



Review

Cellular stress responses, hormetic phytochemicals and vitagenes in aging and longevity[☆]

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ABSTRACT

Modulation of endogenous cellular defense mechanisms represents an innovative approach to therapeutic intervention in diseases causing chronic tissue damage, such as in neurodegeneration. This paper introduces the emerging role of exogenous molecules in hormetic-based neuroprotection and the mitochondrial redox signaling concept of hormesis and its applications to the field of neuroprotection and longevity. Maintenance of optimal long-term health conditions is accomplished by a complex network of longevity assurance processes that are controlled by vitagenes, a group of genes involved in preserving cellular homeostasis during stressful conditions. Vitagenes encode for heat shock proteins (Hsp) Hsp32, Hsp70, the thioredoxin and the sirtuin protein systems. Dietary antioxidants, such as polyphenols and L-carnitine/acetyl-L-carnitine, have recently been demonstrated to be neuroprotective through the activation of hormetic pathways, including vitagenes. Hormesis provides the central underpinning of neuroprotective responses, providing a framework for explaining the common quantitative features of their dose response relationships, their mechanistic foundations, their relationship to the concept of biological plasticity as well as providing a key insight for improving the accuracy of the therapeutic dose of pharmaceutical agents within the highly heterogeneous human population. This paper describes in mechanistic detail how hormetic dose responses are mediated for endogenous cellular defense pathways including sirtuin, Nrfs and related pathways that integrate adaptive stress responses in the prevention of neurodegenerative diseases. This article is part of a Special Issue entitled: Antioxidants and Antioxidant Treatment in Disease.

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1. Introduction

In recent years, studies have shown that aging (more correctly defined as longevity) is due to a complex genetic and cellular process that seems to be partly regulated by sirtuins [1,2] the human and murine homologs of the *Saccharomyces cerevisiae* Sir2 that control both replicative and overall lifespan [3,4]. Findings have also shown that this gene family regulates longevity in *Caenorhabditis elegans* and *Drosophila melanogaster* [1,2], suggesting that there is an evolutionary

need in many different complex species to preserve these proteins in the cells. Sirtuins, which are classified as class III histone deacetylases, differ from traditional class I and II histone deacetylases [5,6]. They differ from conventional HDACs in that their substrates range from metabolic enzymes to structural proteins and histones [7–9].

The activity, stability and intracellular location of nearly all proteins depend on post-translational modifications (PTMs) that include phosphor-ylations, acetylations, sumoylations, ubiquitinations, ADP-ribosylations and nitrations [10]. Most of these highly dynamic processes occur as a result of opposing enzyme activity (e.g. kinases vs. phosphatases, acetylases vs. deacetylases, etc.). Numerous studies have demonstrated that PTM signaling pathways play a significant role in crosstalk between the cell and its environment and in enabling cells to become flexible towards change [10]. For this reason, nicotinamide adeninedinucleotide (NAD) has aroused new interest as it is an

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important substrate for a number of enzymes that catalyze a set of post-translational modifications, such as deacetylation or ADP-ribosylation. The role of NAD in these regulatory processes differs from its involvement in energy metabolism since it is based on the ability to act as an ADP-ribose donor, thus requiring NAD re-synthesis to avoid depletion of the intracellular NAD pool [11]. The fundamental role of NAD in energy metabolism and protein modification has an important physiological influence on the control of cell metabolism, cell death and longevity [12,13].

Since transient post-translational modifications such as acetylation, phosphorylation and ubiquitination determine a rapid and efficient cell response to intra- and extracellular stimuli, they play a central role in cell signaling cascades. Recent research has identified a PTM involving the attachment of a SUMO peptide, a process frequently referred to as SUMOylation that occurs in signaling pathways both within the nucleus and other parts of the cell. SUMO attachment is thought to be involved in a number of cell processes, e.g. transcription, nuclear transport, DNA repair, mitochondrial activity, plasma membrane ion channels, cell cycle and chromatin structure. Although its function is as diverse as its substrates, modification of a protein substrate by SUMO generally alters its interactions with other protein and DNA molecules [14].

A prediction of oxidative stress theory of aging is that, among species, differential rates of aging may be apparent on the basis of intrinsic differences in oxidative damage accrual. Although widely accepted, exceptions to this theory, mostly related to the specificity of species, strain and even tissues of investigation, are occurring more frequently. Proteins are a principal target for oxidative damage and cysteine residues are highly sensitive to reversible and irreversible oxidation. To adapt and survive, cells and organisms need to sense proteotoxic insults and to protect themselves by coordinating cellular stress response pathways and chaperone networks related to protein quality control and stability. The mis-assembly or aggregation of proteins or peptides, in any cell type leads to toxic effects or proteotoxicity. Despite the abundance and apparent capacity of chaperones and other components of homeostasis to re-establish folding equilibrium, cells do not appear to adapt well to chronic proteotoxic stress which increases in cancer, metabolic and neurodegenerative diseases. The use of drugs to modulate cellular stress response pathways is emerging as a treatment for human diseases such as neurodegenerative disorders, cardiovascular disease and cancer. For medical intervention to be successful, the dose must be right, but it can be extremely difficult to achieve this goal on account of human inter-individual variation in age, gender, diet, exercise, genetic factors and health status. The past decade has witnessed considerable progress in the nature of the dose response in and adjacent to the therapeutic zones. Long-standing ideas about the nature of the dose–response in a low dose zone, have been challenged by the hormetic dose–response which could significantly influence the design of pre-clinical studies, clinical trials and optimal patient dosing strategies in the treatment of numerous diseases. The broad cytoprotective properties of the heat shock response have aroused strong interest in discovering and developing pharmacological agents that can induce stress responses, including carnitines. This paper illustrates the mechanisms by which hormetic dose responses are mediated for endogenous cellular defense pathways. These include the possible signaling mechanisms through which, by interplaying metabolism, mitochondrial energetics and activation of critical vitagenes, the carnitine system modulates signal transduction cascades that provide cytoprotection against chronic degenerative damage associated with aging and neurodegenerative disorders.

2. The mitochondrial theory of aging

Mitochondria are membrane-enclosed organelles found in eukaryotic cells where they generate ATP as a source of chemical

energy. ATP synthesis occurs through the respiratory or electron transport chain (ETC) located at the inner mitochondrial membrane, and consists of five protein complexes (Complexes I–V) [15–18].

Besides supplying ATP, mitochondria are involved in many other cell functions including the biosynthesis of heme, cholesterol and phospholipids [16] and initiation of the apoptotic process [18]. According to the endosymbiosis theory, mitochondria are organelles that evolved from purple bacteria approximately 1.5 billion years ago. Mitochondria have their own genome [17,19] and can replicate and transcribe their DNA semi-autonomously. Mitochondrial DNA (mtDNA), like nuclear DNA, is constantly exposed to DNA damaging agents. For many years it was thought, in mtDNA repair, that excessively damaged mtDNA molecules were simply degraded and replaced by newly-generated successors copied from undamaged genomes. However, findings now indicate that mitochondria possess the machinery needed to repair genome damage caused by endogenous or exogenous harmful agents. Harman [20,21] suggested that free radicals are involved in the aging process and that mitochondria-derived ROS may influence cellular aging [21]. The treatment of IMR-90 fibroblasts with *N*-tertbutyl hydroxylamine (an antioxidant recycled by the mitochondrial electron-transport chain), initially gave support to the theory of mitochondrial involvement in cellular senescence. *N*-tert-butyl hydroxylamine extends fibroblast replicative capacity and delays changes in mitochondrial function due to age by reducing ROS production, preservation of mitochondrial membrane potential and by increasing the cellular GSH/GSSG ratio [16]. A more recent study has demonstrated that mitochondria derived ROS play an important and direct role in the shortening of telomeres and the onset of senescence [16]. Other findings have proposed that mitochondrial dysfunction leads to mitochondrial biogenesis, thereby increasing the number of cell sites for the production of ROS that causes telomere shortening [15,18].

On account of its elevated mutagenic propensity, accumulation of mtDNA during life is thought to be a major cause of age-related disease. The lack of introns and protective histones, limited nucleotide excision and recombination DNA repair mechanisms, location in proximity of the inner mitochondrial membrane with exposure to an enriched free radical milieu are all factors contributing to a mutation rate that is 10-fold higher in the mtDNA than in the nuclear DNA (nDNA) [22]. Furthermore, considerable evidence suggests that mtDNA mutations increase as a function of age, reaching the highest levels in brain and muscle. It has been reported that more than twenty different types of deletions accumulate in aging human tissues. The first report on an age-related increase in a mtDNA deletion was found in elderly brain and in Parkinson's disease [22]. This deletion, called the “common deletion”, was observed between 13-bp sequence repeats beginning at nucleotides 8470 and 13447, removing almost a 5-kb region of mtDNA between ATPase 8 and the ND5 genes. The deletion takes place during replication of the mtDNA, the missing sequence encodes for six essential polypeptides of the respiratory chain and 5 tRNAs, and has been associated with several clinical diseases, such as chronic progressive external ophthalmoplegia and Kearns Sayre syndrome. In a comparison with age-matched controls, an association was found between numerous age-related disorders and higher levels of mtDNA mutations. In the central nervous system (CNS), patients with Parkinson's disease were found to have a 17 times higher level of the common deletion in the striatum, compared to age-matched controls. There is also evidence of higher levels of this deletion in patients with Alzheimer's disease, paralleling increased levels in the oxidized nucleotide 8-OH-dG [15]. More than 100 mutations of mtDNA have been associated with human diseases [23]. Phenotypic manifestation of mtDNA mutations is extremely broad ranging from oligosymptomatic patients with isolated deafness, diabetes, ophthalmoplegia, etc., to complex encephalomyopathic disorders that may include dementia, seizures, ataxia, stroke-like episode and so on. There is also a wide range of genotype variants, with

rearrangements (deletions, duplications) and point mutations affecting protein coding genes, tRNAs and rRNAs. Although there are some broad genotype/phenotype correlations, considerable overlap also occurs. Further research is needed to fully understand the pathogenetic mechanisms involved in the expression of mtDNA mutations. Recent studies have identified mutations of nuclear genes encoding subunits of the respiratory chain, particularly those of complex I. These principally involve infant onset disease with early death. Moreover recent research has shown that the function of the respiratory chain may be impaired by mutations affecting other mitochondrial proteins or as a secondary phenomenon to other intracellular biochemical derangements. An example is Friedreich ataxia where a mutation of a nuclear encoded protein (frataxin), probably involved in iron homeostasis in mitochondria, results in severe deficiency of the respiratory chain in a pattern indicative of free radical mediated damage [22,24–26].

Mutations of nuclear encoded proteins involved in cytochrome oxidase assembly and maintenance have been identified. As predicted, they are associated with severe deficiency of cytochrome oxidase and Leigh syndrome. Defects of intracellular metabolism, particularly with excess-free radical generation including nitric oxide or peroxynitrite, may cause secondary damage to the respiratory chain. This probably plays an important role in Huntington disease, motor neuron disease (amyotrophic lateral sclerosis) and Wilson disease. It is important to consider however that mutation and ROS induced DNA damage are different, and the diseases mentioned above are mostly dependent on genetics and not a causative factor of oxidative stress. Since a common pathway in the pathogenesis of these disorders appears to be defective oxidative phosphorylation, treatments designed to improve respiratory chain function may bring amelioration in the progression of these disorders [23]. These findings establish that a key feature of the aging process is the relationship between age-associated accumulation of mtDNA mutations and bioenergy dysfunction, at least in tissues predominantly composed of postmitotic cells, such as CNS and skeletal muscle.

With regard to mitochondrial bioenergetics, a significant decrease in state 3/state 4 ratio has been seen to occur in brain during aging [24]. Since this ratio relates to the coupling efficiency between electron flux through the electron transport chain and ATP production, an increase in state 4 would reduce mitochondrial complexes and consequently increase free radical species production. A decrease in state 3/state 4 respiration during aging has been associated with a significant decrease in cardiolipin content in brain mitochondria [27]. This loss may play a vital role in age-related reduction in mitochondrial function, and appears to be associated with both quantitative and qualitative region-specific protein changes such as decrease of the inner membrane surface, smaller and fewer cristae, decreased fluidity and increased fragility. It is known that functional changes in brain mitochondria are correlated with modifications in cardiolipin composition. Acetylcarnitine fed to old rats increased cardiolipin to levels found in young rats and restored protein synthesis both in the inner mitochondrial membrane and cellular oxidant/antioxidant balance, suggesting that cellular bioenergetics in aged rats may benefit from administration of this compound [28]. It is interesting to note that caloric restriction, a dietary regimen that increases life-span in rodents, maintains the levels of 18:2 acyl side chains and inhibits cardiolipin composition changes [29]. It has also been shown to retard aging associated changes in oxidative damage, mitochondrial oxidant generation and antioxidant defenses observed during aging [18,30].

Recent finding indicates that calorie restriction and specifically reduced glucose metabolism induce mitochondrial metabolism to extend life span in a number of model organisms including *S. cerevisiae*, *D. melanogaster*, *C. elegans* and possibly mice. In contrast with Harman's free radical theory of aging, these effects may be caused by an increase in ROS formation within the mitochondria leading to an adaptive response that culminates in increased stress

resistance thought to ultimately cause a long-term reduction in oxidative stress [31]. This type of retrograde response, termed mitochondrial hormesis or mitohormesis, may be involved in the health-promoting effects of physical exercise in humans and, hypothetically, impaired insulin/IGF-1-signaling in model organisms. Abrogation of this mitochondrial ROS signal by antioxidants consistently impairs the lifespan-extending and health-promoting capabilities of glucose restriction and physical exercise, respectively [32]. In short, ROS are essential signaling molecules needed to promote health and longevity. The concept of mitohormesis therefore provides a common mechanistic denominator for the physiological effects of physical exercise, reduced calorie uptake and glucose restriction [33].

3. Redox regulation of mitochondrial dynamics fusion and fission: role in cell survival

In eukaryotic cells, mitochondria are the principal source of energy (ATP and NAD⁺). Neuronal processes such as energy production, Ca²⁺ regulation, maintenance of plasma membrane potential, protein folding by chaperones, axonal and dendritic transport and the release and re-uptake of neurotransmitters at synapses all depend on mitochondrial function. Mitochondria help neurons to satisfy the high energy demand required for proper neuronal function since, unlike other cell types, they cannot switch to glycolysis when oxidative phosphorylation becomes limited. In response to changing energy demands, cells continually adjust the rate of mitochondrial fission and fusion to aid the distribution of mitochondria [18,30]. Mitochondria also produce large amounts of superoxide anion radical by abstracting an electron from oxygen in the so-called electron transport chain at the inner mitochondrial membrane [15]. Mitochondrial respiration accounts for about 90% of the oxygen consumed in the cell. Most of this is reduced to water through four consecutive one-electron reductions in which a small proportion of the oxygen molecules (1–2%) is converted to superoxide anion radicals. Complexes I and III are recognized the main sites of superoxide production in the respiratory chain [15] and recent studies have detected up to nine submitochondrial reactive oxygen species (ROS) generating sites [16]. The resulting superoxide then dismutates either spontaneously or enzymatically due to superoxide dismutase to form hydrogen peroxide [17] which may then diffuse through the cell and decompose to form harmful hydroxyl radicals, thus damaging the cell through interactions with macromolecules. These processes cause mitochondria to become a major source of physiological or endogenous ROS production [18]. Many studies have focused on the harmful effects of ROS, but it is now clear that mitochondrially generated ROS are also involved in regulating intracellular signal transduction pathways that result in cell activities such as proliferation [18]. Mitochondria can produce superoxide in relatively constant amounts, or elicit spontaneous or environmentally-induced “superoxide flashes” [18] and it is generally assumed that the main sites of superoxide production in the mitochondrial respiratory chain are complexes I and III [18]. Consequently any impairment in the electron transfer process causes a reduction in upstream carriers, thus filling Q cycle electron pool. The cytochrome (cyt)2 bc1 complex (EC 1.10.2.2) (cyt bc1 complex), situated 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. 1). 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 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 [34]. Three enzymatic binding sites participate in catalysis on the cyt bc1 complex, the quinol oxidase (Qo) site, quinol reductase (Qi) site, and a docking site for soluble cyt c on cyt c1.

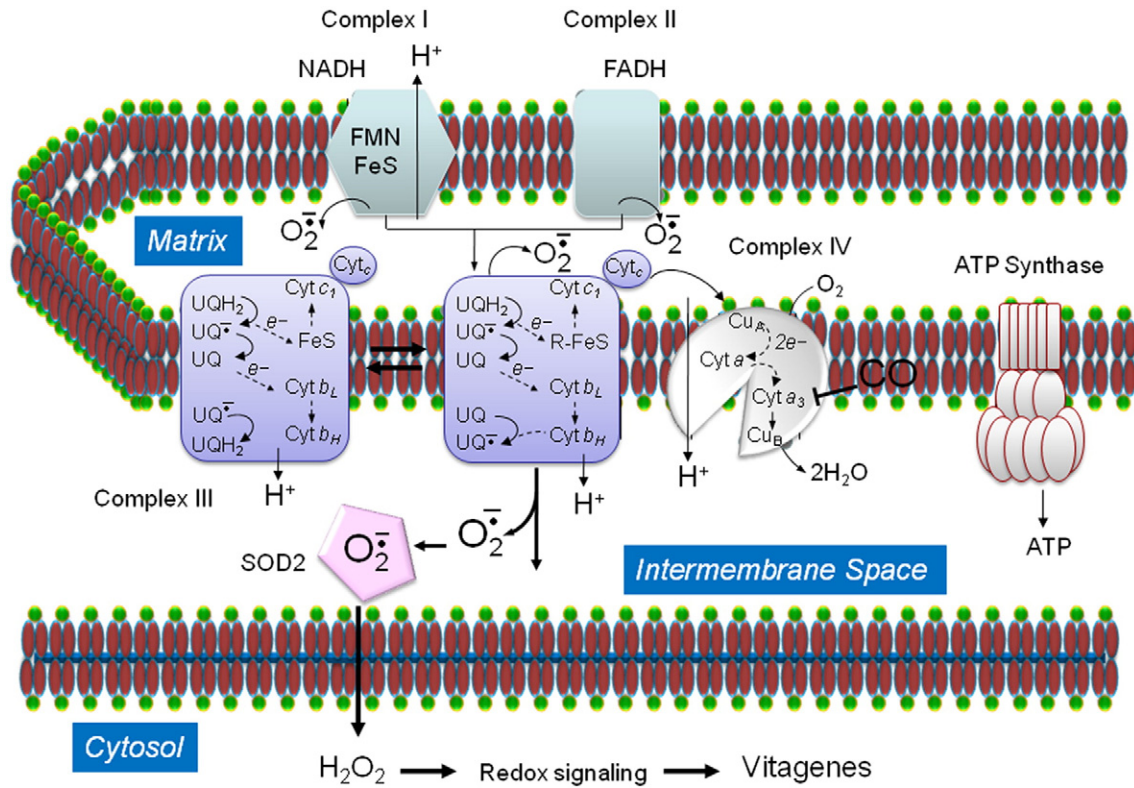


Fig. 1. The ubiquinone (Q) cycle. The ubiquinone (Q) cycle is initiated when one electron from ubiquinol (QH₂) is donated to the Rieske-iron sulfur (R-FeS) protein and the second electron is donated to cytochrome b. The intermediate moiety is the free radical ubisemiquinone (Q_o), which can donate electrons 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 pathway of vitagenes network, which results in preconditioning.

The Q_o site is positioned toward the positively charged side of the membrane, where protons are released during enzyme turnover. The Q_i site is located toward the negatively charged membrane surface, 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. A key reaction in the Q-cycle may be the “bifurcated” electron transfer at the Q_o site, but the exact mechanism has not yet been clarified [34]. 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, while 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 Q_i site. Two turnovers of the Q_o site are required to reduce a Q_i site Q to a QH₂. Under some conditions, the Q-cycle can be short-circuited by various “bypass” reactions, some of which yield the superoxide that is physiologically harmful [18]. The bypass reactions are typically observed *in vitro* under “partially inhibited” conditions, e.g. in the presence of antimycin A, or under high proton motive force, where the (2Fe2S) cluster may oxidize QH₂ to a semiquinone (SQ), but processing of electrons by the low potential chain is impeded, thus generating the accumulation of a SQ intermediate, which in turn can reduce O₂ to superoxide [18].

The electron transport chain consumes as much as 90% of the oxygen taken up by a cell, of which ~1% is transformed into superoxide under normal physiological conditions [35]. The rate of mitochondrial electron transfer from semi-reduced Q to molecular O₂ to form superoxide is proportional to the product of the semi-reduced Q and O₂ concentrations. Estimates indicate that under pathophysiological conditions, superoxide production occurs at 2–10% of the uninhibited rates. Superoxide dismutases, in turn, generate the more stable

hydrogen peroxide before it is transformed into water by catalase or, if metals are present, into highly reactive hydroxyl radicals. Thus, SOD2, which directs H₂O₂ out of the mitochondrion, performs two functions: the classical scavenging of superoxide and its signalization as ROS-dependent signaling pathways. The binding of CO to cytochromes found in complex IV (i.e., cytochrome a₃) enhances reduction in the respiratory carriers in the cytochrome bc1 region of complex III, associated with increased leakage of superoxide and H₂O₂ production.

Much of the superoxide produced is efficiently converted to hydrogen peroxide by the activity of superoxide dismutases that include the cytoplasmic (SOD1) and mitochondrial (SOD2) enzymatic forms. As the superoxide is a highly reactive free radical, it can damage molecules (DNA, proteins and lipids). Its conversion to hydrogen peroxide therefore protects cells, but if Fe²⁺ or Cu⁺ are present even in very low concentrations, this peroxide can generate hydroxyl radical that induces membrane lipid peroxidation [36]. Moreover, nitric oxide (generated in response to activation of the enzyme nitric oxide synthase by Ca²⁺/calmodulin) can interact with mitochondrial superoxide to produce the highly reactive free radical peroxynitrite [37]. Antibodies that selectively recognize proteins modified by the lipid peroxidation product 4-hydroxynonenal and by nitration have been particularly useful instruments for detecting and quantifying oxidative damage to proteins, lipids and DNA [38]. In addition, researchers have made use of fluorescent probes for imaging relative levels of overall mitochondrial redox status and superoxide in order to define the role of mitochondrial ROS in a number of physiological and pathological processes [39]. An excessive ROS production is thought to contribute to damaging effects in a range of diseases such as cancers, cardiovascular disease and inflammatory conditions such as arthritis [40]. Neurons may be particularly vulnerable to mitochondrial ROS on account of their high energy demands, excitability, and the fact that they are postmitotic and therefore irreplaceable in most cases [64]. On the contrary, lower subtoxic

levels of mitochondrial ROS can activate signaling pathways that protect cells against injury and disease [18,38].

New findings suggest that modifications in mitochondrial activity can induce morphological adaptations of the mitochondrial network. Moreover, clinical studies indicate that molecular defects affecting mitochondrial dynamics bring about pathology. This suggests that there is a link between energy status and organellar network configuration. In this context, a balance between fusion and fission events, mediated by specific proteins that might be involved in the modulation of energy production, occurs controlling mitochondrial configuration [41]. Physiologically, mitochondria undergo continuous fission and fusion (known as mitochondrial dynamics) to generate smaller organelles or rather elongated structures, respectively (Fig. 2). During this process of mitochondrial biogenesis, in which new mitochondria are formed, defective mitochondrial DNA is repaired by redistributing mitochondria to high energy demand sites. In neurons, the fission/fusion proteins, regulated by a mechanism involving large dynamin-related GTPases, maintain mitochondrial integrity and ensure that they are present at critical sites such as the synapse where high concentrations of ATP are required [42]. These proteins include Drp1 and Fis1 that act as fission proteins, and Mitofusin (Mfn) and Opa1 that have the function of fusion proteins. Since mitochondria play a critical role in neurons, impaired mitochondrial dynamics is increasingly considered to be a factor in neurodegenerative disorders such as Parkinson's, Alzheimer's and Huntington's diseases [43].

Dysfunction in mitochondrial dynamics may be due either to genetic mutations in fission- or fusion-related genes or to post-translational changes in the fission or fusion proteins. Hereditary mutations in the mitochondrial fusion GTPases optic atrophy-1 (OPA1) and mitofusin-2 (Mfn2) occur in Charcot-Marie-Tooth Disease (CMT), a peripheral neuropathy characterized by muscle weakness and axonal degradation of sensory and motor neurons. It is also observed in hereditary motor and sensory neuropathy type VI, clinically similar to CMT, with the addition of optic atrophy and visual impairment, as well as in Autosomal Dominant Optic Atrophy. In addition, two proteins, PTEN-induced kinase 1 (PINK1) and Parkin, which are mutant in familial forms of PD have been reported in association with increased mitochondrial fission in Parkinson's disease (PD) models. Furthermore, mutant huntingtin, the disease-causing protein in Huntington's disease (HD), alters mitochondrial morphology and dynamics [43]. Rotenone, a pesticide and inducer of PD symptoms, and amyloid- β (A β) peptide, which has been causally linked to Alzheimer's disease (AD), are able to initiate mitochondrial fission [42].

Post-translational modifications due to nitrosative/oxidative stress may be the pathogenic cause of common sporadic cases of neurodegeneration, which seem to stem from intricate interactions of multiple genes and/or their products with environmental factors. According to Nakamura et al., three post-translational modifications – phosphorylation, sumoylation and ubiquitination – appear to regulate mitochondrial fission and fusion proteins [42]. Protein Kinase A (PKA, cAMP-dependent protein kinase) phosphorylates Drp1, which results in a significant decrease in GTPase activity and inhibition of mitochondrial fission. Interestingly, mitosis-promoting factor (MPF, Cdk1/cyclin B) phosphorylates Drp1 at Ser585 in rats during mitosis. Unlike phosphorylation by PKA, Cdk1/cyclin B phosphorylation seems to stimulate mitochondrial fission during mitosis. However, phosphorylation of Drp1 at different amino acids causes opposite effects [44,45]. Drp1 regulation also occurs through sumoylation. SUMO1 (small ubiquitin-related modifier-1) interacts with Drp1 and associates with mitochondria at fission sites before and after fission [45]. Over expression of SUMO1 results in mitochondrial fragmentation and apoptosis. This probably occurs because SUMO1 protects Drp1 from degradation, stabilizing Drp1 and promoting binding to mitochondria. Ubiquitination has also been associated with mitochondrial dynamics [45] since MARCH-V or MARCH5, a mitochondrial outer

membrane protein, ubiquitinates Drp1, thus promoting fission. Finally, the functional link between mitochondrial dynamics and apoptosis is suggested by the fact that Bax and Bak, two pro-apoptotic regulators, interact with mitochondrial fission and fusion GTPases. Bax associates with Drp1 and Mfn2 on mitochondria during apoptosis. Furthermore, neurons under nitrosative stress exhibit Bax foci on mitochondria undergoing mitochondrial fission. Inhibition of Drp1 function delays mitochondrial fission and is related to Bax foci formation and neuronal cell death [45].

Another potentially harmful effect of increased ROS levels is chronic mitochondrial fission. In an animal model of stroke, nitrosative stress causes profound mitochondrial fission in neurons prior to the onset of neuronal loss and treatment with antioxidants has been shown to reduce mitochondrial fission [45]. Amyloid-beta peptide, possibly a key mediator of AD pathogenesis, engenders S-nitrosylation and thus hyperactivation of the mitochondrial fission protein Drp1. This activation results in excessive mitochondrial fragmentation, bioenergetic compromise, and synaptic damage in models of AD [42]. Expression of Mfn or dominant-negative Drp1 partially prevented mitochondrial fission and neuronal cell death by nitric oxide, which is known to be largely caspase independent. Another study demonstrated that mitochondrial fission in cerebellar granule neurons was enhanced by oxidative stress and that Mfn2 expression was protective [45]. Whether oxidative stress directly regulates the mitochondrial fission and fusion GTPases is not yet clear. However, since mitochondrial integrity is essential to cellular homeostasis, when the energetic demand is low, excess mitochondria are not needed and can generate excessive ROS [46]. Furthermore, if uncoupled, they can consume ATP, thus their elimination by autophagy is an efficient cytoprotective response. Uncoupled or unstable mitochondria may release ROS and a number of apoptosis-promoting factors, including cytochrome c, AIF, SMAC/DIABLO, thus promoting damage to neighboring mitochondria [47].

Mitochondrial biogenesis is another important event in mitochondrial biology. It is a phenomenon that relies on a space and time coordinated synthesis and the import of approx. 1000 nuclear-encoded proteins, some of which are assembled with mitochondrial-encoded proteins within newly synthesized inner and outer mitochondrial phospholipid membranes. Both replication of mitochondrial DNA and mitochondrial fusion and fission mechanisms must also be synchronized with these processes [48]. Oral acetylcarnitine supplementation increases mitochondrial content together with nuclear transcripts of factors involved in mitochondrial biogenesis (PGC-1 α , NRF-1, TFAM). This event is associated with increased levels of mitochondrial transcripts for COX I, ATP6 and ND6, and also 16S rRNA. This is consistent with the notion that peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α) positively modulates several genes involved in ROS detoxification and in turn PGC-1 α is sensitive to ROS and/or RNS [49]. Acetylcarnitine also by stimulating the vitagen system, especially HO-1, leads to the production of carbon monoxide which, according to the model proposed by Piantadosi [50], can bind to the reduced heme a3 in the cytochrome c oxidase complex, thus promoting a parallel increase in superoxide formation from complex III. Superoxide then induces MnSOD overexpression with formation of hydrogen peroxide and signaling of so-called preconditioning. As signaling molecule H₂O₂ activates protein kinase B (Akt), which deactivates glycogen synthase kinase-3 β , allowing the nuclear translocation of Nrf2. Nuclear Nrf2, which binds to antioxidant response elements in the HO-1, MnSOD, and NRF-1 gene promoters, amplifies the initial signal and drives the transcription of TFAM and other genes that have promoter binding sites for NRF-1 [18,37,38,51,52]. These genes play a role in the control of mitochondrial transcription, protein synthesis, mitochondrial protein import, and oxidative phosphorylation [52]. This was confirmed by research demonstrating that acetylcarnitine reversed the age-related decrease in complex III activity and oxidative phosphorylation through complexes III and IV, and increased the amount of

cytochrome b and aa3 hemes in mitochondria isolated from old rats [48]. Of note is the fact that both cytochrome b and aa3 proteins are encoded by the mitochondrial genome, suggesting that acetylcarnitine promotes either mtDNA transcription, the stability of mitochondrial mRNA, or mitochondrial protein synthesis [51].

4. Autophagy pathway

Autophagy is a lysosomal pathway for the degradation and recycling of long-lived proteins and organelles, i.e. a kind of ‘self digestion’ cell pathway. During autophagy, cytoplasmic constituents are taken into double-membraned autophagosomes and delivered to lysosomes to be degraded. This process generates nucleotides, amino acids and fatty acids, which are recycled to be used for ATP generation and macromolecular synthesis [53] (Fig. 3).

While the unfolding protein response (UPR) regulates the degradation of smaller units of unfolded or misfolded proteins through chaperone transcription, larger aggregates are detoxified via autophagy. Autophagy involves the encapsulation of cargo on a double-membrane vesicle to form the autophagosome. Autophagosomes then fuse with lysosomes to form the autolysosome where the cargo degradation occurs. Hybrid intermediates, known as amphisomes, are also formed when autophagosomes fuse with endosomes or MVEs, before fusing with lysosomes. In eukaryotic cells, the ubiquitin–proteasome and autophagy–lysosome pathways represent the two principal routes of protein clearance [53]. Proteasomes mainly degrade short-lived nuclear and cytosolic proteins. However, substrates must be unfolded in order to pass through the narrow pore of the proteasome barrel, so clearance of aggregated proteins observed in many neurological disorders is excluded [54]. On the contrary, lysosomal-related compartments are able to degrade substrates such as protein complexes and

organelles. Recent evidence indicates that protein clearance via autophagy is linked to their propensity to aggregate. Since findings suggest that a number of different diseases may be caused by aggregate-prone proteins gaining function mechanisms, attempts have been made to find therapeutic strategies that reduce the levels of this type of proteins, at either the synthesis or degradation stage [54]. Recent studies suggest that autophagy has a crucial role in protein quality control, and a clear function in balancing homeostasis in the nervous system [55]. Notably, p62 (also termed sequestosome 1) interplays between polyubiquitination and the autophagy-degradative mechanisms, thus shuttling substrates of the proteasome system. Autophagy is less selective than the latter mechanism, but recent findings indicate that it is finely regulated and specific for diverse target organelles, such as mitochondria (mitophagy) endoplasmic reticulum (reticulophagy), peroxisomes (pexophagy), ribosomes (ribophagy), granules (crinophagy), and pathogens (xenophagy). Cytosol, cytoskeleton, nuclei (nucleophagy), and protein aggregates (aggrephagy) can also be removed by autophagy.

Studies have defined several upstream regulators of autophagy [53]. The class III PI3 kinase complex, including Beclin-1/Atg6, is involved in the generation of pre-autophagosome structures. The mammalian target of rapamycin (mTOR), a nutrient-sensing kinase complex that controls cell growth and survival, prevents autophagy during nutrient-rich conditions by inhibiting the Atg1 complex, which is present in the initiation stages of autophagic activity. Upstream activators of mTOR (NF- κ B, class I PI3 kinase, Akt and NF- κ B) block autophagy, whereas inhibitors of this pathway such as PTEN (phosphatase and tensin homologue) induce autophagy. For this reason, *in vivo* experiments have shown that rapamycin, a classical mTOR inhibitor, alleviates neurodegeneration in models of Huntington’s disease, associated with reduced neuronal loss, improved survival and degradation of toxic protein aggregates [55]. Atg

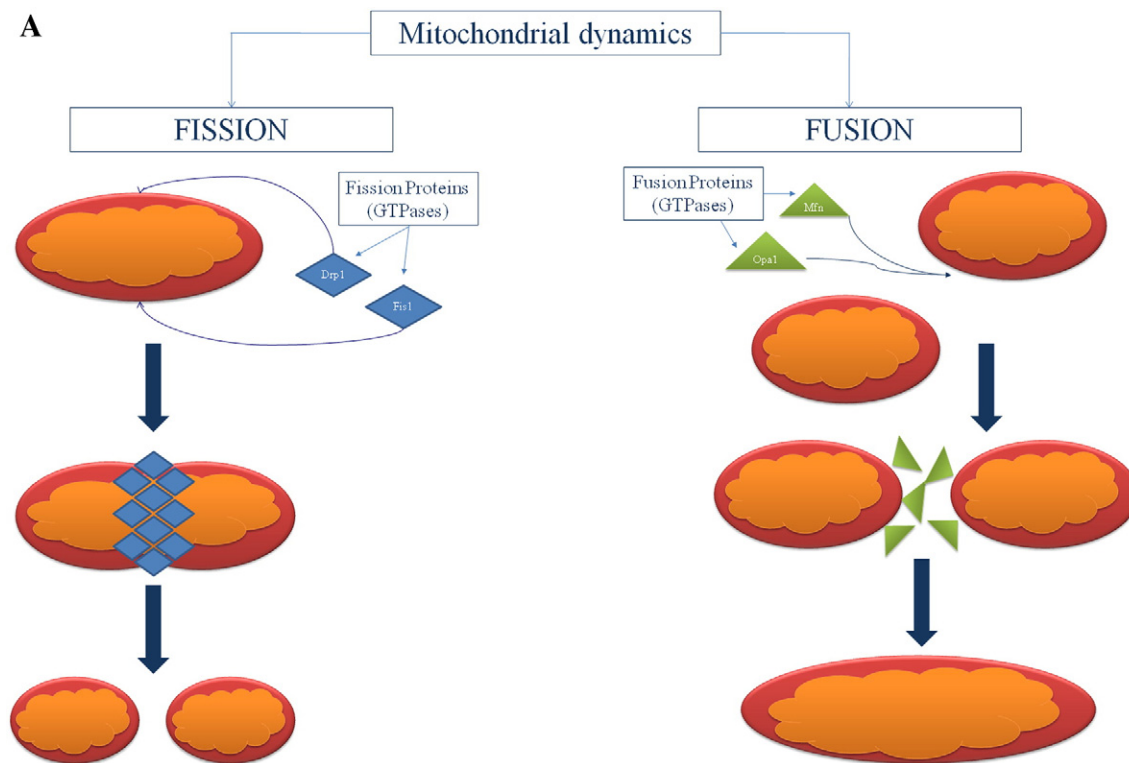


Fig. 2. Mitochondrial dynamics. Role of fission (Drp1 and Fis1) and fusion (Mfn and Opa1) proteins in the mitochondrial dynamics (A). Post-translational modifications of fission and fusion proteins (B). The phosphorylation of dynamin-related protein (Drp1) by the Protein Kinase A (PKA) inhibits mitochondrial fission decreasing GTPase activity. On the contrary, the phosphorylation of Drp1 by mitosis-promoting factor seems to stimulate mitochondrial fission during mitosis. Moreover, the activity of Drp1 is regulated by the mechanism of sumoylation. The small ubiquitin-related modifier-1 (SUMO1) interacts with Drp1 at fission sites and its overexpression leads to mitochondrial fragmentation and apoptosis. Mitochondrial fission is promoted also by the ubiquitination of Drp1 by the human membrane-associated RING-CH (MARCH)-V. Finally, there is evidence of a functional link between mitochondrial dynamics and apoptosis. In fact, the Bcl-2-associated X protein (Bax) associates with Drp1 and mitofusin (Mfn2) on mitochondria during apoptosis and neurons under nitrosative stress exhibit Bax foci on mitochondria undergoing mitochondrial fission.

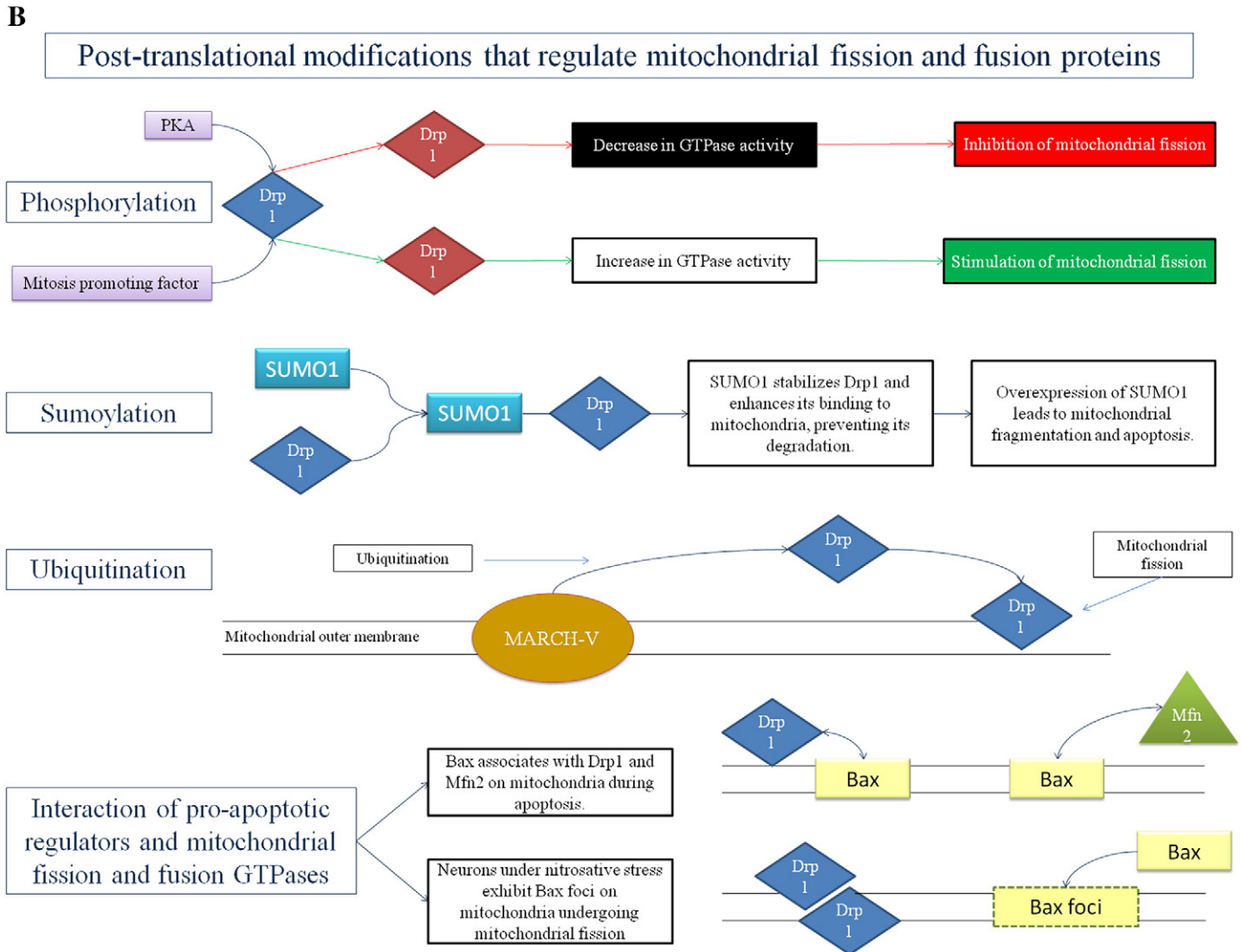


Fig. 2 (continued).

proteins regulate sequential steps in the autophagy process, centrally organized around two ubiquitin-like conjugation systems. When Atg12 is activated, it forms a covalent bond with Atg5, which then interacts with other components to form a multimeric complex that translocates to membranes of early autophagosomes. Alternatively, LC3 is cleaved and then conjugated to phosphatidylethanolamine (PE) via Atg7 and Atg3 (another ubiquitin carrier protein (E2)-like protein), generating LC3-II. The unconjugated LC3-I remains in the cytosol while the conjugated LC3-II form targets to the autophagosomal membrane after the formation of the active Atg12–Atg5 complex [56]. Following elongation of the autophagosomal membrane, cytoplasmic proteins and organelles are taken into double-membraned autophagosomes, delivered to lysosomes and degraded [53]. Autophagy is essential for maintaining neuronal homeostasis and plays a role in basal elimination of misfolded proteins in the nervous system. Brain specific ablation of the autophagy-related genes *atg5* and *atg7* results in spontaneous neurodegeneration typical of Alzheimer's and Parkinson's disease with pathological features such as neurological/motor dysfunction, accumulation of poly-ubiquitinated protein aggregates, neuronal loss and premature death [54]. As in UPR, autophagy is believed to play a dual role in cell survival or cell death. Autophagic degradation of cellular materials produces amino acids and fatty acids, which can be recycled for protein synthesis and ATP generation during stressful conditions such as starvation. It can also remove protein aggregates (which can trigger apoptosis) and damaged mitochondria (source of

apoptotic proteins and toxic ROS), thus becoming a protective mechanism. On the contrary, prolonged autophagy can lead to cell death through excessive self-digestion or activation of apoptosis. In fact, there is evidence of prosurvival autophagy in response to starvation, growth factor withdrawal, ischemia/reperfusion injury, and various chemotherapeutic drugs [57,58], while autophagic cell death has been reported in response to hypoxia, oxidative stress, radiation, GF withdrawal, lipopolysaccharide, overexpression of smARF, and various chemotherapeutic drugs [57].

5. NF- κ B signal pathway: cell death vs cell survival

Nuclear factor- κ B (NF- κ B) is a family of transcription factors that have a crucial role in inflammation, immunity, cell proliferation, development, survival and apoptosis. Consequently, NF- κ B dysregulation has been implicated in diverse human pathologies ranging from autoimmune diseases to cancers.

In mammalian cells, the NF- κ B/Rel family contains five members: RelA (p65), c-Rel, RelB, NF- κ B (p50; p105), and NF- κ B2 (p52; p100) [58]. These proteins possess a structurally conserved 300 amino acid sequence called the REL region, which contains the dimerization, nuclear localization, and DNA-binding domains. Three of the family members, RelA, c-Rel, and RelB, have a transactivation domain at the C-terminus. NF- κ B1/p105 and NF- κ B2/p100 are the inactive precursors of the p50 and p52 proteins, respectively; in an unstimulated

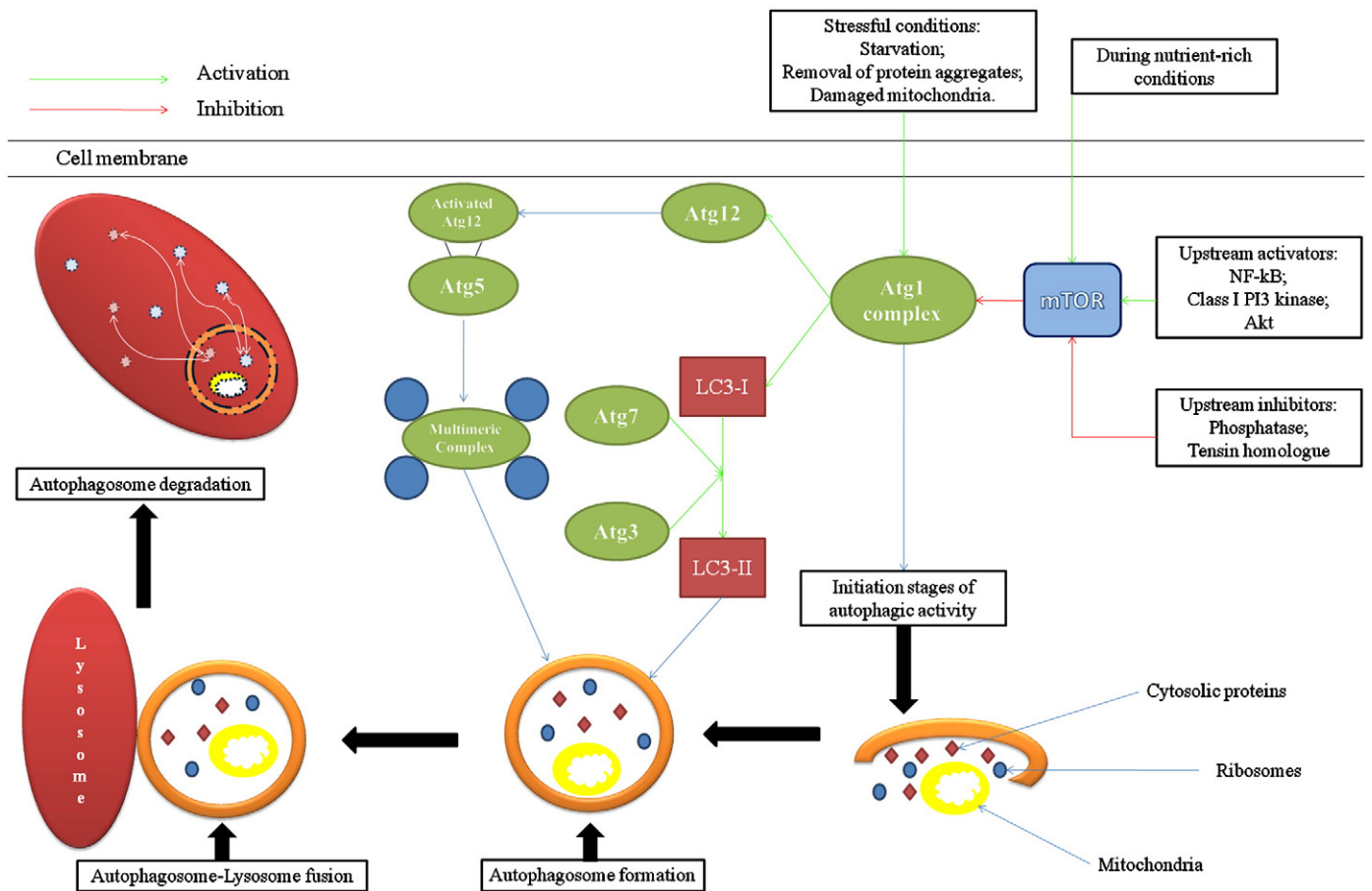


Fig. 3. Autophagy. Autophagy pathway: Role of the Atg proteins in the initiation stages of autophagic activity (Atg1 Complex) and autophagosome formation (Atg12 and LC3) and subsequent autophagosome-lysosome fusion and autophagosome degradation.

state, these proteins are localized to the cytoplasm. Proteolytic processing removes the C terminal inhibitory domains, allowing the resulting proteins to enter the nucleus [59].

NF- κ B is induced by a variety of extra-cellular stimuli such as pro-inflammatory cytokines, bacterial lipopolysaccharides, viral RNA and DNA via the activation of membrane and cytosolic receptors [60,61]. It is also induced by low and high doses of ionizing radiation and numerous DNA damaging drugs [62,63]. Under normal conditions, NF- κ B is present within the cytoplasm in an inactive state, bound to its inhibitory protein κ B- α (I κ B- α). This stimulation initiates an intracellular signaling cascade, resulting in the phosphorylation of I κ B- α on serine residues 32 and 36 by I κ B kinase (IKK). Phosphorylation and subsequent ubiquitination of these residues target I κ B- α for degradation by the 26S-proteasome complex. Once liberated from its inhibitory protein, NF- κ B translocates into the nucleus, where it orchestrates the transcription of a number of cytokines and chemokines, growth regulatory and survival genes [64,65].

NF- κ B has been shown to activate, via transcription, the genes encoding pro-inflammatory cytokines [tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-12], cell adhesion molecules [vascular cell adhesion molecule (VCAM)-1 and intercellular cell adhesion molecule (ICAM)-1], inducible nitric oxide synthase (iNOS), cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) [66,67]. These, together with nitric oxide (NO) derived from iNOS and prostaglandin E₂ (PGE₂) produced by both COX-1 and COX-2, play important roles in the pathogenesis of acute and chronic inflammation [68]. The production of reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxide and hydroxyl radicals, as well as peroxynitrite, also contribute to the tissue injury observed during inflammation [68,69]. ROS and peroxynitrite also cause DNA

damage [70,71], which results in the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP), depletion of NAD⁺ and ATP and ultimately cell death [70].

It should be noted that pathogenic stimuli like excessive production of toxic reactive oxygen and nitrogen species, e.g., the oxidative stress and nitrosative stresses, and many genotoxic factors activate nuclear factor kappa beta (NF κ B) signaling pathways which in turn stimulates the activation of aging-related genes [72]. The NF κ B trigger genes that block the cell death (by apoptosis or necrosis) resulting in aging of the immune system or “immunosenescence”, muscle atrophy, and inflammation [73].

Interestingly, the master of inflammation, NF- κ B signaling, is also a key player in anti-apoptotic signaling [74,75]. For instance, NF- κ B signaling activates the expression of c-FLIP, Bcl-xL, c-IAP1, c-IAP2, and XIAP proteins, all well-known inhibitors of apoptosis (IAPs). A few studies have focused on the effect of aging on the expression of IAP proteins, e.g.-chromosome-linked IAP (XIAP) level has been shown to increase in the brain during aging [76].

Additionally, there is considerable interest in the contribution of NF- κ B-mediated chronic inflammation in aging. The age-related “constitutive activation of NF- κ B” has been verified in various tissues during aging [77]. In mice and rats, NF- κ B activity has consistently been shown to be increased with age and in a variety of tissues, including heart, liver, kidney, and brain [78]. In humans, NF- κ B protein concentrations were found to be fourfold higher in elderly human muscles compared with those of young people [79].

Interestingly, the function of the NF- κ B complex can be regulated by the acetylation and phosphorylation of the p65 component [80]. The acetylation of p65 transactivator protein especially increases the duration and efficiency of the NF- κ B transcriptional response and

hence the abundance of target gene mRNA transcripts [81]. Histone deacetylases, such as HDAC3 and SIRT1, can regulate the acetylation level of p65 protein and reduce the efficiency of NF- κ B-dependent signaling [82]. The observations of Adler et al. [83] indicate that this kind of posttranscriptional NF- κ B activation occurs during aging.

SIRT1 is the most-widely studied of the Sirtuins and is known to regulate the function of several important transcription factors, such as p53, FoxOs and NF- κ B, and is also a mammalian longevity gene [82]. Yeung et al. [82] were the first to show that SIRT1 can interact with RelA/p65 protein in the NF- κ B complex and specifically deacetylates lysine 310, which has been earlier demonstrated to potentiate the transactivation capacity of the NF- κ B complex [81]. Several studies have indicated that SIRT1 is a potent inhibitor of NF- κ B transcription [82]. The signaling link between SIRT1 and NF- κ B is especially interesting with respect to aging since according to the articles of Yeung et al. [82] and Adler et al. [83]. It seems that SIRT1 acts to extend the lifespan by inhibiting NF- κ B signaling. Accordingly, it is proposed that the signaling cascades mediated via Sirtuins and FoxO represent the life-span extending, anti-aging type of regulation. Effects of sirtuin overexpression on aging, however, have been recently re-examined after standardization of genetic background and the use of appropriate controls both *C. elegans* and *Drosophila* suggesting that although a role for sirtuins in determination of metazoan lifespan cannot be ruled out, but doubt on the robustness of the previously reported effects of sirtuins on lifespan in *C. elegans* and *Drosophila* are questioned [84,85].

6. Heat shock proteins and neuroprotection

Heat shock response (HSR), in which a set of heat shock proteins (Hsps) are induced and take a key role in cell repair and protective mechanisms, represents one possible type of cell stress response [86,87]. This response is regulated at the transcriptional, translational and post-translational levels by a family of heat shock transcription factors (HSFs) that are expressed and maintained in an inactive state under non-stress conditions. Of the three functionally different HSFs in humans, HSF type 1 (HSF1) is the principal regulator of the HSR genes. Its task is to mediate signaling of stress-induced stimuli, such as elevated temperatures, as well as those involved in development and many patho-physiological conditions such as cancer, ischemia-reperfusion injury, diabetes, and aging [88,89]. 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 [90]. Upon exposure to heat shock and other types of stresses, which result in 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 [91]). 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 monomeric state [92]. Recent studies have also shown that inducible acetylation negatively regulates DNA binding activity [86,93]. Mammalian genomes encode three homologues of HSF (HSF1, HSF2 and HSF4) regulating HSP expression. HSF1 is believed to be the paralog responsible for regulating the heat-induced transcriptional response [86,93], while HSF2 has also been reported to contribute to inducible expression of heat shock genes through interplay with HSF1 [87]. However, regulation of the mammalian HSR is a more complex phenomenon than was previously thought. Microarray and chromatin immunoprecipitation analysis, while confirming

that many known heat-inducible genes have HSF1-binding sites in their promoters, have identified another class of genes that recruit HSF1 to their HSE promoter elements but are not induced by heat shock [88]. However, these studies have also proven that, in some cases, HSF1 can also control the expression of genes with non-chaperone function [94], such as superoxide dismutase, multiple-drug resistance genes, lactate dehydrogenase, and the T-cell death associated gene 51 [94]. Neurons appear to be deficient in the heat shock response while retaining the ability to express such HSF proteins [87]. Furthermore, activation of HSF1 does not occur in motor neurons even when microinjected with plasmids encoding an HSF1 expression vector, suggesting that the HSF1 signal transduction pathways are blocked in these cells [95]. Under non-stress conditions, HSF1 is repressed by a complex containing Hsp90 and other proteins. In this inactive state, HSF1 is a monomer that is unable to bind cis-acting heat shock elements (HSE) in the promoters of HSP genes [96]. During protein stress and the consequent generation of misfolded proteins which displace HSF1 from the inhibitory chaperone complex, HSF1 trimerizes, becomes 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 [88,94]. Activation of HSF1 by heat shock is a multi-step process, involving multiple inducible phosphorylation, dephosphorylation, acetylation, deacetylation and sumoylation, which together result in the transcription of HSP genes. 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 [96]. Akt regulates HSF1 at least in part through modulating its association with the phosphoserine binding scaffold protein [96].

The inducible expression of heat shock proteins is triggered not only by thermal stress, but also by environmental redox changes or exposure to electrophiles which cause trimerization and DNA binding of HSF1 [89,92], demonstrating that the cysteine redox state is critical for the activation of this transcription factor. Thus, an intermolecular disulfide bond formation between C36 and C103 within HSF1 induces trimerization and DNA binding, whereas an intramolecular disulfide bond formation (in which C153, C373 and C378 participate) inhibits the activity of the transcription factor [97]. Strategies for preventing damage to brain cells following cerebral ischemia or the progression of neurodegenerative diseases might be found in the manipulation of HSR [92]. Hsps are evolutionarily conserved and exist in all cell compartments [98,99]. Some of the major chaperones (Hsc70, Hsp90, small Hsps) are present at high concentrations in non-stressed cells reaching 1–5% of total cellular protein, indicating an important role for chaperones in cellular homeostasis. Hsps are classified according to their molecular weight [100]. Hsp70, the 70 kDa family of stress protein, has been extensively studied and includes Hsc70 (heat shock cognate, the constitutive form), Hsp72 (the inducible form, also referred to as Hsp72) and GRP-75 (a constitutively expressed glucose-regulated protein found in the endoplasmic reticulum) [101]. Hsp70s, which are involved in co- and post-translational folding and the quality control of misfolded proteins [102], participate in the folding and assembly of newly synthesized proteins into macromolecular complexes; aggregation prevention; dissolution and refolding of aggregated proteins; as well as protein degradation [103–106]. Recent findings demonstrate that the heat shock response decreases in aging cells and weakens when the life of organisms is prolonged beyond the mature adult stage [107]. Cells are no longer able to activate the transcriptional pathways leading to HSP synthesis. A decline in protein quality control in neuronal tissues has been strongly hypothesized, as the etiology of a number of diseases involve aggregation-prone proteins that form inclusion bodies known to be linked to pathology. Hsp70 has been extensively associated with the pathogenesis of misfolding disease [96]. The use of transgenic animals and gene transfer allowing over-expression of the gene encoding for

Hsp70, have demonstrated that overproduction of this protein leads to protection in several different models of nervous system pathology [22]. Overexpression of Hsp70 and/or its co-chaperones suppresses huntingtin aggregation and toxicity in yeast and mammalian cell models of misfolding disease [105]. Increased Hsp70 levels led respectively to reduced aggregation and toxicity of tau and A β – two components associated with Alzheimer's disease [101]. Similarly, overexpression of Hsp70 reduces toxicity and accumulation of α -synuclein in high molecular weight and detergent-insoluble deposits [101]. Increased expression of Hsp70 has been linked to a reduction in apoptotic cell death, an increase in the expression of the antiapoptotic protein Bcl-2, a suppression of microglial/monocyte activation, and a reduction in matrix metalloproteinases. Up-regulation of Hsp70 likewise reduced apoptosis and the formation of co-aggregates of the prion disease protein, PrP [107]. In cases of focal cerebral ischemia, Hsp70 mRNA is synthesized in most ischemic cells except in areas blood flow is very low, 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 [108–110]. This neuronal expression of Hsp70 outside an infarct might be used to define the ischemic penumbras, 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 ischemia, it has been demonstrated that overexpression of Hsp70 reduces the overall lesion size and also limits the tissue damage within the lesion [110]. Numerous findings now point to a correlation between nitrosative stress mechanisms and Hsp induction. *In vitro* and *in vivo* experiments have shown that cytokine-induced nitrosative stress is associated with an increase in the synthesis of Hsp70 stress proteins in the brain [111–113]. The molecular mechanisms that control NO-induced activation of the heat-shock signal seem to be associated with cellular oxidant/antioxidant balance, maintained principally by the glutathione status and antioxidant enzymes [61,114–116].

7. Heme oxygenases

Heme oxygenases (HO) are known to be dynamic sensors of cell oxidative stress and modulators of redox homeostasis throughout the phylogenetic spectrum. Located within the endoplasmic reticulum, they act together with NADPH cytochrome P450 reductase to oxidize heme to biliverdin, free ferrous iron and carbon monoxide (CO). Biliverdin reductase further catabolizes biliverdin to the bile pigment, bilirubin [38,107,117,118], a linear tetrapyrrole which has been shown to effectively counteract nitrosative stress due to its interaction with NO and RNS [37,38,51,119]. Bilirubin is then conjugated with glucuronic acid and excreted [120]. In the past two decades, much experimental evidence has demonstrated that bilirubin is an endogenous cytoprotective molecule. In 1987, Stocker and colleagues were the first to report the antioxidant activity of bilirubin when they proposed bilirubin as a chain-breaker because it was able to scavenge peroxy radicals, transforming into a stable carbon-centered radical [121]. Later, after observing that bilirubin, in the nanomolar range, may protect cortical neurons from the toxicity elicited by ~10,000 times higher levels of hydrogen peroxide, Snyder and colleagues [122], proposed a 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, the importance of this mechanism of action remains controversial since only a small fraction of the bile pigments undergo this redox cycle. Other studies have shown that bilirubin acts as an endogenous scavenger for both NO and RNS, which possibly alter the redox status of the cell and generate nitrosative stress [123]. Although bilirubin possesses these important antioxidant properties, if produced in excess (e.g. in haemolytic

anemia or sepsis), unconjugated bilirubin becomes neurotoxic on account of multiple mechanisms involving disruption of the cell membrane structure, reduction of mitochondrial transmembrane potential and activation of the apoptotic cascade.

Although HO-1 and HO-2 have the same substrate and cofactor characteristics, the isoforms are encoded by distinct genes, which share only 43% amino acid sequence homology in humans, and differ substantially in molecular weight, electrophoretic mobility, tissue distribution, regulation, and antigenicity [120]. Several stimuli associated with oxidative and/or nitrosative stress, such as, dopamine analogs, H₂O₂, hyperoxia, UV light, heavy metals, heme, A prostaglandins, NO, peroxynitrite, Th1 cytokines, oxidized lipid products and lipopolysaccharide, as well as certain growth factors can induce HO-1 [120,124–127]. HO-1 gene expression, which is controlled by factors such as pro-oxidant states or inflammation [126,127], is regulated mainly by two upstream enhancers, E1 and E2 [118,119,128]. Both enhancer regions contain multiple stress (or anti-oxidant) responsive elements (StRE, also called ARE) that also conform to the sequence of the Maf recognition element (MARE) [120,128,129] with a consensus sequence (GCnnnGTA) similar to that of other antioxidant enzymes [51]. Polymorphisms in the lengths of GT repeats [11–40] within the 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. Short-GT polymorphisms which may protect against atherosclerosis-linked conditions (e.g. coronary artery disease) are accompanied by significantly higher HO-1 activity, whereas the malignant behavior of various neoplasms is fairly consistently enhanced by the short-GT form [120]. Interest has focused on the role of HO-1 in AD, a neurodegenerative disorder involving a chronic inflammatory response associated with oxidative brain injury and A β -associated pathology. Significant increases in HO-1 levels have been reported in AD brains associated with neurofibrillary tangles. An increase in HO-1 mRNA was also found in AD neocortex and cerebral vessels; the HO-1 increase was also found together with senile plaques and glial fibrillary acidic protein-positive astrocytes in AD brains [120]. The dramatic increase in HO-1 in AD could be the result of a direct response to an increase in free heme concentrations, associated with neurodegeneration, during which brain cells try to convert the highly toxic heme into the antioxidants CO and bilirubin [25]. The protective role played by HO-1 and its products in AD has focused attention on the possibility of using natural substances capable of increasing HO-1 levels, as potential drugs for the prevention and treatment of AD. Polyphenolic compounds found in some herbs and spices, e.g. curcumin, appear promising in this context [119,130–133]. Curcumin is the active anti-oxidant principle in *Curcuma longa*, a coloring agent and food additive commonly used in Indian dishes. This polyphenolic substance can not only inhibit lipid peroxidation and effectively intercept and neutralize ROS and RNS [124,132,133], but it can also significantly increase HO-1 in astrocytes and vascular endothelial cells [134,135]. Epidemiological studies suggest that curcumin, which is widely used by the Indian population in nutritional and medicinal compounds, is responsible for a much lower (4.4- fold) prevalence of AD in India compared to the United States [132,136,137]. Subsequently, Lim and colleagues have demonstrated that dietary curcumin given to an AD transgenic APPSw mouse model (Tg2576) for 6 months reduced indices of inflammation and oxidative damage in the brain of these mice [138]. Further studies indicate that by inhibiting NF κ B activation, curcumin efficiently prevents cell death [139,140].

In the past few years, significant progress has been made in determining the diverse roles of HO-1 in brain senescence, aging-related human neurodegenerative disorders and other CNS pathologies. Although the acute induction of HO-1 in neural and other tissues has a predominantly cytoprotective outcome, protracted or repeated

up-regulation of the Hmox1 gene in astrocytes, oligodendroglia and possibly neurons may continue cellular dysfunction and demise in many chronic degenerative and neuroinflammatory conditions even when provocative stimuli have ceased [141,142]. In fact increasing evidence indicates that in normal brain aging, AD, PD, and other senescent neurodegenerations, iron sequestration in tissues, intracellular oxidative stress and mitochondrial dysfunction may produce pathogenic momentum that acts 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 the cerebral cortex and hippocampus neurons and astrocytes compared to age-matched, cognitively intact controls and co-localizes to senile plaques, neurofibrillary tangles, and corpora amylacea. In PD, HO-1 is markedly over-expressed in astrocytes of the substantia nigra and Lewy bodies in affected dopaminergic neurons. HMOX1 is also reported to be up-regulated in glial cells located around human cerebral infarcts, hemorrhages and contusions, within multiple sclerosis plaques, and in other human CNS disorders involving oxidant degeneration and inflammatory conditions. Within this context, heme-derived free ferrous iron, CO, and biliverdin/bilirubin are all biologically active substances that can improve or exacerbate neural injury according to the specific disease conditions. For example, in 'stressed' astroglia, HO-1 hyperactivity promotes mitochondrial sequestration of non-transferrin 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 also affect cell survival and neuroplasticity through modulation of brain sterol metabolism and proteosomal degradation of neurotoxic protein aggregates [143].

8. Hormesis

In hormesis dose response is characterized by a low dose stimulation and a high dose inhibition. Graphically it can be represented by either an inverted U-shaped dose response or by a J- or U-shaped dose response (Fig. 4). The term hormesis first appeared in 1943 when Southam and Ehrlich reported that low doses of extracts from the Red Cider tree enhanced the proliferation of fungi with the overall shape of the dose response being biphasic. However, it was in fact Hugo Schulz who demonstrated the occurrence of hormesis experimentally in 1888 [144] by observing biphasic dose responses in yeast following exposure to a large number of toxic agents. The work of Schulz encouraged researchers in many different fields to ascertain whether such low dose effects were a general feature of biological systems. In fact, similar types of dose response were observed by numerous researchers assessing chemicals [145] and radiation [146–157]. Despite the bulk of historical literature on hormetic dose responses, this concept was incorporated into routine safety assessment and pharmacological investigations with difficulty, principally because (i) it required more accurate evaluation in the

low dose zone, (ii) investigators failed to understand its clinical significance (iii) the quantitative features of the hormetic dose response were not appreciated (iv) the limitations of its implications for commercial applications in agriculture as well as medicine were not well understood (v) during most of the 20th century interest focused on responses at relatively high doses (vi) there was a continuing, yet inappropriate, tendency to associate the concept of hormesis with homeopathy [158–160]. However, after the late 1970s [161,162] biomedical sciences in general showed a growing interest in hormetic-like biphasic dose responses due to the possibility of measuring progressively lower doses of drugs and chemicals, the adoption of cell culture methods that allowed more efficient testing of numerous doses, the need to re-examine the validity of linearity at low dose modeling of cancer risks as well the astute observations of independent investigators and their capacity to generalize their findings across biological systems [162,163].

These research initiatives undertaken in extremely diverse biomedical areas demonstrated that hormetic dose responses are common and highly generalizable, irrespective of biological model, endpoints measured and chemical class and/or physical agent studied [146,154–157,164–166]. This finding caused surprise in mainstream branches of toxicology and pharmacology as hormetic responses were often considered to be paradoxical, not commonly expected and unreliable because of the lack of capacity for replication. During the mid decades of the last century, the hormesis concept was largely absent from the leading toxicological and biomedical textbooks, but this situation has radically changed as hormesis is now included into all leading toxicology textbooks (e.g. [166] encyclopedias [159,167] and other leading monographs. In fact, while the terms hormetic and hormesis appeared only about 160 times during the entire decade of the 1980s, in the Web of Science database, in 2010 alone, these terms were cited nearly 3200 times (Fig. 5).

Observations indicating that these broad ranging dose response relationships also shared the same general quantitative features were of further significance. More specifically, the low dose stimulation which is manifested immediately below the pharmacological and toxicological thresholds is modest in magnitude, being not more than 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). The hormetic dose response may result from either a direct stimulation or through an overcompensation stimulatory response following disruption in homeostasis [145,154]. The quantitative features of hormetic dose responses are similar, regardless of the way in which stimulation occurs as is confirmed by copious data in the literature ranging from plants to humans [160,165], and involving numerous receptor systems [158,168–171]. As a result of these findings, nearly 60 biomedical scientists have suggested that biological stress responses, including those of pre- and post-conditioning, should be

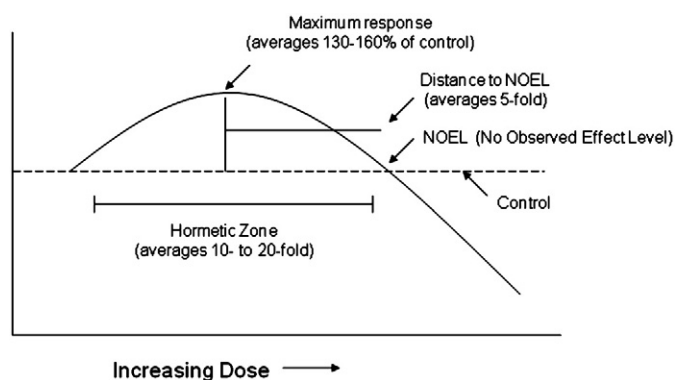


Fig. 4. Dose–response curve depicting the quantitative features of hormesis.

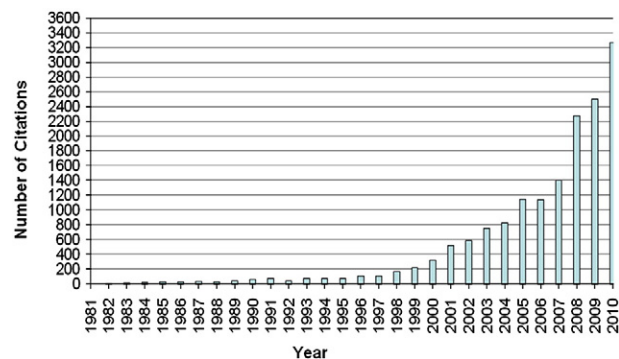


Fig. 5. Citations of Hormesis/Hormetic in Web of Science Database in the period 1981–2010.

integrated within a hormetic context, and a specific terminology should be based within an interdisciplinary framework [172].

The hormetic dose response requires a different 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 falls below the threshold, the low dose stimulation more likely represents a manifestation of an adaptive response that conforms to a measure of biological performance visible in modest increases in cognition, growth, longevity, bone density and other biomedical endpoints of interest.

Since hormetic findings display broad consistency, this strongly implies that this dose response may demonstrate the plasticity of biological systems. That nearly all biological models respond to imposed stress with the same quantitative features of the dose response is a relatively innovative and significant finding within the biological sciences. 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 providing resistance to environmental stress, but it also helps to regulate the allocation of biological resources in a manner that ensures cell and organismal stability.

These quantitative features of the hormetic dose response have important medical implications since constraint is imposed 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 no further increase in this level could be obtained by using a new drug combination. Numerous studies on hormesis and drug interaction have corroborated this concept. Flood [173–176] demonstrated that the hormetic response for memory was limited to the 30–60% increase even when several drugs were used in combination to maximize memory outcome. Similar response magnitude constraint has also been reported for immune stimulation, bacterial growth, increases in hair growth, plant growth, decrease in anxiety, decreases in tumor incidence and many other endpoints [177].

This limitation in the magnitude of the stimulatory response, which is documented by extensive findings, is a critical feature of the hormesis dose response concept and defines what pharmaceutical companies can expect to achieve with drugs designed to enhance performance. However, the limitation in the magnitude of response is also potentially important as regards the possibility of detecting a desirable response. Although this may not be an important issue when using highly inbred animal models or cell cultures where experimental conditions can be highly controlled, measuring a low dose hormetic stimulation in clinical trials can be problematic. Since human response to a drug can vary considerably, responsiveness of the test population may be 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 probably explains 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. It is of note that investigators may have to modify doses according to the sensitivity or susceptibility of the subjects. Calabrese and Baldwin [178] have shown that the hormetic dose response is often expressed in a broad range of subjects regardless of their susceptibility. As expected, those individuals that 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 above normal susceptibility would have their hormetic response shifted to the left. The hormetic dose response therefore presents considerable challenges for the biomedical community that develops drugs that aim to improve human performance.

The hormetic dose response can also have undesirable effects as may be most readily seen in the case of drugs designed to suppress

growth or kill cells or organisms at higher doses. For example, considerable evidence now indicates that low doses of many antitumor drugs can stimulate the proliferation of such cells at lower concentrations [179]. This also occurs with antibiotics, including penicillin [180] and streptomycin [180,181]. The same phenomenon has also been observed with selected cardiac glycosides that have effects on non-target tissues such as the prostate where it can enhance smooth muscle cell proliferation by about 30% with clinically relevant doses [182,183]. However, this 30% increase in prostate smooth muscle was thought likely to impede urination in males. If the possibility of the hormetic response is not taken into consideration, this can lead not only to failure to recognize a desirable drug-induced response, but it can also 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 that can activate hormetic pathways in order to produce a desirable clinical effect without risks associated with exposure as in the case of certain chemical agents and radiation.

8.1. Hormesis and adaptive stress response

The ability to sense oxidative and proteotoxic insults and to coordinate defensive stress response are basic elements for cellular adaptation and survival [184]. Over the past decade, an increasing interest has emerged on the hormetic mechanisms leading to adaptive responses of cells and organisms to moderate stress [51,185,186]. Mild oxidative or nitrosative stress (characterized by environmental insults, such as metabolic or physical stress, heavy metals, or pathophysiological conditions of oxidant-antioxidant balance perturbation), is able to induce the activation of cellular defense signaling pathways. These are part of a programmed cell life survival setting and serve to balance more severe stress conditions [51]. Typically, stressor elicited molecular responses do not only protect the cell against higher doses of the same agent, but also cross react and function against other specific stressors including oxidative, metabolic, and thermal stress. According to the hormetic principles, low doses of drugs, toxins, and natural substances may elicit a positive response in terms of adaptation to or protection from the stressor, whereas at higher concentration the toxic effect prevails [25,154,187]. The hormetic dose-response can occur through different mechanisms: as a direct stimulatory response; after an initial disruption in homeostasis followed by the modest overcompensation response; or as a response to an “adapting” or “preconditioning” dose that is followed by a more massive challenging dose [51].

Recent findings have elucidated the cellular signaling pathways and molecular mechanisms that mediate hormetic adaptive response which typically involve the synthesis of various stress resistance proteins as the products of “vitagenes”, a group of genes strictly involved in preserving cellular homeostasis during stressful conditions. The vitagene family is composed of the heat shock proteins (Hsp) HO (Heme-oxygenase)-1/Hsp32, Hsp70, Hsp60, by the thioredoxin system [22,188,189] and by sirtuin proteins. In this context, as detailed in the subsequent paragraphs, there is growing evidence that low concentrations of ROS may be beneficial since they are able to induce the expression of antioxidant enzymes or other defense mechanisms, while higher doses may be dramatically deleterious for the cellular redox homeostasis. Moreover, as an interesting emerging point of scientific debate, is the role of exogenous small molecules, including resveratrol, carnosic acid, sulphoraphane, dymethyl fumarate in activating hormetic mechanisms in response to deleterious stress conditions, such as during neurodegeneration and cancer [190,191].

As hormetic paradigmatic cases, they may induce adaptive stress response at subtoxic doses while may be toxic to mammalian cells at high concentrations [18]. From a molecular point of view, they

are able to 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 [22,25,37,190,192,193] (Fig. 6). They resulted also inducers of the Keap1/Nrf2/ARE pathway. Transcription factor Nrf2 has shown the ability, both in vitro and in vivo experiments, to activate a series of vitagenes, such as heme-oxygenase-1 (HO-1) [51,119,124,132,134,194–196], coordinately with other cytoprotective proteins [124,195,197–204]. Interestingly, from an hormetic perspective, HO-1 exerts protective role by degrading the intracellular levels of pro-oxidant heme, producing biliverdin, the precursor of bilirubin, and carbon monoxide (CO), molecules with potent antioxidant-anti-nitrosative and cytoprotective features at low concentrations [51,111,118,189,205–207]. However, upregulation of HO-1 (i.e., HO/BVR axis) is not always beneficial for cells: the heme depletion and accumulation of CO and bilirubin it causes are potentially toxic. Therefore, new pharmacological modulators of HO/BVR activity must act in a dose-dependent manner. This would allow dose titration to achieve a desired pharmacologic effect without producing toxicity. Unfortunately, this goal is more complicated than it seems because toxicity has to be defined in terms of each of the main products of heme metabolism. Furthermore, sensitivity to the therapeutic/toxic effects of these products is likely to be tissue- or cell-type specific. The solution may lie in the use of novel drug-delivery systems that allow targeted delivery of low doses of the HO/BVR modulator to selected tissues [208,209]. Consistent to this notion, biliverdin reductase-A (BVR-A) is emerging as a pleiotropic enzyme involved in cellular stress responses. It not only transforms biliverdin-IX alpha into the antioxidant bilirubin-IX alpha but through its serine/threonine/tyrosine kinase activity is able to modulate cell signaling networks. BVR-A's involvement in neurodegenerative disorders such as Alzheimer disease (AD) and amnesic mild cognitive impairment (MCI) was previously described. Statins significantly increased BVR-A protein levels, phosphorylation and activity in parietal cortex, which significantly correlated with β -secretase protein levels in the brain, suggesting a possible role for BVR-A in A β formation [210–212]. Thus, a better knowledge of the vitagene defense network and of the leading hormesis mechanisms may support the stimulation of various maintenance and repair pathways through exogenous interventions, such as mild stress or compounds targeting the vitagene network, as a novel approach to delay various alterations in cells, tissues and organs and potentially to prevent and treat many different diseases [25,186].

8.2. Mitostress vs mithormesis and neuroprotection

Recent findings have revolutionized the previous belief that mitochondrial ROS have only a negative impact on cell function and survival since it is now clear that mitochondrial superoxide and hydrogen peroxide play important roles in a range of cellular functions and are able to activate signaling pathways that promote cell survival and disease resistance. Exposure of hippocampal neurons to subtoxic levels of hydrogen peroxide triggers the release of Ca^{2+} from the endoplasmic reticulum by causing the opening of both IP_3 and ryanodine receptor channels [213]. 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 [214]. Mitochondrial ROS may also be involved in recovery from injury as superoxide can stimulate neurite outgrowth by directly activating protein kinase C [215]. Moreover, peroxynitrite can promote the phosphorylation and nitration of regulatory sites within receptor tyrosine kinases thereby activating cell survival signaling pathways including those involving PI3 kinase – Akt and mitogen-activated protein (MAP) kinases [18]. Nitration of proteins involved in synaptic vesicle trafficking can enhance glutamate release, suggesting that superoxide and peroxynitrite have a potential role in regulating neurotransmission [18]. Mitochondrial superoxide production is known to contribute to neuron damage in pathological conditions such as chronic cerebral hypoxia or Alzheimer's disease [18]. Nevertheless, abundant evidence shows that transient neuron exposure to low levels of superoxide which are converted into hydrogen peroxide can protect them against a subsequent exposure to a level of stress that what would otherwise have been lethal. This neuroprotective effect due to a subtoxic increase in cellular oxidative stress is known as “preconditioning” and clearly falls under the paradigm of hormesis [18]. Although studies have revealed the involvement of oxidants in many signaling pathways, cell strategies for conferring pathway specificity to such reactive molecules still requires elucidation. Recent research suggests that cells may spatially restrict oxidant production to allow microdomain-specific signaling [216]. The specific molecular mechanisms by which mitochondrial ROS elicit hormetic responses in neurons are not fully understood, but recent findings suggest that important roles can be attributed to certain transcription factors with regulatory effects. For example, NF- κ B is activated in neurons in response to oxidative stress and plays a critical part in the adaptive response that protects neurons against more severe oxidative stress,

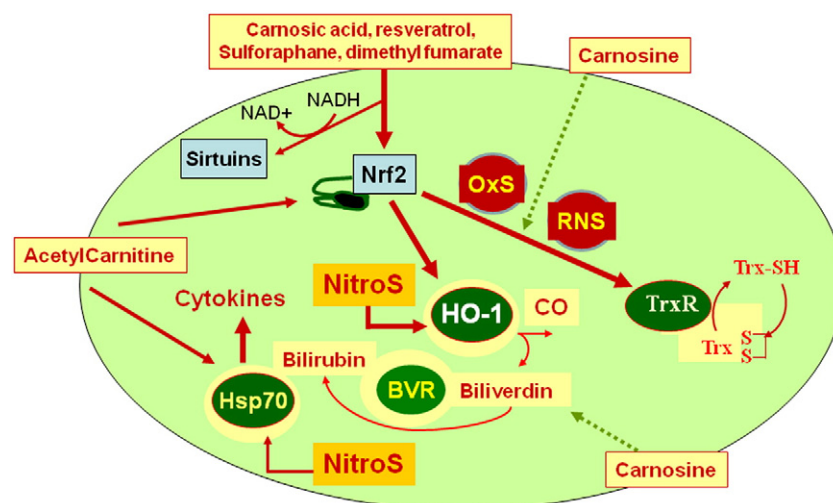


Fig. 6. Vitagenes and the pathway of cellular stress response. Nutritional antioxidants, including carnosic acid, resveratrol, sulforaphane, dimethyl fumarate, acetyl-L-carnitine or carnosine are able to activate vitagenes, such as heme oxygenase, Hsp70, thioredoxin reductase and sirtuins which represent an integrated system for cellular stress tolerance. Phytochemicals and Acetyl-L-carnitine act through the activation of transcription factor Nrf2, which after binding to the ARE (antioxidant responsive element) in the HO-1 gene, up-regulates both HO-1 and TrxR, thus counteracting pro-oxidant conditions. In addition, carnosine has been shown to inhibit the induction of both HO-1 and Hsp70 following strong nitrosative conditions.

by promoting the expression of protective genes including SOD2 and Bcl-2 [217], TNF also up-regulates the expression of Mn-SOD with an NF- κ B-mediated mechanism, thereby protecting neurons against excitotoxic, ischemic and oxidative injuries [218]. While mitochondrial H₂O₂ may activate adaptive stress response pathways in neurons, they may also exert a neuroprotective effect by acting on other cell types in the nervous system. For example, microglial activation in response to increased oxidative stress can have either positive or detrimental effects on neurons and neural progenitor cells according to the type and amounts of cytokines and growth factors secreted by the microglia [219]. Oxidative stress can also promote angiogenesis in the brain [18], which is of vital importance in restoring normal neuronal perfusion during the days and weeks after a stroke. This latter effect is an example of “trans-cellular” hormesis mediated by ROS [220].

8.3. The neuroprotective effects of hormetic phytochemicals that activate the KEAP1/NRF2/ARE pathway

An elaborate network of protective mechanisms allows all eukaryotic organisms to defend themselves against the damaging effects of oxidants and electrophiles, the principal agents responsible for the pathogenesis of cancer, atherosclerosis, neurodegeneration, and aging [221]. Endogenously-formed and exogenously-encountered electrophiles and oxidants are counteracted by a group of functionally diverse cytoprotective proteins which are regulated by the Keap1/Nrf2/ARE pathway (Fig. 7). Based on numerous biochemical studies and global gene expression profiling, it is now evident that the family of cytoprotective proteins is characterized by extraordinary diversity and includes: (i) conjugating enzymes (e.g. glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases); (ii) export pumps (e.g., multidrug resistance proteins (MRPs)), (iii) antioxidant enzymes (e.g., heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1)); (iv) enzymes that participate in the synthesis and regeneration of glutathione, the principal small molecule antioxidant in the

cells (e.g., γ -glutamylcysteine ligase, glutathione reductase); (v) enzymes that promote the synthesis of reducing equivalents, i.e., NADPH (e.g., glucose-6-phosphate dehydrogenase, malic enzyme); (vi) proteins that do not have enzymatic activities, but possess an enormous capacity to protect against metal overload (e.g., ferritin); (vii) enzymes that inhibit inflammation (e.g., leukotriene B₄ dehydrogenase); (viii) proteins that participate in the repair and removal of damaged proteins (e.g., Hsp40, Hsp70, p62 and subunits of the 26S proteasome) and DNA (e.g., O⁶-methylguanine-DNA methyltransferase). The basis for grouping them into one “family” is the fact that they share common transcriptional regulation, are inducible, and catalyze reactions that, in almost all cases, collectively result in protection against electrophiles and oxidants, and in enhanced survival [222–225].

The upstream regulatory regions of the genes coding for cytoprotective proteins contain single or multiple copies of the antioxidant response element (ARE, also known as electrophile response element, EpRE) with the consensus sequence 5'-A/CTGAC/GNNNGCA/G-3' [226–231] (Fig. 8). Nuclear factor erythroid 2-related factor 2 (Nrf2), a basic leucine zipper protein, is the major transcription factor that binds to the ARE [232]. Activation of transcription requires that Nrf2 binds to the ARE as a heterodimer with members of the small Maf family of transcription factors [233]. Under homeostatic conditions the expression of cytoprotective genes is low due to the low intracellular levels of Nrf2 which are maintained by Kelch-like ECH-associated protein 1 (Keap1) [234]. Keap1 binds to the E3 ubiquitin ligase Cullin3-RING box1 (Cul3-Rbx1) and presents Nrf2 for ubiquitination and subsequent proteosomal degradation [235–238]. However, the pathway is activated by various stress conditions (e.g., shear stress, hyperoxia), and also by many small-molecules (inducers). This activation renders the cell well-equipped to meet a subsequent challenge due to the transcriptional upregulation of a large number (more than 100) and extraordinary functional versatility of cytoprotective proteins.

Inducers of the Keap1/Nrf2/ARE pathway belong to ten distinct chemical classes: (i) oxidizable diphenols, phenylenediamines, and

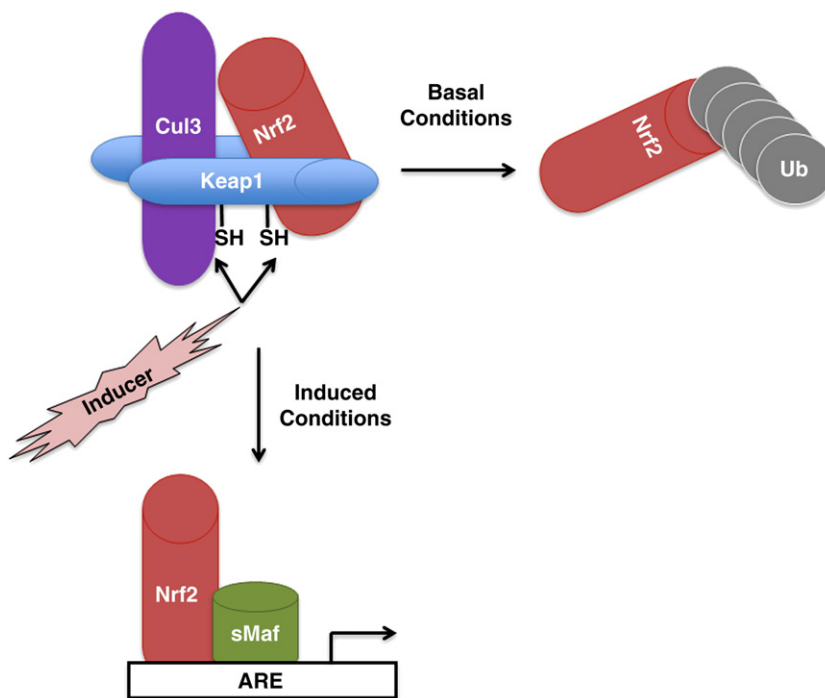


Fig. 7. The Keap1/Nrf2/ARE pathway. Under basal conditions, Keap1 binds and targets transcription factor Nrf2 for ubiquitination and proteasomal degradation via association with the Cullin 3 (Cul3)-based E3 ubiquitin ligase. Under induced conditions, small-molecule inducers chemically modify specific reactive cysteine residues (-SH) of Keap1 which consequently loses its substrate adaptor function. This leads to increased stabilization of Nrf2, its nuclear translocation, binding to the ARE (as a heterodimer with small Maf), and ultimately transcriptional activation of the expression of cytoprotective genes.

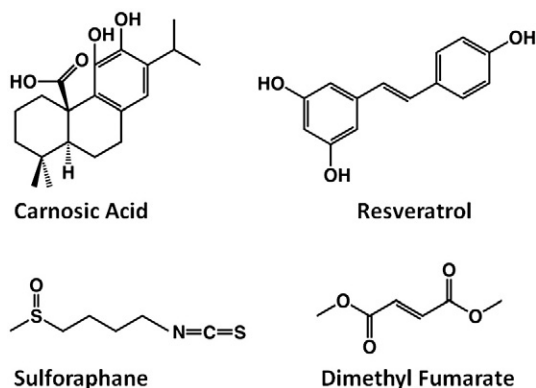


Fig. 8. Structures of some phytochemical inducers of the Keap1/Nrf2/ARE pathway.

quinones; (ii) Michael acceptors (olefins or acetylenes conjugated to electron-withdrawing groups); (iii) isothiocyanates; (iv) thiocarbamates; (v) trivalent arsenicals; (vi) dithiolethiones; (vii) hydroperoxides; (viii) vicinal dimercaptans; (ix) heavy metals; and (x) polyenes. They all have a common property: chemical reactivity with sulfhydryl groups by oxido-reduction, alkylation, or disulfide interchange [239–243]. It is now widely accepted that exogenous and endogenous inducers chemically modify specific reactive cysteine residues of Keap1, which in addition to serving as an adaptor protein for the ubiquitination and degradation of Nrf2, also functions as the intracellular sensor for inducers [224,244]. The cysteine modification(s) within Keap1 result in conformational changes that abrogate its substrate-adaptor function, ultimately resulting in Nrf2 stabilization, binding to the ARE and recruitment of the basal transcriptional machinery to activate transcription of cytoprotective genes (reviewed in 197,199,230,231,245,246).

Many inducers of the Keap1/Nrf2/ARE pathway are natural products. Plants have evolved a versatile array of molecules (phytochemicals) with potent activities (i.e., ultraviolet radiation-filtering, antimicrobial, antifungal, antioxidant) which allow their successful adaptation and survival in an environment full of challenges, such as solar radiation, toxins, pathogens and infectious agents. Some of these phytochemicals (e.g., the phytoalexins) are synthesized de novo in response to pathogen attack [247]; others (e.g., the isothiocyanates) exist as metabolic precursors which are “activated” by enzymes that normally are expressed in highly specialized cells and can only be in direct contact with their substrates following plant injury [248]; yet a third category (e.g., malondialdehyde) has the capacity to potently induce transcriptional upregulation of the plant's own defense mechanisms [249]. The now compelling evidence for biphasic dose response effects of environmental “toxins” in biological systems suggests the possibility that phytochemicals from edible plants might exert biphasic dose responses and could contribute to the well-recognized health benefits from plant-rich diets. There are currently multiple lines of evidence on the protective effects of sulforaphane, curcumin, and resveratrol, all present in edible plants. Although the antioxidant activity of certain phytochemicals might be partly responsible for their health benefits, most people do not consume fruits and vegetables at quantities that are sufficient to achieve the relatively high concentrations of the antioxidants that are required to scavenge free radicals directly. Furthermore, the clinical trials have not demonstrated benefits of dietary supplementation with antioxidants such as vitamin E and vitamin C. Emerging evidence suggests that activation of the Keap1/Nrf2/ARE pathway, rather than direct free radical scavenging activity, may better account for the health benefits of phytochemicals. Indeed, many phytochemicals have been shown to activate adaptive cellular stress response pathways that induce the expression of networks of genes encoding antioxidant enzymes, protein chaperones, neurotrophic factors and other cytoprotective

proteins. In contrast to direct antioxidants (such as vitamin C) which have short half-lives, activators of the Keap1/Nrf2/ARE pathway have long-lasting effects because their action is based on induction of transcription-mediated signaling [225,250]. Here, we give examples of some of the most studied phytochemicals that activate the Keap1/Nrf2/ARE pathway, focusing on their neuroprotective activities.

8.3.1. Carnosic acid

Compounds with hydroquinone and catechol moieties that are abundant in plants, activate the Keap1/Nrf2/ARE pathway, and have been shown to be neuroprotective [251]. In order to activate the Keap1/Nrf2/ARE pathway, hydroquinones and catechols have to be oxidized to their corresponding electrophilic quinones [252–254]. As such they can be viewed as “pro-drugs” that are converted to the ultimate inducers “via the pathological activity that they are intended to combat” [255]. One catechol-containing phytochemical is carnosic acid (Fig. 8), a compound found in rosemary (*Rosmarinus officinalis*) which accounts for 5% of the dry weight of the leaves of this plant [256]. A recent investigation in rats has demonstrated good bioavailability of carnosic acid following oral administration [257]. Treatment of cells with carnosic acid was reported to cause chemical modification of cysteine residues of Keap1 and induce Nrf2-dependent genes, resulting in protection of neurons against oxidative stress and excitotoxicity [258]. The same study showed that carnosic acid crosses the blood–brain barrier, increases the level of reduced glutathione in the brain of C57BL/6 mice, and protects against middle cerebral artery ischemia/reperfusion. A subsequent study in murine hippocampal HT22 cells confirmed the role of increased glutathione levels in the neuroprotective mechanisms of carnosic acid [259]. This catechol was also shown to induce expression of the neurotrophin nerve growth factor (NGF) promoting neurite outgrowth and differentiation [260]. Most recently, it was reported that enhanced expression of nerve growth factor by carnosic acid also occurs in normal human astrocytes, and that this induction is dependent on activation of Nrf2 [261]. Increased brain neurotrophin levels were also observed following intranasal administration of carnosic acid to Sprague–Dawley rats [262].

8.3.2. Sulforaphane

The isothiocyanate sulforaphane (1-isothiocyanato-(4R)-methylsulfanyl)butane) (Fig. 7) is one of the most potent naturally occurring inducers of the Keap1/Nrf2/ARE pathway. Sulforaphane was isolated from extracts of the commonly consumed cruciferous vegetable broccoli (*Brassica oleracea*) based on its ability to induce the classical Nrf2-target gene NQO1 [263]. In the nearly 20 years subsequent to its discovery, the protective effects of sulforaphane have been demonstrated in various cell culture systems and animal models. Thus, sulforaphane treatment caused Nrf2 activation and upregulated Nrf2-dependent gene expression in astrocytes [264–267]. This activation resulted in protection against the damaging effects of transient exposure to O₂ and glucose deprivation [266], as well as of the toxicities of hydrogen peroxide and glutamate, and only in cell cultures isolated from wild-type, but not Nrf2-knockout animals [265]. Reintroduction of Nrf2 restored both the upregulation of Nrf2-dependent genes and the neuroprotection, implying activation of Nrf2 as the main mechanism of protection [265]. Importantly, the neuroprotective effects of this isothiocyanate are long-lasting: following sulforaphane exposure of astrocytes for 4 h the levels of NQO1 and HO-1 mRNA remained elevated for 24 h, and the protein levels for at least 48 h; repeated exposures resulted in even further accumulation of NQO1 and sustained protection against oxidative stress [267]. In dorsal root ganglion neurons, sulforaphane treatment caused Nrf2 nuclear translocation and upregulation of the activities of GST and NQO1 [268]. In rat organotypic nigrostriatal cocultures, sulforaphane protected against the toxicity of 6-hydroxydopamine [269]. Similarly,

neuroprotective effects by sub-micromolar concentrations of sulforaphane were observed when primary murine cortical neurons were treated with the endogenous neurotoxin S-S-cysteinyl dopamine; protection was dependent on induction of Nrf2-dependent genes, and also, on activation of ERK1/2 and PI3K/Akt signaling [270]. Cultured embryonic hippocampal neurons were also protected by sulforaphane against the toxicities of hemin or of exposure to oxygen in combination with glucose deprivation [271]. In motor neurons grown in organotypic cultures of rat spinal cord, sulforaphane reduced extracellular accumulation of glutamate and protected against glutamate-mediated excitotoxicity caused by threo-hydroxyaspartate [272]. Kainate-induced cell death was reduced upon treatment with sulforaphane in hippocampal slices of wild-type, but not Nrf2-knockout mice [273]. When mitochondria were isolated from brain of Fischer 344 rats that had been treated with sulforaphane (10 mg/kg, *i.p.*, 40 h prior to isolation of mitochondria), they were found to be more resistant to oxidative stress-induced Ca^{2+} -dependent permeability transition pore opening than brain mitochondria from control animals [274].

Administration of sulforaphane (5 mg/kg, *i.p.*) to Long–Evans rats, increased the levels of HO-1 mRNA and protein in brain; increases were detected in both neurons and astrocytes [194]. In a model of focal cerebral ischemia, when sulforaphane was administered 15 min after the onset of ischemia, the infarct volume (evaluated 3 days later) was reduced by ~50% [194]. In a model of traumatic brain injury in Sprague–Dawley rats, sulforaphane administered 6 h post-injury attenuated the loss of the water channel aquaporin-4 (AQP4) in the injury core and increased the levels of AQP4 in the penumbra region [275]. In a subsequent study, a similar treatment with sulforaphane reduced the blood–brain barrier permeability and the loss of endothelial cell markers and tight junction proteins, and preserved the blood–brain barrier function [195]. The protective effects of sulforaphane were also evident in C57BL/6 wild-type mice, but not in Nrf2-knockout animals, implicating activation of Nrf2 as the underlying mechanism of protection.

In a model of intracerebral hemorrhage in Sprague–Dawley rats, sulforaphane induced Nrf2-dependent genes and reduced markers of oxidative damage in the perihematoma area [202]. Three days following intracerebral hemorrhage, in the sulforaphane-treated animals, the perihematoma neutrophil count and the total hemorrhage-affected striatum neutrophil count were reduced compared to the same parameters in control animals. Importantly, 10 days after the hemorrhage, the neurologic deficits were substantially reduced in the sulforaphane-treated animals. In close agreement with the traumatic injury model, Nrf2-knockout animals had exacerbated neurologic deficits than wild-type mice, and the protective effects of sulforaphane were lost. Interestingly, sulforaphane was also shown to increase the levels of haptoglobin in oligodendroglia correlating with reduction in brain damage following intracerebral hemorrhage [276]. Haptoglobin which is synthesized in the brain is important for protecting neurons and oligodendrocytes against damage caused by intracerebral hemorrhage as it binds and neutralizes hemoglobin that could otherwise cause cytotoxicity. Sulforaphane treatment (5 mg/kg, *i.p.*, administered at 0.5, 12, and 36 h following blood injection) was recently reported to reduce brain edema, cortical apoptosis, blood brain barrier impairment, and motor deficits in a model of early brain injury after subarachnoid hemorrhage in Wistar rats [277].

In another study of traumatic brain injury, sulforaphane treatment caused activation of Nrf2 and upregulation of Nrf2-dependent genes, whereas there was reduction in oxidative damage, neuronal death, contusion volume, and neurological deficits [278]. Compared to wild-type, Nrf2-knockout mice showed greater severity of damage and were not protected by the sulforaphane treatment [279]. In addition to its immediate beneficial effects, sulforaphane treatment was also shown to improve long-term cognitive function following

traumatic brain injury; however, this improvement only occurred when sulforaphane was administered 1 h, but not 6 h, post-injury [279]. In a rat model of neonatal hypoxic-ischemic injury evoked by carotid artery ligation and hypoxia, sulforaphane administration 30 min prior to injury activated Nrf2 and reduced oxidative stress markers and the infarct size in the brain [280].

Notably, sulforaphane, and inducers of the Keap1/Nrf2/ARE pathway in general, also have anti-inflammatory properties. Thus, in primary co-cultures of rat microglial and astroglial cells sulforaphane induces Nrf2-dependent genes and attenuates LPS-stimulated production of TNF α , IL-1 β , IL-6 and NO [281]. In C57BL/6 mice that received endotoxin injection, sulforaphane treatment decreased microglial activation and the upregulation of inflammatory markers (iNOS, IL-6, and TNF- α) [282]. Compared to wild-type, Nrf2-knockout mice were hypersensitive to LPS-induced neuroinflammation, strongly suggesting that even the basal expression of Nrf2-dependent genes provides protection. In the methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease, sulforaphane protected nigral dopaminergic neurons against cell death, reduced astrogliosis, microgliosis, and release of pro-inflammatory mediators in basal ganglia; in contrast to wild-type mice, no protection by sulforaphane was observed in Nrf2-knockout animals [283–285].

Similarly to the damaging effects of microgliosis to the brain, spinal cord microglia activation following peripheral nerve injury also causes tissue damage. Intrathecal administration of sulforaphane was shown to activate Nrf2 in the spinal cord, markedly reduce oxidative stress and pro-inflammatory cytokine expression caused by spinal nerve transection, and to inhibit the development of neuropathic pain [286]. Very recently, two studies reported the neuroprotective effects of post-injury treatment with sulforaphane in spinal cord injury models in Fischer rats and ICR mice; again, it is clear that both induction of Nrf2-dependent genes and anti-inflammatory activity play critical roles in the protective mechanism(s) [287,288].

Sulforaphane, through the Nrf2-dependent upregulation of cytoprotective proteins, also has the potential to accelerate the metabolism and elimination of neurotoxic chemicals. Thus, sulforaphane treatment before exposure to methylmercury decreased mercury accumulation in the brain and liver of wild-type, but not Nrf2-knockout mice most likely by facilitating the metabolism and excretion of methylmercury [289]. Indeed, studies in primary hepatocytes showed that exposure to sulforaphane led to activation of Nrf2, causing upregulation of γ -glutamylcysteine ligase (and consequently increased levels of GSH), GSTA1, and MRP2, thus reducing mercury accumulation and cytotoxicity [289].

8.3.3. Dimethyl fumarate

Fumaric acid is found in shepherd's purse (*Capsella bursa-pastoris*), a cruciferous weed which is used as a traditional herbal medicine. Before the discovery of Nrf2, it had been shown that inclusion of the methyl ester of fumaric acid, dimethyl fumarate (Fig. 8), in the diet of CD-1 mice and Sprague–Dawley rats elevated the activities of GSTs and NQO1 in many organs [204,290]. In primary co-cultures of rat microglial and astroglial cells, dimethyl fumarate induced Nrf2-dependent genes, and lowered the LPS-induced formation of TNF- α , IL-1 β , IL-6 and NO [281]. In a model of chronic experimental autoimmune encephalomyelitis in C57BL/6 mice, dimethyl fumarate treatment increased blood levels of IL-10 and lowered macrophage infiltration in the spinal cord [291]. Orally administered dimethyl fumarate to mice increased Nrf2 positivity in neuronal subpopulations in the striatum and the motor cortex in models of Huntington's disease; Nrf2 activation correlated with preservation of morphologically intact neurons in these areas [292]. In a model of multiple sclerosis, dimethyl fumarate upregulated NQO1 activity in liver and cerebellum and had protective effects on oligodendrocytes, myelin, axons and neurons [293]. In a model of experimental autoimmune encephalomyelitis, dimethyl fumarate increased Nrf2 levels in oligodendrocytes, astrocytes, and in neurons of the

motor cortex and the brainstem [293]. In comparison to wild-type, Nrf2-knockout mice had exacerbated pathology, and the beneficial effects of dimethyl fumarate were lost [293]. Because fumaric acid salts and esters are already used as therapeutic agents [294–296], human studies have been recently conducted in patients with relapsing-remitting multiple sclerosis. In a pilot study, oral dimethyl fumarate reduced the formation of new inflammatory lesions [297]. These encouraging results were further confirmed and extended in a multicentre, randomized, double-blind, placebo-controlled, dose-escalation, Phase IIb clinical trial [298]. Currently, dimethyl fumarate is in a Phase III clinical trial [299].

8.3.4. Resveratrol

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) (Fig. 8) was identified as the active principle of an extract derived from a nonedible Peruvian legume (*Cassia quinquangulata* Rich.) in an activity-guided fractionation based on its ability to inhibit cyclooxygenase; in the same study, it was also found to induce the classical Nrf2-dependent enzyme NQO1 in Hepa1c1c7 cells, and, remarkably, to be able to inhibit the process of carcinogenesis at all of its stages [300]. Resveratrol is present in more than 70 plant species. Its quantity is especially high in grapes (50–100 µg of resveratrol per gram of fresh grape skin) and red wine [300,301]. There are currently more than 4000 publications on resveratrol, demonstrating the phenomenal amount of scientific interest fueled by the extraordinary broad spectrum of biological activities that have been attributed to this phytochemical [302].

One very widely-used experimental model demonstrating the neuroprotective effects of resveratrol is the rat pheochromocytoma cell line PC12. Thus, exposure of PC12 cells to resveratrol has been shown to cause activation of Nrf2 and upregulation of HO-1 [273], and to protect against the toxicities of hydrogen peroxide [196,303], β-amyloid [303,304], ethanol [305] oxidized lipoproteins [306,307], 4-hydroxy-2-nonenal [308], oxygen-glucose deprivation [309], and the combination of Fe²⁺ and *t*-butyl hydroperoxide [310]. Furthermore, in PC12 cells that express α-synuclein under the control of an inducible promoter, resveratrol activated the AMPK/SIRT1 pathway, induced autophagy and enhanced the degradation of α-synuclein [311]. Additionally, resveratrol was reported to inhibit the polymerization of β-amyloid *in vitro* [312]. In cultured dorsal root ganglion neurons, resveratrol treatment caused Nrf2 nuclear translocation and upregulation of the activities of GST and NQO1 [268]. Resveratrol increased glutamate uptake and intracellular glutathione levels, and protected against the toxicity of hydrogen peroxide in C6 astroglial cells, primary astrocytes and hippocampal slices [313–315]. In human neuroblastoma SH-SY5Y cells, resveratrol induced autophagy and protected against rotenone-induced apoptosis [311].

In a *Drosophila* model of Huntington's disease, resveratrol activated ERK [316] and suppressed neurodegeneration [317]. Neuroprotection by resveratrol has also been demonstrated in *in vivo* models of Alzheimer's disease and Parkinson's disease, with activation of SIRT1 being implicated as the protective mechanism (reviewed in 318). In a rat model of spinal cord injury, resveratrol improved functional recovery, reversed the spinal cord injury-induced decrease in activity of superoxide dismutase, and reduced the levels of markers of oxidative stress (malondialdehyde), inflammation (TNF-α, IL-1β, and myeloperoxidase), and apoptosis (caspase-3) [319]. In several other models of spinal cord injury, resveratrol protected against oxidative stress (as evidenced by lower malondialdehyde, nitric oxide, xanthine oxidase, and higher glutathione levels), reduced tissue injury, and improved neurological recovery [320–323]. Reduction in infarct volume and neuroprotective effects of resveratrol were also seen in rats subjected to focal cerebral ischemia injury [324,325]. Another study of cerebral ischemia injury reported that resveratrol treatment improved neurological scores and reduced infarct volume and brain water content [326]. The levels of malondialdehyde were lower in the resveratrol-treated animals,

whereas the activity of superoxide dismutase was restored. These effects were accompanied by Nrf2 activation, upregulation of HO-1, and downregulation of caspase-3. A recent investigation using a rat model of fetal alcohol spectrum disorders also reported the Nrf2-dependent protective effects of resveratrol: the authors found that ethanol increased the levels of oxidative stress and inhibited the activation of Nrf2 in the cerebellum; administration of resveratrol prior to ethanol exposure restored the levels and the DNA-binding activity of Nrf2 and the expression of its downstream cytoprotective proteins NQO1 and SOD, increased the levels of reduced glutathione, reduced oxidative stress, and enhanced survival of cerebellar granule neurons [327].

It is becoming increasingly apparent that the neuroprotective activities that have been attributed to the phytochemicals discussed in this section in a great number of experimental systems are due to the fact that they are not *single-target* agents, but quite the opposite: all of these molecules have the ability to affect *numerous* cellular components and processes. It is also clear that many of these diverse protective effects can be attributed to the capacity to these molecules to mobilize endogenous defense mechanisms by providing two major triggers: (i) activation of transcription factor Nrf2-mediated gene expression, and (ii) suppression of pro-inflammatory responses. It is then the ability of the cell to mount and orchestrate its own versatile protective responses that determines its successful adaptation and survival.

8.4. A focus on the hormetic role of resveratrol

Due to its remarkably broad range of effects, including mostly antioxidant and anti-inflammatory activities, immunomodulation, chemoprevention, cardiovascular protection and longevity, resveratrol has attracted numerous researchers investigating the hormetic mechanisms underlining its biological responses in cells and organisms [168]. The emerging body of evidence indicates that the wide range of resveratrol induced end-points displayed a biphasic dose response with quantitative features consistent with the hormetic relationship.

Interestingly, many *in vitro* studies demonstrated the resveratrol ability to hormetically induce and inhibit cell proliferation in numerous human cancer cell lines, at low and high concentrations, respectively. Several cell lines were affected, such as breast [328–335], prostate [336–339], leukemia [340,341], colon [342,343], uterus [344], and lung [345] tumor cell lines. These results suggest that resveratrol may prevent or enhance tumor development, according to the extent of the dose applied. It is a crucial point of discussion in consideration of both the relevance given to resveratrol potential chemopreventive effects, to the implications that resveratrol dietary amount may have for human health and the potential therapeutic dose management [168,334].

Similarly a consistent hormetic biphasic dose–response relationship was detected by Gu et al. [346] for endothelial human cell proliferation, and also for cell migration, as well as cell adherence and eNOS expression/concentration in injured arteries. These *in vitro* findings were supported also in subsequent *in vivo* studies with a rat model. Such studies demonstrated that a low dose of resveratrol enhanced the mobilization of endothelial cells, facilitated re-endothelialization, reduced the occurrence of neointimal formation and up-regulated the expression of eNOS following an induced balloon injury. These findings were not only supported in subsequent research of Xia et al. [347] which corroborated the hormetic-like response of resveratrol on endothelial progenitor cell proliferation and cell migration, but further extended other observations by linking these responses to telomerase activity via AKT-dependent mechanisms. As in the case of the endothelial cell parameters measured, the resveratrol-induced alterations in telomerase activity were also indicative of hormetic responses.

Szende et al. [342] demonstrated that resveratrol biphasically affected proliferation with a low-concentration stimulation and a high-concentration inhibition in human endothelial cells. Using

bovine aortic endothelial cells (BAECs), In et al. [348] showed that the resveratrol biphasically affected endothelial cell migration. While the authors acknowledged the capacity of resveratrol to induce a biphasic concentration response, they emphasized that their data revealed that high concentrations of resveratrol might have utility as a potent anti-angiogenesis drug. However, at lower concentrations, the response could switch to angiogenic effects.

Resveratrol has anti-inflammatory properties such as shown with the inhibition of COX-1 [300] and COX-2 [349], down-regulation of prostaglandin biosynthesis and suppression of carrageenan-induced paw edema [300,350]. Since these collective findings suggested a possible effect on immune response, Falchetti et al. [351] explored, in detail, the effects of resveratrol on multiple immune functions of human T-cells in vitro. These included the development of cytokine-producing CD4+ and CD8+ T cells by stimulating peripheral blood mononuclear cells (PBMC) with anti-CD3/antiCD28, specific antigen-induced generation of cytotoxic T lymphocytes and natural killer (NK) activity of peripheral blood mononuclear cells. These authors reported that there was a hormetic-like biphasic dose response for each endpoint assessed. According to Falchetti et al. [351] these findings suggested a regulatory effect of resveratrol on the immune response. Gao et al. [352,353] revealed that resveratrol inhibited the Con A induced proliferation of the spleen cells but only at high concentrations. At lower concentrations, the proliferative response was significantly increased. These findings generally supported the conclusion that resveratrol has the capacity to suppress or upregulate immune response depending on the concentration.

Interestingly, a 2005 study by Juan et al. [354] provided such a mechanistic insight into how resveratrol affects a hormetic response in human aortic smooth muscle cells (HASMCs). Resveratrol induced HO-1 expression in a manner that conformed to the hormetic dose response, markedly enhancing HO-1 expression at low concentrations whereas at higher concentrations, this response was diminished. Resveratrol induction of the HO-1 gene was mediated via the NF- κ B pathway. In fact, two separate NF- κ B inhibitors abolished the capacity of resveratrol to induce HO-1 expression and the activity of the HO-1 promoter. Low concentrations of resveratrol enhanced NF- κ B-binding activity based upon experiments assessing electrophoretic mobility shifts. At these low concentrations, the resveratrol trans-activated the NF- κ B and this activation process enhanced the transmigration of NF- κ B into the nucleus, which then led to the modulation of HO-1 gene expression.

It was demonstrated also that resveratrol had chemopreventive effects with respect to several conditions such as cardiovascular disease [355], Alzheimer's disease [304], osteoporosis [356] and gastric ulcers [357]. From a hormetic perspective, in these conditions, low doses of resveratrol were shown to confer protection whereas higher doses showed adverse health effects. However, in relation to digestive disorders, there are contradictory results that identified resveratrol as a beneficial agent at different doses, without detecting any biphasic dose responses [358].

Various parasitic diseases were also hormetically influenced by resveratrol. Animal models showed that elevated doses protected against Leishmaniasis and Trichinella, while low concentrations consistently enhanced their proliferation [359,360].

Globally viewing, low concentrations of resveratrol can be potentially beneficial or harmful, depending on the endpoint investigated. In this regard, low doses of resveratrol would have the capacity to increase the risk of tumor development of a number of organs and in contrast to be significantly cardio-protective. This type of conflict between beneficial or harmful effects is not uncommon and it is maybe related to the different biological system, tissue and chemical agent investigated.

Resveratrol is probably the most studied molecule of a series of phytochemicals, including sulforaphane, curcumin, fumaric acid and other chemicals (as previously detailed), that, as hormetic

paradigmatic cases, may induce adaptive stress response at subtoxic doses while may be toxic to mammalian cells at high concentrations [18]. However, even if the evidence regarding the hormetic dose-response induced by resveratrol is quite strong, the molecular mechanisms underlining this phenomenon, at present, are not fully understood and deserve greater attention in future research. Moreover, future studies should be focused in putting the findings described into a realistic biological framework to determine whether or not these effects are relevant for human health [361].

8.5. The potential hormetic role of carnitine and carnosine

In the fight against oxidative and nitrosative stress, a great scientific interest has been attracted by the ability of the natural peptides L-carnitine (and its acyl-carnitine ester, Acetyl-L-carnitine (ALC) (Fig. 9), and carnosine (Fig. 9) to decrease cellular oxidative stress, associated with aging, improving mitochondrial bioenergetics, scavenging radicals or functioning as proton buffers and metal chelators [25,362,363].

Although, as previously described for the phytochemicals, the antioxidant activity of these molecules might be partly responsible for their health benefits, recent findings support the activation of a series of vitagene pathways such as networks of genes encoding antioxidant enzymes, protein chaperones, neurotrophic factors and also the activation of the Keap1/Nrf2/ARE pathway as leading cytoprotective mechanisms [225,250].

The emerging evidence for biphasic dose response effects of environmental "toxins" in biological systems and the analog mechanisms of action demonstrated for phytochemicals, carnitine and carnosine suggests the possibility that these latter exert their function according to a biphasic dose response relationship.

8.5.1. Carnitine

L-Carnitine (LC) is a nonessential nutrient first described in the beginnings of the 20th century [364]. Mammals can synthesize LC in the liver, kidney and brain from two essential amino acids, lysine and methionine [365,366]. In humans, 75% of the total body carnitine comes from dietary sources, particularly red meat and dairy products [367]. L-Carnitine main role is in cellular energy metabolism. It has two major functions: (1) transport of long-chain fatty acids in the form of acylcarnitines from the cytosol into the mitochondria for their subsequent use as a source of energy (via acetyl-CoA formation in the process known as β -oxidation) and (2) the removal of short- and medium-chain fatty acids formed as a consequence of normal metabolism, preventing a toxic accumulation of these compounds in the mitochondria and leading to an increase of free CoA. In this context, L-Carnitine is essential in the equilibrium between acetyl-CoA (plus carnitine) and CoA (plus acetylcarnitine) (acetyl-CoA/CoA ratio) which is crucial for mitochondrial metabolism.

Both a decline in mitochondrial energetics and an increase in oxidative stress are some of the effects of aging. Regarding the oxidative insult, in vitro experiments reported antioxidant properties of acetyl-carnitine mediated by its iron chelating properties [368]. Comparatively, as a cellular antioxidant defense mechanisms, ALC decreased brain lipid peroxidation in old rats [369]. ALC may also prevent the age-associated changes to proteins. Oxidative modification to proteins appears to increase with age, as evidenced by increased levels of protein carbonyls, 3-nitrotyrosine and 4-hydroxynonenal [370]. Supplementing ALC to the older rats decreased many of these parameters in most of the brain regions investigated [370,371]. Pre-treatment of cortical neurons with ALC significantly reduced the HNE-associated cytotoxicity, protein and lipid oxidation, and apoptosis in a dose-dependent manner as well as increased cellular reduced glutathione and Hsps as compared to controls [372,373]. ALC treatment also leads to the activation of phosphoinositol-3 kinase (PI3K), PKG, and ERK1/2 pathways that are important in neuronal cell

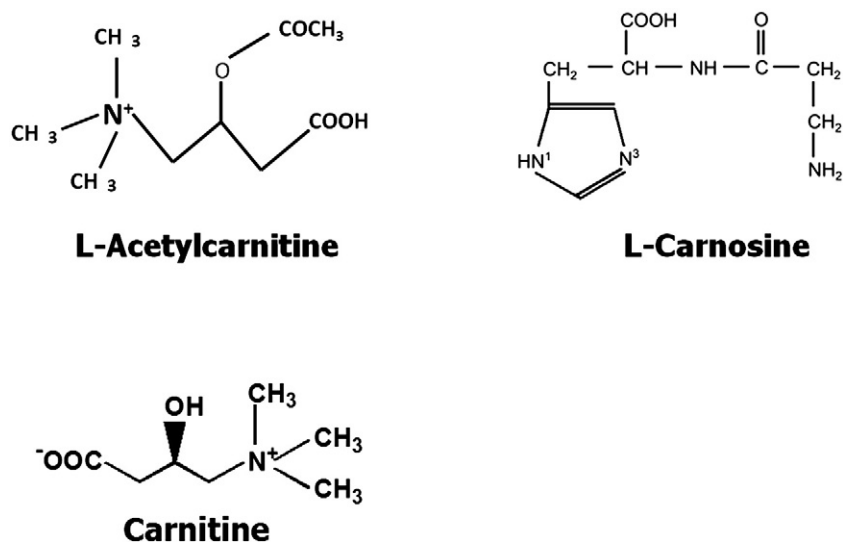


Fig. 9. Chemical structures of the L-carnitine, acetyl-L-carnitine and L-carnosine.

survival and differentiation [373]. ALC has also been found to protect against lipid peroxidation and membrane breakdown [374,375]. In streptozotocin-induced diabetic rats, treatment with ALC improved nerve conduction velocities (the speed of signal through the nerves), and this was associated with a reduction in elevated malonyldialdehyde (MDA) content, an indicator of lipid peroxidation [376]. When administered via the perfusate to ischemia-reperfused rat hearts, carnitine derivatives were able to scavenge peroxy or superoxide radicals [377].

As an ulterior confirm of the ALC stimulated endogenous cellular antioxidant defense, treatment of astrocytes with acetylcarnitine increased the amount and activity of heme oxygenase-1 (HO-1) [378]. In addition, pre-incubation of astrocytes with acetylcarnitine before the initiation of a nitrosative stress with lipopolysaccharide and interferon, prevented the decrease in complex IV activity, protein nitration and restored the reduced glutathione/oxidized glutathione ratio [378].

One of the mechanisms responsible of the decrease in oxidative stress is the protective effect of ALC on mitochondrial structure and function. ALC has been considered as a mitochondrial nutrient that protect mitochondrial dysfunction by different mechanisms, such as preventing oxidant production or scavenging free radicals (processes that prevent oxidative stress in mitochondria); enhancing mitochondrial antioxidant defenses; enhancing mitochondrial metabolism to facilitate both the repair of less damaged and the degradation of more damaged mitochondria, and protecting mitochondrial enzymes and/or stimulating enzyme activity as enzyme substrates and cofactors [379]. In this regard, pretreatment of pancreatic β -cells with micromolar concentrations of acetylcarnitine was demonstrated to protect the cells from oleic acid-induced mitochondrial dysfunction and decrease ROS production [380]. ALC increased hepatocellular oxygen consumption and partially reversed the decrease in mitochondrial membrane potential associated with aging, while reducing MDA levels and restoring hepatocellular ascorbate levels [362]. A recent study showed that acetylcarnitine reversed the age-related decrease in the activity of complex III and oxidative phosphorylation through complexes III and IV and increased the amount of cytochrome b and aa3 hemes in cardiac mitochondria isolated from old rats [381]. Of interest, both cytochrome b and aa3 proteins are encoded by the mitochondrial genome, suggesting that acetylcarnitine enhances either mtDNA transcription, the stability of mitochondrial mRNA, and mitochondrial protein synthesis. ALC has been demonstrated to improve mitochondrial respiration in neurons and to be neuroprotective through a variety of other effects such as the

increase in protein kinase-C activity [382]. Similarly to the phytochemicals above described, also ALC has been found to up-regulate HO-1 expression reducing the A β toxicity in primary cortical neuronal cultures [383] and to prevent age-related changes in mitochondrial respiration through the induction of HO-1, Hsp70, Hsp60 and Thioredoxin Reductase, and decreasing oxidative stress biomarkers in senescent rats [370–372,378,383–386]. The HO-1 up-regulation has been linked to activation of the transcription factor Nrf2 [378], implying the possibility that the ALC mediated acetylation of DNA-binding proteins, can induce post-translational modifications of critical target proteins involved in DNA competence and transactivating activity [37,51,371,378].

Interestingly, ALC and LC have been shown to reduce apoptosis through the mitochondrial pathway [388,390]. This anti-apoptotic effect has been observed in different cells, including neurons [390], myocytes [391], teratoma cells [392], hepatocytes [393,394] and lymphocytes [395]. Particularly, in neurons [396], cellular death induced by methamphetamine was reduced by high concentrations of acetylcarnitine. Acetylcarnitine protected also the dopaminergic system against the intraventricular injection of 1-methyl-4-phenylpyridinium (MPP⁺) in rats [397]. Less apoptosis was demonstrated in mouse fibroblasts treated with different concentrations of acetylcarnitine [389]. ALC and LC promoted neuronal survival and mitochondrial activity in addition to having anti-apoptotic effects in serum-deprived primary culture neurons [390]. In another tissue culture study, ALC treatment of TRX2-knockout DT40 cells suppressed oxidative stress in mitochondria, which prevented the mitochondrial signaling pathway leading to apoptosis [398]. The antiapoptotic effect of acetylcarnitine may be related to the overexpression and activation of HO-1, which increases the level of antiapoptotic bcl-2 protein and inactivates the pro-apoptotic transcription factor p53 in neurons [130]. Orally-supplemented acetylcarnitine in rats has been reported to decrease caspase activation by increasing the level of X-linked inhibitor of apoptosis protein (XIAP), thus limiting the mitochondrial-induced apoptosis in peripheral neurons [399]. Neither a protective effect on apoptosis induction nor a decrease in XIAP level was observed by these authors after carnitine administration, suggesting that the acetyl groups of acetylcarnitine have a fundamental role in protecting against mitochondrial-induced apoptosis.

ALC treatment has been found to produce several changes in gene expression [400–404]. An important role in the regulation of nuclear DNA transcription was recently attributed to the acetylation and deacetylation of core histone tails at lysine residues in consideration of the activation and repression of the gene transcription induced by

the two reactions, respectively [405,406]. The limiting factor for histone acetylation is the acetyl-CoA availability in the nucleocytoplasmic pool [407]. From the analysis of the Poly(A)⁺ RNAs isolated from brains of rats treated with ALC the expression of two genes resulted modified: the isoform c 14-3-3 protein, implicated in cell differentiation and growth [401,408,409] was up-regulated and the precursor mitochondrial P3 of ATP synthase lipid-binding protein, involved in transmembrane proton conduction, [410] was down-regulated. In addition, the gene coding for Hsp72 was up-regulated by treatment with ALC, which appears to help establish a cytoprotective state in inflammation, neurodegenerative disorders, and aging [385,401]. In addition to acetylation of histone proteins, site-specific acetylation of non-histone proteins plays an important role in transcriptional regulation. In particular, high mobility group (HMG)-box proteins are acetylated [411]. The mitochondrial transcription factor A (TFAM) required for mitochondrial DNA replication, contains two HMG-box-like domains. The total amount of TFAM increases in the liver, cerebellum and kidney with aging [412].

Acetylation can control the activity of mitochondrial enzymes, and possibly *de novo* synthesis of acetyl-CoA in mitochondria. Mitochondrial matrix acetyl-CoA synthetase is reversibly acetylated at a lysine residue in the active site of the enzyme. The deacetylation of this site induced by the nicotinamide adenine nucleotide (NAD⁺)-dependent deacetylase silent information regulators (sirtuin, SIRT3) activates the enzyme [48,413]. It is conceivable that this modification can alter protein conformation and consequently its function. Moreover, glutamate dehydrogenase is another target for both SIRT3 [414] and SIRT4 [415] and is inhibited upon deacetylation. As a result, cytochrome b content increases, leading to increased activity of electron transport chain (ETC.) complexes, associated with stimulation of oxidative phosphorylation [416].

Oral acetylcarnitine supplementation in rats increases soleus muscle mitochondrial content, nuclear transcripts of factors involved in mitochondrial biogenesis (PGC-1 α , NRF-1, TFAM), as well as the level of mitochondrial transcripts (COX I, ATP6, ND6, 16S rRNA), and prevents the unloading-induced downregulation of mRNA levels of kinases able to transduce metabolic (AMPK) and neuronal stimuli (CaMKII β) [400,417]. The activity and amount of HO-1 in cell culture increased by ALC treatment in a dose- and time-dependent manner [378]. Further, HO-1 was shown to increase mitochondrial biogenesis in cardiomyocytes via the transcriptional control of the nuclear respiratory factor-1 and 2 (NRF-1, 2) [52]. As a support of the ALC role in stabilizing mitochondrial transcripts or mitochondrial protein synthesis, recent data demonstrated that the decreased levels of a ribosomal RNA (12SrRNA) and a messenger RNA (mRNA for the subunit I of complex IV) were reversed in the brain and cardiac muscle of old rats 1 h after acetylcarnitine administration [418]. In addition, ALC treatment was able to contrast in heart mitochondria the reduction of cardiolipin content, the alteration of cholesterol/phospholipid ratio, the decrease of transport activity of adenylic nucleotides, pyruvate, phosphate and acylcarnitines [419–421]. Furthermore, the increase in the content of TFAM, which controls mitochondrial DNA transcription and translation, remains in skeletal muscle one month after the withdrawal from chronic acetylcarnitine supplementation, indicating the long-lasting effect of acetylcarnitine on mitochondria [422]. However, even if interesting findings are emerging relative to the protective action of carnitine and ALC, particularly in relation to leading role of different vitagenes in many of these effects, further research is needed to fully clarify the ALC mechanisms in balance oxidative stress according to a hormetic dose–response relationship and its potential cytoprotective role. In this context, the interesting study carried out by Calabrese et al. [378] demonstrated the ability of ALC to increase the expression of HO-1 in astrocytes both in absence or presence of an oxidative stress stimulus and this does not allow to extrapolate a definite conclusion regarding the role of ALC as a defense mechanism after an external damage of the homeostatic equilibrium

or as itself a preconditioning agent. Very importantly, the ALC capability to potentiate cellular stress response through the HO-1 pathway, appears to be a promising alternative therapeutic approach for those pathophysiological conditions where stimulation of this response is warranted [378].

8.5.2. Carnosine

Carnosine is a simple dipeptide, β -alanyl-L-histidine, that was isolated from Liebig's meat more than a century ago [423] and was subsequently classified as a histidine containing dipeptide by Krimberg [424,425], who demonstrated the hydrolysis of carnosine to its constituent amino acids (β -alanine and histidine) [426]. Carnosine is endogenously formed mainly in muscle and brain tissue, by bonding histidine and β -alanine in a reaction catalyzed by carnosine synthase [426]. It is present in vertebrate brain, cardiac muscle, kidney, stomach and high millimolar concentrations are found in the olfactory bulbs and in skeletal muscle [427–434]. Diet influences tissue carnosine concentrations [435–437]. Within the organism, carnosine breakdown is catalyzed by a specific carnosinase and a less specific homocarnosinase which are present in many tissues, with the exception of skeletal muscle [427,438,439]. The products of carnosine hydrolysis are metabolized and excreted in the urine. Many hypotheses have been made regarding the cellular action of carnosine as anti-oxidant and oxygen free radical scavenger, physiological buffer, histidine source, regulator of enzyme activity, metal-ion chelator, carbonyl scavenger, antiglycator, neurotransmitter, wound healing agent and immunostimulant [430,440–442]. Among antioxidant mechanisms reported for carnosine, its ability to inactivate reactive oxygen species, scavenge free radicals, such as hydroxyl, superoxide radicals and singlet molecular oxygen, and chelate pro-oxidative metals, like copper and iron has been reported [441,443–447]. It has been shown to delay aging in cultured human fibroblasts [448] and to be neuroprotective because of its ability to counteract both oxidative and nitrosative stress related to several pathological conditions including ischemia [449–452], methamphetamine neurotoxicity [453] and neurodegenerative disorders [454,455]. Regarding the mechanisms leading to oxidative stress response, carnosine, administered to astrocytes prior to the LPS/INF γ stimulus, prevented the up-regulation of iNos and the induction of both HO-1 and Hsp70 [387]. These data are in line with recent findings demonstrating that HO-1 can be repressed following oxidant conditions. In contrast to the general agree that HO-1 overexpression was a common feature during oxidative stress, recent works demonstrated that exposure to hypoxia, thermal stress, and interferon- γ was able to induce a marked HO-1 repression in human cells [51,141,456–458]. The usefulness of the HO-1 repression relies on the possibility to maintain an efficient metabolic balance during stressful conditions, decreasing the energy costs necessary for heme degradation, reducing the accumulation of CO and biliverdin which can become toxic when in excess, and increasing the intracellular content of heme necessary for the preservation of vital functions [459]. Unfortunately, the limited number of studies and the lack of data regarding the role of carnosine alone on the HO-1 induction prevent to fully clarify the role of this protein in the stress response.

A rather unusual reported antioxidant property of carnosine was its ability to reduce concentrations of thiobarbituric acid reactive substances (TBARS) when added to previously oxidized lipids [441,460,461]. If carnosine could interact with aldehydic lipid oxidation products, this could potentially help protect biological tissues from oxidation, since aldehydes can form adducts with DNA, proteins, enzymes, and lipoproteins, causing alterations in their biological activity [441]. In this regard, the protective anti-peroxidation function of carnosine has been demonstrated on a variety of biomolecules including proteins [447,462–465], lipids [464,466,467], DNA [466] and DNA bases [443]. Initial studies demonstrated that carnosine was an efficient hydrogen ion (H⁺) buffer over the physiological pH range

[426,468]. In muscle, where its concentration is highest, carnosine makes an important contribution to the maintenance of intracellular pH, which is vital for normal muscle function during intense exercise [469,470]. At weakly alkaline pH, carnosine is easily able to suppress lipid peroxidation [435,428]. This allows carnosine to maintain its suppression of peroxidation. In addition, carnosine also exhibits heavy metal ion binding properties, which inhibit some enzymatic reactions [427].

Carnosine has also been demonstrated to be an effective anti-glycating agent, at least in model systems and cultured cells [432]. The structure of carnosine closely resembles that of preferred glycation sites in proteins, that is, a target amino group with proximal imidazole and carboxyl groups. Carnosine itself could, sacrificially, react with many potential glycation agents thereby protecting other potential targets against glycation [442,471]. The carnosine ability to react with toxic aldehydes (e.g. acetaldehyde, formaldehyde, malondialdehyde and methylglyoxal), and deoxyribose has been shown to protect cultured human fibroblasts and lymphocytes, rat brain endothelial cells and Chinese hamster ovary cells, by inhibiting the protein–protein and DNA–protein cross linking induced by those substances [442,463,471–474]. Interestingly, the dipeptide has been shown to inhibit formation of protein carbonyls generated by the actions of oxygen free radicals and related species (reactive oxygen species) as well as aldehydes or ketones on amino acid side chains [471,475]. Moreover, it resulted also able to react with (i.e. carnosinylate) protein carbonyls to form protein–carbosyl–carnosine adducts, inhibiting further cross-linking to normal proteins [476]. Finally, carnosine can suppress AGE formation [463] and AGE-induced protein modification [473]. Proteotoxic stress, characterized by an accumulation of altered proteins derived from biosynthetic errors and/or deleterious post-synthetic polypeptide modifications, is a typical feature of aging conditions [87,477–480]. Carnosine is able to exert anti-aging actions, induces rejuvenating effects [448,481], and protects against telomere shortening in cultured human fibroblasts [482].

The dipeptide also extended the life-span of senescence-accelerated mice [483]. There are many possible mechanisms by which carnosine exert these anti-aging actions [463]. Among these, the suppression of mRNA translation initiation [484] lowers production of error-proteins, reduces the formation of protein carbonyls and increases the relative availability of chaperone and proteolytic activities for the recognition and elimination of altered proteins arising from deleterious post-synthetic modification [108]. Carnosine has the potential to inhibit much deleterious posttranslational polypeptide modification caused by oxidative [443,485–487], nitroxidative [387,488] and glycoxidative agents [461,463,472–474,489–493]. Moreover, carnosine may improve cellular ability to deal with aberrant proteins through the nitric oxide mediated upregulation of certain proteolytic functions [494–501,487–494]. It was demonstrated in carnosine-treated cultured human fibroblasts [463], cardiomyoblasts [502] and in cell-free extracts of rat brain [503]. Stress/chaperone proteins participate in the recognition and proteolytic elimination of altered proteins and their upregulation is associated with increased organism longevity and suppression of some age-related diseases [25,87,504]. Carnosine-zinc complexes (polaprezinc) stimulate expression of certain stress (heat-shock) proteins [505–507], which could improve cellular ability to deal with altered proteins and may again contribute to the dipeptide's apparent anti-aging activity.

In consideration of the carnosine properties described, the therapeutic role of carnosine in managing secondary diabetes complications, such as cataractogenesis and atherosclerosis should be deeply investigated [463,508]. In fact, these complications result from protein glycation and oxidation [509,510] mediated by agents and processes against which carnosine may, theoretically, protect [473]. Some preliminary supportive evidence from animal studies has been obtained [432,511–513]. Glycoxidation management is also

important in neurodegenerative conditions [514–520]. Chen et al. [521] suggest that carnosine could supplement glyoxalase's action, both by its aldehyde-scavenging action and its glyoxalase-mimetic activity. This capacity could also suppress aldehyde-mediated tau modification and consequent aggregation in a mouse model of Alzheimer's disease [522]. Carnosine treatment reduced inflammation and tissue damage in a mice animal model of spinal cord injuries [523]. Other experimental observation provided some support for the suggestion that carnosine could be useful in ameliorating aspects of Parkinson's, Alzheimer's diseases and also the consequences of dis-circulatory brain ischemia [455,523–532]. It has been suggested to be potentially useful for treating also autism [533], Down's syndrome [534], epilepsy [535] and aggregation processes. Further refinements have led to the synthesis of new carnosine derivatives able to survive to carnosinase hydrolytic activity and endowed with cytoprotective effects [536–538]. Interestingly, the different molecular behavior of carnitine and carnosine in response to similar stress stimuli, particularly in relation to the activation/repression of the Keap1/Nrf2/ARE pathway, should merit confirmation and deep future investigations. These differences, in fact, although not fully understood, may suggest that several factors could interplay in influencing the molecular mechanisms at the basis of the cellular defense response. Nevertheless, the discoveries that molecules, which are toxic at high doses, play fundamental or protective roles in cellular signaling or metabolism at low concentrations, suggest that evolution triggered organisms to include environmental toxins for an advantageous use [51]. Thus, it seems important to plan future researches to deeply investigate the leading mechanisms induced by exogenous small molecules in response to stressful conditions. It may be a valid tool to develop interventions able to activate hormetic signaling pathways in cells as new approaches for the prevention and treatment of a wide range of pathological disorders.

9. Conclusion and future directions

Due to considerable improvement in medical and environmental factors, in the past few hundred years, average human lifespan has increased substantially, bringing demographic change in most industrialized countries. Nevertheless, despite our achievements in average lifespan, the maximal lifespan of humans remains unchanged as the latter may be genetically programmed or determined by inevitable deleterious cell changes that occur with age. These changes are typical of the senescent phenotype and eventually lead to death. Many types of interventions, including genetic manipulations and caloric restriction, appear to increase the maximal life span in animal models, but it is not yet clear whether the results from these animal experiments will contribute to lengthening the human life span. Among the hypotheses put forward to explain the biological processes underlying senescence, one theory suggests that ROS formation is principally responsible for cell damage and senescence [539,540]. The deleterious effects of ROS could slowly accumulate over years and lead to dysfunction of a number of cell functions. The mitochondrion is the main organelle for the production of ROS. Although energy generation in the mitochondrion is extremely important for survival, mitochondrial ROS production also has some negative consequences for age-related intracellular changes. However, ROS is not only a waste of oxidative phosphorylation in the respiratory chain but is also highly regulated signal molecules involved in cellular stress response [541]. Cells and organisms, subjected to chronic proteotoxic stress associated with diseases, undergo a global decline in cellular function with negative consequences on viability. This increases the levels of damaged proteins, resulting in a decline in multiple biosynthetic and repair activities and, over time, negatively affects health and aging of the organism. Much evidence in the literature supports the theory that molecular determinants of longevity influence proteotoxicity, suggesting that the integrity of the

proteome is a vital mechanism for ensuring the efficient functioning of the cell and the healthy state of biological processes throughout the life span of the organism. This is achieved by a complex network of longevity assurance processes which are controlled by vitagenes, a group of genes involved in preserving cellular homeostasis during stressful conditions [542]. This network responds to the age related mitochondrial dysfunction and is modulated by mitochondrially produced ROS [51,543–545]. Thus, by sensing the nutritional status of the whole organism as well as the intracellular nutrient and energy status, functional state of mitochondria, and mitochondrial ROS concentration, the longevity network regulates life span across species by coordinating information flow along its signaling pathways. Given the unique roles of HSF1 and Nrf2/keap1 in stress biology and proteostasis, enhanced activity of these principal regulators during development and early adulthood is important for the stability of the proteome and the health of the cell. To this end, the crosstalk between distinct HSFs as well as between HSF1 and Nrf2/Keap1 pathway, only recently uncovered, raises obvious questions about the stoichiometry between the components in different complexes residing in different cellular compartments, and the mechanisms by which the factors interact with each other. Interaction between members of the vitagene system, HSFs and Nrf2/Keap1-related, could lead to new therapeutic approaches for protein-folding diseases, metabolic disorders and cancer, as well as healthy aging.

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