CHAPTER SIX

PRACTICAL APPROACHES TO INVESTIGATE REDOX REGULATION OF HEAT SHOCK PROTEIN EXPRESSION AND INTRACELLULAR GLUTATHIONE REDOX STATE

Vittorio Calabrese,* Anna Signorile,† Carolin Cornelius,* Cesare Mancuso,‡ Giovanni Scapagnini,§ Bernardo Ventimiglia,‖ Nicolo’ Ragusa,* and Albena Dinkova-Kostova

Contents

1. Introduction 84

2. Nitric Oxide and Cellular Stress Response: Role of Vitagenes 87

2.1. The vitagene system 87

2.2. Heme oxygenase: Regulation by nitrosative stress 88

2.3. Hsp70 and nitrosative stress 90

2.4. Induction of vitagenes by small molecules via the Keap1/Nrf2/ARE pathway 91

3. Material and Methods 93

3.1. Chemicals 93

3.2. Cell cultures and treatment 93

3.3. Western blot analysis of heme oxygenase-1, Hsp70, protein carbonyls, and 4-hydroxy-nonennals 94

3.4. Preparation of nuclear extract and Western blot for Nrf 2 94

3.5. Real-time quantitative polymerase chain reaction 95

3.6. Total reduced and oxidized glutathione assay 96

3.7. Determination of reduced glutathione and oxidized glutathione in the cytosol 96

3.8. Determination of total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) in mitochondria 97

* Department of Chemistry, Clinical Biochemistry and Clinical Molecular Biology Chair, University of Catania, Italy
† Department of Biochemistry, University of Bari, Italy
‡ Institute of Pharmacology, Catholic University School of Medicine, Roma, Italy
§ Department of Health Sciences, University of Molise, Campobasso, Italy
* Department of Science of Senescence, Urology and Neuro-Urology, University of Catania, Italy
‖ Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Scotland, UK
and Department of Pharmacology and Molecular Sciences and Department of Medicine, Johns Hopkins University, Baltimore, MD, USA.
3.9. Determination of protein 98
3.10. Statistical examination 98
4. Results 98
5. Discussion 99
Acknowledgment 103
References 104

Abstract
The products of vitagenes such as heat shock protein 32 (Hsp32, heme oxygenase 1) and Hsp70, the family of inducible cytoprotective proteins regulated by the Keap1/Nrf2/ARE pathway, and small molecule antioxidants such as glutathione provide the cell with powerful means to counteract and survive various conditions of stress. Among these protective systems, the heat shock proteins represent a highly conserved and robust way for preservation of correct protein conformation, recovery of damaged proteins, and cell survival. Their regulation is dependent on the redox status of the cell, thus redox regulation is rapidly evolving as an important metabolic modulator of cellular functions, and is being increasingly implicated in many chronic inflammatory and degenerative diseases. Protein thiols play a key role in redox sensing, and regulation of cellular redox state is crucial mediator of multiple metabolic, signalling and transcriptional processes in the brain. Nitric oxide, and reactive nitrogen species induce the transcription of vitagenes and Keap1/Nrf2/ARE-dependent genes whose functional products protect against a wide array of subsequent challenges. Emerging interest is now focusing on exogenous small molecules that are capable of activating these systems as a novel target to minimize deleterious consequences associated with free radical-induced cell damage, such as during neurodegeneration. This chapter describes methods that can be used to assess the expression of heat shock proteins and the cellular glutathione redox status and discusses their relevance to mechanisms modulating the onset and progression of neurodegenerative diseases.

1. Introduction
The importance of oxidative stress in the pathogenesis of several diseases, such as neurodegenerative disorders, diabetes, and atherosclerosis, has been well established (Kaneto et al., 2007; Madamanchi and Runge, 2007; Mancuso et al., 2007). Reactive oxygen species (ROS), which are generated as a result of a number of physiological and pathological processes (Bellomo et al., 2006), include superoxide anion ($O_2^{-}$), hydroxyl radical ($OH^*$), singlet oxygen ($^1O_2$), and hydrogen peroxide ($H_2O_2$) (Bergamini et al., 2004). Each of these ROS is highly reactive and unstable because of an unpaired electron in their outer electron shell (Bergamini et al., 2004). This increased reactivity promotes the ability of ROS to interact rapidly with cellular macromolecules such as proteins, lipids, and nucleic acids...
If there is an increased level of ROS, as in the case of inflammation, or the intracellular level of antioxidant molecules is low, oxidative stress damage occurs. As a result of oxidative stress, protein, lipid, and DNA oxidation are common features. Oxidative stress can ultimately induce neuronal damage, modulate intracellular signaling, and lead to neuronal death by apoptosis or necrosis (Loh et al., 2006). Interestingly, the propensity or sensitivity of cells to oxidative stress appears to be cell type specific, with cells exhibiting dramatic differences with regards to their sensitivity to accumulate oxidized molecules and cope with toxicity during periods of high ROS exposure. The basis for this cell type specificity is poorly understood but is clearly an important topic for aging, hepatic, cardiovascular, cancer, and neuroscience research.

There are several mechanisms by which ROS may be generated, including aerobic respiration, nitric oxide synthesis, and NADPH oxidase pathways during inflammation. In aerobic respiration, the mitochondrial respiratory chain produces ROS as it transfers electrons during the reduction of molecular oxygen to water. During this process, some electrons escape the electron transport chain and interact with oxygen to generate superoxide, hydrogen peroxide, or hydroxyl radical (Calabrese et al., 2007a; Mancuso et al., 2007; Papa et al., 2006a). Activated neutrophils can also release the enzyme myeloperoxidase, which produces the highly active oxidant hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions (Winterbourn et al., 2006). In addition to these biological mechanisms of ROS generation, there are also exogenous sources of free radicals, including pollutants, environmental toxins, cigarette smoke (Churg, 2003).

The nitric oxide synthase (NOS) enzymes produce nitric oxide (NO) via oxidation of the terminal guanidine nitrogen of L-arginine to L-citrulline (Calabrese et al., 2007b). NO is not highly reactive per se but can interact with other intermediates such as oxygen, superoxide, and transition metal generating products that affect the functionality of macromolecules. The term “nitrosative stress” has been used to indicate the cellular damage elicited by nitric oxide and its congeners peroxynitrite, N2O3, nitroxy lamin, and nitrosonium (all can be indicated as reactive nitrogen species or RNS) (Mancuso et al., 2006; Ridnour et al., 2004; Kroncke, 2003).

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 and flavonoids), enzymes [heat shock protein (HSP)-32, superoxide dismutase, catalase, glutathione peroxidases, thioredoxin reductase], and redox-sensitive protein transcriptional factors (AP-1, NFkB, Nrf2, HSF) (Calabrese et al., 2004a, 2007a; Mancuso et al., 2007) (Fig. 6.1). Heat shock proteins (Hsps) are one of the most studied defense system active against cellular damage. Consistently, integrated responses exist in the cells to detect and control diverse forms of stress. This is accomplished by a complex network of the so-called longevity assurance processes, which...
are composed of several genes termed vitagenes (Calabrese et al., 2004a, 2006a, 2007a; Mancuso et al., 2007). Among these, heat shock proteins form a highly conserved system responsible for the preservation and repair of the correct protein conformation. Paradoxically, damage to cells can engage one of two opposing responses: apoptosis, a form of cell death that removes damaged cells to prevent inflammation, and the heat shock or stress response that prevents damage or facilitates recovery to maintain cell survival. Studies have shown that the heat shock response contributes to establishing a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging, and neurodegenerative disorders. 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 (Calabrese et al., 2004a, 2007a; Mancuso et al., 2007). In this paper we describe recent methods employed to measure cellular stress response in a neuronal cell line, as well as the key role played by the heat shock response, particularly the heme oxygenase-1 (also referred to Hsp32) and Hsp70 pathways in brain stress tolerance. Increasing evidence underscores the high potential of the Hsp system as a target for new neuroprotective strategies, especially those aimed at minimizing deleterious consequences associated with oxidative stress, such as in neurodegenerative disorders and oxidative stress and oxidant disorders

Antioxidant vitamins
Polyphenols
Lipoic acid
Carnitine
Carnosine
GSH
Cytoprotective gene products
Antioxidant genes
Vitagenes
SOD Catalase GPx Hsp70 TRXr HO-1

Figure 6.1 Multiple intracellular pathways activated in the fight against oxidative stress. Under free radical attack, significant changes in the intracellular milieu occur and several intracellular pathways are activated. Among these, it is noteworthy to mention antioxidant vitamins (A, C and E), glutathione (GSH) and enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). A main role is also played by the vitagenes heat shock protein 70 (Hsp70) and heme oxygenase-1 (HO-1). All these intracellular systems, by acting in concert to modulate the pro-oxidant/antioxidant balance confer cytoprotection.
brain aging. This chapter also reviews evidence for the emerging role of redox-dependent mechanisms in the regulation of vitagenes in the brain as a potential mechanism to potentiate brain stress tolerance.

2. NITRIC OXIDE AND CELLULAR STRESS RESPONSE: ROLE OF VITAGENES

2.1. The vitagene system

The term vitagenes refers to a group of genes that are strictly involved in preserving cellular homeostasis during stressful conditions. The vitagene family is actually composed of the heat shock proteins Hsp32 and Hsp70 (Calabrese et al., 2004a, 2006a, 2007a; Mancuso et al., 2007) (Fig. 6.2). Among these genes, heme oxygenase-1 (HO-1), is receiving considerable attention because of its major role in counteracting both oxidative and nitrosative stress. In fact, HO-1 induction is one of the earlier events in the cell response to stress. Heme oxygenase-1 exerts a protective role by degrading the intracellular levels of the prooxidant heme and by producing

Cytokines, \( \beta \)-amyloid

\[
\text{ROS/RNS} \quad \text{Red/Ox}\quad \text{Hsp70} \quad \text{HO-1}
\]

\[
\text{Caspases NF-kB MAPK} \quad \text{ERKs} \quad \text{p38} \quad \text{CO} \quad \text{BV} \quad \text{BR} \quad \text{BVR}
\]

Figure 6.2  The adaptive stress response to oxidative/nitrosative stress and the role of vitagenes. In response to pro-oxidant stimuli, such as cytokines or \( \beta \)-amyloid, free radicals (FR) which include reactive oxygen species (ROS), nitric oxide (NO) and reactive nitrogen species (RNS), induce the expression and/or activity of both heat shock protein 70 (Hsp70) and heme oxygenase-1 (HO-1). Hsp70 inhibits caspasas and NFkB, while activating members of the MAPK family, such as ERKs and p38. Conversely, HO-1 converts heme into carbon monoxide (CO) and biliverdin (BV) which is then reduced by biliverdin reductase (BVR) into bilirubin (BR). This latter is a well known scavenger for FR thus allowing a negative feedback to prevent uncontrolled formation of ROS, NO and RNS. Notably, Hsp70 induces HO-1, thus potentiating cytoprotective action. Straight arrows, activation; dashed arrows, inhibition/scavenging.
biliverdin, the precursor of bilirubin; the latter is an endogenous molecule with powerful antioxidant and antinitrosative functions (Calabrese et al., 2007a; Mancuso et al., 2003, 2004, 2006a,b, 2007).

Heat shock protein 70 (Hsp70) is induced in the nervous system following a variety of oxidative injuries, including cerebral ischemia or neurodegenerative disorders (Calabrese et al., 2004a; Giffard et al., 2004). The intracellular mechanisms by which Hsp70 exerts neuroprotection are related to the inhibition of the NF-κB signaling pathway, as well as to the inhibition of both extrinsic and intrinsic apoptotic pathways (Chan et al., 2004; Feinstein et al., 1996; Matsumori et al., 2006). Furthermore, Hsp70 induction has been associated with activation of the ERK1/2 and p38 members of the MAPK system in the rat cerebellum and hippocampus (Maroni et al., 2003) (Fig. 6.2).

2.2. Heme oxygenase: Regulation by nitrosative stress

Heme oxygenase exists in two main isoforms: the inducible one (HO-1) and the constitutive one (HO-2). Heme oxygenase-1 is induced by several stimuli, including oxidative and nitrosative stress, heat shock, lipopolysaccharide, cytokines, inorganic metals, phenolic compounds (i.e., curcumin and ferulic acid) and the neuroprotective agent neotrofin (Fig. 6.2). In contrast, the constitutive HO-2 is responsible for physiologic heme metabolism and responds only to glucocorticoids. Interestingly, evidence suggests a novel role for HO-2 as an endogenous sensor for gaseous signaling molecules such as oxygen CO and NO (Calabrese et al., 2006a; Maines 2005, 1997; Mancuso 2004). The central nervous system is endowed with high HO activity under basal conditions, mostly accounted for by HO-2, with the latter being expressed in neuronal populations in forebrain, hippocampus, hypothalamus, midbrain, basal ganglia, thalamus, cerebellum, and brain stem. The inducible isoform is instead present in small amounts and is localized in sparse groups of neurons, including ventromedial and paraventricular nuclei of the hypothalamus (Maines, 1997; Mancuso, 2004). Heme oxygenase-1 is also found within cells of glial lineage, where its gene expression can be induced by oxidative stress (Mancuso, 2004). In 1997, Maines and her group described a third HO isoform called HO-3 (McCoubrey et al., 1997). In the brain, this isoform is constitutively expressed in astrocytes of the hippocampus, cerebellum, and cortex (Scapagnini et al., 2002). The regulation of HO-3 gene expression and its synthesis is not well understood and its possible role in physiology and pathology remains to be further clarified. With regard to modulation of HO by NO it is important to distinguish between the two HO isoforms and the tissues where this interaction occurs.

Nitric oxide and RNS induce the HO-1 gene and protein in different conditions by a mechanism not fully understood (Motterlini et al., 2002). However, taking into consideration the strong prooxidant activity of NO and RNS, it is plausible to conclude that HO-1 induction has to be considered
as a mechanism by which cells can react to stressful conditions. In fact, HO-1 induction by NO is important in selected cells, such as macrophages, for two reasons: first, because HO-1 activity depletes heme from cells; heme is toxic if in excess. Second, the production of bilirubin (BR) and CO through HO activity ensures an efficient scavenging of ROS and RNS and a further inhibition of NADPH-oxidase and inducible NOS (iNOS), thus contributing to the resolution of oxidative conditions (Srisook et al., 2005). In addition, peroxynitrite and nitroxyl anion have been shown to increase, in a dose-dependent manner, HO-1 expression in endothelial cells and human colorectal adenocarcinoma cells (Foresti et al., 1999; Hara et al., 1999; Motterlini et al., 2000; Naughton et al., 2002). In brain cells, NO has been shown to induce HO-1 expression in rat astrocytes and microglia (Kitamura et al., 1998b; Son et al., 2005), as well as in rat hippocampus (Kitamura et al., 1998a). Nitric oxide inhibits or stimulates HO activity, and this differential modulation depends on the tissue or cell line. In particular, studies carried out on endothelial or smooth muscle cells have shown that NO is able to increase HO activity (Durante et al., 1997; Motterlini et al., 1996; Sammut et al., 1998), whereas Willis et al. (1995, 1996) demonstrated that NO (released by sodium nitroprusside) reduced HO activity in rat brain and spleen homogenates. The reason for this dual effect of NO on HO activity was clearly explained by Maines (1997) on the basis of the chemical structure of NO: because of its free radical nature, NO can reduce HO activity either by inactivating proteins, in particular those rich in thiol groups such as HO-2, or by forming nitrosyl-heme, which prevents oxygen binding to HO, which is mandatory for its activation. By virtue of these actions, NO can reduce HO activity; this effect is particularly relevant in brain because of the abundance of neuronal HO-2. Meanwhile, the free radical nature of NO can induce HO-1 protein and HO activity, and this biochemical event is important in those cells (endothelial and smooth muscle cells) in which HO-1 is predominant. Furthermore, NO can regulate HO activity by modulating the activity of δ-aminolevulinic acid synthase, the rate-limiting enzyme in heme synthesis, or ferritin, the iron storage protein (Maines, 1997). Moreover, peroxynitrite and nitroxyl anion share with NO the dual effect on HO activity because the first has been shown to decrease HO activity in rat brain or spleen microsomal preparations (Kinobe and Nakatsu, 2004) and increase oxygenase activity in endothelial cells (Foresti et al., 1999), whereas the second increased HO activity in vascular cells (Naughton et al., 2002). Taken together, these data demonstrated that the role of NO and RNS in regulating HO activity strictly depends on the cell type and HO isoform. An interesting corollary emerged by these studies: it has been demonstrated that BR is able to interact with NO and RNS and, as a result of this interaction, formation of a N-nitrosated product of BR or the oxidized product BV occurs (Mancuso et al., 2003, 2006a,b). The ability of BR to scavenge NO and RNS is quite important in the brain. In fact, the brain lacks BR conjugating enzymes, allowing the bile pigment to accumulate from neuronal HO-2 activity.
(Ewing and Maines, 1992; Snyder and Barañano, 2001). This finding becomes more intriguing in light of the evidence that neurons have relatively low concentrations of glutathione (GSH) (Raps et al., 1989; Slivka et al., 1987; Sun et al., 2006), a tripeptide involved in the detoxification of ROS that is very abundant in almost all mammalian tissues; this suggests a possible role of BR as an alternative endogenous antioxidant molecule in neurons (Baranano and Snyder, 2001). Biliverdin shares with BR this scavenging effect, even if the biological importance of the BV–NO interaction is limited because of the rapid transformation of BV in BR by biliverdin reductase (BVR). Furthermore, even CO, the gaseous product of HO activity, inhibits NO-mediated vasodilation in the adult rat cerebral microcirculation, and this effect is probably a consequence of the photoreversible gas binding to the prosthetic heme of NOS (Ishikawa et al., 2005). Therefore, it is possible to hypothesize a negative feedback between HO products and NO/RNS: in this frame, CO, BV, and BR could act in concert to reduce an unnecessary stimulation of HO by NO (Fig. 6.2).

2.3. Hsp70 and nitrosative stress

The 70-kDa family of stress proteins is one of the most extensively studied. To this family belong the constitutive form or heat shock cognate (Hsc70), the inducible Hsp70 (also referred to as Hsp72), and a constitutively expressed glucose-regulated protein found in the endoplasmic reticulum (GRP75) (Calabrese et al., 2006a). After a variety of central nervous system (CNS) insults, Hsp70 is synthesized at high levels and is present in the cytosol, nucleus, and endoplasmic reticulum (Yenari et al., 1999). A high expression of Hsc70 was observed in neuronal populations in the dentate gyrus, CA1 and CA2 regions of the hippocampus, and Purkinje neurons of the cerebellum (Belay and Brown, 2006). Whether or not stress proteins are neuroprotective has been the subject of much debate, as it has been speculated that these proteins might be merely an epiphenomenon unrelated to cell survival. Only recently, however, with the availability of transgenic animals and gene transfer, has it become possible to overexpress the gene encoding Hsp70 to test directly the hypothesis that stress proteins protect cells from injury, and it has been demonstrated that overproduction of Hsp70 leads to protection in several different models of nervous system injury (Fink et al., 1997; Kelly et al., 2002; Narasimhan et al., 1996). A large body of evidence now suggests a correlation between mechanisms of oxidative and/or nitrosative stress and Hsp induction (Calabrese et al., 2002; Lai et al., 2005; Sultana et al., 2005). Current opinion also holds the possibility that the heat shock response can exert its protective effects through inhibition of the NF-κB signaling pathway (Heneka et al., 2000, 2001) (Fig. 6.2). We have demonstrated that cytokine-induced nitrosative stress in astroglial cell cultures is associated with an increased synthesis of Hsp70 stress proteins (Calabrese et al., 2000, 2005a). The molecular
mechanisms regulating the NO-induced activation of the heat shock signal seems to involve the cellular oxidant/antioxidant balance, mainly represented by the glutathione status and the antioxidant enzymes (Calabrese et al., 2000, 2004b). Induction of Hsp70 under stress conditions is often accompanied by the induction of other Hsps, which act in concert to protect neuronal cells from oxidative damage (Fig. 6.2). This paradigm has been recently confirmed in a recent study from our laboratory (Sultana et al., 2005) demonstrating that ferulic acid ethyl ester protects cortical neurons from β-amyloid toxicity by acting at three different levels: (i) inducing HO-1 and Hsp70 proteins; (ii) decreasing the neuronal 3-nitrotyrosine levels and, therefore, iNOS activity; and (iii) by the well-known direct free radical quenching activity (Kanski et al., 2002; Sultana et al., 2005). These data provide consistent evidence that a profound interplay between Hsps exists and further sustain the importance of Hsps in mediating neuroprotective effects.

A growing body of literature has unraveled the antioxidant and anticarcinogenic activities of polyphenolic compounds, such as curcumin. Curcumin, a well-known spice used commonly in India to make foods colored and flavored, is also used in traditional medicine to treat mild or moderate human diseases. Based on the ability of this compound to regulate a number of cellular signal transduction pathways, it is emerging as a potential therapeutic drug for the treatment of neurodegenerative disorders. Particularly interesting is the interaction of curcumin with the vitagene system. In particular, curcumin increased the expression of HO-1 in human cardiac myoblasts, hepatocytes, monocytes, and endothelial cells (Abuarqoub et al., 2007; Jeong et al., 2006; McNally et al., 2006; Rushworth et al., 2006), rat neurons, and astrocytes (Scapagnini et al., 2006), as well as porcine endothelial cells (Balogun et al., 2003). In several rodents and human cells, the curcumin-induced HO-1 overexpression was correlated with the production of mitochondrial ROS, activation of transcription factors Nrf 2 and NF-κB, induction of MAPK p38, and inhibition of phosphatase activity (Andreadi et al., 2006; McNally et al., 2007; Rushworth et al., 2006). Moreover, curcumin upregulated Hsp70 in human colorectal carcinoma cells, proximal tubule cells (Chen et al., 1996, 2001; Rashmi et al., 2003; Sood et al., 2001), and rat glioma cells (Kato et al., 1998).

2.4. Induction of vitagenes by small molecules via the Keap1/Nrf2/ARE pathway

It is noteworthy that the levels of glutathione and HO-1 can be upregulated by small molecules (inducers) via the Keap1/Nrf2/ARE system which is now a very well recognized stress-response pathway that leads to induction of a battery of cytoprotective proteins (Pretera et al., 1995; Motohashi and Yamamoto, 2004; Kobayashi and Yamamoto, 2006; Kensler et al., 2007). One of the recently discovered inducers is nitric oxide (Buckley et al., 2008;
Calabrese et al. 2007b). Inducers belong to 10 structurally distinct chemical classes and have a single common property: the ability to react with sulfhydryl groups (Prestera et al., 1993; Dinkova-Kostova et al., 2001; 2005). Upon entry into the cell they modify highly reactive cysteine residues of the protein sensor Keap1 (Dinkova-Kostova et al., 2002), which then loses its ability to target transcription factor Nrf2 for ubiquitination and proteasomal degradation (McMahon et al., 2003). Consequently, Nrf2 undergoes nuclear translocation where it binds in heterodimeric combinations with a small Maf protein to the antioxidant response elements (AREs) that are present in the promoter regions of phase 2 proteins and activates their transcription (Figure 6.3). In addition to HO-1, g-glutamatescysteine ligase, the enzyme catalyzing the rate-limiting step in the glutathione biosynthesis, glutathione reductase, and thioredoxin reductase, enzymes involved in maintaining glutathione in its reduced state, are all inducible through the Keap1/Nrf2/ARE pathway and such induction correlates with protection against various cytotoxic insults (Li et al., 2007; Dinkova-Kostova and Talalay, 2008).

Because both heme oxygenase 1 and the thioredoxin/thioredoxin reductase system can be upregulated in an Nrf2/ARE-dependent manner, the questions arise whether: (i) the third member of the vitagene family,
Hsp70, is also inducible by other inducers, and (ii) there could be a common regulatory mechanism. Indeed, many inducers of Nrf2-dependent genes have been shown to increase the protein levels of Hsp70. Among them are the cyclopentenone prostaglandin 15-deoxy-12,14-prostaglandin J2 (15dPGJ2), and the vicinal dithiol reagent phenylarsine oxide, (Zhang et al., 2004; Rokutan et al., 2000). Importantly, all of these compounds react with sulfhydryl groups and the transcriptional activation of both Nrf2 and heat shock factor 1 (HSF1), the major activator of Hsp70 gene expression, depend on cysteine modification either within the Nrf2 regulator Keap1 (Wakabayashi et al., 2004; Dinkova-Kostova et al., 2002), or within HSF1 itself (Liu et al., 1996).

3. Material and Methods

3.1. Chemicals

5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), 1,1,3,3-tetraethoxypropane, purified bovine blood SOD, NADH, reduced GSH, oxidized glutathione disulfide (GSSG), β-NADPH (type I, tetrasodium salt), glutathione reductase (type II from baker’s yeast), Nω-monomethyl-L-arginine (a nonisoform-specific NOS inhibitor), and glucose oxidase (which generates hydrogen peroxide in the culture medium) are from Sigma Chemicals Co. (St. Louis, MO). Zinc protoporphyrin IX, a specific inhibitor of HO activity, is from Porphyrin Product (Logan, UT). Acetylcarnitine (99.99% pure) was a generous gift from Sigma Tau Co. (Pomezia, Italy). All other chemicals are from Merck (Germany) and of the highest grade available.

3.2. Cell cultures and treatment

Human neuroblastoma SH-SY5Y cells are seeded at $1 \times 10^4$ cells per 1-cm$^2$ plastic wells with cultured Dulbecco’s modified Eagle’s medium (DMEM/F-12 Ham) supplemented with 10% fetal calf serum, 1.0 mM glutamine, and 100 units/ml penicillin plus 10 μg/ml streptomycin and incubated in 5% CO$_2$ humidified at 37 °C. The medium is replaced every 3 days. Undifferentiated confluent cells are treated with 0.5 to 3 mM 3-morpholinosydnonimine (SIN-1) for 7 to 24 h. After treatments, cells are harvested in phosphate-buffered saline (154 mmol/liter of NaCl, 0.61 mmol/liter of Na$_2$HPO$_4$, and 0.38 mmol/liter of KH$_2$PO$_4$, pH 7.4) containing 0.04% (wt/vol) EDTA, washed three times, and stored as pellets at -70 °C until use. The cellular pellet is resuspended in 0.32 M sucrose, 1 mM EDTA, 10 mM Tris (pH 7.4), and 0.5 mM phenylmethylsulfonyl fluoride and homogenized.
3.3. Western blot analysis of heme oxygenase-1, Hsp70, protein carbonyls, and 4-hydroxy-nonenals

Samples of human neuroblastoma SH-SY5Y cells are analyzed for HO-1 and Hsp70 protein expression, as well as protein carbonyl (DPNH) and 4-hydroxynonenal (4-HNE) levels, by using a Western immunoblot technique. Briefly, an equal amount of proteins (30 μg) for each sample is separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred overnight to nitrocellulose membranes, and the nonspecific binding of antibodies is blocked with 3% nonfat dried milk in phosphate-buffered saline (PBS). Immunodetection of HO-1 and Hsp70 protein expression is performed with a polyclonal rabbit anti-HO-1 antibody (SPA-895; Stressgen Biotechnologies, Glenford, Victoria, Canada) and a monoclonal mouse anti-Hsp70 antibody (SPA-810; Stressgen Biotechnologies), respectively. When probed for 4-HNE levels and for DPNH content, membranes are incubated with a rabbit anti-4-hydroxynonenal (HNE11-S, Lost Lane, San Antonio, TX) and rabbit anti-2,4-dinitrophenol antibodies (V0401, Dako, Denmark). A goat polyclonal antibody specific for β-actin is used as a loading control (sc-1615, Santa Cruz Biotechnology). Blots are then visualized with either an amplified horseradish peroxidase–conjugated antirabbit immunoglobulin G (sc-2030, Santa Cruz Biotechnology) when probing for HO-1, 4-HNE, and DPNH, a goat antimouse IgG when probing for Hsp70 (SAB-100, Stressgen Biotechnologies), and donkey antigoat IgG in the case of β-actin (sc-2020, Santa Cruz Biotechnology). Immunoreactive bands are scanned by a laser densitometer (LKB Ultroscan XL). Molecular weights of the proteins detected are determined by using a standard curve obtained with proteins of known molecular weight.

3.4. Preparation of nuclear extract and Western blot for Nrf 2

Samples of human neuroblastoma SH-SY5Y cells are washed twice with PBS, harvested in 1 ml PBS, and centrifuged at 3000 rpm for 3 min at 4 °C. The cell pellet is carefully resuspended in 200 μl of cold buffer A, consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), and complete protease inhibitor cocktail (Roche, Mannheim, Germany). The pellet is then incubated on ice for 15 min to allow cells to swell. After this time, 15 μl of 10% NP-40 is added and the tube is vortexed for 10 s. The homogenate is then centrifuged at 3000 rpm for 3 min at 4 °C. The resulting nuclear pellet is resuspended in 30 μl of cold buffer B consisting of 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 μM DTT, and protease inhibitors. The pellet is then incubated on ice for 15 min and vortexed for 10 to 15 s every 2 min. The nuclear extract is finally centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatant containing the nuclear proteins is loaded on a SDS–polyacrylamide
gel, and Western blot analysis using Nrf 2 antibodies (1:1000 dilution) is performed as described earlier.

3.5. Real-time quantitative polymerase chain reaction

Total RNA from cell cultures is extracted using Trizol (Sigma, St. Louis, MO) and is treated with RNase-free DNase to remove any residual genomic DNA. Single-stranded cDNAs are synthesized by incubating total RNA (1 μg) with SuperScript II RNase H reverse transcriptase (200 U), oligo (dT)₁₂₋₁₈ primer (100 nM), dNTPs (1 mM), and RNase inhibitor (40 U) at 42 °C for 1 h in a final volume of 20 μl. The reaction is terminated by incubating at 70 °C for 10 min. Forward (F) and reverse (R) primers used to amplify HO-1 were, respectively, HO-1-F: TCTCTTGCTGGCTTCCCTTA and HO-1-R: ATGCCTGGATGTGCTTTC (GenBank accession no. NM_002133), and the expected amplification products for HO-1 was 132 bp. To control the integrity of RNA and for differences attributable to errors in experimental manipulation from tube to tube, primers for rat phosphoglycerate kinase 1 (PGK 1) a housekeeping gene that is consistently expressed in brain cells, were used in separate PCR reactions (PGK-F: AGGTGCTCAACAACATGGAG, PGK-R: TACCAGAGCCACACAGTAGCT, GenBank accession no. M31788), and the expected amplification products for this gene was 183 bp, or HO-2 (Figure 6.8) (HO2-F: CCCCCTTCTACGCTGCTGAAC, and HO-2-R: TGCTGTCAGAGGAGGCTCTA (GenBank accession no. NM_002134) and the expected amplification products for HO-1 was 317 bp. Aliquots of cDNA (0.1 μg) and known amounts of external standard (purified PCR product, 10² to 10⁸ copies) are amplified in parallel reactions using the forward and reverse primers. Each PCR reaction (final volume, 20 μl) contains 0.5 μM of primers, 2.5 mM Mg²⁺, and 1× Light Cycler DNA Master SYBR Green (Roche Diagnostics, Indianapolis, IN). PCR amplifications are performed with a Light Cycler (Roche Molecular Biochemicals) using the following four cycle programs: (i) denaturation of cDNA (one cycle: 95 °C for 10 min); (ii) amplification (40 cycles: 95 °C for 0 s, 58 °C for 5 s, 72 °C for 10 s); (iii) melting curve analysis (one cycle: 95 °C for 0 s, 70 °C for 10 s, 95 °C for 0 s); and (iv) cooling (one cycle: 40 °C for 3 min). The temperature transition rate is 20 °C/s except for the third segment of the melting curve analysis, where it is 0.2 °C/s. The fluorimeter gain value is 6. Real-time detection of fluorimetric intensity of SYBR Green I, indicating the amount of PCR product formed, is measured at the end of each elongation phase. Quantification is performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards. For this analysis, fluorescence values measured in the log-linear phase of amplification are considered using the second derivative maximum method of the Light Cycler Data Analysis software (Roche Molecular Biochemicals). Specificity of PCR products obtained is
characterized by melting curve analysis followed by gel electrophoresis, visualized by ethidium bromide staining, and DNA sequencing.

3.6. Total reduced and oxidized glutathione assay

Total (i.e., cytosolic and mitochondrial) reduced GSH and total GSSG are measured by the NADPH-dependent GSSG reductase method, modified as follows. Cells are homogenized on ice for 10 s in 100 mM potassium phosphate, pH 7.5, which contains 12 mM disodium EDTA. For reduced glutathione, aliquots (0.1 ml) of homogenates are immediately added to 0.1 ml of a cold solution containing 10 mM DTNB and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The samples are mixed by tilting and are centrifuged at 12,000g for 2 min at 4°C. An aliquot (50 μl) of the supernatant is added to a cuvette containing 0.5 U of GSSG reductase in 100 mM potassium phosphate and 5 mM EDTA, pH 7.5 (buffer 1). After 1 min of equilibration, the reaction is initiated with 220 nmol of NADPH in buffer 1 for a final reaction volume of 1 ml. Formation of a GSH–DTNB conjugate is then measured at 412 nm. The reference cuvette contains equal concentrations of DTNB, NADPH, and enzyme, but not sample. For assay of total GSSG, aliquots (0.5 ml) of homogenate are immediately added to 0.5 ml of a solution containing 10 mM N-ethylmaleimide (NEM) and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The sample is mixed by tilting and is centrifuged at 12,000g for 2 min at 4°C. An aliquot (500 μl) of the supernatant is passed at one drop per second through a Sep-Pak C18 column (Waters, Framingham, MA) that has been washed with methanol followed by water. The column is then washed with 1 ml of buffer 1. Aliquots (865 μl) of the combined eluates are added to a cuvette with 250 nmol of DTNB and 0.5 U of GSSG reductase. The assay then proceeds as in the measurement of total GSH. GSH and GSSG standards in the ranges between 0 and 10 nmol and 0.010 and 10 nmol, respectively, added to control samples are used to obtain the relative standard curves, and results are expressed in nanomoles of GSH or GSSG, respectively, per milligram of protein.

3.7. Determination of reduced glutathione and oxidized glutathione in the cytosol

Cells are harvested from petri dishes with 0.05% trypsin, 0.02% EDTA and washed in phosphate-buffered saline, pH 7.4, with 5% fetal bovine serum. Cells are collected by centrifugation at 500g for 3 min at room temperature, and the pellet (≈5 × 10^6 cells) is resuspended in 0.5 ml of 100 mM potassium phosphate buffer, pH 7.5, containing 5 mM EDTA (buffer 1). The cell suspension is homogenized by five strokes; the temperature of the
suspension is maintained at 4 °C during homogenization and all subsequent steps. The homogenate is divided into two aliquots; for the total glutathione (GSH + GSSG) assay, 0.25 ml of homogenate is added to an equal volume of 100 mM potassium phosphate buffer, pH 7.5, containing 17.5 mM EDTA and 10 mM DTNB (sample SS1). For the oxidized glutathione (GSSG) assay, 0.25 ml of homogenate is added to 100 mM potassium phosphate buffer, pH 6.5, containing 17.5 mM EDTA and 10 mM NEM (sample SS2). The samples are centrifuged at 800g for 20 min, and the supernatant fractions are then centrifuged at 10,000g for 30 min. The supernatants of SS1 and SS2 represent the cytosolic fractions and are used for the spectrophotometric assay of total or oxidized glutathione. Before spectrophotometric determination, a 0.25-ml aliquot of the SS2 sample is passed through a C18 Sep-Pak cartridge (Waters, Watford, UK) to remove excess NEM and washed with 0.5 ml of buffer (100 mM potassium phosphate buffer, pH 7.5, containing 5 mM EDTA). The spectrophotometric assay of glutathione is performed by adding the samples to a cuvette containing 0.5 unit of glutathione reductase, 0.2 mM DTNB in a final volume of 1 ml of 100 mM potassium phosphate buffer, pH 7.5, 5 mM EDTA and the reaction is initiated by adding NADPH (220 nmol). The change in absorbance at 412 nm is recorded over a period of 5 min for the SS1 sample or 10 min for the SS2 sample using a reference cuvette containing equal concentrations of NADPH, DTNB, and enzyme. The GSH and GSSG content, expressed as nanomoles per milligram of protein, is determined by comparison with a standard curve obtained with GSH and GSSG solution.

3.8. Determination of total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) in mitochondria

The pellet of SS1 and SS2 samples obtained after centrifugation at 10,000g is utilized for the determination of total and oxidized glutathione in mitochondria. The SS1 pellet is resuspended in 0.3 ml of 100 mM potassium phosphate buffer, pH 7.5, 10 mM EDTA, 5 mM DTNB. The SS2 pellet is resuspended in 0.5 ml of 100 mM potassium phosphate buffer, pH 6.5, containing 10 mM EDTA and 5 mM NEM. Mitochondria are mixed, sonicated, and centrifuged at 10,000g for 11 min, and the supernatants are used for the spectrophotometric determination assay. Before spectrophotometric determination, 0.25 ml of the SS2 sample is passed through a C18 Sep-Pak cartridge to remove excess NEM and is washed with 0.5 ml of buffer 100 mM potassium phosphate buffer, pH 7.5, containing 5 mM EDTA.

Spectrophotometric assay of glutathione is performed by adding the samples to a cuvette containing 0.5 unit of glutathione reductase, 0.2 mM DTNB in a final volume of 1 ml of 100 mM potassium phosphate buffer, pH 7.5, 5 mM EDTA, and the reaction is initiated by adding NADPH
The change in absorbance at 412 nm is recorded over a period of 5 min for the SS1 sample or 10 min for the SS2 sample using a reference cuvette containing equal concentrations of NADPH, DTNB, and enzyme. Protein concentration is determined in the samples with NEM, according to the Bradford method, using bovine serum albumin as standard. The GSH and GSSG content, expressed as nanomoles per milligram of protein, is determined by comparison with a standard curve obtained with known concentrations of GSH and GSSG solution.

3.9. Determination of protein

Proteins are estimated by the bicinchoninic acid reagent.

3.10. Statistical examination

Results are expressed as means ± SEM of at least eight separate experiments. Statistical analyses are performed using the software package SYSTAT (Systat Inc., Evanston, IL). The significance of the differences, evaluated by two-way ANOVA, followed by Duncan’s new multiple range test, is considered significant at \( P < 0.05 \). Correlation analysis is considered statistically significant if the coefficient of determination \( R \) is ≥0.8.

4. Results

As mentioned previously, tissues, particularly brain, counteract oxidative stress by several mechanisms, the most important of which are antioxidant molecules, such as GSH, and antioxidant enzymes, including HO-1 and Hsp70. In a human neuroblastoma cell line (SH-SY5Y), nitrosative stress induced by the administration of SIN-1 (1 mM), which produces both NO and superoxide, thus resulting in increased peroxynitrite formation, resulted in a significant decrease in the total content of GSH associated with significantly increased GSSG levels (Fig. 6.4). A further dissection of this effect demonstrated that SIN-1 affected the GSH/GSSG balance, particularly in the cytosol, whereas mitochondria were not a target for this compound (Fig. 6.4). Furthermore, SIN-1 (0.5–2 mM for 7 or 24 h) increased other indices of oxidative stress, such as protein carbonyls (DNPH) and lipid peroxidation products (4-HNE) (Figs. 6.5 and 6.6). This documented prooxidant effect of SIN-1 was paralleled by upregulation of both HO-1 and Hsp70 protein levels in SH-SY5Y cells (Fig. 6.7). The increased protein expression of HO-1 in SH-SY5Y cells exposed to SIN-1 was accompanied by increased message expression (Fig. 6.8). Results shown in Fig. 6.8 demonstrate that at the concentration used, SIN-1 is sufficient to increase the expression of HO-1 at
the mRNA (measured at 3 h from the beginning of the treatment, Figs. 6.8D and 6.8F) and protein levels and is sufficient to increase the activity of HO-1 (data not shown). All this thus enforces the hypothesis that cells upregulate vitagenes to effectively counteract oxidative and nitrosative stress.

### 5. Discussion

Mitochondrial dysfunction is characteristic of several neurodegenerative disorders, and evidence for mitochondria being a site of damage in neurodegenerative disorders is partially based on decreases in respiratory chain complex activities in such diseases (Calabrese et al., 2001; Mancuso et al., 2007; Papa et al., 2006; Scacco et al., 2006). Such defects in respiratory complex activities, possibly associated with oxidant/antioxidant balance perturbation, are thought to underlie defects in energy metabolism and induce cellular degeneration. Evidence that mitochondrial dysfunction

---

**Figure 6.4** Effect of SIN-1 treatment on GSH and GSSG content in SH-SY5Y cells. Human neuroblastoma cells (SH-SY5Y) were treated with SIN-1 (1 mM) for 7 h. At the end of incubation, cells were collected and lysed, and the amount of total, cytosolic, and mitochondrial GSH and GSSG was analyzed as described in the text. Data are expressed as mean ± SEM of eight independent experiments.
**Figure 6.5** SIN-1 increases protein oxidation in neuronal cells. Human neuroblastoma cells (SH-SY5Y) were treated with SIN-1 (0.5–2 mM) for 7 to 24 h. At the end of incubation, cells were collected, lysed, and immunoblotted using a specific antibody against DNPH as described in the text. Each experiment was performed at least three times. A representative gel is shown.

**Figure 6.6** SIN-1 increases lipid oxidation in neuronal cells. Human neuroblastoma cells (SH-SY5Y) were treated with SIN-1 (0.5–2 mM) for 7 to 24 h. At the end of incubation, cells were collected, lysed, and immunoblotted using a specific antibody against 4-HNE as described in the text. Each experiment was performed at least three times. A representative gel is shown.
may be a mechanism for NO-mediated neurotoxicity arises from different studies that indicate excessive production of NO, a free radical that has several important messenger functions within the CNS, leads to the formation of peroxynitrite anion (ONOO⁻) by reacting with the superoxide anion (Calabrese et al., 2007b). This extremely potent oxidizing agent can interact at the binuclear center of cytochrome oxidase, leading to inhibition of the respiratory rate and ATP stores (Calabrese et al., 2001). NO can also stimulate the S-nitrosylation of protein and nonprotein thiols and also binds to the nonheme iron of ribonucleotide reductase to inhibit DNA synthesis (Gegg et al., 2003).

Efficient functioning of the maintenance and repair process seems to be crucial for both survival and physical quality of life. This is accomplished by a complex network of the so-called longevity assurance processes, which are composed of several genes termed vitagenes (Calabrese et al., 2004a, 2006a, 2007a). 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. Heat shock proteins and molecular chaperones have been known to protect cells against a wide variety of toxic conditions, including extreme temperatures, oxidative stress, virus infection, and exposure to heavy metals or cytotoxic drugs (Calabrese et al., 2004a, 2006a, 2007a). Chaperone-buffered silent mutations may be activated during the aging process and lead to the phenotypic exposure of previously hidden features and contribute to the onset of polygenic diseases, such as age-related disorders, atherosclerosis, and cancer.

**Figure 6.7** SIN-1 upregulates vitagenes. Human neuroblastoma cells (SH-SY5Y) were treated with SIN-1 (0.5–2 mM) for 7 to 24 h. At the end of incubation, cells were collected, lysed, and immunoblotted using specific antibodies against HO-1 and Hsp70 as described in the text. Each experiment was performed at least three times. A representative gel is shown.
Figure 6.8 Real-time quantification of HO-1 and HO-2 mRNA levels by RT-PCR in SH-SY5Y cells treated with SIN-1. Specific primers for HO-1 and HO-2 were used to amplify human brain RNA (Fig. 6.8 A–E). Total RNA from different samples and known amounts of external standards (purified PCR product, 102 to 108 copies) were amplified in parallel reaction. Fluorimetric intensity of SYBR Green I, indicating the amount of PCR product formed, was measured at the end of each elongation phase. Quantification was performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards (A). Fluorescence values measured in the log-linear phase of amplification were measured by the second derivative maximum method and used to produce standard curves (C) that were used to estimate the concentration of unknown samples. The specificity of the products amplified was evaluated by melting curve analysis (E). Cellular expression of HO-1 transcript relative to the expression of HO-2 (mean ± SEM) after treatment with SIN-1 1mM is shown (D). *p < 0.05 versus 0 mM SIN-1. The amplified fragments for HO-1 was run on 2% agarose gel (B). A 100-bp DNA ladder is shown at the left of the gel (M), with bands labelled in bp units. Exposure of SH-SY5Y cells to SIN-1 significantly (p < 0.05) up-regulates mRNA levels measured 3 hours after SIN-1 addition (panels D,F).
(Soti and Csermely, 2003). Hence, Hsp induction is not only a signal for the detection of physiological stress, but is utilized by the cells in the repair process following a wide range of injuries to prevent damage resulting from the accumulation of nonnative proteins (Kelly and Yenari, 2002).

Involvement of the heme oxygenase pathway in antidegenerative mechanisms, especially those operating in Alzheimer’s disease, has been demonstrated: overexpression of HO-1 has been observed in association with neurofibrillary tangles and senile plaques (Premkumar et al., 1995; Schipper, 2000; Takeda et al., 2000). HO induction, which occurs together with the induction of other Hsp during various physiopathological conditions by generating the vasoactive molecule CO and the potent antioxidant BR, represents a protective system potentially active against brain oxidative injury (Mancuso et al., 2004, 2006a,b, 2007). The HO-1 gene is redox regulated; this is supported by the fact that the HO-1 gene has a heat shock consensus sequence as well as AP1, AP2, and NF-κB binding sites in its promoter region. In addition, heme oxygenase-1 is upregulated rapidly by oxidative and nitrosative stress, as well as by glutathione depletion (Mancuso et al., 2007; Motterlini et al, 2002).

All this evidence emphasizes the well-established concept of the cellular stress response to oxidative insults as a crucial mechanism operating against neurodegenerative damage (Calabrese et al., 2004b, 2006a, 2007a; Mancuso et al., 2007). However, relatively new is the notion that pharmacological or nutritional intervention can lead to the same cytoprotective cellular responses (Butterfield et al., 2002; Calabrese et al., 2003a,b). Acetyl-l-carnitine (LAC) is proposed as a therapeutic agent for several neurodegenerative disorders, as well as an agent protective in numerous disease paradigms; however, the mechanism of protection in brain disorders still remains elusive (Calabrese et al., 2007b). Furthermore, experimental evidence exists that upregulation of HO-1 involves the transcription factor Nrf 2 (see earlier discussion). These data, along with evidence that LAC induces Nrf 2 in rat astrocytes (Calabrese et al., 2005b), suggest the potential impact on dietary antioxidants in the HO-1/Nrf 2 axis. Therefore, this highly inducible system should be seriously considered as a target for novel therapeutic interventions focusing on the capability that compounds such as antioxidant polyphenols or acetylcarnitine have to upregulate the vitagene system as a means to limit the deleterious consequences of oxidative and nitrosative stress associated with aging and age-related disorders.

ACKNOWLEDGMENT

This work was supported by grants of PRIN 2005, FIRB RBRN07BMCT, and ICT-E1 Comune di Catania.
REFERENCES


