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Mitochondrial dysfunction, free radical generation and cellular stress response in neurodegenerative disorders

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1. ABSTRACT

Protein conformational diseases, such as Alzheimer's, Parkinson's and Huntington's, affect a large portion of aging population. The pathogenic dysfunctional aggregation of proteins in non-native conformations is associated with metabolic derangements and excessive production of reactive oxygen species. Reduction of cellular expression and activity of antioxidant proteins result in increased oxidative stress. Free-radicals derived from mitochondrial dysfunction and from the cyclooxygenase enzyme activity play a role in oxidative damage of brain. Cyclooxygenase also mediates in neuro-inflammmation by the production of pro-inflammatory prostaglandins which contribute to brain injury. The pathogenic role of cyclooxygenase has been demonstrated in Alzheimer and Parkinson diseases. The brain responses to detect and control diverse forms of stress are accomplished by a complex network of "longevity assurance processes" integrated to the expression of genes termed *vitagenes*. Heat shock proteins are a highly conserved system responsible for the preservation and repair of correct protein conformation. Heme oxygenase-1, a inducible and redox-regulated enzyme, is currently considered as having an important role in cellular antioxidant defense. A neuroprotective effect, due to its heme degrading activity, and tissue-specific pro-oxidant effects, due to its products CO and free iron, are under debate. There is a current interest in dietary compounds that can inhibit, retard or reverse the multi-stage pathophysiology of Alzheimer disease, with a chronic inflammatory response, brain injury and beta-amyloid associated pathology. Curcumin and ferulic acid, two powerful antioxidants, the first from the curry spice turmeric and the second a major constituent of fruit and vegetables, have emerged as strong inducers of the heat shock response. Food supplementation with curcumin and ferulic acid is considered a nutritional approach to reduce oxidative damage and amyloid pathology in Alzheimer disease.

2. INTRODUCTION

It is well established that living cells are constantly challenged by conditions which cause acute or chronic stress. Oxidative stress is characterized by an overproduction of reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Within the cells, ROS are physiologically present at minimal concentration as by-products of aerobic metabolism as well as second messengers in many signal transduction pathways and, in normal conditions, there is a steady-state balance between pro-oxidants and antioxidants which is necessary to ensure optimal efficiency of antioxidant defenses (1-4). However, when the rate of free radical generation exceeds the capacity of antioxidant defenses, oxidative stress ensues with consequential severe damage to DNA, protein and lipid (5-7). Recently the term "nitrosative stress" has been used to indicate the cellular damage elicited by nitric oxide and its congeners peroxynitrite, N_2O_3, nitroxyl anion and nitrosonium (all of these indicated as reactive nitrogen species or RNS) (8-10).

Oxidative stress has been implicated in mechanisms leading to neuronal cell injury in various brain pathological conditions, such as Alzheimer's disease (11-15). In fact, brain has a limited capacity to counteract oxidative stress due to: a) an high content of easily oxidizable substrates, such as polyunsaturated fatty acids and catecholamines; b) relatively low levels of glutathione and vitamin E, as well as of the antioxidant enzymes glutathione peroxidase, catalase and superoxide dismutase; c) the endogenous generation of free radicals through specific reactions such as those catalyzed by cyclooxygenase; d) the inability of neuronal cells to replicate and e) the elevated content of iron in specific areas of the human brain, such as *globus pallidus* and *substantia nigra* (SN), while cerebrospinal fluid has very little iron-binding capacity owing to its low content of transferrin (16). One of the more important system devoted to the antioxidant defense in brain is represented by the so called "heat shock response" sustained by the "heat shock proteins" (Hsps) (17). In mammalian cells Hsps 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 (18,19). Although prolonged exposure to conditions of extreme stress is harmful and can lead to cell death, induction of Hsps 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 cytoprotection in a variety of metabolic disturbances and injuries, including stroke, epilepsy, cell and tissue trauma, neurodegenerative disease and aging (20,21). Among the Hsps family, an emerging role has been attributed to heme oxygenase-1 (HO-1 or Hsp32) which is responsible of the transformation of the heme moieties into carbon monoxide (CO) and biliverdin (BV) (22,23).

There is increasing evidence that mitochondrial dysfunction plays a pivotal role in the pathogenesis of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and Friedreich ataxia (FRDA). A mutation, whether inherited or acquired, leads to impaired electron transfer chain (ETC) (24). Impaired electron transfer, in turn, leads to decreased ATP production, increased formation of toxic free radicals, and altered calcium homeostasis. These toxic consequences of ETC dysfunction may sustain further mitochondrial damage, including oxidation of mitochondrial DNA, proteins, and lipids, and opening of the mitochondrial permeability transition pore, an event associated with cell degeneration and death (25). Moreover, mutations in the mitochondrial genome may play an essential role in neurodegenerative diseases, such as FRDA (26). It is generally recognized that, in addition to the nuclear genome, each human cell contains multiple copies of a small double-stranded mitochondrial genome. Mitochondrial DNA (mtDNA) disorders possess tissue specificity, characterized by the fact that even if a mitochondrial DNA mutation is present in all tissues, only some will be affected and express a pathology. Due to the coexistence in cells of both normal and mutated mtDNA, (a situation termed heteroplasmy), the levels of mutation can vary considerably between mitochondria, cells and even tissues. The precise sequence of events in FRDA pathogenesis is uncertain. However, impaired intramitochondrial metabolism associated with increased free iron and the consequent oxidative stress are considered as a possible pathogenic mechanism (16).

In this paper, we discuss the role of mitochondrial dysfunction in the pathogenesis of neurodegenerative disorders. Further, we will focus our attention on cyclooxygenase (COX), as an enzyme involved in neuroinflammation and a potential free radical generating system and on heat shock proteins, in particular HO-1 and Hsp70, as proteins primarily devoted to the brain first line of defense.

3. ENERGY THRESHOLDS IN BRAIN MITOCHONDRIA: IMPLICATION FOR NEURODEGENERATIVE DISORDERS

Human cells contain from a few hundreds to more than a thousand mitochondria; each mitochondrion in turn has 2-10 copies of mtDNA, thus, several thousands copies of the mitochondrial genome can be present within a single cell. Importantly, unique to mtDNA is that it is maternally inherited, and that it may exist in many different copies in the oocyte cytoplasm. This implies that no mtDNA recombination occurs at fertilization and only a sequential accumulation of mutations from the maternal lineage account for mtDNA variations. Moreover, mtDNA is particularly prone to mutation, being estimated as 10 times greater than nuclear DNA (27), owing to the absence of protective proteins (such as histones) and of a high-efficiency repair system. Thus, mutant and wild-type (normal) mtDNA can coexist within a cell in any proportion and this situation is termed heteroplasmy. It is becoming increasingly evident that the mitochondrial genome plays an important role in the pathogenesis of

neurodegenerative diseases, and evidence for mitochondria being a site of damage in neurodegenerative disorders is based in part on the observed decreases in the respiratory chain complex activities in the main neurodegenerative diseases, such as Parkinson's, Alzheimer's and Huntington's. Such defects in respiratory complex activities, possibly associated with oxidant/antioxidant imbalance, are thought to underlie defects in energy metabolism and induce cellular degeneration. That the pathogenesis of these defects is mitochondrial in origin is also supported by the results of experiments with cytoplasmic hybrid, or "cybrid" cells (28), which reproduce the respiratory chain defects present in diseased patients. These cybrids are created by the transfer of mtDNA to clonal neuronal-like cells which have been depleted of their endogenous mtDNA by application of a low concentration of ethidium bromide over a long term. Host cells are then polyethylene glycol (PEG) fused with platelets, containing no nuclear DNA, from a control or diseased patient. This system allows investigators to specifically investigate the role of mtDNA in cellular pathology. The first hint that mitochondria play a role in human disease did not emerge until 1958, when mitochondrial dysfunction was identified in a Swedish patient who had severe perspiration, polydipsia, polyphagia, weight loss and weakness (29). Laboratory studies showed a basal metabolic rate 200% above normal, very low weight (37 kg) and basal temperature reaching 38°C. Biochemical studies revealed that the patient had a partially uncoupled mitochondrial respiration which accounted for the generation of excessive heat and high calorie consumption. Although the primary etiologic event in Luft's disease remains to be identified, the disease is associated with release of mitochondrial calcium stores, abnormal calcium cycling and sustained stimulation of loosely coupled respiration. The discovery of Luft's disease cleared the path for fertile investigations and since 1960s, over 120 human mitochondrial diseases have been discovered, many of which involve selected populations in the central nervous system consisting of postmitotic, highly energy-dependent cells. Many of these diseases have been associated with specific inherited mitochondrial DNA mutations and respiratory chain deficiencies. Point mutations may involve either the RNA or protein-encoding genes, and rearrangements may take the form of deletions or duplications. In the presence of heteroplasmy there is a critical ratio of mutant to wild-type mitochondrial genomes that is necessary before the disease becomes both biochemically and clinically apparent. As might be expected mtDNA disorders are phenotypically diverse given the ubiquitous presence of mitochondria and the variation in the levels of heteroplasmy in the body. Yet many have predominant neurologic and muscular symptoms, including dementia, seizures, ataxic syndromes, peripheral neuropathies, and progressive myopathy. Postmitotic tissues typically show increased levels of mutant mitochondria due to the inability of these tissues to select against cells containing mutant mtDNA genomes. Central nervous system (CNS) imaging of patients with mtDNA disorders often reveals moderate degrees of cerebral or cerebellar atrophy that are consistent with neurodegeneration, often comparable with the pathologies associated with the brain in senescence or in dementia (30).

Several mechanisms have been proposed to explain the variability of the phenotypic expression of a mtDNA mutation, such as sporadic mutation or mitotic segregation. However all these hypotheses incorporate a unique feature of mitochondrial genetics and pathologies: that of the heteroplasmic concept of mtDNA mutations. Levels of mutation can vary considerably between mitochondria, cells, and even tissues within the same individual. Consequently, the expression of a mutation in mtDNA can be thought as a function of the heteroplasmy degree. In general, whether or not a metabolic defect expresses itself as a recognizable clinical disease will depend upon the extent to which it affects the metabolic pathway in question and this can lead to a threshold expression of the disease state. One of the most important features recognized in mitochondrial diseases is the existence of a threshold in the degree of a mitochondrial deficit for the expression of the disease, and these were shown by Wallace (31) to be related to the balance between normal and mutant mtDNA. Accordingly, it has been reported that only 10% of wild type DNA is enough to maintain a normal respiratory rate and also that 80-90% deletion in mtDNA must be achieved before complex IV activity is compromised (32,33). All this represents compelling evidence that there is a threshold in the heteroplasmy of mutation of about 90% before a pathological consequence become manifest. Below this threshold the flux of respiration and of ATP synthesis are at a level which does not compromise normal metabolism. As consequence, there are at least four levels at which threshold effects occur in mitochondrial metabolism, with respect to their possible involvement in the pathogenesis of neurodegeneration. The first is the expression of the heteroplasmy of mtDNA at the level of a given enzymatic step, whereby mitochondria from patients might exhibit a particular ratio of defective DNA compared to normal DNA. The second is the threshold effect observed in the mitochondrial metabolism as a result of a decrease in a given mitochondrial activity. The third may occur in the expression of defective mitochondria with respect to the whole cellular metabolism. The fourth is the fact that the control coefficient of a given step may vary depending on different types of mitochondria, which leads to the observation that the threshold value for a given complex of the electron transport chain can vary according to the threshold in the energy demand of different tissues. At each level the threshold effect will reinforce the others, as interpreted by the *double threshold hypothesis* (34), whose predictions are now beginning to be documented. Data from studies of rat brain mitochondria of non synaptic origin have shown that thresholds exist whereby complex activities need to be reduced by at least 60% before major changes in ATP synthesis and O_2 consumption occur. Interestingly, in synaptic mitochondria, titration of various complexes with specific inhibitors generated threshold curves showing that complex I, III and IV activities had to be decreased 25, 80 and 70%, respectively before major changes in rates of oxygen consumption and ATP synthesis were observed (35) (Figure 1). These results suggest that in mitochondria of synaptic origin complex I activity has a major control of oxidative phoshorylation, such that when a threshold of 25% inhibition is exceeded, energy metabolism is compromised, and reduction in ATP

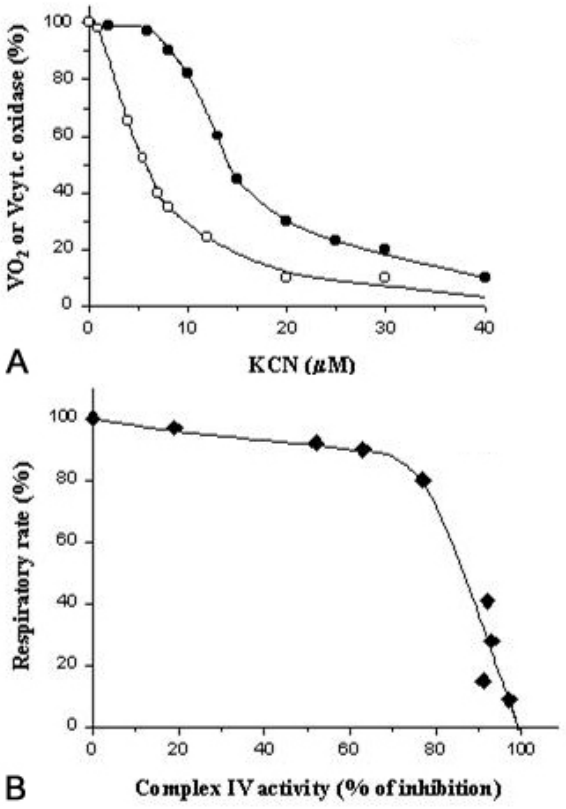

Figure 1. Energy thresholds in brain mitochondria. In (a) the effect of KCN titration on respiration (filled circle) and complex IV activity (empty circle) is shown. In (b) the respiratory rate as a function of complex IV inhibition is plotted. "Reprinted from Calabrese V. et al., J. Neurol. Sci. 233, 145-162, 2005., with permission from Elsevier".

synthesis ensues. Moreover, the same study reported that depletion of glutathione, which has been reported to be a primary event in idiopathic Parkinson's disease, abolished the threshold for complex I, providing experimental evidence that antioxidant status is critically involved in maintaining energy thresholds in mitochondria (35).

Other data are also consistent with these findings, as it has been shown both in a patient with cytochrome c oxidase deficiency and in an animal model of copper deficiency that more than a 50% deficit in complex IV activity did not affect the respiratory flux (34). It is possible to explain these findings within the framework of the *metabolic control theory* (36). According to this theory, which investigates the effects of infinitesimally small parameter perturbations on the variables of metabolic systems, a crucial stage in the expression of a threshold for a clinical disease is, at molecular level, the impact that a localized defect in a given step has on the global flux of a metabolic network (36). In this theory an important parameter is the *control coefficient* which quantitatively expresses the fractional change in pathway flux of a metabolic network, under steady-state conditions, induced by a fractional change in the individual step under consideration (35). For the oxidative flux (respiration) in mitochondria it can be determined according to Eq.1:

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

where C is the flux control coefficient of the mitochondrial complex under investigation, $dVc/d(\text{Inhibitor})$ is the rate of change of complex activity (individual step) and $dJO_2/d(\text{Inhibitor})$ is the rate of change of respiration (global flux), at low concentrations of the complex inhibitor. In determining the control coefficients of the various steps of oxidative phosphorylation on respiratory flux with the inhibitory titration method, two very differently shaped curves are observed, for the isolated step and the whole flux. Figure 1a shows the effect of KCN titration on respiration and complex IV activity. It can be seen that even at 50% cytochrome oxidase inhibition, there is only 20% inhibition of the whole flux, and 90% of inhibition of the isolated step is required to achieve a significant reduction of the respiration, corresponding to global flux. This is apparent from Figure 1b, obtained by plotting the inhibition of the respiratory flux as a function of the complex IV activity, given the same KCN concentration. Generation of a threshold curve is evident, and the complex activity must be decreased by 70% before a rapid decline in the rate of respiration occurs. This pattern is a direct consequence of the summation theorem of metabolic control theory (34), which states that the sum of control coefficients of a defined metabolic pathway is equal to 1 with the result that most of the control coefficients are low. Consistently, a control coefficient of 0.1 for a given complex, implies a 10% perturbation in the activity of this complex and can result in an inhibition of respiratory rate by as little as 1%. This implies that in the case of oxidative phosphorylation each single control coefficient is close to zero producing at the beginning a quasi horizontal slope; at very low activity of the step both curves must meet again, due to the fact that the flux becomes zero and the step is completely inactivated. (37,38).

4. MITOCHONDRIAL DAMAGE, REACTIVE NITROGEN SPECIES, AND NEURODEGENERATIVE DISORDERS

Increasing evidence sustain the hypothesis that mitochondrial energy metabolism underlie the pathogenesis of neurodegenerative diseases. Decreased complex I activity is reported in the *substantia nigra* of *post-mortem* samples obtained from patients with PD (39). Similarly, impaired complex IV activity has been demonstrated in AD (40). Increased free radical-induced oxidative stress has been associated with the development of such disorders (41) and a large body of evidence suggest that NO play a central role. Cytokines (IFN-gamma) which are present in normal brain are elevated in numerous pathological states, including PD (42), AD (43), MS, ischemia, encephalitis and central viral infections (44). Accordingly, as cytokines promotes the induction of NOS in brain, a possible role for a glial-derived NO in the pathogenesis of these diseases has been suggested (45,46). Excessive formation of NO from glial origin has been evidenced in a study in which NADPH diaphorase (a cytochemical marker of NOS activity)

positive glial cells were identified in the *substantia nigra* of *post-mortem* brains obtained from individuals with PD (47). Loss of nigral GSH is considered an early and crucial event in the pathogenesis of PD (48) and as a consequence, decreased peroxynitrite ($ONOO^-$) 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. Moreover, 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 (49).

5. CYCLOOXYGENASE

Cycloxygenase (COX), also referred to as prostaglandin (PG)H_2 synthase (PGHS), catalyzes the conversion of arachidonic acid (AA) to the unstable endoperoxide intermediate prostaglandin H_2(PGH_2), the common precursor of prostaglandins (PGs) and thromboxanes. These bioactive lipids are involved in many patho-physiological conditions including inflammation, mitogenesis, apoptosis, angiogenesis, hemostasis, regulation of kidney function, and maintenance of gastric mucosal integrity (50). COX products play important roles in patho-physiological processes within the CNS. It is well known their key role in pain and fever. Recently, they have been also involved in the development of neurodegenerative disorders, in particular AD, since long-term use of COX inhibitors has been shown to reduce the risk of this disease (51). This finding prompted the researchers to study the therapeutic implications of COX inhibitors in AD patients. To date, clinical trials showed that COX inhibitors have no, or minimal, therapeutic benefit in AD patients (52,53). However, the possible beneficial effects of non-steroidal anti-inflammatory drugs (NSAIDs) in preventing AD or in early stages of this disease are still under debate.

5.1. Cyclooxygenase regulation and distribution

COX exists as two main different isoforms: a constitutive one, called COX-1, and an inducible one, called COX-2. These isoforms are homodimers with a molecular weight of about 70 kDa per monomer. Each monomer contains a heme molecule as prosthetic group (50). COX isoforms are bi-functional enzymes, as they catalyze two sequential chemical reactions in separate, but functionally coupled, active sites. The cyclooxygenase activity is responsible for the synthesis of PGG_2 from two molecules of O_2 and one molecule of AA, whereas the peroxidase activity catalyses a net two electron reduction of the 15-hydroperoxyl group of PGG_2 yielding PGH_2 (54,55), with the release of an oxidizing radical (56).

COX-1 is constitutively expressed and is involved in physiological processes that require constant regulation (e.g. platelet aggregation and renal blood flow). On the contrary, COX-2 expression is very low, but can be induced by several factors including lipopolysaccharide (LPS), pro-inflammatory cytokines, neurotransmitters, growth factors, calcium, and phorbol esters (57). This drastic distinction has been recently re-evaluated based on new evidence showing that COX-1 expression can be induced during stress conditions including nerve injury, while many tissues, such as brain and kidney, constitutively express COX-2 (58).

Immunohistochemical analysis showed COX-like immunoreactivity (LI) in neurons of neocortex, enthorinal cortex, hippocampus, amygdala, hypothalamus, cerebellum and brainstem nuclei. COX-2-LI is particularly localized in neurons, within postsynaptic cell bodies and dendritic spines (55,59,60). Weak COX-LI is also found within cells of glial lineage diffusely in the brain. In physiological conditions, the contribution of glial cells to brain PGs production seems to be quite low (60). On the contrary, PGs production increases during recovery from hypoxic-ischemic or traumatic brain injury, and in AD as a result of long-term accumulation of COX-1-expressing microglia (61).

5.2. Cyclooxygenase and neuro-inflammation

Along with their physiological role, PGs are also implicated in many pathological conditions affecting the nervous system, in particular of immuno-inflammatory origin (62). Most of the data about the pro-inflammatory role of PGs in the brain have been obtained by using the experimental models of cultured rat glial cells challenged with bacterial LPS. It is generally agreed that LPS exerts most of its pathological effects by the activation of a specific receptor, named LPS binding protein/CD14/toll-like receptor-4. Although early studies suggested that these receptors were specific for immuno-inflammatory cells such as monocytes, later studies showed that they are also expressed in rat astrocytes (63). It has been recently suggested that LPS is able to increase the intracellular content of ROS through the activation of NADPH oxidase. ROS, in turn, serve as second messengers to enhance gene expression induced by LPS through the receptor pathway (64). As a consequence, cultured glial cells challenged with LPS are currently considered a valuable *in vitro* model to investigate the inflammatory processes of the brain.

Microglia is the major source of prostaglandins in the brain (62). Microglial cells are mainly endowed with COX-1 activity. Proinflammatory cytokines, *i.e.* interleukin-1beta, IL-6 and TNF-alpha, do not induce COX-2 expression in microglial cells. The fact that LPS induces COX-2 expression suggests that this isoform can be induced in microglial cells only in severe conditions. On the contrary, COX-1 expression in microglia is not modulated during neuropathological conditions. Thus, significant increases in PGs synthesis and release occur in neurodegenerative diseases only due to strong accumulation of microglial cells (62).

Incubation up to 24 h with LPS significantly increases PGE_2 release from primary cultures of hypothalamic astrocytes. The increase occurs early, after 1 hour of incubation. Experiments with selective COX inhibitors showed that about 80% of PGE_2 release induced by 1-hour incubation with LPS is accounted for by COX-1 activity. This production decreases to about 30 % after a 24-h treatment. With this duration of LPS incubation, most

of the increase in PGE_2 levels is due to increased AA availability through phospholypase A_2 (PLA_2) and COX-2 induction (65). Nuclear factor kappaB (NFkappaB) appears to play a role in the activation of COX-2 induced by LPS, since inhibitors of this transcription factor partially antagonize LPS-induced PGE_2 release (66). The fact that LPS induced COX-2 expression with a long latency lead to hypothesize that this event could be mediated by the other factors, such as the pro-inflammatory cytokine interleukin-1beta (IL-1beta) and NO formed by the inducible isoform of NO synthase (iNOS), both of which are active in the brain (64,67). The experimental results showed that (*i*) IL-1beta and NO are able *per se* to increase PGE_2 production and (*ii*) IL-1ra, an IL-1beta antagonist, and aminoguanidine, a relatively selective iNOS inhibitor, counteract LPS-induced PGE_2 release from rat hypothalamic astrocytes (62,67).

Recently, some authors reported that neuronal COX activity is able to trigger neuronal damage by itself, independently of any glial cell contribution. It has been clearly demonstrated that selective COX-2, but not COX-1 inhibitors protected neurons from injury caused by excitotoxicity or anoxia. The ability of COX-2 to trigger cell death could be explained, at least in part, by the evidence that the enzymatic activity of this isoform, but not of COX-1, is coupled to the production of cyclopentanone-PGs (CyPGs). The latter are ligands of peroxisome proliferators-activated receptor gamma (PPARgamma) and have been shown to induce apoptosis in different cell types, including neurons (68).

5.3. Cyclooxygenase and neurodegenerative disorders

During last years, increasing evidence has demonstrated the relevance of COX-1 and COX-2 in the pathogenesis of AD. In particular, increased COX-1 and COX-2 levels have been found in AD brain compared with age-matched controls, and immunohistochemical studies have shown that the immunoreactivity was restricted to microglia and neurons, respectively (69). The mechanism by which COX could contribute to the onset and progression of AD has been attributed, among others, to the pro-inflammatory activity of prostanoids such as PGE_2. In fact, *in vitro* studies demonstrated that non-selective COX inhibitors, such as indomethacin and flurbiprofen, decreased levels of pro-inflammatory molecules, such as PGE_2 and IL-1beta, produced by microglia and astrocytes (69). However, other studies showed that PGE_2 can play either neuroprotective or neurotoxic roles, depending on its concentrations. While low concentrations are neuroprotective, neurotoxic effects are observed with high concentrations (70). Besides the effects induced by PGs generated by COX, a direct connection between ROS, COX-2 and neurodegeneration has been also proposed. Oxidative stress from abnormal generation of free radicals has been shown to play a central role in neuronal damage in various neurodegenerative diseases and following ischemia-reperfusion and brain trauma (71,72). Free radicals can be generated through enzymatic or non-enzymatic (*i.e.*, iron-catalyzed Fenton-like reactions) mechanisms. COX is numbered among free radical forming enzymes, sharing this activity with other enzymes such as lipoxygenases, xanthine oxidase and peroxidases. During COX catalytic activity, no oxygen radicals are formed (73,74). Instead, carbon-centered radicals are generated during the cyclooxygenase reaction. Their generation has been shown to be sensitive to COX-2 selective inhibitors (73). In addition, carbon-centered radicals generated from COX-2 enzymatic activity induce phosphatidylserine oxidation, which is blocked by COX-2 selective inhibitors (nimesulide and NS-398) (73). In a model of excytotoxicity produced by N-methyl-D-aspartate (NMDA), it has been shown that the activation of NMDA receptors in the rat hippocampus increases both PGE_2 and 8-epi-PGF_2-alpha as indices of COX activity and free radical-dependent lipid peroxidation, respectively (70). Both COX-1 and COX-2 are responsible for radical generation and lipid peroxidation, even though COX-2 plays a major role (70). The two isoforms differ only for their temporal contribution to PGE_2 and 8-epi-PGF_2-alpha formation, COX-1 being more important in increasing 8-epi-PGF_2-alpha levels during the late phase after NMDA infusion (70). These findings, from different labs, can be put together by speculating that 8-epi-PGF_2-alpha is generated through peroxidation of phospholipids by carbon-centered radicals produced through the catalytic activity of both COX-1 and COX-2 (70). These observations leave open the possibility that also COX-1 can be a therapeutic target in neurodegenerative diseases. It is noteworthy to mention at this point that other mechanisms can be at the basis of the neuroprotective effect of NSAIDs shown in epidemiological studies. Other targets of NSAIDs include NFκappaB and PPARgamma. Modulation of these pathways could explain the epidemiological findings on NSAIDs use and reduced AD risk. As a last remark, direct amyloid-beta-lowering effects have been attributed to some NSAIDs (ibuprofen, indomethacin and flurbiprofen) used at high doses. NSAIDs used in clinical trials on AD patients (naproxen, rofecoxib and celecoxib) have much less amyloid-beta-lowering properties in experimental models. This fact could explain why clinical trials failed to show a therapeutic benefit of NSAIDs in AD patients. If this hypothesis holds true, the inflammatory pathogenic theory, and the theory of damage from free radical deriving from COX activity, in AD should be re-evaluated (69).

The involvement of COX-2 has also been hypothesized in PD. A marked increase in COX-2 has been observed both in PD patients and in mice treated with the neurotoxin MPTP, whereas transgenic mice deficient in COX-2 showed a reduced degree of neurodegeneration after MPTP administration (62,75,76). Interestingly, the inhibition of COX-2 did not reduce neuroinflammation and do not have any protective effect in mice (77).

6. HEME OXYGENASE

Heme oxygenase (HO) is a microsomal enzyme that catalyzes the degradation of heme in a multistep, energy-requiring system. The reaction catalyzed by HO consistes in the alpha-specific oxidative cleavage of heme moieties to form equimolar amounts of ferrous iron, CO and BV. This reaction requires both O_2 and reducing equivalents provided by cytochrome-P-450 reductase. In

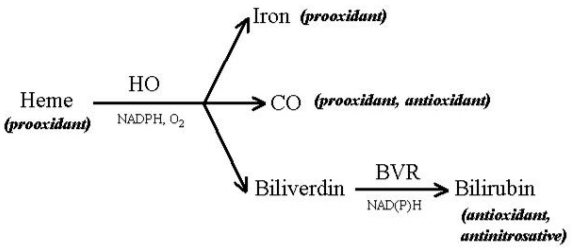

Figure 2. The heme degradation pathway *via* the heme oxygenase/biliverdin reductase system. Heme oxygenase cleaves the heme-moieties at the alpha-methene bridge yielding equimolecular amounts of ferrous iron, carbon monoxide (CO) and biliverdin. Reducing equivalents are provided by the NADPH-cytochrome P450-reductase system. In mammals, biliverdin is further reduced by biliverdin reductase (BVR) into bilirubin.

mammals, BV is then reduced by the cytosolic enzyme biliverdin reductase (BVR) in bilirubin (BR) which is then conjugated, by the liver, with glicuronic acid and excreted (23) (Figure 2).

6.1. Heme oxygenase regulation and distribution

Heme oxygenase-1, also referred to as heat shock protein-32, is induced by various stimuli including oxidative and nitrosative stress, ischemia, heat shock, LPS, hemin and the neuroprotective agent Neotrofin. Furthermore, in cultured human cells, HO-1 expression is repressed by hypoxia or by the treatment with interferon-gamma or desferrioxamine. On the other hand HO-2, the constitutive form, is responsive to developmental factors, opiates, adrenal glucocorticoids and NO. Although HO-1 and HO-2 catalyze the same reaction, they play different roles in protecting tissues against injuries. Based on several lines of evidence, the more convincing hypothesis suggests that HO-1 induction is one of the earlier cellular response to tissue damage and is responsible for the rapid transformation of the pro-oxidant heme into CO and BR, two molecules with anti-inflammatory and anti-oxidant activity. On the contrary HO-2, which is constitutively expressed, is primarily involved in maintaining cellular heme homeostasis and preventing NO mediated damage (78).

This characteristic inducibility of HO-1 gene strictly relies on its configuration: the 6.8-kb gene is organized into 4 introns and 5 exons. A promoter sequence is located approximately 28 base pairs upstream from the transcriptional site of initiation. In addition, different transcriptional enhancer elements, such as heat shock element and metal regulatory element reside in the flanking 5' region. Also, inducer-responsive sequences have been identified in the proximal enhancer located upstream the promoter and, more distally, in two enhancers located 4kb and 10 kb upstream the initiation site (79). The molecular mechanism that confers inducible expression of HO-1 gene 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 (80). 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) (81) with a consensus sequence (GCnnnGTA) similar to that of other antioxidant enzymes (82). 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 gene through these MAREs (81). A possible model, centered on Nrf2 activity, suggests that the HO-1 gene 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 (80), random interaction of activators with the HO-1 gene 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 gene expression by Bach1 and heme, is that expression of HO-1 gene is regulated through antagonism between transcription activators and the repressor Bach1. Under normal physiological conditions expression of HO-1 is repressed by Bach1/Maf complex, while increased levels of heme displace Bach1 from the enhancers and allow activators, such as heterodimer of Maf with NF-E2 related activators (Nrf2), to interact with the transcriptional promotion of HO-1 gene (80). 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 gene 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 metallothionein-1 gene, which respond to heavy metals (Cd and Zn) only after recruitment of another fragment located upstream, between –3.5 and 12 kb (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 (83). The distal enhancer regions are important in regulating HO-1 in inflammation, since it has been demonstrated that they are responsive to endotoxin. In the promoter region also 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 (84,85).

It is noteworthy to point out that HO-1 gene expression is also regulated by BV reductase activity. In fact, during oxidative stress, the accelerated degradation of BV in BR is responsible for the induction of HO-1 gene expression and increased heme degradation activity; moreover, BR, accumulated by BV reductase activity, negatively regulates the reductase with a following increase in BV levels which is responsible for the inhibition of the oxygenase. This regulatory loop permits the return to normal heme degrading activity (86).

Heme oxygenase-1 is ubiquitous and particularly abundant in reticuloendothelial organs such as liver and spleen, whereas HO-2 is localized in specific organs such as brain, kidney and testis. The central nervous system is characterized by very high HO activity under basal conditions, mostly accounted for by HO-2, the latter being expressed in neuronal populations in forebrain, hippocampus, hypothalamus, midbrain, basal ganglia, thalamus, cerebellum and brainstem. The inducible isoform is instead present in very small amounts and is localized in sparse groups of neurons, including the ventromedial and paraventricular nuclei of the hypothalamus (78). This finding indicates that the activation of HO-1 and the following formation of CO can be induced by many noxious stimuli within the nuclei that are primarily involved in the central regulation of the stress response. In fact neurons located within the parvicellular part of the paraventricular nucleus release both corticotropin releasing hormone (CRH) and arginin vasopressin (AVP), the neuropeptides that initiate the endocrine response to a stressor stimulating the release of pituitary adrenocorticotropin hormone (ACTH). Heme oxygenase-1 is also found in cells of glial lineage, where its gene expression is induced by oxidative stress (78). In 1997, Mahin Maines and her group described a third HO isoform called HO-3, as a protein of 33 kDa encoded by a single transcript of 24 kb and constitutively expressed in rat liver, spleen, kidney and brain (87). In a very interesting paper Scapagnini *et al*. investigated the regional brain expression of HO-3 and found that this isoform is expressed mainly in astrocytes of hippocampus, cerebellum and cortex (82). The regulation of HO-3 gene expression and its synthesis is poorly understood and its possible role in physiology and pathology remains to be further clarified.

6.2. Heme oxygenase-1, oxidative stress and neurodegenerative disorders

The mechanism(s) responsible for neuronal death is(are) not completely elucidated, even if many studies suggest that ROS are primarily involved in the genesis of neurodegenerative disorders (11,12,88-90). As far as the contribution of HO-1 in neurodegeneration, there is not a consensus in literature. In fact, it is no matter of question that HO-1 is neuroprotective, but there is evidence of a detrimental effect of this enzyme in neural tissues probably due to the possible toxic effects of CO and iron (91). Due to its strong antioxidant properties and wide distribution within the CNS, HO-1 has been proposed as a key enzyme in the prevention of brain damage (22,78,92). In a very interesting study (93), Panahian *et al*. using transgenic mice over-expressing HO-1 in neurons, demonstrated the neuroprotective effect of this enzyme in a model of ischemic brain damage. The neuroprotective effects of over-expressed HO-1 can be attributed to: (*i*) increase in cGMP and bcl-2 levels in neurons; (*ii*) inactivation of p53, a protein involved in promoting cell death; (*iii*) increase in antioxidant sources and (*iv*) increase in the iron sequestering protein, ferritin (93). Particularly interesting is the role played by HO-1 in AD, a neurodegenerative disorder which involves a chronic inflammatory response associated with both oxidative brain injury and beta-amyloid associated pathology. Significant increases in the levels of HO-1 have been observed in AD brains in association with neurofibrillary tangles and also HO-1 mRNA was found increased in AD neocortex and cerebral vessels (94,95). HO-1 increase was not only in association with neurofibrillary tangles, but also co-localized with senile plaques and glial fibrillary acidic protein-positive astrocytes in AD brains (96). In addition Takeda *et al*. explored the relationship between HO-1 and *tau* protein, this latter being the major component of intraneuronal neurofibrillary tangles in AD. In transfected neuroblastoma cells overexpressing HO-1, the activity of this enzyme was increased, and conversely, the level of *tau* protein was significantly decreased when compared with antisense HO-1 or vector transfected cells (94). The suppression of *tau* protein expression was almost completely counteracted by zinc-deuteroporphyrin, a specific inhibitor of HO activity (94). The activated forms of ERKs (extracellular signal-regulated kinases) were also decreased in cells overexpressing HO-1 although no changes in the expression of total ERKs were observed (94).

Taken together all these findings do not allow to single out a product of HO activity as the main neuroprotective factor; rather a complex puzzle of regulatory interactions between heme degradation products and cellular pathways involved in cell death/survival is hypothesized.

The protective role played by HO-1 in AD raised new possibilities regarding the possible use of natural substances, which are able to increase HO-1 levels, as potential drugs for the treatment of AD. In this light, very promising are the phenolic compounds contained in some herbs and spices, *e.g.* curcumin (97-99). Curcumin is the active anti-oxidant principle in *Curcuma longa*, a colouring agent and food additive commonly used in Indian culinary preparations (Figure 3). This polyphenolic substance has the potential to inhibit lipid peroxidation and to effectively intercept and neutralize ROS and RNS (100). In addition, curcumin has been shown to significantly increase HO-1 in astrocytes and vascular endothelial cells (99,101). This latter effect on HO-1 can explain, at least in part, the strong antioxidant properties of curcumin, in particular keeping in mind that HO-1-derived BR has the ability to efficiently scavenge both ROS and RNS (102-107). Epidemiological studies suggested that curcumin, as one of the most prevalent nutritional and medicinal compounds used by the Indian population, is responsible for the significantly reduced (4.4- fold) prevalence of AD in India compared to United States (108). Based on these findings, Lim and colleagues have provided convincing evidence that dietary

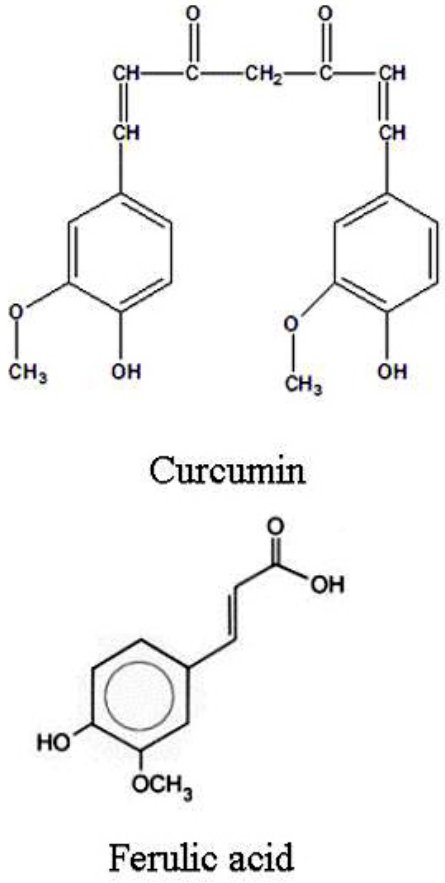

Figure 3. Chemical structures of the phenolic compounds curcumin and ferulic acid.

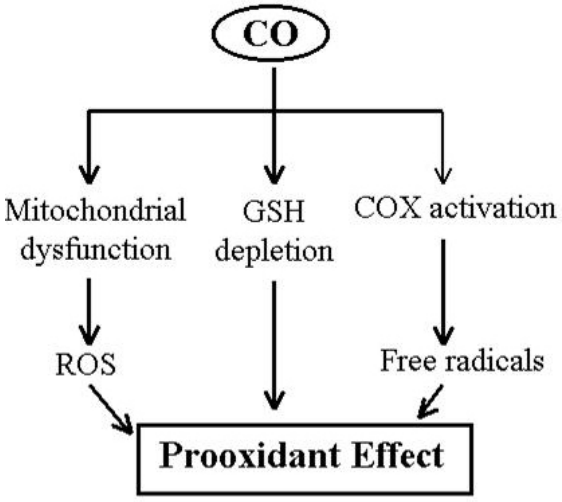

Figure 4. Cellular pathways involved in the prooxidant effects of carbon monoxide. Carbon monoxide (CO) causes mitochondrial dysfunction, reduction in the intracellular GSH content and activates cyclooxygenase (COX). This latter is a well known free radical-producing enzyme. All these mechanisms can trigger oxidative stress.

curcumin, given to an Alzheimer transgenic APPSw mouse model (Tg2576) for 6 months, resulted in a suppression of indices of inflammation and oxidative damage in the brain of these mice (109). Furthermore, in a human neuroblastoma cell line it has recently been shown that curcumin inhibits NFkappaB activation, efficiently preventing neuronal cell death (100).

Ferulic acid (FA) is another phenolic compound (Figure 3) and a major constituent of fruit and vegetables with strong antioxidant and antinfammatory properties. Recently, it has been demonstrated that FA ethyl ester (FAEE), naturally occurring and a more hydrophobic form of FA, protects synaptosomal membrane system and neuronal cell culture systems against hydroxyl and peroxyl radical oxidation (110,111) as well as mice against beta-amyloid-induced microglial activation (112). Other than this direct antioxidant property, FAEE has been shown to increase HO activity either in rat astrocytes or neurons (97) thus corroborating the hypothesis that HO activation is a common pathway through which phenolic compounds can exert neuroprotective effects (see paragraph 7.1).

6.3. Carbon monoxide, mitochondria and cellular stress response

Carbon monoxide (CO) is the gaseous product of HO activity and it has been found to play a role in several biological phenomena, including hippocampal long-term potentiation, non-adrenergic non-cholinergic gastrointestinal relaxation and vasodilatation, and is currently regarded as a neuromodulator in the peripheral and central nervous system (for a review on CO and its functions in the nervous system see refs. 78 and 113).

A direct connection between CO, mitochondrial impairment and brain damage has been postulated. Rat exposed to exogenous CO exhibited an increased production of H_2O_2 in forebrain mitochondria, probably due to an oxidative damage at the cytochrome oxidase level, together with a reduction in GSH/GSSG *ratio* (114,115). These results have been confirmed by recent studies which reported that CO, either released by CO-releasing molecules (CO-RMs) or produced directly following HO-1 induction, inhibited mitochondrial respiratory chain in renal mitochondria and cultured smooth muscle cells (116,117). As a consequence, an increase in the intracellular content of mitochondria-derived ROS was observed. This result, along with the inhibitory effect of this gas on NAD(P)H oxidase, another enzyme involved in the redox regulation of the intracellular *milieu*, can explain the antiproliferative effects of CO seen in different cell lines (117). The overproduction of mitochondrial ROS following CO exposure together with the evidence that this gas is able to induce HO-1 protein (118) and acutely reduce the intracellular GSH content, allowed to hypothesize a prooxidant, other than antioxidant effect of this gas (Figure 4). These prooxidant effects of CO are in good agreement with previous data obtained in rat brain. In fact, *in vitro* and *in vivo* studies suggested that the HO-CO pathway is involved in the modulation of the neuroendocrine mechanism of stress. Thus, increased CO generation is clearly associated with the inhibition of K^+ stimulated arginine vasopressin (AVP) and

oxytocin release from rat hypothalamic explants, whereas the inhibition of HO activity significantly potentiates the LPS-induced increase in AVP circulating levels while reducing the hypothalamic content of this neuropeptide (119-121). With regards to corticotropin-releasing hormone (CRH), the effects of CO on the release of this hormone are contradictory, since increases in CO generation induced by two HO substrates, hematin and hemin, were associated with reduced or enhanced CRH release respectively, in two different *in vitro* models (122,123). Therefore, the ability of this gas to blunt the increase of CRH and AVP in response to different stimuli (thus reducing the production and release of glucocorticoid by the adrenal gland), put CO in the "pro-inflammatory" molecules arena.

Further evidence regarding the proinflammatory role of CO is provided by studies about the induction of stress fever in rat. Papers by Luiz Branco's group clearly demonstrated that CO is a mediator of the pyrogenic response to stressors. In support of this hypothesis, the administration of HO inhibitors decreases LPS-induced fever in the rats (124,126), while heme overload causes a rise in body temperature (124-126). In the light of these findings, Steiner *et al*. studied the relationship between HO-CO system and the physico-emotional stress using the experimental system of restraint-induced stress in the rats. The authors showed that i.c.v. administration of the HO inhibitor Zn-deuteroporphyrin-bis-glycol does not affect the body temperature of euthermic rats, but markedly attenuates the restraint-induced fever (127). These results confirm that HO-CO pathway is not involved in the tonic regulation of body temperature but when this enzyme is activated by a specific stimulus CO becomes a pyrogenic neuromodulator.

It is generally agreed that CO exerts its intracellular actions by activating the cytosolic form of guanylyl cyclase (sGC) which in turn increases intracellular cGMP levels (22). However, during the last ten years many studies in the literature reported that CO signals through the activation of alternative intracellular signal transduction pathways. *In vitro* evidence suggested that the activation of another hemoprotein, COX, plays a significant role in CO signaling in the rat hypothalamus. In these studies it has been demonstrated that hemin, the precursor of CO *via* HO, dose-dependently increases PGE_2 production from rat hypothalamus *in vitro* and this effect is specifically due to CO because it is counteracted by the HO inhibitor Sn-mesoporphyrin-IX and oxyhemoglobin, the latter being a well known scavenger for CO (128). The direct evidence about the stimulatory role of CO on PGs production was obtained incubating hypothalami directly in CO saturated solutions and measuring significantly increased PGE_2 levels with respect to control tissue (129). This relationship between CO and increased COX activity, once more, strengthens the hypothesis of a proinflammatory role of this gas in the brain (Figure 4).

7. HEAT SHOCK PROTEIN 70

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).

Only recently, the availability of transgenic animals and gene transfer allowed to over-express the gene encoding for Hsp70, thus demonstrating that overproduction of this protein leads to protection in several different models of nervous system injury (130,131). Following focal cerebral ischemia, Hsp70 mRNA is synthesized in most ischemic cells except in areas of very low blood flow, due to scarce ATP levels. Hsp70 proteins is 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 (132).

7.1. Hsp70 and neurodegeneration

As mentioned above, Hsps with cytoprotective function are induced in many neurodegenerative disorders. Hsp72 was overexpressed in *post-mortem* cortical tissue of AD patients and an increase in Hsp70 mRNA was found in cerebellum, hippocampus and cortex of AD patients during the agonal phase of the disease (133-135). Recently Kakimura *et al*. demonstrated that Hsp70 induces IL-6 and TNF-alpha in microglial cells and this event is associated with an increased phagocytosis and clearance of amyloid-beta peptides (136). The same authors hypothesize that Hsps could activate microglial cells through a NFkappaB and p-38 MAPK-dependent pathways (136).

A large body of evidence now suggest a correlation between mechanisms of nitrosative stress and Hsps induction. We have demonstrated in astroglial cell cultures that cytokine-induced nitrosative stress is associated with an increased synthesis of Hsp70 stress proteins. The molecular mechanisms regulating the NO-induced activation of heat-shock signal seems to involve cellular oxidant/antioxidant balance, mainly represented by the glutathione status and the antioxidant enzymes (18,19).

Induction of Hsp72 under stress conditions is often accompanied by the induction of other heat shock proteins and they act in concert to protect neuronal cells from oxidative damage. This paradigm has been recently confirmed in a very interesting study by Sultana *et al* (137). These authors demonstrated that FAEE, protects cortical neurons from beta-amyloid toxicity by acting at three different levels: (*i*) inducing HO-1 and Hsp72 proteins, (*ii*) decreasing the neuronal 3-nitrotyrosine levels and, therefore, inducible NOS activity; and (*iii*) by the well known direct free radical quenching activity (137) (Figure 5). These data provide consistent evidence that a profound interplay between Hsps exists and further sustain the importance of Hsps in mediating neuroprotective effects.

8. CONCLUSIONS

Modulation of endogenous cellular defense mechanisms *via* stress response signaling represents an

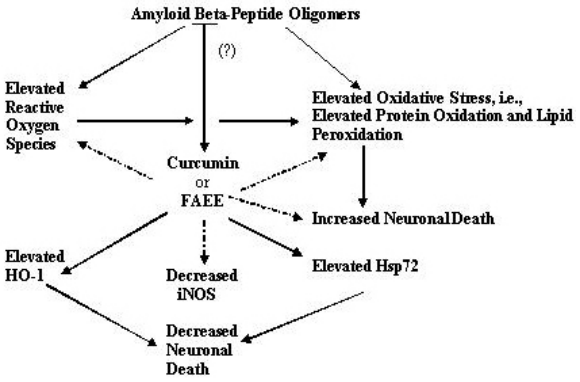

Figure 5. Proposed mechanism(s) through which phenolic compounds can exert neuroprotective effects. Either curcumin or ferulic acid ethyl ester (FAEE) can activate many pro-survival pathways such as Hsps system (HO-1 and Hsp-72) as well as reduce iNOS levels. The global effect is an increase in neuronal survival. Straight arrows: activation/induction; dotted arrows: inhibition. (?): Possible interaction, but not yet proven.

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 can be possible to delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy life span (3,4,138). Alzheimer disease is one of the most important neurodegenerative disorders and it is characterized by a progressive 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 (98,139). This will pose an immense economic and personal burden on the people of this country. There is now strong evidence to suggest that factors such as oxidative stress, mitochondrial dysfunction and disturbed protein metabolism and their interaction in a vicious cycle are central to AD pathogenesis. Brain-accessible antioxidants, potentially, may provide the means of implementing this therapeutic strategy of delaying the onset of AD, and more in general all degenerative diseases associated with oxidative stress (97). Unfortunately, the very promising results obtained in *in vitro* studies regarding the potential benefits of NSAIDs in the prevention or delay of AD were not confirmed by large clinical trials (51,52). On the contrary, natural substances such as curcumin, FA and its derivatives, vitamin A and vitamin E prove to be much more efficient in counteracting this neurodegenerative disorder. In fact, clinical studies suggested a decreased risk and a diminished oxidative stress in AD in persons taking high dietary dose or perhaps supplemental vitamin E (and vitamin C to regenerate vitamin E from the tocopherol radical). If proven to be true by additional studies, vitamin E or newer analogues of this antioxidant may prove beneficial in this dementing disorder associated with oxidative stress (13,14). Conceivably, dietary supplementation with vitamin E or with phenolic agents, such as curcumin and FAEE, can forestall the development of AD, consistent with a major "metabolic" component to this disorder. Nutritional biochemical research is providing optimism that this devastating brain disorder of aging may be significantly delayed and/or modulated.

With regard to the HO-1, its contribution in the development of neurodegenerative disorders is still under debate. Although there is no doubt about the effect of this enzyme in preventing neurodegenerative disorders, primarily by degrading prooxidant heme, it is important to isolate the contribution of its product CO and BR. In particular, the ability of this gas to (*i*) increase intracellular mitochondrial-derived ROS and COX-derived free radicals, (*ii*) induce HO-1 and acutely reduce intracellular GSH content and (*iii*) blunt the increase of ACTH secretagogues such as CRH and AVP in response to different stimuli, suggest a prooxidant and proinflammatory role of CO (Figure 4). This hypothesis is in contrast with other data which show the antioxidant and antinflammatory role of CO in several tissues and cell lines through the modulation of intracellular signaling pathways including the mytogen activated protein kinase (MAPK) system (140-143). It is our opinion that, to date, it is not possible to give any "label" to CO, but the anti- *versus* pro-inflammatory features of this gas strictly depend on the cell type, tissue and function regulation. On the other hand BR, the product of the HO/BVR activities, is a very important endogenous antioxidant in situations involving either oxidative stress or nitrosative stress (102-107). The ability of BR to scavenge not only ROS but also NO and its congeners is quite important in the brain, because brain lacks BR conjugating enzymes, allowing the bile pigment to accumulate from neuronal HO-2 activity (144,145). This finding together with the evidence that neurons have relatively low concentrations of GSH (146,147), a tripeptide involved in detoxification of ROS and very abundant in almost all mammalian tissues, suggests a role of BR as a valid endogenous antioxidant molecule in neurons (105,148). Keeping in mind all these results, it is reasonable to conclude that the overall effect of HO-1 stimulation in neuronal death/survival is the result of the balance between negative factors such as the release of CO and excess iron and positive factors including the heme degrading activity, production of antioxidant BR and stimulation of cellular pathways involved in cell survival (bcl-2, sGC).

9. PERSPECTIVE

Protein conformational diseases, such as Alzheimer's, Parkinson's and Huntington's, affect a large portion of our aging population. Cells have evolved mechanisms for rescuing and recycling misfolded proteins, but these systems are not perfect. Chaperones can rescue misfolded proteins by breaking up aggregates and assisting in the refolding process. Proteins that cannot be rescued by refolding can be delivered to the proteasome by chaperones

to be recycled. In general, conformational diseases are conditions that arise from the dysfunctional aggregation of proteins in non-native conformations. This often is associated with multiple metabolic derangements that result in the excessive production of ROS and oxidative stress. These ROS set in motion a host of redox reactions which can result in unstable nitrogen and thiol species that contribute to additional redox stress. The ability of a cell to deal with ROS and oxidative stress requires functional chaperones, antioxidant production, protein degradation and a cascade of intracellular events collectively known as the unfolded protein response, a form cell stress response. It is known that the beta conformation in proteins is particularly susceptible to perturbations in the quality control system and that ROS play an important role in the development and/or pathogenetic progression in aging and neurodegenerative diseases Oxidative stress and increased ROS production contribute to endoplasmic reticulum stress, protein misfolding, and induction of the unfolded protein response. As the cell's quality control system becomes overwhelmed, conformational changes occur to amyloid polypeptide intermediates, generating stable oligomers with an anti-parallel crossed beta-pleated sheet structure that eventually accumulate as space-occupying lesions within neurons. Anfinsen showed that a protein's fold is specified by its sequence. Although it is clear why mutant proteins form amyloid, it is harder to rationalize why a wild-type protein adopts a native conformation in most individuals, but it misfolds in a minority of others, in what should be a common extracellular environment. This discrepancy suggests that another event likely triggers misfolding in sporadic amyloid disease. One possibility is that an abnormal metabolite, generated only in some individuals, covalently modifies the protein or peptide and causes it to misfold. Candidate metabolites are suggested by the recently recognized links between AD and atherosclerosis, in which known chronic inflammatory metabolites, may play a critical pathogenic role. If this holds true, then new targets are disclosed for a prevention strategy brought about through nutritional antioxidants.

Presented here is strong evidence that a crosstalk between stress response genes is critical for cell stress tolerance, highlighting compelling reason for a renewed effort to understand the central role of this most extraordinary defence 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 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. Hence, by maintaining or recovering the activity of vitagenes can be possible to delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy life span.

10. ACKNOWLEDGEMENTS

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