ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 5, 2004

Forum Review

Redox Regulation in Neurodegeneration and Longevity: Role of the Heme Oxygenase and HSP70 Systems in Brain Stress Tolerance

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ABSTRACT

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," among these, the heat shock system, a highly conserved mechanism responsible for the preservation and repair of cellular macromolecules, such as proteins, RNAs, 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, cancer, as well as metabolic and neurodegenerative disorders. Recently, the involvement of the heme oxygenase (HO) pathway in antidegenerative mechanisms has received considerable attention, as it has been demonstrated that the expression of HO is closely related to that of amyloid precursor protein. HO induction occurs together with the induction of other heat shock proteins during various physiopathological conditions. The vasoactive molecule carbon monoxide and the potent antioxidant bilirubin, products of HO-catalyzed reaction, represent a protective system potentially active against brain oxidative injury. Given the broad cytoprotective properties of the heat shock response, molecules inducing this defense mechanism appear to be possible candidates for novel cytoprotective strategies. Particularly, manipulation of endogenous cellular defense mechanisms, via the heat shock response, through nutritional antioxidants or pharmacological compounds, may represent an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration. Consistently, by maintaining or recovering the activity of vitagenes, it is feasible to delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy life span. *Antioxid. Redox Signal.* 6, 895–913.

INTRODUCTION

PERTURBATION OF THE CELLULAR OXIDANT/ANTIOXIDANT BALANCE has been suggested to be involved in the neuropathogenesis of several disease states, including stroke, Parkinson's disease (PD), Alzheimer's disease (AD), as well as "normal" physiological aging (60). Reactive oxygen species (ROS) are constantly produced in the course of aerobic metabolism, and in normal conditions there is a steady-state balance between prooxidants and antioxidants. Most of the reactive species produced by healthy cells result from "leakage" or short circuiting of electrons at several specific locations within the cell, which then become sources of free radical production. These include the mitochondrial respiratory chain, the enzyme xanthine dehydrogenase, and, to a lesser extent, arachidonic acid metabolism and autooxidation of catecholamines or hemoproteins. However, when the rate of free radical generation exceeds the capacity of antioxidant

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defenses, oxidative stress ensues, causing extensive damage to DNA, proteins, and lipids (14, 19, 23).

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* (25, 29). Among these, chaperones are highly conserved proteins responsible for the preservation and repair of the correct conformation of cellular macromolecules, such as proteins, RNAs, and DNA. Chaperone-buffered silent mutations may be activated during the aging process and may 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 (28, 136). 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-1, the inducible isoform of HO, is closely related to that of amyloid precursor protein (APP) (42, 114). HO-1 induction, which occurs together with the induction of other heat shock proteins (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 modulated by redox-sensitive transcription factors that recognize specific binding sites within the promoter and distal enhancer regions of the HO-1 gene. These include Fos/Jun [activator protein-1 (AP-1)], nuclear factor-κB (NFκB), and the more recently identified nuclear factor–erythroid 2 (NF-E2)-related factor 2 (Nrf2) proteins (1, 2, 116). HO-1 gene is redox-regulated depending on the presence in the promoter region of two upstream enhancers, E1 and E2 (143). 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) (140). There is now evidence to suggest that heterodimers of 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 (2). In addition, HO-1 is rapidly up-regulated by oxidative and nitrosative stresses, as well as by glutathione depletion (105, 152). 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 (5, 16, 17, 104, 129).

AD is a progressive disorder with cognitive and memory decline, speech loss, personality changes, and synapse loss. Many approaches have been undertaken to understand AD, 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 AD pathogenesis (13–15, 17–19). Recently, increasing interest has been 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 β-amyloid-associated pathology. 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. Such an outcome would provide optimism that the signs and symptoms of this devastating brain disorder of aging may be largely delayed and/or modulated.

NITRIC OXIDE (NO) AND CO: GASEOUS MOLECULES THAT PROMOTE ADAPTIVE RESPONSES

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 nitric oxide synthase 2 (NOS2) and/or NO are functionally interrelated in mediating their protective effects. In some situations, CO can activate the expression of NOS2 and, in others, inhibits expression of NOS2 and consequently NO (11, 23, 50). NO up-regulates HO-1 with production of CO (50, 51). We have recently found evidence for a functional relationship between CO and NO. In endotoxic shock, the salutary action of CO in rat brain appears to depend sequentially on the activation of NFκB, which triggers transcription of NOS2 with production of NO, and subsequently on the up-regulation of HO-1. In the absence of any of these steps, the beneficial effect of CO is lost (127). This has been also demonstrated in mice in the treatment of hepatitis induced by tumor necrosis factor-α (TNFα) and D-galactosamine (111). To what extent CO and NO act interdependently in other physiopathological conditions that are responsive to CO and/or NO is unknown.

NOS 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 NOΠ. NOS, localized in the CNS and in the periphery (16), is present in three well characterized isoforms: (a) neuronal NOS (nNOS, type I), (b) endothelial NOS (eNOS; type III), and (c) inducible NOS (iNOS, type II). Activation of different isoforms of NOS requires various factors and 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 functional active dimer exhibits NOS activity, which depends also on cofactors such as tetrahydrobiopterin, FAD, FMN, and NADPH (12, 68). nNOS has a predominant cytosolic localization, whereas the eNOS is bound to the plasma membrane by N-terminal myristylation (41). In contrast to nNOS and eNOS, iNOS can bind to calmodulin even at very low concentration of intracellular calcium; thus, iNOS can exert its activity in a calcium-independent manner. iNOS, usually present only in the cytosol, also requires NADPH, FAD, FMN, and tetrahydrobiopterin, for full activity. eNOS expressed in cerebral endothelial cells critically regulates cerebral blood flow. However, a small population of neurons in the pyramidal cells of CA1, CA2, and CA3 subfields of the hippocampus and granule cells of the dentate gyrus express eNOS. nNOS, which is expressed in neurons, is

critically involved in synaptic plasticity, neuronal signaling, and neurotoxicity. Activation of nNOS forms part of the cascade pathway triggered by glutamate-receptor activation that leads to intracellular cyclic GMP (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 (41). Activation of iNOS requires gene transcription, and the induction can be influenced by endotoxin and cytokines [interleukin (IL)-1, IL-2, lipopolysaccharide (LPS), interferon-γ (IFN-γ), TNF]. This activation can be blocked by antiinflammatory drugs (dexamethasone), inhibitory cytokines (IL-4, IL-10), prostaglandins (PGA_2), tissue growth factors, or inhibitors of protein synthesis, *e.g.,* cycloheximide (23).

NO 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 (139). 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 reacts chemically with a target substrate. NO when produced in small quantities can regulate cerebral blood flow and local brain metabolism (25), 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 (41).

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 the following: (a) 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 (134); (b) interaction of NOΠ (through its congener NO^+) with the thiol group on the *N*-methyl-D-aspartate (NMDA) receptor with consequent down-regulation and inhibition of calcium influx (138); (c) inactivation of caspases (124); (d) activation of a cGMP-dependent survival pathway, as demonstrated in PC12 cells (105); (e) inducing expression of cytoprotective proteins, such as HSPs (22, 31, 51, 103); and (f) inhibition of NFκB activation or glyceraldehyde-3-phosphate dehydrogenase, whose activity appears to be required in one paradigm of neuronal apoptosis (114). 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 (122). In addition, it has been proposed that NO might inhibit T-cell activation and cell trafficking across the blood–brain barrier, hence limiting the setting of the autoimmune cascade associated with degenerative damage (41).

The difficulty in delineating a mechanistic involvement of NO as a 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 (105). 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 (139). In contrast to NO, which contains one unpaired electron in the outer orbital, the 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, NOS, and *S*-nitrosothiols (RSNO). A fundamental aspect of NO biochemistry is the attachment of NO groups to sulfhydryl centers to form *S*-nitrosyl derivatives or RSNO (138). This chemical process, known as *S*-nitrosation, has been suggested to represent a refined endogenous tool to stabilize and preserve NO biological activity (105). It has been speculated that low-molecular-weight RSNO, such as *S*-nitrosoglutathione or nitrosocysteine, may also represent a mechanism for storage *in vivo* of NO (90, 91, 105). In this regard, glutathione becomes an important determinant of the reactivity and fate of NO because this cysteine-containing tripeptide is very abundant in most tissue and biological fluids. In addition, *S*-nitrosation is also an important process in modulating the activity and function of several enzymes and proteins. However, deleterious and oxidative modification of protein structure and function may occur when reactive nitrogen species (RNS) reach a critical threshold, and hence nitrosative stress may ensue (63). At the cellular level, nitrosative stress has been linked to inhibition of cell growth and apoptosis, and implicated in NO pathogenesis (91). 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 (26, 91, 105).

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 descibed in bacteria where regulatory proteins such as OxyR was discovered as an activator of antioxidant and stress responsive genes. The OxyR is a homotetramer that is activated by hydrogen peroxide (H_2O_2) and RSNOs. 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 Cys199 being a likely candidate site of posttranslational modification (105). The expression of these protective genes renders the bacteria more resistant to oxidant damage (36). As the cytoprotective mechanism triggered by SoxR in *E. coli* includes the expression of critical antioxidant defensive proteins, such as superoxide dismutase (59), the emerging concept is that an analogous system might operate in mammalian cells. In eukaryotes, typical examples are genes such as the HO gene, thioredoxin, and detoxificant enzymes (manganese superoxide dismutase, glutathione *S*-transferase, NADPH:quinone reductase), cytokine, immunoreceptors, and growth factors. That the antioxidant protein HO can "sense" NO and, thus, protecting against ROS and RNS insults, is supported by the following findings: (a) NO and NO-related species induce HO-1 expression and increase HO activity in human glioblastoma cells, hepatocytes, and aortic vascular cells; (b) cells pretreated with various NO-releasing molecules acquire increased resistance to H_2O_2-mediated cytotoxicity at the time HO is maximally activated; and (c) bilirubin, one of the end products of heme degradation by HO, protects against the cytotoxic effects caused by strong oxidants H_2O_2 and peroxynitrite ($ONOO^-$) (37, 105).

The concept 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 (127). In rat glial cells, treatment with LPS and IFN-γ results in a rapid increase in both iNOS expression and nitrite levels followed by enhancement of HO-1 protein (22). 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 the *in vivo* rat model of septic shock (127). 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 (103, 105).

Taken together, these findings point to the central role of the NO as a signaling molecule that, 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 (17, 105). 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), NFκB, and the more recently identified Nrf2 proteins (2, 5, 116). Importantly, both AP-1 and NFκB contain cysteine residues whose interaction with oxidant or nitrosant species might be crucial for determining the binding activity to DNA (105). 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 (105, 109).

NO as signaling molecule for induction of neuroprotective HSPs

It is well known that living cells are continually challenged by conditions that cause acute or chronic stress. To adapt to environmental changes and survive different types of injuries, eukaryotic cells have evolved networks of different responses that detect and control diverse forms of stress (25). 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 and unfavorable conditions (116). In mammalian cells, HSP synthesis is induced not only after hyperthermia, but also following alterations in the intracellular redox environment, exposure to heavy metals, amino acid analogues, or cytotoxic drugs (24, 29). Although 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, aging, and neurodegenerative diseases (16, 29). This has opened new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear as possible candidates for novel cytoprotective strategies (17). In mammalian cells, the induction of the heat shock response requires the activation and translocation to the nucleus of one or more heat shock transcription factors that control the expression of a specific set of genes encoding cytoprotective HSPs. We have recently demonstrated in astroglial cell cultures that cytokine-induced nitrosative stress is associated with an increased synthesis of HSP70 stress proteins. Increase in HSP70 protein expression was also found after treatment of cells with the NO-generating compound sodium nitroprusside, thus suggesting a role for NO in inducing HSP70 proteins (22). *In vivo* experiments performed in our laboratory have also demonstrated that the redox glutathione status is a critical factor for induction of cytoprotective HSP70 (24, 31).

Mitochondrial damage, RNS, and neurodegenerative disorders

Increasing evidence sustains the hypothesis that mitochondrial energy metabolism underlie the pathogenesis of neurodegenerative diseases. Decreased complex I activity is reported in the substantia nigra of postmortem samples obtained from patients with PD (6). Similarly, impaired complex IV activity has been demonstrated in AD (64). Increased free radical-induced oxidative stress has been associated with the development of such disorders, and a large body of evidence suggests that NOΠ plays a central role (53). Cytokines

(IFN-γ), which are present in normal brain, are elevated in numerous pathological states, including PD (53, 94), AD (13–15, 17, 19, 101), multiple sclerosis (4, 21, 30), ischemia, encephalitis, and central viral infections (25). Accordingly, as cytokines promote the induction of NOS in brain, a possible role for a glial-derived $NO^{\cdot}$ in the pathogenesis of these diseases has been suggested (53, 64, 149). Excessive formation of NOΠ from glial origin has been evidenced in a study in which NADPH diphorase (a cytochemical marker of NOS activity) positive glial cells have been identified in the substantia nigra of postmortem brains obtained from individuals with PD (67). Loss of nigral GSH is considered an early and crucial event in the pathogenesis of PD (6), and as a consequence decreased $ONOO^-$ 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 nNOS prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism in experimental animals (40, 101).

HO SYSTEM: A PUTATIVE VITAGENE TARGET FOR NEUROPROTECTION

HSP32 or HO is the rate-limiting enzyme in the production of bilirubin. 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 a cytosolic enzyme, biliverdin reductase. Biliverdin complexes with iron until its final release (29, 34, 35).

HO isoforms

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 (34, 35), HO-2 or constitutive isoform (46, 47, 96), and the recently discovered HO-3, cloned only in rat to date (39, 128). They are all products of different genes; however, unlike HO-3, which is a poor heme catalyst, both HO-1 and HO-2 catalyze the same reaction (*i.e.*, degradation of heme), but differ in many respects and are regulated under separate mechanisms. The most relevant similarity between HO-1 and HO-2 consists in a common 24-amino acid domain (differing in just one residue) called the "HO signature" that renders both proteins extremely active in their ability to catabolize heme (89). They have different localization, similar substrate and cofactor requirements, and different molecular weights. They also display different antigenicity, electrophoretic mobility, inducibility, and susceptibility to degradation. The proteins for HO-1 and HO-2 are immunologically distinct and, in humans, the two genes are located on different chromosomes, *i.e.,* 22q12 for HO-1 and 16q13.3 for HO-2 (96).

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 >70% of HO activity is accounted for by HO-2, whereas in the testes the pattern of HO isoenzyme expression differs according to the cell type, although HO-1 expression predominates after heat shock. This also occurs in brain tissue, where HO isoforms appear to be distributed in a cell-specific manner and HO-1 distribution is widely apparent after heat shock or oxidative stress. Although previous reports from our and other groups have not found detectable levels of HO-1 protein in normal brain (26, 47), we have recently demonstrated that HO-1 mRNA expression is physiologically detectable in the brain and shows a characteristic regional distribution, with high level of expression in the hippocampus and the cerebellum (39, 128). This evidence may suggest the possible existence of a cellular reserve of HO-1 transcript quickly available for protein synthesis and a posttranscriptional regulation of its expression.

HO isoenzymes also 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 endothelium exhibits the same isoform to colocalize with eNOS. 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 (38).

HO-3, the third isoform of HO, shares a high homology with HO-2, at both 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 (66). Although the biological properties of this isoenzyme still remain to be elucidated, the presence of two HRM motifs in its amino acidic sequence might suggest a role in cellular heme regulation (89). Studying the HO-3 mRNA sequence (GenBank accession no. AF058787), we have observed that its 5′ portion corresponds to the sequence of an L1 retrotransposon, a member of a family of retrotransposons recently involved in evolutionary mechanisms (75). Based on the close similarity to a paralogous gene (HO-2) and the preliminary data from our group demonstrating no introns in the HO-3 gene (127), it is possible that this last could have originated from the retrotransposition of the HO-2 gene. In addition, this genetic mutation in rat may represent a species-specific event because no other sequence in the available databases match that of the rat HO-3.

Induction of HO-1 gene could be used clinically. However, the GT length polymorphism in the promoter of the gene encoding HO-1 that regulates the magnitude of the HO-1 response to a given stress signal can render this approach difficult for those individuals with the long GT repeats that are associated with low HO-1 responsiveness. This polymorphism appears to be of functional significance in that short

repeats, which are associated with high responsiveness, seem to be also associated with lesser likelihood of restenosis after angioplasty (111).

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 (89). As in the classic view of metabolic control, expression of HO-1 is induced by the substrate heme (72). 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 (78). Thus, HO-1 is an essential antioxidant defense enzyme that converts toxic heme into antioxidants and is fundamental to cope with various aspect of cellular stress and to regulate iron metabolism (116). In clinical conditions, HO-1 expression has been associated with increased resistance to tissue injury, thus leading to a gene therapy approach using HO-1 (110, 112).

The HO-2 gene consists of five exons and four introns. HO-2 has a molecular mass of 34 kDa and exhibits 40% homology in amino acid sequence with HO-1. It is generally considered a constitutive isoenzyme; however, *in situ* hybridization studies have shown increases in HO-2 mRNA synthesis, associated with increased HO-2 protein and enzyme activity, in neonatal rat brain after treatment with corticosterone (119). The organization of the HO-2 gene needs to be fully elucidated, although a consensus sequence of the glucocorticoid response element has been demonstrated in the promoter region of the HO-2 gene (86). In addition, endothelial cells treated with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) and HO inhibitor zinc mesoporphyrin exhibited a significant up-regulation of HO-2 mRNA.

The HO-1 gene is induced by a variety of factors, including metalloporphyrins and hemin, as well as ultraviolet A irradiation, H_2O_2, prooxidant states, or inflammation (103, 151). This characteristic inducibility of the 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 ~28 bp upstream from the transcriptional site of initiation. In addition, different transcriptional enhancer elements, such as heat shock element and metal regulatory element, reside in the flanking 5′ region. Also, inducer-responsive sequences have been identified in the proximal enhancer located upstream of the promoter and, more distally, in two enhancers located 4 kb and 10 kb upstream of the initiation site (65). 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 (143). Both enhancer regions contain multiple StRE (also called ARE) that also conform to the sequence of the MARE (140) with a consensus sequence (GCnnnGTA) similar to that of other antioxidant enzymes (5, 128). There is now evidence to suggest that heterodimers of 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 (55, 140). A possible model, centered on Nrf2 activity, suggests that the *ho-1* locus is situated in a chromatin environment that is permissive for activation. As 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 (143), 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 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 the *ho-1* gene is regulated through antagonism between transcription activators and the repressor Bach1. Whereas under normal physiological conditions expression of *ho-1* is repressed by the Bach1/Maf complex, increased levels of heme displace Bach1 from the enhancers and allow activators, such as heterodimer of Maf with NF-E2-related activators (Nrf2), to the transcriptional promotion of the *ho-1* gene (143). To our knowledge, the Bach1–*ho-1* system is the first example in higher eukaryotes that involves a direct regulation of a transcription factor for an enzyme gene by its substrate. Thus, regulation of *ho-1* involves a direct sensing of heme levels by Bach1 (by analogy to *lac* repressor sensitivity to lactose), generating a simple feedback loop whereby the substrate effects repressor–activator antagonism.

The promoter region also contains two metal responsive elements, similar to those found in metallothionein-1 gene, which respond to heavy metals (cadmium and zinc) only after recruitment of another fragment located upstream, between −3.5 and 12 kbp (CdRE). In addition, a 163-bp fragment containing two binding sites for HSF-1, which mediates the HO-1 transcription, are located 9.5 kb upstream of the initiation site (5). The distal enhancer regions are important in regulating HO-1 in inflammation, because they have been shown to be responsive to endotoxin. In the promoter region also resides a 56-bp fragment that responds to the STAT-3 (signal transducer and transcription activator) acute-phase response factor, involved in the down-regulation of the HO-1 gene induced by glucocorticoid (86, 119).

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 (17). A compromised GSH system in the brain has been connected with the oxidative stress occurring in neurological diseases (18). Recent data demonstrate that, besides intracellular functions, GSH has also important extracellular functions in brain. In this respect, astrocytes appear to play a key role in the GSH metabolism of the brain, because astroglial GSH export is essential for providing GSH precursors to neurons (45). 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 the export of both GSH and GSSG from astrocytes (45). 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 m*M* depending on the cell type (16). 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 (22, 24). There is now evidence to suggest that a direct link between a decrease in glutathione levels by oxidant stress and rapid up-regulation of HO-1 mRNA and protein exists in a variety of cells, including rat brain, human fibroblasts, endothelial cells, and rat cardiomyocytes (50, 103). This finding is supported by the fact that *N*-acetylcysteine (a precursor of glutathione) abolishes oxidative stress-mediated induction of the HO-1 gene (10, 121). 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 (22). 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 (50, 52). 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 (103). 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 (52, 103). All this evidence corroborates the notion that generation of ROS and RNS is an important signal transduction mechanism linking HO-1 activation to cell stress tolerance (90).

HO 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 (35, 82). Induction of HO occurs together with the induction of other HSPs in the brain during various experimental conditions, including ischemia (42). Injection of blood or hemoglobin results in increased expression of the gene encoding HO-1, which has been shown to occur mainly in microglia through brain (25). 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 positive microglia/macrophages at the hemorrhagic lesion was detected as early as 6 h post trauma and was still pronounced after 6 months (9).

There is now evidence that oxidative stress contributes to secondary injury after spinal cord trauma. Induction of HO-1 in the hemisected spinal cord, a model that results in reproducible degeneration in the ipsilateral white matter, was found in microglia and macrophages from 24 h to at least 42 days after injury. Within the first week after injury, HO-1 was induced in both the gray and the white matter. Thereafter, HO-1 expression was limited to degenerating fiber tracts. Interestingly, HSP70 was consistently colocalized with HO-1 in the microglia and macrophages, indicating that long-term induction of HO-1 and HSP70 in microglia and macrophages occurs long after traumatic injury and is correlated with Wallerian degeneration and remodeling of surviving tissue (93).

As the expression of HSPs is closely related to that of APP, HSPs have been studied in brain of patients with AD. Significant increases in the levels of HO-1 have been observed in AD brains in association with neurofibrillary tangles (145, 147), and also HO-1 mRNA was found increased in AD neocortex and cerebral vessels (118). The HO-1 increase was not only in association with neurofibrillary tangles, but also colocalized with senile plaques and glial fibrillary acidic protein-positive astrocytes in AD brains (133). 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 (131).

Up-regulation of HO-1 in the substantia nigra of PD subjects has been demonstrated. In these patients, nigral neurons containing cytoplasmic Lewy bodies exhibited in their proximity maximal HO-1 immunoreactivity (132). New evidence showed a specific up-regulation of HO-1 in the nigral dopaminergic neurons by oxidative stress (160).

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 (3, 154). 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 (4, 20, 21, 27). The expression of HO-1 is increased in the CNS of mice and rats with experimental allergic encephalomyelitis (EAE), an animal model of MS (30, 32). To investigate the role of HO-1 in EAE, tin protoporphyrin IX (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 with 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 (33). On the contrary, in another study, high expression of HO-1 in lesions of EAE was enhanced by hemin treatment, a procedure associated with attenuation of clinical signs of pathology, whereas tin mesoporphyrin, an inhibitor of HO-1, markedly exacerbated EAE (87). 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 (27, 30, 116). All these findings can open up new therapeutic perspectives, as molecules activating these defense mechanisms appear to be possible candidates for novel neuroprotective strategies (17, 29).

CO: a signaling molecule endowed with antiinflammatory properties

The first detection of a combustible gas in the blood occurred in 1894 by Grehant (59). This gas was supposed by de Saint Martin and Nicloux to be CO. However, it was not until 1949 that Sjorstrand discovered that endogenously produced CO arose from the degradation of hemoglobin released from senescing erythrocytes (136). Greater than 75% of CO produced in humans arises from erythrocyte turnover generated as a by-product of heme metabolism. In 1969, the source of endogenous CO was discovered, as Tenhunen and collaborators described and characterized 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 (148). Since then, supported by a large body of experimental evidence, CO is proving to be an extraordinary signaling molecule generated by the cell that is vital in the regulation of cellular homeostasis. In the brain, CO is emerging as a chemical messenger molecule that can influence physiological and pathological processes in the CNS and PNS. 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 exocitosis.

It seems likely that CO is involved in the mechanism of cell injury (150). This is evidenced by the fact that CO binds to heme in guanylyl cyclase to activate cGMP (114). 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 (88). Based on endogenous distribution of HO in the CNS, it has been suggested that CO can influence neurotransmission like NO (155). CO appears to be involved as a retrograde messenger in long-term potentiation and also is involved in mediating glutamate action at metabotropic receptors (57). This is evident from the fact that metabotropic receptor activation in brain regulates the conductance of specific ion channels via a cGMP-dependent mechanism that is blocked by HO inhibitors (54). Experimental evidence suggests that CO plays a role similar to that of NO in the signal transduction mechanism for the regulation of cell function and cell-to-cell communication (88). 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 C-terminal half of NOS (25). 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 the glutamate effect at NMDA receptors, whereas CO is primarily responsible for glutamate action at metabotropic receptors. Taken together, it appears that CO and NO play an important role in the regulation of CNS function; thus, impairment of CO and NO metabolism results in abnormal brain function (23). Much evidence suggests a possible role of CO in regulating nitrergic transmission. Endogenous CO has been suggested to control constitutive NOS activity. Moreover, CO may interfere with NO binding to guanylyl cyclase, and this is in addition to the important role of HO in regulating NO generation, owing to its function in the control of heme intracellular levels, as part of the normal protein turnover (50). This hypothesis is sustained by recent findings showing that HO inhibition increases NO production in mouse macrophages exposed to endotoxin (150).

CO may also act as signaling effector molecule, by interacting with targets different from guanylyl cyclase. Notably, it has been recently demonstrated that Ca^{2+}-activated K^+ channels are activated by CO in a cGMP-independent manner (157) and also that CO-induced vascular relaxation results from the inhibition of the synthesis of the vasoconstrictor endothelin-1 (38). Little, however, is known about how CO is sensed on a biological ground. Interestingly, the photosynthetic bacterium *Rhodospirillum rubrum* has the ability to respond to CO through the heme protein CooA, which, upon exposure to CO, acquires DNA-binding transcriptional activity for the CO dehydrogenase gene, thereby encoding for the CO dehydrogenase, which is the key enzyme involved in the oxidative conversion of CO to CO_2. Remarkably, heart cytochrome *c* oxidase possesses CO-oxygenase activity, thus metabolizing CO to CO_2 (25). 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 (111). 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 (mouse *hmox*$^{-/-}$), or because HO-1 activity is inhibited with a selective inhibitor tin protoporphyrin (SnPPIX), the hearts are rejected rapidly. HO-1 expression in the transplanted heart is essential to prevent rejection in this model. Surprisingly, if the donor and recipient are both treated with 250 ppm CO, a heart that cannot express HO-1 activity still survives indefinitely (111). In this scenario, CO appears to be able to substitute for HO-1 in suppressing the proinflammatory response that is the leading cause of graft rejection. CO emerges as a powerful antiinflammatory promoting agent act-

ing at level of the macrophage cell line, a cell that probably controls the balance of inflammation in many conditions. Macrophages stimulated with bacterial LPS produce several proinflammatory cytokines, such as TNFα. The antiinflammatory cytokine IL-10 is also produced (112). If macrophages overexpress HO-1 or are exposed to CO *in vitro* before stimulation with LPS, the proinflammatory response, and consequently TNFα, are 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: (a) CO prevents platelet aggregation and the consequent thrombosis (111); (b) CO downmodulates the expression of plasminogen activator inhibitor type 1; and (c) CO prevents apoptosis in several cell types, including endothelial cells, fibroblasts, hepatocytes, and β-cells of the pancreas (111). In addition, CO suppresses the proliferative response of smooth muscle cells that contribute to neointimal proliferation associated with inflammatory lesions *in vivo.* Many of the observed effects of CO have been obtained by exposing cells or animals to gaseous CO by inhalation. Interestingly, the recently discovered 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 (106, 107).

Bilirubin and biliverdin: an endogenous antioxidant system

Supraphysiological levels (>300 μ*M*) of nonconjugated bilirubin, as in the case of neonatal jaundice, are associated with severe brain damage. This is a plausible reason whereby bilirubin has generally been recognized as a cytotoxic waste product. However, in recent years, its emerging role as a powerful antioxidant has received wide attention. The specific role of endogenously derived bilirubin, as a potent antioxidant, has been demonstrated in hippocampal and cortical neurons where accumulation of this metabolite due to phosphorylative-dependent enhancement of HO-2 activity protected against H_2O_2-induced cytotoxicity (90, 141). Moreover, nanomolar concentrations of bilirubin resulted in a significant protection against H_2O_2-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 (44). 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 (79). Similarly, a decreased risk for coronary artery disease is associated with mildly elevated serum bilirubin, with a protective effect comparable to that of high-density lipoprotein cholesterol (44). 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 into biliverdin, then rapidly converted back to bilirubin by biliverdin reductase, which in brain is present in large functional excess, suggesting a mechanism to amplify the antioxidant effect (116). Remarkably, the rapid activation of HO-2 by protein kinase C (PKC) phosphorylation parallels the disposition of nNOS. Both are constitutive enzymes localized to neurons, and nNOS is activated by calcium entry into cells binding to calmodulin on nNOS. Similarly, PKC phosphorylation of HO-2 and the transient increase in intracellular bilirubin would provide a way for a rapid response to calcium entry, 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 H_2O_2. Exposure of bilirubin and biliverdin to agents that release NO or nitroxyl resulted in a concentration- and time-dependent loss of bilirubin and biliverdin. Increasing concentrations of thiols prevented bilirubin and biliverdin consumption by nitroxyl, indicating that bile pigments and thiol groups can compete and/or synergize the cellular defense against NO-related species. In view of the high inducibility of HO-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 (74).

NEUROPROTECTIVE HSP70 AND SMALL HSPS

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. Denaturated proteins are thought to serve as a stimulus for induction (23, 25, 29). These denaturated proteins activate heat shock factors (HSFs) within the cytosol by dissociating other HSPs that are normally bound to HSF (5). Freed HSF is phosphorylated and forms trimers, which enter the nucleus and bind to heat shock elements within the promoters of different heat shock genes, leading to transcription and synthesis of HSPs (76). After heat shock, for instance, the synthesis of HSP70 increases to a point to where it becomes the most abundant single protein in a cell (17, 157). Once synthesized, HSP70 binds to denaturated proteins in an ATP-dependent manner. The N-terminal end contains an ATP-binding domain, whereas the C-terminal region contains a substrate-binding domain. HSPs serve as chaperones that bind to other proteins and regulate their conformation, regulate the protein movement across membranes or through organelles, or and the availability of a receptor or activity of an enzyme. A large body of evidence now suggests a correlation between mechanisms of oxidative and/or nitrosative stress and HSP induction (77). Current opinion holds also the possibility that the heat shock response can

exert its protective effects through inhibition of the NFκB signaling pathway (23, 29). We have recently demonstrated in astroglial cell cultures that cytokine-induced nitrosative stress is associated with an increased synthesis of HSP70 stress proteins. Increase in HSP70 protein expression was also found after treatment of cells with the NO-generating compound sodium nitroprusside, thus suggesting a role for NO in inducing HSP70 proteins. The molecular mechanisms regulating the NO-induced activation of heat-shock signal seem to involve cellular oxidant/antioxidant balance, mainly represented by the glutathione status and the antioxidant enzymes (23, 26, 103).

In the nervous system, HSPs are induced in a variety of pathological conditions, including cerebral ischemia, neurodegenerative disorders, epilepsy, and trauma (76). Expression of the gene encoding HSPs has been found in various cell populations within the nervous system, including neurons, glia, and endothelial cells (77). HSPs consist of both stress-inducible and constitutive family members. There is now strong evidence that overproduction of HSP70 leads to protection in several different models of nervous system injury (77, 158). 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 the cells that are most resistant to ischemia, in glial cells at the edges of infarcts, and in neurons outside the areas of infarction. It has been suggested that this neuronal expression of HSP70 outside an infarct can be used to define the ischemic penumbras, which means the zone of protein denaturation in the ischemic areas (116). 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 (108). Transfection of cultured astrocytes with HSP70 protects them from ischemia or glucose deprivation (48). In addition to the possibility that the heat shock response can exert its protective effects through inhibition of the NFκB signaling pathway, HSP70 has been demonstrated to inhibit caspase-3 activation caused by ceramide, and also to affect JUN kinase and p38 kinase activation (97, 102). In addition, HSP70 binds to and modulates the function of BAG-1, the bcl-2 binding protein (97). Hence, HSP70 appears to act upstream in some apoptotic cascade, thereby modulating some, but not all, types of apoptosis-related cell death (Fig. 1).

Ubiquitin is one of the smallest HSPs and is expressed throughout brain in response to ischemia. It is involved in targeting and chaperoning of proteins degraded in proteasomes, which include NFκB, cyclins, HSFs, hypoxia-inducible factor, some apoptosis-related proteins, FOS, TNF, and erythropoietin receptors (94).

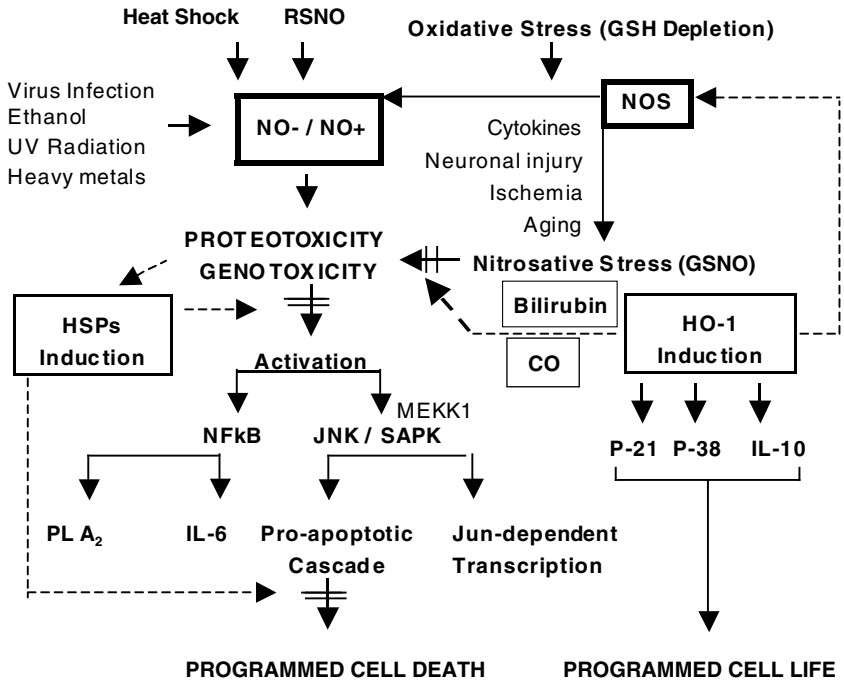

FIG. 1. Redox regulation of gene expression involving the vitagene system. Shown is the proposed role for the vitagene member HSPs in the modulation of cellular redox state and cell stress tolerance. Various proteotoxic (or genotoxic) conditions cause depletion of free HSPs that lead to activation of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

HSP27 is synthesized mainly in astrocytes in response to ischemic situations or to kainic acid administration. It chaperones cytoskeletal proteins, such as intermediate filaments, actin, or glial fibrillary acidic protein following stress in astrocytes. It also protects against Fas-Apo-1, staurosporine, TNF, and etoposide-induced apoptotic cell death, as well as H_2O_2-induced necrosis (8). HSP47 is synthesized mainly in microglia following cerebral ischemia and subarachnoid hemorrhage (153).

HSP60, 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 (70, 151).

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 (16, 29). Spices and herbs contain phenolic substances with potent antioxidative and chemopreventive properties (129). The active antioxidant principle in *Curcuma longa*, a coloring agent and food additive used in Indian culinary preparations, has been identified as curcumin (diferuloylmethane). Due to the presence in its structure of two electrophilic α,β-unsaturated carbonyl groups that, by virtue of Michael reaction, can react with nucleophiles such as glutathione, curcumin has the potential to inhibit lipid peroxidation and to effectively intercept and neutralize reactive oxygen and NO-based free radicals (19). This agent is a potent inhibitor of tumor initiation *in vivo* and possesses antiproliferative activities against tumor cells *in vitro* (116). Recent epidemiological studies (13) 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. Based on these findings, compelling evidence has shown that dietary curcumin given to an Alzheimer transgenic APPSw mouse model (Tg2576) for 6 months resulted in a suppression of indices of inflammation and oxidative damage in the brain of transgenic APPSw mice (29). Furthermore, in a human neuroblastoma cell line, it has recently been shown that curcumin inhibits NFκB activation, effectively preventing neuronal cell death (16). Remarkably, recent evidence has demonstrated that curcumin is a potent inducer of HO-1 in vascular endothelial cells (105). 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 (129). The similarity of CAPE to curcumin is striking because CAPE is also a Michael reaction acceptor, endowed with antiinflammatory, antioxidant, and anticancer effects (17, 104). These agents all appear capable of transcriptionally activating a gene battery that includes antioxidant enzymes and HO (29). Gene induction occurs through the ARE (1, 2). Thus, increased expression of genes regulated by the ARE in cells of the CNS may provide protection against oxidative stress.

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

The first half of the 20th century saw a rapid increase in the expectation of life in industrialized nations due to improved sanitation, public health, housing, nutrition, and medical technology/pharmaceuticals. The second half of this century has been characterized by a growing concern with the challenge produced by the increasing prevalence of old people in the society. Aging is a very common feature in living organisms and can be described as the total effect of those intrinsic changes in an organism that adversely affect its vitality and that render it more susceptible to the many factors that can cause death. Typically, the mortality rate accelerates with time, but it is not clear whether this effect is the result of external or internal causes of death. The full extent of aging in a population becomes apparent when most important external hazards are removed, such as under captive or laboratory conditions, when average longevity is usually greatly extended (25). 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, as natural selection is effective only through the reproductive output of individuals, the strength of natural selection decreases with age (25).

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 (61) and Szilard (145), respectively. A synthesis of evolutionary and mechanistic theories occurred in 1977 within the frame of the soma theory of aging postulated by Kirkwood (80). This theory provides a direct connection between evolutionary and physiological aspects of aging, by recognizing the primary importance of the allocation of metabolic energy resources between growth, somatic maintenance, and reproduction. It is suggested that longevity is determined through the setting of longevity assurance mechanisms so as to provide an optimal compromise between investments in somatic maintenance (including stress resistance) and in reproduction. As a corollary, increasing maintenance promotes the survival and longevity of the organism only at the expense of significant metabolic investments that could otherwise be used to accelerate processes such as growth and reproduction. The "disposable soma" theory of the evolution of aging also proposes that a high level of accuracy is maintained in immortal germ line cells, or alternatively, that any defective germ cells are eliminated. The evolution of an increase in longevity in mammals may be due to a concomitant reduction in the rates of growth and reproduction, the so-called "essential life," and an increase in the accuracy of synthesis of macromolecules. The theory can be tested by measuring accuracy in germ line and somatic cells and also by comparing somatic cells from mammals with different longevities. Notably, the HO gene is evolutionarily different in birds and mammals, with the biliverdin reductase–bilirubin step present in the latter case, but absent in the former group. Consistently, the organism sacrifices the potential for indefinite

survival in favor of earlier and more prolific fecundity. From an evolutionary perspective, aging is a nonadaptive phenomenon, because 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 rather little credence. A clear prediction is that the actual mechanisms of senescence are stochastic, involving most likely processes such as random accumulation of somatic mutations or oxidative damage to macromolecules. In the word of an anonymous poet, "we are born as copies, but we die as originals."

It is becoming increasingly clear that genetic factors are prominently involved in aging. The major lines of empirical evidence include the following: (a) the life span in human populations shows significant heritability; (b) different species have different intrinsic life spans due to genomic differences; (c) human populations possess inherited progeroid disorders, such as Werner's syndrome, a disease characterized by premature age-related disorders, including atherosclerosis, type II diabetes, osteoporosis, and cancers; and (d) clear evidence of genetic effects on life span have been demonstrated in invertebrate model systems, such as *Drosophila melanogaster* and *Caenorhabditis elegans*. In the latter organism, five different genomic regions appear to be associated with longevity, as assessed by quantitative genetic analysis (125). Also, in *Saccharomyces cerevisiae*, 13 longevity genes have been identified and cloned. Of these 13 genes, 11 have human homologues (125). At least three categories of genes are predicted to affect aging and longevity: (a) genes that regulate levels of somatic maintenance and repair; (b) pleiotropic genes, whose expression involves trade-offs between early-life fitness benefits and late-life fitness disadvantages, which do not encompass somatic maintenance; and (c) late-acting deleterious mutations that have escaped elimination as a consequence of the decline in the force of natural selection at old ages (125). Efficient functioning of maintenance and repair processes seems to be crucial for both survival and physical quality of life. This complex network of the so-called longevity assurance processes is composed of several genes, termed vitagenes (Table 1). The homeodynamic property of living systems is a function of such a vitagene network. Because aging is characterized by the failure of homeodynamics, a decreased efficiency and accuracy of the vitagene network can influence gerontogenic processes. It is not clear how various components of the vitagene network operate and influence each other in a concordant or a discordant manner. As 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.

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 (25, 29, 116) and 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 acquirement of major resistance to the effect of stressful and pathogenic conditions. HO-1 activity seems to be required for the action of several other therapeutic molecules. In each case, the expression of HO-1 or administration of one of its metabolic products substitutes for the actions of the other protective molecule (111).

In many inflammatory situations, the ability of IL-10 to suppress TNFα expression in macrophages requires the presence of HO-1 and the generation of CO; HO-1 expression or CO administration has the same effects as IL-10 (83). In concert with this conceivable possibility, the protective effect of IL-10 in a lethal endotoxic shock mouse model is strongly dependent on the expression of HO-1 and the generation of CO (83). Moreover, rapamycin appears not to exert its antiproliferative effects on smooth muscle cells unless HO-1 is present (156), and it has been proven that, in order for NO to protect mouse livers from hepatitis induced by TNFα and galactosamine, up-regulation of HO-1 seems to be essential (112). Also, alcohol has antiinflammatory effects in that TNFα is suppressed and IL-10 is increased (144). However, protection is lost when HO-1 is blocked (144). In addition, the antiinflammatory effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 has been shown to require the activity of HO-1 (84). Notably, during heat shock, which leads to up-regulation 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

TABLE 1. POLYGENIC CONTROL OF LONGEVITY BY THE HOMEODYNAMIC VITAGENE NETWORK

Somatic maintenance and repair functions	
Molecular control	*Cellular control*
Antioxidant defense	Cell proliferation
DNA repair systems	Cell differentiation
Transfer of genetic information	Stability of cell membrane
Stress protein synthesis	Stability of intracellular milieu
Proteasomal function	Macromolecular turnover
Tissue and organ control	*Physiological control*
Removing of toxic chemicals	Neuronal response and synaptic plasticity
Tissue regeneration	Hormonal response
Tumor suppression	Thermoregulation
Cell death and cell replacement	HO pathway and cell stress response

events associated with degenerative damage, particularly polyphenols such as curcumin, caffeic acid, and ferulic acid (all capable of exerting powerful antiinflammatory action), have been shown to function by up-regulation of HO-1 (116, 129). The fact that, in all these situations, specific molecules or biological phenomena appear to lose most, if not all, of their effect when HO-1 is absent, represents compelling evidence that the HO-1 system may represent a final common mediator of many biological events associated with 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.

CONCLUSIONS

Modulation of endogenous cellular defense mechanisms via the stress response signaling represents an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration. Efficient functioning of maintenance and repair processes seems to be crucial for both survival and physical quality of life. 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-21st century in the absence of effective interventions (16). This will pose an immense economic and personal burden on the people of this country. Similar considerations apply worldwide, except in sub-Sahara Africa, where HIV infection rates seem to be leading to decreased incidence of AD (17). 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 generally all degenerative diseases associated with oxidative stress. As one potentially successful approach, potentiation of endogenous secondary antioxidant systems can be achieved by interventions that target the HO-1/CO and/or HSP70 systems. In this review, the importance of the stress response signaling and, in particular, the central role of HO-1 together with the redox-dependent mechanisms involved in cytoprotection are outlined. The beneficial effects of HO-1 induction result from heme degradation and cytoprotective regulatory functions of biliverdin/bilirubin redox cycling. Thus, HO-1 can amplify intracellular cytoprotective mechanisms against a variety of insults. Consequently, induction of HO-1, by increasing CO and/or biliverdin availability, can be of clinical relevance.

CO has been studied for >100 years and, until the last few years, has been touted as a molecule to avoid, owing to its toxic effects exerted mostly on hemoglobin and cytochrome oxidase functions (110). However, these toxic effects are seen at concentrations of CO well above concentrations used experimentally. Beneficial effects are obtained with relatively low doses of CO (250 ppm for one to few hours) in rodents (112). Carboxyhemoglobin levels generated in such a model are not too different from those of heavy smokers. If this beneficial effect is confirmed also in human, limited exposure of patients to CO might be considered as therapy for various syndromes, particularly to prevent restenosis after angioplasty or 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 (112). Interestingly, the recently discovered CORMs appear to afford similar protective action, thereby providing an alternative therapeutic approach for those pathophysiological conditions where CO administration is warranted (106, 107). Furthermore, administration of biliverdin or bilirubin after the first few weeks of life is proven not to have toxicity, and doses as much as 2.5 mg/dl used in experimental paradigms are only slightly above normal levels, yet endowed with cytoprotective effects. Although clinical application of the HO system should be fully considered, a better understanding of how HO mediates its action will guide therapeutic strategies to enhance or suppress HO effects. Remarkably, the recently envisioned role of HSP70 as a vehicle for intracytoplasmic and intranuclear delivery of fusion proteins or DNA to modulate gene expression (159), along with the evidence that binding of HO protein to HO-1 DNA modifies HO expression via nonenzymatic signaling events (158) associated with CO and p38-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 (Fig. 1), depending on the relative extent of activation.

Presented here, and confirmed by other vascular (1, 5, 111, 158) and neurobiologists (34, 35, 88, 89), is strong evidence that a cross talk between stress response genes is critical for cell stress tolerance, highlighting compelling reasons 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 (116). Hence, by maintaining or recovering the activity of vitagenes (25), 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.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the Wellcome Trust and FIRB RBNE01ZK8F (V.C.), and by grants from the National Institutes of Health (D.A.B.). The authors

acknowledge helpful discussions with Roberto Motterlini and Roberta Foresti (Northwick Park Institute for Medical Research, Harrow, U.K.).

ABBREVIATIONS

AD, Alzheimer's disease; AP-1, activator protein-1; APP, amyloid precursor protein; ARE, antioxidant responsive element; CAPE, caffeic acid phenethyl ester; cGMP, cyclic GMP; CO, carbon monoxide; CORMs, CO-releasing molecules; EAE, experimental allergic encephalomyelitis; eNOS, endothelial nitric oxide synthase; GRP, glucose-regulated protein; GSH, reduced glutathione; GSSG, oxidized glutathione; HO, heme oxygenase; H_2O_2, hydrogen peroxide; HRM, heme-responsive motif; HSF, heat shock factor; HSP, heat shock protein; INF-γ, interferon-γ; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MARE, Maf recognition element; MS, multiple sclerosis; NF-E2, nuclear factor–erythroid 2; NF-κB, nuclear factor-κB; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; Nrf2, NF-E2-related factor 2; $ONOO^-$, peroxynitrite; PD, Parkinson's disease; PKC, protein kinase C; RNS, reactive nitrogen species; ROS, reactive oxygen species, RSNO, *S*-nitrosothiols; SnPPIX, tin protoporphyrin IX; StRE, stress responsive element; TNF, tumor necrosis factor.

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Received for publication May 20, 2004; accepted June 13, 2004.

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