Review

Nitric Oxide and Cellular Stress Response in Brain Aging and Neurodegenerative Disorders: The Role of Vitagenes

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Abstract. *Nitric oxide and other reactive nitrogen species appear to play crucial roles in the brain such as neuromodulation, neurotransmission and synaptic plasticity, but are also involved in pathological processes such as neurodegeneration and neuroinflammation. Acute and chronic inflammation result in increased nitrogen monoxide formation and nitrosative stress. It is now well documented that NO and its toxic metabolite, peroxynitrite, can inhibit components of the mitochondrial respiratory chain leading to cellular energy deficiency and, eventually, to cell death. Within the brain, the susceptibility of different brain cell types to NO and peroxynitrite exposure may be dependent on factors such as the intracellular reduced glutathione and cellular stress resistance signal pathways. Thus neurons, in contrast to astrocytes, appear particularly vulnerable to the effect of nitrosative stress. Evidence is now available to support this scenario for neurological disorders such as Alzheimer's disease, amyothrophic lateral sclerosis, Parkinson's disease, multiple sclerosis and Huntington's disease, but also in the brain damage following ischemia and reperfusion, Down's syndrome and mitochondrial encephalopathies. To survive different types of injuries, brain cells have evolved integrated responses, the so-called longevity assurance processes, composed of several genes termed vitagenes and including, among others, members of the HSP system, such as HSP70 and HSP32, to detect and control diverse forms of stress. In particular, HSP32, also known as heme oxygenase-1 (HO-1), has received considerable attention, as it has been recently demonstrated that HO-1 induction, by generating the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin, could represent a protective system potentially active against brain oxidative injury. Increasing evidence suggests that the HO-1 gene is redox-regulated and its expression appears closely related to conditions of oxidative and nitrosative stress. An amount of experimental evidence indicates that increased rate of free radical generation and decreased efficiency of the reparative/degradative mechanisms, such as proteolysis, are factors that primarily contribute to age-related elevation in the level of oxidative stress and brain damage. Given the broad cytoprotective properties of*

Abbreviations: Alzheimer's disease: (AD); Parkinson's disease: (PD); amyotrophic lateral sclerosis: (ALS); Multiple sclerosis: (MS); Huntington's disease: (HD); Huntingtin: (Htt); electron transport chain: (ETC); activator protein-1: (AP-1); stress-activated protein kinase (SAPK); c-jun N-terminal kinase: (JNK); nuclear factor kappa-B: (NFkB); heat shock protein: (HSP); mitogen-activated protein kinase: (MAPK); signal transducer and transcription activator: (STAT); tumor necrosis factor; (TNF); janus kinase: (JAK); N-methyl-D-aspartate: (NMDA); substantia nigra: (SN); Central nervous system: (CNS); phospholipase A_2: $(PLA)_2$; N-ethylmaleimide: (NEM); reduced glutathione: (GSH); oxidized glutathione: (GSSG); N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: (MPP^+); reactive oxygen species: (ROS); superoxide dismutase: (SOD); glutathione peroxidase: (GPX); cyclo-oxygenase: (COX-2); intraneuronal fibrillary tangles: (NFT); amyloid beta-peptide: (Aβ); advanced glycosylation end products: (AGEs); nitric oxide synthase: (NOS).

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the heat shock response there is now strong interest in discovering and developing pharmacological agents capable of inducing such a response. These findings have led to new perspectives in medicine and pharmacology, as molecules inducing this defense mechanism appear to be possible candidates for novel, cytoprotective strategies. Particularly, manipulation of endogenous cellular defense mechanisms such as the heat shock response, through nutritional antioxidants or pharmacological compounds, represents an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration. Consistent with this notion, maintenance or recovery of the activity of vitagenes may possibly delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy life span.

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 (1). 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 results 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 oxidase 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 defenses, oxidative stress ensues causing extensive damage to DNA, proteins and lipids (2).

The brain has a large potential oxidative capacity (3) due to the high level of tissue oxygen consumption. However, the ability of the brain to combact oxidative stress is limited for the following anatomical, physiological and biochemical reasons: a) high content of easily oxidizable substrates, such as polyunsaturated fatty acids and catecholamines; b) the relatively low levels of antioxidants such as glutathione and vitamin E and antioxidant enzymes (such as GSH peroxidase, catalase and superoxide dismutase); c) the endogenous generation of reactive oxygen free radicals *via* several specific reactions; d) the elevated content of iron in specific areas of the human brain, such as *globus pallidus* and *substantia nigra*. However, cerebrospinal fluid has very little iron-binding capacity owing to its low content of transferrin; e) CNS contains non-replicating neuronal cells which, once damaged, may be permanently dysfunctional or committed into programmed cell death (apoptosis).

An increasing body of evidence suggests that dysfunction of cell energy metabolism is an important factor in NO-mediated neurotoxicity and that the intracellular content of thiols is crucial in determining the sensitivity of cells to oxidative and nitrosative stress (4). Nitric oxide (NO) derived from neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase appears to have an important role in neural signaling and immunomodulation, as well as in the regulation of mitochondrial respiratory chain complex IV activity. The activity of inducible nitric oxide synthase (iNOS) is much higher than nNOS or eNOS, and the induction of iNOS, producing greater amounts of NO than nNOS or eNOS, is usually associated with cellular pathology. The actions of NO can be either direct, resulting from reactions between NO and specific biological molecules, or indirect, resulting from reactions of NO-derived reactive nitrogen species; for instance, the reaction of NO with superoxide produces the peroxynitrite anion and represents an important pathway of NO reactivity. Peroxynitrite is a powerful oxidant and can nitrate aromatic amino acid residues such as tyrosine to form nitrotyrosine. Nitration to form 3-nitrotyrosine can occur on either free or protein-bound tyrosine. Since the half-life of peroxynitrite at physiological pH is short the detection of 3-nitrotyrosine in tissues is often used as a biological marker of peroxynitrite generation *in vivo*. Not only is 3-nitrotyrosine a marker for peroxynitrite production, but it appears that the nitration of specific proteins by peroxynitrite may be relevant to brain pathophysiology (3, 5).

In contrast to the conventional idea that reactive species serve as a trigger for oxidative damage of biological structures, we now know that low physiologically relevant concentration of oxidants can regulate a variety of key molecular mechanisms. In light of this, the significance of oxidants in various aspects of biology and medicine needs to be revisited. Oxidants may function as cellular messengers that regulate a variety of signal transduction pathways (6). Mild oxidative stress in fact has been shown to modify the expression of most important antioxidant enzymes as well as to enhance expression and DNA binding of numerous transcription factors, including AP-1, fos, jun, myc, erg-1, SAPK, nuclear factor-kB (NFkB), heat shock factor (HSF) and NF-E2-related factors 2 (Nrf2) (6,7).

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 (8). Moreover, efficient functioning of maintenance and repair processes seems to be crucial for survival of brain cells under conditions of oxidative damage. This is accomplished by a complex network of the so-called longevity assurance processes, which are composed of several genes termed *vitagenes* (3). 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 (9) may be activated
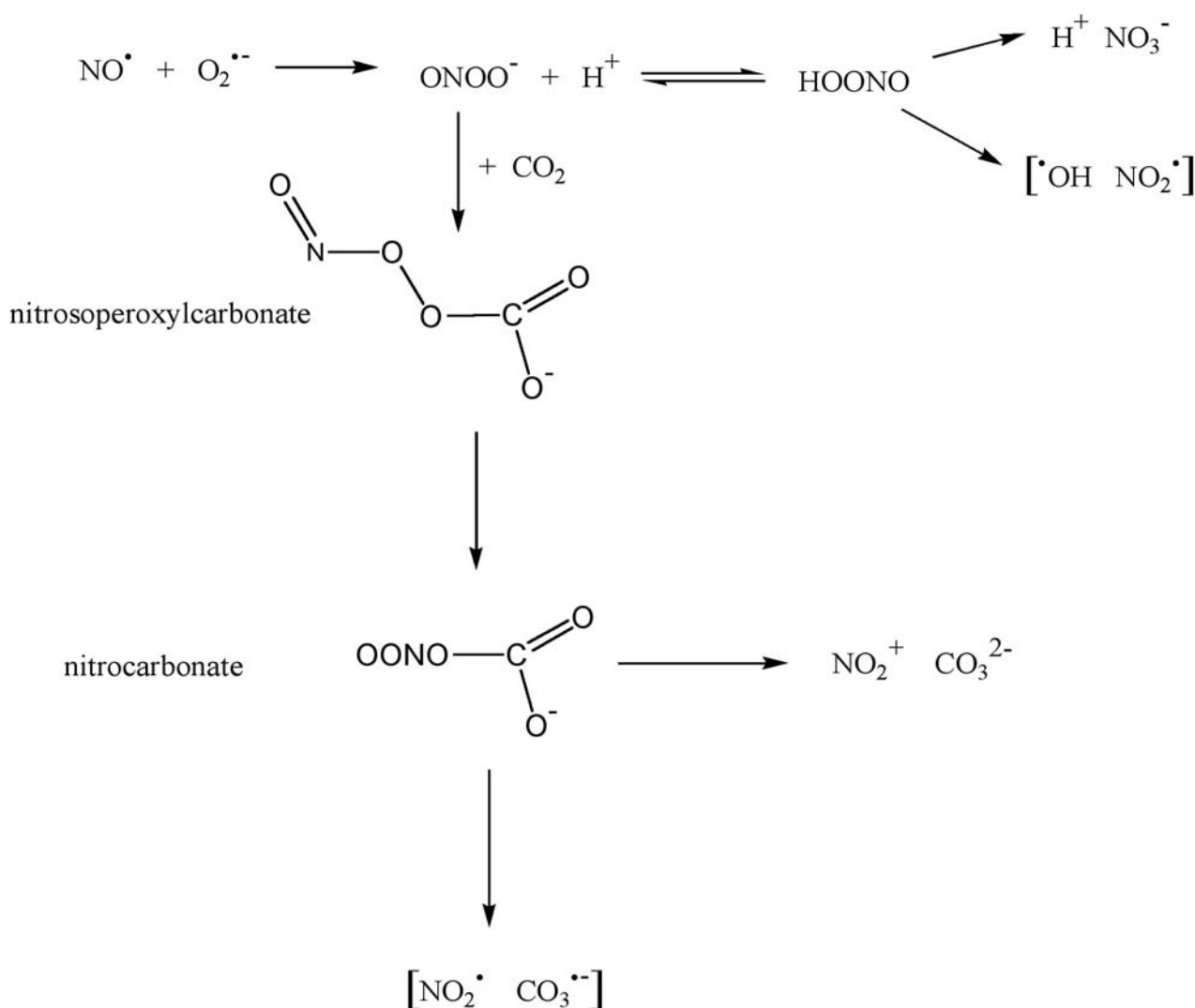
Figure 1. *Formation of peroxynitrite from nitric oxide and superoxide anion and reaction products with carbon dioxide*

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 (3).

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 heat shock proteins (HSPs). There is now strong evidence that heat shock proteins are critically involved in protection from nitrosative and oxidative stress (10-11). Thus, the heat shock response contributes to the establishment of a cytoprotective state in a wide variety of human diseases, including ischemia and reperfusion

damage, fever and inflammation, metabolic disorders, cell and tissue trauma, aging, infection and cancer. HSPs consist of both stress-inducible and constitutive family members (12). Constitutively expressed HSPs perform housekeeping functions. However, many are also up-regulated by stress. Inducible HSPs prevent protein denaturation and incorrect polypeptide aggregation during exposure to physiochemical insults. HSPs can prevent protein unfolding and, hence, enhance cell survival. Denatured proteins are thought to serve as the stimulus for stress protein induction. The 70-kDa family of stress proteins is one of the most extensively studied. Included in this family are HSC70 (constitutive form) and HSP70 (the inducible form, also referred to as HSP72). We have recently demonstrated in astroglial cell
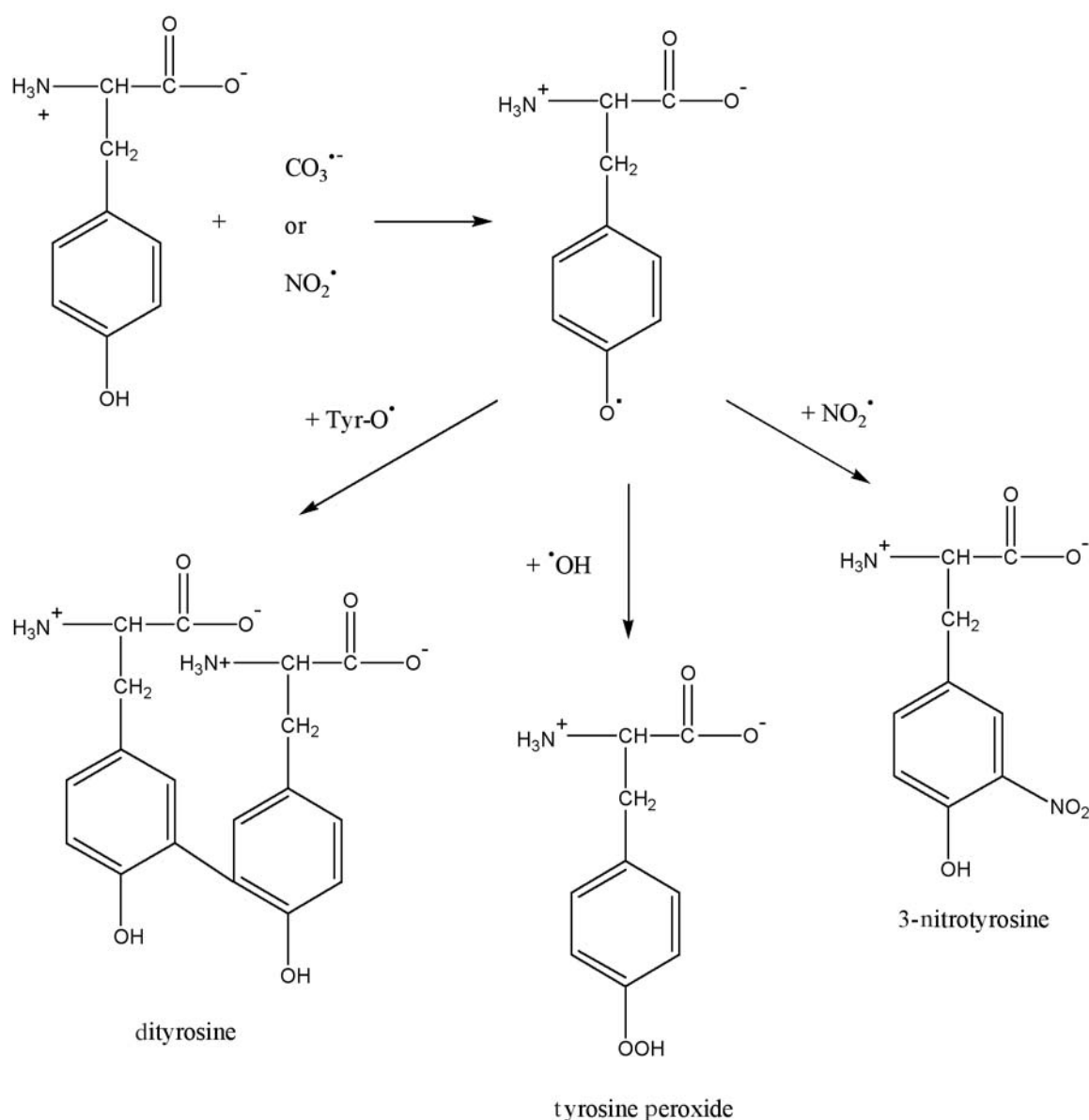
Figure 2. *Formation of 3-nitrotyrosine, dityrosine and tyrosine peroxide.*

cultures that cytokine-induced nitrosative stress is associated with an increased synthesis of HSP70 stress proteins. Increased 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 (10). *In vivo* experiments performed in our laboratory have also demonstrated that the redox glutathione status is a critical factor for induction of cytoprotective HSP70 (11). Another important family of heat shock protein is represented by the HSP32 or heme oxygenase (HO), which include the constitutive form HO-2

and the stress inducible HO-1. This last protein family, through generation of the antioxidant bilirubin from endogenous heme, contributes to the antioxidant cellular status and, in addition, produces carbon monoxide, a molecule involved in the regulation of the cellular NO signal pathways (13).

Recently, the involvement of the pathway in anti-degenerative mechanisms operating in AD has received considerable attention, as it has been demonstrated that the expression of HO is closely related to that of amyloid precursor protein (APP) (14-15). HO induction generally

occurs together with the induction of other HSPs during various physiopathological conditions and represents a protective system potentially active against brain oxidative injury. The HO-1 gene is redox-regulated, depending on the presence in the promoter region of two upstream enhancers, E1 and E2 (16). 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) (17). 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 (18). In addition, heme oxygenase-1 is rapidly up-regulated 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 (9, 19).

Alzheimer's disease (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 (19). Recently, increasing interest has been focused on identifying dietary compounds that can inhibit, retard or reverse the multi-stage pathophysiological events underlying AD pathology (20). Alzheimer's disease, in fact, involves a chronic inflammatory response associated with both brain injury and β-amyloid associated pathology. Conceivably, dietary supplementation with antioxidant compounds, such as vitamin E or polyphenolic agents (curcumin and its derivatives), can forestall the development of AD, consistent with a major "metabolic" component to this disorder (20). 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.

The NO system

Chemistry of nitric oxide. Nitric oxide (NO.) is produced from the catalytic conversion of arginine to citrulline by nitric oxide synthase (NOS) (21). Nitric oxide is neither a strong oxidant nor a strong reducant. Typically, nitric oxide does not react rapidly with the exception of reaction with superoxide anion ($O_2 \cdot^-$) (22,23). Nitric oxide reacts with superoxide anion at a diffusion controlled rate to produce peroxynitrite ($ONOO^-$). The concentration of nitric oxide within the biological system is regulated by the activity of

Table I. *Neurodegenerative disorders associated with free radical damage*

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1.	Amyothrophic lateral sclerosis
2.	Alzheimer's disease
3.	Parkinson's disease
4.	Multiple sclerosis
5.	Down's syndrome
6.	Huntington's Disease
7.	Ischaemia / Reperfusion

NOS, while the concentration of superoxide anion is regulated by the activity of superoxide dismutase (SOD). The reaction is first order with respect to both reactants; consequently, the kinetics of the reaction of nitric oxide with superoxide anion depend on the activities of NOS and SOD. Cells can regulate the concentration of superoxide anion and, hence, the intracellular formation of peroxynitrite, *via* cytosolic CuZnSOD and mitochondrial MnSOD. The formation of extracellular peroxynitrite is controlled by secreted or extracellular SOD (EC-SOD) located on the cell surface membrane (24).

$$NO. + O_2 \cdot^- \rightarrow ONOO^-$$
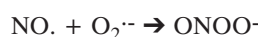
Peroxynitrite can exist in two forms: the nucleophilic peroxynitrite anion ($ONOO^-$), or the protonated peroxynitrous acid (HONOO) (25).
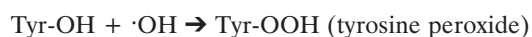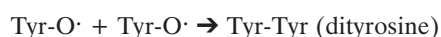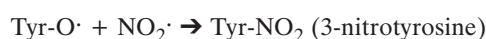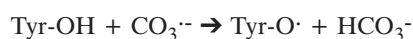
$$ONOO^- + H^+ \; HOONO$$

Peroxynitrite is highly reactive with a half-life of less than one second. Peroxynitrite can undergo a variety of chemical reactions depending upon its cellular environment and the availability of reactive targets such as DNA, proteins, and lipids (26). In addition to the first order decay to form nitrate (27), peroxynitrous acid can undergo homolytic decomposition to form a hydroxyl radical and a nitrite radical (28).

$$HOONO \rightarrow \cdot OH + NO_2 \cdot$$
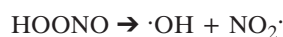
Peroxynitrite can react with carbon dioxide faster than the uncatalyzed decomposition of peroxynitrite; hence, the possibility exists that the *in vivo* oxidative damage attributed to peroxyinitrite may actually be mediated by the reactive intermediates from the reaction of peroxynitrite and carbon dioxide rather than peroxynitrite itself (29). However, there are some instances in which the presence of carbon dioxide actually inhibits the reaction of peroxynitrite with a

biotarget, such is the case with the reaction of peroxynitrite with glutathione (30). The reaction of peroxynitrite and carbon dioxide proceeds *via* the ONOO⁻/CO$_2$ reactive pair (31). The first intermediate in the reaction of peroxynitrite and carbon dioxide is nitrosoperoxycarbonate, which rearranges to form the second intermediate nitrocarbonate. Homolysis of nitrocarbonate is not favored, but can occur to generate a nitrite radical (NO$_2$·) and a carbonate radical anion (CO$_3$·⁻) (24). These nitrating and oxidizing species can then react with biomolecules forming modifications such as 3-nitrotyrosine. 3-Nitrotyrosine is a covalent protein modification that has been used as a marker of nitrosative stress under a variety of cellular conditions especially diseased *versus* control states (5, 25, 32). Dityrosine and tyrosine peroxide may also be formed from the reaction conditions described above (reviewed in 33, 34).

Tyr-OH + CO$_3$·⁻ ➜ Tyr-O· + HCO$_3$⁻

Tyr-O· + NO$_2$· ➜ Tyr-NO$_2$ (3-nitrotyrosine)

Tyr-O· + Tyr-O· ➜ Tyr-Tyr (dityrosine)

Tyr-OH + ·OH ➜ Tyr-OOH (tyrosine peroxide)

Alternatively, nitrocarbonate can undergo heterolytic cleavage resulting in the formation of NO$_2$⁺ and CO$_3$²⁻. NO$_2$⁺ can nitrate tyrosine residues in proteins in hydrophobic environments by electrophilic substitution (35). Figures 1 and 2 provide a summary of the reactions discussed above.

Peroxynitrite can also react with sulfhydryl compounds intracellularly due to the high concentration of free thiols within the cell (36). Glutathione (GSH) is a likely target of reaction with peroxynitrite resulting in the oxidation of GSH to GSSG which can be recycled by glutathione reductase (26). Sulfhydryls can also react *via* S-nitrosylation with NO to form a nitrosothiol (RSNO). Cysteine residues are preferentially nitrosylated due to favorable reaction kinetics (37). In 1997, Gow *et al.* proposed a mechanism for the formation of S-nitrosothiols *in vivo*. The proposed mechanism involved the direct reaction of NO with free sulfhydryls to form a reactive intermediate that is reduced by an electron acceptor, such as molecular oxygen (O$_2$), to produce RSNO. It was determined that cysteine accelerates the consumption of NO under physiological conditions. Likewise, the consumption of O$_2$ was also determined to increase in the presence of cysteine and NO. Additionally, the reaction of NO and cysteine, in the presence of CuZnSOD, produced H$_2$O$_2$. Taken together, these results suggest that the reaction of cysteine and NO leads to the reduction of O$_2$ to O$_2$·⁻ and the production of a nitrosothiol (38). Such nitrosothiols have been shown to be involved in intracellular signaling, ion channel regulation, immunology and apoptosis (37).

Nitric oxide as a new 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 signalling transduction mechanisms (39). Once generated, the cell cannot regulate the local concentration of NO, therefore another way to influence NO activity is to control its synthesis. The activity of NO also terminates when it chemically reacts with a target substrate. Nitric oxide, when produced in small quantities, can regulate cerebral blood flow and local brain metabolism (3), neurotransmitter release and gene expression, and play a key role in morphogenesis and synaptic plasticity. NO is a major component in signalling transduction pathways controlling smooth muscle tone, platelet aggregation, host response to infection and a wide array of other physiological and pathophysiological processes. In contrast, under conditions of excessive formation, NO is emerging as an important mediator of neurotoxicity in a variety of disorders of the nervous system (Table I).

Nitric oxide synthase (NOS) and its isoforms in the CNS. The enzyme responsible for NO synthesis is the nitric oxide synthase (NOS) family of enzymes, which catalyse the conversion of arginine to citrulline and NO·. NOS, localized in the CNS and in the periphery (40), is present in three well characterised isoforms: (a) neuronal NOS (nNOS, type I), (b) endothelial NOS (eNOS; type III), and (c) inducible NOS (iNOS, type II). Activation of different isoforms of NOS requires various factors and co-factors. In addition to a supply of arginine and oxygen, an increase in intracellular calcium leads to activation of eNOS and nNOS, and formation of calcium/calmodulin complexes is a prerequisite before the functional active dimer exhibits NOS activity. This complex depends also on cofactors such as tetrahydrobiopterin (BH4), FAD, FMN and NADPH (41). 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 BH4 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
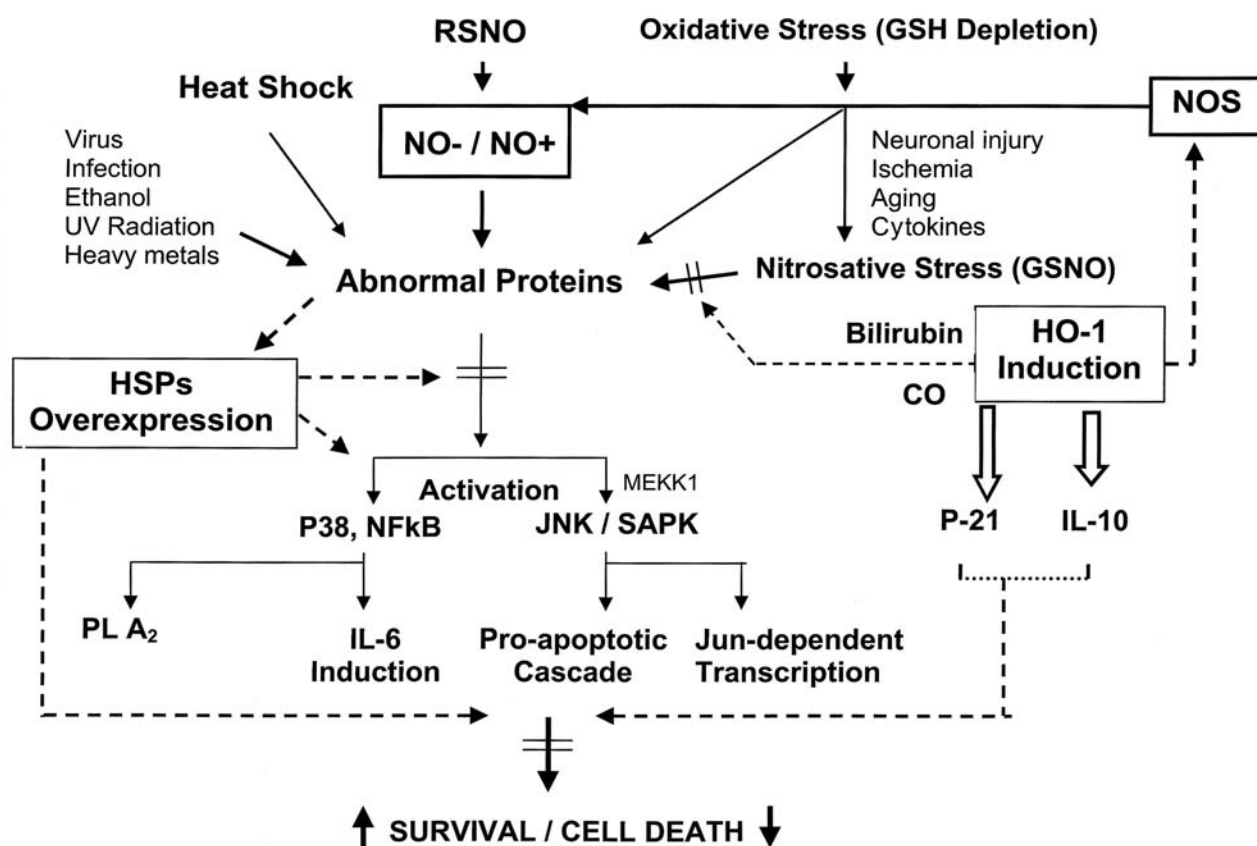
Figure 3. *Redox regulation of gene expression involving NO.*
Proposed role for the vitagene member HSPs, in modulation cellular redox state and cell stress tolerance. Various proteotoxic conditions cause depletion of free HSPs that lead to activation of stress kinase and proinflammatory and apoptotic signaling pathway. HSP70 prevents stress-induced apoptosis by interfering with the SAPK/JNK signaling and by blocking caspase proteolytic cascade. Nitrosative-dependent thiol depletion triggers HO-1 induction, and increased HO-1 activity is translated into augmented production of carbon monoxide and the antioxidant bilirubin. These molecules may counteract increased NOS activity and NO-mediated cytotoxicity. In addition, HO-1 may directly decrease NO synthase protein levels by degrading the cofactor heme. (PLA$_2$: phospholipase A$_2$; IL : interleukin; AP-1: activator protein-1; SAPK: stress-activated protein kinase; JNK: c-jun N-terminal kinase; NFkB: nuclear factor kappa-B; GSNO: S-nitrosoglutathione; HO-1 (Heme oxygenase-1).

the dentate gyrus express eNOS. nNOS, which is expressed in neurons, is critically involved in synaptic plasticity, neuronal signalling and neurotoxicity (42). Activation of nNOS forms part of the cascade pathway triggered by glutamate-receptor activation that leads to intracellular cyclic GMP elevation (42). 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 (42). Activation of iNOS requires gene transcription, and the induction can be influenced by endotoxin and cytokines (Interleukin-1, interleukin-2, lipopolysaccharide, interferon-γ, tumor necrosis factor). This activation can be blocked by anti-inflammatory drugs (dexamethasone), inhibitory cytokines (interleukin-4, interleukin-10,) prostaglandins (PGA2), tissue growth factors or inhibitors of protein synthesis, *e.g.*, cycloheximide (2).

Arginine, in brain cells, is supplied by protein breakdown or extracted from the blood through cationic amino acid transporters and represents a common substrate of NOS and arginase. Arginine can also be recycled from citrulline produced by NOS activity, through argininosuccinate synthetase and argininosuccinate lyase activities, and metabolized by arginase. NOS, argininosuccinate synthetase and argininosuccinate lyase constitute the so-called citrulline-NO cycle. In rat brain cells expressing nNOS all appear to express the entire citrulline-NO cycle, whereas numerous cells expressing argininosuccinate lyase do not express argininosuccinate synthetase 920. The differential expression of these genes within the same anatomical structure could indicate that intercellular exchanges of citrulline-NO cycle metabolites are relevant (2). Two different isoforms (I and II) have been isolated and exhibit

approximately 60% homology at the nucleotide level. Arginase II mRNA was co-induced with iNOS mRNA in murine macrophage-like RAW 264.7 cells by LPS. Thus, arginase may have an important role in down-regulating NO synthesis in murine macrophages by decreasing the availability of arginine (2). Whether this is the case also for brain arginase II remains to be elucidated.

Role of NOS and NO in brain pathophysiology. NO can react with carrier molecules and release oxidised (NO^+) or reduced (NO^-) forms (39). All these chemical states are found in brain and seems to account for the different controversial effects of NO in CNS. NO reacts with the superoxide anion ($O_2\bullet^-$) to produce the potent oxidant, peroxynitrite ($ONOO^-$) (43). The rate of this reaction is three times faster than the rate of superoxide dismutase (SOD) in catalyzing the dismutation of the superoxide anion to hydrogen peroxide. Therefore, when present at appropriate concentrations, NO effectively competes with SOD for $O_2\bullet^-$. Peroxynitrite is a strong oxidant capable of reacting with sulfhydryl groups, such as those of proteins, or directly nitrate aromatic aminoacids and possibly affect their participation in signal transduction mechanisms (44). In addition, peroxynitrite oxidizes lipids, proteins and DNA (45). Thiols are commonly assumed to be a major target for NO. Nitrosothiols with biological relevance have been isolated and characterized, including S-nitrosoglutathione and the nitrosothiols of serum albumin (44). The biological role for NO in the S-nitrosylation of many proteins is emerging as an important regulatory system (46). For instance, the NMDA receptor is inactivated by nitrosylation, hence NO may modulate glutamatergic neurotransmission by this mechanism (3). NO has been demonstrated to stimulate the auto-ADP ribosylation of glyceraldehyde-3-phosphate dehydrogenase (GADPH) by reacting with a critical cysteine with resulting binding of NAD to the catalytic cysteine, inhibition of GADPH activity and depression of glycolysis (2). Through formation of S-nitrosoglutathione (GSNO) NO 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 (see below) (4).

One of the most significant biological reactions of NO is with transition metals resulting in NO-metal complexes, such as occurs with iron in the heme moiety of guanylate cyclase (47). This interaction through induction of conformational changes in the heme moiety results in activation of the enzyme with rise in cGMP levels. Other heme protein targets for NO are catalase, cytochrome c, hemoglobin and peroxidase. NO also reacts with non-heme iron, such as iron-sulfur clusters present in numerous enzymes including, NADH-ubiquinone oxidoreductase, cis-aconitase and NADH: succinate oxidoreductase (48). In contrast with the reversible reaction of NO with heme, binding of NO to non-heme iron results in irreversible enzyme inactivation. Through this mechanism NO (a) irreversibly inactivates the enzyme ribonucleotide reductase (thereby inhibiting DNA (synthesis), (b) moves iron from iron-storage proteins such as ferritin, and (c) mobilizes Cu^+ from ceruloplasmin and metallothionein. NO can also influence iron metabolism at the post-transcriptional level by interacting with cytosolic aconitase, which after binding NO, functions as an iron responsive binding protein diminishing its aconitase activity (49).

NO-mediated neurotoxicity. When NO is present at higher than normal (physiological) levels, this paramagnetic gas molecule can initiate a neurotoxic cascade similar to glutamate. A large body of evidence indicates that glutamate-induced activation of NMDA receptors mediates cell death in focal cerebral ischemia, and also similar mechanisms are evoked in mediation of cell damage and cell death occurring in neurodegenerative diseases, such as AD and Huntington's disease (HD) (50). The activation of NMDA receptors, in fact, leading to increase in cytosolic calcium levels, presumably initiates glutamate neurotoxicity through activation of calcium-dependent enzymes, such as NOS. Treatment of cortical cultures with NOS inhibitors or removal of arginine from the media blocks NMDA neurotoxicity (51). In addition reduced hemoglobin, a quencher for NO, prevents NMDA-induced neuronal damage (52). However, brief exposure of neuronal cultures to glutamate-receptor agonists only transiently elevates intracellular calcium, which returns to basal levels within seconds. It is conceivable that this short-term calcium elevation may initiate processes that contribute to delayed neurotoxicity such as that involved in the aetiology of neurodegenerative diseases (51).

Defects in mitochondrial energy metabolism have long been considered to underlie the pathology of neurodegenerative disorders (53). Increasing evidence indicate that mitochondrial dysfunction may be a mechanism for the NO-mediated neurotoxicity (54). In isolated synaptosomes NO reversibly inhibits oxygen consumption at the level of complex IV in a competitive manner with oxygen (55). Interaction of NO with complex IV leads to formation of a nitrosyl-heme complex by donating one electron to ferric cytochrome a_3 and then interacting with the Cu^{2+}_B center (56). These mechanisms explain NO competition with oxygen resulting in reversible inhibition of activity. Accordingly, secondary to reversible complex IV inhibition, increased superoxide radical formation may occur *via* the mitochondrial respiratory chain, resulting in $ONOO^-$ production. This event may, in turn, inhibit complex I (57). However, complex I appears sensitive to $ONOO^-$ only in conditions of severe GSH depletion (58). Conversely, inhibition of complex II-III activity appears to be a consistent feature in neurons or astrocytes exposed to $ONOO^-$, and this also occurs in isolated

brain mitochondria (4). In contrast to reversible complex IV damage mediated by NO, irreversible complex IV damage, due to ONOO⁻ formation, may contribute to neurotoxicity, ATP depletion and cell death (54). Prolonged exposure of cells to NO-derived free radical species promotes peroxidation. Cardiolipin, an inner mitochondrial membrane lipid that is specifically required for maximal complex IV catalytic activity, is susceptible to free radical damage thereby compromising the catalytic activity of the complex IV. In support of this idea, Trolox, a vitamin E analogue and inhibitor of lipid peroxidation, protects complex IV activity from NO-induced damage (4).

It is important to point out that within the brain there are differences in the susceptibility of different cell types to nitric oxide. The factors responsible for this include the inner mitochondrial membrane lipid composition and/or the oxidant/antioxidant balance, particularly manganese superoxide dismutase and /or heat shock protein activity and expressions, as well as the glutathione status. These views are supported by experimental evidence indicating that exposure to ONOO⁻ results in a dramatic drop in intracellular GSH levels which lead to mitochondrial damage and cell death in neurons, but not in astrocytes (58). In this cell type, it has been demonstrated that mitochondrial damage and cell death occur only in GSH-depleted astrocytes subsequently exposed to ONOO⁻ (59). Whether NO-mediated mitochondrial damage causes neurotoxicity *via* necrosis or apoptosis is still matter of debate. However, it seems plausible that an extreme insult leading to severe mitochondrial damage might cause necrosis, possibly because of a massive energy failure. In contrast, glutamate neurotoxicity proceeds *via* apoptosis only if there is functional mitochondrial activity and ATP availability for the apoptotic program to become activated. This also suggests that irreversible complex IV activity inhibition may be neurotoxic by initiating apoptosis (60).

The NO hypothesis of brain aging. In aging, a decrease of NADPH diaphorase-positive neurons (*i.e.*, containing nNOS) has been described (61). However, this initial reduction in NO could finally lead to the expression of NADPH-diaphorase or NOS activity in other neurons that are normally negative (62). In addition, neuronal atrophy and neurodegenerative lesions could activate iNOS in microglial cells and astrocytes to produce toxic amounts of NO, thereby contributing to neurodegenerative changes observed during aging. Experimental evidence exists which indicates that brain cortex during aging shows appearance of iNOS⁻-positive neurons associated with considerable increase in nitrotyrosine immunoreactivity (62). Moreover, spontaneous expression of inducible NOS in the hypothalamus and other brain regions of aging rats, as well as in senescence accelerated mouse (SAM) brain, has been documented (63).

Although the endogenous factors that induce iNOS expression in the aging brain are unknown, by analogy with what is known on experimental iNOS induction (3), it can be postulated that this may occur through an increment in either the circulating levels or the local tissue release of cytokines. It has been reported, in fact, that TNF-γ in the cerebrospinal fluid and peripheral circulation, and IL-1β and interferon-γ in monocytes are increased by aging (64). Cytokines are also synthesized *in situ* in the hypothalamus (65), and their exogenous administration can block the NO-dependent control of GnRH release both *in vitro* and *in vivo*. All this evidence prompted the NO hypothesis of brain aging, as postulated recently by McCann (66). Accordingly, the fact that injection of moderate amounts of LPS to mimic the effect of bacterial infection induces increased numbers of IL-1α immunoreactive neurons in the region of the thermosensitive neurons in the preoptic-hypothalamic region, coupled to increased IL-β mRNA and iNOS mRNA expression in the paraventricular nucleus, arcuate nucleus, median eminence, choroid plexus and meninges, and a massive increase in the anterior pituitary and pineal, suggest that toxic amounts of NO could exist in these regions during moderate infections, even though there is no direct involvement of the brain. Destruction of neurons in the temperature regulating centers and in the paraventricular and arcuate median-eminence region following multiple infections over a life-span may be responsible for the reduced fibrile response and pituitary hormone secretion in response to infection, respectively. Repeated bouts of infections throughout life could lead to neuronal loss in the hippocampus, and cerebellar and cortical dysfunction could also ensue. Even more important is the massive increase in iNOS mRNA occurring in the pineal gland with age, which should result in high levels of toxic NO derived free radical species and considerable decrease in melatonin secretion, leading to impaired resistence to free radicals that are normally scavenged by melatonin. Obviously, experimental work needs to be conducted to verify this hypothesis, but it strengthens the possibility that treatment with free radical scavengers, such as vitamin C, E and melatonin might delay age-related changes.

Mitochondrial damage, reactive nitrogen species and neurodegenerative disorders. Decreased complex I activity is reported in the substantia nigra of postmortem samples obtained from patients with Parkinson's disease (PD) (67). Similarly, impaired complex IV activity has been demonstrated in Alzheimer's disease (68). Increased free radical-induced oxidative stress has been associated with the development of such disorders (69), and a large body of evidence suggests that NO· plays a central role. Cytokines (INF-γ), which are present in normal brain, are elevated in numerous pathological states, including PD (69) AD(70-74),

multiple sclerosis (75-78), ischemia, encephalitis and central viral infections (3). Accordingly, as cytokines promote the induction of NOS in brain, a possible role for a glial-derived NO· in the pathogenesis of these diseases has been suggested (79). Excessive formation of NO· from glial origin has been evidenced in some studies 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 (80). Loss of nigral GSH is considered an early and crucial event in the pathogenesis of PD (81) 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. Moreover, in support of this notion, 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 (82,83). The glial-derived factor S 100, which is overexpressed in AD (84,85), has also been shown to induce NOS in astrocytes (86) suggesting a role for S 100-mediated NO formation in the pathology of this disorder.

NO-mediated neuroprotection

Redox activities elicited by NO. In recent years a number of studies have shown a protective effect of nitric oxide in a variety of paradigms of cell injury and cell death. These include: a) direct scavenging of free radicals, such as superoxide (87); b) effects on intracellular iron metabolism, including interaction with iron to form nitrosyl-iron complexes (88), thus preventing release of iron from ferritin (89); c) interaction of NO· (through its congener NO⁺) with thiol group on the NMDA receptor with consequent down-regulation and inhibition of calcium influx (90); d) inactivation of caspases (91); e) activation of a cyclicGMP-dependent survival pathway, as demonstrated in PC12 cells (92); f) induction of expression of cytoprotective proteins, such as heat shock proteins (10,12,13); (g) inhibition of nuclear factor-kβ activation (93) or GADPH (94), whose activity appears to be required in one paradigm of neuronal apoptosis (95). In general, current opinion holds that the intracellular redox state is the critical factor determining whether in brain cell NO is toxic or protective (96). 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 multiple sclerosis (97).

The difficulty in delineating a mechanistic involvement of NO as pro-inflammatory or antiinflammatory agent and the controversy arising on whether excessive NO elicits cytoprotective or cytotoxic actions are better appreciated by recognizing the complexity of NO chemistry when applied to biological systems. As detailed by Stamler and colleagues,

the reactivity of the NO group is dictated by the oxidation state of the nitrogen atom, which enables the molecule to exist in different redox-activated forms (43). In contrast to NO, which contains one unpaired electron in its π^*-antibonding 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, neuronal NOS and S-nitrosothiols (RSNO). Thus, a broad range of chemical reactions can be predicted to arise once NO and other RNS are generated in the vicinity of proteins or biomolecules.

RSNO and nitrosative stress. A fundamental aspect of NO biochemistry is the attachment of NO groups to sulphydryl centers to form S-nitrosyl derivatives or RSNO (39). This chemical process, known as S-nitrosation, has been suggested to represent a refined endogenous tool to stabilize and preserve NO biological activity. Indeed, proteins such as albumin, tissue-type plasminogen activator, and hemoglobin that are nitrosylated at specific cysteine residues can exert, similarly to NO (40), vasorelaxation and inhibition of platelet aggregation. Low-molecular weight RSNO, such as S-nitrosoglutathione or nitrosocysteine may also represent a mechanism for storage *in vivo* of NO (46). 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. S-nitrosation is also an important process in modulating the activity and function of several enzymes and proteins, the best exmple of which is provided by S-nitrosohemoglobin. Hemoglobin, whose cysteine 93 (Cys-93) in the β chain is charged with a nitroso group when passing through the lung, delivers NO in arterioles, thereby regulating their diameter in response to the need for flow, as sensed by oxygen tension. In analogy to the accepted evidence that an excessive production of oxidants and ROS leads to oxidative damage, alteration of protein functions may occur when RNS reach a critical threshold. This phenomenon, which is driven by uncontrolled nitrosative reactions, has been termed nitrosative stress (98). The intriguing aspect in the parallellism between the effects mediated by increased RNS and ROS is the ability of cells to respond to these two types of stress; most notably, depending on the severity of the nitrosative/oxidative insult, this response may result in both adaptation and resistance to toxicity (99).

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 common in prokaryotes. The most elegant example is provided by the transcription factor

OxyR, an activator of antioxidant genes that is responsive to oxidative stress. It has been shown that oxidized but not reduced, OxyR activates transcription *in vitro* and that, upon treatment with oxidants, the conversion between the two forms of OxyR is rapid and reversible (100). The expression of these protective genes renders bacteria more resistant to oxidant damage (101). Studies have confirmed that, prior to the binding to specific promoters, OxyR must undergo a conformational change in which the molecular mechanism appears to involve either the oxidation of Cys-199 to the corresponding sulfenic acid (102) or the formation of a reversible disulfide bond (103). Interestingly, S-nitrosation of OxyR, following treatment of *E. coli* with high concentration of RSNO under anaerobic conditions, has been recently proposed as an alternative mechanism of transcriptional regulation (98). This mechanism appears to: (a) be independent of oxidant-mediated stress; (b) involve the depletion of intracellular glutathione and accumulation of endogenous RSNO; and (c) lead to resistance to nitrosative stress.

The cytoprotective mechanism triggered by SoxR in *E. Coli* includes the expression of critical antioxidant defensive proteins, such as superoxide dismutase (104). The emerging concept from this elegant study was that an analogous system might operate in mammalian cells. A possible candidate is the antioxidant protein heme oxygenase, as it could "sense" NO and act effectively as a pivotal player in cytoprotection against ROS and RNS insults. This hypothesis is corroborated by the following findings (105): (a) NO and NO-related species induce HO-1 expression and increase heme oxygenase activity in human glioblastoma cells, hepatocytes and aortic vascular cells; (b) cells pretreated with various NO-releasing molecules acquire increased resistance to H_2O_2-mediated cytotoxicity at the time heme oxygenase is maximally activated; and (c) bilirubin, one of the end products of heme degradation by heme oxygenase, protects against the cytotoxic effects caused by the strong oxidants H_2O_2 and $ONOO^-$ (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 heme oxygenase activity, in a variety of tissues, including brain cells (106). In rat glial cells, treatment with lipopolysaccaride (LPS) and interferon-γ results in a rapid increase in both iNOS expression and nitrite levels followed by enhancement of HO-1 protein (10). 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

(106). 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 (13). Collectively, these findings point to the central role of the NO molecule as signal triggering expression of cytoprotective genes, such as HO-1, which by virtue of its by-products CO, biliverdin and bilirubin may lead to adaptation and resistance of brain cells to subsequent, eventually more severe, nitrosative and oxidative stress insults (20). The exact molecular mechanism(s) by which both exogenously and endogenously formed NO (or NO-related species) modulate the induction of the *HO-1* gene remains obscure. It has been suggested that NO displaces the heme prosthetic group from the P450 proteins leading to an increase in intracellula heme pool, which would ultimately promote *HO-1* induction and heme oxygenase activation (39). Based on the evidence that NO is known to activate guanylate cyclase (40), some authors reported an increase in HO-1 transcript and protein levels after expoure of cells to high intracellular levels of cGMP analogues (107), although others have found that activation of the HO-1 pathway is a cGMP-independent process (108). Recent evidence suggests that a translation-independent stabilization of HO-1 mRNA occurs upon exposure of human fibroblasts to NONOates (109) and that these NO-releasing agents increase, in rat smooth muscle cells, HO-1 gene expression by enhancing both gene transcription and mRNA stability (110). 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. Notably, redox-sensitive transcription factors that recognize specific binding sites within the promoter and distal enhancer regions of the *HO-1* gene include: Fos/Jun [activator protein-1 (AP-1)], nuclear factor-kB (NFkB) and the more recently identified Nrf2 proteins (7, 8, 16, 17). Of major interest is the notion that both AP-1 and NFkB contain cysteine residues whose interaction with oxidant or nitrosant species might be crucial for determining the binding activity to DNA (39). 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 (111).

NO as signaling molecule for induction of neuroprotective heat shock proteins. It is well known that living cells are continually challenged by conditions which cause acute or chronic stress. To adapt to environmental changes and survive different types of injuries, eukaryotic cells have evolved networks of different responses which detect and

control diverse forms of stress (3). 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 unfavourable conditions (112). 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 analogs or cytotoxic drugs (9). 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 a cytoprotective state in a variety of metabolic disturbances and injuries, including stroke, epilepsy, cell and tissue trauma, neurodegenerative disease and aging (19, 112). This has opened new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear as possible candidates for novel cytoprotective strategies (8). 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 heat shock proteins. 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 (10). *In vivo* experiments performed in our laboratory have also demonstrated that the redox glutathione status is a critical factor for induction of cytoprotective HSP70 (11, 113) [see Figure 3].

The HO system: a putative *vitagene* target for neuroprotection

HSP32 or heme oxygenase is the rate-limiting enzyme in the production of bilirubin. In the last decade the heme oxygenase (HO) system has been extensively studied for its potential significance in maintaining cellular homeostasis. HO is found in the endoplasmic reticulum in a complex with NADPH cytochrome c P450 reductase, and it catalyzes the degradation of heme in a multistep, energy-requiring system. The reaction catalyzed by HO is the α-specific oxidative cleavage of the heme molecule to form equimolar amounts of biliverdin and carbon monoxide (CO). Iron is reduced to its ferrous state through the action of NADPH cytochrome c P450 reductase. 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 (9,13, 112, 113).

Heme oxygenase isoforms. HO is present in various tissues with the highest activity in the brain, liver, spleen and testes. There are three isoforms of heme oxygenase, HO-1 or inducible isoform (114), HO-2 or constitutive isoform (115) and the recently discovered HO-3, cloned only in rat to date (116). They are all products of different genes and, unlike HO-3, which is a poor heme catalyst, both HO-1 and HO-2 catalyze the same reaction (*i.e.*, degradation of heme) but differ in many respects and are regulated under separate mechanisms. The most relevant similarity between HO-1 and HO-2 consists of 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 (117). HO-1 and HO-2 have different localization, similar substrate and cofactor requirements, while presenting different molecular weight. These enzymes also display different antigenicity, electrophoretic mobility, inducibility as well as susceptibility to degradation. HO-1 and HO-2 are immunologically distinct and, in humans, the two genes are located on different chromosomes, *i.e.*, 22q12 for HO-1 and, 16q13.3 for HO-2, respectively (118).

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 brain tissue, where HO isoforms appears 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 (99, 119), 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 (115, 116). This evidence may suggest the possible existence of a cellular reserve of HO-1 transcript quickly available for protein synthesis and a post-transcriptional regulation of its expression.

HO isoenzymes are also seen to co-localize with different enzymes dependent on the cell type. In the kidney HO-1 colocalizes with erythropoietin, whereas in smooth muscle cells HO-1 colocalizes with nitric oxide synthase. In neurons HO-2 co-localizes with NOS, whereas in endothelium the same isoform co-localizes with NOS III. The cellular-specificity of this pattern of co-localization lends further

support to the concept that CO may serve a function similar to that of NO. Furthermore, the brain expression pattern shown by HO-2 protein and HO-1 mRNA overlaps with distribution of guanylate cyclase (120), the main CO functional target.

HO-3, the third isoform of heme oxygenase, shares a high homology with HO-2, both at the nucleotide (88%) and protein (81%) levels. Both HO-2 and HO-3, but not HO-1, are endowed with two Cys-Pro residues considered the core of the heme-responsive motif (HRM), a domain critical for heme binding but not for its catalysis (121). Although the biological properties of this isoenzyme still remains to be elucidated, the presence of two HRM motifs in its amino acidic sequence might suggest a role in cellular heme regulation (117). Studying HO-3 mRNA sequence (GenBank accession n.: AF058787), we have observed that its 5' portion corresponds to the sequence of an L-1 retrotransposon, a member of a family of retrotransposons recently involved in evolutionary mechanisms (122). 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 (116), it is possible that this last could have originated from the retrotransposition of the *HO-2* gene. In addition, this genetic mutation in rat may represent a *species*-specific event since no other sequence in the public databases match the rat *HO-3*.

Regulation of HO genes. Coupling of metabolic activity and gene expression is fundamental to maintain homeostasis. Heme is an essential molecule that plays a central role as the prosthetic group of many heme proteins in reactions involving molecular oxygen, electron transfer and diatomic gases. Although heme is integral to life, it is toxic because of its ability to catalyze the formation of reactive oxygen species and, consequently, oxidative damage to cellular macromolecules. In higher eukaryotes, the toxic effects of heme are counteracted by the inducible HO-1 system (117). As in the classic view of metabolic control, expression of HO-1 is induced by the substrate heme (123). In addition, expression of HO-1 is robustly induced in mammalian cells by various proinflammatory stimula, such as cytokines, heavy metals, heat shock and oxidants that induce inflammatory damage (124). Thus, HO-1 is an essential antioxidant defense enzyme that converts toxic heme into antioxidants and is fundamental to cope with various aspects of cellular stress and to regulate iron metabolism (125). 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 (126, 127).

The *HO-2* gene consists of five exons and four introns. HO-2 has a molecular weight of 34 kDa and exhibits 40% homology in aminoacid 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 (128). 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 (129). In addition, endothelial cells treated with the NOS inhibitor L-NAME and the HO inhibitor zinc mesophorphyrin exhibited a significant *up*-regulation of HO-2 mRNA.

The *HO-1* gene is induced by a variety of factors, including metallophorphyrins and hemin, as well as ultraviolet A (UVA) irradiation, hydrogen peroxide, prooxidant states or inflammation (13,130). This characteristic inducibility of the *HO-1* gene strictly relies on its configuration: the 6.8-kilobase gene is organized into 4 introns and 5 exons. A promoter sequence is located approximately 28 bases pairs upstream from the transcriptional site of initiation. In addition, different transcriptional enhancer elements, such as the heat shock element and the 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 4kb and 10 kb upstream of the site (131).

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 mechnisms controlling the mouse and human *HO-1* genes. The induction of *HO-1* is regulated principally by two upstream enhancers, E1 and E2 (16). Both enhancer regions contain StRE or ARE that also conform to the sequence of the MARE (17) with a consensus sequence (GCnnnGTA) similar to that of other antioxidant enzymes (8, 114). There is now evidence to suggest that heterodimers of Nrf2-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 (17, 133). 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 (16) 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 non-specific 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 the *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 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 *HO-1* gene (16). 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 affects 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 the HO-1 transcription, are located 9.5 kb upstream of the initiation site (8). The distal enhancer regions are important in regulating HO-1 in inflammation. Also in the promoter region resides a 56 bp fragment which responds to the STAT-3 acute-phase response factor, involved in the down-regulation of HO-1 gene induced by glucocorticoid (128, 129).

Glutathione and RSNO: intracellular modulators of HO-1 expression. The antioxidant glutathione (GSH) is essential for the cellular detoxification of reactive oxygen species in brain cells (20). A compromised GSH system in the brain has been connected with the oxidative stress occuring in neurological diseases (134). Recent data demonstrate that, in addition to intracellular functions, GSH has also important extracellular functions in brain. In this respect astrocytes appear to play a key role in the GSH metabolism of the brain, since astroglial GSH export is essential for providing GSH precursors to neurons (135). Of the different brain cell types studied *in vitro,* only astrocytes release substantial amounts of GSH. In addition, during oxidative stress astrocytes efficiently export oxidized glutathione (GSSG). The multidrug resistance protein 1 participates in both the export of GSH and GSSG from astrocytes (135). Glutathione plays an essential role in maintaining the intracellular environment in a tightly controlled redox state (19). 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 (10, 11). It is then not surprising that, in response to oxidation of glutathione and alteration of the thiol redox state, certain antioxidant genes, including *HO-1* gene are activated. Data in the literature support evidence for a direct link between a decrease in glutathione levels by oxidant stress and rapid up-regulation of HO-1 mRNA and protein in a variety of cells, including rat brain, human fibroblasts, endothelial cells and rat cardiomyocytes (13, 137). The strong correlation that exists between changes in thiol redox state and activation of the heme oxygenase pathway is validated by the findings that oxidative stress-mediated induction of *HO-1* gene is suppressed by the glutathione precursor, N-acetyl-cysteine (138, 139). In addition to oxidants, 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 (10). Of major interest is that an important role for glutathione metabolism and RSNO formation in the regulation of HO-1 expression by NO has been established (13, 137). Specifically, elevation of intracellular glutathione prior to exposure of endothelial cells to NO donors almost completely abolishes activation of the heme oxygenase pathway, which suggests that thiols can antagonize the effect of NO and NO-related species on HO-1 induction (140). In a model of hypoxia induced in endothelial cells, the expression of HO-1 and the consequent elevation of the heme oxygenase activity are associated with a transient decrease in the GSH/GSSG ratio and with formation of endogenous RSNO, as a consequence of early induction of the iNOS gene (13). Thus, in conditions of low oxygen availability, both oxidative and nitrosative reactions may participate in the stimulation of HO-1 (141). All this evidence supports the notion that generation of ROS and RNS are important signal transduction events involved in HO-1 activation and that nitrosation and oxidation are not necessarily mutually exclusive phenomena.

Heme oxygenase in brain function and dysfunction. In the brain the HO system has been reported to be active, and its modulation seems to play a crucial role in the pathogenesis of neurodegenerative disorders. The heme oxygenase pathway, in fact, has been shown to act as a fundamental defensive mechanism for neurons exposed to an oxidant challenge (142-144). Induction of HO occurs together with the induction of other HSPs in the brain during various experimental conditions including ischemia (14). 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 brain (3). 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 (145).

Since the expression of heat shock proteins is closely related to that of amyloid precursor protein (APP), heat-shock proteins have been studied in brain of patients with AD. Significant increases in the levels of HO-1 have been observed in AD brains in association with neurofibrillary tangles (146, 147), and HO-1 mRNA was found increased in AD neocortex and cerebral vessels (148). 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 (149). 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 (150). Alternatively, the elevated HO-1 expression may reflect increased oxidative stress in AD brain (72,74).

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 maximum HO-1 immunoreactivity (151). As with AD (72, 74), up-regulation of HO-1 in the nigral dopaminergic neurons by oxidative stress was shown (152).

Hemin, an inducer of HO-1, inhibited effectively experimental autoimmune encephalomyelitis (EAE), an animal model of the human disease, multiple sclerosis (MS) (153). In contrast, tin mesoporphyrin, an inhibitor of HO-1, markedly exacerbated EAE. These results suggest that endogenous HO-1 plays an important protective role in EAE and MS.

All these findings have led to new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear to be possible candidates for novel therapeutic neuroprotective strategies (20, 112).

Carbon monoxide effects on brain function in health and diseases. Increasing evidence indicates CO as an emerging chemical messenger molecule which can influence physiological and pathological processes in the central and peripheral nervous system. This gaseous molecule is now considered a putative neurotransmitter, owing to its ability to diffuse freely from one cell to another, thereby influencing intracellular signal transduction mechanisms. However, unlike a conventional neurotransmitter, CO is not stored in synaptic vescicles and is not released by membrane depolarization and exocitosis. It seems likely that CO is involved in the mechanism of cell injury (154). This is evidenced by the fact that CO binds to heme in guanylyl cyclase to activate cGMP (155). It has been found that CO is responsible for maintaining endogenous levels of cGMP. This effect is blocked by potent HO inhibitors but not NO inhibitors (156). Based on endogenous distribution of HO in the CNS it has been suggested that CO can influence neurotransmission similar to NO (120). CO appears to act as a retrograde messenger in LTP and also is involved in mediating glutamate action at metabotropic receptors (157). This is evident from the fact that metabotropic receptor activation in brain regulates the conductance of specific ions channels *via* a cGMP-dependent mechanism that is blocked by HO inhibitors (158). Experimental evidence suggests that CO plays a similar role to NO in the signal transduction mechanism for the regulation of cell function and cell to cell communication (156). HO resembles NOS in that the electrons for CO synthesis are donated by cytochrome P450 reductase which is 60% homologous at the aminoacid level to the carboxyterminal half of NOS (159). 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 effects at NMDA receptors, while CO is primarily responsible for glutamate action at metabotropic receptors. Taken together, it appears that CO and NO play an important role in the regulation of CNS function, thus impairment of CO and NO metabolism results in abnormal brain function (2).

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 related 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 (137). This hypothesis is sustained by recent findings showing that HO inhibition increases NO production in mouse macrophages exposed to endotoxin (160). CO may also act as a signaling effector molecule, by interacting with targets different from guanylate cyclase. Notably, it has been recently demonstrated that K_{Ca} channels are activated by CO in a GMPc-independent manner (161) and also that CO-induced vascular relaxation results from the inhibition of the synthesis of the vasoconstrictor endothelin-1 (162). 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.

Bilirubin: an endogenous antioxidant derived from heme oxygenase. Supraphysiological levels (> 300 μM) of non-conjugated bilirubin, as in the case of neonatal jaundice, are associated with severe brain damage. This is a plausible reason why 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 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 phosphorylation-dependent enhancement of HO-2 activity, protected against hydrogen peroxide-induced cytotoxicity (105,164). Moreover, nanomolar concentrations of bilirubin resulted in a significant protection against hydrogen peroxide-induced toxicity in cultured neurons as well as in glial cells following experimental subarachnoid hemorrhage. In addition, neuronal damage following middle cerebral artery occlusion was substantially worsened in HO-2 knock-out mice (165). Bilirubin can become particularly important, as a cytoprotective agent for tissues with relatively weak endogenous antioxidant defenses such as the central nervous system and the myocardium. Interestingly, increased levels of bilirubin have been found in the cerebrospinal fluid in AD, which may reflect the increase of degraded bilirubin metabolites in the AD brain derived from the scavenging reaction against chronic oxidative stress (166). 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 (165). 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 biliverdine reductase, which in brain is present in large functional excess. Remarkably, the rapid activation of HO-2 by protein kinase C (PKC) phosphorylation parallels the disposition of nNOS. Both are constitutive enzymes localized to neurons, and nNOS is activated by calcium entry into cells binding to calmodulin on nNOS. Similarly, PKC phosphorylation of HO-2 and the transient increase in intracellular bilirubin would provide a way for a rapid response to calcium entry, a major activator of PKC. Recent evidence has demonstrated that bilirubin and biliverdin possess strong antioxidant activities toward peroxyl radicals, hydroxyl radicals and hydrogen peroxide (167). 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 induction of heme oxygenase-1 by NO-releasing agents in different cell types, these findings highlight novel anti-nitrosative characteristics of bilirubin and biliverdin, suggesting a potential function for bile pigments against the damaging effects of uncontrolled NO production (167).

Peroxynitrite and Alzheimer's disease. Nitrosative stress has been implicated in a variety of neurodegenerative diseases including AD. Reactive nitrogen species such as peroxynitirite ($ONOO^-$) are capable of causing protein, lipid, and DNA oxidation (25). Such oxidative modifications have been reported in AD (168) and have been shown to alter the function of cellular components (169, 170, 171). Increased levels of 3-nitrotyrosine (3-NT) have been reported in AD brain (172, 173) and CSF (174). Likewise, amyloid β-peptide (1-42) [Aβ(1-42)] has been implicated in AD and results in an increase in 3-NT levels in primary neuronal cell culture (175). Research in our laboratory has provided additional evidence for the role of nitrosative stress in AD.

In vitro models such as synaptosomes and rat hippocampal cell culture have been used to demonstrate the oxidative stress induced by $ONOO^-$. Treatment of synaptosomes with $ONOO^-$ leads to elevated markers of protein oxidation and decreased activity of glutamine synthetase (GS) (176), all of which have been reported in AD brain (169). The decreased activity of GS may be due to nitration of tyrosine residues within this protein, as it has been shown that treatment of GS with $ONOO^-$ leads to the irreversible nitration of tyrosine residues, which can prevent tyrosine phosphorylation by protein kinases (177).

$ONOO^-$ oxidizes sulfhydryls (26); consequently, thiol-containing compounds are potential scavengers of $ONOO^-$. Several antioxidant interventions have been tested for protection against peroxynitrite-induced oxidative stress including glutathione. Glutathione (GSH) is a tripeptide (γ-glutamic acid-cysteine-glycine) containing a free thiol moiety that is present in millimolar concentration in the brain. GSH has been shown to attenuate the oxidative stress and toxicity induced by peroxynitrite in both synaptosomal and cell culture models (176, 178, 180). Additionally, elevation of endogenous GSH levels by agents such as N-acetylcysteine (NAC) and γ-glutamyl cysteine ethyl ester (GCEE) have been shown to provide protection against peroxynitrite-induced oxidative stress (179, 178). NAC provides cysteine, the limiting amino acid in the synthesis of GSH. Conversely, GCEE provides the limiting substrate of GSH synthesis, γ-glutamyl cysteine while the ethyl ester moiety allows the compound to cross the blood-brain barrier following intraperitoneal injection. GCEE has also been shown to increase mitochondrial GSH levels and protect against peroxynitrite-induced oxidation of mitochondrial proteins (180). Likewise, GSH-mimetics such as D609 have been

shown to prevent the Aβ(1-42)-induced increase in 3-NT (175). Furthermore, tellurium-containing compounds have been studied. Tellurium exhibits chemistry similar to sulfur, but is more nucleophilic than sulfur, hence, compounds containing tellurium can react faster with electrophilic compounds such as free radicals. One such compound, 3-[4-(N,N-dimethylamino)benzenetellurenyl]propanesulfonic acid (NDBT) prevented ONOO⁻ induced oxidation of neuronal proteins and lipids as well as cell death by acting as a ONOO⁻ scavenger. Additionally, NDBT prevented ONOO⁻-mediated changes in protein conformation (181).

Proteomics techniques have been used in our laboratory to determine which proteins are post-translationally modified to 3-NT in AD brain. Six proteins were identified which exhibited increased specific 3-NT immunoreactivity: α-enolase, triosephosphate isomerase, neuropolypeptide h3, β-actin, L-lactate dehydrogenase, and γ-enolase. Three of the proteins, α-enolase, triosephosphate isomerase, and neuropolypeptide h3 were significantly increased in 3-NT immunoreactivity (5). α-enolase had previously been identified as specifically oxidized in AD brain (182), and is one of the subunits composing the enzyme enolase. Enolase catalyzes the reversible conversion of 2-phosophoglycerate to phosphoenolpyruvate in glycolysis. Taken together with the increased nitration of triosephosphate isomerase, which interconverts dihydroxyacetone phosphate and 3-phophoglyceraldehyde in glycolysis, these results indicate a possible mechanism to explain the altered glucose tolerance and metabolism exhibited in AD (183, 184). Neuropolypeptide h3, also known as phosphatidylethanolamine-binding protein (PEBP), hippocampal cholinergic neurostimulating prptide (HCNP), and raf-kinase inhibitor protein (RKIP), has a variety of functions in the brain. Among them is *in vitro* up-regualtion of the production of choline acetyltrasferase in cholinergic neurons following NMDA receptor acivation (185). Choline acetyltransferase activity is known to be decreased in AD (186), and cholinergic deficits are prominent in AD brain (187, 188). Nitration of neuropolypeptide h3 may help to explain the decline in cognitive function due to lack of neurotrophic action on cholinergic neurons of the hippocampus and basal forebrain.

Conclusion and future perspectives

Increasing evidence supports the concept that impairment of mitochondrial energy metabolism may underlie the pathology of aging and of the most important neurodegenerative disorders, although the exact mechanisms involved still remain unknown. Evidence that mitochondrial dysfunction may be a mechanism for NO-mediated neurotoxicity arises from different studies which indicate that excessive production of NO, a free radical that has several important messenger functions within the CNS, can react with the superoxide anion to form ONOO⁻, thereby causing nitrosative stress. NO· can also stimulate the S-nitrosylation of numerous proteins to modify these proteins, and thus serves as a powerful signal for modulating the expression of protective genes. As HSPs are produced in all cell types by a wide variety of stressful stimuli, this observation implies that HSP modulation might provide a means of protecting the brain against a wide variety of pathological processes including ischemia, seizures or neurodegenerative disorders. All these considerations strongly sustain the idea that efficient functioning of maintenance and repair processes are crucial for the survival of brain cells under conditions of oxidative and/or nitosative damage and that brain stress tolerance can be achieved through modulation of complex polygenic systems, such as the *vitagene* network (3, 9, 94, 95). Importantly, the pharmacological or nutritional manipulation of endogenous cellular defense mechanisms represents an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration, suggesting potential novel therapeutic strategies relying upon the simultaneous activation of cytoprotective genes of the cell life program and down-regulation of proinflammatory and pro-oxidative genes involved in programmed cell death.

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