6 Aging and Oxidative Stress Response in the CNS

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Abstract: Cellular oxidant/antioxidant balance has become the subject of intense study, particularly focused on brain aging and neurodegenerative disorders. There is now evidence to suggest that reduction of cellular expression and activity of antioxidant proteins and the resulting increase of oxidative stress are fundamental causes for both the aging processes and neurodegenerative diseases. However, to survive different types of injuries, brain cells have evolved networks of different responses, which detect and control diverse forms of stress. 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 assurance processes, which are composed of several genes termed "vitagenes." Among these, heat-shock proteins (Hsps), proteasome, and mitochondrial uncoupling protein systems are highly conserved mechanisms responsible for the preservation and repair of the correct conformation of cellular macromolecules, such as proteins, RNA, and DNA.

Recent studies have shown that the heat-shock response contributes to establishing a cytoprotective state in a wide variety of human diseases, including ischemia and reperfusion damage, inflammation, metabolic disorders, cancer, infection, trauma, and aging. Among the various Hsps, Hsp32 also known as heme oxygenase I (HO-1), has received considerable attention, as it has been recently demonstrated that HO-1 induction, by generating the vasoactive molecule carbon monoxide (CO) and the potent antioxidant bilirubin, could represent a protective system potentially active against brain oxidative injury. The major neurodegenerative diseases, Alzheimer's 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. 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 perspectives in medicine and pharmacology, as molecules inducing this defense mechanism appear to be possible candidates for novel cytoprotective strategies. Particularly, modulation of endogenous cellular defense mechanisms such as the heat-shock response, and the proteasomal system, through nutritional antioxidants or pharmacological compounds may represent an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration. Moreover, by maintaining or recovering the activity of vitagenes, it would be possible to delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy life span.

List of Abbreviations: AD, Alzheimer's disease; AP-1, activator protein-1; Aβ, amyloid beta-peptide; CNS, central nervous system; GSH, reduced glutathione; GSSG, oxidized glutathione; Hsp, heat-shock protein; JAK, janus kinase; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-B; NFT, intraneuronal fibrillary tangles; NOS, nitric oxide synthase; PD, Parkinson's disease; PLA_2, phospholipase A_2; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; STAT, signal transducer and transcription activator; TNF, tumor necrosis factor

1 Introduction

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 (Mattson et al., 2002). Numerous theories have been suggested to explain the aging process (*Table 6-1*). However, current thoughts generally propose that senescence results from various extrinsic events that lead progressively to cell damage and death and/or characteristic intrinsic events related to the genome-based theory. These general theories have been presented in various ways. It is agreed that aging is a combination of several theories, the free radicals and mitochondrial theories presumably being the most important, while others may play a definite, although less critical, role. However, it should be emphasized that no single theory is entirely satisfactory.

Although several lines of evidence suggest that accumulation of oxidative molecular damage is a primary causal factor in senescence, it is 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

Table 6-1
Theories of aging

A. Stochastic (random event)
1. Somatic mutation
2. Error catastrophe
3. Protein glycosylation
B. Developmental
1. Immune
2. Neuroendocrine
C. Genome-based
1. Intrinsic mutagenesis
2. Programmed
D. Free radical and mitochondrial dysfunction

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) (Calabrese et al., 2001a). 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. Among these, chaperones are highly conserved proteins responsible for the preservation and repair of the correct conformation of cellular macromolecules, such as proteins, RNA, 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 (Soti and Csermely, 2002). 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 correlated to that of amyloid precursor protein (APP) (Dore, 2002; Perry et al., 2003). HO induction, which occurs together with the induction of other Hsps during various physiopathological conditions, by generating the vasoactive molecule carbon monoxide (CO) and the potent antioxidant bilirubin, represents a protective system potentially active against brain oxidative injury. 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 activator proteins (AP)-1, -2, and nuclear factor kappa-B (NF-κB) binding sites in its promoter region. In addition, HO-1 expression is rapidly upregulated by oxidative and nitrosative stresses, as well as by glutathione depletion. 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 (Butterfield et al., 2002a). In the present chapter, we discuss the role of free radicals and mitochondria in brain aging and neurodegenerative disorders, then review the role of NO and CO gases and that of the heat-shock system in brain stress tolerance and their relevance to mechanisms of longevity.

2 The Free-Radical Hypothesis of Aging

As one of the most prominent current theories of aging, the free-radical theory postulates that free radicals generated through mitochondrial metabolism can act as a 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 life span would eventually play a major role in aging (Knight, 2000; Fries, 2002; Anisimov et al., 2003; Sastre et al., 2003). 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.

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 (Cu/Zn-SOD) and catalase in transgenic *Drosophila melanogaster* prolongs the life span, retarding the age-associated accumulation of oxidative damage (Biesalski, 2002). Among the correlative evidence supporting the involvement of oxidative stress are the following: (1) oxidative damage to DNA and proteins increases exponentially with age, and concomitantly, the rates of mitochondrial $O_2^{\bullet -}$ and H_2O_2 generation as well as the susceptibility of tissues to experimentally induced oxidative stress are increased; (2) experimental regimens that extend life span, such as caloric restriction in mammals and reduction of metabolic rate in insects, decrease the accumulation rates of oxidative damage; and (3) mitochondria make two rather contradictory contributions to cell survival. The classically recognized function is the synthesis of ATP for energizing endergonic reactions and the other is generation of reactive oxygen species (ROS) that 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.

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 ROS 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 (Harris et al., 2003). 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 (Sharman and Bondy, 2001).

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 (Kalyuzhny, 2002). 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. 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 neurodegenerative conditions such as AD, and 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 AD (Mecocci et al., 1998). Levels of the oxidized nucleotide 8-hydroxy-deoxyguanosine (8-OH-dG), a biomarker of DNA damage, have also been shown to accumulate with aging. In several tissues, including brain and muscle, levels of 8-OH-dG in mitochondrial DNA (mtDNA) exceed that of nuclear DNA (nDNA) some 16-fold, although as yet there have been no studies performed using absolutely pure mtDNA (Halliwell, 1999). 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 and coworkers (1997) found that 8-OH-dG significantly correlates with increases in levels of a 7.4-kb deletion in the human brain.

The major DNA product formed by methylating agents in vitro and in vivo is 7-methylguanine, and it has been shown that in nDNA of normal mouse brains 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 (Gaubatz and Tan, 1993). This repair-resistant fraction of damage was greater in DNA from old tissues, and it was suggested that although DNA repair enzymes are present and active in senescent postmitotic tissues such as the brain, changes in the structure and function of "old" chromatin somehow decrease the capacity of the DNA repair enzymes present in the nucleus to repair oxidatively damaged DNA (Gaubatz and Tan, 1994). In addition, single-strand and double-strand breaks in DNA accumulate in the aging brain, and on exposure of neurons isolated from young and aged rats to an excitotoxic insult, more extensive DNA breaks were measured in neurons isolated from older rats (Mandavilli and Rao, 1996). During the course of normal metabolism in the brain there is production of ROS such as superoxide and hydroxyl radicals, as well as the production of reactive nitrogen species (RNS) such as nitric oxide (NO) 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 important to maintain normal brain functions.

There are several enzymes systems that have been found to repair damage to DNA caused by oxidizing species (Demple and Harrison, 1994). These include endonucleases, exonucleases, thymine glycol glycolases, and DNA polymerases. DNA polymerases so far detected in the mammalian brain (α, β, δ, and ϵ) 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 and somatic and mtDNA mutations, increases with age (Aviv et al., 2003). Levels of oxidative damage in mtDNA isolated from various brain regions appear to be at least tenfold higher than those of nDNA (Bohr and Dianov, 1999), although owing to technical difficulties there has as yet been no definitive study of oxidative damage to mtDNA (Beckman and Ames, 1998). This increase correlates with the 17-fold higher evolutionary mutation rate in mtDNA compared with nDNA. 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 activations of genes involved in error-prone DNA repair (Schapira, 1998). The age-associated accumulation of oxidative damage to mtDNA correlates with the level of mtDNA deletions found in various tissues composed of postmitotic cells (Floyd and Hensley, 2002). 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 (Calabrese et al., 2003a).

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 (GS) and glucose-6-phosphate dehydrogenase (G-6-PDH), has been demonstrated to occur in the brain during aging (Stadtman, 2001). A number of experimental evidence indicates that increased rate of free-radical generation and decreased efficiency of the reparative/degradative mechanisms, such as proteolysis, are the two factors that primarily contribute to age-related elevation in the level of oxidative stress and brain damage. 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 (Calabrese et al., 2000a). 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 proteins 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 (Calabrese et al., 2001a). The age-related increase in oxidized protein is accompanied by a loss of GS and G-6-PDH activities, and by 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-α-phenylnitrone (PBN) resulted in normalization of the several biochemical parameters to those characteristic of the young animals; coincidentally, the short-term memory index was restored to the values seen in young animals (Carney et al., 1991). 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 been proposed that a primary mechanism leading to neuronal cell death in aging and common neurodegenerative disorders is interference with proteasome function (Hyun et al., 2003).

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. The major neurodegenerative diseases, Alzheimer's 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 (Hyun et al., 2002). 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 patients with ALS associated with mutation in the gene encoding Cu/Zn-SOD (Halliwell, 2002). Even in the more common sporadic version 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 also a wide range of other proteins. Most of them are oxidized and nitrated. Similar damage has been described for proteins in Lewy bodies in sporadic PD. Oxidative as well as nitrosative 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 proteasome (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," which binds in an ATP-dependent manner and confers specificity for the degradation of polyubiquitinated proteins (Halliwell, 2001). 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 ensue a decreased clearance of oxidatively modified biomolecules. Oxidized protein levels in the central nervous system (CNS) tend to increase with age (Friguet, 2002), consistent with several reports that proteasome activity decreases with age and in neurodegenerative disorders (Mc Naught et al., 2001).

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 (Ojaimi et al., 1999), and increased likelihood of superoxide generation. Studies on isoprostanes, the end product of lipid peroxidation that can be measured in the 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 binding of cytotoxic aldehyde such as HNE to proteins, and by glycosidation of proteins (Drake et al., 2003a). The content of protein carbonyls in Alzheimer's brain samples is greater than in age-matched controls (Butterfield, 2002), 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 (Butterfield, 2002). Importantly, the accumulation of oxidation products in particular regions of the brain seems to be related to specific cognitive defects (Forster et al., 1996). In support of this, administration of the spin-trap PBN to gerbils retards both protein oxidation and such neurological defects (Carney et al., 1991).

2.1 The Mitochondrial Theory of Aging

Harman in 1972 first proposed that mitochondria may have a central role in the process of aging (Harman, 1981). According to this theory, free radicals generated through mitochondrial metabolism can act as a causative factor of abnormal function and cell death. Mitochondria are the cell's most significant source of oxidants and in vitro studies have indicated that approximately 1%–2% of electron flow through the electron transport chain (ETC) results in the univalent generation of superoxide (Calabrese et al., 2000a). Moreover, various toxins in the environment can injure mitochondrial enzymes, leading to increased generation of free radicals that over the life span would eventually play a major role in aging (Calabrese et al., 2004a). 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, has been shown to decrease with age (Paradies et al., 2002). 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 (Calabrese et al., 2001a).

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. As discussed before, the lack of introns and protective histones, limited nucleotide excision and recombination DNA repair mechanisms, and location in proximity of the inner mitochondrial membrane which expose it to an enriched free-radical milieu are all factors contributing to a tenfold higher mutation rate occurring in the mtDNA than in the nDNA. Moreover, a large body of evidence indicates that mtDNA mutations increase as a function of age, reaching the highest levels in the brain and muscle. More than 20 different types of deletions have been documented to accumulate in aging human tissues. The first report on an age-related increase in mtDNA deletion was found in brains from elderly subjects and in PD (Calabrese et al., 2001a). 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 the ATPase 8 and the ND5 genes. 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 have been shown to be linked to higher levels of mtDNA mutations than age-matched controls. In the CNS, 17 times higher levels of the common deletion in the striatum of patients with PD have been demonstrated, compared with age-matched controls. Evidence also exists indicating higher levels of this deletion in patients with AD, which parallel increased levels in the oxidized nucleotide 8-OH-dG (Bohr and Dianov, 1999).

A major feature of mtDNA disease in humans is the presence of cells with low cytochrome *c* oxidase activity, and evidence exists that indicates that the mechanism for these changes is likely to be clonal expansion of individual mtDNA deletions within single cells (Schapira, 1998). 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 neurons have been demonstrated to exist in abundance in the CNS of patients with mitochondrial disorders (Cottrell et al., 2001). These findings establish the relationship between age-associated accumulation of mtDNA mutations and bioenergy dysfunction as a key feature of the aging process, at least in tissues predominantly composed of postmitotic cells, such as the 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 during aging (Calabrese et al., 2001a). Since this ratio relates to the coupling efficiency between electron flux through the ETC 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 has been found associated with a significant decrease in cardiolipin content in brain mitochondria (Cottrell and Turnbull, 2000). 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, which 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 is 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 (Portero-Otin et al., 2001; Quiles et al., 2002). 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 (Paradies et al., 1999), suggesting that administration of this compound may improve cellular bioenergetics in aged rats (Hagen et al., 1998a, b). Interestingly, caloric restriction, a dietary regimen that extends life span in rodents, maintains the levels of 18:2 acyl side chains and inhibits the cardiolipin composition changes (Selman et al., 2003). In addition, caloric restriction was shown to retard the aging-associated changes in oxidative damage, mitochondrial oxidant generation, and antioxidant defenses (Mattson, 2003).

2.2 Mitochondrial Damage, Reactive Nitrogen Species, and Neurodegenerative Disorders

Increasing evidence sustains the hypothesis that mitochondrial energy metabolism underlies the pathogenesis of neurodegenerative diseases (Papa and Skulachev, 1997; Genova et al., 2004). Decreased complex I activity is reported in the substantia nigra of postmortem samples obtained from patients with PD (Beal, 1998). Similarly, impaired complex IV activity has been demonstrated in AD (Heales et al., 1999). Increased free-radical-induced oxidative stress has been associated with the development of such disorders and a large body of evidence suggests that $NO^{\bullet}$ plays a central role (Stamler and Hausladen, 1998). Cytokines (INF-γ) that are present in the normal brain are elevated in numerous pathological states, including PD (Mayer, 2003), AD (Moore et al., 2003), multiple sclerosis (MS) (Calabrese et al., 1994, 1998, 2002a, 2003b; Bagasra et al., 1995), ischemia, encephalitis, and viral infections of the CNS (Calabrese et al., 2001a). Accordingly, as cytokines promote the induction of nitric oxide synthase (NOS) in the brain, a possible role for a glial-derived $NO^{\bullet}$ in the pathogenesis of these diseases has been suggested (Stamler and Hausladen, 1998). Excessive formation of $NO^{\bullet}$ from glial origin has been evidenced in some study in which NADPH diaphorase (a cytochemical marker of NOS activity)-positive glial cells have been identified in the substantia nigra of postmortem brains obtained from individuals with PD (Hyun et al., 2003). Loss of nigral GSH is considered an early and crucial event in the pathogenesis of PD (Beal, 1998, 2003) 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 PD. In support of this it has been reported that the selective inhibition of neuronal NOS (nNOS) prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism in experimental animals (Dawson and Dawson, 2002; Moore et al., 2003).

3 Oxidative Stress and Brain Stress Tolerance: Role of Vitagenes

There is now evidence to suggest that reduction of cellular expression and activity of antioxidant proteins and the resulting increase of oxidative stress are fundamental causes for both aging process and neurodegenerative diseases (Ferri et al., 2003; Genova et al., 2003). However, to survive different types of injuries, brain cells have evolved networks of different responses, which detect and control diverse forms of stress. 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 assurance processes, which are composed of several genes termed vitagenes. 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 were discovered as an activator of antioxidant-and stress-responsive genes. As the cytoprotective mechanism triggered by SoxR in *E. coli* includes the expression of critical antioxidant defensive proteins, such as superoxide dismutase, the emerging concept is that an analogous system might operate in mammalian cells (Calabrese et al., 2001a, 2003a, 2004a). In eukaryotes, typical examples are the HO and Hsp genes, thioredoxin, detoxificant enzymes (Mn-SOD, glutathione-*S*-transferase, NADPH/quinone reductase), cytokines, immunoreceptors and growth factors (Calabrese et al., 2003c; Colombrita et al., 2003).

3.1 Neurogasobiology of Nitric Oxide and Carbon Monoxide: Two Molecules That Promote Adaptive Responses in the CNS

CO is the second gas discovered in the last 25 years to have salutary effects, the first being NO. 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 the expression of NOS2 and consequently of NO (Motterlini et al., 2002a). NO upregulates HO-1 with production of CO (Maines, 1997). We have recently found evidence for a functional relationship between CO and NO. In endotoxic shock, the salutary action of CO in the rat brain appears to depend sequentially on the activation of NF-κB, 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 (Scapagnini et al., 2002a). This has been also demonstrated in mice treated for hepatitis induced by TNF-α and D-galactosamine (Otterbein et al., 2003a). To what extent CO and NO act interdependently in other physiopathological conditions that are responsive to CO and/or NO is unknown.

3.2 Nitric Oxide Synthase and Its Isoforms in the CNS

The enzyme responsible for NO synthesis is the NOS family of enzymes, which catalyze the conversion of arginine to citrulline and $NO^{\bullet}$. NOS, localized in the CNS and in the periphery (Calabrese et al., 2000a), is present in three well-characterized isoforms: (1) neuronal NOS (nNOS; type I), (2) endothelial NOS (eNOS; type III), and (3) inducible NOS (iNOS; type II). Activation of different isoforms of NOS requires various factors and cofactors. 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 functionally active dimer exhibits NOS activity, which depends also on cofactors such as tetrahydrobiopterin (BH_4), FAD, FMN, and NADPH (Dawson and Dawson, 1995). nNOS has a predominant cytosolic localization whereas the eNOS is bound to the plasma membrane by N-terminal myristaglation (Calabrese et al., 2000a). In contrast to nNOS and eNOS, iNOS can bind to calmodulin even at very low concentrations 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 cGMP 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 (Bredt, 1999). Activation of iNOS requires gene transcription, and the induction can be influenced by endotoxin and cytokines (Calabrese et al., 2000a) (IL-1, IL-2, lipopolysaccharide, IFN-γ, TNF). This activation can be blocked by antiinflammatory drugs (dexamethasone), inhibitory cytokines (IL-4, IL-10), prostaglandins (PGA_2), and tissue growth factors or inhibitors of protein synthesis, e.g., cycloheximide.

3.3 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 (Stamler and Hausladen, 1998). Once generated, the cell cannot 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. NO when produced in small quantities can regulate cerebral blood flow, local brain metabolism (Calabrese et al., 2001a), and 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 (Heales et al., 1999).

3.4 Redox Activities Elicited by NO

In the last several years, a number of studies have shown a protective effect of NO in a variety of paradigms of cell injury and cell death. These include (1) direct scavenging of free radicals, such as superoxide with effects on intracellular iron metabolism, including interaction with iron to prevent, through formation of nitrosyl-iron complexes, release of iron from ferritin (Sergent et al., 1977); (2) interaction of $NO^{\bullet}$ (through its congener NO^{+}) with thiol group on the NMDA receptor with consequent downregulation and inhibition of calcium influx (Ignarro, 2002); (3) inactivation of caspases (Ignarro, 2002); (4) activation of a cGMP-dependent survival pathway, as demonstrated in PC12 cells (Motterlini et al., 2002a); (5) inducing expression of cytoprotective proteins, such as heat-shock proteins (Hsps) (Motterlini et al., 2000a); and (6) inhibition of NF-κB activation or GADPH, whose activity appears to be required in one paradigm of neuronal apoptosis (Piantadosi et al., 1997). 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 (Rosenberg et al., 1999). 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 (Dawson and Dawson, 1995). The difficulty in delineating a mechanistic involvement of NO as proinflammatory 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 (Motterlini et al., 2002a). 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 (Stamler and Hausladen, 1998). 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. Whereas NO^{+} can be transferred reversibly between cysteine residues (transnitrosation), NO^{-} can be formed by hemoglobin, nNOS, 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 (Ignarro, 2002). This chemical process, known as S-nitrosation, has been suggested to represent a refined endogenous tool to stabilize and preserve NO biological activity (Rosenberg et al., 1999; Motterlini et al., 2002a). It has been speculated that low-molecular weight RSNO, such as *S*-nitrosoglutathione or nitrosocysteine, may also represent a mechanism for storage of NO in vivo (Stamler et al., 1992; Rosenberg et al., 1999). 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 tissues 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 RNS reach a critical threshold, and hence nitrosative stress may ensue (Hausladen et al., 1996). At the cellular level, nitrosative stress has been linked to inhibition of cell growth and apoptosis, and implicated in NO pathogenesis (Sergent et al., 1977). The intriguing aspect in the parallelism 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 (Calabrese et al., 2002b).

3.5 Regulation of Gene Expression 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. 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. Recent studies suggested 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 (Rosenberg et al., 1999; Motterlini et al., 2002a). The expression of these protective genes renders bacteria more resistant to oxidant damage (Motterlini et al., 2002b). As the cytoprotective mechanism triggered by SoxR in *E. coli* includes the expression of critical antioxidant defensive proteins, such as superoxide dismutase (Motterlini et al., 2003), the emerging concept is that analogous systems might operate in mammalian cells. In eukaryotes, typical examples are genes such as the HO gene, thioredoxin and detoxificant enzymes (Mn-SOD, glutathione-*S*-transferase, NADPH/quinone reductase), cytokines, immunoreceptors, and growth factors. That the antioxidant protein HO could "sense" NO, and thus protecting against ROS and RNS insults, is supported by the following findings: (1) NO and NO-related species induce HO-1 expression and increase HO activity in human glioblastoma cells, hepatocytes, and aortic vascular cells; (2) cells pretreated with various NO-releasing molecules acquire increased resistance to H_2O_2-mediated cytotoxicity when HO is maximally activated; and (3) bilirubin, one of the end products of heme degradation by HO, protects against the cytotoxic effects caused by strong oxidants such as H_2O_2 and $ONOO^-$ (Rosenberg et al., 1999; Motterlini et al., 2002a). 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 HO activity, in a variety of tissues, including brain cells (Scapagnini et al., 2002a). In rat glial cells, treatment with lipopolysaccharide (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 (Calabrese et al., 2000b). 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 an in vivo rat model of septic shock (Scapagnini et al., 2002a). 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 (Motterlini et al., 2000a, 2002a). Taken together, these findings point to the central role of 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 (Butterfield et al., 2002b). 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 (AP-1), NF-κB, and the more recently identified Nrf2 proteins (Balogun et al., 2003; Poon et al., 2004a). Importantly, both AP-1 and NF-κB contain cysteine residues whose interaction with oxidant or nitrosant species might be crucial for determining the DNA-binding activity (Rosenberg et al., 1999; Motterlini et al., 2002a). 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 (Butterfield et al., 2002a; Poon et al., 2004b).

We have demonstrated in astroglial cell cultures that cytokine-induced nitrosative stress is associated with an increased synthesis of Hsp70 stress proteins, which was also found after treatment of cells with the NO-generating compound sodium nitroprusside (SNP), thus suggesting a role for NO in inducing Hsp70 protein expression (Calabrese et al., 2000b). In vivo experiments performed in our laboratory have also demonstrated that the redox glutathione status is a critical factor for induction of cytoprotective Hsp70 (Calabrese et al., 2000c, 2002c, 2004a; Balogun et al., 2003).

3.6 Carbon Monoxide: A Signaling Molecule Endowed with Antiinflammatory Properties

Grehan first detected a combustible gas in the blood in 1894 (Grehan, 1894). This gas was supposed by de Saint Martin and Nicloux to be CO. However, it was not until 1949 that Siorstrand discovered that

endogenously produced CO arose from the degradation of hemoglobin released from senescing erythrocytes (Siorstrand, 1949). 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 (1969) described and characterized HO 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 by-products (Tenhunen et al., 1969). Since then, supported by a large body of experimental evidence, CO is proving to be an extraordinary signaling molecule generated by the cell, which 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 the central and peripheral nervous systems. This gaseous molecule is now considered a putative neurotransmitter, owing to its capability to diffuse freely from one cell to another, thereby influencing intracellular signal transduction mechanisms. However, unlike conventional neurotransmitters, CO is not stored in synaptic vesicles and is not released by membrane depolarization and exocytosis. It seems likely that CO is involved in the mechanism of cell injury (Turcanu et al., 1998). This is evidenced by the fact that CO binds to iron of heme of the enzyme guanylyl cyclase to activate cGMP (Piantadosi et al., 1997). Indeed, it has been found that CO is responsible for maintaining endogenous levels of cGMP. This effect is blocked by potent HO inhibitors but not by NO inhibitors (Maines, 1997). On the basis of endogenous distribution of HO in the CNS, it has been suggested that CO can influence neurotransmission like NO (Verma et al., 1993). CO appears to be involved as a retrograde messenger in LTP and also in mediating glutamate action at metabotropic receptors (Graser et al., 1990). This is evident from the fact that metabotropic receptor activation in the brain regulates the conductance of specific ions channels via a cGMP-dependent mechanism, which is blocked by HO inhibitors (Glaum and Miller, 1993). Experimental evidence suggests that CO plays a similar role like NO in the signal transduction mechanism in regulating cell function and cell-to-cell communication (Maines, 1997). 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 half carboxyterminal of NOS (Calabrese et al., 2001a). 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, therefore impairment of CO and NO metabolism results in abnormal brain function (Calabrese et al., 2000a). A number of evidence suggest 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 (Calabrese et al., 2003a). This hypothesis is sustained by recent findings showing that HO inhibition increases NO production in mouse macrophages exposed to endotoxin (Turcanu et al., 1998). CO may also act as a signaling effector molecule, by interacting with targets other than guanylate cyclase. Notably, it has been recently demonstrated that $K_{(Ca)}$ channels are activated by CO in a cGMP-independent manner (Wang and Wu, 2003) and also that CO-induced vascular relaxation results from the inhibition of the synthesis of the vasoconstrictor endothelin-1 (Coceani et al., 1997). 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 that, upon exposure to CO, acquires DNA-binding transcriptional activity for the CO dehydrogenase gene, thereby encoding for CO dehydrogenase, which is the key enzyme involved in the oxidative conversion of CO to CO_2. Remarkably, heart cytochrome *c* oxidase possesses CO-oxygenase activity, thus metabolizing CO to CO_2 (Calabrese et al., 2001a). Whether this occurs also in brain mitochondria remains to be elucidated. Aside from the CNS, 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 (Otterbein et al., 2003a). 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 as a consequence of HO-1 activity being 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, even a heart that cannot express HO-1 activity still survives indefinitely (Otterbein, 2002). In this scenario CO appears to be able to substitute for HO-1 in suppressing the proinflammatory response, which is the leading cause of graft rejection. CO emerges as a powerful antiinflammatory promoting agent acting at the level of the macrophage cell line, a cell that probably controls the balance of inflammation in many conditions. Macrophages stimulated with bacterial LPS produce proinflammatory cytokines such as TNFα and an inflammatory cytokine interleukin-10 (IL-10) is also produced (Otterbein et al., 2003b). 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 (Otterbein et al., 2003a); (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 pancreatic β-cells (Otterbein et al., 2003a). In addition, CO suppresses the proliferative response of smooth muscle cells, which 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 and its subsequent inhalation. Interestingly, the recently discovered CO-releasing molecules (CORMS) appear to afford similar protective action, thereby providing an alternative therapeutic approach for those pathophysiological conditions where CO administration is warranted (Motterlini et al., 2002b, 2003).

3.7 The Heat-Shock Pathway of Brain Stress Tolerance

It is well known that living cells are continually challenged by conditions that cause acute or chronic stress. To adapt to environmental changes and survive different types of injuries, eukaryotic cells have evolved networks of different responses, 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 (Calabrese et al., 2001a; Mattson et al., 2002). This has opened new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear as possible candidates for novel cytoprotective strategies (Scapagnini et al., 2002b; Soti and Csermely, 2002; Colombrita et al., 2003). 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, which control the expression of a specific set of genes encoding cytoprotective Hsps. Some of the known Hsps include ubiquitin, Hsp10, Hsp27, Hsp32 (or HO-1), Hsp47, Hsp60, Hsc70, Hsp70 (or Hsp72), Hsp90, and Hsp100/105. Most of the proteins are named according to their molecular weight.

HSP70. The 70-kDa family of stress proteins is one of the most extensively studied. Included in this family are Hsc70 (heat-shock cognate, the constitutive form), Hsp70 (the inducible form, also referred to as Hsp72), and GRP75 (a constitutively expressed glucose-regulated protein found in the endoplasmic reticulum). After a variety of 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 stimuli for induction. These denatured proteins activate heat-shock factors (HSFs) within the cytosol by dissociating other Hsps that are normally bound to HSF (Balogun et al., 2003; Calabrese et al., 2003a; Poon et al., 2004a, b). Freed HSF is phosphorylated and forms trimers, which enter the nucleus and bind to heat-shock elements (HSEs) 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 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. Hsps serve as chaperones that bind to other proteins and regulate their conformation, regulate 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 (Kelly et al., 2002). 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 (Wang and Wu, 2003). Following focal cerebral ischemia, mRNA encoding Hsp70 is synthesized in most ischemic cells except in 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 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 is the zone of protein denaturation in the ischemic areas (Balogun et al., 2003). 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 (Narasimhan et al., 1996). Transfection of cultured astrocytes with Hsp70 protects them from ischemia or glucose deprivation (Fink et al., 1997). Hsp70 has been demonstrated to inhibit caspase-3 activation caused by ceramide, and also affects JUN kinase and p38-kinase activation (Mosser et al., 1997). In addition, Hsp70 binds to and modulates the function of BAG-1, the bcl-2 binding protein (McLaughlin et al., 2003), thus modulating some type of apoptosis-related cell death.

A large body of evidence now suggests a correlation between mechanisms of oxidative and/or nitrosative stress and Hsp induction. Current opinion holds also the possibility that the heat-shock response can exert its protective effects through inhibition of NF-κB-signaling pathway (Calabrese et al., 2001a). We have demonstrated in astroglial cell cultures that cytokine-induced nitrosative stress is associated with an increased synthesis of Hsp70. Increase in Hsp70 protein expression was also found after treatment of cells with the NO-generating compound SNP, thus suggesting a role for NO in inducing Hsp70 proteins. The molecular mechanisms regulating the NO-induced activation of the heat-shock signal seems to involve cellular oxidant/antioxidant balance, mainly represented by the glutathione status and the antioxidant enzymes (Calabrese et al., 2000a, 2003a; Motterlini et al., 2000a).

Ubiquitin. 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 NF-κB, cyclins, HSFs, hypoxia-inducible factor, some apoptosis-related proteins, tumor necrosis factor, and erythropoietin receptors (Mayer, 2003).

Hsp27. Hsp 27 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 etoposide-induced apoptotic cell death as well as H_2O_2-induced necrosis (Bechtold and Brown, 2003).

Hsp47. Hsp47 is synthesized mainly in microglia following cerebral ischemia and subarachnoid hemorrhage (Valentim et al., 2003).

Hsp60, glucose-regulated protein 75 (*GRP75*), *and Hsp10.* 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 (Turner et al., 1999). Hsp60 is encoded in

the nucleus and resides mainly in the mitochondria (Calabrese et al., 2003c, 2004a, 2005). Hsp60 forms the chaperonin complex, which is implicated in protein folding and assembly within the mitochondria under normal conditions (Izaki et al., 2001). Most mitochondrial proteins are synthesized in the cytosol and must be imported into the organelles in an unfolded state (Izaki et al., 2001). 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 (Okubo et al., 2000). 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 mechanism targeted by nutritional interventions leading to restoration of mitochondrial respiratory complex function compromised by oxidative stress (Calabrese et al., 2004a, 2005; Perluigi et al., 2005; Poon et al., 2005; Sultana et al., 2005).

Hsp32. Hsp32 or HO is the rate-limiting enzyme in the production of bilirubin. There are three isoforms of HO, HO-1 or inducible isoform, HO-2 or constitutive isoform, and the recently discovered HO-3 (Scapagnini et al., 2002c).

3.8 HO System: A Putative Vitagene Target for Neuroprotection

In the last decade the 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 CO. Iron is reduced to its ferrous state through the action of NADPH cytochrome *c* P450 reductase. 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 biliverdin reductase. Biliverdin complexes with iron until its final release (Calabrese et al., 2003a). HO is present in various tissues with the highest activity in the brain, liver, spleen, and testes. There are three isoforms of HO, HO-1 or inducible isoform (Calabrese et al., 2004b), HO-2 or constitutive isoform (Ewing and Maines, 1992; Hon et al., 2000), and the recently discovered HO-3, cloned only in rat to date (Scapagnini et al., 2002c). They are all products of different genes and, unlike HO-3, which is a poor heme degrading 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 similarity between HO-1 and HO-2 consists of a common 24 amino acid domain (differing in just one residue) called the "HO signature," which renders both proteins extremely active in their ability to catabolize heme (Calabrese et al., 2004b). They have different localization, similar substrate and cofactor requirements, while presenting different molecular weights. 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 chromosome arms i.e., 22q12 for HO-1 and 16q13.3 for HO-2 (Ewing and Maines, 1992).

Various tissues have different 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 more than 70% of HO activity is accounted for by HO-2, whereas in the testes the pattern of HO isoenzyme expression differs according to the cell type, although HO-1 expression predominates after heat shock. This also occurs in the 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 us and other groups have not found detectable levels of HO-1 protein in the normal brain (Ewing and Maines, 1992), 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 cerebellum (Scapagnini et al., 2002c). This evidence may suggest the possible existence of a cellular reserve of HO-1 transcripts quickly available for protein synthesis and a posttranscriptional regulation of its expression.

HO isoenzymes are also seen to colocalize with different enzymes depending on the cell type. In the kidney, HO-1 colocalizes with erythropoietin, whereas in smooth muscle cells HO-1 colocalizes with NOS. In neurons, HO-2 colocalizes with NOS, whereas the endothelium exhibits the same isoform that colocalizes with NOS III. The cellular specificity of this pattern of colocalization lends further support to the concept that CO may serve a function similar to that of NO. Furthermore, the brain expression pattern shown by HO-2 protein and HO-1 mRNA overlaps with the distribution of guanylate cyclase, the main CO functional target (Coceani et al., 1997).

HO-3, the third isoform of HO, 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 (Hon et al., 2000).

Although the biological properties of this isoenzyme still remains to be elucidated, the presence of two HRM motifs in its amino acidic sequence might suggest a role in cellular heme regulation (Maines, 2000). Studying the HO-3 mRNA sequence (GenBank accession no.: AF058787), we have observed that its 5′-portion corresponds to an L-1 retrotransposon sequence, a member of a family of retrotransposons recently found to be involved in evolutionary mechanisms (Kazazian, 2000). On the basis of the close similarity to a paralogous gene (HO-2) and on the preliminary data from our group demonstrating the absence of introns in the HO-3 gene (Scapagnini et al., 2002c), it is possible that this 5′-portion could have originated from the retrotransposition of the HO-2 gene. In addition, this genetic mutation in the rat may represent a species-specific event since no other sequence in the public databases match that of the rat HO-3.

Induction of HO-1 gene could be used for clinical diagnosis. However, the length of the GT 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 long GT repeats, which are associated with low HO-1 responsiveness. This polymorphism appears to be of functional significance in the short repeats, which are associated with high responsiveness and seem to be also associated with lesser likelihood of re-stenosis after angioplasty (Otterbein et al., 2003a).

3.9 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 ROS, and consequently oxidative damage to cellular macromolecules. In higher eukaryotes, toxic effects of heme are counteracted by the inducible HO-1 system (Maines, 2000). As in the classic theory of metabolic control, expression of HO-1 is induced by the substrate heme (Kanakiriya et al., 2003). 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 (Keyse and Tyrrell, 1989). Thus, HO-1 is an essential antioxidant defense enzyme that converts toxic heme into antioxidants and is fundamental for coping with various aspects of cellular stress and for regulating iron metabolism (Poon et al., 2004a). 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 (Otterbein, 2002).

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 the neonatal rat brain after treatment with corticosterone (Raju et al., 1997). The organization of the HO-2 gene needs to be fully elucidated, although a consensus sequence of the glucocorticoid response element (GRE) has been demonstrated in the promoter region of the HO-2 gene (Liu et al., 2000). In addition, endothelial cells treated with the NOS inhibitor L-NAME and HO inhibitor zinc mesoporphyrin exhibited a significant upregulation of HO-2 mRNA.

HO-1 gene is induced by a variety of factors, including metalloporphyrins and hemin, as well as ultraviolet A (UVA) irradiation, hydrogen peroxide, prooxidant states, or inflammation (Tyrrell, 1999). This characteristic inducibility of HO-1 gene strictly relies on its configuration: the 6.8-kb gene is organized into four introns and five exons. A promoter sequence is located approximately 28 base pairs upstream from the transcriptional site of initiation. In addition, different transcriptional enhancer elements such as the HSE 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 kb and 10 kb upstream the initiation site (Hill-Kapturczak et al., 2003). 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 (Sun et al., 2002). 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) (Martin et al., 2004) with a consensus sequence (GCnnnGTA) similar to that of other antioxidant enzymes (Balogun et al., 2003). 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 (Gong et al., 2002). 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 (Sun et al., 2002), 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 Nrf2, to promote the transcription of *HO-1* gene (Sun et al., 2002). 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 the metallothionein-1 gene, which respond to heavy metals (cadmium and zinc) only after recruitment of another fragment located upstream, between −3.5 and 12 kbp (CdRE). In addition, a 163-bp fragment containing two binding sites for HSF-1, which mediates HO-1 transcription, are located 9.5 kb upstream of the initiation site (Balogun et al., 2003). The distal enhancer regions are important in regulating HO-1 in inflammation, as they have been demonstrated to be responsive to endotoxin. In the promoter region, there also resides a 56 bp fragment that responds to the STAT-3 acute-phase response factor, involved in the downregulation of HO-1 gene induced by glucocorticoid (Raju et al., 1997).

3.10 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

ROS in brain cells (Butterfield et al., 2002b). A compromised GSH system in the brain has been associated with the oxidative stress occurring in neurological diseases (Butterfield et al., 2002c). Recent data demonstrate that, besides intracellular functions, GSH has also important extracellular functions in the 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 (Dringen and Hirrlinger, 2003). 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 (Dringen and Hirrlinger, 2003). Glutathione exists in either a reduced (GSH) or oxidized (GSSG) form and participates 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 mM depending on the cell type (Drake et al., 2003b). 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 (Calabrese et al., 2000b; Balogun et al., 2003; Catania et al., 2003). 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 the rat brain, human fibroblasts, endothelial cells, and rat cardiomyocytes (Foresti and Motterlini, 1999). This finding is supported by the fact that *N*-acetyl-cysteine (a precursor of glutathione) abolishes oxidative stress-mediated induction of HO-1 gene (Foresti et al., 1997, 2001). 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 increasing GSSG, and this effect was abolished by pretreatment of glial cells with NOS inhibitors (Calabrese et al., 2000b). Moreover, elevation of intracellular glutathione prior to exposure of endothelial cells to NO donors almost completely abolishes activation of the HO pathway, which suggests that thiols can antagonize the effect of NO and NO-related species on HO-1 induction (Calabrese et al., 2000b). 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 (Motterlini et al., 2000a). 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 (Motterlini et al., 2000b). All these evidence corroborate the notion that generation of ROS and RNS are important signal transduction mechanisms linking HO-1 activation to cell stress tolerance (Mancuso et al., 2003; Pocernich et al., 2005).

3.11 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 HO pathway, in fact, has been shown to act as a fundamental defensive mechanism for neurons exposed to an oxidant challenge (Chen et al., 2000). Induction of HO occurs together with the induction of other Hsps in the brain during various experimental conditions including ischemia (Dore, 2002). Injection of blood or hemoglobin results in increased expression of the gene encoding HO-1, which has been shown to occur mainly in microglia throughout the brain (Calabrese et al., 2001a). 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 (Beschorner et al., 2000).

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 grey and the white matter. Thereafter, HO-1 expression was limited to degenerating fiber tracts. Interestingly, Hsp70 was consistently colocalized 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 remodeling of surviving tissue (Mautes et al., 2000).

Since the expression of Hsps is closely related to that of APP, Hsps proteins have been studied in the brain of patients with AD. Significant increases in the levels of HO-1 have been observed in AD brains in association with neurofibrillary tangles (NFTs) (Takeda et al., 2000), and also HO-1 mRNA was found increased in AD neocortex and cerebral vessels (Premkumar et al., 1995). HO-1 increase was not only in association with NFTs but also colocalized with senile plaques and glial fibrillary acidic protein-positive astrocytes in AD brains (Schipper et al., 2000). 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 (Calabrese et al., 2004b).

Upregulation of HO-1 in the substantia nigra of patients with PD has been demonstrated. In these patients, nigral neurons containing cytoplasmic Lewy bodies exhibited in their proximity maximum HO-1 immunoreactivity (Ewing and Maines, 1992). New evidence showed a specific upregulation of HO-1 by oxidative stress in the nigral dopaminergic neurons (Poon et al., 2004a).

Multiple sclerosis (MS) is a common, often disabling disease of the 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 Hsps, thereby initiating the MS lesion (Aquino et al., 1977; Maines, 2000). However, cytokines, immunoglobulins, and complement complexes may elicit a survival response in the oligodendrocytes, involving the induction of endogenous Hsps and other protective molecules, which indicates that redox systems and therefore the oxidant/antioxidant balance in these cells are of great importance in MS (Calabrese et al., 1994, 1998, 2002a, 2003b; Bagasra et al., 1995). The expression of HO-1 is increased in the CNS of mice and rats with experimental allergic encephalomyelitis (EAE), an animal model of MS (Catania et al., 2003). To investigate the role of HO-1 in EAE, SnPPIX was administered to SJL mice during active disease. SnPPIX (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 (Chakrabarty et al., 2003). On the contrary, in another study, high expression of HO-1 in lesions of EAE was enhanced by hemin treatment, a procedure that resulted in the attenuation of clinical signs of pathology, whereas tin mesoporphyrin, an inhibitor of HO-1, markedly exacerbated EAE (Liu et al., 2001). 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 MS. We have recently shown that thiol disruption and nitrosative stress are associated in active MS with induction of Hsp70 and HO-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 (Poon et al., 2004a). All these findings can open up new therapeutic perspectives, as molecules activating these defense mechanisms appear to be possible candidates for novel neuroprotective strategies (Calabrese et al., 2000c).

3.12 Bilirubin and Biliverdin: An Endogenous Antioxidant System

Supraphysiological levels (>300 μM) of nonconjugated bilirubin, as in the case of neonatal jaundice, are associated with severe brain damage. This is a plausible reason whereby bilirubin has generally been recognized as a cytotoxic waste product. However, only in recent years its emerging role as a powerful antioxidant has received wide 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 owing to phosphorylative-dependent enhancement of HO-2 activity protected against hydrogen peroxide-induced cytotoxicity (Stocker et al., 1987; Mancuso et al., 2003). 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 (Dore et al., 2000). Bilirubin can become particularly important as a cytoprotective agent for tissues

with relatively weak endogenous antioxidant defenses such as the CNS and the myocardium. Interestingly, increased levels of bilirubin have been found in the cerebrospinal fluid in AD, which may reflect the increase of degraded bilirubin metabolites in the AD brain, derived from the scavenging reaction against chronic oxidative stress (Kimpara et al., 2000). 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 (Dore et al., 2000). 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 to biliverdin, then rapidly converted back to bilirubin by biliverdin reductase, which in the brain is present in large functional excess, suggesting a mechanism to amplify the antioxidant effect (Poon et al., 2004b). Remarkably, the rapid activation of HO-2 by protein kinase C (PKC) phosphorylation parallels the availability of nNOS. Both are constitutive enzymes localized in neurons, and nNOS is activated by calcium entry into cells binding to calmodulin. Similarly, PKC phosphorylation of HO-2 and the transient increase in intracellular bilirubin would provide a way for a rapid response to calcium entry, this being 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 defence against NO-related species. In view of the high inducibility of heme 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 (Kaur et al., 2003).

4 Caloric Restriction and Endogenous Oxidative Stress: Relevance to Aging and Cell Survival

Caloric restriction in mammals has been recognized as the best characterized and most reproducible strategy for extending maximum life span, 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 for 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 (Hursting et al., 2003). 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 induce this effect are currently unknown. General consensus, however, has been achieved that caloric restriction feeding regimes reduce 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 (Mattson et al., 2002). 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 depending 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 (Calabrese et al., 2001a). It has been also demonstrated that mitochondria from calorie-restricted animals produce less ROS per nanomole 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 (Mattson et al., 2003). 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 bioenergetics of individual mitochondria or the mitochondrial number and turnover rate. High-density oligonucleotide array 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 (Martin et al., 2003; Strauss, 2003; Calabrese et al., 2004b).

4.1 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 (Butterfield et al., 2002a). Spices and herbs contain phenolic substances with potent antioxidative and chemopreventive properties (Scapagnini et al., 2002d). The active antioxidant principle in *Curcuma longa*, a coloring agent and food additive used in Indian culinary preparations, has been identified as curcumin (diferuloylmethane). Because of the presence in its structure of two electrophilic α, β-unsaturated carbonyl groups which, by virtue of the Michael reaction, can react with nucleophiles such as glutathione, curcumin has the potential to inhibit lipid peroxidation and effectively to intercept and neutralize reactive oxygen and NO-based free radicals (Poon et al., 2004a). This agent is a potent inhibitor of tumor initiation in vivo and possesses antiproliferative activities against tumor cells in vitro (Butterfield et al., 2002b). Recent epidemiological studies (Ganguli et al., 2000) 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 with the United States. On the basis of these findings, compelling evidence has been provided 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 these mice (Lim et al., 2001). Furthermore, in a human neuroblastoma cell line it has recently been shown that curcumin inhibits NF-κB activation, effectively preventing neuronal cell death (Poon et al., 2004a). Remarkably, recent evidence has demonstrated that curcumin is a potent inducer of HO-1 in vascular endothelial cells (Balogun et al., 2003). 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 (Scapagnini et al., 2002d). The similarity of CAPE to curcumin is striking because CAPE is also a Michael reaction acceptor, endowed with antiinflammatory, antioxidant, and anticancer effects (Butterfield et al., 2002a). These agents all appear capable of transcriptionally activating a gene battery that includes antioxidant enzymes and HO (Dinkova-Kostova et al., 2001). Gene induction occurs through the antioxidant-responsive element (ARE) (Alam, 2002; Alam and Cook, 2003). Thus, increased expression of genes regulated by the ARE in cells of the CNS may provide protection against oxidative stress.

5 Major CNS Disorders Associated with ROS/RNS-Mediated Damage

The major CNS disorders associated with ROS/RNS-mediated damage are listed in *Table 6-2*.

Table 6-2
Neurodegenerative disorders associated with free-radical damage

1. Amyotrophic lateral sclerosis
2. Alzheimer's disease
3. Parkinson's disease
4. Multiple sclerosis
5. Friedreich's ataxia
6. Down's syndrome
7. Huntington's disease
8. Ischemia/reperfusion

5.1 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 a distinct possibility. 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 (Calabrese et al., 2004b). Familial ALS patients heterozygous for SOD mutations have less than 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 arise 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 (Brwon, 1994). These studies suggest that the enzyme is endowed with neurotoxic properties. What the neurotoxic function might be remains to be elucidated. With regard 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 constitute the primary pathogenic event leading to protein nitration, which slowly injures the motor neurons (Beckman et al., 1993). 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 (Heales et al., 1999). Immunocytochemistry studies have also revealed, in the neurofilament aggregates associated with ALS, a close association between SOD-1 and NOS activity (Chou et al., 1996). Since light neurofilaments are 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 (Beal et al., 1997). 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 (Fujita et al., 1996). 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 (Collard et al., 1995).

5.2 Alzheimer's Disease

AD affects over two million Americans and is the major cause of admission to nursing homes. AD, which rarely occurs before the age of 50 years, usually becomes clinically apparent as a subtly impaired cognitive function or as affectivity disturbance. With time there is a progressive memory loss and disorientation, 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 NFTs, 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 (Calabrese et al., 2001a). 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 posttranslationally modified proteins 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 (Christen, 2000). Oxidative stress increases the frequency of hydroxyl radical-induced autoxidation of unsaturated membrane lipids. Reactive aldehydes, resulting by metal ion-mediated fragmentation of 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 (Butterfield, 2004). 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 (Calabrese et al., 2000a). Second, increased concentrations of iron would result in increased protein modifications, which are catalyzed by metal ions and reducing sugars (Calabrese et al., 2000a). Third, microglia are activated and increased in number in AD and represent a major source of free radicals (El Khoury et al., 1998). 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 the cytoskeleton and activation of specific degradative enzymes (Markesbery, 1997; Drake et al., 2004). A decrease of complex IV activity has been reported in the cerebral cortex of individuals who died of AD (Kish et al., 1992). 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 (Butterfield, 2002). In addition, there is now evidence to suggest that NO metabolism is affected in AD. The glial-derived factor, S-100-β, which is overexpressed in this condition, causes induction of iNOS in astrocytes associated with NO-mediated neuronal cell death in a co-culture system (Hu et al., 1997). Furthermore, β-amyloid is reported to activate NOS in a substantia nigra/neuroblastoma hybrid cell line (Heales et al., 1999). Analysis of postmortem material has revealed in AD brain the presence of tyrosine, as result of the reaction of $ONOO^-$ and nitrotyrosine residues in protein, which was not detectable in age-matched control brains (Smith et al., 1997). In addition, using antibodies specifically directed against iNOS, the presence of this isoform in neurofibrillary tangle-bearing neurons was demonstrated (Sayre et al., 2000). 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 (Corregidor and De Pasamonte, 1996). 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 considered to be 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 (Butterfield et al., 2002a). 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 (Castegna et al., 2004; Drake et al., 2004). In this model, Aβ-associated free radicals initiate lipid peroxidation, protein oxidation, 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 (Butterfield et al., 1997). Consistent with this model, this free-radical scavenger was shown to block Aβ-initiated lipid peroxidation in cortical synaptosomes (Butterfield et al., 1999). 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 (Koppal et al., 1998). 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 $A\beta_{1-42}$ and $A\beta_{1-40}$ was shown to be critical to the oxidative stress properties of these peptides (Butterfield and Kanski, 2002). Human $A\beta_{1-42}$, expressed in vivo in transgenic *Caenorhabditis elegans* nematodes, led to protein oxidation in the living animal, and methionine was important in this process as well (Yatin et al., 1999).

A risk factor for AD is the presence of allele 4 of apolipoprotein E (apoE) (Roses, 1996). Synaptosomes from apoE-knockout mice, containing no gene for apoE, show increased susceptibility to oxidative stress induced by Aβ, (Lauderback et al., 2001), while synaptosomes from knockout mice containing human apoE4 with no mouse background show significantly increased Aβ-induced oxidative stress compared with synaptosomes from human apoE2 or apoE3 knockin mice (Lauderback et al., 2002). Thus, apoE may serve an antioxidant function, but apoE4 may be less able than apoE2 or apoE3 to do so (Lauderback et al., 2002). 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 (Veinbergs et al., 2000). The sum of these studies suggests a decreased risk for and diminished oxidative stress in AD 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. Hsps are proteins serving as molecular chaperones involved in the protection of cells from various forms of stress. Increasing interest has focused on identifying dietary compounds that can inhibit, retard, or reverse the multistage pathophysiological events underlying AD pathology. AD, in fact, involves a chronic inflammatory response associated with both brain injury and Aβ-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. Spice 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 (Butterfield et al., 2002a, c; Calabrese et al., 2004b).

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.

5.3 Parkinson's Disease

PD is a progressive neurodegenerative disorder that increases in frequency after the age of about 50 years. The major clinical disturbances in PD result from dopamine depletion in the striatum, because of nigral neuronal loss. Although, a number of hypothesis, including defective DNA repair mechanisms, specific genetic defects, viral disorder, lack of a neurotrophic hormones, 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 supports the role of free radicals in the pathogenesis of the disease (Hyun et al., 2003). Levels of lipid hydroperoxides are increased tenfold in the substantia nigra in PD (Hyun et al., 2002).

Decreased glutathione peroxidase and catalase activities associated to increased SOD activity leads to increased levels of hydrogen peroxide (Dexter et al., 1991). 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 (Mattson et al., 2002). Dexter and coworkers (1991) reported a 31%–35% increase in the total iron content in parkinsonian SN compared with control tissue, which was associated with decreased levels of the iron storage protein ferritin, contrasting with a significant decrease of the levels of iron-binding protein in the CSF. A shift of iron II/iron III ratio in the substantia nigra from almost 2:1 in the normal brain to 1:2 in the parkinsonian brain is also served (Li and Dryhurst, 1997). 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, have been demonstrated to be cytotoxic in vitro, presumably by formation of covalent bonds between their quinone forms and macromolecules of vital importance, primarily represented by thiol groups (Spencer et al., 2002). 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 considerable levels of the highly reactive hydroxyl radical, which on reacting with free thiol groups may contribute to the decreased levels of GSH and a corresponding increase in GSSG found in the SN (Calabrese et al., 2002b, 2004a). This is of special importance considering that nigral cells also contain neuromelanin, a pigmented substance related to lipofuscin and derived from dopamine. Neuromelanin has been demonstrated to have 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 (Spencer et al., 1994) 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 substantia nigra could result in a position of increased demand and decreased synthetic capability, and hence contribute to the high vulnerability of this region to peroxidative injury (Calabrese et al., 2001a, 2002b, 2004a). This is confirmed by the study of Perry and coworkers (1982), which showed that GSH levels in the SN were significantly lower than in other brain regions. Moreover, a 40% decrease in GSH in the SN of PD, associated with significant increase in oxidized glutathione, has been also reported (Sian et al., 1994). Recently, it has been demonstrated in PD patients that the proportion of dopaminergic neurons with immunoreactive NF-κB in their nuclei was more than 70-fold than that in control subjects (Hunot et al., 1997). A possible relationship between the nuclear localization of NF-κB 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 (France-Lanord et al., 1997). Data suggest that this oxidant-mediated apoptogenic transduction pathway may play a role in the mechanism of neuronal death in PD (Schapira et al., 1990; Mc Naught et al., 2001; Dawson and Dawson, 2002; Moore et al., 2003). Moreover, a potential role for excitotoxic processes in PD has been strengthened by the observation that there appears to be a mitochondrially encoded defect in complex I activity of the ETC (Schapira et al., 1990). An impairment of oxidative phosphorylation will enhance vulnerability to excitotoxicity (Xin et al., 2000). 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 nNOS, which can then lead to the generation of peroxynitrite (Bechtold and Brown, 2003). Consistent with such a mechanism, studies of MPTP neurotoxicity in both mice and primates have shown that inhibition of nNOS exerts neuroprotective effects, raising the prospect that excitatory amino acid antagonists for nNOS inhibitors might be useful in the treatment of PD (Dawson and Dawson, 1995, 2002).

5.4 Multiple Sclerosis

MS is a common often disabling disease of the 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 (Risch and Merikangas, 1996). It is generally accepted that vascular factors, metabolic alterations, virus infections of the CNS, 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 T_H cells against CNS protein components is likely to be crucial for this development. Once triggered, the immune system attacks and destroys myelin and the myelin-forming cells (Calabrese et al., 1998). Evidence exists which indicate that oligodendrocytes and their secreted products respond to the attack by immune cells through modulation of its metabolism and gene expression (Lindsey et al., 1997). 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 Hsps, thereby initiating the MS lesion (Calabrese et al., 2002a). In addition, cytokines, immunoglobulins, and complement complexes may elicit a survival response in the oligodendrocytes, involving the induction of endogenous Hsps and other protective molecules, which indicates that redox systems and therefore the oxidant/antioxidant balance in these cells are of great importance in MS (Calabrese et al., 2003b). A variety of studies support a role for oxidative stress in MS. These include studies on increased serum peroxide levels in MS relative to control (Toshniwal and Zarling, 1992; Calabrese et al., 1994). Patients with MS in acute exacerbation exhibit significantly higher levels of pentane and exane (products of lipid peroxidation) in expired breath compared with either MS patients in remission or control subjects (Toshniwal and Zarling, 1992). Moreover, recent clinical and animal studies suggest that NO and its reactive derivative peroxynitrite are implicated in the pathogenesis of MS (Bagasra et al., 1995). Patients dying with MS demonstrate increased astrocytic iNOS activity as well as increased levels of iNOS mRNA and nitrotyrosine residues (Bagasra et al., 1995; Cross et al., 1998). In EAE, both astrocytes and microglia express iNOS (Tran et al., 1997). 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 (Stamler and Hausladen, 1998; Heales et al., 1999), 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 (Calabrese et al., 2001a). A local autoimmune process involving activation of glia is likely to be crucial in the development of this damage (Tran et al., 1997).

Activated glia secrete RNS products of 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 $t_{1/2}$ of less than 1 s. One of the fastest reactions of $ONOO^-$ is with (3 to 5.8 $\times$ 10^4 M^{-1} s^{-1} at 37°C). Together with the high concentrations of CO_2 (~1.3 mM) and HCO_3^- (~25 mM) this reaction is the most probable pathway of $ONOO^-$ decomposition in vivo (Calabrese et al., 2003b).

RNS can cause nitrosative stress, which results in the destruction of myelin and (myelin-forming) oligodendrocyte cells (Calabrese et al., 2002a). 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 (Calabrese et al., 2002a). In addition, conduction in demyelinated and early remyelinated axons is particularly sensitive to block by NO (Heales et al., 1999). 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 (Heales et al., 1999). 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 (Gegg et al., 2003).

Recent clinical and animal studies also indicate that NO and $ONOO^-$ play a central role in the pathogenesis of MS (reviewed in Calabrese et al., 2001a). It has also been shown that in CSF and plasma nitrite + nitrate (stable end products of NO metabolism) levels are elevated in patients with MS (Giovannoni, 1998).

ROS and RNS have a major role in the mediation of cell damage, and free sulfhydryl groups are vital in cellular defence against endogenous or exogenous oxidants (Calabrese et al., 2000a, 2001b). The possible links between MS and oxidant/antioxidant balance in cell perturbation may be suggested by several factors, including increased incidence of MS in populations consuming high proportions of animal fat, (Calabrese et al., 1994), increased malonaldehyde levels in blood, and decreased glutathione peroxidase activity in MS erythrocytes, lymphocytes and granulocytes (Calabrese et al., 1998) and, in addition, an inappropriate expression of Hsps on oligodendrocytes (Calabrese et al., 2002a). This last event could represent a possible initiating factor at the 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 (Calabrese et al., 2002a). Nitrosative stress in isolated astrocytes in vitro causes modifications in the endogenous thiol pool associated with induction of Hsp32 or HO-1, which is prevented by antioxidants, suggesting a biochemical link between nitrosative stress, sulfhydryl function, and the heat-shock pathway (Scapagnini et al., 2002d; Calabrese et al., 2004b). 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 (Thompson, 1988), and we have recently demonstrated in MS patients decreased levels of protein sulfhydryl groups associated with an increase in RNS and peroxidative products (Calabrese et al., 2002a, 2003b). More recently, we have provided experimental evidence that increased levels of RNS are present in the CSF of MS patients, and this is associated with increased nitrosylation of sulfhydryl moieties. Our results are consistent with evidence indicating increased protein nitrosylation in MS patients (Cross et al., 1997) 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 factors in addition to demyelination. In favor of this possibility is the evidence that NO donors are capable of blocking conduction in rat demyelinated axons (Garthwaite et al., 2002). All this would suggest a broader potential role for NO in the symptomatic manifestations of MS. Whether or not NO is central to the pathogenesis of MS remains to be clarified, owing to its role of being a double-edged sword.. Consistently, a recent study (Sellebjerg et al., 2002) has demonstrated an association between high CSF levels of NO metabolites with severe disease activity in relapsing/remitting MS, and 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 activity, and development of cortical atrophy on MRI. (Yuceyar et al., 2001). 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 GSH, with increased levels of GSSG and nitrosothiols (Calabrese et al., 2002a). 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 and GSH/GSSG ratio. In addition, urinary nitrites, which were higher in MS patients than in controls, decreased significantly after treatment with acetylcarnitine. Several studies have shown the capability of carnitines to interfere with changes in oxidant/antioxidant balance and metabolism induced by oxidants (Hagen et al., 1998a, b). Although, so far, the exact mechanisms of action of acetylcarnitine are still unknown, current research points to its ability to enhance neuronal mitochondrial bioenergetics (Calabrese and Rizza, 1999), which in turn may influence cellular oxidant/antioxidant balance (Calabrese et al., 2002d). We have recently shown in astrocytes exposed to LPS and INFγ-induced nitrosative stress that acetylcarnitine protects against cytokine-mediated mitochondrial chain respiratory complex impairment and the associated increase in protein and lipid peroxidation. The increase in astroglial antioxidative potential observed after acetylcarnitine treatment involves a secondary line of antioxidant defenses, represented by stress-responsive genes, such as HO-1 and the mitochondrial Hsp60

and SOD (Calabrese et al., 2005). Furthermore, as a brain energy enhancer, acetylcarnitine could improve survival of damaged neurons (Scapagnini et al., 2002b), 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.

MS 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 MS, 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.

5.5 Friedreich's Ataxia

FA is an autosomal recessive neurodegenerative disorder involving both the central and peripheral nervous systems. Patients also show a systemic clinical picture presenting heart disease and diabetes mellitus or glucose intolerance. The disease is caused by mutations in the FA gene mapped on chromosome arm 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 (Durr et al., 1996). 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 (Harding, 1981). 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 that of normal subjects (Campuzano et al., 1996). 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 FA patients are attributed to low levels of ATP produced in these energy-intensive tissues (Durr et al., 1996). Point mutations are described in compound heterozygous subjects with one expanded allele. A two-step model of GAA normal alleles toward 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 counseling, and prenatal diagnosis. Many approaches have been undertaken to understand FA, 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 FA pathogenesis. Brains of FA 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 FA 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, causing oxidative damage, which may be considered a possible mechanism that compromises cell viability. Recent evidence suggests that frataxin might detoxify ROS via activation of glutathione peroxidase and elevation of thiols, and in addition, decreased expression of frataxin protein is associated with FA.

Recent studies have shown that frataxin acts as a chaperone for Fe(II) and a storage compartment for excess iron (Babcock et al., 1997). 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. In mouse models, deletion of the frataxin gene results in embryonic lethality (Radisky et al., 1999) 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 (Bradley et al., 2000).

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 (Radisky et al., 1999). Deletion of the yeast frataxin homolog *YFH1* results in a tenfold increase in iron within the mitochondria along with increased ROS production (Lodi et al., 2002). This leads to loss of mitochondrial function and the appearance of a petite phenotype in nearly all strains that have been examined (Radisky et al., 1999). Bradley and colleagues (2000) demonstrated an impaired oxidative phosphorylation system with severe and significant deficiencies of mitochondrial respiratory chain complexes I and II/III and aconitase activity in cardiac muscle from patients with FA; mtDNA levels were reduced in FA heart and skeletal muscle and increased iron deposition was present in FA heart, liver, and spleen in a pattern consistent with a mitochondrial location. In addition, there is the appearance of nDNA damage (Bradley et al., 2000). 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 deposition and enzyme deficiencies have been reported in postmortem heart and brain tissues (Foury and Talibi, 2000) of FA patients. The role of oxidative damage in the pathogenesis of FA 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 FA patients (Lodi et al., 2002).

Upregulation of protein manganese superoxide dismutase (MnSOD) fails to occur in FA fibroblasts exposed to iron. This finding, together with the absence of activation of the redox-sensitive factor NF-κB, suggests that NF-κB-independent pathway which may not require free-radical signaling is responsible for the reduced 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 the treatment of FA. Drugs that reduce oxidative stress have a limited effect on the progression of the disease pathology, probably because these cannot properly remove iron accumulation. The potential role of iron chelator analogues (e.g., 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH)) as agents to remove mitochondrial iron deposits have been recently under investigation (Jauslin et al., 2003). 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 FA patients, probably because of its hydrophilicity that prevents mitochondrial access. Indeed, standard chelation regimens will probably not work in FA, as these patients do not exhibit gross iron loading. Considering that there is no effective treatment for FA, it is essential that the therapeutic potential of iron chelators focusses on the mitochondrial iron pools as their primary target. Remarkably, in an in vitro model of regulated human frataxin overexpression, it was shown that downregulation of the expression of mitogen-activated protein kinase kinase 4 was 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 FA cells, revealing in FA patients a significantly higher activation of caspase-9 after serum withdrawal compared with controls. These findings suggest the presence, in FA patient cells, of a "hyperactive" stress-signaling pathway. The role of frataxin in FA pathogenesis could be explained, at least in part, by this hyperactivity. Pilot studies have shown the potential effect of antioxidant therapy using idebenone or coenzyme Q10 with vitamin E administration and provide a strong rationale for designing larger randomized clinical trials (Lodi et al., 2003). There is now strong evidence to suggest that mitochondrially localized antioxidant ameliorates cardiomyopathy in FA patients, as well other lipophilic antioxidants can protect FA cells from cell death, indicating novel treatment strategies for FA and presumably for other neurodegenerative diseases with mitochondrial impairment. Antioxidants targeted to mitochondria appear a promising approach to effectively slow disease progression.

5.6 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 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 located on the short arm of chromosome 4 that 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. 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 (Calabrese et al., 2004b). 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. Most of the ATP produced in the brain is used by energy-dependent pumps to restore transmembrane potential following synaptic transmission (Calabrese et al., 2004b). 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 (Jenkins et al., 1993). This increase well correlates with the duration of the disease, implying that normal energy metabolism is progressively impaired by the disease process. This might arise by the fact that when oxidative phosphorylation is no longer sufficient in supplying cellular energy demands, cells resort to reducing pyruvate by NADH in order 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 have evidenced defects in succinate dehydrogenase as well as pyruvate dehydrogenase activities in the striatum of HD patients, and also these defects have been found as a function of illness duration. Further evidence supporting a mitochondrial defect in HD has been provided by an NMR spectroscopy study demonstrating 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 with controls (Browne, 1997).

5.7 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 (Calabrese et al., 2000a). DS patients have an extra chromosome 21 (trisomy 21) and the recent assignment to chromosome 21 of the gene for Cu/Zn-SOD together with the observation of increased SOD activity in red blood cells in DS patients has directed the interest on the role played by free-radical species in the pathogenesis of the disease (Calabrese et al., 2000a). DS patients have a very high predisposition to develop 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 increase in SOD activity might be detrimental. In addition, increased SOD activity results in decreased steady-state levels of superoxide anion, which also plays a role in terminating the chain reactions of lipid peroxidation. All these evidence highlight the importance of oxidant/antioxidant balance as a critical determinant, and with this the conceivable possibility that the use of exogenous antioxidants can slow the progression of the disease (Halliwell, 2002).

5.8 Ischemia/Reperfusion

Ischemic brain damage is accompanied by an energy deficiency state and selective neuronal loss (Heales et al., 1999). 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, 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 (Heales et al., 1999). 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 (Powell and Jackson, 2003). Reperfusion was associated with restoration of activity of these mitochondrial components, followed after 2 h by a dramatic loss of complex IV activity (Brooks et al., 2002). The exact mechanism for this loss of complex IV activity is not known, but could involve the oxidative and/or nitrosative stress-mediated reactions (Brooks et al., 2002). In fact, ischemia is accompanied by the formation of gliotic scar, principally comprised of reactive astrocytes, which in large amount express iNOS (Heales et al., 1999). 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 (Bolanos et al., 1998); in addition, administration of an NOS inhibitor to the mothers prevented impairment of brain energy metabolism in the hypoxic pups (Bolanos et al., 1998). Notably, ischemic preconditioning, which has been demonstrated to increase Hsp expression, preserves brain mitochondrial functions during middle cerebral artery occlusion (Zhang et al., 2003).

6 Genetics of Human Longevity: Role of Vitagenes in Prolongation of Healthy Life Span

The first half of the twentieth century has seen a rapid increase in the life expectancy of individuals in industrialized nations because of improved sanitation, public health, housing, nutrition, medical technology, and 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 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 captive or laboratory conditions, and average longevity is usually greatly extended (Calabrese et al., 2001a). 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 (Calabrese et al., 2001a).

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 (1956) and Szilard (1977), respectively. A synthesis of evolutionary and mechanistic theories occurred in 1977 within the framework of the soma theory of aging postulated by Kirkwood (1977). 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, defective germ cells, if any, 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 but absent in the former.. 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, since it limits 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 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 words of an anonymous poet, "we are born as copies, but we die as originals."

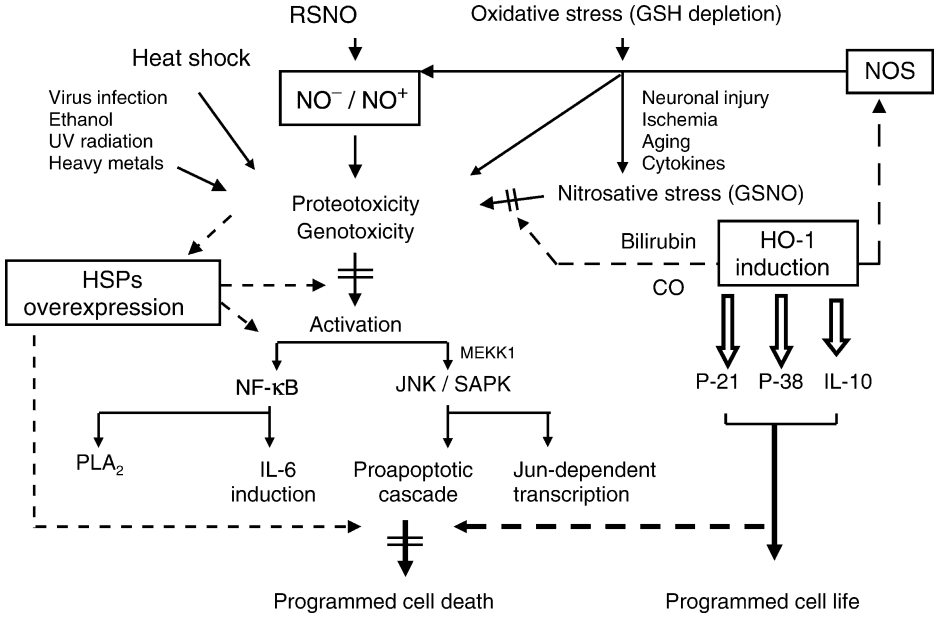
It is becoming increasingly clear that genetic factors are prominently involved in aging, the major lines of empirical evidence being: (1) the life span which in human populations shows significant heritability; (2) different species have different intrinsic life spans due to genomic differences; (3) 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; and (4) clear evidence of genetic effects on life span have been demonstrated in invertebrate model systems, such as *D. melanogaster* and *C. elegans*. In this organism, five different genomic regions appear to be associated with longevity, as assessed by quantitative genetic analysis (Rothschild and Jazwinski, 1988). Also, in *S. cerevisiae* 13 longevity genes have been identified and cloned. Of these 13 genes, 11 have human homologues (Rothschild and Jazwinski, 1988). At least, three categories of genes are predicted to affect aging and longevity. They are: (1) genes that regulate levels of somatic maintenance and repair; (2) pleiotropic genes, whose expression involves trade-offs between early-life fitness benefits and late-life fitness disadvantages, which do not encompass somatic maintenance; and (3) late-acting deleterious mutations that have escaped elimination as consequence of the decline in the force of natural selection at old ages (Calabrese et al., 2001a). 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 6-2*). 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. Since aging is characterized by a progressive failure of maintenance and repair, it is reasoned that genes involved in homeodynamic repair pathways, such as the HO-1 or Hsp70 genes, are the most likely candidate vitagenes.

7 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 (Butterfield et al., 2002a, b; Calabrese et al., 2004b) 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 HO/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 acquiring major resistance to the effects of stressful and pathogenic conditions (*Figure 6-1*). 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 (Otterbein et al., 2003a).

Figure 6-1

Redox regulation of gene expression involving the vitagene system. Proposed role for the vitagene member heat-shock proteins (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 (CO) 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. Abbreviations: PLA_2, phospholipase A_2; IL-6, interleukin-6; AP-1, activator protein-1; SAPK, stress-activated protein kinase; JNK, c-jun N-terminal kinase; NF-κB, nuclear factor kappa-B; GSNO: *S*-nitrosoglutathione; HO-1, heme oxygenase-1



In many inflammatory situations, the ability of IL-10 to suppress TNF-α 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 (Lee and Chau, 2002; Soares et al., 2004). In concert with this conceivable possibility, the protective effect of IL-10 in a lethal endotoxic shock mice model is strongly dependent on the expression of HO-1 and the generation of CO (Lee and Chau, 2002; Soares et al., 2004). Moreover, rapamycin appears not to exert its antiproliferative effects on smooth muscle cells unless HO-1 is present (Lee and Chau, 2002; Akamatsu et al., 2004), and it has been proven that, in order for NO to protect mice livers from hepatitis induced by TNF-α and galactosamine, upregulation of HO-1 seems to be essential (Otterbein et al., 2003b). Also, alcohol has antiinflammatory effects as TNF-α is suppressed and IL-10 is increased (Otterbein et al., 2003b; Yamashita et al., 2004). However, protection is lost when HO-1 is blocked (Foresti et al., 1997). In addition, the antiinflammatory effect of 15-deoxy-Δ-12,14-prostaglandin J_2 has been shown to require the activity of HO-1 (Lee and Chau, 2002; Otterbein et al., 2003b). Notably, during heat shock, which leads to upregulation of several Hsps endowed with cytoprotective actions, entire cytoprotection is lost if HO-1 is blocked with SnPPIX. Last, relevant to brain physiopathology, dietary and medicinal phytochemicals that can inhibit, retard, or reverse the multistage pathogenic events associated with degenerative damage,

particularly polyphenols such as curcumin, caffeic acid, and ferulic acid, all capable of exerting powerful antiinflammatory actions, have been shown to function by upregulating HO-1 (Scapagnini et al., 2002d, 2004; Poon et al., 2004a). The fact that in all these situations specific molecules or biological phenomena appear to lose most, if not all, of their effects when HO-1 is absent represents a 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 a critical vitagene, which links redox-dependent pathways of stress tolerance to a versatile biological program of cell life.

8 Conclusion

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 (Calabrese et al., 2005; Sultana et al., 2005). Efficient functioning of maintenance and repair processes seems to be crucial for both survival and physical quality of life. This is accomplished by a complex network of the so-called longevity assurance processes, which are composed of several genes termed vitagenes. Consistently, by maintaining or recovering the activity of vitagenes it 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, 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-twent-first century in the absence of effective interventions (Butterfield et al., 2002a). This will pose an immense economic and personal burden on the people of this country. Similar considerations apply worldwide, except in sub-Saharan Africa where HIV infection rates seem to be leading to decreased incidence of AD (Butterfield et al., 2002b). There is now strong evidence to suggest 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 chapter, 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 (Otterbein, 2002). 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 (Otterbein et al., 2003b). Carboxyhemoglobin levels generated in such a model are not too high from those of heavy smokers. If this beneficial effect is confirmed also in humans, limited exposure of patients to CO might be considered as therapy for various syndromes, particularly to prevent re-stenosis after angioplasty or 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 (Otterbein et al., 2003b; Akamatsu et al., 2004). Interestingly, the recently discovered CORMS appears to afford similar protective action, thereby providing an alternative therapeutic approach for those pathophysiological conditions where CO administration may prove to be beneficial (Motterlini et al., 2002b, 2003). 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 (Yamashita et al., 2004). 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 recent envisioned role of Hsp70 as a vehicle for intracytoplasmic and intranuclear delivery of fusion proteins or DNA to modulate gene expression (Wheeler et al., 2003), along with the evidence that binding of HO protein to HO-1 DNA modifies HO expression via nonenzymatic signaling events (Weng et al., 2003) associated to CO and P-38-dependent induction of Hsp70, opens 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 (*Figure 6-1*), depending on the relative extent of activation.

Presented here is strong evidence that a cross talk between stress response genes is critical for cell stress tolerance, highlighting a 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 also 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 (Poon et al., 2004a, b). Hence, by maintaining or recovering the activity of vitagenes (Calabrese et al., 2001a, 2004b, 2005) it can be possible to delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy life span.

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