

Cellular Stress Responses, The Hormesis Paradigm, and Vitagenes: Novel Targets for Therapeutic Intervention in Neurodegenerative Disorders

1. Calabrese V, Cornelius C, Dinkova-Kostova AT, Calabrese EJ, Mattson MP. Cellular Stress Responses, The Hormesis Paradigm, and Vitagenes: Novel Targets for Therapeutic Intervention in Neurodegenerative Disorders. *Antioxidants & Redox Signaling*. 2010;13(11):1763-1811.

I. Introduction

The adaptation and survival of cells and organisms requires the ability to sense proteotoxic insults and to coordinate protective cellular stress response pathways and chaperone networks related to protein quality control (310). The toxic effects that stem from the misassembly or aggregation of proteins or peptides, in any cell type, are collectively termed proteotoxicity, whereas neurotoxicity is a term that refers to general toxic effects observed in neurons. To understand the relevance of proteotoxicity to neurotoxicity and to neurodegeneration is important, a comprehensive understanding of the mechanisms that lead to the development of neurodegenerative diseases. To date, these mechanisms are poorly understood; however, it is clear that protein aggregation is tightly linked to the emergence and development of neurodegenerative diseases. Molecular chaperones are known to disrupt aggregates and this represents the basis of the therapeutic potential of heat shock proteins (Hsps), which prevent protein misfolding and aggregation. However, despite the abundance and apparent capacity of chaperones and other components of homeostasis to restore folding equilibrium, the cell appears poorly adapted for chronic proteotoxic stress that increases in cancer and in metabolic and neurodegenerative diseases (311). In these conditions, a decline in biosynthetic and repair activities that compromises the integrity of the proteome is strongly influenced by protective genes called vitagenes that control aging, thus linking stress and protein homeostasis with the health and life span of the organism (99, 100). The disruption of protein folding quality control results in the accumulation of a nonnative protein species that can form oligomers, aggregates, and inclusions indicative of neurodegenerative disease (312). Pharmacological modulation of cellular stress response pathways has emerging implications for the treatment of human diseases, including neurodegenerative disorders, cardiovascular disease, and cancer (102).

A critical key to successful medical intervention is getting the dose right. Achieving this goal can be extremely challenging due to human interindividual variation as affected by age, gender, diet, exercise, genetic factors, and health status. Getting the dose right can also be affected by exposure to other drugs as well as factors such as circadian rhythms. For instance, it has been shown that exercise promotes longevity and ameliorates type 2 diabetes mellitus and insulin resistance. However, exercise also increases mitochondrial formation of presumably harmful reactive oxygen species (ROS). Consistent with the concept of mitohormesis, exercise-induced oxidative stress ameliorates insulin resistance and causes an adaptive response promoting endogenous antioxidant defense capacity. Importantly, recent finding indicates that

supplementation with antioxidants may preclude these health-promoting effects of exercise in humans (372). Another issue that can affect clinical success is the nature of the dose response in and adjacent to the therapeutic zone (58,60). Over the past decade considerable advances have been made in the understanding of the nature of the dose response, especially in the low-dose zone (88, 116, 204, 280, 291, 292, 398). These findings challenge previous concepts of the dose response, suggesting that the commonly accepted threshold and linear dose–response models upon which society has based its toxicological and pharmacological predilections for drug and chemical effects often fail to provide reliable estimates of responses in the low-dose zone, that is, below the threshold (55, 61, 69–71, 90, 91). Failure to accurately predict the effects of drugs and chemicals can lead to failed clinical trials, inadequate patient care, and potentially harmful governmental regulations (40, 73, 135, 143). While traditional dose–response models have failed to accurately predict responses in the low-dose zone in large-scale studies, the hormetic dose response performed extremely well, lending considerable support to other studies (56, 57, 65, 66, 73–86, 92) that have suggested that the process of drug development and chemical hazard/risk assessment could be improved in significant ways by a consideration of the hormetic dose response in the design, execution, and analysis of toxicological and pharmacological investigations. Consequently, this article will introduce the hormetic dose–response concept, including its scientific foundations, toxicological and pharmacological implications, and its applications to the field of neuroprotection and their mechanistic foundations.

II. Hormesis

Hormesis is a dose–response phenomenon characterized by a low-dose stimulation and a high-dose inhibition (Fig. 1). It may be graphically represented by either an inverted U-shaped dose response or by a J- or U-shaped dose response. The term hormesis was first presented in the published literature in 1943 by Southam and Ehrlich, who reported that low doses of extracts from the red cedar tree enhanced the proliferation of fungi with the overall shape of the dose response being biphasic. However, credit for experimentally demonstrating the occurrence of hormesis goes to Hugo Schulz (396), who reported biphasic dose responses in yeast after exposure to a large number of toxic agents. The work of Schulz inspired a large number of investigators in diverse fields to assess whether such low-dose effects may be a general feature of biological systems. In fact, similar types of dose–response observations were subsequently reported by numerous researchers assessing chemicals (49) and radiation (41, 50–53, 246, 307, 313, 367, 381, 397, 431, 432) with investigators adopting different names such as the Arndt–Schulz Law, Huppe's Rule, and other terms to describe these similar dose–response phenomena (368). Despite the rather substantial historical literature concerning hormetic dose responses, this concept had a difficult time being incorporated into routine safety assessment and pharmacological investigations, principally because it (a) required more rigorous evaluation in the low-dose zone, (b) failure of investigators to understand its clinical significance, (c) failure to appreciate the quantitative features of the hormetic dose response, (d) failure to understand the limitations of its implications for commercial applications in agriculture as well as medicine, (e) because of the predominant interest in responses at relatively high doses during most of the 20th century, and (f) the continuing, yet inappropriate, tendency to associate the concept of hormesis with the medical practice of homeopathy (64, 89, 91). However, from the late 1970s (423, 433) there has been a growing interest in hormetic-like biphasic dose responses across the broad spectrum of biomedical sciences. This resurgence of interest resulted from a variety of factors,

including the capacity to measure progressively lower doses of drugs and chemicals, the adoption of cell culture methods, which has permitted more efficient testing of numerous doses and the need to reexamine the validity of linearity at low-dose modeling of cancer risks due to their enormous cost implications for regulations (379), as well the astute observations of independent investigators and their capacity to generalize their findings across biological systems (267, 423).

[View larger version\(178K\)](#)

FIG. 1. Dose-response curve depicting the quantitative features of hormesis. NOAEL, No Observed Adverse Effect Level; ZEP, zero equivalent point.

What has emerged from these research initiatives from highly diverse biomedical areas is the recognition that hormetic dose responses were common and highly generalizable, being independent of biological model, endpoints measured, and chemical class and/or physical agent studied (50–54, 68, 306, 448). This was an unexpected finding as hormetic responses were often considered by many in the so-called mainstream branches of toxicology and pharmacology to be paradoxical, not commonly expected and being of questionable reliability with a lack of capacity for replication. The casual dismissal of the hormesis concept during the mid decades of the last century is reflected in the general absence of the hormesis concept from the leading toxicological and biomedical textbooks. This situation has radically changed such that hormesis is now incorporated into all leading textbooks of toxicology (*e.g.*, ref. 167) encyclopedias (89, 94) and other leading monographs. In fact, while the terms hormetic and hormesis were cited only about 160 times during the entire decade of the 1980s within the Web of Science database, in 2008 alone these terms were cited nearly 2300 times.

Of further significance were observations that these broad-ranging dose-response relationships also shared the same general quantitative features. More specifically, the low-dose stimulation that becomes manifested immediately below the pharmacological and toxicological thresholds is modest in magnitude, being at most only about 30%–60% greater than the control group response. The width of the hormetic stimulation is usually about 10–20-fold starting immediately from the zero equivalent dose (*i.e.*, estimated threshold) (Fig. 1). The hormetic dose response may result from either a direct stimulation or *via* an overcompensation stimulatory response after disruption in homeostasis (49, 50). Regardless of the mode of action by which the stimulation occurs, the quantitative features of hormetic dose responses are similar. These observations are based on copious data derived from the published literature ranging from plants to humans (68, 93), involving numerous receptor systems (61–64). These findings have recently led to nearly 60 biomedical scientists recommending that biological stress responses, including those of pre- and postconditioning, be integrated within an hormetic context, along with the adoption of a terminology that would be based within an interdisciplinary framework (72).

The hormetic dose response confers a new set of interpretations for the dose response. At high doses within a toxicological setting, the typical endpoints measured indicate cellular damage. However, as the dose decreases below the threshold, the low-dose stimulation more likely represents a manifestation of an adaptive response that conforms to a measure of biological

performance as may be seen in the cases of modest increases in cognition, growth, longevity, bone density, and other biomedical endpoints of interest (424).

The consistency of the vast array of hormetic findings suggests strongly that this dose response may be a manifestation of the plasticity of biological systems. That essentially all biological models respond to imposed stress with the same quantitative features of the dose response is a central finding within the biological sciences that has not been previously recognized. These findings suggest that the hormetic dose response would have been broadly selected for and highly conserved. This adaptive response not only enhances survival by conferring resistance to environmental stress, but also represents a way to regulate the allocation of biological resources in a manner that ensures cellular and organismal stability. The consistency of the hormetic dose response across the vast array of biological models, at different levels of biological organization, regardless of the type of stress and health status of the individual, indicates that hormesis provides a quantitative index of biological plasticity across multiple levels of biological organization, making it a central biological element for enhancing survival.

These quantitative features of the hormetic dose response have important medical implications. Most significantly, the hormetic dose response imposes constraints upon the magnitude of a drug to induce a desired effect. For example, if a drug increased cognitive performance in an elderly patient by approximately 25%–30%, the hormetic model suggests that this level of performance could not be further increased using a new drug combination. This concept has been supported in a variety of studies on hormesis and drug interaction. Flood (173–176) has demonstrated that the hormetic response for memory was bounded by the 30%–60% increase even when several drugs were used in combination that were designed to maximize memory outcome. This response magnitude constraint has been reported for immune stimulation, bacterial growth, increases in hair growth, plant growth, decrease in anxiety, decreases in tumor incidence, and numerous other endpoints (73).

This limitation in the magnitude of the stimulatory response is a critical implication of the hormesis dose–response concept. It is an observation that is based on extensive findings, and it is a controlling feature that defines what pharmaceutical companies can expect to achieve with drugs that are designed to enhance performance. However, the limitation in the magnitude of response is also potentially important with respect to the capacity to detect a desirable response. This may not be a particularly important issue when using highly inbred animal models or cell cultures where experimental conditions can be highly controlled. However, attempting to measure a low-dose hormetic stimulation within the context of a clinical trial can be problematic. Given the likelihood of considerable human variation in response to a drug, it is possible that the test population may have their responsiveness distributed over a range of responses that includes toxicity, optimal response, and a group in which the dose is ineffective. The data from all subjects in such studies would normally be averaged together leading to a marked dilution of an overall positive treatment effect in the optimal response zone subgroup. This suggests a possible reason why drugs that were very successfully tested in preclinical studies with highly inbred strains of animals could and often have failed during the clinical trial. Of particular note is that investigators may have to modify doses based upon the sensitivity or susceptibility of the subjects. Calabrese and Baldwin (59) have shown that the hormetic dose response is often expressed in the broad range of subjects independent of their susceptibility. As expected, those

individuals who are very resistant to the drug or chemical treatment would have their hormetic response shifted to the right on the dose–response graph, whereas those individuals with greater-than-normal susceptibility would have their hormetic response shifted to the left. The hormetic dose response therefore imposes considerable challenges to the biomedical community that is interested in the development of drugs that are concerned with improvements in human performance.

The hormetic dose response can also have undesirable effects. This may be most readily seen in the case of drugs that are designed to suppress growth or kill cells or organisms at higher doses. For example, there is now substantial evidence that low doses of many antitumor drugs can stimulate the proliferation of such cells at lower concentrations (67). This has also been shown to be the case with antibiotics, including penicillin (366) and streptomycin (366, 465). This phenomenon has also been reported with selected cardiac glycosides that have effects on nontarget tissues such as the prostate, where it is able to enhance the proliferation of smooth muscle cells by about 30% with clinically relevant doses (1, 127). Such a 30% increase in prostate smooth muscle was considered likely to impede urination in males. The failure to consider the possibility of the hormetic response not only can lead to a lack of recognition of a desirable drug-induced response but also can result in failure to prevent an adverse effect of drug treatment.

Since hormetic dose responses have now been widely reported in biological systems, there is the desire to develop a new subfield of hormetic mimetics. These mimetics are agents that can activate hormetic pathways with the intention of producing a desirable clinical effect. For example, it would be desirable to develop agents that can induce the desirable adaptive hormetic response without having risks associated with the exposures as in the case of certain chemical agents and radiation.

In the ensuing sections of this article we consider several of the major cellular and molecular systems that mediate adaptive stress responses/hormesis, with a focus on the nervous system in health and disease.

III. Membrane Radical Dynamics

To elucidate the roles of hormesis in protecting cells and organs against disease, it is important to understand the nature of the potentially damaging stresses to which cells are subjected. While our focus in this article is on neurodegenerative conditions, many of the mechanisms we describe are also operative in disorders of other organ systems. The plasma membrane serves as the proximate sensor of extrinsic stressors. Membranes serve vital functions in all cells, not only by maintaining the ionic and molecular (proteins, nucleic acids, *etc.*) compositions of subcellular compartments, but also by serving as signal transduction platforms for a range of extracellular (hormones, growth factors, neurotransmitters, lipid messengers, and others) and intracellular (inositol phospholipids, kinases and their substrates, *etc.*) signaling molecules. Lipids that comprise membranes (phospholipids, cholesterol, sphingomyelins, and others) and the various proteins associated with membranes (receptors, ion channels, cell adhesion proteins, and others) encounter a range of ROS including hydrogen peroxide, hydroxyl radical, superoxide, nitric oxide (NO), and others. Excessive attack by free radicals can impair fundamental functions of

membranes, resulting in cell damage and death. Such oxidative damage to membranes is believed to play major roles in a range of disorders, including myocardial infarction (172), stroke and traumatic brain injury (254), and Alzheimer's disease (AD) (286). However, as described below, lower amounts of membrane-associated oxidative stress may activate adaptive stress response pathways.

IV. Lipid Peroxidation and Sphingomyelin Metabolism

The unsaturated (double) bonds in the fatty acid chains of the membrane lipid bilayer are prone to attack by free radicals, particularly hydroxyl radical and peroxynitrite (282, 459). Hydroxyl radical is formed from hydrogen peroxide in the Fenton reaction that is catalyzed by Fe^{2+} or Cu^{+} , whereas peroxynitrite is generated when superoxide interacts with NO (150). Both hydroxyl and peroxynitrite radicals can abstract a hydrogen atom from a fatty acid, quenching the original radical but generating a lipid radical that interacts with oxygen to form a lipid peroxyl radical. The peroxyl radical can then interact with neighboring fatty acids, thereby setting in motion a self-propagating lipid peroxidation reaction. Lipid peroxidation can occur in any cellular membranes (mitochondria, endoplasmic reticulum [ER], nucleus, *etc.*), but for the purposes of the present article we will focus on the plasma membrane. Lipid peroxidation generates lipid cleavage products, including aldehydes of various chain lengths and isoprostanes. Among the products of lipid peroxidation, 4-hydroxynonenal (HNE) has received considerable attention for its possible contributions to the pathogenesis of several major disorders, including cardiovascular, renal, and neurodegenerative diseases (226, 252, 411). For example, evidence from studies of experimental models of Alzheimer's disease has shown that HNE can impair the function of ion-motive ATPases (sodium and calcium pumps) (277), glucose transporters (279), and GTP-binding proteins coupled to muscarinic acetylcholine receptors (37) in neurons.

In addition to lipid peroxidation, membrane-associated oxidative stress can activate sphingomyelinases (basic and acidic sphingomyelinases), which cleave sphingomyelin to generate ceramides (140) (Fig. 2). Sphingomyelinases are also activated by tumor necrosis factor (TNF) and some growth factors. Ceramides can activate signaling pathways involved in the regulation of cell growth and survival. However, excessively high amounts of ceramide can trigger a form of programmed cell death called apoptosis (415). Ceramides generated in response to membrane-associated oxidative stress are implicated in the dysfunction and death of cells in a range of disorders, including diabetes (428), emphysema (352), ischemic stroke (476), Alzheimer's disease (141), and amyotrophic lateral sclerosis (142).

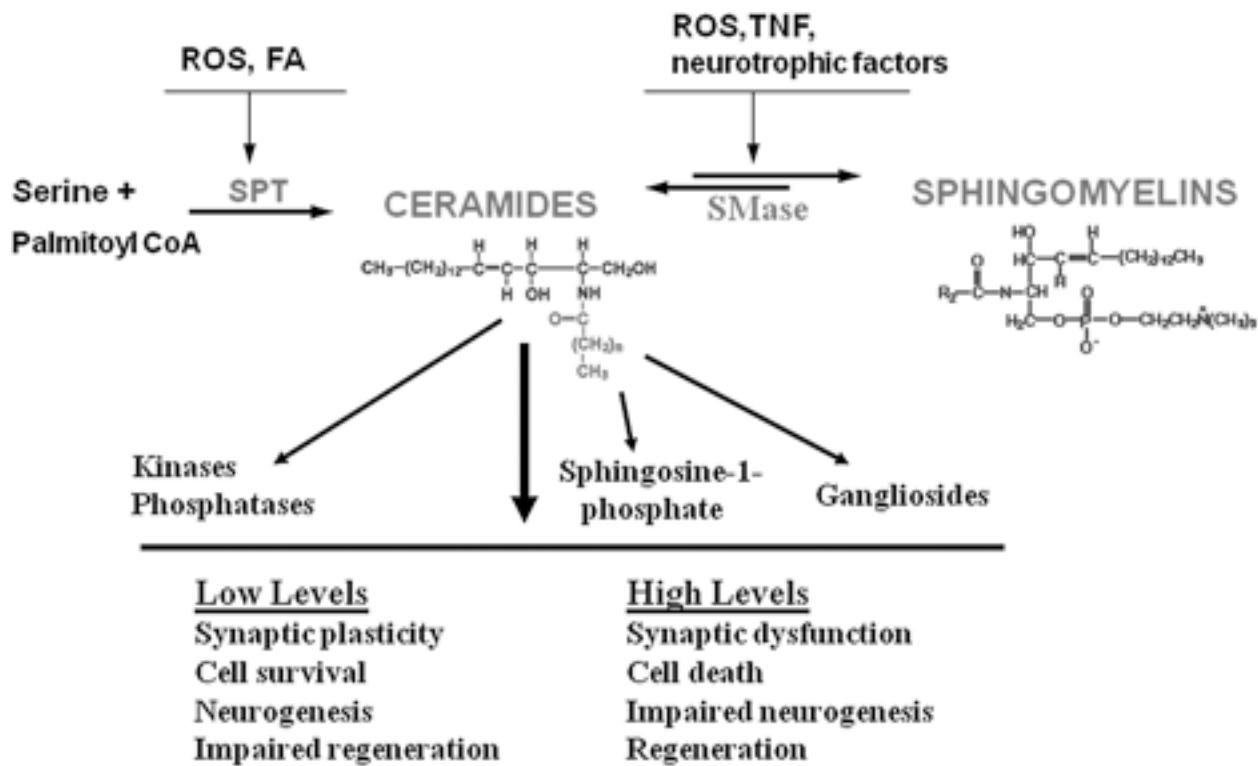


FIG. 2. Membrane sphingomyelin and ceramide metabolism pathways involved in adaptive stress responses and neurodegenerative conditions. Modified from Cutler *et al.* (142). FA, fatty acids; ROS, reactive oxygen species; SMase, sphingomyelinase; SPT, serine palmitoyl CoA transferase; TNF, tumor necrosis factor.

V. Plasma Membrane Redox System

An understudied redox system of enzymes is associated with the plasma membrane where it is involved in modulating levels of oxidative stress (210). The plasma membrane redox system (PMRS) includes the following enzymes reduced nicotinamide adenine dinucleotide (Fig. 3): (a) reduced nicotinamide adenine dinucleotide (NADH)-ascorbate free radical reductase; (b) NAD (P)H-quinone oxidoreductase 1 (NQO1), (c) NADH-ferrocyanide reductase; (d) NADH-coenzyme Q10 reductase; and (e) NADH-cytochrome *c* reductase. In addition, the lipophilic antioxidants α -tocopherol and coenzyme Q10 are involved. The PMRS is therefore similar to the redox system of the inner mitochondrial membrane, which is involved in electron transport and energy metabolism. The PMRS plays an important role in the response of cells to membrane-associated oxidative stress by transferring electrons from reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H] and ascorbate to extracellular free radicals/oxidants. Within the membrane, coenzyme Q10 (CoQ) can be reduced by either NQO1 or NADH-cytochrome *b*5 reductase. Dietary CoQ is important for the maintenance of sufficient levels of reduced CoQ and α -tocopherol in the plasma membrane, thereby protecting against lipid peroxidation. Expression and activity of the PMRS enzymes can be altered in response to physiological challenges. For example, activity and protein levels of NADH-ascorbate free radical reductase; NQO1, NADH-ferrocyanide reductase; and NADH-coenzyme Q10 reductase are decreased in brain cells during normal aging and are increased in response to dietary energy restriction (211). The PMRS is therefore ideally poised

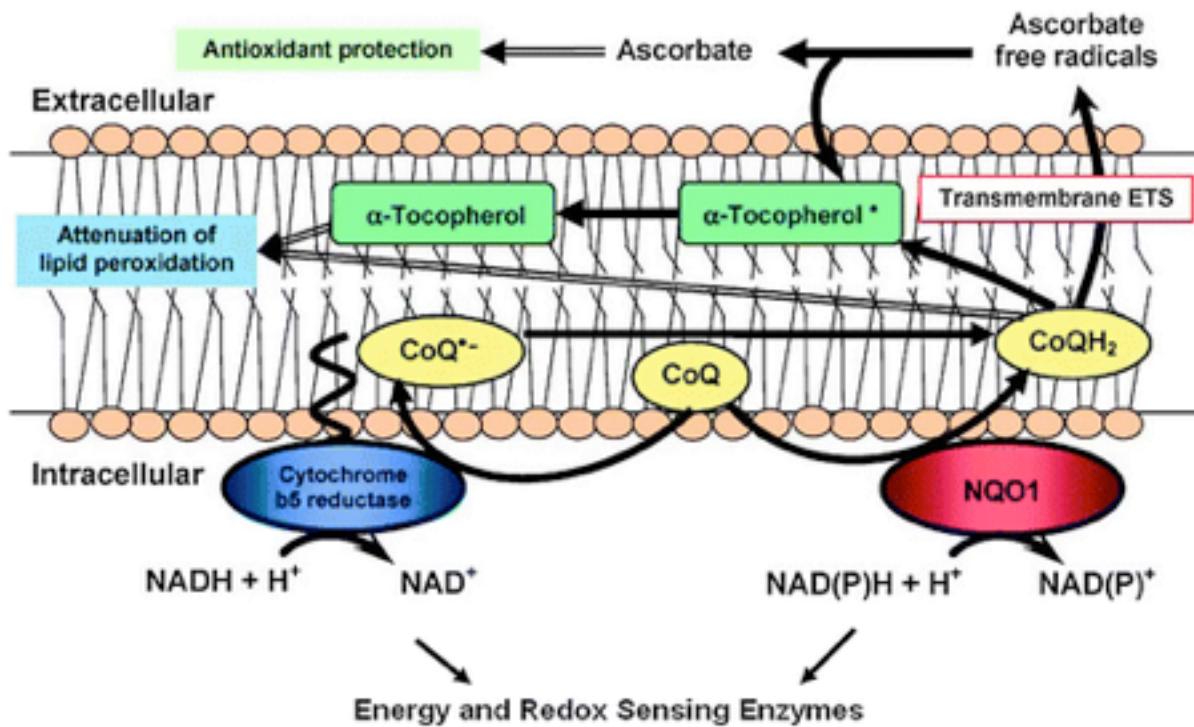


FIG. 3. The plasma membrane redox system is a conserved sensor of cellular redox status and energy metabolism. Modified from Hyun *et al.* (210). CoQ, coenzyme Q10; ETS, electron transport system; NAD⁺, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NAD(P)H, reduced nicotinamide adenine dinucleotide phosphate; NQO1, NAD(P)H quinone oxidoreductase 1.

VI. Membrane-Related Hormesis Mechanisms

Evidence is emerging to support hormetic roles for low and transient increases in membrane oxidative stress. Levels of membrane lipid peroxidation are relatively low under most normal conditions. However, in some types of cells, lipid peroxidation increases considerably during periods of increased energy demand. For example, during vigorous physical exercise there is a marked increase in production of superoxide and hydrogen peroxide, hydroxyl radical, peroxynitrite, and lipid peroxidation (377). Evidence suggests that free radicals and products of lipid peroxidation generated during moderate exercise play important roles in hormetic effects of exercise on muscles, including changes in energy metabolism pathways, mitochondrial biogenesis, and upregulation of protein chaperones and antioxidant systems (377). Benefits of exercise on the cardiovascular system may also involve membrane oxidative stress-related mechanisms. Thus, it was reported that HNE activates nuclear factor erythroid 2-related factor 2 (Nrf2) and antioxidant gene expression in vascular cells (412). HNE may also activate other adaptive stress response pathways that promote the survival and plasticity of cells (349).

Ceramide is also believed to mediate hormetic effects of moderate/transient increases in membrane-associated oxidative stress. For example, pretreatment of neurons with subtoxic concentrations of ceramide results in increased resistance of the neurons to subsequent high levels of oxidative stress (191). Other studies have provided evidence for a pivotal role for ceramide in the cardioprotective effect of preconditioning ischemia in animal models of myocardial infarction (16, 149). Preconditioning ischemia is a classic example of hormesis, wherein exposure of cells to a moderate transient stress protects them against more severe stresses. Changes in the PMRS in response to stress may also allow cells to adapt to potentially

damaging conditions. A dramatic example comes from a study in which the mitochondria of cells were rendered dysfunctional, and the cells were able to survive because of a compensatory upregulation of PMRS enzyme activities (212).

VII. Mitochondria: A Hub of Cellular Redox Processes

Mitochondria are the major source of energy (ATP and oxidized nicotinamide adenine dinucleotide [NAD⁺]) production in eukaryotic cells, and produce correspondingly large amounts of superoxide anion radical through abstraction of an electron from oxygen in the so-called electron transport chain at the inner mitochondrial membrane (291). Mitochondria may produce superoxide in relatively constant amounts, or may elicit spontaneous or environmentally induced superoxide flashes (462). Much of the superoxide produced is efficiently converted to hydrogen peroxide *via* the activity of superoxide dismutases, including cytoplasmic (SOD1) and mitochondrial (SOD2) forms of the enzyme. Being a highly reactive free radical, superoxide can damage molecules (DNA, proteins, and lipids), and so its conversion to hydrogen peroxide protects cells. However, in the presence of even very low concentrations of Fe²⁺ or Cu⁺, hydrogen peroxide can generate hydroxyl radical, which is a potent inducer of membrane lipid peroxidation (Fig. 3). In addition, NO (which is generated in response to activation of the enzyme NO synthase by Ca²⁺/calmodulin) can interact with mitochondrial superoxide to generate the highly reactive free radical peroxynitrite. Methods have been developed to detect and quantify oxidative damage to proteins, lipids, and DNA, with antibodies that selectively recognize proteins modified by the lipid peroxidation product 4-hydroxynonenal (37, 242, 279) and by nitration (124, 220) being particularly useful. In addition, fluorescent probes for imaging relative levels of overall mitochondrial redox status and superoxide have been used to elucidate roles for mitochondrial ROS in a range of physiological and pathological processes (178, 243, 261, 462). The damaging effects of excessive production of ROS are believed to contribute to a wide range of diseases, including cancers, cardiovascular disease, and inflammatory conditions such as arthritis (2, 250, 296). Neurons may be particularly vulnerable to mitochondrial ROS because of their high energy demands and their excitability, and because they are postmitotic and are therefore in most cases irreplaceable (284, 291). However, lower subtoxic levels of mitochondrial ROS can activate signaling pathways that protect cells against injury and disease.

VIII. Hormesis, Mitochondria, and Neuroprotection

Recent findings have overturned the long-held belief that mitochondrial ROS have only a negative impact on cell function and survival. It is now clear that mitochondrial superoxide and hydrogen peroxide play important roles in a range of cellular functions, and can also activate signaling pathways that promote cell survival and disease resistance. Exposure of hippocampal neurons to subtoxic levels of hydrogen peroxide triggers the release of Ca²⁺ from the ER by causing the opening of both IP₃ and ryanodine receptor channels (184). Interestingly, superoxide enhances long-term potentiation of synaptic transmission in hippocampal CA1 neurons by a mechanism requiring activation of ryanodine receptors and extracellular regulated kinases (207). Mitochondrial ROS may also play roles in recovery from injury. For example, superoxide can stimulate neurite outgrowth by directly activating protein kinase C (192). Moreover, peroxynitrite can promote the phosphorylation and nitration of regulatory sites within receptor tyrosine kinases, thereby activating cell survival signaling pathways, including those involving phosphatidylinositol 3-kinase (PI3K)–protein kinase B (Akt) and mitogen-activated protein (MAP) kinases (38). Nitration of proteins involved in synaptic vesicle trafficking can enhance

glutamate release, suggesting a potential role for superoxide and peroxynitrite in regulating neurotransmission (151).

Mitochondrial superoxide production is believed to contribute to damage of neurons in conditions ranging from chronic intermittent cerebral hypoxia (402) to Alzheimer's disease (225). However, it has been widely reported that transient exposure of neurons to low levels of superoxide that are converted into hydrogen peroxide can protect the neurons against a subsequent exposure to what would have otherwise been a lethal level of stress. This neuroprotective effect of a subtoxic increase in cellular oxidative stress has been termed "preconditioning" by neuroscientists who study stroke (162), but clearly falls under the broad umbrella of hormesis (72). Although the involvement of oxidants in many signaling pathways is well documented, the cellular strategies for conferring pathway specificity to such reactive molecules have remained more recondite. Recent studies now suggest that cells may spatially restrict oxidant production to allow microdomain-specific signaling (446). The specific molecular mechanisms by which mitochondrial ROS elicit hormetic responses in neurons are poorly understood, but emerging evidence suggests important roles for certain transcriptional regulators. For example, we showed that the transcription factor NF κ B is activated in neurons in response to oxidative stress and plays a pivotal role in the adaptive response that protects the neurons against more severe oxidative stress [see ref. (288) for review]. NF κ B induces expression of genes encoding several proteins that protect mitochondria against oxidative stress, including SOD2 and Bcl-2 (46, 115, 281). We also found that, contrary to the view that TNF exerts only detrimental effects on neurons, TNF upregulates expression of Mn-SOD *via* an NF κ B-mediated mechanism and can thereby protect neurons against excitotoxic, ischemic, and oxidative injury (45, 47).

While mitochondrial H₂O₂ may activate adaptive stress response pathways in neurons, they may also play neuroprotective roles by acting on other cell types in the nervous system. For example, microglial activation, which occurs in response to increased oxidative stress, can have either beneficial or detrimental effects on neurons and neural progenitor cells depending upon the type and amounts of cytokines and growth factors secreted by the microglia (168). A final example of *trans*-cellular hormesis mediated by ROS comes from studies showing that oxidative stress can stimulate angiogenesis in the brain (217, 295), a process that could be very important in restoring blood flow to neurons during the days and weeks after a stroke.

IX. Proteotoxicity, Cellular Stress Response, and the Vitagene Network

Protein thiols play a key role in redox sensing, and regulation of cellular redox state is a crucial mediator of multiple metabolic signaling and transcriptional processes (97, 108). More in general, protein quality control, which is a critical feature of intracellular homeostasis (100), is maintained by a highly complex network of molecular interactions that balances protein biosynthesis, folding, translocation, assembly/disassembly, and clearance (98, 312). The ability to ensure proper protein folding is critical for cellular function and organismal viability. In cells undergoing division, damaged and oxidized proteins can be sequestered and retained in mother cells, enabling daughter cells to have a pristine, undamaged proteome. However, in postmitotic cells, such as most neurons, protein quality control must be maintained by other, possibly more complex, mechanisms. Further, particular neuronal populations are more vulnerable to proteotoxicity, whereas others are more able to tolerate the misfolding and aggregation of disease

proteins. Thus, the cellular environment must play a significant role in determining whether disease proteins are converted into toxic or benign forms. The endomembrane neuronal network divides brain cells into different subcellular compartments that possess distinct sets of molecular chaperones and protein interaction networks (164). Cells buffer proteotoxic events related to intracellular protein misfolding *via* chaperone-mediated partitioning of nonnative conformers between pathways for proper folding, inclusion body formation, and degradation. Chaperones, in fact, act as agonists and antagonists of disease protein aggregation to prevent the accumulation of toxic intermediates in the aggregation pathway. Interacting partners can also modulate the conformation and localization of disease proteins and thereby influence proteotoxicity. Thus, interplay between these protein homeostasis network components can modulate the self-association of disease proteins and determine whether they elicit a toxic or benign outcome (164, 203). Polypeptides that fail to fold properly, along with damaged and oxidized mature proteins, are targeted for degradation by specialized cellular degradation machineries. A failure to prevent the misfolding and aggregation of one protein can destabilize the proteome, resulting in uncontrolled aggregation of other polypeptides. When conformationally challenged aggregation-prone proteins are expressed, the resulting unfolded or misfolded proteins are rapidly degraded *via* the ubiquitin–proteasome pathway. However, in some cases, protein aggregation leads to the development of the so-called conformational diseases. Among the conformational diseases are the human neurodegenerative diseases, such as Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD). In AD, dual digestion of amyloid precursor protein (APP) releases the aggregation-prone peptides that are collectively termed amyloid- β (A β). The accumulation and aggregation of A β (particularly the highly aggregative A β 1–42) is associated with AD. Similarly, aberrant aggregation of α -synuclein is associated with the emergence of PD, whereas Huntington and other CAG triplet diseases are typified by the accumulation of polyglutamine-containing aggregates. This also includes prion diseases such as Creutzfeldt–Jakob disease with accumulation of misfolded prion protein, type II diabetes with accumulation of islet amyloid polypeptide, and amyotrophic lateral sclerosis with aggregated superoxide dismutase-1 (186). One of the most characteristic features present in most brain oxidant disorders is the occurrence of extra- or intracellular fibrillar aggregates containing misfolded proteins with beta-sheet conformation. These aggregates are composed of distinct proteins in each neurodegenerative disease. As already mentioned, toxicity deriving from misfolded proteins or peptides are collectively termed proteotoxicity, and it has become evident that protein aggregation is tightly linked to the emergence and development of neurodegenerative diseases. Moreover, the mechanisms that have been found to counter toxic protein aggregation in different cell types and different organisms are highly conserved. Thus, it is likely that proteotoxicity that is associated the expression of neurodegeneration-linked aggregation-prone proteins in any tissue can provide insights into the neuronal defence mechanisms. Many of the insights that have been obtained from invertebrate-based or nonneuronal studies await confirmation in mammalian neuronal systems and clinical observations (132). Recent data suggest that small oligomeric aggregating structures, termed also protofibrils, are the underlying cause, rather than high-molecular-mass aggregates as previously assumed. *In vitro* studies on fragments of amyloidogenic proteins and synthetic peptides have established that the tendency for a protein to form amyloid is often limited to a short sequence of the full protein, known as an SRE (selfrecognition element). SREs form the core of amyloid fibrils. These amyloidogenic sequences constitute hotspots for aggregation of the native protein into amyloid fibrils and are often the sites of mutations leading to early-onset amyloidosis. In the case of tau-paired helical filaments, which accumulate in the

neurofibrillary tangles characteristic of AD and other neurodegenerative diseases, it has been shown that only three residues (Val-Tyr-Lys) are sufficient for fibril formation. Similarly, short sequences forming the core domain of various amyloid fibrils have been identified for A β (amyloid β -peptide), calcitonin, IAPP (islet amyloid polypeptide), insulin, and α -synuclein. It may be possible to delete residues freely on either side of an SRE while retaining the ability to form amyloid (11). Genetic and age-related factors, as it is known, act as vicious cycle increasing the amounts of pathogenic proteins in AD, where the increase in A β 42 levels is caused by (a) mutations in amyloid precursor protein or presenilins (e.g., γ -secretase), (b) by reactive oxygen species, and (c) by reductions in A β -degrading enzymes (A β DE), such as neprilysin and insulin-degrading enzyme, as well as increases in tau concentrations are influenced by oxidant damage, phosphorylation, and calcium (278); in PD, where increased levels of α -synuclein caused by triplication of its gene or mutations in parkin, DJ1, ubiquitin carboxy-terminal hydrolase 1 (UCHL1), phosphatase and tensin homolog induced kinase 1 (PINK1), or leucine-rich repeat kinase 2 (LRKK2) are associated with proteasome impairment and oxidative stress; and in HD, with polyglutamine expansions in huntingtin (HTT). The protein aggregation process itself is enhanced by increasing protein concentration; the action of transglutaminases; protein chaperone insufficiency; mutations in α -synuclein (PD) and polyglutamine expansions in huntingtin (HD); and/or posttranslational modifications, such as oxidations induced by, for example, hydrogen peroxide (H₂O₂), Fe²⁺, and Cu⁺, and phosphorylation. Although the proteins involved can differ, there is considerable overlap in the mechanisms by which they damage and kill neurons. Oligomers of A β , α -synuclein, and HTT might damage and kill neurons by inducing membrane-associated oxidative stress (MAOS), thereby impairing mitochondrial function and thus causing degenerative cell death (287).

Cellular stress response is the ability of a cell to counteract stressful conditions (Fig. 4). This phenomenon, which includes heat shock response (HSR), represents an ancient and highly conserved cytoprotective mechanism (270, 312, 416). Production of Hsps, including protein chaperones, is essential for the folding and repair of damaged proteins, serving thus to promote cell survival conditions that would otherwise result in apoptosis (20). The term “molecular chaperone” denotes a large family of ubiquitous proteins that function as part of an ancient defense system in our cells. Chaperones promote cell survival by sequestering damaged proteins and preventing their aggregation. During stressful conditions, such as elevated temperature, they prevent protein aggregation by facilitating the refolding or elimination of misfolded proteins. The stress-induced response to damaged proteins is helped by a sophisticated regulatory system, which shuts down most cellular functions and, in parallel, induces the synthesis of several chaperones and other survival-promoting proteins. Therefore, many of the chaperones are also called stress or heat shock proteins in reference to the archetype of cellular stress, heat shock. Besides their role during stress, chaperones have multiple roles under normal conditions. They promote the transport of macromolecules (e.g., proteins and RNA) and participate in remodeling events involving larger protein complexes, including signaling, transcription, cell division, migration, and differentiation. Molecular chaperones both in the cytosol (heat-shock proteins, crystallins, prefoldin, and Hsc70) and in the ER (Bip, Grp94, calnexin, and calreticulin) form large complexes and have a large number of cochaperones to regulate their activity, binding properties, and function (380). These chaperone complexes regulate local protein networks, such as the mitochondrial protein transport apparatus and the assembly and substrate specificity of the major cytoplasmic proteolytic system, the proteasome (380). For instance, mood and anxiety

disorders are considered stress-related diseases characterized by an impaired function of mineralocorticoid and glucocorticoid receptors (MR and GR, respectively), the major regulatory elements of the hypothalamus–pituitary–adrenocortical (HPA) axis. Hence, a number of chaperone proteins moderate the function of these receptors. Genetic variations in one of these chaperones, FKBP5, have been associated with antidepressant treatment response and with a major risk factor for the development of posttraumatic stress disorder (395). Chaperones are well known to protect the cell nucleus after stress. Consistent with this, Hsp70 was shown to drive damaged nuclear proteins to the nucleolus, clearing other nuclear components of misfolded proteins and decreasing the danger of their widespread aggregation (139). In agreement with these findings, chaperones promote the transport of ribosomal subunits and the mobility of steroid receptors inside the nucleus (139). Molecular chaperones regulate both the activation and the disassembly of numerous transcriptional complexes (380). Thus, chaperones emerge as regulators of the transcriptional network (139, 272). Stress-induced nuclear translocation of chaperones may preserve nuclear remodeling capacity during environmental damage, and thus protect the integrity of DNA. Consistently, there is significant interest in the discovery and development of small molecules that modulate HSRs and parallel stress response pathways for therapeutic purposes (99, 106, 108, 309, 452).

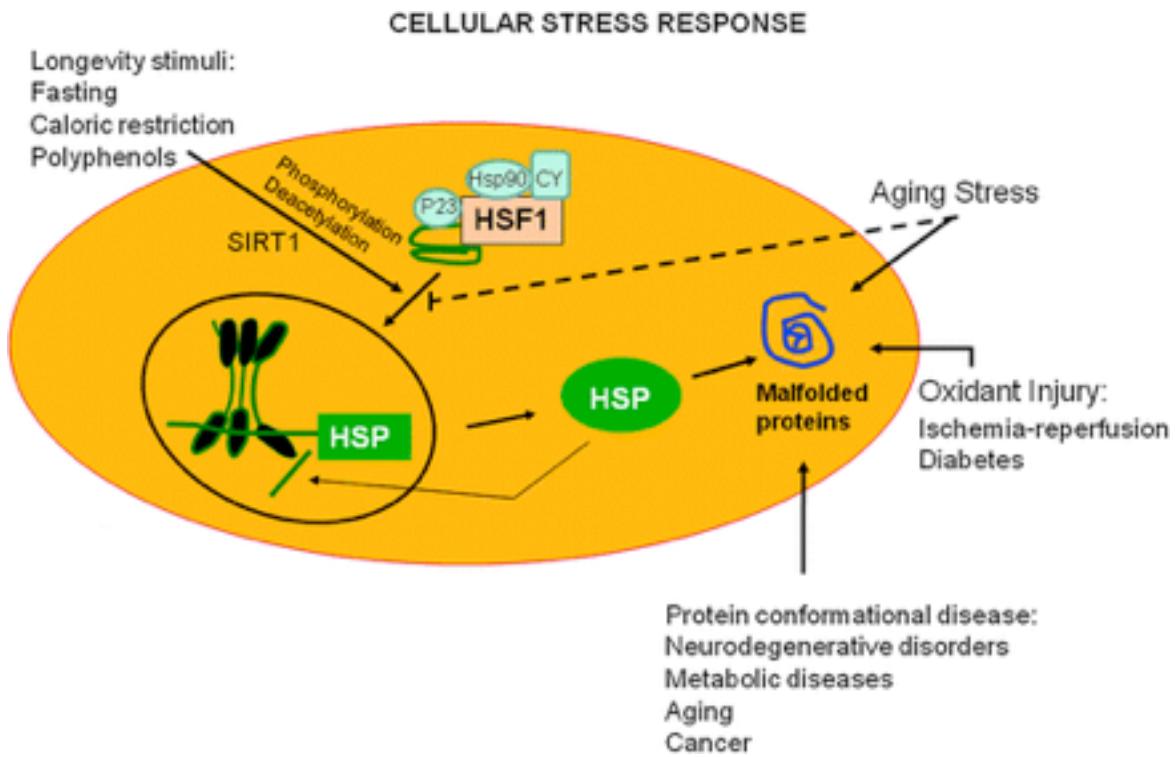


FIG. 4. Cell stress responses. Expression of HS genes including chaperones and components of the clearance machinery is induced in response to physiological and environmental stress conditions, including longevity stimuli, such as fasting, caloric restriction, or polyphenol antioxidants, and protein conformational diseases. HSF1 can also be directly stimulated by longevity stimuli such as the histone deacetylase SIRT1 that directly activates HSF1 by deacetylation, thus fostering longevity. The increased flux of damaged or misfolded proteins in

response to proteotoxic environmental conditions (stress) is the trigger for the induction of the cellular stress response. Aging, however, is associated with a gradual decline in potency of the heat shock response and this may prevent repair of protein damage, leading to degeneration and cell death. HSF, heat shock transcription factor; SIRT, silent information regulator two.

Cellular stress response ([Fig. 4](#)) requires the activation of pro-survival pathways that, under control of protective genes called vitagenes ([99](#), [100](#), [106–108](#)), result in the production of molecules (Hsps, glutathione, and bilirubin) endowed with antioxidant and antiapoptotic activities. Generally, molecular chaperones help hundreds of signaling molecules to keep their activation-competent state, and regulate various signaling processes ranging from signaling at the plasma membrane to transcription. In addition to these specific regulatory roles, recent studies have revealed that chaperones act as genetic buffers stabilizing the phenotypes of various cells and organisms ([43](#)). Protein function is regulated by the proteostasis network ([20](#)), an integrated biological system that generates and protects the protein fold. Proteostasis refers to controlling the concentration, conformation, binding interactions (quaternary structure), and location of individual proteins making up the proteome by readapting the innate biology of the cell, often through transcriptional and translational changes. Proteostasis thus influences specific cellular functions and enables differentiated cells to change their physiology for successful organismal development and aging in the face of constant intrinsic and environmental challenges to prevent disease onset. Proteostasis is influenced by the chemistry of protein folding/misfolding and by numerous regulated networks of interacting and competing biological pathways that influence protein synthesis, folding, trafficking, disaggregation, and degradation. The composition of the proteostasis network is regulated by signaling pathways, including the unfolded protein response (UPR), the HSR, the ubiquitin proteasome system (UPS), together with epigenetic programs ([185](#)). The same networks are preferentially remodeled in various diseases and aging, which may help us to design novel therapeutic and antiaging strategies ([312](#)). Among the cellular pathways involved in the so-called programmed cell life conferring protection against oxidative stress, a key role is played by the products of vitagenes ([97–100](#), [105–108](#), [281](#)). These include members of the Hsp family, such as heme oxygenase-1 and Hsp72, sirtuins, and the thioredoxin/thioredoxin reductase system ([103](#), [106](#), [107](#)). Recent studies have shown that the HSR 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 HSR, there is now strong interest in discovering and developing pharmacological agents capable of inducing the HSR ([99](#)). Molecular chaperones are known to disrupt aggregates actually promoted active aggregation when the concentration of the aggregating protein is high. Consistent with this notion, it has been proposed that, although protein aggregation is hazardous under certain circumstances, the creation of apparently less-toxic large aggregates is protective ([335](#)). This hypothesis is the basis of the therapeutic potential of Hsps, which prevent protein misfolding and aggregation. Transgenic animal models of the diseases have demonstrated that induction or overexpression of Hsps can suppress neuronal dysfunction and degeneration. Hsp70 can reduce the amount of misfolded and aggregated a-Syn species *in vivo* and *in vitro*, and protect neuronal cells from a-Syn-dependent neurotoxicity ([120](#)). Hsp104 reduced the phosphorylation of a-Syn inclusions and prevented the nigrostriatal dopaminergic neurodegeneration induced by mutant a-Syn (A30P) ([235](#)). Recent findings regarding the pathogenic species generated during fibril formation have highlighted some of the beneficial and problematic aspects of Hsp-based therapy.

X. Sirtuins and the Integration of Adaptive Stress Responses in Neurons

Sirtuins are a family of histone deacetylases that, in humans, includes at least seven members (silent information regulator two [SIRT]1–7) that exhibit different cellular and subcellular localizations and substrate specificities [see ref. (144) for review]. The best studied sirtuin is SIRT1, an NAD⁺-dependent enzyme that deacetylates several different protein substrates involved in the regulation of cellular energy metabolism and redox state, thereby influencing cell survival and plasticity (Fig. 5). In yeast, worms, and flies, the SIRT1 homolog (Sir2) has been shown to play a major role in lifespan determination and stress resistance [see ref. (34) for review]. Similarly, emerging evidence suggests important roles for SIRT1 in hormetic responses of cells to a range of metabolic and oxidative stressors. In addition to roles in chromatin remodeling effected by deacetylating histones, SIRT1 has been shown to deacetylate and thereby activate a transcriptional regulator called peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC-1 α). PGC-1 α is believed to play a pivotal role in adaptive responses of cells to dietary energy restriction and exercise (30, 183), effectively increasing cellular stress resistance by upregulating expression of a set of genes that encode proteins (peroxisome proliferators activated receptor- γ , pyruvate dehydrogenase kinase isoform 4, and estrogen-related receptor- γ) that control the production of antioxidant and detoxifying enzymes (Fig. 5). PGC-1 α has also been shown to increase the proliferation of mitochondria in neurons (362). In comparison with SIRT1, very little is known concerning the functions of SIRTs 2–7, but their differential subcellular localization is providing interesting clues; for example, SIRT3 is associated with mitochondria, where it may deacetylate protein substrates involved in energy metabolism (3).

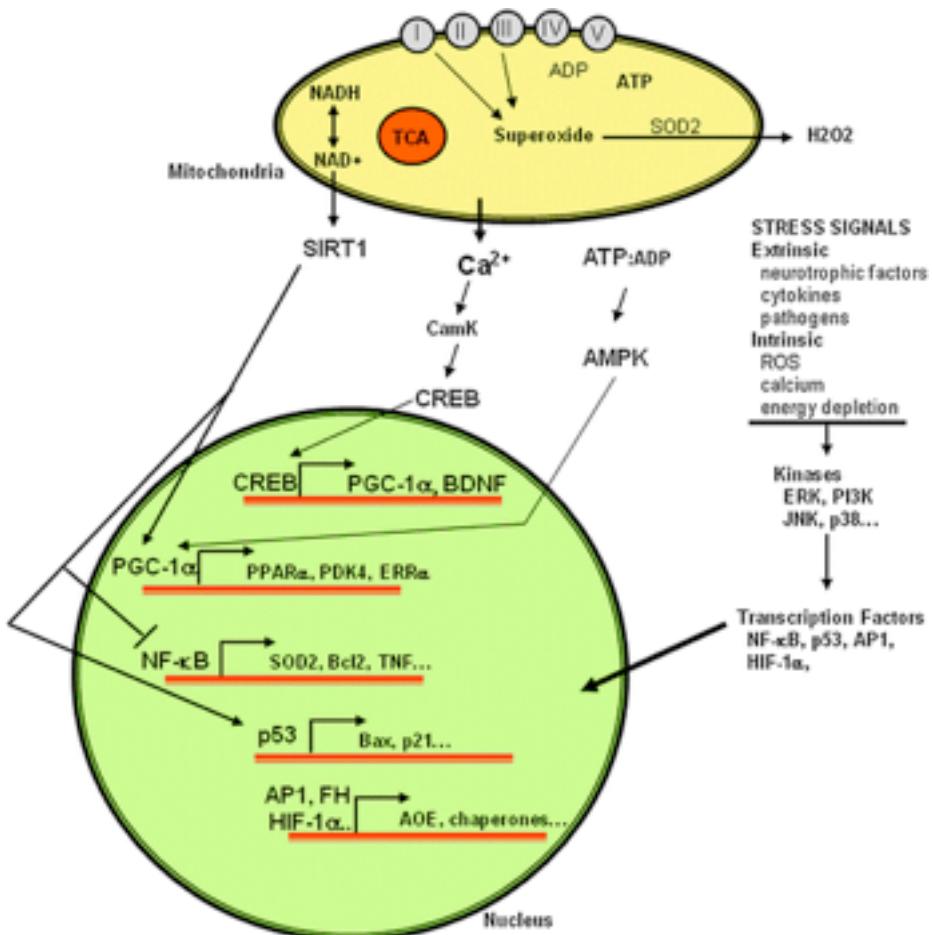


FIG. 5. SIRT1-based and related hormetic signaling pathways in neurons are coupled to transcriptional regulators that control expression of genes involved in neuronal plasticity and cell death. AMPK, adenosine monophosphate-activated protein kinase; AOE, antioxidant enzyme; BDNF, brain-derived neurotrophic factor; CREB, cyclic AMP response element binding protein; ERK, extracellular signal regulated kinase; ERR, estrogen-related receptor; FH, forkhead transcription factor; HIF, hypoxia inducible factor; JNK, jun *N*-terminal kinase; PDK, pyruvate dehydrogenase kinase; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1 α ; PPAR, peroxisome proliferator activated receptor; SOD, superoxide dismutase.

Together with DNA methylation, acetylation of histones and transcription factors plays roles in regulating gene expression in neurons [see ref. (285) for review]. Through these activities, sirtuins are shown to regulate important biological processes, such as apoptosis, cell differentiation, energy transduction, and glucose homeostasis (262, 289, 361). In cultured mammalian cells, Sirt1 is activated in response to growth factor deprivation and increased oxidative stress. Changes in the cellular redox state as reflected by pyridine nucleotide homeostasis, specifically concentrations of NAD $^{+}$ or the ratio of the concentration of NAD $^{+}$ and its reduced form NADH, control the deacetylase activity of Sir2 and its homologs. During hypoxic stress, redox changes in cellular metabolism occur that activate Sirt1 and integrate also with hypoxia-inducible factors (HIFs) which are transcriptional regulators that control genes induced during hypoxia and other stresses (Fig. 5). Activation of the founding HIF member, HIF-1a, is increased when oxygen concentrations are reduced. The second HIF alpha member, endothelial known as HIF-2a, is closely related to HIF-1a in structure and is likewise activated during hypoxia. HIF-2a target genes identified from mouse knockout studies include Sod2 encoding the mitochondrial-localized manganese superoxide dismutase, VegfA encoding the proangiogenic regulator vascular endothelial growth factor A, and Epo encoding the cytokine erythropoietin. Sirt1 augmentation of HIF-2 signaling is conferred through formation of a Sirt1/HIF-2a complex as well as through Sirt1-mediated deacetylation of acetylated HIF-2a (161). Some studies have provided evidence that activation of SIRT1 can protect neurons against degeneration, whereas others suggest that SIRT1 promotes neuronal death. In a mouse model of peripheral nerve degeneration, nicotinamide prevents axonal degeneration and SIRT1 is a pivotal downstream mediator axonal protection (15). Overexpression of nicotinamidase in flies increases their lifespan in a Sir2-dependent manner, and nicotinamidase overexpression in cultured human neurons increases their resistance to oxidative stress, also in a sirtuin-dependent manner (19). The function of Sir2 deacetylases is negatively regulated by nicotinamide, a by-product feedback inhibitor in deacetylation reactions involving NAD $^{+}$ -dependent deacetylases and also a metabolite in the nucleotide salvage pathway. Reactions performed by these enzymes can rapidly deplete cellular NAD and generate nicotinamide, which acts as a potent feedback inhibitor of the NAD $^{+}$ -dependent deacetylases (199). Thus, in the absence of nicotinamide, sirtuin protein activity is enhanced. Salvage enzymes, such as nicotinamidase that deaminates nicotinamide into metabolites that can be recycled back to NAD, increase activity of Sir2 deacetylases, thus

exerting, in yeast, effects on life span. All this is corroborated in mammals, by the finding that increased nicotinamide clearance in cells provides positive effects on organism life span and cellular response to oxidative stress. This supports the hypothesis that manipulation of nicotinamide metabolism through genetic approaches or pharmacological agents in vertebrates could yield similar beneficial results (125). D'Mello and his colleagues showed that SIRT1 can protect cultured cerebellar neurons against apoptosis induced by potassium deprivation, apparently by a mechanism independent of its deacetylase activity (352). In contrast, SIRTs 2, 3, and 6 induced apoptosis, suggesting opposing actions of different sirtuins, at least in this particular cell culture model.

Interestingly, emerging evidence suggests that sirtuins may be involved in the regulation of synaptic plasticity (22, 303), and adaptive responses of brain cells to a range of stressors (Figs. 4 and 5). For example, decreased activities of histone deacetylases (HDAC1 and HDAC2), whose activity is not dependent on NAD⁺, during early synaptic development enhance excitatory synapse maturation associated with increased synapse numbers, whereas in mature neurons a decrease in HDAC2 levels attenuates basal excitatory neurotransmission without a change in the numbers of synapses (4). SIRT1 may also play important roles in the regulation of neural progenitor cell fate decisions because SIRT1 is upregulated in neural progenitor cells in response to mild oxidation, and suppression of SIRT1 expression in neural progenitors prevents oxidative stress-induced suppression of neurogenesis (360). Because of their roles in cellular stress responses, sirtuins would be expected to play particularly important roles in adaptive responses of neural cells to stress and, presumably, the enhancement of synaptic plasticity and neurogenesis in response to exercise, dietary energy restriction, and other hormetic environmental factors (291). In support of this notion, SIRT1 has been shown to interact either directly or indirectly with several pathways known to be involved in adaptive neural plasticity, including the Ca²⁺-calmodulin-cyclic adenosine monophosphate (AMP) response element binding protein (409) and NF-κB (382) systems (Fig. 5).

It has also been reported that the SIRT1 activator resveratrol can protect neurons against degeneration in experimental models of Alzheimer's disease and amyotrophic lateral sclerosis (229). Similarly, resveratrol treatment protected neurons against the toxic effects of polyglutamine proteins in animal models of Huntington's disease (348). Dietary energy restriction can protect neurons against dysfunction in mouse models of Alzheimer's disease (197), and recent evidence suggests a role for SIRT1 in this neuroprotective action of caloric restriction (363).

Resveratrol was recently shown to affect the activity of SIRT1 *in vitro* although its effects seem to depend on the nature of the substrate for deacetylation (6). However, *in vivo*, resveratrol has been shown to exert effects dependent on sirtuin orthologs—extension of lifespan in yeast, *C. elegans*, and *Drosophila*, and metabolic effects on mammalian cells (378). In addition, resveratrol protects *C. elegans* neurons expressing a fragment of the Huntington-disease-associated protein huntingtin and mammalian neurons from mutant polyglutamine cytotoxicity in an HdhQ111 knock-in mouse model of Huntington disease (144). Two recent studies have also shown that deleterious effects of high-fat, high-caloric diets in mice were mitigated by resveratrol feeding. In one study, the shortening of lifespan by the high-fat diet was reversed (305). In a second study, resveratrol increased SIRT1 activation, PGC-1α deacetylation, and

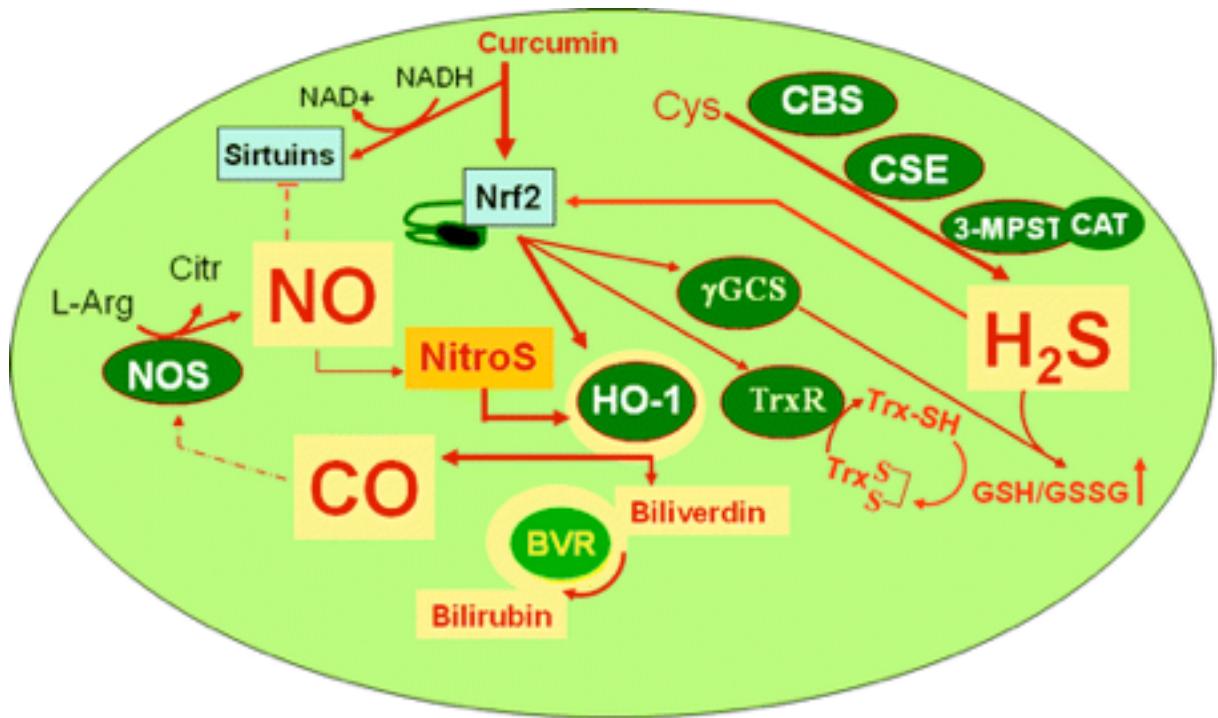
mitochondrial biogenesis in muscle (247). Although resveratrol has been claimed to be a *bona fide* SIRT1 activator, whereby offering a promising approach for treating metabolic disorders, with a potential to change the practice of medicine, recent reports indicate that this finding might be an experimental artifact and need to be clarified (26). It has been suggested that metabolism of the redox couple NAD/NADH provides a link between sirtuin activity and the control of cell senescence and organism life-span: NAD-dependent protein deacetylation helps maintain the juvenile phenotype, whereas inhibition of deacetylation activity by NADH or nicotinamide, or by NAD unavailability, promotes the onset of cellular aging and decrease organism lifespan (6, 100). Raising NAD levels, or lowering NADH levels by increasing its oxidation also promote sirtuin activation, with concomitant beneficial effects on cell survival (201). There exists an interrelationship and overlap between sirtuin regulation, generation of altered proteins, and mitochondrial activity, exerted by metabolic effects on NAD and NADH levels (Fig. 5). It has been proposed that when protein synthesis is strongly upregulated (e.g., during growth and sirtuin-enhanced mitogenesis) there is a concomitant stimulation in chaperone protein synthesis and proteolytic potential, to ensure rapid removal of erroneously synthesized/misfolded polypeptide chains. This hypothesis is sustained by recent finding that heat-shock protein expression (466) and autophagy (383) are enhanced by NAD⁺-activated SIRT1 activity, thereby facilitating recognition and elimination of altered proteins. Further, sirtuin-mediated stimulation of aerobic mitochondrial activity, for example, in response to caloric restriction (14), will also facilitate NADH oxidation back to NAD⁺, thereby improving NAD⁺ availability and decreasing the potential for generation and accumulation of altered proteins (202). In the Wallerian degeneration slow (Wlds) mouse model, SIRT1 activation protects axons against neuronal injury (144). This Wlds mouse bears in fact a dominant mutation producing an overexpressed chimeric Wlds protein composed of the ubiquitin assembly protein Ufd2a and the NAD⁺ salvage pathway enzyme NMNAT1. Decreasing SIRT1 activity reduces the axonal protection originally observed, whereas SIRT1 activation by resveratrol decreases the axonal degeneration after neuronal injury (430). This suggests that the neuroprotection in the Wlds mouse model is achieved by increasing the neuronal NAD⁺ reserve and/or SIRT1 activity (144). In addition, SIRT1 activation significantly decreases neuronal cell death induced by amyloid-beta (A β) peptides through inhibition of NF κ B signaling (144). Specific brain hSIRT1 overexpression in transgenic animals induces a significant increase in the α -secretase activity, an enzyme that cleaves the amyloid precursor peptide (APP) within the A β peptide, thereby mediating the nonamyloidogenic pathway of the APP processing (144). These studied animal models have indicated that SIRT1 could contribute to the pathogenesis of some complex diseases. In line with this hypothesis, genetic variants (single nucleotide polymorphisms) in the human SIRT1 gene have been shown to be tightly associated with energy expenditure (247).

While SIRT1 may promote neuronal survival and plasticity in some settings, it can contribute to the death of neurons in experimental models of some neurodegenerative disorders (353). For example, sirtuin inhibitors protect neurons against α -synuclein-mediated toxicity in models of Parkinson's disease (309). Others have reported that nicotinamide treatment restores cognitive performance in Alzheimer's disease transgenic mice *via* a mechanism involving sirtuin inhibition and reduction of levels of hyperphosphorylated tau (194). Another study provided evidence that SIRT1 may promote neuronal death by activating a insulin-like growth factor-1 signaling pathway (258), although other findings suggest that insulin-like growth factor-1 signaling is itself neuroprotective (123). Our own findings suggest that SIRT1 can promote neuronal survival

under conditions where cellular energy levels are sufficient, but may endanger neurons by depleting NAD⁺ under conditions of marginal cellular energy levels (262, 263). This biphasic action of SIRT1 depending upon cellular NAD⁺ levels is similar to that previously described for PARP [poly-ADP(ribose) polymerase], which can protect neurons against apoptosis when energy levels are sufficient, but contributes to energy failure in conditions such as ischemia and hypoxia (169). While the roles of SIRT1 in neurodegenerative conditions therefore appear complex, the development of agents that selectively activate or inhibit SIRT1 is being actively pursued with the expectation of novel therapeutic treatments for a range of neurological disorders (248). Moreover, it has been recently shown that SIRT2 inhibition, through nicotinamide (NAM), O-acetyl-ADP-ribose (O-AA-ribose), and AGK, prevented α -synuclein cytotoxicity and modulated its aggregation in cultured cells; ameliorated mutant α -synuclein neurotoxicity in rat primary dopamine-positive neurons; and rescued degeneration of dopaminergic neurons from α -synuclein toxicity in a Drosophila animal PD model (341). These results suggested that modulation of α -synuclein aggregation pathway could be one of the sirtuin neuroprotective mechanisms (340). The exact mechanism whereby SIRT2 inhibition affects α -Synuclein aggregation remains uncertain. Increased α -tubulin acetylation is associated with microtubule stabilization, and has been reported to interact with α -tubulin as well as the microtubule-binding proteins MABP1 and tau. One possibility is that the increase in acetylated α -tubulin resulting from SIRT2 inhibition may stimulate aggregation of α -Synuclein through its affinity to microtubules. Moreover, microtubule stabilization itself could be an important factor contributing to neuroprotection (341). Sirtuins hold a great potential as therapeutic targets in neurodegeneration; however, the development of therapeutic activators and inhibitors against the various sirtuin isoforms is necessary to assess the therapeutic potential of these targets in rodent models of neurodegenerative diseases, so that the development of lead-candidate compounds for human clinical trials can be expedited.

XI. The Kelch-Like ECH-Associated Protein 1/Nrf2/Antioxidant Response Element Pathway
As mentioned above, a central regulator in gene expression of Hsps is heat shock transcription factor 1 (HSF1) (310). In addition, some of the vitagenes are also upregulated as part of the phase 2 response, also known as the electrophile counterattack response, a cytoprotective response that protects against various electrophiles and oxidants (97, 104–106, 146) (Figs. 6 and 7). Examples include heme oxygenase 1, thioredoxin, and thioredoxin reductase, all of which can be upregulated by the transcription factor Nrf2 (Nuclear factor-erythroid 2 p45-related factor 2) coordinately with a battery of cytoprotective proteins, such as glutathione S-transferases (GSTs), UDP-glucuronosyltransferase, NAD(P)H quinone oxidoreductase 1 (NQO1), epoxide hydrolase, ferritin, γ -glutamylcysteine synthetase, glutathione reductase, aldo-keto reductases, and glutathione conjugate efflux pumps (106). This elaborate network of protective mechanisms allows eukaryotic organisms to counteract the damaging effects of oxidants and electrophiles, major agents involved in the pathogenesis of cancer, atherosclerosis, neurodegeneration, and aging (227). Gene expression of this group of functionally diverse proteins is regulated by the Kelch-like ECH-associated protein 1 (Keap1)/Nrf2/antioxidant response element (ARE) pathway (Fig. 7). Long before this pathway was discovered, it had been reported that exposure to low doses of carcinogens protects against the toxicity of a subsequent encounter with high doses of carcinogens [reviewed in ref. (205)], illustrating the phenomenon of hormesis, or preconditioning. Thus, as far back as the late 1920s Berenblum showed that topical application

of dichlorodiethyl sulfide reduced skin tumor incidence in mice painted with tar (31). Similar were the findings of Lacasagne and colleagues and Riegel and colleagues, who reported that weakly carcinogenic hydrocarbons (e.g., 1,2,5,6-dibenzofluorene and 1,2,5,6-dibenzoacridine) protected against the carcinogenicity of highly active carcinogenic hydrocarbons 1,2,5,6-dibenzo(a)anthracene and 3-methylcholanthrene (246, 371). Importantly, the protection was much more effective with pretreatment and simultaneous treatment compared to simultaneous treatment alone. The studies of Richardson and Cunningham, who showed that intravaginal administration of 3-methylcholanthrene could inhibit liver carcinogenesis caused by orally administered 3'-methyl-4-dimethyl-aminoazobenzene, demonstrated that protection could be achieved even when the protector and the carcinogen were administered by two different routes (370) and thus excluded the possibility of a direct interaction between the protector and the carcinogen. The first insights into the protective mechanism(s) were provided by James and Elizabeth Miller and their colleagues, who demonstrated that 3-methylcholanthrene was an inducer of enzymes that catalyze the conversion of carcinogens to inactive (detoxification) products (134, 304).



IG. 6. Endogenous biosynthetic pathways of (a) NO, involving NOSs, (b) CO, involving HO isoforms (HO-1, HO-2), and (c) H₂S, involving CBS, CSE, and MPST. Methionine, which is derived from alimentary sources, is converted to S-adenosylmethionine by methionine adenosyltransferase. S-adenosylmethionine is subsequently hydrolyzed to homocysteine by glycine *N*-methyltransferase. Cystathione β -synthase catalyses the production of cystathione by transferring serine to homocysteine. Cystathione γ -lyase, a pyridoxal 5'-phosphate-dependent enzyme, subsequently converts cystathione to cysteine. In the mitochondria, cysteine can get converted to 3-mercaptopyruvate by aspartate aminotransferase, which can then

be converted to H₂S by MPST. CBS, cystathionine β -synthase; CO, carbon monoxide; CSE, cystathionine γ -lyase; HO, Heme oxygenase; H₂S, hydrogen sulfide; MPST, 3-mercaptopyruvate sulfur transferase; NO, nitric oxide; NOS, NO synthase.

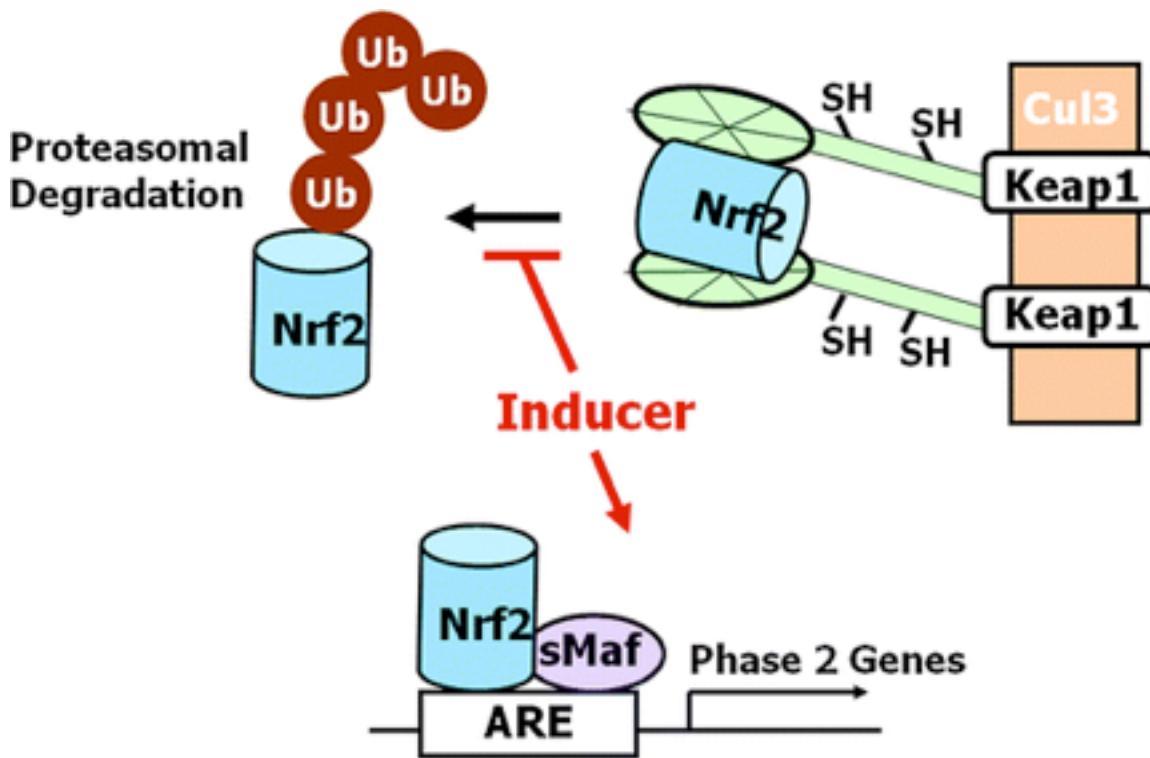


FIG. 7. The Keap1/Nrf2/ARE pathway. Under basal conditions transcription factor Nrf2 is bound to a cytoplasmic repressor Keap1, which targets Nrf2 for ubiquitination and proteasomal degradation *via* association with the Cullin 3-based E3 ubiquitin ligase complex. Small molecule inducers modify highly reactive (sensor) cysteine residues of Keap1, which loses its ability to target Nrf2 for degradation. This results in stabilization of Nrf2, binding to the ARE (in heterodimeric combinations with a small Maf transcription factor), and activation of the transcription of cytoprotective (phase 2) genes. ARE, antioxidant response element; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor-erythroid 2 p45-related factor 2.

In the 1960s Charles Huggins and his colleagues reported that small doses of various aromatic hydrocarbons protected against the toxicity and carcinogenicity of subsequent exposure to large doses of 7,12-dimethylbenz(*a*)anthracene (208, 209). The phenomenon was called induced protection. Importantly, protection only occurred when the protector was given before the carcinogen and protection was long-lasting (in some cases up to 6 days), indicating that some synthetic process was of critical importance. This hypothesis was further strengthened by the finding that protein synthesis was required for protection; protection was abolished by the protein

synthesis inhibitor ethionine and could be specifically restored only by methionine, but not by any other amino acid (208). This study led to the discovery that NQO1 was induced by the protective agents in several tissues, that is, in liver, lung, adrenal, and mammary gland (208). It is now widely recognized that NQO1 is a cytoprotective enzyme (152, 439, 440). A highly quantitative and robust bioassay based on the ability to induce NQO1 in murine hepatoma (Hepa1c1c7) cells is used to screen for potent small molecule inducers. Importantly, inducers identified using this screen have subsequently been confirmed to protect against tumor development, and to confer cardiovascular and neuroprotective effects in various animal models (155, 170, 222, 357).

In the 1960s Frankfurt (180) and Wattenberg (463) found independently that the phenolic antioxidants BHA and BHT that are widely used as food preservatives and therefore consumed by humans protected experimental animals against the toxicity and carcinogenicity of 7,12-dimethylbenz(*a*)anthracene and other carcinogens. The findings of Talalay, Bueding, and their colleagues that supplementation of the diet of rodents with BHA resulted in dramatic reduction of the mutagenic metabolites of benzo(*a*)pyrene, which was accompanied by selective induction (by enhanced transcription) of several cytoprotective proteins (*i.e.*, GSTs, uridine diphosphate [UDP]-glucuronosyltransferases, and NQO1) without significant effects on enzymes that catalyze the activation of this pro-carcinogen (*e.g.*, cytochrome P450 enzymes), provided a mechanistic explanation for the observed protection [reviewed in refs. (435, 436)]. Subsequent studies involving many inducers some of which are related to BHA, but many of which belong to a wide variety of structurally unrelated chemical classes, led to two crucial discoveries: (a) all inducers share a common chemical property, namely, the ability to react with sulfhydryl groups (437); (b) inducers activate the transcription of cytoprotective genes that occurs in the liver and many peripheral tissues [reviewed in ref. (356)] ensuring fitness for meeting subsequent challenges, and ultimately survival. Again, these discoveries represented an illustration of the hormesis phenomenon and the designation of the Electrophile Counterattack response was proposed (356). It is now recognized that due to their functional versatility (Table 1) the transcriptional induction of cytoprotective genes is a highly effective strategy for achieving protection against a variety of chronic conditions, such as neoplasia, atherosclerosis, hypertension, and neurodegeneration (205, 219, 228, 329, 438).

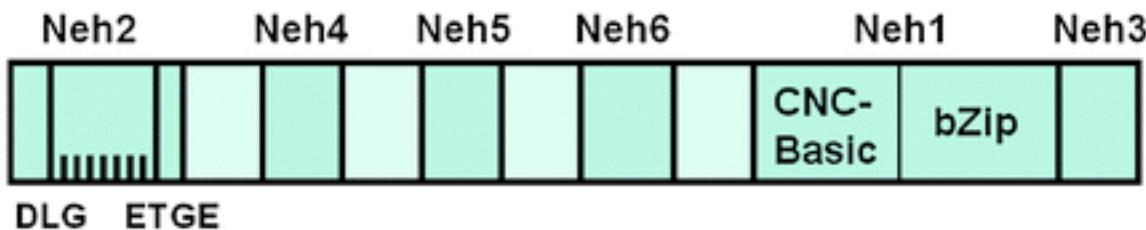
Gene expression of these cytoprotective proteins is coordinately regulated by a common molecular mechanism that involves the Keap1/Nrf2/ARE pathway (Fig. 7). The upstream regulatory regions of these genes contain single or multiple copies of the antioxidant/electrophile response elements (ARE and EpRE) with the consensus sequence 5'-A/CTGAC/GNNNGCA/G-3' (182, 324, 375, 376). The major transcription factor that binds to the ARE is Nrf2, a basic leucine zipper transcription factor. Activation of gene expression requires that Nrf2 binds to the ARE in heterodimeric combinations with members of the small Maf family of transcription factors (314). Under basal conditions the pathway operates at low levels due to the repressor function of the cytosolic protein Keap1, which binds to the E3 ubiquitin ligase Cullin3-RING box1 and presents Nrf2 for ubiquitination and subsequent proteosomal degradation (200, 236, 324).

Inducers of the Keap1/Nrf2/ARE pathway belong to at least 10 distinct chemical classes: (a) oxidizable diphenols, phenylenediamines, and quinones; (b) Michael acceptors (olefins or

acetylenes conjugated to electron-withdrawing groups); (c) isothiocyanates; (d) thiocarbamates; (e) trivalent arsenicals; (f) dithiolethiones; (g) hydroperoxides; (h) vicinal dimercaptans; (i) heavy metals; and (j) polyenes. The only common property among them is their chemical reactivity with sulphydryl groups by oxido-reduction, alkylation, or disulfide interchange. (153, 155, 356, 358, 421, 438). This common property led Talalay and his colleagues to propose that the sensor for inducers must be a molecule (perhaps a protein) endowed with exquisitely reactive cysteine residues (160, 438). Indeed, there is now compelling experimental evidence that exogenous and endogenous inducers chemically modify specific and highly reactive cysteine residues of Keap1, which, in addition to being a repressor for Nrf2, also functions as the intracellular sensor for inducers (157, 216). This reaction leads to conformational changes in Keap1 that abrogate its ability to repress Nrf2, ultimately resulting in Nrf2 stabilization, binding to the ARE and recruitment, the basal transcriptional machinery to activate transcription of cytoprotective genes (200, 237, 315). Whereas many aspects of the model are still controversial and the intricate details of the regulation of the pathway are not fully understood (200, 324, 345), there is voluminous experimental evidence for a mechanism of regulation of Keap1 activity through its reactive cysteine residues. Of note, even though Nrf2 is regulated primarily at the level of protein stability, the consequences of its stabilization are due to its downstream target genes that have relatively long half-lives (on the order of days). Thus, the evaluation of the effects of inducers on the levels of the Nrf2-target gene expression or activity rather than on Nrf2 levels themselves represents a much more biologically meaningful measure of cellular protection against damage.

Murine Nrf2 is composed of 597 amino acids and has a molecular weight of 60 kD (Fig. 8). Nrf2 is a Cap 'n' Collar (CNC) transcription factor that belongs to the class of basic leucine zipper (b-Zip) proteins. These proteins form heterodimers with members of the Maf family of transcription factors. Keap1 is a multidomain homodimeric protein that comprises 624 amino acids and has a molecular weight of 69 kD. Murine Keap1 contains 25 cysteine residues (its human homologue has 27), 9 of which (*i.e.*, C23, C38, C151, C241, C273, C288, C297, C319, and C613) are flanked by basic amino acids, which in turn are known to lower the pKa values of the cysteines, favoring the formation of the thiolate anions at neutral pH, thus making them potentially highly reactive. We and others have reported that C151, C273, and C288 are of particular importance in the function of Keap1 as a sensor for inducers and as a repressor of Nrf2 (154, 253, 461, 477).

Nrf2



Keap1

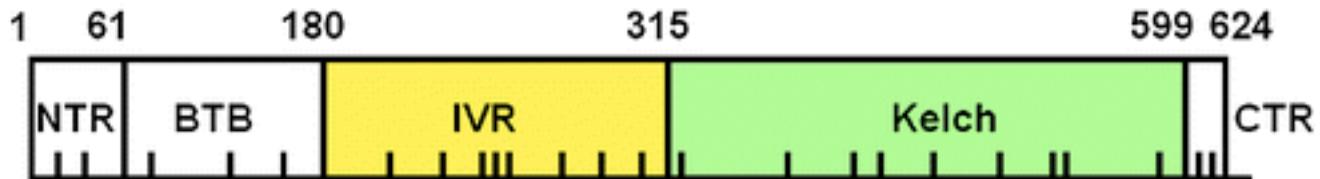


FIG. 8. Overall structures of Nrf2 and Keap1 showing the different domains. In Nrf2, the DLG motif (amino acids 27–32) and the ETGE motif (amino acids 77–82) of the Neh2 domain comprising the two Keap1-binding sites are indicated and the black bars represent the lysine residues that are ubiquitinated by Cul3-Rbx. The Neh1 and Neh3 domains form the DNA-binding site of the transcription factor. In Keap1, the BTB domain is the dimerization domain and the site of interaction with Cul3. The Kelch domain is the Nrf2-binding domain. The black bars indicate the distribution of the cysteine residues of Keap1.

The BTB domain of Keap1 represents a protein–protein interaction motif (Fig. 8). Indeed, Keap1 exists as a homodimer through the BTB domains of its subunits. Two groups have reported the crystal structure of the Kelch domain showing the formation of a 6-bladed β -propeller structure, with residues at the C-terminus forming the first strand in the first blade (257, 344, 449). It is through the Kelch domain that Keap1 binds to the Neh2 domain of Nrf2. For binding between Keap1 and Nrf2 to occur, two motifs within Nrf2 are critically important: the ETGE and the DLG. On the basis of a series of elegant studies, McMahon *et al.* (299, 450, 451) proposed that, first, the ETGE motif of Nrf2 binds to an arginine triad on one of the Keap1 subunits. This binding then positions the DLG motif of Nrf2 in the vicinity of the triad on the second subunit of Keap1. This so-called fixed-ends or hinge-and-latch model explains the observation that the binding affinity of Keap1 for the ETGE motif is much higher than its affinity for the DLG motif and predicts that the lysine residues in Nrf2 targeted for ubiquitination must be positioned between the two Keap1-binding sites. Indeed, all seven lysine residues of Nrf2 (out of a total of 27) that have been implicated in ubiquitination are located between the ETGE and DLG motifs, a portion of the protein that is largely helical (451).

Whereas Keap1 is the major repressor of Nrf2 and the sensor for inducers of Nrf2-dependent genes, DJ-1 has been recently implicated as a necessary factor required for Nrf2 stability and activity (130), even though up to date no evidence for a physical interaction between DJ-1 and either Nrf2, Keap1, or Cullin 3 has been reported. Nonetheless, the ability of DJ-1 to stabilize Nrf2 may be an essential contributor to the importance of DJ-1 for cellular protection against oxidative and ER stress. Conversely, the loss of DJ-1 may represent an important factor in the decreased inducibility of Nrf2-dependent genes that has been observed under certain disease conditions (275) and with aging (133). Thus, loss-of-function mutations in *PARK7*, the gene encoding DJ-1, are associated with autosomal recessive early onset Parkinsonism (39).

Another protein that interacts with Keap1 resulting in Nrf2 activation is p62 (238). Both genetic and pharmacological activation of Nrf2 leads to accumulation of p62, which can then further activate Nrf2. Conversely, impaired p62 expression could lead to decreased Nrf2 activity and may further explain the association between low p62 levels, a common occurrence in many neurodegenerative disorders (165), and decreased Nrf2-dependent inducibility. Importantly, mice that lack p62 have increased oxidative DNA damage in brain as well as biochemical and cognitive defects that resemble Alzheimer's disease (342, 365).

The generation of the *nrf2*-knockout mouse (117, 215) provided an unequivocal demonstration of the cytoprotective role of Nrf2-dependent gene battery [see ref. (227) for a scholarly review]. Under standard laboratory conditions, *nrf2*-knockout mice do not display any obvious phenotypic abnormalities. In contrast to wild-type mice, however, they fail to adapt to

environmental challenges and are much more sensitive to the toxicity and carcinogenicity of electrophiles and oxidants. Unlike their wild-type littermates, *nrf2*-knockout mice have low basal levels of cytoprotective proteins and cannot be protected by inducers. Conversely, liver-specific *keap1*-knockout mice and *keap1*-knockdown mice have constitutively high basal levels of cytoprotective proteins and are much more resistant to acute drug toxicity (332, 333, 336, 369, 370, 471).

The neuroprotective effects of upregulation of the Keap1/Nrf2/ARE pathway have been demonstrated in several *in vitro* and *in vivo* models. Thus, adenovirus-mediated overexpression of Nrf2 was shown to upregulate ARE-dependent genes in primary cultures of neurons and astrocytes that were derived from the cortex of the rat (407) or the mouse (249) brain. Importantly, this upregulation of the Keap1/Nrf2/ARE pathway protected against the toxicity of glutamate and H₂O₂. Similar protection was also observed when the small molecule inducers *tert*-butyl hydroquinone (tBHQ) or sulforaphane were used instead of Nrf2 overexpression (240, 407). Strikingly, the protective effects of induction of the Keap1/Nrf2/ARE pathway in astrocytes extend beyond this cell type and also provide protection to neurons. Even though the intricate mechanisms are not fully understood, one very important player is undoubtedly reduced glutathione, the secretion of which from the astrocytes increases after Nrf2 activation. It has been hypothesized that this effect is the primary factor leading to neuroprotection of both cortical and motor neurons in culture (219). In addition, the upregulation of proteins that are concerned with the synthesis, use, and transport of reduced glutathione (GSH), including xCT cystine/glutamate antiporter, γ -glutamatecysteine ligase, glutathione S-transferases, glutathione reductase, and the multidrug resistance protein 1, in the astrocyte were shown to be critical for neuronal protection. However, contrary to the expectations based on the *in vitro* experiments, *nrf2*-knockout mice did not differ from their wild-type littermates in terms of basal activity of the xCT cystine/glutamate antiporter in the ventral striatum, cocaine-induced behaviors either after acute or repeated exposure, or dopamine depletion after methamphetamine-induced oxidative stress (343).

Compared to their wild-type counterparts, neurons isolated from *nrf2*-knockout mice were much more sensitive to the cytotoxic effects of the mitochondrial toxins 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine and rotenone (249). Oligonucleotide microarray analysis on primary neuronal cultures isolated from wild-type and *nrf2*-knockout mice revealed differences in several functional categories of genes: (a) detoxification/antioxidant/reducing potential; (b) calcium homeostasis; (c) receptor/channel/carrier proteins; (d) growth factors; (e) cell signaling; (f) defense/immune/inflammation; and (g) neuron-specific proteins (249). Of special interest are the genes playing roles in calcium homeostasis. Indeed, neuronal cultures isolated from *nrf2*-knockout mice were more sensitive to increases in intracellular Ca²⁺ levels caused by ionomycin- and 2,5-di-(*t*-butyl)-1,4-hydroquinone (249). Importantly, adenoviral-vector-mediated overexpression of Nrf2 recovered ARE-dependent gene expression in *nrf2*-knockout neuronal cultures and protected *nrf2*-knockout neurons from rotenone- or ionomycin-induced cell death.

The exciting findings using mixed primary cultures of neurons and astrocytes showing that activation of the Keap1/Nrf2/ARE pathway in astrocytes provides protection to neurons prompted subsequent *in vivo* studies. A large number of *in vivo* experiments have demonstrated the neuroprotective role of the Keap1/Nrf2/ARE pathway and suggested the potential use of Nrf2 inducers for achieving protection against neurodegenerative diseases. Thus, in comparison to

wild-type mice, *nrf2*-knockout mice are hypersensitive to the mitochondrial complex II inhibitors 3-nitropropionic acid and malonate, which induce lesions in the striatum (111, 406).

Hypersensitivity was both time and dose dependent such that motor deficits and striatal lesions developed more rapidly in homozygous null mice than in animals that are either homozygous or heterozygous for *nrf2* (406). Importantly, striatal succinate dehydrogenase activity, the target of 3-nitropropionic acid, was inhibited to the same extent in all genotypes, indicating that the absence of Nrf2 did not lead to alterations in metabolism and brain concentration of the drug.

The toxicity of 3-nitropropionic acid was lower when the diet of wild-type but not *nrf2*-knockout mice was supplemented with the Nrf2 inducer tBHQ, suggesting that protection was dependent on Nrf2. Indeed, similar protective effects were achieved *via* intrastratal adenovirus-mediated Nrf2 overexpression, which significantly reduced lesion size compared with green fluorescent protein-overexpressing controls. tBHQ, administered intracerebroventricularly or intraperitoneally, was also protective against the occurrence of cortical damage and sensory and motor deficits at 24 h and even at 30 days after ischemia-reperfusion in rats (408). In mice, tBHQ attenuated neuronal death caused by intracortical endothelin-1 microinjection (a penumbral model of stroke), but was ineffective in *nrf2*-knockout animals (408). *Nrf2*-knockout mice are also more susceptible to seizures, neuronal damage, and microglial infiltration in hippocampus induced by kainic acid exposure (241). Nrf2-dependent protection against excess release of dopamine was also recently reported (405).

Transplanted Nrf2-overexpressing astrocytes into the mouse striatum before challenge with malonate provided dramatic protection against malonate-induced neurotoxicity; remarkably, brain hemispheres receiving Nrf2-producing astrocyte transplants were essentially resistant to malonate toxicity, whereas hemispheres receiving control astrocytes were no different from untransplanted controls (111). Recently, transgenic mice overexpressing Nrf2 selectively in astrocytes have been generated using the glial fibrillary acidic protein (GFAP) promoter (456). These mice were crossed with two ALS-mouse models. It was found that overexpression of Nrf2 in astrocytes significantly delayed disease onset and extended survival. In a model of Parkinson's disease, *nrf2*-knockout mice were found to be more sensitive than wild-type mice to the toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (48, 122). Further, whereas MPTP challenge led to upregulation of the Keap1/Nrf2/ARE pathway in substantia nigra of wild-type mice (indicative of a counterattack in response to the toxic challenge), such upregulation was absent in *nrf2*-knockout mice, demonstrating that the absence of Nrf2 results in failure of an adaptive stress response (122). Importantly, activation of the Keap1/Nrf2/ARE pathway by oral administration of the Nrf2 inducer 3H-1,2-dithiole-3-thione to wild-type mice resulted in partial protection against MPTP-induced neurotoxicity, but had no effect when administered to *nrf2*-knockout mice (48). Strikingly, the toxicity of MPTP was abolished in GFAP-Nrf2 transgenic mice (overexpressing Nrf2) on both *nrf2*-wild-type and *nrf2*-knockout backgrounds. Because the GFAP-Nrf2 transgenic mice on an *nrf2*-knockout background express Nrf2 only in astrocytes, this finding confirmed the previous observations that Nrf2 expression restricted to astrocytes is sufficient to protect the brain against MPTP toxicity. In a model of permanent cerebral ischemia it was shown that whereas absence of Nrf2 appears to have no significant effect on cortical infarction at 24 h, it exacerbates tissue injury at 7 days after permanent ischemia, as demonstrated by the large increase (twofold) in infarct volume at the 7-day time point (408). In contrast, there was no increase in infarct size between 24 h and 7 days in wild-type animals. On the basis of this observation, the authors proposed a model in which Nrf2-dependent gene

expression in surviving glial cells restricts infarct expansion in wild-type, but is unable to do so in *nrf2*-knockout mice (408). It was recently reported that *nrf2*-knockout mice develop spongiform leukoencephalopathy characterized by degeneration of myelin in the white tracts of the brain accompanied by widespread reactive gliosis (206). In this study, the mean age of the mice with vacuolar leukoencephalopathy was 8.3 ± 2.6 months (mean \pm SE), and the prevalence at the age of 1 year or older was 100% (11 of 11 animals). In the age- and gender-matched wild-type controls, only one animal had a locally extensive area of mild vacuolar degenerative changes in the cerebellar white tracts. These findings show the importance of Nrf2 in supporting myelin integrity and suggest that antioxidant responses mediated through the Keap1/Nrf2/ARE pathway are essential to prevent oxidative injury within the myelin sheath. Taken together, these studies convincingly demonstrate the neuroprotective effect of the Keap1/Nrf2/ARE pathway and it was recently proposed that it represents a novel neuroprotective pathway that confers resistance to a large variety of oxidative-stress-related and neurodegenerative insults (112, 219, 327).

It is noteworthy that the levels of markers of the Keap1/Nrf2/ARE pathway such as NQO1, GST, and GSH in brain do not differ as dramatically as they do in liver of wild-type and *nrf2*-knockout mice (406). Thus, *nrf2*-knockout mice have only 25% of the liver NQO1 activity of wild-type controls. In sharp contrast, the brain NQO1 activity in *nrf2*-knockout mice is 85% of the wild type. Liver glutathione levels in *nrf2*-knockout mice are 30% lower than in wild-type mice, whereas there is no significant difference between the two genotypes in terms of total glutathione content in the brain. The implications of these finding are at least twofold. First, there must be other Nrf2-independent pathways of regulation of cytoprotective genes in brain. Curiously, the roles of the other members of the Nrf family of transcription factors (Nrf1 and Nrf3) have not been investigated in the brain even though the expression levels of brain Nrf1 are much higher than those of Nrf2 (298). Second, because *nrf2*-knockout mice are clearly much more sensitive than their wild-type littermates to all of the above mentioned challenges, differences in expression of cytoprotective genes as small as 10%–20% seem to have a very significant impact on the sensitivity of the brain to damage, and especially on its ability to cope with the long-term consequences of such damage. Because the battery of cytoprotective genes is so large (>100) and their functions so diverse, it is perhaps not surprising that very small differences in a large number of proteins could have a big overall impact.

Table 1. Examples of the Versatility of Functions of Cytoprotective Proteins

Conjugation and export
Glutathione transferases
UDP-glucuronosyltransferases
Multidrug resistance proteins
Glutathione homeostasis
γ -Glutamylcysteine ligase
Glutathione reductase
Thioredoxin
Thioredoxin reductase
Antioxidant

Heme oxygenase 1
Ferritin
Catalase
Antiinflammatory
Leukotriene B₄ dehydrogenase
Synthesis of reducing equivalents:
Glucose-6-phosphate dehydrogenase
Maleate dehydrogenase
Repair and removal of damaged macromolecules
Heat shock protein 40
Heat shock protein 70
26S proteosome subunits
*O*⁶-methylguanine-DNA methyltransferase
UDP, uridine diphosphate.

XII. Hormetic Phytochemicals and the Neuroprotective Effects of Pharmacological Activators of the Keap1/Nrf2/ARE Pathway

Phytochemicals include compounds with various biological properties (*i.e.*, antimicrobial, antifungal, antioxidant, and antiproliferative) that have presumably evolved, in part, to allow plants to cope with environmental challenges, including exposure to radiation and toxins, and defense against pests and infectious agents. These chemicals, or their metabolic precursors, are particularly concentrated in the seeds, the growing buds, and the skin of fruits and function as natural pesticides. From an evolutionary perspective, the noxious properties of such phytochemicals play an important role in dissuading insects and other pests from eating the plants. However, at the relatively small doses ingested by humans who consume the plants, the phytochemicals are not toxic and instead induce mild cellular stress responses (319). The now compelling evidence for biphasic dose–response effects of environmental toxins in biological systems suggests the possibility that chemicals in foodstuffs, particularly from plants, might also exert biphasic dose responses with health benefits resulting from ingestion of the chemicals in doses within the hormetic range. Whereas at high concentrations phytochemicals can be toxic to mammalian cells, subtoxic doses may induce adaptive stress responses. Thus, there is voluminous evidence on the protective effects of isothiocyanates, curcumin, and resveratrol, all present in edible plants. In spite of this, however, high doses of these phytochemicals can also be toxic to some types of cells (289, 291, 293). Although in the past it was assumed that the direct free radical scavenging activity of phytochemicals might be responsible for their direct health benefits, to date it is unclear, if not unlikely, whether humans consume fruits and vegetables to an extent sufficient to achieve the high (micromolar) concentrations of the phytochemicals required to scavenge free radicals directly. Indeed, epidemiological studies and clinical trials have failed to demonstrate benefits of dietary supplementation with antioxidants such as vitamin E and vitamin C. Within this context, emerging evidence suggests that hormetic mechanisms may account for the health benefit of phytochemicals (330). One general mechanism of action of phytochemicals is that they activate adaptive cellular stress response pathways, including kinases and transcription factors that induce the expression of genes that encode antioxidant enzymes, protein chaperones, phase 2 enzymes, neurotrophic factors, and other cytoprotective proteins

(294). Sulforaphane, curcumin, and resveratrol are inducers of the Keap1/Nrf2/ARE pathway. Resveratrol activates the sirtuin–FOXO pathway, resulting in increased expression of antioxidant enzymes and cell survival-promoting proteins (294). Ingestion of other phytochemicals may activate the hormetic transcription factors cyclic AMP response element binding protein, resulting in the induction of genes encoding growth factors and antiapoptotic proteins (289, 293).

A. Tert-butylhydroquinone

Several pharmacological activators (inducers) of the Keap1/Nrf2/ARE pathway have been shown to be neuroprotective (Fig. 9). Most of them are either natural products or synthetic compounds that are present in the human diet. As already discussed, tBHQ is one of the most widely used inducers of the Keap1/Nrf2/ARE pathway in both *in vitro* and *in vivo* studies. tBHQ is also one of the very early inducers that was shown to elevate cytoprotective enzymes (NQO1 and GST) in several tissues of CD-1 mice and Hepa1c1c7 murine hepatoma cells (145, 359, 360) and to reduce the number of gastric tumors in female ICR/Ha mice treated with benzo[a]pyrene (464) in a series of elegant studies carried out more than a decade before the discovery of the Keap1/Nrf2/ARE pathway. Among several phenolic antioxidants, tBHQ is more potent as an inducer compared to compounds in which the phenolic groups are alkylated. Further, its oxidation-reduction lability was identified as an essential feature for its inducer ability (359). It was recently suggested that compounds with hydroquinone and catechol moieties could constitute novel neuroprotective agents based on their ability to activate the Keap1/Nrf2/ARE pathway (388). It was pointed out that such molecules have an advantage over classical antioxidants (such as ascorbic acid) because their action is sustained and amplified by transcription-mediated signaling pathways (385). Further, because hydroquinones and catechols are not electrophilic themselves, they could be viewed as pro-drugs that are converted to the ultimate inducers by oxidation reactions (29). Critically, because redox imbalance is a recognized component of many neurodegenerative states, a novel strategy against neurodegenerative disorders has been proposed, namely, activation of such pro-drugs *via* the pathological activity that they are intended to combat (260). Thus, the catechol-containing carnosic acid is a compound abundant in rosemary (*Rosmarinus officinalis*) that accounts for 5% of the dry weight of the leaves (239). Carnosic acid induces Nrf2-dependent genes by binding to specific cysteine residues of Keap1 located in its BTB and/or IVR domains (386). Further, carnosic acid crosses the blood–brain barrier, increases the level of reduced glutathione in the brain, and protects against ischemia/reperfusion (386). Electrophilic neurite outgrowth-promoting prostaglandin derivatives have been shown to be taken up preferentially into neurons, where they bind to Keap1 leading to Nrf2 activation, binding to the ARE and upregulation of ARE-dependent genes (387). These electrophilic neurite outgrowth-promoting prostaglandin are neuroprotective *in vitro* against glutamate-related excitotoxicity as well as *in vivo* in a model of cerebral ischemia/reperfusion injury. Because of their neuroprotective effects, there are currently efforts toward the targeted development of neuroprotective electrophilic drugs, including prostaglandin derivatives and hydroquinones that exert their activity through activating the Keap1/Nrf2/ARE pathway (385).

[View larger version\(480K\)](#)

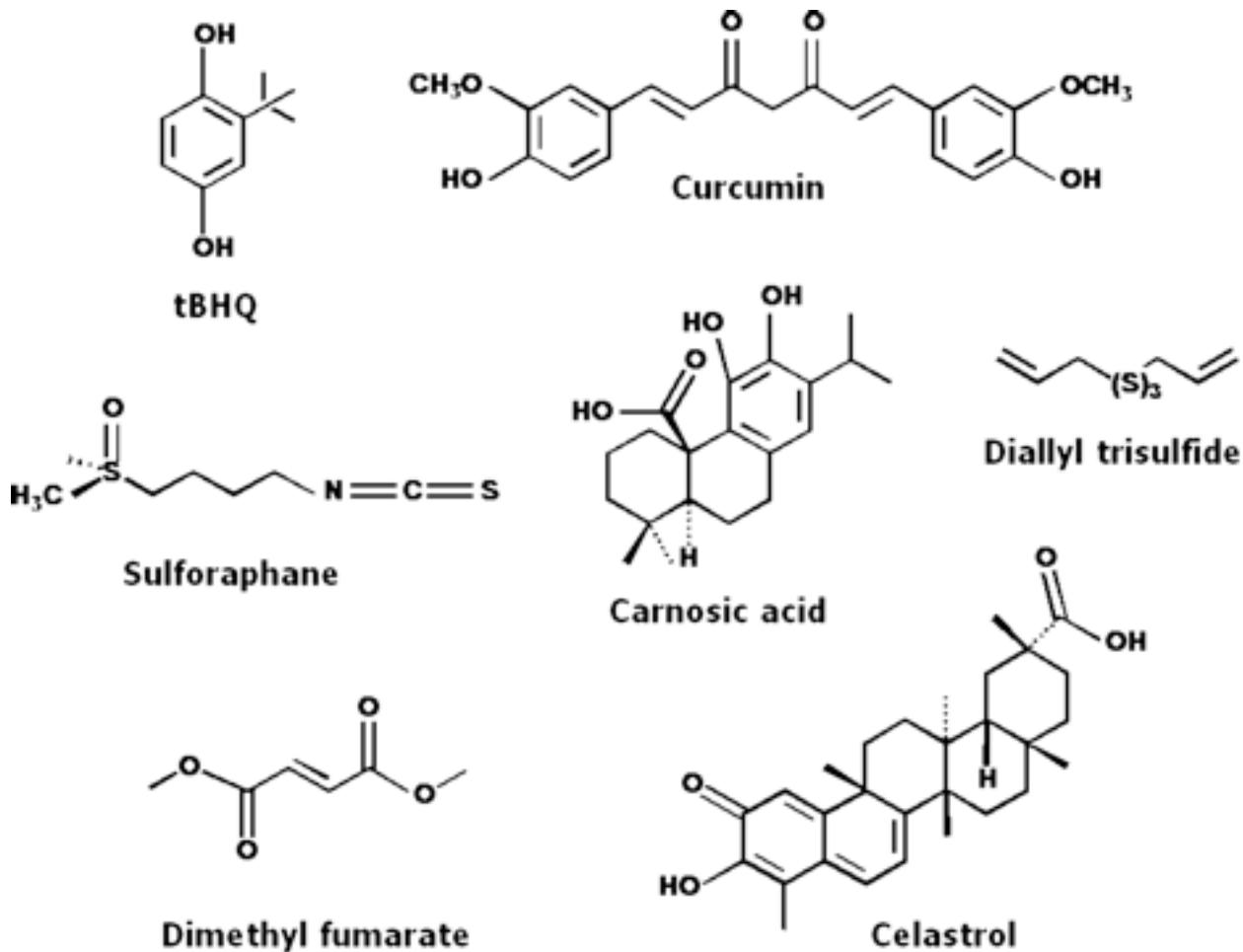


FIG. 9. Chemical structures of inducers of the Keap1/Nrf2/ARE pathway for which neuroprotective activities have been demonstrated.

B. Sulforaphane

One of the most potent naturally occurring inducers of the Keap1/Nrf2/ARE pathway is the isothiocyanate sulforaphane, and it is therefore not surprising that the interest in its development as a protective agent in many chronic conditions, including neurodegeneration, is growing rapidly. The protective activities of sulforaphane in various animal models of carcinogenesis have been recently reviewed (159). The focus here is on its neuroprotective activities.

Sulforaphane (Fig. 9) was isolated from extracts of the common cruciferous vegetable broccoli (*Brassica oleracea*) via an activity-guided fractionation using the Prochaska test (see above), a bioassay based on induction of the prototypical ARE-dependent gene NQO1 (480, 481). In the brain of male Long-Evans rats, the levels of heme oxygenase-1 (HO-1) mRNA were elevated after intraperitoneal administration of sulforaphane (5 mg/kg) (482). The protein expression of HO-1 was increased by 1.5-fold in both neurons and astrocytes. In a model of brain ischemia/reperfusion, administration of sulforaphane 15 min after the onset of ischemia reduced the infarct volume (which was evaluated 3 days later) by 50%. The same dose of sulforaphane administered

to Sprague-Dawley rats 6 h after traumatic brain injury attenuated the loss of the water channel aquaporin-4 in the injury core, and additionally, it also increased the levels of aquaporin-4 in the penumbra region (483). The protective effects were long-lasting, and even 3 days postinjury there was a significant, although modest, reduction in brain edema. The mRNA levels of several cytoprotective genes (*i.e.*, HO-1, NQO1, and GST) in the parietal cortex and brain microvessels were increased 24 h after dosing (484). Postinjury (6 h later) administration of sulforaphane reduced the loss of endothelial cell markers and tight junction proteins and preserved the blood brain barrier function. The protective effects were evident only in wild-type but not in *nrf2*-knockout mice. Moreover, compared to their wild-type counterparts, *nrf2*-knockout mice were hypersensitive to injury-induced blood–brain barrier permeability.

In a model of intracerebral hemorrhage in Sprague-Dawley rats, sulforaphane administration caused induction of several Nrf2-dependent genes (catalase, superoxide dismutase, GST, and NQO1) in brain 3 h later, concomitantly with reduction in markers of oxidative damage (3'-nitrotyrosine and 4-hydroxynonenal) in the perihematoma area (485). Three days after intracerebral hemorrhage, the perihematoma neutrophil count was reduced by 60%, and the total intracerebral hemorrhage-affected striatum neutrophil count was reduced by 33%. Again, the protective effects were long-lasting; 10 days after intracerebral hemorrhage, the neurologic deficits (a composite score from several behavioral tests, including foot fault, forelimb placing, postural reflex, cylinder, and circling) were substantially reduced. Conversely, Nrf2 deficiency resulted in exacerbated neurologic deficits and loss of the protection. In addition, treatment of organotypic nigrostriatal cocultures with tBHQ or sulforaphane protected dopaminergic neurons against 6-hydroxydopamine-induced toxicity (410).

Interestingly, sulforaphane as well as many other inducers of the Keap1/Nrf2/ARE pathway also have antiinflammatory properties. Further, there is a linear correlation spanning more than 6 orders of magnitude between Nrf2 inducer potency and potency in inhibiting pro-inflammatory responses among small molecules that belong to structurally diverse chemical classes (158, 264). In cultured murine macrophages sulforaphane induces NQO1 and inhibits the upregulation of inducible NO synthase (iNOS) and Cox-2 caused by lipopolysaccharide (LPS) or interferon (IFN)- γ and TNF α (158, 264). Similarly, in primary cocultures of rat microglial and astroglial cells, sulforaphane induces Nrf2-dependent genes and attenuates LPS-stimulated production of TNF α , interleukin (IL)-1 β , IL-6, and NO (466). The protective effects of sulforaphane against brain inflammation were recently reported in C57BL/6 mice that received endotoxin injection (213). Sulforaphane treatment decreased microglial activation and the upregulation of inflammatory markers (inducible NO synthase, IL-6, and TNF α) in response to LPS. The same study also showed that compared to wild-type mice, *nrf2*-knockout mice were hypersensitive to LPS-induced neuroinflammation. Although the mechanistic relationship between Nrf2 inducer and antiinflammatory activities of sulforaphane (and other Nrf2 inducers) are not clear at present, there is no doubt that both of these activities contribute to the cytoprotective effects of these small molecules (441).

C. Dimethyl fumarate

Fumaric acid is the active principal of shepherd's purse (*Capsella bursa-pastoris*), a cruciferous weed that has been widely used as a traditional herbal medicine. In the 1940s Crabtree (137, 138) demonstrated that fumaric acid and several related compounds delay or prevent the development

of skin tumors caused by benzo(*a*)pyrene in mice. Fumaric acid is also protective against the carcinogenic effects of a nitrofuran on the forestomach and lung in mice and against the toxicity of mitomycin C (244, 245). It was shown nearly 20 years ago that addition of dimethyl fumarate to the diet of female CD-1 mice and female Sprague-Dawley rats at 0.2%–0.5% concentrations elevated cytosolic GST and NQO1 activities in many organs (420). In primary cocultures of rat microglial and astroglial cells, dimethyl fumarate attenuated LPS-induced production of TNF α , IL-1 β , IL-6, and NO, in addition to inducing Nrf2-dependent genes (468). In a model of chronic experimental autoimmune encephalomyelitis in C57BL/6 mice, treatment with dimethyl fumarate strongly reduced macrophage inflammation in the spinal cord and increased blood levels of IL-10 (391). In humans, fumaric acid salts and esters are already used as therapeutic agents, for example, ferrous fumarate for iron deficiency (487) and alkyl esters for psoriasis (10, 324). In human keratinocytes and peripheral blood mononuclear cells stimulated with IFN- γ or LPS, dimethyl fumarate (Fig. 9) inhibits the production of chemokines that may be critically involved in the development and persistence of psoriatic lesions (426). In patients with relapsing-remitting multiple sclerosis, dimethyl fumarate reduced the formation of new inflammatory lesions in both a pilot study (392) and a multicenter, randomized, double-blind, placebo-controlled, dose-escalation, Phase IIb study (224).

D. Diallyl trisulfide

The volatile organosulfur compounds from *Allium* (garlic) plants are also inducers of the Keap1/Nrf2/ARE pathway (155). The unsaturated allyl or propenyl functionalities in their structures (Fig. 9) are critical determinants of inducer potency (318, 438). In the 1980s such compounds were found to elevate glutathione S-transferase activity in the forestomach and to protect against benzo[*a*]pyrene-induced carcinogenesis (419). Very recently using rat spinal cord explants, it was shown that pretreatment for 48 h with diallyl trisulfide elevates NQO1 and protects motor neurons against glutamate-induced excitotoxicity (429).

E. Celastrol

The quinone methide triterpene celastrol (Fig. 9) is derived from the traditional Chinese medicinal plant known as Thunder of God Vine (*Tripterygium wilfordii*). Celastrol is an inducer of NQO1 (Albena T. Dinkova-Kostova and Paul Talalay, unpublished observations) and a potent inhibitor of NF κ B (136, 400). Celastrol decreases induced expression of class II MHC molecules by microglia (9). Moreover, administration of celastrol to rats significantly improved their performance in memory, learning, and psychomotor activity tests. In addition, celastrol activates heat shock gene transcription synergistically with other stresses and exhibits cytoprotection against subsequent exposures to lethal heat stress (465). Celastrol administration significantly attenuated the loss of dopaminergic neurons and the depletion in dopamine concentration after MPTP treatment (131). In a model of Huntington's disease, celastrol decreased the striatal lesion volume induced by 3-nitropropionic acid (131). Hsp70 within dopaminergic neurons was induced by celastrol, whereas TNF- α and NF κ B and astrogliosis were suppressed. Structure-activity studies revealed a remarkable specificity in activating HSF1 with kinetics resembling those of heat shock, as determined by induction of HSF1 binding to DNA and increased transcription of heat shock genes (466, 467). Moreover, celastrol induced HSR synergistically with other stressors and this induction was protective against subsequent exposure to otherwise lethal forms of stress.

F. Curcumin

The neuroprotective role of curcumin ([Fig. 9](#)), another naturally occurring inducer of the Keap1/Nrf2/ARE pathway ([151](#), [153](#)), is also a subject of intense investigations ([101](#), [102](#), [106](#)).

Curcumin (diferuloylmethane) is a component of turmeric, a yellow spice extracted from the rhizome of the East Indian herb *Curcuma longa* L., that is widely used as a food flavoring and coloring agent (*e.g.*, in curry). Thus, treatment of astrocytes with curcumin induced the cytoprotective proteins HO-1, NQO1, and GST and provided protection against the damaging effects of glucose oxidase-mediated toxicity ([390](#)). Further, curcumin was reported to bind to amyloid plaques, and to reduce amyloid levels and plaque burden in aged Tg2576 mice (a model of familial Alzheimer's disease) with advanced amyloid accumulation ([469](#)). Extensive research within the past two decades has shown that curcumin mediates its antiinflammatory effects through the downregulation of inflammatory transcription factors (such as nuclear factor- κ B [NF κ B]), enzymes (such as cyclooxygenase 2 and 5 lipoxygenase), and cytokines (such as TNF, IL-1 β and IL-6) ([12](#), [444](#)), as well as upregulation of cytoprotective vitagene ([102](#), [106](#), [107](#)).

Curcumin has antiinflammatory, antioxidant, and anticancer activities. One of the most challenging tasks concerning cancer is to induce apoptosis in malignant cells; thus, more focus has been given on natural products to modulate apoptotic signaling pathways. Curcumin has chemopreventive properties, which are mainly due to its ability to arrest cell cycle and to induce apoptosis. The anticarcinogenic properties of curcumin in animals have been demonstrated by its inhibition of tumor initiation and tumor promotion ([12](#)). The number of signaling pathways and molecular targets involved is continuously growing and consequently the picture is becoming more and more complex, not least because the results often appear to be cell type specific and dose dependent ([12](#)).

Curcumin is quite stable at acidic pH, and upon ingestion almost 40%–80% of this compound remains in the gastrointestinal tract. However, curcumin undergoes a marked first-pass metabolism that limits its systemic bioavailability (60%) as demonstrated in humans and rodents ([102](#), [109](#)). Interestingly, to increase its bioavailability, the coadministration of curcumin with piperine or its complexation with phospholipids to form a curcumin-phospholipids complex has been proposed ([12](#)). Preclinical studies have shown that administration of 1 g/kg of curcumin to the rat allows the polyphenol to reach plasma concentrations around 1.3 μ M. Early studies have shown that curcumin has strong antioxidant activity. In fact, the beta-diketone moiety, especially the phenolic hydroxyl group, is critical for the antioxidant activity of curcumin and its analogs ([109](#), [151](#), [206](#)). Very recently, many articles have appeared in the literature demonstrating that curcumin and its metabolites interact with several intracellular systems such as transcription factor NF κ B, iNOS, and HIF-1 and members of the vitagene family (see below). This complex array of interactions is in agreement with the well-known ability of curcumin to serve not only as an antioxidant but also as antiinflammatory and anticarcinogenic molecule ([264](#)). In lung epithelial cells, curcumin exerted anticarcinogenic activity and prevented the cigarette-smoke-induced NF κ B activation through inhibition of I κ B α kinase activation, I κ B α phosphorylation, and degradation ([264](#)). The inhibition of the NF κ B activation was paralleled by the suppression of many NF κ B-related genes, including cyclin D1, cyclooxygenase-2, and matrix metalloproteinase-9 ([264](#)). Particularly interesting is the interaction of curcumin with the vitagene system. Notably, curcumin increased expression of HO-1 in human cardiac myoblasts, hepatocytes, monocytes, and endothelial cells ([316](#)), as well as rat neurons and astrocytes ([390](#)). In several rodents and human cells, the curcumin-induced HO-1 overexpression was correlated

with production of mitochondrial ROS, activation of transcription factors Nrf2 and NFkB, induction of mitogen-activated protein kinase (MAPK) p38, and inhibition of phosphatase activity (300). Moreover, curcumin upregulated Hsp70 in human colorectal carcinoma cells, proximal tubule cells, and leukemia cells (443). Quite different is the effect of curcumin on thioredoxin reductase, as it has been shown that curcumin irreversibly inhibits thioredoxin reductase activity. As a consequence, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity is stimulated with overproduction of ROS (171). This latter paradoxical effect may explain, at least in part, the cancer chemopreventive activity of curcumin (171).

G. Ferulic acid

Ferulic acid is another phenolic compound (Fig. 9) and a major constituent of fruit and vegetables with strong antioxidant and antinflammatory properties. Recently, it has been demonstrated that ferulic acid ethyl ester, a more hydrophobic form of ferulic acid, protects synaptosomal membrane system and neuronal cell culture systems against hydroxyl and peroxy radical oxidation, as well as mice against A β -induced microglial activation (97, 351). In addition to this direct antioxidant property, ferulic acid ethyl ester has been shown to increase HO activity in rat astrocytes and neurons (106, 389). Recent finding indicates that ferulic acid promotes nuclear translocation of Nrf2, whereby increasing the transcription of HO-1 together with other antioxidant related genes, such as GCLC (glutamate-cysteine ligase catalytic subunit), GCLM (glutamate-cysteine ligase regulatory subunit), and NQO1. These changes involve increase of the intracellular GSH and activation of PI3K and extracellular signal regulated kinase signaling pathways (268). All this corroborates the hypothesis that HO activation is a common pathway through which phenolic compounds can exert neuroprotective effects (106).

XIII. Heme Oxygenases

The heme oxygenases have been recognized as dynamic sensors of cellular oxidative stress and modulators of redox homeostasis throughout the phylogenetic spectrum. Heme oxygenases are located within the ER where they act in association with NADPH cytochrome P450 reductase to oxidize heme to biliverdin, free ferrous iron, and carbon monoxide (CO) (Fig. 6). Biliverdin reductase further catabolizes biliverdin to the bile pigment, bilirubin (104, 105, 108, 274), a linear tetrapyrrole that has been shown to effectively counteract nitrosative stress due to its ability to interact with NO and reactive nitrogen species (RNS) (99, 106–108). Bilirubin is then conjugated with glucuronic acid and excreted (393). In the last 20 years, many research articles have demonstrated that bilirubin is an endogenous cytoprotective molecule. The first evidence about the antioxidant activity of bilirubin was provided by Stocker and colleagues in 1987, who suggested that bilirubin may act as chain-breaker because of its ability to scavenge peroxy radicals transforming itself in a stable carbon centered radical (425). Later, Snyder and colleagues based on the evidence that bilirubin, in the nanomolar range, may protect cortical neurons from the toxicity elicited by 10,000 times higher levels of hydrogen peroxide (163), proposed a novel mechanism based on an amplification cycle whereby bilirubin is oxidized to bilirubin by reactive oxygen species and then recycled by biliverdin reductase back to bilirubin. However, only a small fraction of the bile pigments undergo this redox cycle; therefore, the relevance of this mechanism of action is still debated. Finally, bilirubin has been shown to serve as an endogenous scavenger for both NO and reactive nitrogen species, which may alter the redox status of the cell and originate nitrosative stress (276). Despite this important antioxidant

properties, if produced in excess, as in the case of haemolytic anaemia or sepsis, unconjugated bilirubin becomes neurotoxic through multiple mechanisms involving the disruption of cell membrane structure, the reduction of mitochondrial transmembrane potential, and the activation of the apoptotic cascade.

Mammalian cells express at least two isoforms of HO, HO-1 and HO-2. A third protein, HO-3, determined to be a retrotransposition of the HO-2 gene (pseudogene), has been found unique to rats (390). Heme oxygenase-1, also referred as Hsp32, is induced by various stimuli, including oxidative and nitrosative stress, ischemia, heat shock, LPS, hemin, phenolic compounds, and the neuroprotective agent Neotrofin. Further, in cultured human and rodent cells, HO-1 expression can be repressed by hypoxia, cigarette smoke, or by treatment with IFN- γ or desferrioxamine. On the other hand, HO-2, the constitutive form, is responsive to developmental factors and adrenal glucocorticoids. Although HO-1 and HO-2 catalyze the same reaction, they play different roles in protecting tissues against injury. A convincing hypothesis suggests that HO-1 induction is one of the earlier cellular response to tissue damage and is responsible for the rapid clearance of the intracellular pro-oxidant heme and its transformation into CO and biliverdin, the latter being the precursor of the antioxidant bilirubin. On the contrary, constitutively expressed HO-2 is primarily involved in maintaining cell heme homeostasis and in the sensing of intracellular levels of gaseous compounds including NO and CO.

Although HO-1 and HO-2 exhibit identical substrate and cofactor specificities, the isoforms are encoded by distinct genes, which share only 43% amino acid sequence homology in humans, and exhibit significant differences with regard to molecular weight, electrophoretic mobility, tissue distribution, regulation, and antigenicity (393). HO-1 contains a destabilizing proline-glutamic acid-serine-threonine sequence at the carboxy terminus that renders the peptide sensitive to rapid proteolysis, a characteristic not exhibited by HO-2. The half-lives of HO-1 mRNA and protein have been estimated to be 3 and 15–21 h, respectively (148). The HO-1 gene is now well defined in its redox regulation as it contains the ARE in its promoter region, similar to other antioxidant enzymes (223, 273). HO-1, in fact, can be induced by several stimuli associated with oxidative and/or nitrosative stress, such as heme, A β , dopamine analogues, H₂O₂, hyperoxia, UV light, heavy metals, prostaglandins, NO, peroxynitrite, Th1 cytokines, oxidized lipid products, and LPS, as well as certain growth factors (148, 273, 393). HO-1 gene expression is regulated by a variety of factors such as pro-oxidant states or inflammation (273). The molecular mechanism that confers inducible expression of *ho-1* in response to numerous and diverse conditions has remained elusive. One important clue has recently emerged from a detailed analysis of the transcriptional regulatory mechanisms controlling the mouse and human *ho-1* genes, which in humans is located on chromosome 22q12. The induction of *ho-1* is regulated principally by two upstream enhancers, E1 and E2 (5, 106, 273). 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 (5, 325, 393) with a consensus sequence (GCnnnGTA) similar to that of other antioxidant enzymes (107) (Fig. 6). Polymorphisms in the lengths of GT repeats [11–40] within the *ho-1* gene (HMOX1) promoter appear to be an important determinant of HO-1 expression and function in humans. Long GT sequences code for relatively unstable (Z-conformational) DNA with attenuated transcriptional activity and diminished baseline and stimulated HO-1 protein expression profiles. Significantly higher HO-1 activity is associated with the short-GT polymorphisms, which may protect against atherosclerosis-linked conditions

(e.g., coronary artery disease), whereas the malignant behavior of various neoplasms was fairly consistently enhanced by the short-GT form (393). 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 (106, 107). The neuroprotective effects of overexpressed HO-1 can be attributed to (a) increase in cGMP and bcl-2 levels in neurons; (b) inactivation of p53, a protein involved in promoting cell death; (c) increase in antioxidant sources, and (d) increase in the iron sequestering protein, ferritin (346). Specifically, the interaction between HO-1, p53, and Bcl2 could involve the heme-regulating motifs of HO-2 (297), which could modulate gene expression by promoting oxygen radical formation and acting as a sink for NO. When HO-1 levels are increased, as in Tg mice, a decreased level of heme for binding to HO-2 heme-regulating motifs may alter heme/oxygen gene-regulating events. bcl-2 and p53 may well be among those genes whose expression would be affected as the consequence of the change in the oxidoreductase state of the cell brought about by altered heme-regulating motif interactions with oxygen and/or NO.

Particularly interesting is the role played by HO-1 in AD, a neurodegenerative disorder that involves a chronic inflammatory response associated with both oxidative brain injury and A β -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; the HO-1 increase also colocalized with senile plaques and glial fibrillary acidic protein-positive astrocytes in AD brains (393).

It is plausible that the dramatic increase in HO-1 in AD may be a direct response to an increase in free heme concentrations, associated with neurodegeneration, and can be considered as an attempt of brain cells to convert the highly toxic heme into the antioxidants CO and bilirubin (100). The protective role played by HO-1 and its products 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 prevention and treatment of AD. In this light very promising are the polyphenolic compounds contained in some herbs and spices, for example, curcumin (102, 106, 109). Curcumin is the active antioxidant principle in *Curcuma longa*, a coloring agent and food additive commonly used in Indian culinary preparations. This polyphenolic substance has the potential to inhibit lipid peroxidation and to effectively intercept and neutralize ROS and RNS (102, 105, 109, 390). In addition, curcumin has been shown to significantly increase HO-1 in astrocytes and vascular endothelial cells (316, 317). This latter effect on HO-1 can explain, at least in part, the antioxidant properties of curcumin, in particular keeping in mind that HO-1-derived bilirubin has the ability to scavenge both ROS and RNS (316). 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 (102, 259, 469). On the basis of these findings, Lim and colleagues have provided convincing evidence that dietary curcumin given to an AD 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 (258). Further, increasing evidence indicates that curcumin inhibits NF κ B activation, efficiently preventing cell death (12, 384).

In the absence of elevated intracellular heme or oxidative stress, the basic region leucine zipper transcriptional regulator BACH1 binds HMOX1 AREs and represses transcription. Conversely, increased intracellular heme or sulphydryl oxidation inactivates BACH1, permitting

transcriptional induction of HMOX1. Although it is generally agreed that increased HO-1 expression is a common feature during oxidative stress, recent articles demonstrated that HO-1 can be repressed during oxidative stress conditions. In particular, human cells exposed to hypoxia, thermal stress, and IFN- γ showed a marked HO-1 repression, and this effect seems to be peculiar for human, because rodent cells increased HO-1 expression when exposed to the same stimuli (232). The importance of HO-1 repression has been corroborated by the discovery of Bach1/Bach2 as heme-regulated transcription factors for HO-1 gene (232). In fact, Bach1 is broadly expressed in mice and human tissues; in human cells, it is induced by the same stimuli that are able to repress HO-1 gene (5, 325). Current hypothesis suggests that HO-1 repression is useful for the cell because it (a) decreases the energy costs necessary for heme degradation, (b) reduces the accumulation of CO and bilirubin, which can become toxic if produced in excess, and (c) increases the intracellular content of heme necessary for the preservation of vital functions such as respiration (5, 126, 232). Delineation of the diverse roles of HO-1 in brain senescence, aging-related human neurodegenerative disorders, and other CNS conditions has progressed substantially in these last years. Whereas the acute induction of this enzyme in neural and other tissues is predominantly cytoprotective in nature, protracted or repeated upregulation of the Hmox1 gene in astrocytes, oligodendroglia, and possibly neurons may perpetuate cellular dysfunction and demise in many chronic degenerative and neuroinflammatory conditions long after provocative stimuli have dissipated. In the case of normal brain aging, AD, PD, and other late-life neurodegenerations, there is converging evidence that the associated tissue iron sequestration, intracellular oxidative stress, and mitochondrial dysfunction may constitute a single pathogenic *momentum* acting downstream from the sustained action of HO-1 within the astrocytic compartment. In AD and mild cognitive impairment, immunoreactive HO-1 protein is overexpressed in neurons and astrocytes of the cerebral cortex and hippocampus relative to age-matched, cognitively intact controls and colocalizes to senile plaques, neurofibrillary tangles, and corpora amylocitae. In PD, HO-1 is markedly overexpressed in astrocytes of the substantia nigra and Lewy bodies in affected dopaminergic neurons. HMOX1 is also upregulated in glial cells located around human cerebral infarcts, hemorrhages, and contusions, within multiple sclerosis plaques, and in other oxidant degenerative and inflammatory human CNS disorders. Within this context, heme-derived free ferrous iron, CO, and biliverdin/bilirubin are all biologically active substances that can either ameliorate or exacerbate neural injury contingently to the specific disease conditions, such as intensity and duration of HO-1 expression and/or the nature of the resulting redox milieu. In stressed astroglia, HO-1 hyperactivity promotes mitochondrial sequestration of nontransferrin iron and macroautophagy and may thereby contribute to the pathological iron deposition and bioenergetic failure found in most age-related oxidant neurodegenerative disorders. Glial HO-1 expression may impact also cell survival and neuroplasticity by modulating brain sterol metabolism and proteasomal degradation of neurotoxic protein aggregates (394).

XIV. Adaptive ER Stress Responses: Calcium and Protein Chaperones

Two major functions of the ER are the biosynthesis and quality control of proteins, and the regulation of cellular calcium homeostasis (302, 478). Several key proteins that control these two ER functions are responsive to cellular stress, including metabolic and oxidative stress. One of the first ER stress-responsive proteins discovered is glucose-regulated protein 78 (GRP78 or Bip), which is a protein chaperone that protects nascent proteins from misfolding and damage

(326). The expression of GRP78 is highly sensitive to metabolic stress, and its upregulation under such adverse conditions has been shown to protect cells against death (326). Several other such chaperones, with complementary functions, have been identified, including GRP94, GRP170, and calreticulin. Interestingly, even relatively mild physiological stressors can upregulate ER chaperones, including dietary energy restriction (17) and exercise (454). ER Ca^{2+} -regulating proteins, including IP_3 receptors, ryanodine receptor, and the sarco-/endo-plasmic reticulum Ca^{2+} -ATPase, are also influenced by and, in turn, modify cellular stress. For example, activation of IP_3 receptor channels in the ER of nerve cells leads to activation of the transcription factor NF κ B, which, in turn, induces expression of proteins that protect the neurons against stress, including Mn-superoxide dismutase and Bcl-2 (188).

A very important stereotyped sequence of events is now known as the UPR (320). Eukaryotic cells respond to unfolded proteins in their ER stress, amino acid starvation, or oxidants by phosphorylating the alpha subunit of translation initiation factor 2 (eIF2 α). This adaptation inhibits general protein synthesis while promoting translation and expression of the transcription factor ATF4. $\text{Atf4}(-/-)$ cells are impaired in expressing genes involved in amino acid import, glutathione biosynthesis, and resistance to oxidative stress (198). Under low stress conditions, GRP78 is associated with several different ER proteins, including protein kinase-like ER kinase (PERK), inositol requiring element-1 (IRE-1), and activating transcription factor 6 (ATF6). When cells are under oxidative and metabolic stress, GRP78 is recruited to oxidatively damaged and misfolded proteins, and PERK, IRE-1 and ATF-6 are mobilized to serve the complementary roles in the UPR. PERK suppresses protein translation by phosphorylating eIF2 α , and also phosphorylates and thereby activates the transcription factor Nrf2, resulting in the production of antioxidant and phase 2 enzymes. IRE-1 activation results in the production and nuclear translocation of the transcriptional regulator XBP-1, which, in turn, induces expression of GRP78 and GRP94. During the UPR, ATF-6 is cleaved to generate an active transcription factor that translocates into the nucleus and induces expression of several cytoprotective proteins. Examples of stimuli that activate the UPR include nutrient deprivation, altered protein glycosylation, oxidative stress, and increased intraluminal Ca^{2+} levels.

Until recently, the ER was viewed as a protein synthesis factory that was not involved in signal transduction processes or cellular stress responses. We now know that the ER plays pivotal roles in regulating cellular Ca^{2+} homeostasis and transduces Ca^{2+} -mediated signals from extracellular signals (excitatory neurotransmitters and growth factors) and signals emanating from internal organelles, including mitochondria and the nucleus (456). Indeed, the ER is believed to be involved in the integration of signaling events within axon terminals and dendrites of neurons (283, 347, 413). When stimulated by IP_3 or Ca^{2+} itself, Ca^{2+} is released from the ER through opened IP_3 receptor and ryanodine receptor channels, respectively (Fig. 10). An ER membrane Ca^{2+} ATPase is responsible for transporting Ca^{2+} into the ER. Excessive ER stress and dysregulation of ER Ca^{2+} homeostasis is believed to contribute to the dysfunction and death of neurons in ischemic stroke (412), AD (32, 283, 290), and Parkinson's disease (233). The ER is also an important sensor of cellular stress. In response to energetic, oxidative, and ionic (particularly Ca^{2+}) stress, protein synthesis may be decreased, and signals are sent from the ER to the nucleus that result in the upregulation of expression of genes encoding ER protein chaperones such as GRP78 and GRP94, and antioxidant enzymes (320). Recently, an ER

membrane-associated protein called Herp was shown to stabilize cellular Ca^{2+} homeostasis in neurons during ER stress (118).

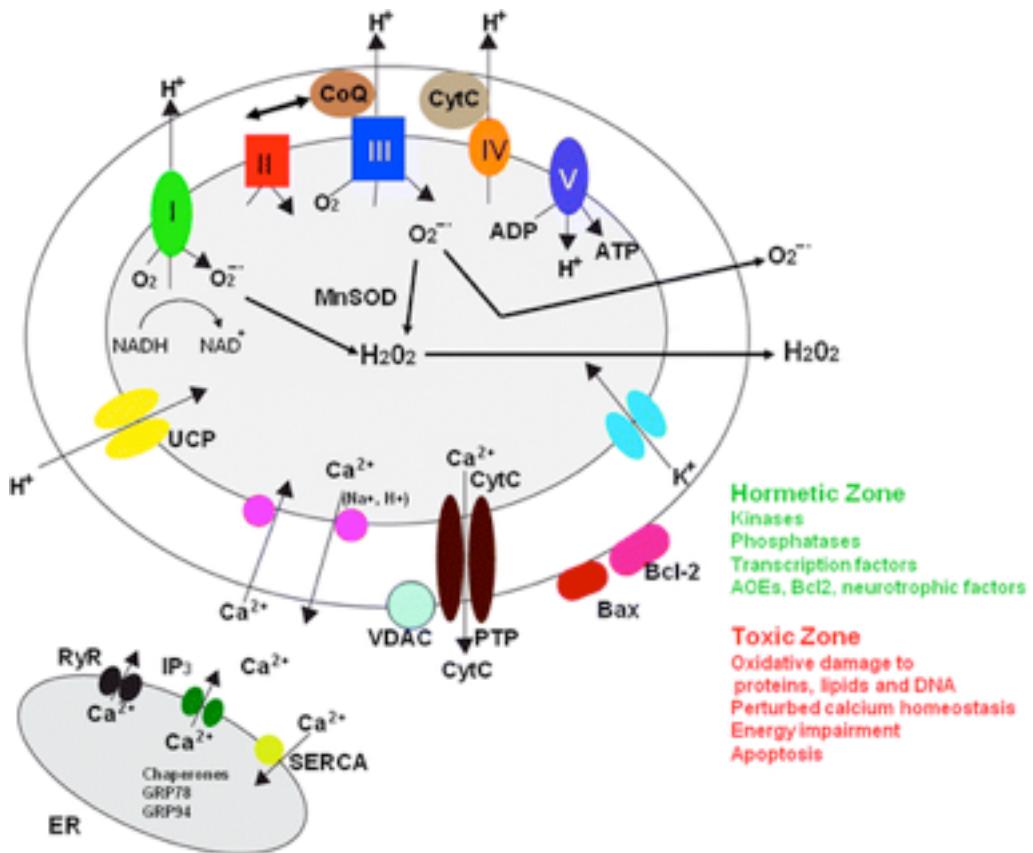


FIG. 10. Regulatory systems in mitochondria and the endoplasmic reticulum involved in hormetic responses of neurons to a range of environmental factors. Modified from Mattson *et al.* (292). CytC, cytochrome c; ER, endoplasmic reticulum; GRP, glucose regulated protein; PTP, permeability transition pore; RyR, ryanodine receptor; SERCA, sarco endoplasmic reticulum calcium ATPase; TCA, tricarboxylic acid cycle; UCP, uncoupling protein; VDAC, voltage-dependent anion channel.

The ER plays important roles in hormetic responses to stress in neurons. Thapsigargin is an agent that inhibits the ER Ca^{2+} -ATPase resulting in depletion of ER Ca^{2+} stores and activation of the UPR. Treatment of cultured cerebellar neurons with thapsigargin increased their survival under culture conditions (low extracellular K^+ concentration) in which they would otherwise undergo apoptosis (470). The mechanism by which ER Ca^{2+} mobilization can promote neuron survival is poorly understood, but emerging data suggest the involvement of both the released Ca^{2+} itself and a protein(s) released from the ER. In either case, it appears that transcription factors are activated, resulting in the upregulation of cell-survival-promoting proteins. Glazner *et al.* (188) showed that a cell survival signal is released from the ER in response to emptying of IP₃-

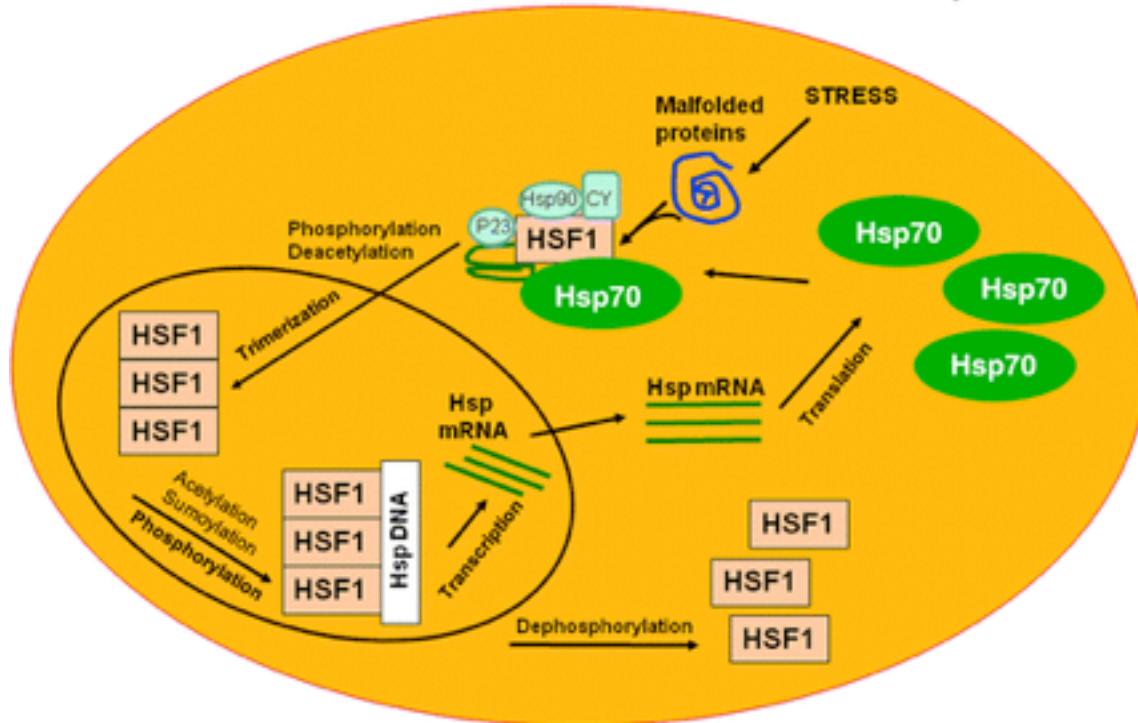
sensitive Ca^{2+} stores; the signal, which may be a protein, activates NF κ B. Another study showed that the immediate early gene pip92 and mitogen-activated protein kinase are activated in response to ER Ca^{2+} mobilization (129). Interestingly, Bcl-2 can decrease ER Ca^{2+} uptake, resulting in enhanced Ca^{2+} signaling and activation of cyclic AMP response element binding protein, thereby promoting the regeneration of injured axons (218). Moreover, Mendes *et al.* (301) showed that an ER-mediated signal associated with the UPR enhances antioxidant defenses and inhibits caspase-dependent cell death. Thus, mild ER stress can elicit multiple hormetic responses in neurons.

A better understanding of adaptive responses to ER stress may lead to novel therapeutic approaches for both acute and chronic neurodegenerative conditions. As evidence, it has been reported that, by activating ryanodine receptors in the ER, paraxanthine (the major metabolite of caffeine) can prevent the degeneration of dopaminergic neurons in a model of Parkinson's disease (196). Induction of ER protein chaperones is another therapeutic approach that is supported by experimental data showing that GRP78 (474), GRP94 (21), and ORP150 (234) can protect neurons against excitotoxic and ischemic death. Mild ER stress responses may be elicited by factors that are known to be neuroprotective including mild energetic stress (dietary energy restriction and 2-deoxyglucose treatment) (166, 475) and exercise (454).

XV. Hsps and Neuroprotection

The HSR is one possible cellular stress response where a set of Hsps are induced, playing important roles in cellular repair and protective mechanisms (311, 312). HSR is regulated at the transcriptional, translational, and posttranslational levels by a family of heat HSFs that are expressed and maintained in an inactive state under nonstress conditions. Among three functionally different HSFs in humans, HSF1 represents the major regulator of the HSR genes, mediating signaling of stress-induced stimuli, such as elevated temperatures, as well as those involved in development and in many pathophysiological conditions, including cancer, ischemia-reperfusion injury, diabetes, and aging (Figs. 4 and 11) (452, 466). HSF1 is generally found in the cytoplasm as an inert monomer lacking transcriptional activity; both DNA-binding and transcriptional transactivation domains are repressed through intramolecular interactions and constitutive serine phosphorylation (308). Upon exposure to heat shock and other types of stresses, which cause protein damage, HSF1 is derepressed in a stepwise process that involves oligomerization of HSF1 monomers to a trimeric state, localization to the nucleus, inducible phosphorylation and sumoylation, binding of nuclear-localized trimers to DNA, and transcription of HS genes [reviewed in ref. (13)]. The main targets for HSF1 are specific promoter elements composed of repeats of the pentameric sequence nGAAn (heat shock elements [HSE]) located upstream of HS genes. High rates of transcription are maintained only when HSF1 trimers remain bound to the HSE; when either the stress signal is removed or damaged proteins are no longer generated, the HSR attenuates rapidly, with subsequent conversion of HSF1 back to the

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monomeric state (310). Inducible acetylation has also been recently shown to negatively regulate DNA binding activity (42, 311).

FIG. 11. Damaged or misfolded proteins titrate away heat shock proteins that are bound to HSF1 and maintain it in a repressed state before stress, resulting in its activation.

Multistep activation of HSF1 involves posttranslational modifications, such as hyperphosphorylation and deacetylation, which allow HSF1 to trimerize and translocate into the nucleus, where inducible acetylation, phosphorylation, and sumoylation occur before binding of nuclear-localized trimers to DNA, and HS genes are transcribed. In particular, upon activation, HSF1 is transiently sumoylated on lysine 298, which requires the phosphorylation of serine 303 adjacent to the consensus site (13). Hence, small ubiquitin-related modifier modification is elaborately regulated, and the small ubiquitin-related modifier substrate specificity can be determined by regulatory elements outside the consensus site. Hsp, heat shock protein.

Mammalian genomes encode three homologs of HSF (HSF1, HSF2, and HSF4) regulating Hsp expression. Among these HSF1 is considered to be the paralog responsible for regulating the heat-induced transcriptional response (42, 311, 312). HSF2 has also been reported to contribute to inducible expression of heat shock genes through interplay with HSF1 (373). Whereas it is well established that HSF1 regulates inducible Hsp expression, recently it has become evident that the regulation of the mammalian HSR is a more complex phenomenon than previously thought. Microarray and chromatin immunoprecipitation analysis, while confirming that many known heat-inducible genes have HSF1-binding sites in their promoters, have defined another class of genes that recruit HSF1 to their HSE promoter elements but that nevertheless are not induced by heat shock (373). On the other hand, these studies have provided evidence that, in

some cases, HSF1 may also control expression of genes with nonchaperone function (373), such as superoxide dismutase, the multiple-drug resistance genes, lactate dehydrogenase, and the T-cell death-associated gene 51 (373). Neurons appear to be deficient in the HSR while retaining the ability to express such HSF proteins (312). Further, HSF1 fails to be activated in motor neurons even when microinjected with plasmids encoding an HSF1 expression vector, suggesting a block to the HSF1 signal transduction pathways in these cells (24). HSF1 is repressed under nonstress conditions by a complex containing Hsp90 and other proteins (Fig. 11). In this inactive state, HSF1 is a monomer that lacks the ability to bind *cis*-acting HSE in the promoters of Hsp genes (110). During protein stress and the consequent generation of misfolded proteins that displace HSF1 from the inhibitory chaperone complex, HSF1 trimerizes, become phosphorylated, and is translocated to the nucleus, where it is acetylated and sumoylated before being able to bind to the heat shock element of Hsp genes (310, 466). Activation of HSF1 by heat shock is a multistep process, involving multiple inducible phosphorylation, dephosphorylation, acetylation, deacetylation, and sumoylation steps, the sum of which results in the transcription of Hsp genes (Fig. 11). Extracellular signal input during heat shock involves tyrosine phosphorylation upstream of HSF1, involving the receptor tyrosine kinase HER2 and launching downstream signaling cascades through intracellular kinase Akt (110). (Fig. 11) Akt regulates HSF1 at least in part through modulating its association with the phosphoserine binding scaffold protein (110).

In addition to thermal stress, the inducible expression of Hsps is also triggered by environmental redox changes or exposure to electrophiles, which cause trimerization and DNA binding of HSF1 (310, 452), pointing to the importance of the cysteine redox state for the activation of this transcription factor. Thus, an intermolecular disulfide bond formation between C36 and C103 within HSF1 causes trimerization and DNA binding, whereas an intramolecular disulfide bond formation (in which C153, C373, and C378 participate) is inhibitory for the activity of the transcription factor (266).

Manipulation of HSR may offer strategies to protect brain cells from damage that is encountered after cerebral ischemia or during the progression of neurodegenerative diseases (309). Hsps are evolutionarily conserved and present in all cellular compartments (417, 418). Some of the major chaperones (Hsc70, Hsp90, and small Hsps) are present at high concentrations in nonstressed cells reaching 1%–5% of total cellular protein, consistent with an important role for chaperones in cellular homeostasis. Hsps are classified according to their molecular weight (270). Hsp70, the 70 kDa family of stress proteins, is one of the most extensively studied. Included in this family are Hsc70 (heat shock cognate, the constitutive form), Hsp72 (the inducible form, also referred to as Hsp72), and GRP75 (a constitutively expressed glucose-regulated protein found in the ER) (269). Hsp70s function in co- and posttranslational folding and the quality control of misfolded proteins (139). More specifically, Hsp70s participate in folding and assembly of newly synthesized proteins into macromolecular complexes, aggregation prevention, dissolution and refolding of aggregated proteins, as well as protein degradation (43). Hsp70s have an N-terminal ATP-binding domain (NBD) and a C-terminal substrate-binding domain (SBD), which are both critical for chaperone function. Nonnative substrates with exposed hydrophobic stretches within an accessible polypeptide backbone associate transiently with Hsp70 via its SBD. ATP binding to the NBD triggers opening of the SBD binding pocket, decreasing affinity for polypeptide substrates, thereby accelerating both on and off rates. Reciprocally, substrate binding induces

ATP hydrolysis, closing the SBD and thus stabilizing the substrate-Hsp70 complex (43). It is this cycle of rapid but controlled binding and release of the substrate that fosters folding and assembly with partner proteins while preventing aggregation of substrates; however, detailed mechanistic understanding of how Hsp70 accomplishes these feats is not yet available (43). Numerous hypotheses have been put forth to explain the molecular mechanism of Hsp70-induced structural conversion of substrate proteins. For example, an entropic pulling mechanism has been proposed, whereby Hsp70 binding stabilizes peptide segments in an unfolded state, causing local unfolding, thereby facilitating disaggregation and allowing refolding upon Hsp70 release (189). Cofactors, such as the nucleotide exchange factors and cochaperones, are crucial regulatory components of the Hsp70 cycle that confer versatility and specificity to the Hsp70 chaperone machine (43). The Hsp40 cochaperone targets substrates to Hsp70 while stimulating ATP hydrolysis; nucleotide exchange factors like Bag-1 (BCL2- associated athanogene 1) and Hsp110 reinitiate the Hsp70 cycle by facilitating ADP release and rebinding of ATP (380). Moreover, Bag-1 has the additional ability to bind to the 26S proteasome, and another BAG isoform, the Bag-3 cochaperone, links Hsp70 to the macroautophagic degradation pathway during aging (110). Carboxy terminus of HSC70-interacting protein, a cochaperone of Hsp70 that also has E3 ubiquitin ligase activity, cooperates with Bag-1, and possibly Bag-3, to facilitate degradation of terminally misfolded substrate proteins. Notably, mutations in Hsp70 cofactors are lethal (271) or may be associated with neurodegenerative disease (269, 271).

Recent studies indicate that the HSR declines in aging cells and becomes weaker as organisms live beyond the mature adult stage (104). Cells lose the capacity to activate the transcriptional pathways leading to Hsp synthesis (Fig. 4). In neuronal tissues, decline in protein quality control has been widely predicted, as the etiology of a number of diseases involves aggregation-prone proteins that form inclusion bodies whose occurrence is linked to pathology. Hsp70 has been extensively implicated in the pathogenesis of misfolding disease (110). Numerous studies indicate that Hsp70 and components of the ubiquitin–proteasome system associate with inclusion bodies/plaques characteristic of misfolding diseases, indicating a general activation of the cellular quality control machinery in an attempt to circumvent the accumulation of misfolded species (201). In these conditions the Hsp70 system is unable to refold disease-related proteins, causing perturbation of protein homeostasis associated with disease onset. Several hypotheses account for this apparent disruption of the balance between the production of misfolded proteins and Hsp70 activity. As misfolded disease proteins accumulate, these can overwhelm the capacity of the Hsp70 system to control the cellular folding milieu (312). Progressive reduction in protein levels and/or activity of Hsp70 and other components of the quality control network may exacerbate this imbalance, permitting further accumulation of toxic misfolded proteins. Such reduction could be due to the aging process, as transcription of Hsp70 decreases during aging of the human brain (43). Alternatively, disease processes themselves might cause, or worsen, chaperone deficiency. Inclusions have been proposed to sequester Hsp70 and other proteins in a nonfunctional state, inhibiting their essential function in cellular processes (43).

The availability of transgenic animals and gene transfer allowing overexpression of the gene encoding for Hsp70 has revealed that overproduction of this protein leads to protection in several different models of nervous system pathology (97). Overexpression of Hsp70 and/or its cochaperones suppresses huntingtin aggregation and toxicity in yeast and mammalian cell models of misfolding disease (380). Increased Hsp70 levels caused reduced aggregation and

toxicity of tau and A β , respectively, two components associated with Alzheimer's disease (269). Similarly, overexpression of Hsp70 reduces toxicity and accumulation of α -synuclein in high-molecular-weight and detergent-insoluble deposits (269). Increased expression of Hsp70 has been reported to be associated with a decrease in apoptotic cell death, an increase in expression of the antiapoptotic protein Bcl-2, a suppression of microglial/monocyte activation, and a reduction in matrix metalloproteinases. Upregulation of Hsp70 likewise reduced apoptosis and the formation of coaggregates of the prion disease protein, PrP (104). Numerous studies have also shown that Hsp70 overexpression reduces polyQ toxicity. Results obtained *in vitro* have elucidated the mechanism of action of Hsp70 against misfolding and thus toxicity of disease proteins. Purified Hsp70 acts preferentially on monomers or oligomers, rather than fibrillar aggregates of A β , huntingtin, and α -synuclein species modulating the aggregation process (104). Hsp70 inhibits the aggregation of A β and α -synuclein species even at substoichiometric levels, suggesting that Hsp70 can recognize multimeric protein assemblies (43). As mentioned before, the effect of Hsp70 on aggregation, which requires its ATPase activity, is enhanced by the cochaperone Hsp40. Thus, Hsp70, together with Hsp40, stabilizes huntingtin in a monomeric conformation and prevents accumulation of spherical oligomers, which are the toxic species for fibril formation (403). As a result, mutant huntingtin is deviated from the potentially toxic, fibrillar aggregation pathway and instead accumulates in amorphous aggregates, or other benign conformers. Sequestered in these conformers, mutant huntingtin may no longer participate in heterotypic interactions known to inactivate essential cellular machinery, such as polyQ-containing transcription factors (311, 403).

After 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 (479). It has been suggested that this neuronal expression of Hsp70 outside an infarct can be used to define the ischemic penumbra, which means the zone of protein denaturation in the ischemic areas; consistently in *in vivo* transgenic mice overexpressing Hsp70, compared to wild-type mice in a middle cerebral artery occlusion model of permanent cerebral ischaemia, it has been demonstrated that overexpression of Hsp70 reduces the overall lesion size and also limits the tissue damage within the lesion (479). Hsp72 overexpression has been documented in postmortem cortical tissue of AD patients (95, 270). Consistently, the use of agents that limit microglial activation and inflammation in AD has recently emerged as an attractive therapeutic strategy for this disease. For instance, the vasoactive intestinal peptide has been shown to prevent A β -induced neurodegeneration, through inhibition of major pathways involved in the production of inflammatory mediators, such as the p38 MAPK, p42/p44 MAPK, and NF κ B cascades, in activated microglial cells (147, 221).

A large body of evidence now suggests a correlation between mechanisms of nitrosative stress and Hsp induction. We have demonstrated *in vitro* and *in vivo* that cytokine-induced nitrosative stress is associated with an increased brain synthesis of Hsp70 stress proteins. The molecular mechanisms regulating the NO-induced activation of heat-shock signal seem to involve cellular oxidant/antioxidant balance, mainly represented by the glutathione status and the antioxidant enzymes (27, 98, 99).

A. Neuroprotective effects of extracellular Hsps

Hsps are transferred between cell types in the nervous system. Thus, stress tolerance in neurons is not solely dependent on their own Hsps, but can be supplemented by additional Hsps transferred from adjacent glial cells. Therefore, supplying exogenous Hsps at neural injury sites could be an effective strategy to maintain neuronal viability. This idea has been tested in a number of model systems. Injection of Hsc/Hsp70 into the vitreous chamber of the eye protected retinal photoreceptors from photodamage. Application of exogenous Hsc/Hsp70 to the cut end of the sciatic nerve reduced cell death in sensory and motor neurons. Extracellular Hsp70 protected spinal cord motor neurons deprived of trophic support *in vitro* or undergoing cell death *in vivo*. Thus, exogenous application of Hsps has potential as a therapeutic strategy for acute injury in the nervous system. Hsps are released into the blood stream after stressful stimuli and this may represent an important feature of the stress response. Exercise stress has been reported to induce the release of Hsp70 from the human brain into the blood stream *in vivo*. The biological significance of this neural release is yet to be determined (44).

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XVI. Neuro Gas Biology and the Roles of CO, NO, and Hydrogen Sulfide in Brain Physiopathology

A. Carbon monoxide

The first detection of a combustible gas in the blood occurred in 1894 by Grehant (195). This gas was supposed by de Saint Martin and Nicloux to be CO. However, it was not until 1949 that Sjorstrand discovered that endogenously produced CO arose from the degradation of hemoglobin released from senescent erythrocytes (414). Greater than 75% of CO produced in humans arises from erythrocyte turnover generated as a by-product of haem metabolism. In 1969, the source of endogenous CO was discovered, as Tenhunen and collaborators described and characterized heme oxygenase as the enzyme responsible for breaking down heme in the body, demonstrating that heme catalysis resulted in the subsequent release of CO and free iron as by-products (445) (Fig. 6). Since then, a large body of experimental evidence has demonstrated that CO is an extraordinary signaling molecule playing an important role in the regulation of cellular homeostasis, especially in the brain, where CO can influence physiological and pathological

processes both in the central and peripheral nervous systems (Fig. 12). Due to its capacity to freely diffuse from cell to cell, together to the fact that is not stored in synaptic vesicles from which is released through membrane depolarization processes, it is a nonconventional neurotransmitter capable of influencing signal transduction mechanisms. CO binds to heme in guanylyl cyclase to activate cGMP, a process responsible for maintaining endogenous levels of cGMP (354). This effect is blocked by potent HO inhibitors but not NO inhibitors (274). Consistent with endogenous distribution of HO in the brain, it has been suggested that CO can influence neurotransmission like NO (457). CO is involved as a retrograde messenger in LTP as well as in mediating glutamate effects on metabotropic receptors (193). This is corroborated by the finding that HO inhibitors block the conductance of specific ion channels associated with metabotropic receptor activation in the brain regulates *via* a cGMP-dependent mechanism (187, 255). Experimental evidence indicates that CO plays a role similar to that of NO in signal transduction processes. HO resembles NOS in that the electrons for CO synthesis are donated by cytochrome P450 reductase, which is 60% homologous at the amino acid level to the carboxy-terminal half of NOS (96, 458). Like NO, CO binds to iron in the heme moiety of guanylyl cyclase. However, while NO mediates glutamate's effects primarily at *N*-methyl-d-aspartate (NMDA) receptors, CO is involved in the effects of glutamate at a level of metabotropic receptors. As CO and NO play important roles in the regulation of CNS function, impairment of CO and NO metabolism results in abnormal brain function (Fig. 12) (97, 99, 337).

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FIG. 12. Schematic signal transduction pathways underlying the interfunctional role of CO, NO, and H₂S in the modulation of cell survival. NO exerts neuroprotection through stimulation of the sGC/cGMP/PKG system as well as through S-nitrosylation. As a consequence of S-nitrosylating reactions, inhibition of NF κ B and caspase-3 activity and a decrease in cell death occur. NO-mediated transcriptional activation of the Keap1/Nrf2/ARE pathway, associated with activation of K_{Ca++} channel and inhibition of mitochondrial energy transductions at level of complex IV (Cox), activates mitochondrial-dependent H₂O₂-mediated redox signaling, leading to upregulation of pro-survival mechanisms, such as vitagenes HO-1 and heat shock protein 70, Bcl2, and mitochondrial biogenesis. The latter results from activation of coactivator PGC-1 α and mtTFA. CO-mediated activation of mitochondrial redox signaling results in antiinflammatory, antiproliferative, and antiapoptotic effects, which confer neuroprotection. This occurs through activation of multiple pathways, including PPAR γ , PGC-1 α , and mtTFA, which induce mitochondrial biogenesis; P38 MAPK, HIF1 α , K_{Ca++} channel, PI3K-Akt, and Nrf2, as well as inhibition of ERK-1/2. H₂S is a highly reactive, strong reducing molecule that easily reacts with ROS and RNS, thus providing antioxidant activity and, in addition, activates ATP-sensitive potassium channels. Known cellular targets of H₂S include cytochrome c oxidase (complex IV, Cox) and carbonic anhydrase (CA). This gas H₂S has also been demonstrated to regulate cellular signal transduction pathways, including thioredoxin reductase, HO-1, and glutamyl-cysteine synthetase, resulting in cytoprotection. K_{ATP}, ATP-dependent K⁺; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase.

CO, is a primordial gas, produced and metabolized in almost all organisms from prokaryotes to mammals, and serves as an important signaling molecule owing to its capability to bind to

reduced transition metal centers in heme proteins (354). Both exogenous CO and endogenous CO produced by heme oxygenase generate a pro-oxidant milieu in aerobic mammalian cells that initiates signaling *via* ROS formation. Signaling promoted by CO and mediated by ROS presumably is dependent on the CO concentration and the time of exposure, on the localization of heme proteins (*e.g.*, mitochondrial or nonmitochondrial sites), or on the specificity of redox reactions. Activation of redox-sensitive transcription factors or stress-activated kinases, which induce expression of cytoprotective enzymes, thus conferring adaptations and tolerance to oxidative stress, represent a fundamental response to CO. However, elevated or protracted CO exposures elicit responses that generally are nonspecific, favoring biological oxidations to an extent that disrupt cellular homeostasis (354). It is generally recognized that CO exposures that avoid macromolecular damage should stand in a range of tissue CO concentrations that are below those produced at 20%–25% carboxyhemoglobin, the conventional threshold in mammals for serious poisoning. Beneficial for health are slow CO-releasing agents that do not exceed a concentration of 50 micromolar within a cell, as well as inhaled CO concentrations that acutely do not exceed 500 ppm for 1 h or 50 ppm continuously, in rodents. In humans, exposure limits of 50 ppm for 8 h and exposure concentration maximum of 200 ppm appear safe and may induce physiological effects (354).

The normal rate of endogenous CO production results in cellular CO concentrations, ranging from picomolar to nanomolar levels. This physiological concentration, however, can increase several fold depending on the heme turnover, whereas cellular CO content can increase 20–50-fold after exogenous CO administration. At high CO levels, the physiology is dominated by a range of adaptive stress redox-related responses, overlapping those observed during hypoxia. In the past, mitochondrial CO effects in living tissues were considered minor because the α_3 heme is primarily in the oxidized state. However, even at physiological PO_2 , CO does bind cytochrome oxidase, as some α_3 does remain in the reduced state, especially in metabolically active tissues like the brain and the heart. In this milieu, bound CO reduces the cytochrome bc_1 region of the chain respiratory complexes, thereby promoting significant mitochondrial ROS production (Fig. 13), which disrupts mitochondrial glutathione redox status. CO-dependent mitochondrial ROS generation has also been demonstrated for endogenous CO (427). Recently, signalization through mitochondrial ROS production by CO has become an emerging area and mitochondrial ROS are now implicated in various cellular regulatory processes, including cell proliferation, expression of angiogenesis factor, metalloproteinase mitochondrial biogenesis (328), and stabilization of hypoxia-inducible factor-1 (HIF-1). Low physiological levels of CO such as those generated by HO-1 induction have been demonstrated to elicit strong proliferative and antiapoptotic effects mediated by various signaling pathways, including PI3K-Akt, signal transducer and activator of transcription, and MAPK-Erk (33) (Figs. 10 and 13). Consistent to the metal-binding properties of CO, all heme-containing proteins are possible candidates with biological significance as CO sensors. These include proteins with heme as prosthetic group, such as hemoglobin, myoglobin, sGC, cyclooxygenase, cytochrome p450, cytochrome c oxidase, NOS, NADPH oxidase (Nox), transcription factors, neuronal PAS domain protein 2, and Bach-1 and Bach-2 (33).

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FIG. 13. The ubiquinone (Q) cycle is initiated when one electron from ubiquinol (QH₂) is donated to the Rieske-iron sulfur protein and the second electron is donated to cytochrome

b. The intermediate moiety is the free radical ubisemiquinone (Q_o), which can donate electrons to molecular oxygen to generate superoxide. Mitochondrial electron transport chain generates superoxide at complexes I, II, and III. Complexes I and II generate superoxide within the mitochondrial matrix. Complex III can generate superoxide in both the intermembrane space and the matrix. Release of superoxide from complex III into the cytosol is followed by conversion to H₂O₂ and subsequent activation of oxidant-dependent (redox) signaling pathways, which results in preconditioning. R-FeS, Rieske-iron sulfur.

Although CO at high concentrations is toxic, generation of lower amounts of ROS in response to exogenous low-dose CO might affect cellular respiration eventually resulting in adaptation (33). This is consistent with the general notion that the mitochondrial respiratory chain complexes I and III are the main sites of superoxide production (177). In this context, any impairment in the electron transfer process determines that upstream carriers become more reduced, thus filling Q cycle electron pool. The cytochrome (cyt)2 bc1 complex (EC 1.10.2.2) (cyt bc1 complex), localized in the inner membrane of mitochondria, couples the oxidation of a substrate quinol (QH₂) with the generating proton motive force across the energy transducing membrane system, where the energy is ultimately stored in the form of ATP (Fig. 13). Cytochrome bc1 complexes contain four redox-active metal centers, arranged in two separate chains. The high potential chain consists of the Rieske iron-sulfur [2Fe2S] cluster (Fig. 13) and cyt c1. The low potential chain, which binds to the cyt b subunit of ubiquinol oxidizing complexes, consists of two b-type hemes, cyt bL and bH, labeled for their relatively lower and higher electrochemical potentials (177). Three enzymatic binding sites participate in catalysis on the cyt bc1 complex: the quinol oxidase (Q_o) site, quinol reductase (Qi) site, and a docking site for soluble cyt c on cyt c1. The Q_o site is located toward the positively charged side of the membrane, where protons are released during turnover of the enzyme (Fig. 13). The Qi site is located toward the negatively charged surface of the membrane, where protons are taken up during catalysis. The water-soluble cyt c docking site is located on the c-type cyt representing the terminal electron carrier within the cyt bc1 complex on the positive side of the membrane. Cyt bc1 complex catalysis is thought to occur by a Q-cycle mechanism (Fig. 13). Conceivably, a key reaction in the Q-cycle is the bifurcated electron transfer at the Q_o site, although the exact mechanism is still controversial (177). In the bifurcated reaction, QH₂ is oxidized at the Q_o site, with one electron being transferred through the high potential chain to reduce cyt c, and the other electron is transferred through the low potential chain, to reduce a quinoid species (Q or SQ, depending on the state of the two-electron gate) at the Qi site. Two turnovers of the Q_o site are required to reduce a Qi site Q to a QH₂ (Fig. 13). Under some conditions, the Q-cycle can be short-circuited by various bypass reactions, some of which yield the physiologically deleterious superoxide (177). The bypass reactions are typically observed *in vitro* under partially inhibited conditions, for example, in the presence of antimycin A, or under high proton motive force, where it is possible that the [2Fe2S] cluster can oxidize QH₂ to a semiquinone (SQ), but processing of electrons by the low potential chain is hindered, resulting in the accumulation of an SQ intermediate, which in turn can reduce O₂ to superoxide (427).

Physiologically, electron transport chain consumes up to 90% of the oxygen taken up by a cell, of which 1% is transformed in superoxide under normal physiological conditions (33). The rate

of mitochondrial electron transfer from semireduced Q to molecular O₂ to form superoxide is proportional to the product of the concentrations of semireduced Q and O₂. It is estimated that under pathophysiological conditions, superoxide production can occur at 2%–10% of the uninhibited rates. Superoxide dismutases, in turn, generate the more stable hydrogen peroxide before it is transformed by catalase in water or, depending on the presence of metals, into highly reactive hydroxyl radicals. Thus, SOD2, which directs H₂O₂ out of the mitochondrion, accomplishes two functions: the classical scavenging of superoxide, as well as its signalization as ROS-dependent signaling pathways. The binding of CO to cytochromes localized within complex IV (*i.e.*, cytochrome a₃) promotes reduction in the respiratory carriers in the cytochrome bc₁ region of complex III, associated with increased leakage of superoxide and H₂O₂ production (Fig. 13).

ROS-dependent signaling mediated by CO may involve other pathways, such as xanthine oxidase and Nox. The latter is assembled at the membrane only after stimulation. This confers biological significance to the cytosolic compartment, where the NADPH membrane-dependent system generating radicals represent the primary site of action for the CO molecule, rather than oxidases, which are located in the more distal mitochondrial milieu. Increasing evidence indicates that CO modulates mitochondrial ROS production. Mitochondrial H₂O₂ production as consequence of CO binding to Cox leads to activation of PI3K-Akt pathway with subsequent activation of mitochondrial biogenesis (33). Further, CO increases RNA expression of Nrf1, Nrf2, and peroxisome proliferator-activated receptor-gamma coactivator-1 α , the transcription factors involved in the regulation and activation of mitochondrial biogenesis (33). In addition, CO upregulates mitochondrial transcription factor A, SOD2, Bad, as well as activates KCa channels and modulates vascular relaxation through inhibition of endothelin-1 (422). Of note, in *Rhodospirillum rubrum*, a photosynthetic bacterium, CO binds to CooA, which, upon exposure to CO, acquiring DNA-binding transcriptional activity for the CO dehydrogenase gene, promotes the oxidative conversion of CO to CO₂. Consistently, heart cytochrome c oxidase is able to metabolize CO to CO₂, *via* its oxygenase activity (473). However, whether this occurs also in brain mitochondria remains to be determined.

CO was also found to be protective through an antiinflammatory action, as demonstrated initially in a model of endotoxic shock, as well as mouse cardiac xenotransplantation (336). Accordingly, mouse hearts transplanted into rats treated with cyclosporine A and cobra venom factor as immunosuppressants survive indefinitely. However, in these experimental conditions, inhibition of HO activity or ablation of gene expression for HO-1 causes acute rejection, unless the donor and recipient received CO at a concentration of 250 ppm, similar to that found in heavy smokers, in which case grafts survive indefinitely (338). In this experimental paradigm, CO appears to be able to substitute for HO-1 in suppressing the pro-inflammatory response that causes graft rejection (339). These seminal experiments launched CO as a powerful antiinflammatory agent acting at a level of macrophages, to control the balance of pro-inflammatory and antiinflammatory molecular events. Inflammation is a complex dynamic process initiated by the body in response to tissue injury or infective agents. In this context macrophages are central for initiation, maintenance, and resolution of inflammatory process, through a series of steps involving antigen presentation, phagocytosis, and immunomodulation *via* cytokines and growth factors production (338). Activation signals include cytokines, such as IFNs, granulocyte-macrophage colony-stimulating factor, and TNF α as well as bacterial LPS, extracellular matrix

proteins, and other chemical mediators. Macrophages recognize distinct pathogen-associated molecular patterns by Toll-like receptors (TLRs) that activate signaling pathways inducing expression of pro-inflammatory genes (33, 334). Consequently, inhibition of inflammatory events is a necessary process when the noxious stimulus promoted by invading cells and pathogens has been removed, so that survival of host cells is ensured. During the inflammatory process, secretion of antiinflammatory cytokines such as IL-10 and transforming growth factor β promoted by CO leads to neutralization of activated macrophages, as demonstrated *in vivo* and *in vitro* after LPS administration (121). Notably, IL-10 itself induces HO-1 expression, thus generating a feedback mechanism whereby a persistent antiinflammatory effect promoted by CO is maintained (33). In conclusion, CO modulates inflammatory processes through the (a) prevention of platelet aggregation, thus exerting antithrombotic affects, (b) downregulation of plasminogen activator inhibitor type 1 expression, and (c) antiapoptotic action in several cell types, including endothelial cells, fibroblasts, hepatocytes, and β -cells of the pancreas (25). In addition, CO inhibits smooth muscle cell proliferation associated with neointimal thickening in inflammatory lesions *in vivo* (339). Many of the observed effects of CO have been obtained by exposing cells or animals to gaseous CO by inhalation; interestingly, the recently discovered CO-releasing molecules appear to afford similar protective action, thereby providing an alternative therapeutic approach for those pathophysiological conditions where CO treatment is warranted (7, 121).

B. NO and NO synthases

NO plays multiple roles in the nervous system (18, 108, 323). In addition to regulating proliferation, survival, and differentiation of neurons, NO is also involved in synaptic activity, neural plasticity, and memory function. NO exerts long-lasting effects through regulation of transcription factors and gene, thus modulating cell differentiation and survival processes in the brain. Signaling by NO is also mediated by reactive nitrogen species through targeted modifications of critical cysteine residues in proteins, including *S*-nitrosylation and *S*-oxidation, as well as by lipid nitration. NO is involved neuroinflammation and neurodegeneration, such as in AD, amyotrophic lateral sclerosis, Parkinson's disease, multiple sclerosis, Friedreich's ataxia, and Huntington's disease. Susceptibility to NO and its reactive nitrogen species is modulated by intracellular reducing potential, mainly reduced glutathione with its related antioxidant enzymes as well as redox-dependent cellular stress resistance signaling pathways. In view of this, neurons, in contrast to astrocytes, appear particularly vulnerable to the effects of nitrosative stress (323).

NO synthesis is catalyzed by the NOS family of enzymes, which convert arginine to citrulline and NO \bullet (Fig. 6). NOS, localized in the CNS and in the periphery (323), is present in three well-characterized isoforms: (a) neuronal NOS (nNOS, type I), (b) endothelial NOS (eNOS; type III), and (c) inducible NOS (iNOS, type II). Activation of different isoforms of NOS requires various factors and cofactors. In addition to a supply of arginine and oxygen, an increase in intracellular calcium leads to activation of eNOS and nNOS, and formation of calcium/calmodulin complexes is a prerequisite before the functional active dimer exhibits NOS activity, which depends also on cofactors such as tetrahydrobiopterin, flavin adenine dinucleotide, flavin mononucleotide, and NADPH (99). nNOS has a predominant cytosolic localization, whereas the eNOS is bound to the plasma membrane by *N*-terminal myristylation (18). In contrast to nNOS and eNOS, iNOS can bind to calmodulin even at very low concentration of intracellular calcium; thus, iNOS can exert its activity in a calcium-independent manner. iNOS, usually present only in the cytosol, also

requires NADPH, flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin for full activity. eNOS expressed in cerebral endothelial cells critically regulates cerebral blood flow. However, a small population of neurons in the pyramidal cells of CA1, CA2, and CA3 subfields of the hippocampus and granule cells of the dentate gyrus express eNOS. nNOS, which is expressed in neurons, is critically involved in synaptic plasticity, neuronal signaling, and neurotoxicity. Activation of nNOS forms part of the cascade pathway triggered by glutamate-receptor activation that leads to intracellular cyclic GMP elevation. The levels of iNOS expression in the CNS are generally very low. However, after noxious stimuli, such as viral infection or trauma, astrocytes and microglia express high levels of induced iNOS (350). Activation of iNOS requires gene transcription, and the induction can be promoted by endotoxin and cytokines (IL-1, IL-2, IFN- γ , and TNF). iNOS induction can be blocked by antiinflammatory drugs (dexamethasone), inhibitory cytokines (IL-4 and IL-10), prostaglandins, tissue growth factors, or inhibitors of protein synthesis, for example, cycloheximide (350).

The discovery of the role of NO as a messenger molecule has revolutionized the concept of neuronal communication in the CNS. NO is a gas freely permeable to the plasma membrane. Thus, NO does not need a biological receptor to influence the intracellular communication or signaling transduction mechanisms (108). Once generated, the cell cannot regulate the local concentration of NO; therefore, the other way to influence NO activity is to control its synthesis. The activity of NO also terminates when it chemically reacts with a target substrate. NO when produced in small quantities can regulate cerebral blood flow and local brain metabolism (8, 99, 149), neurotransmitter release, and gene expression, and play a key role in morphogenesis and synaptic plasticity (181, 288). It is also generally accepted that NO is a major component in signaling transduction pathways controlling smooth muscle tone, platelet aggregation, host response to infection, and a wide array of other physiological and pathophysiological processes (317, 398, 453). Under conditions of excessive formation, NO is emerging as an important mediator of neurotoxicity in a variety of disorders of the nervous system (8, 486).

A large body of evidence shows cytoprotection by NO in various models of cellular toxicity and death: (a) direct scavenging of superoxide, as well as indirect action on the release of iron from ferritin stores through formation of iron-nitrosyl (399); (b) interaction of NO with cysteine residues on the NMDA receptor and inhibition of calcium influx (321); (c) inactivation of caspases (486); (d) activation of a cyclic GMP-dependent survival pathways (181); and (e) signal for the induction of cytoprotective proteins, such as Hsps (108). Current opinion holds that the intracellular redox state is the critical factor determining whether in brain cells NO is toxic or protective (323). The mechanistic involvement of NO as pro-inflammatory or antiinflammatory agent is dictated by the complexity of NO chemistry when applied to biological systems (190) (Fig. 12). As described in complete mechanistic details by Stamler, the reactivity of the NO depends on the oxidation state of the nitrogen atom, which makes this molecule prone to acquire different redox-active forms (190). In contrast to NO, which contains one unpaired electron in the outer orbital, nitrosonium cation and nitroxyl anion are charged molecules being, respectively, the one-electron oxidation and reduction products of NO. Whereas nitrosonium cation can be transferred reversibly between cysteine residues (transnitrosation), nitroxyl anion can be formed by hemoglobin, nNOS, and S-nitrosothiols (RSNO). Central to the biochemistry of NO is the reaction with sulphhydryl moieties to form S-nitrosyl derivatives or RSNO, a process termed S-nitrosation (28). Conceivably, RSNO, such as S-nitroso-glutathione or nitroso-cysteine,

represents a chemical process for storage, stabilization, and preservation of bioavailable NO *in vivo* (179). In addition, due to its relatively abundant presence, GSH, as the main intracellular free thiol, represents a critical determinant of the reactivity and fate of NO. Thus, through S-nitrosation NO modulates activity and function of several enzymes and proteins (28). However, oxidative modification in protein structure and function may occur when reactive nitrogen species reach a critical threshold, and hence nitrosative stress may ensue (179). At the cellular level, nitrosative stress inhibits cell growth and causes apoptosis, mediating a wide array of pathogenic effects elicited by NO (179). The intriguing aspect of the parallels between the effects mediated by increased RNS and ROS is the ability of cells to respond to these two types of stress, and depending on the severity of the nitrosative/oxidative insult, this response may result in adaptation and resistance to toxicity (108, 179, 281, 292, 317).

C. Hydrogen sulfide

Hydrogen sulfide (H₂S), a gas with the characteristic odor of rotten eggs, is known for its toxicity and as an environmental hazard, inhibition of mitochondrial respiration resulting from blockade of cytochrome c oxidase being the main toxic mechanism. Recently, however, H₂S has been recognized as a signaling molecule of the cardiovascular, inflammatory, and nervous systems; therefore, alongside NO and CO, H₂S is referred to as the third endogenous gaseous transmitter (404, 434, 460) (Fig. 12).

H₂S is an endogenously produced gas in the central nervous system (442) as well as in various peripheral tissues and is present in the blood at an estimated concentration of approximately 1 μ M, which can rise up one order of magnitude in pathological states such as diabetes or hepatic encephalopathy (230, 434). There is some lack of clarity about the various biological forms of H₂S. Chemically, it can be distinguished as free H₂S, acid-labile sulfide (ALS), and dithiothreitol-labile sulfide (DLS) forms, each probably having quite different physiological significance. Free H₂S is the form also known as inorganic sulfide, which can be present as H₂S, HS⁻ (hydrosulfide anion), or S²⁻ (sulfide anion) in the aqueous milieu and as H₂S in the gas phase (breath and flatus). The pKa values for the first and second dissociation steps of H₂S are 7.04 and 11.96, respectively. Therefore, in the aqueous state, such as within a cell or in plasma at physiologic pH 7.4, approximately one-third exists in the undissociated volatile form (H₂S) and the remainder largely as the hydrosulfide anion (HS⁻). Very small amounts of (S²⁻) are present. However, there is considerable ambiguity in literature regarding the terms describing free H₂S. Besides inorganic sulfide, terms such as H₂S, sulfide, and total sulfide are used. We here refer to the sum of H₂S and HS⁻ as total sulfide. Also convenient is to distinguish these forms of H₂S from acid-labile sulfide (ALS) and dithiothreitol-labile sulfide (DLS). ALS is the form from which H₂S is liberated when a biological matrix is treated under acidic conditions, and is contained in iron–sulfur clusters of nonheme iron–sulfur proteins (*e.g.*, ferredoxins). DLS is the form from which H₂S is liberated when a biological matrix is treated with dithiothreitol (DTT). This form belongs to the class of biological sulfane sulfur. Sulfane sulfur atoms are defined as divalent sulfur atoms bonded only to other sulfur, except that they may also bear ionizable hydrogen at some pH values. Labile sulfane compounds are compounds where elemental sulfur is associated with protein, either in the form of persulfides (R–SSH) or polysulfides such as (R–Sn–R) where $n \geq 3$, polythionates (–O₃S–Sn–SO₃²⁻), thiosulfate (SSO₃²⁻), and thiosulfonates (R–S(O₂)S⁻) (443).

H_2S is produced by three enzymes in mammalian tissues, two pyridoxal phosphate-dependent enzymes (cystathione β -synthase [CBS] and cystathione γ -lyase [CSE]), and a newly described enzyme, 3-mercaptopropruvate sulfurtransferase (460) (Figs. 6 and 12). CBS utilizes pyridoxal phosphate as a cofactor (Hishigami 2009) and it catalyzes the production of H_2S from cysteine by β -elimination (214) ($\text{L-cys} + \text{L-Hcy} \rightarrow \text{L-cystathione} + \text{H}_2\text{S}$) or β -replacement reactions ($\text{L-cys} + 2\text{-mercaptoproethanol} \rightarrow \text{S-hydroxyl-L-cysteine} + \text{H}_2\text{S}$) (442). In addition, nonenzymatic H_2S production from organic polysulfides derived from garlic has been suggested as a potential source in the circulation (434).

There are three known pathways of H_2S degradation: mitochondrial oxidation to thiosulfate, which is further converted to sulfite and sulfate; cytosolic methylation to dimethylsulfide; and sulfhemoglobin formation after binding to hemoglobin (434). Similar to NO and CO, H_2S can also bind to hemoglobin—which represents the biochemical sink for these gaseous transmitters. Consequently, saturation with one of these gases might lead to enhanced plasma concentrations and, subsequently, to biological effects of the other gases. H_2S is oxidized into thiosulfate ($\text{S}_2\text{O}_3^{2-}$), sulfite (SO_3^{2-}), and sulfate (SO_4^{2-}) (113) and excreted from the kidney (434).

In the central nervous system, H_2S functions not only as a neuromodulator, but also a neuroprotectant against oxidative stress (455). In the cardiovascular system, H_2S relaxes vascular smooth muscles by the activation of ATP-dependent K^+ channels and inhibits smooth muscle cell proliferation *via* the mitogen-activated protein kinase signaling pathway. These effects are important for maintaining blood pressure and preventing vessel structural remodeling, and identifies H_2S as an important factor in the development of some vascular diseases, such as hypertension. H_2S also shows cardioprotective effects in ischemic myocardium and septic and endotoxin shock (119).

In the CNS, CBS is highly expressed in the hippocampus and the cerebellum (230). CBS is mainly localized to astrocytes (442). CSE is mainly expressed in cardiovascular system, but was also found in microglial cells, spinal cord, and cerebellar granule neurons. 3-Mercaptopropruvate sulfurtransferase is localized to neurons (442). H_2S production in astrocytes is approximately 10-fold higher than in cultured microglial cells, in NT-2 cells, or in SH-SY5Y cells (251). This finding suggests that astrocytes are the main brain cells producing H_2S . H_2S selectively enhances the NMDA receptor-mediated responses and facilitates the induction of hippocampal long-term potentiation (LTP), a synaptic model of memory and learning (433). H_2S may activate NMDA receptor by virtue of its reducing property. One plausible redox modulatory site is the cysteine (Cys) pair (Cys744 and Cys798) located on the extracellular domains of the NR1 subunit (442). Intracellularly, H_2S enhances NMDA receptor-mediated response *via* cAMP production. Exogenous H_2S increases the production of cAMP in primary cultures of rat cerebral and cerebellar neurons, and in some neuronal and glial cell lines (230). cAMP activates cAMP-dependent protein kinase during the initiation and late phase of LTP; consequently, activated cAMP-dependent protein kinase may, in turn, phosphorylate NMDA receptor subunits NR1, NR2A, and NR2B at specific sites and thus enhance NMDA currents, which is essential for LTP induction (442). H_2S also modulates the synaptic responses of serotonergic neurons and regulates the release of corticotropin-releasing hormone from the hypothalamus (364). H_2S upregulates γ -aminobutyric acid B receptor, a G protein-coupled receptor located at pre- and postsynaptic sites (364). In addition, H_2S has been shown to hyperpolarize neurons in the CA1 and dorsal raphe

nucleus by increasing K⁺ efflux probably *via* ATP-dependent K⁺ channels (442). H₂S influences tyrosine and mitogen kinases; receptor tyrosine kinases as well as MAPKs regulate fundamental cellular activities such as apoptosis, differentiation, metabolism, motility, cell division, and survival (442).

In addition to its role in signal transduction, H₂S can protect neuron cells from oxidative stress, not only by increasing the levels of antioxidant glutathione (113), but also by activating ATP-dependent K⁺ channels and Cl⁻ channels (442). H₂S appears to increase GSH levels by activating γ-glutamyl-cysteine synthetase (114). In addition, increased intracellular levels of cysteine were found as consequence of glutamate-independent activation of cystine/glutamate antiporter. This leads to increased cystine uptake that promotes formation of cysteine and glutathione (231). Another important mechanism for the effect of H₂S on GSH may be *via* enhancing glutamate uptake (265). In human cultured neuron cells, H₂S inhibits peroxynitrite, acting as an endogenous peroxynitrite scavenger (119). Thus, H₂S may act as a neuroprotectant against oxidative stress (Fig. 6). Changes in the level of H₂S in the brain have been demonstrated in various CNS pathologies. A 55% decrease in the brains of AD patients has been documented for both H₂S content and CBS activity, which was associated with a parallel loss of AdoMet content, a CBS activator. The CBS activity is decreased in homocysteinuria, febrile seizure frequently occurring in children. Conversely, CBS levels in Down's syndrome brains were approximately three times greater than those found in normal controls (119).

D. H₂S and suspended animation

Suspended animation is a hibernation-like metabolic status characterized by a marked, still reversible, reduction of energy expenditure, which allows nonhibernating species to sustain environmental stress, such as extreme changes in temperature or oxygen deprivation (460). The first line of experiments show that in animals that do not normally hibernate, an hibernation-like state can be obtained, as shown by the Roth laboratory (36, 331, 374). In this work, nematode embryos exposed to anoxic conditions were shown to survive by entering into a suspended animation-like state, which was associated with specific changes in gene expression. This was observed below and above a 10-fold range of hypoxic oxygen concentrations from 0.01% to 0.1%. While embryos exposed outside this range survived completely and progressed to normal development, exposure within this range was associated with loss of protection and cellular damage and death after 24 hours. In these conditions exposure to CO resulted in survival, an effect mediated by a competition with cytochrome oxidase as a target for gas binding resulting in a scavenging of all available oxygen (434). Subsequently, the Roth group provided further evidence that inhaled H₂S can induce a suspended animation-like state in mice. In this condition, animals breathing 80 ppm H₂S exhibited a dose-dependent reduction of respiratory and heart rates, oxygen uptake, and CO₂ production, associated with a drop in body core temperature to levels 2°C above ambient temperature (460). All these effects were totally reversible after H₂S washout, with animals showing a complete recovery and normal behavior (363). Relevant to the finding that a controlled reduction in cellular energetic expenditure is consistent with maintenance of ATP homoeostasis is the notion of an improved outcome during shock states. Notably, pretreatment with inhaled H₂S (150 ppm) for only 20 minutes significantly prolonged survival without apparent damage for mice exposed to lethal hypoxia (5% oxygen), as well as in rats receiving a single intravenous bolus of Na₂S (460). Thus, H₂S can induce adaptive (hormetic) stress responses that protect cells against a range of adverse conditions.

It is generally recognized that hibernation confers cytoprotection, so that tissues from hibernating animals are particularly resistant to various hypoxic and ischaemic insults (460). This is relevant in organ transplantation where, similarly to what was shown for CO, H₂S effects may provide better preservation of transplantable organs. It is noteworthy that, owing to a larger surface area-to-mass ratio than humans, rodents are much more capable of a rapid reduction in core temperature when challenged with a toxic insult than are humans (434). This makes the clinical relevance of some murine models questionable. Mice, in fact, present a body mass 3,000-times less than that of adult humans, and a heart rate 14-times greater and respiratory rates 10-times faster than in humans. In addition, at rest, O₂ consumption is 10 times less in humans than in rodents, so that much of the oxygen consumed in a mouse is used for heat generation. It is possible, therefore, that in mice H₂S-induced reduction of O₂ consumption may largely account for specific ATP-requiring processes, such as mitochondrial uncoupling, whereas ion homeostasis is not drastically compromised. As a consequence, the human window of compromised oxidative phosphorylation is likely to be much smaller than in rodents (460).

E. Functional interrelation of NO, CO, and H₂S gases and their relevance to hormesis

Both CO and NO activate the GC system by binding the active heme and enhancing cGMP production. Although NO is definitely more potent than CO in activating guanylyl cyclase, CO generally operates in the system under specific physiological conditions, as in the case of regulation of vascular smooth muscle cell proliferation during hypoxic conditions (355). Of the two gases, NO is the more reactive, having the same effective size and polarity as the O₂ molecule, and, unlike CO, binds to both Fe²⁺ and Fe³⁺ heme. Moreover, low CO concentrations stimulate NO release and are associated with peroxynitrite generation in vascular cells. The chemistry of CO-mediated NO release depends on the initial distributions of NO and CO in the cell and the differing equilibrium constants for transition-metal binding of the two gases. The association constants for NO binding to Fe²⁺ heme proteins such as myoglobin and hemoglobin are greater than those for CO (355). For hemoglobin, the affinity of Fe²⁺ for NO is at least a thousand times greater than that for CO; however, CO dissociation is much slower than for NO or O₂. Therefore, CO gradually displaces NO from Fe²⁺ heme proteins, requiring minutes or longer for equilibrium even with an excess of CO. NO displacement from Fe²⁺ by CO may also be enhanced in proximity to reduced thiols that serve as a sink for SNO protein formation. (355). HO-1 and/or CO, and NOS2 and/or NO are two systems functionally interrelated. In some situations, CO can induce expression of NOS2 and, in others, inhibits expression of NOS2 and consequently NO. NO upregulates HO-1 with production of CO (316). We have recently found evidence for a functional relationship between CO and NO. In endotoxic shock, the salutary action of CO in rat brain appears to depend on the activation of NF κ B, which triggers transcription of NOS2 with production of NO, and subsequently results in the upregulation of HO-1 (96). In the absence of any of these steps, the beneficial effect of CO is lost (33). To what extent CO and NO act interdependently in other physiological and pathological conditions that involve CO and/or NO is unknown. Endogenous CO has been suggested to control constitutive NOS activity. Moreover, CO may interfere with NO binding to guanylyl cyclase, and this in addition to the important role of HO in regulating NO generation, owing to its function in the control of heme intracellular levels, as part of the normal protein turnover (33). This hypothesis is sustained by recent findings showing that HO inhibition increases NO production in mouse macrophages exposed to endotoxin (128).

H_2S belongs to a family of labile biological mediators termed gasotransmitters, which share many similarities. As a gasotransmitter, H_2S rapidly travels through cell membranes without using specific transporters (434). H_2S can induce an upregulation of heme oxygenase 1 and cytochrome c oxidase subunit V. By upregulating HO1, H_2S can trigger the production of CO, thus providing cytoprotective and antiinflammatory effects. These effects are also mediated by inhibition of the nuclear factor- κB (NF κB) pathway and downregulation of inducible NO synthase (iNOS) expression and NO production by inflammatory stimuli. Expression of HO1 is probably the result of the activation by sulfide of the extracellular signal regulated kinase pathway (434). H_2S can also inhibit cellular respiration, at a level of cytochrome c oxidase *via* a reaction with its copper center. Cytochrome c oxidase is a key regulator of cellular respiration; all three gasotransmitters (NO, CO, and H_2S) have competitive effects on this enzyme. Inhibition of cytochrome c oxidase is central to the regulation of cellular oxygen consumption by H_2S , and has been implicated in the pharmacological effects of H_2S , together with induction of suspended animation (see above).

Moreover, evidence is accumulating to demonstrate that H_2S , as opposed to NO and CO, induces vasorelaxation that is not mediated by the cGMP signaling pathway. This indicates that H_2S is a novel endogenous gaseous modulator of vascular contractility. At the same time, similar to NO and CO, H_2S can inhibit VSMC proliferation and induce apoptosis *in vitro* (23). Using cultured VSMC, exogenous H_2S could dose dependently suppress the proliferation of VSMC through the MAPK signaling pathway. Studies using molecular means to overexpress CSE in cultured VSMC found that endogenous H_2S could also attenuate the rate of cell proliferation and increase the rate of cell apoptosis. The latter effect is *via* the activation of MAPK and caspase-3. This may be relevant to the pathogenesis of atherosclerosis, as well as vascular graft occlusion and restenosis after angioplasty (23). However, the precise role of smooth muscle cell proliferation and apoptosis in the development of diseases, particularly the molecular mechanisms underlying the relationship between the diseases and cell proliferation, remains to be characterized.

As a key early event in the cellular phenotypic changes, smooth muscle cells that are in a quiescent state (G0) in normal uninjured vessels transit through the G1 phase of the cell cycle and enter into the S phase to undergo replication (472). Cell cycle progression is under the control of cyclin-dependent kinases, which phosphorylate different specific target proteins when the cells enter different phases of cell cycle. Synchronized (G0) smooth muscle cells treated with S-diclofenac, a recently discovered H_2S donor, recover at the early cell cycle alteration, a finding associated with a decreased p53 and p21 activity. Interestingly, it has been suggested that both vascular diseases and cancer similarly develop from a clonal proliferation at the sites of local tissue injury and inflammation, with underlying genomic alterations (23).

NO activity in the CNS provides an example of hormesis, being extremely toxic at high concentrations yet neurotrophic at lower concentrations. NO may be released by a variety of cell types (*i.e.*, astrocytes, activated microglia, and macrophages) after injury or disease, becoming 10-fold greater than normal physiological concentrations (35, 322). At such elevated concentrations, NO can facilitate massive cell death *via* apoptotic mechanisms, contributing to the progression of a variety of neurodegenerative diseases, including Alzheimer's disease and ALS. Of significance is that when low doses of NO are administered before a high and neurotoxic dose of NO, the prior preconditioning dose induces a marked protective effect. The

dose–response characteristics of preconditioning doses conform to those of an hormetic dose response, suggesting that preconditioning is a manifestation of hormesis (87). The mechanism inducing the protective response appears to be mediated by the induction of H0-1 and is supported by multiple lines of research, including the use of H01 inhibitors and H01 null mice. H0-1 activation generates CO, bilirubin, and free iron *via* its actions on heme, which is released from NO-damaged heme proteins (35). It is likely that the protective effects induced by NO may be mediated, in part, by low doses of CO, which can induce the synthesis of MnSOD, and facilitate the activation of mitogen-activation protein kinase (MAKP) pathways, including the p38 MAPK pathway, which then activates NF κ B, thereby inhibiting apoptosis (401, 447). These and other related findings have linked CO-induced effects at low and high concentrations with those of NO in an integrated, yet complex series of responses that are hormetic.

As is the case with NO and CO, H₂S also has been reported to induce hormetic dose responses for a broad range endpoints of potential biomedical importance. In fact, the hormetic concept has integratively resolved numerous apparent disputes in the literature in which different groups of researchers reported opposite types of responses when using either high or low concentrations of H₂S. However, when subsequent experiments were conducted that included a far broader dose–response continuum, the hormetic-biphasic dose response was typically found. This has been now widely reported for infarct volume in stroke-related experimental studies, investigations concerning various types of inflammatory processes, vasorelaxation/tension in aortic tissue, cell proliferation/apoptosis, as well as in the case of tumor promotion and inhibition. Since the concentration of H₂S can vary considerably both within and between tissues and under differing conditions, it is likely that H₂S could affect a complex array of biological responses that may challenge the capacity to make definitive biomedical and/or clinical interpretations or predictions. Li and Moore (256) closed their recent insightful review of the role of H₂S in health and disease with a statement probing their articulated biological conundrum of how does one molecule display such widely differing effects and could it be due to different effects at differing concentrations. The answer to this seminal question is found within the framework of an hormetic dose–response interaction that is independent of biological model, tissue, and endpoint.

XVII. Future Directions

The burden of chronic neurodegenerative diseases on our society is expected to continue to increase, largely due to the aging of the population. Although no satisfactory treatments are yet available, it is timely to think about implementing prevention measures that harness the intrinsic cytoprotective mechanisms described in this review. Exploiting the ability of small molecules, many of which are dietary agents and therefore of presumed low toxicity, to mimic various stimuli that lead to the upregulation of these intracellular defenses is an exciting area for future development. As described in this review, oxidizable diphenols, sulforaphane, dimethyl fumarate, celastrol, curcumin, and FA induce the Keap1/Nrf2/ARE pathway and the HSR, which collectively control expression of more than 100 genes that are concerned with cellular protection and survival under various conditions of oxidative and electrophilic stresses. Importantly, this induction correlates with protection against chronic diseases, including neurodegenerative conditions, in many animal models. Since redox regulation and transcription factor cysteine modifications are central to both the Keap1/Nrf2/ARE pathway and HSR, such multitarget

agents, all of which are reactive with sulfhydryl groups, are ideal for simultaneously manipulating these pathways and achieving synergistic protective effects.

Although there are many specific technical questions that could be identified and prioritized for follow-up research activities in the area of neurodegenerative disorders, we believe that the most significant future direction in this area is conceptual. That is, we believe that it is essential that the biomedical community become more informed about the robustness of the data underlying the hormetic dose response and the generalizability of this concept across biological models, endpoints, and chemical classes and its many biomedical and therapeutic implications. We believe that major concepts such as pre- and postconditioning are specific manifestations of the general hormesis concept. The hormesis concept has the capacity to expand the range of biological questions and testable hypotheses, as well as affect how studies are designed, including how results will be analyzed and modeled. The hormesis concept also suggests that therapeutic interventions will be constrained by the limits of biological plasticity that conforms to the quantitative features of the hormetic dose response. This framework would affect the expectations of pharmaceutical companies in their development of new treatment modalities across the broad spectrum of therapeutic activities. Thus, we believe that the hormetic concept is a basic biological concept that needs to be better studied and understood to more successfully treat human diseases and to improve human performance for numerous endpoints. In the future we would strongly encourage the many professional societies that are concerned with issues related to dose response to routinely include technical sessions at annual meetings and specialty conferences on hormesis. We would also recommend that the concept of hormesis be incorporated into the formal biomedical courses in graduate and medical schools. These suggestions are just the start, but these are necessary if the biomedical and clinical fields, including the neurosciences, are going to get the dose right, improving the health and well-being of those individuals we serve.