CHAPTER 4

Heme Oxygenase as a Therapeutic Funnel in Nutritional Redox Homeostasis and Cellular Stress Response:

Role of Acetylcarnitine

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Abstract

Reduction of cellular expression and activity of antioxidant proteins and the consequent

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counteract oxidative stress.[1-3] Within the cell, reactive oxygen species (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.[4-7] 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 lipids.[8-10] Oxidative stress has been implicated in mechanisms leading to neuronal cell injury in various pathological states of the brain, including neurodegenerative disorders such as Alzheimer's disease (AD).[11-15]

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 can be indicated as reactive nitrogen species or RNS).[16-18]

From a molecular point of view, the cell is able to fight against oxidant stress using many resources, including vitamins (A, C and E), bioactive molecules (glutathione, thioredoxin,

Figure 1. Redox regulation of gene expression involving Acetylcarnitine and the Vitagene system. Proposed role for acetylcarnitine and the *vitagene* member HSPs, in modulation cellular redox state and cell stress tolerance. Various proteotoxic (or genotoxic) conditions cause depletion of free HSPs that lead to activation of stress kinase and proinflammatory and apoptotic signaling pathways. HSP70 prevents stress-induced apoptosis by interfering with the SAPK/JNK signaling and by blocking caspase proteolytic cascade. Nitrosative-dependent thiol depletion triggers HO-1 induction, and increased HO-1 activity is translated into augmented production of carbon monoxide and the antioxidant bilirubin. Exogenous non toxic inducers, such as acetylcarnitine or polyphenols can counteract increased NOS activity and NO-mediated cytotoxicity through up-regulation of the HO system. HO-1 may directly decrease NO synthase protein levels by degrading the cofactor heme. (PLA_2: phospholipase A_2; IL-6: interleukin-6; AP-1: activator protein-1; SAPK: stress-activated protein kinase; JNK: c-jun N-terminal kinase; NFκB: nuclear factor kappa-B; GSNO: S-nitrosoglutathione; HO-1: heme oxygenase-1).

that confers inducible expression of *ho-1* in response to numerous and diverse conditions has remained elusive. One important clue has recently emerged from a detailed analysis of the transcriptional regulatory mechanisms controlling the mouse and human *ho-1* genes. The induction of *ho-1* is regulated principally by two upstream enhancers, E1 and E2.[31] 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)[32] with a consensus sequence (GCnnnGTA) similar to that of other antioxidant enzymes.[33] There is 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.[32] 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,[31] random interaction of activators with the *ho-1* enhancers would be expected to cause spurious expression. This raises a paradox as to how cells reduce transcriptional noise from the *ho-1* locus in the absence of metabolic or environmental stimulation. This problem could be

reconciled by the activity of repressors that prevent nonspecific activation. One possible candi-

The promoter region also contains two metal responsive elements, similar to those found in metallothionein-1 gene, which respond to heavy metals (cadmium and zinc) only after recruitment of another fragment located upstream, between -3.5 and 12 kbp (CdRE). In addition, a 163-bp fragment containing two binding sites for HSF-1, which mediates the HO-1 transcription are located 9.5 kb upstream of the initiation site.[34] The distal enhancer regions are important in regulating HO-1 in inflammation, since, as has been demonstrated, 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.[35,36]

HO-1, Oxidative Stress and Neurodegenerative Disorders

The mechanisms responsible for neuronal death are not completely elucidated, even if many studies suggest that ROS are primarily involved in the genesis of neurodegenerative disorders.[11-15,37-39] 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.[21,22,40] Recently, Panahian et al using transgenic mice over-expressing HO-1 in neurons, demonstrated the

because rodent cells over-expressed HO-1 when exposed to the same stimuli.[54-57] The importance of HO-1 repression has been corroborated by the discovery of Bach-1/Bach-2 as heme-regulated transcription factors for the HO-1 gene.[58] In fact, Bach-1 is broadly expressed in mice and human tissues and, in human cells, it is induced by the same stimuli which are able to repress the HO-1 gene.[54,59-61] The reason why the cell should react to an oxidant stress by repressing HO-1 gene is strictly related to the maintenance of a good metabolic balance during stressful conditions. The current hypothesis suggests that HO-1 repression is useful for the cell because this (i) decreases the energy costs necessary for heme degradation; (ii) reduces the accumulation of CO and BR, which can become toxic if produced in excess; and (iii) increases the intracellular content of heme necessary for the preservation of vital functions such as respiration and defense.[60]

Carbon Monoxide and Stress Response

Carbon monoxide (CO) is the gaseous products of HO and it has been found to play a role in several biological phenomena, including hippocampal long-term potentiation,

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 GRP-75 (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.[70,71] 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 are produced mainly in endothelial cells, in the core of infarcts in the cells that are most resistant to ischemia, in glial cells at the edges of infarcts and in neurons outside the areas of infarction[72] It has been suggested that this neuronal expression of Hsp70 outside an infarct can be used to define the ischemic penumbras, i.e., the zone of protein denaturation in the ischemic areas.[72]

As mentioned above, Hsps are induced in many neurodegenerative disorders mainly in the view of its cytoprotective function. 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.[73-75] Recently Kakimura et al[76] demonstrated that Hsp70 induces IL-6 and TNF-α in microglial cells, and this event is associated with an increased phagocytosis and clearance of Aβ peptides. The same authors hypothesize that Hsps could activate microglial cells through NFkB and p-38 MAPK-dependent pathways.[76]

A large body of evidence now suggest a correlation between mechanisms of nitrosative stress and Hsp 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.[77,78]

Acetylcarnitine

Mitochondria are cellular organelles involved in many metabolic processes such as pyruvate oxidation, the tricarboxylic acid cycle, fatty acid β-oxidation and are the common final pathway of oxidative phosphorylation, which generates most of the cellular energetic source, ATP. It has been proposed that accumulation of mitochondrial DNA (mtDNA) during life is a major cause of age-related disease and this is because of its high mutagenic propensity. The lack of introns and protective histones, limited nucleotide excision and recombination DNA repair mechanisms, location in proximity of the inner mitochondrial membrane which exposes mtDNA to an enriched free radical milieu, are all factors contributing to a 10-fold higher mutation rate occurring in the mtDNA than in the nuclear DNA. Relevant to mitochondrial bioenergetics, in fact, is the finding of a significant decrease in state 3/state 4 ratio, which has been observed to occur in brain as function of age.[79] Since this respiratory control ratio relates to the coupling efficiency between electron flux through the electron transport chain and ATP production, an increase in state 4 would result in a more reductive state of mitochondrial complexes and, consequently, to an increase in free radical species production. A decrease in state 3/state 4 respiration during aging has been found associated with a significant decrease in cardiolipin content in brain mitochondria.[80] This loss could play a critically important role in the age-related decrements in mitochondrial function, and appears to be associated with both quantitative and qualitative region-specific protein changes, which are parallel to structural changes, such as decrease of the inner membrane surface, smaller as well as sparser cristae, decreased fluidity and increased fragility. Modifications in cardiolipin composition are recognized to accompany functional changes in brain mitochondria, which include all proteins of the inner mitochondrial membrane that generally require interaction with cardiolipin for optimal catalytic activity.[81]

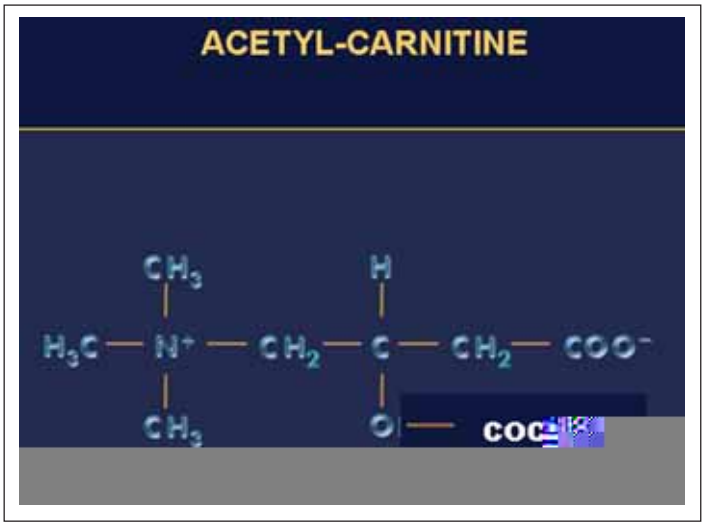

Figure 3. Chemical structure of Acetyl-L-carnitine.

Acetylcarnitine (LAC) (Fig. 3) is an ester of the trimethylated amino acid, L-carnitine, and is synthesised in the brain, liver, and kidney by the enzyme LAC-transferase. LAC facilitates the uptake of acetyl-CoA into the mitochondria during fatty acid oxidation, enhances acetylcholine production, and stimulates protein and membrane phospholipid synthesis.[82] At present, studies have shown that LAC is a compound of great interest for its wide clinical application in various neurological disorders: it may be of benefit in treating Alzheimer's dementia, chronic fatigue syndrome, depression in the elderly, HIV infection, diabetic neuropathies, ischemia and reperfusion of the brain, cognitive impairment of alcoholism, aging.[83-85] The neuroprotective benefits of this compound have been observed in the hippocampus, prefrontal cortex, substantia nigra and muscarinic receptor portions of the brain.[86] These benefits include antioxidant activity, improved mitochondrial energetics, stabilization of intracellular membranes and cholinergic neurotransmission.[87] Promising therapeutic applications of LAC are derived from observations that this compound crosses the blood-brain barrier through a saturable process in a sodium-dependent manner and improves neuronal energetic and repair mechanisms, while modifying acetylcholine production in the CNS.[88] LAC treatment restores the altered neurochemical abnormalities, cerebral energy metabolites in ischemia and aging and, in particular, ammonia-induced cerebral energy depletion.[87] In addition, it increases the responsiveness of aged neurons to neurotrophic factors in the CNS and it has preventive and corrective effects on diabetic neuropathology. Its beneficial effects have been also observed on EEG, evoked potentials and long-term synaptic potentiation in aged animals.[89] Moreover, LAC is commonly used also for the treatment of painful neuropathies: it exerts a potent analgesic effect by up-regulating metabotropic glutamate receptors.[90] There are experimental data that LAC improves memory function in Alzheimer's patients and it influences attention, learning and memory in the rat.[91] Chronic treatment enhances spatial acquisition in a novel environment of rats with behavioral impairments and has a slight effect on retention of the spatial discrimination in a familiar environment.[92] More recently, it has been observed that LAC produces sustained changes of nonassociative learning of sensitization and dyshabituation type in the invertebrate *Hirudo medicinalis*, and it has been suggested that LAC might exerts its effects by means of new protein synthesis, through qualitative and quantitative changes of gene expression. Furthermore, recent evidences have reported that LAC influences expression of glyoxylase 1, a gene involved in

oxidative stress and disturbed protein metabolism and their interaction in a vicious cycle are central to AD pathogenesis. Brain-accessible antioxidants, potentially, may provide the means of implementing this therapeutic strategy of delaying the onset of AD, and more in general all degenerative diseases associated with oxidative stress.[47,102] As one potentially successful approach, potentiation of endogenous secondary antioxidant systems can be achieved by interventions which target the HO-1/CO and/or Hsp70 systems. In this review, the importance of the stress response signaling and, in particular, the central role of HO-1 together with the redox-dependent mechanisms involved in cytoprotection are outlined. The beneficial effects of HO-1 induction result from heme degradation and cytoprotective regulatory functions of biliverdin/bilirubin redox cycling. Thus, HO-1 can amplify intracellular cytoprotective mechanisms against a variety of insults. Consequently, induction of HO-1, by increasing CO and/or biliverdin availability can be of clinical relevance.

Very importantly, HO-1 and CO can suppress the development of atherosclerotic lesions associated with chronic rejection of transplanted organs.[103] Consistently, LAC, as a molecule endowed with the capability of potentating the cellular stress response pathways, appears to afford similar protective action, thereby providing an alternative therapeutic approach valuable for all those pathophysiological conditions where stimulation of the HO pathway becomes a primary target.

Presented here is strong evidence that a crosstalk between stress response genes is critical for cell stress tolerance, highlighting compelling reasons for a renewed effort to understand the central role of this most extraordinary defense system in biology and medicine. All of the above evidence also supports the notion that stimulation of various maintenance and repair

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