toxicity (41, 45, 48, 51).

Signaling mechanisms involved in the control of gene expression: modulation by oxidative and nitrosative stress

Signaling mechanisms adopted by regulatory proteins to control gene expression in response to alterations in the intracellular redox status are very common in prokaryotes. Gene activation by oxidative stress was first described in bacteria where regulatory proteins such as OxyR was discovered as an activator of antioxidant and stress responsive genes. The OxyR is a homotetramer that is activated by hydrogen peroxide and *S*-nitrosothiols. The protein contains six cysteine residues, one of each is absolutely necessary for activity and two are required for maximal activation (91, 118, 179). Recent studies suggest that oxidation of a single thiol to a sulfenic acid may represent a sensor mechanism, whereas the activation mechanism can be ascribed to formation of an intramolecular disulfide, or alternatively to S-nitrosylation of a single cysteine residue, with Cys 199 being a likely candidate site of posttranslational modification (179, 207). The expression of these protective genes renders the bacteria more resistant to oxidant damage (179, 181). As the cytoprotective mechanism triggered by SoxR in *E. coli* includes the expression of critical antioxidant defensive proteins, such as superoxide dismutase (179), the emerging concept is that analogous system might operate in mammalian cells. In eukaryotes, typical examples are genes such as heme oxygenase gene, thioredoxin and detoxificant enzymes (Mn-SOD, glutathione *S*-transferase, NADPH: quinone reductase), cytokine, immunoreceptors, and growth factors. That the antioxidant protein heme oxygenase could "sense" NO and, thus, protecting against ROS and RNS insults, is supported by the following findings: (a) NO and NO-related species induce HO-1 expression and increase heme oxygenase activity in human glioblastoma cells, hepatocytes, and aortic vascular cells; (b) cells pretreated with various NO-releasing molecules acquire increased resistance to H_2O_2-mediated cytotoxicity at the time heme oxygenase is maximally activated; and (c) bilirubin, one of the end products of heme degradation by heme oxygenase, protects against the cytotoxic effects caused by strong oxidants H_2O_2 and $ONOO^{-}$ (179, 207). The conception that NO and RNS can be directly involved in the modulation of HO-1 expression in eukaryotes is based on the evidence that different NO-releasing agents can markedly increase HO-1 mRNA and protein, as well as heme oxygenase activity, in a variety of tissues, including brain cells (216). In rat glial cells, treatment with lipopolysaccaride (LPS) and interferon-γ (IFN-γ) results in a rapid increase in both iNOS expression and nitrite levels followed by enhancement of HO-1 protein (55, 67, 214). In the same study, the presence of NOS inhibitors suppressed both nitrite accumulation and HO-1 mRNA expression. Modulation of HO-1 mRNA expression by iNOS-derived NO following stimulation with LPS has also been reported in different brain regions, particularly in the

hippocampus and substantia nigra *in vivo* rat model of septic shock (214). Moreover, the early increase in iNOS protein levels observed in endothelial cells exposed to low oxygen tension seems to precede the stimulation of HO-1 expression and activity, an effect that appears to be finely regulated by redox reactions involving glutathione (177–179). Taken together, these findings point to the central role of the NO as a signaling molecule which, by triggering expression of cytoprotective genes such as HO-1, may lead to adaptation and resistance of brain cells to subsequent, eventually more severe, nitrosative and oxidative stress insults (28–35). Thus, a direct interaction of NO groups with selective chemical sites localized in transcription proteins that can be activated through nitrosative reactions could effectively contribute to the enhancement of both *HO-1* gene expression and stress tolerance. Recent knowledge concerning the modulation by thiol redox state of the activity of several transcription factors that recognize specific binding sites within the promoter and distal enhancer regions of the *HO-1* gene include: Fos/Jun [activator protein-1 (AP-1)], nuclear factor-κB (NFκB), and the more recently identified Nrf2 proteins (9, 2, 3, 55). Importantly, both AP-1 and NFkB contain cysteine residues whose interaction with oxidant or nitrosant species might be crucial for determining the binding activity to DNA (179, 207). Data in the literature show that NO can either activate or inhibit these transcription factors, and that in many circumstances activation depends on the reversibility of the posttranslational modification elicited by the various RNS (28, 33, 35, 195–198). We have recently demonstrated in astroglial cell cultures that cytokine-induced nitrosative stress is associated with an increased synthesis of HSP70 stress proteins. Increase in HSP70 protein expression was also found after treatment of cells with the NO generating compound sodium nitroprusside, thus suggesting a role for NO in inducing HSP70 proteins (40, 41). *In vivo* experiments performed in our laboratory as well as in other laboratories have also demonstrated that the redox glutathione status is a critical factor for induction of cytoprotective HSP70 (9, 43, 51, 217, 218).

Mitochondrial damage, reactive nitrogen species, and neurodegenerative disorders

Increasing evidence sustain the hypothesis that mitochondrial energy metabolism underlie the pathogenesis of neurodegenerative diseases. Decreased complex I activity is reported in the substantia nigra of postmortem samples obtained from patients with Parkinson's disease (11). Similarly, impaired complex IV activity has been demonstrated in Alzheimer's disease (119). Increased free radical-induced oxidative stress has been associated with the development of such disorders and a large body of evidence suggest that $NO^{\bullet}$ play a central role (234, 235). Cytokines (INF-γ) which are present in normal brain are elevated in numerous pathological states, including Parkinson's disease (168), Alzheimer's disease (175), multiple sclerosis (8, 36, 37, 44, 49), ischemia, encephalitis, and central viral infections (42). Accordingly, as cytokines promotes the induction of NOS in brain, a possible role for a glial-derived $NO^{\bullet}$ in the pathogenesis of these diseases has been suggested (235). Excessive formation of $NO^{\bullet}$ from glial origin has been evidenced in some study in which NADPH diaforase (a cytochemical marker of NOS activity) positive glial cells have been identified in the substantia nigra of postmortem brains obtained from individuals with Parkinson's disease (125, 126). Loss of nigral GSH is considered an early and crucial event in the pathogenesis of Parkinson's disease (10, 11) and as a consequence decreased peroxynitrite scavenging may also occur. Therefore, such perturbations in thiol homeostasis may constitute the starting point for a vicious cycle leading to excessive $ONOO^{-}$ generation in Parkinson's disease. In support of this, it has been reported that the selective inhibition of nNOS prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism in experimental animals (74, 175).

Carbon monoxide in brain signaling, neuroprotection, and neurorestoration

The first detection of a combustible gas in the blood occurred in 1894 by Grehant (108). This gas was supposed to be carbon monoxide (CO). However, it was not until 1949 that Sjorstrand discovered that endogenously produced CO arose from the degradation of hemoglobin released from senescing erythrocytes (227). Greater than 75% of CO produced in humans arises from erythrocyte turnover generated as a by-product of heme metabolism. In 1969, the source of endogenous CO was discovered, as Tenhunen and collaborators described and characterized heme oxygenase as the enzyme responsible for breaking down heme in the body, demonstrating that heme catalysis resulted in the subsequent release of CO and free iron as byproducts (242). Since then, supported by a large body of experimental evidence, CO is proving to be an extraordinary signaling molecule generated by the cell that is vital in the regulation of cellular homeostasis. In the brain, CO is emerging as a chemical messenger molecule which can influence physiological and pathological processes in central and peripheral nervous system. 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 conventional neurotransmitters, carbon monoxide is not stored in synaptic vesicles and is not released by membrane depolarization and exocitosis. It seems likely that carbon monoxide (CO) is involved in the mechanism of cell injury (246). This is evidenced by the fact that CO binds to heme in guanylyl cyclase to activate cGMP (193). It has been found that CO is responsible for maintaining endogenous levels of cGMP. This effect is blocked by potent HO inhibitor but not NO inhibitors (156). Based on endogenous distribution of HO in the CNS it has been suggested that CO can influence neurotransmission like NO (251). CO appears to be involved as retrograde messenger in LTP and also is involved in mediating glutamate action at metabotropic receptors (107). This is evident from the fact that metabotropic receptor activation in brain regulates the conductance of specific ions channels via a cGMP-dependent mechanism which is blocked by HO inhibitors (105). Experimental evidence suggests that CO plays a similar role like NO in the signal transduction mechanism for the regulation of cell function and cell to cell communication (157, 158). 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 carboxyterminal half of NOS (52). CO, like NO, binds to iron in the heme moiety of guanylyl cyclase. However, there are some differences in function between CO and NO. Thus NO mainly mediates glutamate effect 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 (39). A number of evidence suggests a possible role of CO in regulating nitrergic transmission. Endogenous CO has been suggested to control constitutive NOS activity. Moreover, CO may interfere with NO binding to guanylyl cyclase, and this 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 the normal protein turnover (48). This hypothesis is sustained by recent findings showing that HO inhibition increases NO production in mouse macrophages exposed to endotoxin (246). CO may also act as 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 cGMP-independent manner (253) and also that CO-induced vascular relaxation results from the inhibition of the synthesis of the vasoconstrictor endothelin-1 (65). Little, however, 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, thereby encoding for the 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 (12, 42). Whether this also occurs in brain mitochondria remains to be elucidated. Aside from the SNC, the protective effects of CO were initially demonstrated in a model of acute lung injury and endotoxic shock, and subsequently in a mouse cardiac xenotransplantation model (185). Mouse heart transplanted to immunosuppressed rats survive indefinitely. However, if HO-1 activity cannot be expressed in the mouse heart, either as a consequence of absent phenotypical expression of the HO-1 gene (mice *hmox*$^{-/-}$), or because HO-1 activity is inhibited with a selective inhibitor tin protoporphyrin (SnPPIX), the hearts are rejected rapidly. HO-1 expression in the transplanted heart is essential to prevent rejection in this model. Surprisingly, if the donor and recipient were both treated with 250 ppm CO, a heart that cannot express HO-1 activity still survives indefinitely (184). In this scenario CO appears to be able to substitute for HO-1 in suppressing the proinflammatory response that is the leading cause of graft rejection. CO emerges as a powerful antiinflammatory promoting agent acting at level of macrophage cell line, a cell that probably controls the balance of inflammation in many conditions. Macrophages stimulated with bacterial lipopolysaccharide (LPS) produce several proinflammatory cytokines, such as TNFα. The antiinflammatory cytokine interleukin-10 (IL-10) is also produced (186). If macrophages overexpress HO-1 or are exposed to CO *in vitro* before stimulation with LPS, the proinflammatory response, and consequently TNFα, is markedly diminished, whereas the antiinflammatory response, characterized by IL-10 production, is enhanced. At least, three important actions of CO contribute to its antiinflammatory effects: (1) CO prevents platelet aggregation and the consequent thrombosis (217); (2) CO downmodulates the expression of plasminogen activator inhibitor type 1 (PAI-1); and (3) CO prevents apoptosis in several cell types, including endothelial cells, fibroblasts, hepatocytes, and β-cells of the pancreas (185). In addition, CO suppresses the proliferative response of smooth muscle cells that contribute to neointimal proliferation associated with inflammatory lesions *in vivo.* Many of the observed effects of CO have been obtained by exposing cells or animals to gaseous CO by inhalation. Interestingly, the recently discovered carbon monoxide releasing molecules (CORMs) appear to afford similar protective action, thereby providing an alternative therapeutic approach for those pathophysiological conditions where CO administration is warranted (180, 181).

THE HEAT SHOCK PATHWAY OF BRAIN STRESS TOLERANCE

It is well known that living cells are continually challenged by conditions which cause acute or chronic stress. To adapt to environmental changes and survive different types of injuries, eukaryotic cells have evolved networks of different responses which 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 HSP synthesis is induced not only after hyperthermia, but also following alterations in the intracellular redox environment, and 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 (164, 28, 30, 35, 48). This has opened new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear as possible candidates for novel cytoprotective strategies (67, 216, 218, 230, 238).

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 (50, 55). 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), 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. Denatured proteins are thought to serve as stimulus for induction. These denatured proteins activate heat shock factors (HSFs) within the cytosol by dissociating other HSPs that are normally bound to HSF (48, 195–198). Freed 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 denatured 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. Expression of the gene encoding HSPs has been found in various cell populations within the nervous system, including neurons, glia, and endothelial cells (135). 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 epiphenomenon unrelated to cell survival. Only recently, however, with the availability of transgenic animals and gene transfer, it has 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 (59, 88, 247). After focal cerebral ischemia, mRNA encoding HSP70 is synthesized in most ischemic cells except in a areas 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, which means the zone of protein denaturation in the ischemic areas (135). 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 (182). Transfection of cultured astrocytes with HSP70 protects them from ischemia or glucose deprivation (88, 135). HSP70 inhibits caspase-3 activation caused by ceramide, and also affects JUN kinase and p38-kinase activation (176). In addition, HSP70 binds to and modulates the function of BAG-1, the bcl-2 binding protein (171), thus modulating some type of apoptosis-related cell death. *Ubiquitin* is one of the smallest HSPs and is expressed throughout brain in response to ischemia. It is involved in targeting and chaperoning of proteins degraded in proteasomes, which include NFκB, cyclins, HSFs, hypoxia-inducible factor, some apoptosis-related proteins, tumor necrosis factor, and erythropoietin receptors (168).

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 (13). *HSP47* is synthesized mainly in microglia following cerebral ischemia and subarachnoid hemorrhage (249).

HSP60, glucose-regulated protein 75 (GRP75), and HSP10 chaperone proteins 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 overexpression (48). HSP60 is encoded in the nucleus and resides mainly in the mitochondria (46). HSP60 forms the chaperonin complex, which is implicated in protein folding and assembly within the mitochondria under normal conditions (52–55). Most mitochondrial proteins are synthesized in the cytosol and must be imported into the organelles in an unfolded state (230). During translocation, the proteins interact with HSP70. ATP-dependent binding and release of HSP70 provide the major driving force for complete transport of polypeptides into the matrix. Most imported polypeptides are released from soluble HSP70; however, a subset of aggregation-sensitive polypeptides must be transferred from HSP70 to HSP60 for folding (230). Owing to the close functional interaction between this chaperonin system and the HSP70 system, it is likely that upregulation of HSP60 may be a fundamental site targeted by nutritional antioxidants strategies aimed at restoring free radical-driven redox imbalance and the consequent mitochondrial dysfunction (55).

HSP32 or heme oxygenase is the rate-limiting enzyme in the production of bilirubin. There are three isoforms of heme oxygenase, HO-1 or inducible isoform, HO-2 or constitutive isoform and the recently discovered HO-3 (87, 151, 156, 158, 215).

THE HEME OXYGENASE SYSTEM AND ITS FUNCTIONS IN NEUROPROTECTION

HSP32 or heme oxygenase is the rate-limiting enzyme in the production of bilirubin. In the preceding 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 (CO) is released by elimination of the α-methene 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 (142, 146, 158).

Heme oxygenase isoforms

HO is present in various tissues with the highest activity in the brain, liver, spleen, and testes. There are three isoforms of heme oxygenase, HO-1 or inducible isoform (156, 157, 215), HO-2 or constitutive isoform (146–149), and the recently discovered HO-3, cloned only in rat to date (150). 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 AA domain (differing in just one residue) called the "HO signature," that renders both proteins extremely active in their ability to catabolize heme (51–53). They have different localizations, similar substrate and cofactor requirements, while presenting different molecular weight. They also display different antigenicity, electrophoretic mobility, 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) (87).

Various tissues have differing amounts of HO-1 and HO-2. Brain and testes have a predominance of HO-2, whereas HO-1 predominates in the spleen. In the lung not subjected to oxidative stress, >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 and other groups have not found detectable levels of HO-1 protein in normal brain (87), we have recently demonstrated that HO-1 mRNA expression is physiologically detectable in the brain and shows a characteristic regional distribution, with high level of expression in the hippocampus and the cerebellum (45, 215). This evidence may suggest the possible existence of a cellular reserve of HO-1 transcript quickly available for protein synthesis and a post-transcriptional regulation of its expression.

HO isoenzymes are also seen co-localized with different enzymes dependent on the cell type. In the kidney HO-1 co-localizes with erythropoietin, whereas in smooth muscle cells HO-1 co-localizes with nitric oxide synthase. In neurons HO-2 co-localizes with NOS, whereas endothelium exhibit the same isoform to co-localize with NOS III. The cellular specificity of this pattern of co-localization 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, the main CO functional target (65). 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 two Cys-Pro residues considered the core of the heme-responsive motif (HRM), a domain critical for heme binding but not for its catalysis (121).

Although the biological properties of this isoenzyme still remain to be elucidated, the presence of two HRM motifs in its amino acidic sequence might suggest a role in cellular heme regulation (157, 158). Studying HO-3 mRNA sequence (GenBank accession no.: AF058787), we have observed that its 5′ portion corresponds to the sequence of a L-1 retrotransposon, a member of a family of retrotransposons recently involved in evolutionary mechanisms (133). 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 (215), it is possible that this last could have originated from the retrotransposition of the HO-2 gene. In addition, this genetic mutation in rat may represent a *species*-specific event since no other sequence in the public databases match the rat HO-3. Induction of HO-1 gene could be used clinically. However, the GT length polymorphism in the promoter of the gene encoding HO-1 that regulates the magnitude of the HO-1 response to a given stress signal can render this approach difficult for those individuals with the long GT repeats that are associated with low HO-1 responsiveness. This polymorphism appears to be of functional significance in that short repeats, which are associated to high responsiveness, seem to be also associated with lesser likelihood of restenosis after angioplasty (185).

Regulation of HO genes

Coupling of metabolic activity and gene expression is fundamental to maintain homeostasis. Heme is an essential molecule that plays a central role as the prosthetic group of many heme proteins in reactions involving molecular oxygen, electron transfer, and diatomic gases. Although heme is integral to life, it is toxic because of its ability to catalyze the formation of reactive oxygen species and, consequently, oxidative damage to cellular macromolecules. In higher eukaryotes, toxic effects of heme are counteracted by the inducible HO-1 system (156, 158). As in the classic view of metabolic control, expression of HO-1 is induced by the substrate heme (131, 157). In addition, expression of HO-1 is robustly induced in mammalian cells by various proinflammatory stimuli, such as cytokines, heavy metals, heat shock, and oxidants that induce inflammatory damage (136, 248). Thus, HO-1 is an essential antioxidant defense enzyme that converts toxic heme into antioxidants and is fundamental to cope with various aspect of cellular stress and to regulate iron metabolism (195). In clinical conditions, HO-1 expression has been associated with increased resistance to tissue injury thus leading to a gene therapy approach employing HO-1 (185).

HO-2 gene consists of five exons and four introns. HO-2 has a molecular weight of 34 kDa and exhibits 40% homology in 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 (205). 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 HO-2 gene (151). In addition, endothelial cells treated with the NOS inhibitor L-NAME and HO inhibitor zinc mesophorphyrin exhibited a significant upregulation of HO-2 mRNA. 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 (248). This characteristic inducibility of HO-1 gene strictly relies on its configuration: the 6.8-kilobase gene is organized into four introns and five exons. A promoter sequence is located approximately 28 bases pairs upstream from the transcriptional site of initiation. In addition, different transcriptional enhancer elements, such as 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 the promoter and, more distally, in two enhancers located 4 and 10 kb upstream the initiation site (120). The molecular mechanism that confers inducible expression of *ho-1* in response to numerous and diverse conditions has remained elusive. One important clue has recently emerged from a detailed analysis of the transcriptional regulatory mechanisms controlling the mouse and human *ho-1* genes. The induction of *ho-1* is regulated principally by two upstream enhancers, E1 and E2 (239). Both enhancer regions contain multiple stress (or antioxidant) responsive elements (StRE, also called ARE) that also conform to the sequence of the Maf recognition element (MARE) (162) with a consensus sequence (GCnnnGTA) similar to that of other antioxidant enzymes (9). There is now evidence to suggest that heterodimers of NF-E2-related factors 2 (Nrf2) and one or another of the small Maf proteins (i.e., MafK, mafF and MafG) are directly involved in induction of *ho-1* through these MAREs (106). A possible model, centered on Nrf2 activity, suggests that the *ho-1* locus is situated in a chromatin environment that is permissive for activation. Since the MARE can be bound by various heterodimeric basic leucine zipper (bZip) factors including NF-E2, as well as several other NF-E2-related factors (Nrf1, Nrf2, and Nrf3), Bach, Maf and AP-1 families (239) random interaction of activators with the *ho-1* enhancers would be expected to cause spurious expression. This raises a paradox as to how cells reduce transcriptional noise from the *ho-1* locus in the absence of metabolic or environmental stimulation. This problem could be reconciled by the activity of repressors that prevent nonspecific activation. One possible candidate is the heme protein Bach1, a transcriptional repressor endowed with DNA binding activity, which is negatively regulated upon binding with heme. Bach1-heme interaction is mediated by evolutionarily conserved heme regulatory motifs (HRM), including the cysteine-proline dipeptide sequence in Bach1. Hence, a plausible model accounting for the regulation of *ho-1* expression by Bach1 and heme, is that expression of *ho-1* gene is regulated through antagonism between transcription activators and the repressor Bach1. While under normal physiological conditions expression of *ho-1* is repressed by Bach1/Maf complex, increased levels of heme displace Bach1 from the enhancers and allow activators, such as heterodimer of Maf with NF-E2 related activators (Nrf2), to the transcriptional promotion of *ho-1* gene (239). To our knowledge, the Bach1–*ho-1* system is the first example in higher eukaryotes that involves a direct regulation of a transcription factor for an enzyme gene by its substrate. Thus, regulation of *ho-1* involves a direct sensing of heme levels by Bach1 (by analogy to *lac* repressor sensitivity to lactose), generating a simple feedback loop whereby the substrate effects repressor-activator antagonism. The promoter region also contains two metal responsive elements, similar to those found in 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 two binding sites for HSF-1, which mediates the HO-1 transcription are located 9.5 kb upstream of the initiation site (9). The distal enhancer regions are important in regulating HO-1 in inflammation, since has been demonstrated are responsive to endotoxin. In the promoter region also resides a fragment 56 bp which responds to the STAT-3 acute-phase response factor, involved in the downregulation of HO-1 gene induced by glucocorticoid (205).

Glutathione, thiol redox state and RNS: intracellular modulators of HO-1 expression

The major regulator of intracellular redox state is glutathione, a cysteine-containing tripeptide with reducing and nucleophilic properties. This tripeptide (GSH) is essential for the cellular detoxification of reactive oxygen species in brain cells (31, 81–83). A compromised GSH system in the brain has been connected with the oxidative stress occurring in neurological diseases (45, 51, 194–196). Recent data demonstrate that, besides intracellular functions, GSH has also important extracellular functions in brain. In this respect astrocytes appear to play a key role in the GSH metabolism of the brain, since astroglial GSH export is essential for providing GSH precursors to neurons (84). Of the different brain cell types studied *in vitro* only astrocytes release substantial amounts of GSH. In addition, during oxidative stress astrocytes efficiently export glutathione disulfide (GSSG). The multidrug resistance protein 1 participates in both the export of GSH and GSSG from astrocytes (84). Glutathione exists in either a reduced (GSH) or oxidized (GSSG) form and participate in redox reactions through the reversible oxidation of its active thiol. In addition, GSH acts as a coenzyme of numerous enzymes involved in cell defense. In unstressed cells the majority (99%) of this redox regulator is in the reduced form, and its intracellular concentration is between 0.5 and 10 m*M* depending on the cell type (82, 20, 119). Depletion of glutathione has been shown to occur in conditions of moderate or severe oxidative stress and has been associated with increased susceptibility to cell damage (59, 47, 49). There is now evidence to suggest that a direct link between a decrease in glutathione levels by oxidant stress and rapid upregulation of HO-1 mRNA and protein exist in a variety of cells, including rat brain, human fibroblasts, endothelial cells, and rat cardiomyocytes (90). This finding is supported by the fact that *N*-acetyl-cysteine (a precursor of glutathione) abolishes oxidative stress-mediated induction of HO-1 gene (91, 92). In addition, increased production of NO and RSNO can

also lead to changes in intracellular glutathione. In astroglial cell cultures stimulation of iNOS by exposure to LPS and IFN-γ decreases total glutathione while raising GSSG, and this effect is abolished by pretreatment of glial cells with NOS inhibitors (40). Moreover, elevation of intracellular glutathione prior to exposure of endothelial cells to NO donors almost completely abolishes activation of the heme oxygenase pathway, which suggests that thiols can antagonize the effect of NO and NO-related species on HO-1 induction (177). We have recently demonstrated in endothelial cells subjected to hypoxia that induction of HO-1 is associated with a decrease in the GSH/GSSG ratio and with an increase in RSNO levels resulting from early induction of iNOS (177). This implies that in conditions of low oxygen availability, both oxidative and nitrosative reactions may serve as a trigger for induction of the HO-1 gene (178). All this evidence corroborates the notion that generation of ROS and RNS are important signal transduction mechanisms linking HO-1 activation to cell stress tolerance (159).

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 (61). Induction of HO occurs together with the induction of other HSPs in the brain during various experimental conditions including ischemia (80). Injection of blood or hemoglobin results in increased expression of the gene encoding HO-1, which occurs mainly in microglia through brain (54). 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+ microglia/macrophages at the hemorrhagic lesion was detected as early as 6 h post trauma and was still pronounced after 6 months (17).

There is now evidence that oxidative stress contributes to secondary injury after spinal cord trauma. Induction of HO-1 in the hemisected spinal cord, a model that results in reproducible degeneration in the ipsilateral white matter, was found in microglia and macrophages from 24 h to at least 42 days after injury. Within the first week after injury, HO-1 was induced in both the gray and the white matter. Thereafter, HO-1 expression was limited to degenerating fiber tracts. Interestingly, HSP70 was consistently co-localized with HO-1 in the microglia and macrophages, indicating that long-term induction of HO-1 and HSP70 in microglia and macrophages occur long after traumatic injury and are correlated with Wallerian degeneration and remodelling of surviving tissue (167). Since the expression of heat shock proteins is closely related to that of amyloid precursor protein (APP), heat-shock proteins have been studied in brain of patients with Alzheimer's disease. Significant increases in the levels of HO-1 have been observed in AD brains in association with neurofibrillary tangles (241), and also HO-1 mRNA was found increased in AD neocortex and cerebral vessels (201). HO-1 increase was not only in association with neurofibrillary tangles, but also co-localized with senile plaques and glial fibrillary acidic protein-positive astrocytes in AD brains (221). 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 (53, 142, 158). Upregulation of HO-1 in the substantia nigra of Parkinson disease subjects has been demonstrated. In these patients, nigral neurons containing cytoplasmic Lewy bodies exhibited in their proximity maximum HO-1 immunoreactivity (87). New evidence showed a specific upregulation of HO-1 in the nigral dopaminergic neurons by oxidative stress (195, 197).

Multiple sclerosis (MS) is a common, often disabling disease of the central nervous system (CNS). It has been suggested that inappropriate stress response within the CNS could influence both the permeability of the blood-brain barrier and the expression of heat-shock proteins, thereby initiating the MS lesion (5, 157). However, cytokines, immunoglobulins, and complement complexes may elicit a survival response in the oligodendrocytes, involving the induction of endogenous heat shock proteins and other protective molecules which indicates that redox systems and therefore the oxidant/antioxidant balance in these cells are of great importance in MS (8, 36, 37, 44, 49). The expression of heme oxygenase-1 (HO-1) is increased in the CNS of mice and rats with experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis (50, 59). To investigate the role of HO-1 in EAE, tin-protoporphyrin IX (Sn-PP IX), was administered to SJL mice during active disease. Sn-PP IX (200 μmol/kg) attenuated clinical scores, weight loss, and some signs of pathology in comparison to vehicle treatment. Glutathione levels were greater in treated EAE mice than in those receiving vehicle, indicating lower oxidative stress in the former group. These data suggest that inhibition of HO-1 attenuated disease and suppressed free radical production (60).

On the contrary, in another study, high expression of HO-1 in lesions of EAE was enhanced by hemin treatment, a procedure that resulted associated to attenuation of clinical signs of pathology, whereas tin mesoporphyrin, an inhibitor of HO-1, markedly exacerbated EAE (152). These results strongly suggest that endogenous HO-1 plays an important protective role in EAE, and that targeted induction of HO-1 overexpression may represent a new therapy for the treatment of multiple sclerosis. We have recently shown that thiol disruption and nitrosative stress are associated in active multiple sclerosis with induction of HSP70 and heme oxygenase-1 in central and peripheral tissues of MS patients and that acetylcarnitine was able to counteract nitrosative stress-mediated damage, an effect associated with enhancement of HSP stress signaling (195, 197). All these findings can open up new therapeutic perspectives, as molecules activating these defense mechanisms appear to be possible candidates for novel neuroprotective strategies (198, 218, 238).

Bilirubin and biliverdin: an endogenous antioxidant system

Supraphysiological levels (>300 μ*M*) 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 sustain. The specific role of endogenously derived bilirubin, as a potent antioxidant, has been demonstrated in hippocampal and cortical neurons where accumulation of this metabolite due to phosphorylation-dependent enhancement of HO-2 activity protected against hydrogen peroxide-induced cytotoxicity (159, 236). 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 lacking mice (79). Bilirubin can become particularly important, as a cytoprotective agent for tissues with relatively weak endogenous antioxidant defenses such as the central nervous system and the myocardium. Interestingly, increased levels of bilirubin have been found in the cerebrospinal fluid in Alzheimer's disease (AD), which may reflect the increase of degraded bilirubin metabolites in the AD brain derived from the scavenging reaction against chronic oxidative stress (137). Similarly, a decreased risk for coronary artery disease is associated with mildly elevated serum bilirubin, with a protective effect comparable to that of HDL-cholesterol (79). 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 biliverdin reductase, which in brain is present in large functional excess, suggesting a mechanism to amplify the antioxidant effect (174, 197, 257). 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, being this a major activator of PKC. Recent evidence has demonstrated that bilirubin and biliverdin possess strong antioxidant activities toward peroxyl radical, hydroxyl radical and hydrogen peroxide. Exposure of bilirubin and biliverdin to agents that release NO or nitroxyl resulted in a concentration- and time-dependent loss of bilirubin and biliverdin. Increasing concentrations of thiols prevented bilirubin and biliverdin consumption by nitroxyl, indicating that bile pigments and thiol groups can compete and/or synergize the cellular defense against NO-related species. In view of the high inducibility of haem oxygenase-1 by NO-releasing agents in different cell types, these findings highlight novel antinitrosative characteristics of bilirubin and biliverdin, suggesting a potential function for bile pigments against the damaging effects of uncontrolled NO production (132).

Caloric restriction and endogenous oxidative stress: relevance to aging and survival

Caloric restriction in mammals has been recognized as the best characterized and most reproducible strategy for extending maximum survival, retarding physiological aging, and delaying the onset of age-related pathological situations. The overwhelming majority of studies using caloric restriction have used short-lived rodent species, although current work using monkeys should reveal whether this paradigm is also relevant to manipulating the rate of primate aging. The mechanisms by which restricted calorie intake modifies the rate of aging and cellular pathology have been the subject of much controversy, although an attenuation of accumulating oxidative damage appears to be a central feature (124). A major effect of calorie-restricted feeding now appears to be on the rate of production or leak of free radicals from mitochondrial sites, although the details of the adaptation and the signaling pathway that induces this effect are currently unknown. General consensus, however, has been achieved that caloric restriction feeding regimes reduces the rate of accrual of oxidative damage as measured by lipid peroxidation, nuclear and mtDNA damage, and protein carbonyl formation. An analysis of published studies that used a degree of food restriction in the range of 40–50% *ad libitum* intake, revealed a significant positive correlation between survival parameters, such as mean, maximum and average survival time, and duration of caloric restriction. The longer the animals are maintained on low calorie intake during the postweaning period of the life span, the greater is the survival (165, 166). It is unclear whether caloric restriction protects against random oxidative damage *per se* or is protective for those vulnerable proteins of key pathways, such as those containing iron-sulfur centers of the ETC or DNA-binding signaling proteins. This is directly related to the question whether oxidative damage in genomic and mtDNA is primarily random as a function of age or whether there is a specific pattern of distribution of ROS which may vary dependent on the tissue or the state of the cell cycle within any particular cell. It is generally accepted that age-related accrual of ROS-induced damage represents a balance between generation and defences, such as antioxidant enzymes, repair systems, and turnover. It has been demonstrated that caloric restriction reduces cellular injury and improves heat tolerance of old animals by lowering radical production and preserving cellular ability to adapt to stress through antioxidant enzyme induction and translocation of these proteins to the nucleus (197). It has been also demonstrated that mitochondria from calorie-restricted animals produce less reactive oxygen species (ROS) per nanomol of O_2 during state 4 respiration, and recent work on ETC complexes suggests a modification in the K_m for complex III associated with a retention of high-affinity binding sites for complex IV as a possible mechanism operating in reducing superoxide generation (166). It is conceivable that low calorie-induced changes in unsaturated fatty acid composition of the mitochondrial membranes not only may protect against ROS-induced lipid peroxidation but also may influence the binding properties of ETC proteins embedded in the membrane, and the related transport processes.

However, several questions need to be addressed, such as the signaling pathway underlying the adaptive responses triggered by caloric restriction, or the effect of chronic caloric restriction on either the bioenergetic of individual mitochondria, or mitochondrial number and turnover rate. High-density oligonucleotide arrays studies have recently provided compelling evidence that aging results in a differential gene

expression pattern indicative of a marked stress response associated with lower expression of metabolic and biosynthetic genes and also these alterations are either completely or partially prevented by caloric restriction. In addition, the transcriptional patterns of calorie-restricted animals suggest that caloric restriction retards the aging process by causing a metabolic shift toward increased protein turnover and decreased macromolecular damage (53, 163, 237).

Therapeutic potential of nutritional antioxidants

Recently, considerable attention has been focused on identifying dietary and medicinal phytochemicals that can inhibit, retard, or reverse the multistage pathophysiological events underlying AD pathology (29, 30, 32–35). Spices and herbs contain phenolic substances with potent antioxidative and chemopreventive properties (30, 216). The active antioxidant principle in *Curcuma longa*, a coloring agent and food additive used in Indian culinary preparations, has been identified as curcumin (diferuloylmethane). Due to the presence in its structure of two electrophilic α, β-unsaturated carbonyl groups which, by virtue of Michael reaction, can react with nucleophiles such as glutathione, curcumin has the potential to inhibit lipid peroxidation and to effectively intercept and neutralize reactive oxygen and NO-based free radicals (195). This agent is a potent inhibitor of tumor initiation *in vivo* and possesses antiproliferative activities against tumor cells *in vitro* (28). Recent epidemiological studies (99) have raised the possibility that this molecule, as one of the most prevalent nutritional and medicinal compounds used by the Indian population, is responsible for the significantly reduced (4.4-fold) prevalence of AD in India compared to United States. Based on these findings, it has been provided compelling evidence that dietary curcumin given to an Alzheimer transgenic APPSw mouse model (Tg2576) for 6 months resulted in a suppression of indices of inflammation and oxidative damage in the brain of transgenic APPSw mice (149). Furthermore, in a human neuroblastoma cell line it has recently been shown that curcumin inhibits NFκB activation, effectively preventing neuronal cell death (195). Remarkably, recent evidence has demonstrated that curcumin is a potent inducer of HO-1 in vascular endothelial cells (9). We have also recently demonstrated in astroglial cells the role of caffeic acid phenethyl ester (CAPE), an active component of propolis, as a novel HO-1 inducer (216). The similarity of CAPE to curcumin is striking because CAPE is also a Michael reaction acceptor, endowed with anti-inflammatory, antioxidant and anticancer effects (30). These agents all appear capable of transcriptionally activating a gene battery that includes antioxidant enzymes and heme oxygenase (78). Gene induction occurs through the antioxidant responsive element (ARE) (2, 3). Thus, increased expression of genes regulated by the ARE in cells of the central nervous system may provide protection against oxidative stress.

MAJOR CNS DISORDERS ASSOCIATED WITH ROS/RNS-MEDIATED DAMAGE

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a remarkably debilitating disease with inevitable lethal consequences. It typically affects adults in midlife with progressive paralysis and causes death generally within 5 years. It is characterized by degeneration of the motor neurons. These neural components include the anterior horn cells of the spinal cord, motor nuclei of the brain stem, particularly the hypoglossal nuclei, and the upper motor neurons of the cerebral cortex. ALS is currently untreatable and the pathogenesis is unknown, although numerous possible etiologies have been studied including viral, immunologic, and metabolic. However, none of these is considered a serious etiological candidate. On the other hand, the pathogenetic role of oxidative stress has emerged as distinct possibility (190, 196). Recent data from a multicenter study indicate that some but not all cases of inherited ALS arise because of mutations in the gene encoding the cytosolic form of Cu/Zn-SOD (53). Familial ALS patients heterozygous for SOD mutations have <50% of normal SOD activity in their erythrocytes and brains. This defective SOD gene is on chromosome 21 (the gene for mtSOD is on chromosome 6). Thus the implication is that the degeneration of motoneurons in ALS may be initiated by oxidative free radical damage. An alternative hypothesis is that the mutation might impart harmful properties to the enzyme. This gain-of-function theory is based primarily on the fact that familial ALS is dominantly inherited, and thus only one copy of the enzyme is required to cause the disease, which arises from the toxic influence exerted by the abnormal protein. In this regard, transgenic mice containing extra copies of human Cu/Zn-SOD and ALS mutations showed a disorder closely resembling human ALS, whereas overexpression of normal Cu/Zn-SOD alone did not produce the disorder (25). These studies suggest that the enzyme is endowed with neurotoxic properties. What the neurotoxin function might be remains to be elucidated. With regards to the familial form of the disease, one line of evidence suggests that interaction between NO and superoxide by yielding the powerful oxidant $ONOO^-$ might constitutes the primary pathogenic event leading to protein nitration which slowly injures the motor neurons (14). It is possible that the active site of SOD is altered allowing greater access of $ONOO^-$ to the copper center and so favoring the subsequent formation of a nitronium-like species which nitrosylates tyrosine residues (119). Immunocytochemistry studies have also revealed, in the neurofilament aggregates associated ALS, a close association between SOD-1 and NOS activity (63). Since light neurofilament is rich in tyrosine, it is proposed that nitrotyrosine formation occurs which impairs neurofilament assembly and ultimately leads to motoneuron death. Recently, increased nitrotyrosine immunoreactivity has been demonstrated in motor neurons of both sporadic and familial ALS, suggesting that $ONOO^-$-mediated oxidative damage may play a role in the pathogenesis of both forms of the disease (10). Some evidence is now available to suggest that mitochondrial dysfunction is a central event in the disease process. Thus, a significant decrease in complex IV activity is reported in the spinal cord (ventral, lateral, and dorsal regions) of patients with sporadic ALS (98). In addition, studies with a transgenic mouse model of ALS also suggest that axonal transport of organelles, in particular mitochondrial transport, is impaired and may be an important factor in ALS (66).

Alzheimer's disease

Alzheimer's disease (AD) affects over 2 million Americans and is the major cause of admission to nursing homes. Alzheimer's disease, which rarely occurs before the age of 50 years, usually becomes clinically apparent as a subtly impaired cognitive function or a disturbance of affect (134). From this status, clinically defined as mild cognitive impairment (MCI), a progressive memory loss and disorientation occur, which eventually progresses into dementia. Although most cases are sporadic, 5–10% or more are familial. Gross examination of the brain in AD shows a variable degree of cortical atrophy with narrowed gyri and widened sulci most apparent in the frontal, parietal, and temporal lobes. Microscopically, the features include neurofibrillary tangles (NFT), neurite (senile) plaques, amyloid angiopathy, granulovacuolar degeneration, and Hirano bodies. Importantly, all of these changes are present in the brains of nondemented older individuals but to a much lesser extent. The finding that choline acetyltransferase is decreased by 40–90% in the cerebral cortex and hippocampus of patients with AD has led to the hypothesis that AD is consequence of a deficit in the cholinergic system (42). Several lines of evidence now support an important role for free radical-mediated event in the pathogenesis of the disease. Advanced glycosylation end products (AGEs) are a family of complex posttranslational modifications that are initiated by condensation of reducing sugars with proteins amino groups via the Maillard reaction. It has become evident that glycation of proteins occurs *in vivo* in aged individuals (64). Oxidative stress increases the frequency of hydroxyl radical-induced autoxidation of unsaturated membrane lipids. Reactive aldehydes, resulting by metal ion-mediated fragmentation of the lipid hydroperoxides can modify proteins through alteration of protein–protein interactions and intermolecular crosslinking. Age modifications and oxidative stress mechanisms can synergistically accelerate protein damage (26–35). Several potential sources of oxidative stress should be considered in the pathogenesis of AD. First, the concentration of iron, a potent catalyst of oxyradical generation, is increased in NFT-bearing neurons (39). Second, increased concentrations of iron would result in increased protein modifications that are catalyzed by metal ions and reducing sugars (33). Third, microglia cells are activated and increased in number in AD and represent a major source of free radicals (86). Fourth, the increased lipid peroxidation and the resulting membrane disturbances, which are observed in degenerating neurons and neurites, are expected to lead to an influx of calcium, which causes destabilization of cytoskeleton and activation of specific degradative enzymes (83, 161). A decrease of complex IV activity has been reported in the cerebral cortex of individuals who died of AD (139). While the exact mechanism for this loss of activity is not clear, it is known that this enzyme complex is particularly susceptible to oxidative damage (29, 30). In addition, there is now evidence to suggest that NO metabolism is affected in AD. The glial derived factor, S-100-β, which is over expressed in this conditions, causes induction of iNOS in astrocytes associated with NO-mediated neuronal cell death in a co-culture system (122). Furthermore, β-amyloid is reported to activate NOS in a substantia nigra/neuroblastoma hybrid cell line (119). Analysis of postmortem material has revealed in AD brain the presence of nitrotyrosine, as result of the reaction of $ONOO^-$ and nitrotyrosine residues in protein, which was not detectable in age-matched control brains (228). In addition, using antibodies specifically directed against iNOS, the presence of this isoform has been demonstrated in neurofibrillary tangle-bearing neurons (213). Despite evidence for activation of NO metabolism in AD, analysis of the CSF nitrite+nitrate (stable end products of $ONOO^-$ degradation) concentration revealed levels in AD patients comparable to controls (68). While this observation does not dismiss a role for NO/$ONOO^-$ in the etiology of AD, it implies that formation of RNS occurs at a level that not necessarily leads to a rise in CSF RNS concentration.

Amyloid beta-peptide (Aβ), the principal component of senile plaques and the major neuropathological hallmark of AD, is central to the pathogenesis of AD. Aβ is a 40–42 amino acid peptide that accumulates in the neuritic plaques in AD. The AD brain is under extensive oxidative stress (81). These two observations were joined by a model to potentially account for neurodegeneration in AD brain: the Aβ-associated free radical oxidative stress hypothesis of brain cell death in AD (58, 83). In this model, Aβ-associated free radicals initiate lipid peroxidation, protein oxidation, reactive oxygen species (ROS) formation, intracellular and mitochondrial Ca^{2+} accumulation, and eventual death of neurons. A prediction of this model is that the antioxidant vitamin E should prevent or modulate these Aβ-induced effects to neurons (26, 29). Consistent with this model, this free radical scavenger was shown to block Aβ-initiated lipid peroxidation in cortical synaptosomes (27, 28). Further, protein oxidation induced by Aβ in astrocyte cultures and assessed by increased protein carbonyl content was abrogated by the more soluble form of vitamin E, trolox (141). Vitamin E also blocked Aβ-induced inhibition of transmembrane protein function, including ion-motive ATPases, glucose and glutamate transporters, G-protein coupled signal transduction, and the energy-related enzyme creatine kinase, and the methionine residue 35 of Aβ(1–42) and Aβ(1–40) was shown to be critical to the oxidative stress properties of these peptides (32). Human Aβ(1–42), expressed *in vivo* in transgenic *C. elegans* nematodes, led to protein oxidation in the living animal, and methionine was important in this process as well (258). A risk factor for AD is the presence of allele 4 of apolipoprotein E (apoE) (208). Synaptosomes from apoE knock-out mice, containing no gene for apoE, show increased susceptibility to oxidative stress induced by Aβ, (143), while synaptosomes from knock-in mice containing human apoE4 with no mouse background show significantly increased Aβ-induced oxidative stress compared to synaptosomes from human apoE2 or apoE3 knock-in mice (144). Thus, apoE may serve an antioxidant function, but apoE4 may be less able than apoE2 or apoE3 to do so (144). This notion was tested using 1-month-old control and apoE deficient mice. Both received dietary vitamin E for 12 months. Vitamin E-fed animals had better behavioral outcomes of spatial motor activity and decreased levels of lipid peroxidation relative to apoE-deficient mice fed a normal diet (250). The sum of these studies suggests a decreased risk for and diminished oxidative stress in Alzheimer's disease in persons

taking high dose dietary, or perhaps supplemental, vitamin E (and vitamin C to regenerate vitamin E from the tocopherol radical).

Brains of AD patients undergo many changes, such as disruption of protein synthesis and degradation, classically associated with the heat shock response, which is one form of stress response. Heat shock proteins are proteins serving as molecular chaperones involved in the protection of cells from various forms of stress. Increasing interest has been focused on identifying dietary compounds that can inhibit, retard, or reverse the multistage pathophysiological events underlying AD pathology. Alzheimer's disease, in fact, involves a chronic inflammatory response associated with both brain injury and β-amyloid associated pathology. All of the above evidence suggests that stimulation of various repair pathways by mild stress has significant effects on delaying the onset of various age-associated alterations in cells, tissues, and organisms. Spices and herbs contain phenolic substances with potent antioxidative and chemopreventive properties, and it is generally assumed that the phenol moiety is responsible for the antioxidant activity. In particular, curcumin, a powerful antioxidant derived from the curry spice turmeric, has emerged as a strong inducer of the heat shock response. In light of this finding, curcumin supplementation has been recently considered as an alternative, nutritional approach to reduce oxidative damage and amyloid pathology associated with AD (30, 53, 195, 238).

Conceivably, dietary supplementation with vitamin E or with polyphenolic agents, such as curcumin and its derivatives, can forestall the development of AD, consistent with a major "metabolic" component to this disorder. Nutritional biochemical research is providing optimism that this devastating brain disorder of aging may be significantly delayed and/or modulated.

Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder which increases in frequency after the age of about 50 years. The major clinical disturbances in PD result from dopamine depletion in the striatum, due to nigral neuronal loss. Although a number of hypothesis, including defective DNA repair mechanisms, specific genetic defects, viral disorder, lack of a neurotrophic hormone or toxic compounds present in the environment have been proposed, none completely explains the cascade of events responsible for the cause and the course of the disease. A large body of evidence support the role of free radicals in the pathogenesis of the disease (33, 126, 198). Levels of lipid hydroperoxides are increased 10-fold in the SN in PD (125). Decreased glutathione peroxidase and catalase activities associated with increased SOD activity lead to increased levels of hydrogen peroxide (77). This, in dopaminergic cells, is primarily produced by MAO via deamination of dopamine and also nonenzymatically by autoxidation of dopamine. Hydrogen peroxide, by reacting with reduced forms of transition metals [e.g., iron (II) or copper (I)], gives rise to the powerful oxidant hydroxyl radical and oxidative damage to nigral membrane lipids, proteins, and DNA ensues. The role of iron in brain oxidative injury has been extensively considered (164). Dexter *et al.* (77) reported a 31–35% increase in the total iron content in parkinsonian SN compared to control tissue, which was associated to decreased levels of the iron storage protein ferritin, contrasting with a significant decrease of iron binding protein levels in the CSF. An iron II/iron III ratio in the SN shifted from almost 2:1 in the normal brain to 1:2 in the parkinsonian brain (148). Hence, a distinct possibility exists that excessive free radical generation occurs in this region, leading to the death of nigral neurons. In addition, substantia nigra is a dopamine-rich brain area, and catechols, including DOPA and dopamine, are cytotoxic *in vitro*, presumably by formation of covalent bonds between their quinone forms and macromolecules of vital importance, primarily represented by thiol groups (232). In fact, an intermediate in the autoxidation of catechols to quinone is the free radical semiquinone. Both autoxidation steps generate reduced forms of molecular oxygen, such as superoxide anion and hydrogen peroxide, which in addition to hydrogen peroxide produced by the MAO-dependent catabolism of dopamine, contribute to maintain considerably levels of the highly reactive hydroxyl radical, which reacting with free thiol groups may contribute to the decreased levels of GSH and corresponding increase in GSSG found in the SN (45, 53). This is of special importance considering that nigral cells also contain neuromelanin, a pigmented substance related to lipofuscin and derived from dopamine. Neuromelanin has high affinity for iron III, and this iron–melanin interaction might have pathogenetic implications. In fact, the synthesis of neuromelanins from dopamine is known to produce more oxidative damage than the synthesis from other catecholamines (231, 232) and, in addition, neuromelanins polymerize from pheomelanin in a process that requires cysteine for synthesis, thus competing with γ-glutamyl cysteine synthetase which utilizes cysteine for GSH synthesis. Under these circumstances the GSH system in the SN could result in a position of increased demand and decreased synthetic capability, and hence contribute to the highly vulnerability of this region to peroxidative injury (42, 45, 53). This is confirmed by the study of Perry *et al.*, which showed that GSH levels in the SN were significantly lower than other brain regions (191). Moreover, a 40% decrease has been reported in GSH in the SN of PD, associated with a significant increase in oxidized glutathione (226). Recently, it has been demonstrated in PD patients that the proportion of dopaminergic neurons with immunoreactive NFκB (nuclear factor-κB) in their nuclei was more than 70-fold that in control subjects (123). A possible relationship between the nuclear localization of NFκB in mesencephalic neurons of PD patients and oxidative stress in such neurons has been shown *in vitro* with primary cultures of rat mesencephalon, where translocation of NF-κB is preceded by a transient production of free radicals during apoptosis induced by activation of the sphingomyelin-dependent signaling pathway with C2-ceramide (95). The data suggest that this oxidant-mediated apoptogenic transduction pathway may play a role in the mechanism of neuronal death in PD (74, 170, 175, 220).

Moreover, a potential role for excitotoxic processes in Parkinson's disease (PD) has been strengthened by the observations that there appears to be a mitochondrially encoded defect in complex I activity of the electron transport chain (219). An impairment of oxidative phosphorylation will en-

hance vulnerability to excitotoxicity (256). Substantia nigra neurons possess *N*-methyl-D-aspartate receptors and there are glutamatergic inputs into the substantia nigra from both the cerebral cortex and the subthalamic nucleus. After activation of excitatory amino acid receptors, it has been suggested that there is an influx of calcium followed by activation of neuronal nitric oxide (NO) synthase, which can then lead to the generation of peroxynitrite (13). Consistent with such a mechanism, studies of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity in both mice and primates have shown that inhibition of neuronal NO synthase exerts neuroprotective effects, raising the prospect that excitatory amino acid antagonists for neuronal NO synthase inhibitors might be useful in the treatment of PD (74, 75).

Multiple sclerosis

Multiple sclerosis (MS) is a common, often disabling disease of the central nervous system (CNS). Although evidence indicates that MS is a complex trait caused by interaction of genetic and environmental factors, little is known about its cause or the factors that contribute to its unpredictable course (206). It is generally accepted that vascular factors, metabolic alterations, virus infections of the CNS, and/or disturbed immune mechanisms are responsible for the cause and course of MS. The clinical symptoms of MS result from inflammatory damage to the insulating myelin sheath of axons in the CNS and at later stages to axons themselves. A local autoimmune process involving activation of helper T cells against CNS protein components is likely to be crucial in this development. Once triggered, the immune system attacks and destroys myelin and the myelin-forming cell (37). Evidence exist which indicates that oligodendrocytes respond to the attack by immune cells and their secreted products through modulation of its metabolism and gene expression (150). It has been also suggested that inappropriate stress response within the CNS could influence both the permeability of the blood-brain barrier and the expression of heat-shock proteins, thereby initiating the MS lesion (39). In addition, cytokines, immunoglobulins, and complement complexes may elicit a survival response in the oligodendrocytes, involving the induction of endogenous heat shock proteins and other protective molecules, which indicates that redox systems and therefore the oxidant/antioxidant balance in these cells are of great importance in MS (49).

A variety of studies support a role for oxidative stress in MS. These include increased serum peroxide levels in MS relative to control (36, 244). Patients with MS in acute exacerbation exhibit significantly higher levels of pentane and exane (products of lipid peroxidation) in expired breath compared to either MS patients in remission or control subjects (244). Moreover, recent clinical and animal studies suggest that NO and its reactive derivative peroxynitrite are implicated in the pathogenesis of MS (8). Patients dying with MS demonstrate increased astrocytic inducible nitric oxide synthase (iNOS) activity as well as increased levels of iNOS mRNA and nitrotyrosine residues (8). In experimental allergic encephalomyelitis (EAE), both astrocytes and microglia express iNOS (245). All this is consistent with the demonstration that NO derivative species are cytotoxic to oligodendrocytes and neurons by inhibiting the mitochondrial respiratory chain (complex II-III and IV) and certain key intracellular enzymes (119, 235), thereby representing a critical determinant in the etiology of the disease.

MS is a relatively common disease of the CNS, the course of which is often of a progressive but relapsing/remitting nature. The clinical symptoms of MS during relapse (numbness, paralysis, blindness, and a variety of others) are mainly due to conduction block of axonal electrical impulses, caused by a variety of different molecular pathologies, including inflammation and demyelination (42). A local autoimmune process involving activation of glia is likely to be crucial in the development of this damage (245). Activated glia secrete reactive nitrogen species (RNS) products of nitric oxide (NO) metabolism with superoxide radicals ($O_2^{\bullet -}$) to form peroxynitrite anion ($ONOO^-$). At physiological pH, it protonates to its conjugate acid, peroxynitrous acid which decomposes with a t/12 of less than 1 sec. One of the fastest reactions of $ONOO^-$ is with CO_2/HCO_3^- (3–5.8 $\times$ $10^4 M^{-1}$ s^{-1} at 37°C). Together with the high concentrations of CO_2 (~1.3 m*M*) and HCO^{3-} (~25 m*M*) *in vivo* this reaction is the most probable pathway of $ONOO^-$ decomposition *in vivo* (49).

Reactive nitrogen species can cause "nitrosative stress" which results in the destruction of myelin and (myelin-forming) oligodendrocyte cells (45, 49). A direct link between NO and the conduction block that occurs in MS has been suggested, as NO donors cause reversible conduction block in both normal and demyelinated axons of the central and peripheral nervous systems (39, 44). In addition, conduction in demyelinated and early remyelinated axons is particularly sensitive to block by NO (44). This may be due to the direct effects of NO on glutamatergic neurotransmission, as it has been shown that NMDA receptor is inactivated by nitrosylation (119). Furthermore, the formation of S-nitrosoglutathione (GSNO) can cause GSH depletion and hence trigger redox-dependent changes in cellular signaling as well as modification of key intracellular enzymes, such as chain respiratory complex activities (103). Recent clinical and animal studies also indicate that NO and $ONOO^-$ play a central role in the pathogenesis of MS (104, 42). In CSF and plasma, nitrite+nitrate (stable end products of NO metabolism) levels are elevated in patients with MS (104). Reactive oxygen and nitrogen species have a major role in the mediation of cell damage and free sulfhydryl groups are vital in cellular defense against endogenous or exogenous oxidants (37–42). The possible links between MS and oxidant/antioxidant balance cell perturbation may be suggested by several factors, included increased incidence of MS in populations consuming high proportions of animal fat, since vitamin E is predominantly associated with plant lipid in the diet (36, 38); increased malonaldehyde levels in blood, and decreased glutathione peroxidase activity in MS erythrocytes, lymphocytes, and granulocytes (37) and, in addition, an inappropriate expression of heat-shock proteins on oligodendrocytes (52). This last event could represent a possible initiating factor at level of MS lesions capable of modulating the subsequent susceptibility or resistance of cells to oxidative stress. Moreover, a decrease in sulfhydryl groups and increased amounts of lipid peroxidation products, have also been measured in the CSF and plasma of MS patients (44, 49). Ni-

trosative stress in isolated astrocytes *in vitro* causes modifications in the endogenous thiol pool associated with induction of HSP 32 or heme oxygenase-1, which is prevented by antioxidants, suggesting a biochemical link between nitrosative stress, sulfhydryl function, and the heat-shock pathway (53, 215). In addition, this evidence suggests that redox-active compounds such as glutathione and the overall oxidant/antioxidant balance in the CNS are potentially of great importance in MS, although as yet there has been few studies addressing the relationships between NO, $ONOO^-$ and glutathione in MS. The chemical composition of human CSF is considered to reflect brain metabolism (243), and we have recently demonstrated in MS patients decreased levels of protein sulfhydryl groups associated with a increase in the content reactive nitrogen species and peroxidative products (44, 49), also showing that increased levels of nitrogen reactive species are present in the CSF of MS patients and that this is associated with increased nitrosylation of sulfhydryl moieties. Our results are consistent with recent evidence indicating increased protein nitrosylation in MS patients (71) and pose intriguing implications regarding clinical manifestations in MS which are potentially linked to a failure of action potentials to propagate along damaged axons, and involve inflammatory processes as primary causative factor, in addition to demyelination. In favor of this possibility is the evidence that NO donors are capable of blocking conduction in rat demyelinated axons (100).

All this would suggest a broader potential role for NO in the symptomatic manifestations of MS. Whether NO is central to the pathogenesis of MS remains to be clarified owing to the double-edged sword nature of its role. Consistently, a recent study (222) has demonstrated an association between high CSF levels of NO-metabolites with severe disease activity in relapsing-remitting MS, and that high concentrations of NO-metabolites were associated with more pronounced treatment responses after methylprednisolone treatment. However, other studies have shown no significant correlation between NO metabolites and disability score, disease progression index, MRI (magnetic resonance imaging) activity, and development of cortical atrophy on MRI (259). We have also demonstrated in MS patients an increase in nitrosative stress which was associated with a significant decrease of both protein SH groups and reduced glutathione (GSH), and with increased levels of oxidized glutathione (GSSG) and nitrosothiols (44). Interestingly, treatment of MS patients with acetylcarnitine resulted in decreased CSF levels of NO reactive metabolites and protein nitration and in a significantly higher content of both GSH, as well as the GSH/GSSG ratio. In addition, urinary nitrites, which were higher in MS patients than in controls, decreased significantly after treatment with acetylcarnitine (49). Several studies have shown the capability of carnitines to interfere with changes in oxidant/antioxidant balance and metabolism induced by oxidants (38, 109, 110). Although, so far, the exact mechanisms of action of acetylcarnitine are still unknown, current research point to its ability to enhance neuronal mitochondrial bioenergetics (54, 55), which in turn may influence cellular oxidant/antioxidant balance (55). As a brain energy enhancer, acetylcarnitine could offer potential survival to damaged neurons (47, 217), and metabolic studies conducted noninvasively in humans with NMR indicate that acetylcarnitine helps the brain to maintain the constant supply of energy needed for effective homeostasis. Multiple sclerosis is a progressive inflammatory neurodegenerative disease. However, despite increasing research efforts and although several explanations have been proposed for destruction of myelin and oligodendrocytes in multiple sclerosis, there is still no proven mechanism of injury. The possibility of manipulating these complex glial cell functions and controlling their pathologic interactions with immune cells probably will illuminate how myelin damage can be contained and how the injured tissue can be repaired.

Friedreich ataxia

Friedreich ataxia is an autosomal recessive neurodegenerative disorder involving both central and peripheral nervous system. Patients also show a systemic clinical picture presenting heart disease and diabetes mellitus or glucose intolerance. The disease is caused by mutations in the FRDA gene mapped on chromosome 9q13. The product of the gene is frataxin, an 18 kDa soluble mitochondrial protein with 210 amino acids. Crystal structure suggests a new, not previously reported, protein fold (85). The most frequent mutation is the expansion of a GAA trinucleotide repeat located within the first intron of the gene, and represents 98% of the mutations. This triplet motif can adopt a triple helical DNA structure that inhibits transcription (114). The severity of the disease correlates directly with the number of triplet units and consequent decrease in protein levels, with patients having frataxin levels ranging from 6 to 30% of normal (56).

The primary tissues affected in the disease include the large sensory neurons in the dorsal root ganglia and the nucleus dentatus, as well as cardiac and pancreatic cells. The progressive gait and limb ataxia, hypertrophic cardiomyopathy, and diabetes mellitus found in FRDA patients are attributed to lowered levels of ATP produced in these energy intensive tissues (85). Point mutations are described in compound heterozygous subjects with one expanded allele. A two-step model of GAA normal alleles towards premutation alleles, which might generate further full expanded mutations in the population with Indo-European ancestry, has been postulated. Clinical phenotype is variable and an inverse correlation with the GAA expansion size has been observed. Analysis of the GAA triplet is a strong molecular tool for clinical diagnosis, genetic counselling, and prenatal diagnosis. Many approaches have been undertaken to understand FRDA, but the heterogeneity of the etiologic factors makes it difficult to define the clinically most important factor determining the onset and progression of the disease. However, increasing evidence indicates that factors such as oxidative stress and disturbed protein metabolism and their interaction in a vicious cycle are central to FRDA pathogenesis. Brains of FRDA patients undergo many changes, such as disruption of protein synthesis and degradation, classically associated with the heat shock response, which is the most important form of stress response. The precise sequence of events in FRDA pathogenesis is uncertain. The impaired intramitochondrial metabolism with increased free iron levels and a defective mitochondrial respiratory chain will result in increased free

radical generation which will cause oxidative damage may be considered a possible mechanism that compromise cell viability. Recent evidence suggests that frataxin might detoxify ROS via activation of glutathione peroxidase and elevation of thiols, and in addition, that decreased expression of frataxin protein is associated with FRDA.

Recent studies have shown that frataxin acts as a chaperone for Fe(II) and a storage compartment for excess iron, (7). This is consistent with the roles played by frataxin in iron export, Fe-S cluster assembly, heme biosynthesis, and prevention of oxidative stress. Also, frataxin plays a direct role in the mitochondrial energy activation and oxidative phosphorylation. Several model systems have been developed in an effort to understand the disease (54). In mouse models, deletion of the frataxin gene results in embryonic lethality (54, 203) while its selective inactivation in neuronal and cardiac tissues leads to neurological symptoms and cardiomyopathy associated with mitochondrial iron-sulfur cluster-containing enzyme deficiencies and time-dependent mitochondrial iron accumulation. In contrast, a model expressing 25–35% of wild type frataxin levels by virtue of a $(GAA)_{230}$ expansion inserted in the first intron of the mouse gene has no obvious phenotype (21).

Over the last 5 years it has become clear that mitochondrial iron accumulation generates oxidative stress and results in damage to critical biological molecules. Studies using the budding yeast *Saccharomyces cerevisiae* have provided a further understanding of the consequences of frataxin loss (203). Deletion of the yeast frataxin homolog *YFH1* results in a tenfold increase in iron within the mitochondria along with increased ROS production (153). This leads to loss of mitochondrial function and the appearance of a *petite* phenotype in nearly all strains that have been examined (203). Bradley and colleagues (21) have demonstrated an impaired oxidative phosphorylation system with severe and significant deficiencies of mitochondrial respiratory chain complexes I and II/III and aconitase activities in cardiac muscle from patients with FRDA; mitochondrial DNA levels are reduced in FRDA heart and skeletal muscle and increased iron deposition is present in FRDA heart, liver, and spleen in a pattern consistent with a mitochondrial location. In addition, there is the appearance of nuclear DNA damage (21). Moreover, aconitase deficiency is suggestive that oxidative stress may induce a self-amplifying cycle of oxidative damage associated with mitochondrial dysfunction, which may also contribute to cellular toxicity. Iron deposit and enzyme deficiency have been reported in postmortem heart and brain tissues (94) of FRDA patients. The role of oxidative damage in the pathogenesis of FRDA is also supported by the finding that idebenone, an antioxidant similar to ubiquinone, can reduce myocardial hypertrophy and also decrease markers of oxidative stress in FRDA patients (153).

Upregulation of protein manganese superoxide dismutase (MnSOD) fails to occur in FRDA fibroblasts exposed to iron. This finding, together with the observation of absent activation of the redox-sensitive factor NFκB, suggest that the NFκB-independent pathway that may not require free radical signaling is responsible for the reducted induction of MnSOD. This impairment could constitute both, a novel defense mechanism against iron-mediated oxidative stress in cells with mitochondrial iron overload and, conversely, an alternative source of free radicals that could contribute to the disease pathology. Iron chelator drugs and antioxidant drugs have therefore been proposed for Friedreich's treatment. Drugs that reduce oxidative stress have a limited effect on the progression of the disease pathology, probably because they cannot properly remove iron accumulation. The potential role of iron chelators 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH) analogues, as agents to remove mitochondrial iron deposits have been under investigation (128). These ligands have been specifically designed to enter and target mitochondrial iron pools, which is a property lacking in desferrioxamine, the only chelator in widespread clinical use. This latter drug may not have any beneficial effect in FRDA patients, probably because of its hydrophilicity that prevents mitochondrial access. Indeed, standard chelation regimens will probably not work in FRDA, as these patients do not exhibit gross iron-loading. Considering that there is no effective treatment for FRDA, it is essential that the therapeutic potential of iron chelators focuses on the mitochondrial iron pools as primary target. Remarkably, an *in vitro* model of regulated human frataxin overexpression has provided experimental evidence of downregulation of the expression of mitogen-activated protein kinase kinase 4 associated with a decreased phosphorylation of c-Jun N-terminal kinase. In addition, to understand whether this alteration might result in cell death, the caspase pathway was investigated in FRDA cells, revealing in FRDA patients a significantly higher activation of caspase-9 after serum withdrawal compared to controls. These findings suggest the presence, in FRDA patient cells, of a 'hyperactive' stress signaling pathway (54). The role of frataxin in FRDA pathogenesis could be explained, at least in part, by this hyperactivity. Pilot studies have shown the potential effect of antioxidant therapy based on idebenone or coenzyme Q10 plus Vitamin E administration in this condition and provide a strong rationale for designing larger randomized clinical trials (154). There is now strong evidence to suggest that mitochondrially localized antioxidant ameliorates cardiomyopathy in FRDA patients, as well other lipophilic antioxidants that protect FRDA cells from cell death, indicating novel treatment strategies for Friedreich ataxia and presumably for other neurodegenerative diseases with mitochondrial impairment. Antioxidants targeted to mitochondria appear a promising approach to effectively slow disease progression.

Huntington's disease

Huntington's disease (HD) is an autosomal dominant, completely penetrant inherited neurodegenerative disorder, characterized by the insidious progression of severe neuropsychological and motor disturbances. The clinical manifestations of HD primarily involve psychiatric abnormalities, most commonly mood disturbances, followed by the development of involuntary choreiform movements and dementia. Onset of the disease is typically apparent in the fourth or fifth decade of life, and its long duration of up to 15–20 years results in death as a consequence of complicating immobility. The principal neuropathological features of the disease are marked atrophy, neuronal loss, and astrogliosis in the neostriatum. The genetic defect in HD has been recognized in an abnormal expanded trinucleotide (CAG) repeat in a gene lo-

cated on the short arm of chromosome 4 which encodes a protein termed "huntingtin," whose function, so far, remains to be elucidated.

Several lines of evidence indicate that a defect in mitochondrial energy metabolism might underlie the pathogenesis of the selective neuronal death occurring in HD (189). Evidence of bioenergetic defects in HD comes from *in vivo* imaging studies showing a marked hypometabolism, as revealed by PET analysis of [^{18}F]-fluorodeoxyglucose (FDG) utilization, in the caudate and putamen of symptomatic HD patients (52). Recent studies have also identified cortical hypometabolism in symptomatic HD. Alterations in cerebral glucose utilization predominantly reflect changes in neuronal terminal activity, the principal site of energy consumption. As known, most of the ATP produced in brain is used by energy-dependent pumps to restore transmembrane potential following synaptic transmission (52). Consequently, the marked hypometabolism observed in specific brain regions in HD can be related to loss of synaptic density due to the marked atrophy occurring in these regions. This hypothesis has gained further sustain from NMR studies indicating increased lactate concentrations in the basal ganglia of HD patients (129). This increase correlates well with the duration of the disease, implying that normal energy metabolism is progressively impaired by the disease process. This might arise because when oxidative phosphorylation is no longer sufficient in supplying cellular energy demands, cells resort to reduce pyruvate by NADH to recycle NAD for ATP production via the glycolytic pathway. A pathogenic role for mitochondrial dysfunction in HD arises from *in vivo* biochemical studies in postmortem brain tissue, which show defects in succinate dehydrogenase as well as pyruvate dehydrogenase activities in the striatum of HD patients. These defects are also a function of illness duration. Further evidence supporting a mitochondrial defect in HD has been provided by redox proteomics (189). In addition, NMR spectroscopy studies have demonstrated a 60% increase in pyruvate levels in the CSF, and 60% reduction in the activities of complex II and III in the caudate of HD patients, compared to controls (24).

Down's syndrome

The most important pathological features of Down's syndrome (DS) are mental retardation and accelerated aging. Numerous studies link both of these disturbances to free radical-induced damage (39). DS patients have an extra chromosome 21 (trisomy 21) and the recent assignment to chromosome 21 of the gene for copper-zinc SOD, together with the observation of increased SOD activity in red blood cells in DS patients has directed interest to the role played by free radical species in the pathogenesis of the disease (39). DS patients have a very high predisposition for developing the characteristics of AD. Therefore, DS patients provide a genetic model for investigating the role of oxidative stress in AD. In this regard, transgenic mice expressing the human SOD gene preferentially localize SOD in the hippocampus, which is the most vulnerable region in AD. However, these changes are not compensated by corresponding increase in catalase or glutathione peroxidase activities. This provides one possible explanation why increased SOD activity might have detrimental results. In addition, increased SOD activity results in decreased steady state levels of superoxide anion which also play a role in terminating the chain reactions of lipid peroxidation. All this evidence highlights the importance of oxidant/antioxidant balance as critical determinant and the possibility that the use of exogenous antioxidants can slow the progression of the disease (112, 113).

Ischemia/reperfusion

Ischemic brain damage is accompanied by an energy deficiency state and selective neuronal loss (119). Under such conditions, there is an increase in the extracellular concentration of glutamate, which may be neurotoxic due to activation of nNOS. Excess NO generation, as well as causing impairment of energy metabolism and other metabolic processes, may also downregulate glutamate (NMDA) receptors, thereby minimizing the effect of glutamate. In addition, NO can cause vasodilation and hence increase cerebral blood flow to the infarcted area (119). These effects may provide an explanation for the contradictory results that have been obtained when nonspecific NOS inhibitors have been evaluated in various models of ischemia. Reperfusion, following ischemia, may exacerbate the generation of oxidizing species, particularly superoxide. In a model of graded ischemia, loss of brain mitochondrial function, at the levels of complexes I, II, II-III, and ATP synthetase, has been reported (200). Reperfusion was associated with restoration of activity of these mitochondrial components, followed, after 2 h, by a dramatic loss of complex IV activity (23). The exact mechanism for this loss of complex IV activity is not known, but could involve the oxidative and/or nitrosative stress-mediated reactions (23). In fact, ischemia is accompanied by the formation of a gliotic scar, principally composed of reactive astrocytes which in large amount express iNOS (20, 119). Thus excessive generation of glial-derived $ONOO^-$ may constitute an important contributing factor to the mitochondrial damage associated with ischemia. Loss of brain ATP levels and mitochondrial complex II-III and IV activity has been demonstrated in a rat model of perinatal asphyxia (20), where administration of an NOS inhibitor to the mothers prevented impairment of brain energy metabolism in the hypoxic pups (20). Notably, ischemic preconditioning, which increases heat shock protein expression, preserves brain mitochondrial functions during middle cerebral artery occlusion (260).

GENETICS OF HUMAN LONGEVITY: ROLE OF VITAGENES IN PROLONGATION OF HEALTHY LIFE SPAN

The first half of the 20th century saw a rapid increase in the expectation of life in industrialized nations due to improved sanitation, public health, housing, nutrition, and medical technology/pharmaceuticals. The second half of this century has been characterized by a growing concern with the challenge produced by the increasing prevalence of old people in the society. Aging is a very common feature in living organisms and can be described as the total effect of those intrinsic changes in an organism that adversely affect its vitality and that render it more susceptible to the many factors that can cause death. Typically, mortality rate accelerates with

time, but it is not clear whether this effect is the result of external or internal causes of death. The full extent of aging in a population becomes apparent when most important external hazards are removed, such as under captive or laboratory conditions, when average longevity is usually greatly extended (42). Even if an organism is immortal it has nonzero probability of dying because of extrinsic causes, such as starvation, predation, and accidents. The probability of survival decreases in the course of life and, since natural selection is effective only through the reproductive output of individuals, the strength of natural selection decreases with age (42).

The first genetic theories on the evolution of aging were proposed in 1957 by Medawar and Williams almost simultaneously to the mechanistic theories of aging, such as the free radical and the somatic mutation theory, suggested by Harman (115, 116) and Szilard (240), respectively. A synthesis of evolutionary and mechanistic theories occurred in 1977 within the frame of the *soma theory* of aging postulated by Kirkwood (138). This theory provides a direct connection between evolutionary and physiological aspects of aging, by recognizing the primary importance of the allocation of metabolic energy resources between growth, somatic maintenance, and reproduction. It is suggested that longevity is determined through the setting of longevity assurance mechanisms, so as to provide an optimal compromise between investments in somatic maintenance (including stress resistance) and in reproduction. As a corollary, increasing maintenance promotes the survival and longevity of the organism only at the expense of significant metabolic investments that could otherwise be used to accelerate processes such as growth and reproduction. The *disposable soma* theory of the evolution of aging also proposes that a high level of accuracy is maintained in immortal germ line cells, or alternatively, that any defective germ cells are eliminated. The evolution of an increase in longevity in mammals may be due to a concomitant reduction in the rates of growth and reproduction, the so-called "essential life" and an increase in the accuracy of synthesis of macromolecules. The theory can be tested by measuring accuracy in germ line and somatic cells and also by comparing somatic cells from mammals with different longevities. Notably, the HO gene is evolutionarily different in birds and mammals, with the biliverdin reductase-bilirubin step present in the latter case (142, 146), but absent in the former group. Consistently, the organism sacrifices the potential for indefinite survival in favor of earlier and more prolific fecundity. From an evolutionary perspective, aging is a nonadaptive phenomenon and, as such, it can limit the reproductive potential of an individual. For this reason aging should be opposed by natural selection, and hence the argument that it evolved to provide offspring with living space is now receiving rather little credence. A clear prediction is that the actual mechanisms of senescence are stochastic, involving most likely processes such as random accumulation of somatic mutations or oxidative damage to macromolecules. In the word of an anonymous poet, *we are born as copies, but we die as originals.*

It is becoming increasingly clear that genetic factors are prominently involved in aging, the major lines of empirical evidence being: (a) the life span which in human populations show significant heritability; (b) different species have different intrinsic lifespans due to genomic differences; (c) human populations possess inherited progeroid disorders, such as Werner's syndrome, a disease characterized by premature age-related disorders, including atherosclerosis, type II diabetes, osteoporosis, and cancers; (d) clear evidence of genetic effects on lifespan have been demonstrated in invertebrate model systems, such as *Drosophila melanogaster* and *Caenorhabditis elegans*. In this organism five different genomic regions appear to be associated with longevity, as assessed by quantitative genetic analysis (211). Also, in *Saccharomyces cerevisiae* 13 longevity genes have been identified and cloned. Of these 13 genes, 11 have human homologues (211). At least, three categories of genes are predicted to affect aging and longevity: (a) genes that regulate levels of somatic maintenance and repair; (b) pleiotropic genes, whose expression involves trade-offs between early-life fitness benefits and late-life fitness disadvantages, which do not encompass somatic maintenance; (c) late-acting deleterious mutations that have escaped elimination as consequence of the decline in force of natural selection at old ages (48, 52). Efficient functioning of maintenance and repair process seems to be crucial for both survival and physical quality of life. This complex network of the so-called longevity assurance processes is composed of several genes, termed *vitagenes* (Table 1). The homeodynamic property of living systems is a function of such a vitagene network. Because aging is characterized by the failure of homeodynamics, a decreased efficiency and accuracy of the vitagene network can influence gerontogenic processes. It is not clear how various components of the vitagene network operate and influence each other in a concordant or a discordant manner. Aging can be defined as a loss of molecular fidelity (Table 1) due to a progressive failure of maintenance and repair processes. Thus, it is reasonable that genes involved in homeodynamic repair pathways, such as the HO-1, HSP70, thioredoxine reductase, biliverdin reductase, and Mn-SOD genes, are the most likely candidate vitagenes (52, 53, 142, 146, 158).

HO-1 and HSP70 as a therapeutic funnel

A promising approach for the identification of critical vitagene-related processes is represented by the hormesis-like positive effect of stress, including regular muscle exercise (52, 53), caloric restriction, which can result in activation of the HSP signal pathway and, consequently, in stress tolerance. In particular, there is strong evidence that the heme oxygenase/CO and biliverdin-bilirubin redox system might work critically as a therapeutic funnel in a number of physiopathological situations where the sensing of redox active events is coupled to acquirement of major resistance to the effect of stressful and pathogenic conditions. HO-1 activity seems to be required for the action of several other therapeutic molecules. In each case, the expression of HO-1 or administration of one of its metabolic products substitutes for the actions of the other protective molecule (185).

In many inflammatory situations the ability of IL-10 to suppress TNFα expression in macrophages requires the presence of HO-1 and the generation of CO; HO-1 expression or CO administration has the same effects as IL-10 (145, 229). In concert with this conceivable possibility, the protective ef-

Table 1. Polygenic Control of Longevity by the Homeodynamic Vitagene Network

Maintenance and Repair Functions	
Molecular fidelity control	*Cellular control*
Antioxidant defense	Cell proliferation
DNA repair systems	Cell differentiation
Transfer of genetic information	Stability of cell membrane
Stress protein synthesis	Stability of intracellular milieu
Proteasomal function	Macromolecular turnover
Tissue and organ control	*Physiological control*
Removing of toxic chemicals	Neuronal response and synaptic plasticity
Tissue regeneration	Hormonal response
Tumor suppression	Thermoregulation
Cell death and cell replacement	HO-1, BR, TRX, Mn-SOD pathway and cell stress response

fect of IL-10 in a lethal endotoxic shock mice model is strongly dependent on the expression of HO-1 and the generation of CO (185). Moreover, rapamycin appears not to exert its antiproliferative effects on smooth muscle cells unless HO-1 is present (145), and it has been proven that, in order for NO to protect mice livers from hepatitis induced by TNFα and galactosamine, upregulation of HO-1 is essential (186). Also, alcohol has antiinflammatory effects in that TNFα is suppressed and IL-10 is increased (186, 257). However, protection is lost when HO-1 is blocked (90). In addition, the anti-inflammatory effect of 15-deoxy-delta-12,14-prostaglandin J2 requires the activity of HO-1 (145, 186). Notably, during heat shock, which leads to upregulation of several heat shock proteins endowed with cytoprotective actions, entire cytoprotection is lost if HO-1 is blocked with SnPPIX. Last, relevant to brain physiopathology, dietary and medicinal compounds that can inhibit, retard, or reverse the multistage pathogenic events associated with degenerative damage, particularly polyphenols such as curcumin, caffeic acid, ferulic acid, and acetylcarnitine, all capable of exerting powerful antiinflammatory action, function through upregulation of HO-1 (55, 195, 216, 218, 238). The fact that in all these situations specific molecules or biological phenomena appear to lose most, if not all, of their effect when HO-1 is absent, represents compelling evidence that the HO-1 system may represent a final common mediator of many biological events associated to cell stress response and, as such, working as critical *vitagene*, which links redox-dependent pathways of stress tolerance to versatile biological programs of cell life.

CONCLUSIONS

Modulation of endogenous cellular defense mechanisms via the stress response signaling represents an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration. Efficient functioning of maintenance and repair processes seems to be crucial for both survival and physical quality of life (96). This is accomplished by a complex network of the so-called longevity assurance processes, which are composed of several genes termed *vitagenes*. Consistently, maintaining or recovering the activity of vitagenes can be possible to delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy life span. As one of the most important neurodegenerative disorders, Alzheimer's disease (AD) is a progressive disorder with cognitive and memory decline, speech loss, personality changes, and synapse loss. With the increasingly aging population of the United States, the number of AD patients is predicted to reach 14 million in the mid-21st century in the absence of effective interventions (35). This will pose an immense economic and personal burden on the people of this country. Similar considerations apply worldwide, except in sub-Sahara Africa, where HIV infection rates seem to be leading to decreased incidence of AD (30). Strong evidence suggests that factors such as oxidative stress and disturbed protein metabolism and their interaction in a vicious cycle are central to AD pathogenesis. Brain-accessible antioxidants, potentially, may provide the means of implementing this therapeutic strategy of delaying the onset of AD, and more in general all degenerative diseases associated with oxidative stress. As one potentially successful approach, potentiation of endogenous secondary antioxidants systems can be achieved by interventions which target the HO-1/CO and/or HSP70 systems. In this review, the importance of the stress response signaling and, in particular, the central role of HO-1 together with the redox-dependent mechanisms involved in cytoprotection are outlined. The beneficial effects of HO-1 induction result from heme degradation and cytoprotective regulatory functions of biliverdin/bilirubin redox cycling. Thus, HO-1 can amplify intracellular cytoprotective mechanisms against a variety of insults. Consequently, induction of HO-1, by increasing CO and/or biliverdin availability can be of clinical relevance.

CO has been studied for >100 years and, until the last few years, has been touted as a molecule to avoid, owing to its toxic effects exerted mostly on hemoglobin and cytochrome oxidase functions (184). However, these toxic effects are seen at concentrations of CO well above concentrations used experimentally. Beneficial effects are obtained with relative low doses of CO (250 ppm for one to few hours) in rodents (186). Carboxyhemoglobin levels generated in such a model are not too different from those of heavy smokers. If this beneficial effect is confirmed also in human, limited exposure of patients to CO might be considered as therapy for various syndromes, particularly to prevent restenosis after angioplasty or

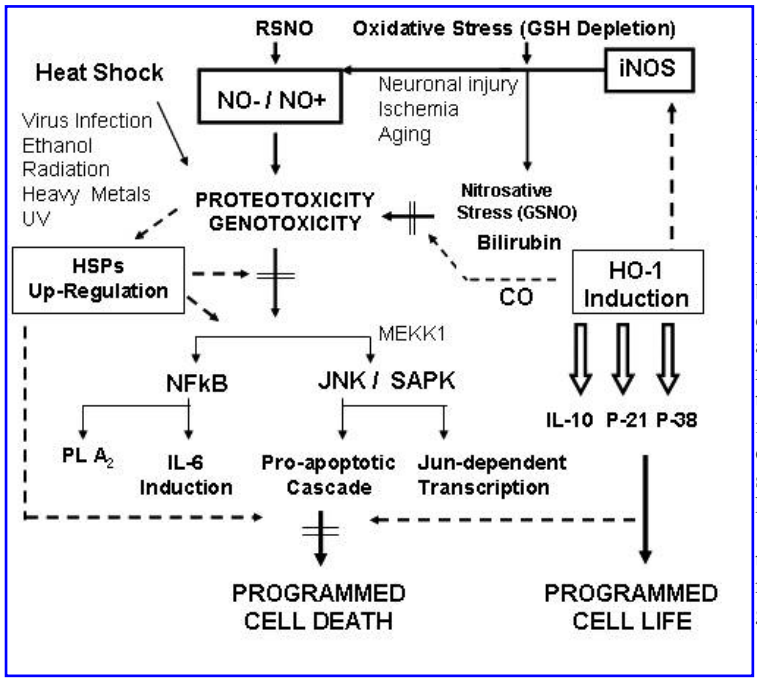

FIG. 2. Redox regulation of gene expression involving the Vitagene system. Proposed role for the *vitagene* member HSPs, in modulating cellular redox state and cell stress tolerance. Various proteotoxic (or genotoxic) conditions cause depletion of free HSPs that lead to activation of stress kinase and proinflammatory and apoptotic signaling pathways. HSP70 prevents stress-induced apoptosis by interfering with the SAPK/JNK signaling and by blocking caspase proteolytic cascade. Nitrosative-dependent thiol depletion triggers HO-1 induction, and increased HO-1 activity is translated into augmented production of carbon monoxide and the antioxidant bilirubin. These molecules may counteract increased NOS activity and NO-mediated cytotoxicity. In addition, HO-1 may directly decrease NO synthase protein levels by degrading the cofactor heme. PLA_2, phospholipase A_2; IL, interleukin; AP-1, activator protein-1; SAPK, stress-activated protein kinase; JNK, c-jun N-terminal kinase; NFκB, nuclear factor kappa-B; GSNO, *S*-nitrosoglutathione; HO-1 (heme oxygenase-1).

treatment of an organ donor and/or the organ to suppress ischemia-reperfusion injury and to prolong allograft survival. Very importantly, HO-1 and CO can suppress the development of atherosclerotic lesions associated with chronic rejection of transplanted organs (1, 186).

Interestingly, the recent discovered carbon monoxide releasing molecules (CORMs) appear to afford similar protective action, thereby providing an alternative therapeutic approach for those pathophysiological conditions where CO administration is warranted (180, 181). Furthermore, administration of biliverdin or bilirubin after the first few weeks of life is proven not to have toxicity, and doses as much as 2.5 mg/dl used in experimental paradigms are only slightly above normal levels, yet endowed with cytoprotective effects. Although clinical application of the HO system should be fully considered, a better understanding of how HO mediates its action will guide therapeutic strategies to enhance or suppress HO effects. Remarkably, the envisioned role of HSP70 as a vehicle for intracytoplasmic and intranuclear delivery of fusion proteins or DNA to modulate gene expression (255), along with the evidence that binding of HO protein to HO-1 DNA modifies HO expression via nonenzymatic signaling events (254) associated to CO and P-38-dependent induction of HSP70, open intriguing perspectives, as it is possible to speculate that synergy between these two systems might impact cell proliferation and apoptotic processes during oxidative stress, hence contributing to programmed cell life or programmed cell death (Fig. 2), depending on the relative extent of activation.

Presented here is strong evidence that a crosstalk between stress response genes is critical for cell stress tolerance, highlighting compelling reason for a renewed effort to understand the central role of this most extraordinary defense system in biology and medicine. All of the above evidence supports the notion that stimulation of various maintenance and repair pathways through exogenous intervention, such as mild stress or nutritional compounds targeting the heat shock signal pathway, may have biological significance as a novel approach to delay the onset of various age-associated alterations in cells, tissues, and organisms (195, 197). The major neurodegenerative diseases—AD, PD, MS, FRDA, HD, and ALS—are protein conformational diseases associated with oxidative stress. The discovery of whether these diseases result from a "toxic gain of function or a loss in function" depends on the scientific pursuit of both normal function and level of regulation that might involve the vitagene system. Hence, maintaining or recovering the activity of vitagenes (52, 142) can be possible to delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy lifespan.

ABBREVIATIONS

Aβ, amyloid beta-peptide; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AP-1, activator protein-1; BR, biliverdin reductase; CNS, central nervous system; FRDA, Friedreich ataxia; GSH, glutathione; GSSG, oxidizxed glutathione; HO-1, heme oxygenase-1; HSP, heat shock protein, JAK, janus kinase; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NFT, intraneuronal fibrillary tangles; NFκB, nuclear factor kappa-B; NOS, nitric oxide synthase; PCD, protein conformational diseases; PD, Parkinson's disease; PLA_2, phospholipase A_2; ROS, reactive oxygen species; RNS, reactive nitrogen species; SAPK, stress-activated protein kinase; STAT, signal transducer and transcription activator; TRX, thioredoxin reductase; TNF, tumor necrosis factor.

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Address reprint requests to:
Professor Mahin Maines
Department of Biochemistry
University of Rochester, School of Medicine
PO Box 712
601 Elmwood Ave
Rochester, NY 14627

E-mail: mahin_maines@urmc.rochester.edu

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