Redox modification of proteins as essential mediators of CNS autophagy and mitophagy

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Abstract

Production of cellular reactive oxygen species (ROS) is typically associated with protein and DNA damage, toxicity, and death. However, ROS are also essential regulators of signaling and work in concert with redox-sensitive proteins to regulate cell homeostasis during stress. In this review, we focus on the redox regulation of mitophagy, a process that contributes to energetic tone as well as mitochondrial form and function. Mitophagy has been increasingly implicated in diseases including Parkinson's, Amyotrophic Lateral Sclerosis, and cancer. Although these disease states employ different genetic mutations, they share the common factors of redox dysregulation and autophagic signaling. This review highlights key redox sensitive signaling molecules which can enhance neuronal survival by promoting temporally and spatially controlled autophagic signaling and mitophagy.

Keywords

1. Introduction

Reactive oxygen species (ROS) and other free radicals are typically associated with stress, DNA and protein damage, and used as a marker of irrevocable damage and cell death. It has become increasingly clear, however, that free radicals function as important signaling molecules in response to stress [1,2] and modify protein structure and function in ways that are essential to adaptation and survival. In this chapter we will discuss ROS production as a consequence of aerobic respiration, how mitophagic containment of injured organelles

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promotes cell survival and how impaired redox regulation of autophagic signaling molecules leads to cell dysfunction.

Eukaryotic cells have evolved symbiotic relationships with mitochondria to enhance energetic status and cellular and organismal complexity. On average, eukaryotic cells contain one billion ATP molecules, and the turnover from ATP to ADP and back occurs approximately three times per minute [3]. The central nervous system (CNS) relies on ATP generated primarily by aerobic respiration and, as reviewed by Erecinska and Silver, the brain at rest utilizes over 20% of total oxygen consumption and 0.3–0.8 μmol of glucose per gram of weight per minute (μmol/g/min). The resulting ATP production in neurons from consumed oxygen and glucose at rest is approximately 25–32 μmol/g/min [4]. Neurons require high levels of ATP – as much as 20–25 μmol/g/min – for maintaining membrane polarity, rapid intra- and inter-cellular signaling, and synaptic transmission. For example, neurons contain 6250 ρmol of the Na^+/K^+ pump/g of tissue (cardiac muscle contains only 1500–2000 ρmol/g) [5] with the highest activity reported in synaptosomes [6]. Neurons need 50–60% of total oxygen delivered to the brain and 12–16 μmol ATP/g/min [4] to maintain ion homeostasis alone. While the liver and heart consume more net ATP, the only organ with comparable levels of energy expenditure for membrane polarity and ionic balance is the kidney [7].

In addition to being the primary source of energy production in the form of ATP, mitochondria produce the majority of free radicals and other ROS. In studies measuring superoxide anions ($O_2^{-\bullet}$) in rat lung slices, about 9% of total oxygen uptake resulted in $O_2^{-\bullet}$ formation, while other studies suggest that about 1–3% of oxygen used in the mitochondria alone form $O_2^{-\bullet}$. Neurons, especially those that are most active and excitatory, have higher oxidative metabolic activity and higher cristae packing density of mitochondria than glia and other cell types [8]. Mitochondria are densely packed in extended dendrites and axon terminals. The fact that neurons are largely post mitotic places these cells under intense pressure to promote appropriate mitochondrial biogenesis fusion, fission, and mitophagy. Seminal work by Chang and Reynolds in 2006 aimed to measure the turnover rate and number of mitochondria at baseline in neurons. Interestingly, they found that mitochondria morphology and trafficking is dependent on the age of neurons [9]. For instance, a mature neuron at 14 days *in vitro* (DIV14) will contain a similar amount of mitochondria as a young neuron at DIV5 (~0.08 mitochondria/μm); however, mitochondria in mature neurons are elongated and form reticular networks, whereas in young neurons, mitochondria are shorter and more motile [9]. This difference in mitochondrial morphology with respect to neuron maturity points to the temporal control of proteins governing mitochondrial dynamics. David Chan pioneered work a decade ago demonstrating that fusion and fission of mitochondria is coordinated by essential proteins which are exquisitely sensitive to genetic mutations and cellular stress [10]. In addition to maladaptive changes in fusion and fission molecules, functional changes in these proteins by redox modification have increasingly been appreciated as central regulators of mitochondrial function.

In the last five years, engulfment of damaged mitochondria by molecules linked to autophagy has played a more prominent role with respect to understanding the pathological cascades associated with Parkinson's disease, Amyotrophic Lateral Sclerosis (ALS),

Alzheimer's disease and other disorders. Autophagy is a highly controlled process in which cells engulf proteins and organelles within intracellular, lipid bilayer compartments and degrade contents after fusion with lysosomes. The process was initially described in yeast and now hundreds of proteins have been associated with this highly complex pathway [11–13]. Autophagic signaling can be promoted in response to a wide variety of stressors including ionic dysfunction, protein aggregation and proteasome failure, energetic stress and oxygen deprivation. Autophagy can also be targeted toward specific organelles – notably the mitochondria – where they can undergo engulfment and digestion; a process that is important given that mitochondria are both one of the primary sources and sensitive targets of ROS. Moreover, mitophagy may be particularly sensitive to ROS given that antioxidants, such as glutathione, can block mitophagy while autophagy continues, as revealed in yeast models [14].

2. Reactive oxygen species

2.1. ROS formation

Free radicals are a class of small molecules that contain one or more unpaired electrons, rendering them highly reactive; those derived from oxygen molecules are called reactive oxygen species (ROS), and those from nitric oxide, reactive nitrogen species (RNS). ROS are generated by a number of reactions promoted by xanthine oxidase, microsomal enzymes, cyclooxygenase and lipoxygenase as well as more abundant mechanisms including ROS produced by NADPH oxidases and the electron transport chain (ETC) [15].

The activity of NADPH oxidases, membrane-associated enzymes that utilize oxygen to catalyze the production of $O_2^{-\bullet}$, is essential for engulfment of injured cells and innate immunity. NADPH oxidase deficiency is associated with X-linked chronic granulomatous disease, a devastating disorder which leaves patients with poor immune function [16]. Assembly of the NADPH complex at lysosomes produces a large increase in oxygen consumption, referred to as a "respiratory burst". In response, superoxide dismutase (SOD) converts $O_2^{-\bullet}$ into hydrogen peroxide (H_2O_2), which kills off the engulfed cells or particles. In addition, high concentrations of catalase to detoxify H_2O_2, as in erythrocytes, are able to protect other cells by absorbing diffused H_2O_2 [17] Another mechanism for $O_2^{-\bullet}$ clearance is via reaction with nitric oxide to produce peroxynitrite, which can subsequently be catalyzed to form hydroxyl radicals.

The major source of ROS in aerobic cells, however, is cellular respiration and oxidative phosphorylation within the mitochondria. In the process of transferring electrons between the protein complexes that mediate phosphorylation of ADP to ATP and reducing oxygen to water, the ETC generates free radicals. Electrons that escape the ETC primarily through mitochondrial complexes I and III transfer directly to oxygen to create $O_2^{-\bullet}$ [18–20]. Due to the high demand of ATP and increased oxidative phosphorylation to meet that demand, neurons are particularly prone to generating concentrated amounts of ROS. The intracellular environment of neurons is thus highly reducing and, as will be further discussed, contains various mechanisms to combat oxidation.

2.2. ROS modify specific amino acids

The location of amino acids within proteins is a central regulator of protein function. Post-translational modifications, such as lipidation or glycosylation, at specific amino acids alter protein behavior through inactivation or enhancing activity or binding with other proteins. Similarly, ROS serve as reversible post-translational modifiers of protein structure and function. Amino acid oxidation can alter protein binding, interaction, and translocation or confer previously unknown properties and binding partners. It is helpful to realize that much like post-translational modification by phosphorylation, the structure and availability of amino acids determines their vulnerability to ROS-mediated modifications.

Thiol groups are organosulfur compound*s* that contain a carbon-bonded sulfhydryl (–C–SH or R–SH) group (where R represents an alkane, alkene, or other carbon-containing group of atoms). Proteins which contain these compounds are most easily oxidized by ROS, RNS, and some metal ions. Oxidized thiol groups can proceed to form thiyl radicals or disulphides. In fact, a third of proteins synthesized by neurons contain disulfide bonds [21] that are catalyzed by protein disulfide isomerases in the endoplasmic reticulum [22].

The amino acids cysteine and methionine contain reactive thiol groups that confer redox sensitivity to proteins. Similarly, the thiol containing amino acid histidine makes it a target for singlet O_2 reactions. Other less reactive amino acids include tyrosine, phenylalanine, valine, proline, arginine, and tryptophan which can be oxidized by oxygen radicals, forming peroxides and peroxyl radicals [23]. Interaction with ROS causes these residues to break down and generate highly reactive intermediate radicals.

While beyond the scope of this review, it should be noted that reactive nitrogen species, such as nitric oxide, are also able to post-translationally modify proteins to regulate their function by S-nitrosylation [24]. Using site-specific mapping of S-nitrosylation of cysteine residues in tissues of healthy mice, a recent study identified a substantial number of oxidatively modified proteins that regulate oxidative phosphorylation, glycolysis, and other pathways for metabolism [25]. The authors report that 20–25% of S-nitrosylated proteins were mitochondrial proteins in brain, liver, and kidney tissues compared to 56% in heart tissue. In addition, when comparing modified proteomes between wildtype and endothelial nitric oxide synthase (eNOS) knockout mice, the group found that mitochondrial proteomes were more than 70% dependent on eNOS activity for S-nitrosylation, suggesting an important role for RNS in normal metabolic regulation. Recent reviews [26,27] can be referenced for more information on these molecules and the link between NO formation and inhibition of autophaghosome formation in neurons [28].

2.3. ROS regulation & redox homeostasis

The tight regulation of ROS is key to energetic status, cell signaling and responses to stress. The ways in which cells regulate redox state include general mechanisms associated with increasing the activity of antioxidants, as well as utilizing redox-sensitive enzymes to decrease ROS (summarized in Table 1). Furthermore, tight spatial constraints on ROS can be enforced by organelles and protein complexes, which localize ROS for efficient removal by co-localized antioxidants, such as thioredoxin and glutathione.

2.3.1. Endogenous non-enzymatic antioxidants reduce intracellular ROS—The tripeptide glutathione (GSH; γ–L-Glutamyl-L-cysteinylglycine) is the most abundant low molecular weight thiol in mammalian cells and constitutes a major cellular defense against ROS. In the process of reduction, GSH is oxidized into glutathione disulfide (GSSG) and can be reduced back to GSH via glutathione reductase. In most cell types, the rate-limiting step for increasing GSH synthesis is the uptake of cysteine. Interestingly, the CNS maintains an enhanced GSH pool through enriched expression of the cysteine/glutamate exchanger (xCT) in meningeal cells, as well as in both neurons and astrocytes in cerebral cortex [29] offering enhanced redox buffering in the CNS. GSH primarily acts as a ROS scavenger, directly reducing hydroxyl radicals and superoxides. In addition, GSH provides an essential function in the maintenance of other reducing enzymes, such as vitamin C and E, by reducing them to their active states.

While the redox status of neurons and other cells tends to be viewed as a homogeneous environment, quite the opposite is true. Thioredoxin (Trx) is a family of proteins that act as endogenous reducing agents with an oxidizable dithiol active site that, after activation, can be reduced back by thioredoxin reductase. Each member of the Trx family exhibits specific intracellular localization. For instance, Trx-1, the most abundant Trx protein in eukaryotes, is present in both the cytosol and nucleus, while Trx-2 localizes to mitochondria. Although they exist at a low intracellular concentration – on the order of μmol – the Trx proteins may be more specific than GSH in its rapid response to certain toxicants. In response to H_2O_2, hypoxia [30], ischemic-reperfusion injury [31,32], radiation [33,34], and other toxicants that increase intracellular free radicals, Trx-1 translocates into the nucleus where it can directly or indirectly associate with transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [35] and activator protein 1 (AP-1) [36]. This compartmentalization of Trx may serve as a discreet mechanism of protection, functioning as a ROS scavenger at the nucleus and as a regulator of gene expression under stress. Also contributing to compartmentalized areas of ROS regulation, the mitochondria-specific Trx-2 interacts with components of the ETC and is thought to play a role in regulating mitochondrial membrane potential [37,38] and mitochondrial permeability transition pore activity [39]. Furthermore, Trx-2 directly reduces H_2O_2 as a peroxiredoxin cofactor, whereas GSH contributes minimally to H_2O_2 clearance [40,41]. Thus, despite minute overall concentration compared to GSH, Trx serves as an essential redox regulator through distinct antioxidant roles in mitochondria and nuclei, as well as in specific peroxide clearance.

2.3.2. Redox-sensitive enzymes catalyze antioxidant processes—In addition to non-catalytic antioxidants, several catalytic proteins also contribute to the regulation of redox homeostasis. The SOD family of enzymes, first discovered by Fridovich and McCord [42] are metal coordinating enzymes that play major roles in the enzymatic reduction of $O_2^{-\bullet}$ into oxygen and H_2O_2. Cytoplasmic copper/zinc superoxide dismutase (CuZnSOD) and mitochondria-localized manganese superoxide dismutase (MnSOD) are both active participants in this process. Both enzymes are highly expressed in neurons, and overexpression of either enzyme decreases tissue damage after focal ischemia in animal models [43,44]. Furthermore, mutations in these enzymes lead to deficiencies in buffering ROS and are linked to familial forms of ALS [45].

Catalases are a group of proteins that consist of four protein subunits that contain a buried heme group. Molecules small enough to travel through narrow channels leading to the heme center are then reduced. Catalases primarily reduce small molecules such as H_2O_2, utilizing a reaction mechanism by which one H_2O_2 is reduced to a water molecule and another oxidized to an oxygen molecule. Catalases are especially active within peroxisomes, which contain enzymes that create H_2O_2. Subcellular localization of catalases at organelles of high H_2O_2 production highlights the ways in which cells tightly regulate redox state. However, mitochondria contain very little catalase, thus any H_2O_2 generated by the mitochondria must either diffuse to peroxisomes or rely on other antioxidant defenses [46].

Another enzymatic antioxidant protein is glutathione peroxidase (GPx) that removes H_2O_2 by reducing it to a water molecule and simultaneously oxidizing a reduced glutathione. Like GSH, GPx is localized primarily to the cytosol but it is also found in mitochondria, which would assist in the removal of H_2O_2 generated from this organelle.

Localized concentrations of antioxidants may also play a significant role in mitophagic signaling. Interestingly, ethacrynic acid-induced depletion of GSH promoted mitophagy in yeast under conditions of starvation [14]. Conversely, increases in the GSH pool by addition of the antioxidant N-acetyl-L-cysteine prevented mitophagy during starvation, but did not impact autophagy [14]. The authors hypothesized that mitophagy was being impacted by the spatial and temporal and localization of GSH.

3. Redox regulation of autophagic signaling

The activation of autophagy and mitophagy is tightly regulated by molecular rearrangement, such as protein conformational changes and complex formation, and protein localization to initiate the transcription of autophagy related genes (Atgs). Increased generation of ROS promotes autophagy via oxidative modification of proteins involved in either the autophagic machinery or in signaling pathways that enhance autophagy.

3.1. Oxidatively-modified proteins change function to promote autophagy

3.1.1. ROS inhibition of PTEN increases Akt signal transduction to regulate FoxO transcription factors—Cellular redox status impacts lipids and protein signal transduction much in the same way that other forms of post-translational modifications do. As modifiers of protein activity, oxidative modifications impact neuronal mitophagy and potentially serve as essential regulators of cell growth, signaling and fate.

One example of the impact of redox status on cell fate decisions is the effect of ROS on growth factor mediated signaling. Molecules such as vascular endothelial growth factor (VEGF) and neuronal growth factor (NGF) activate signaling cascades to promote cell survival signaling by activation of the PI3K/Akt pathway [47,48]. Activation of the PI3K/Akt pathway promotes transcription of pro-survival genes, and the main regulatory member of this cascade is Phosphatase and Tensin homologue (PTEN), a tyrosine phosphatase that normally dephosphorylates PI3K, decreasing Akt signal transduction. Akt is a serine/threonine-specific protein kinase which promotes cell survival by inhibition of

apoptosis and activation of transcription factors that increase expression of genes that regulate cell survival, proliferation, metabolism, and migration [49].

PTEN is oxidatively modified by H_2O_2, which produces a disulfide bond in the PTEN active site. Oxidation of PTEN by H_2O_2 leads to reversible inactivation of PTEN activity, prolonging PI3K and Akt association – an effect that promotes cell survival in the presence of high H_2O_2 concentrations *in vitro* [50] and in *in vivo* models of ischemia where the reperfusion stress of flooding a system with oxygen causes a burst of ROS [51]. Additionally, studies in which Akt signaling is inhibited demonstrate that mitochondrial derived ROS production increases promoting autophagy [52]. Moreover, modification of Akt by ROS can be adaptive by increasing the nuclear export of Forkhead box protein O1 (FoxO1), driving the production of antioxidant enzyme Trx. This signaling loop combats further increases in ROS [50] and enhances the activity of Atg family proteins and facilitating autophagic signaling.

In addition to the indirect effects of PTEN modification on Akt, the Forkhead family of proteins also responds to Akt signaling. The Forkhead transcriptional regulators play diverse roles in eukaryotic cell growth, differentiation, and survival. Indeed, the mammalian FoxO family was initially identified as essential tumor suppressors [53,54] and are direct substrates of Akt. When Akt phosphorylates FoxO transcription factors, both proteins are exported from the nucleus to the cytoplasm via a nuclear export sequence. Following Akt phosphorylation, FoxOs interact with 14-3-3 proteins promoting the cytoplasmic localization of FoxO and alterations in cell signaling independent of DNA binding. Direct interacting partners of FoxOs include other transcription factors, such as β-catenin and Smad, HOX proteins, and hormone receptors [55].

Engagement of FoxO transcription has been increasingly associated with the induction of autophagy-related genes, underscoring the importance of these proteins and the autophagic pathway in mutagenesis and oncogenic transformation [56]. FoxO3 upregulates genes such as Beclin 1, ATG12, ATG4, LC3, and others, with essential roles in autophagic recognition, containment, engulfment and digestion [57]. FoxO3 is a positive regulator of BNIP3 expression – a mitochondrially associated protein that promotes autophagosome formation in cell culture models of skeletal muscle atrophy [57] as well as mitophagy in animal models of hypoxia [58].

In yeast, FoxO1 stimulates autophagy via interaction with Atg7 upon acetylation by Sirtuin-2, a redox sensitive transcription factor [59]. Using mammalian cells, the same group found FoxO3 promotes FoxO1-mediated autophagy, wherein FoxO3 upregulates PI3K subunit expression, thus increasing Akt activity [60]. In consequence, FoxO1 is exported from the nucleus, where it then promotes its pro-autophagic activity [59,60]. Taken together, these data support a model in which redox status of the cell is capable of directly modifying a sentinel protein to promote autophagy via growth factor engagement, kinase activation, transcriptional regulation and subcellular relocalization of proteins and protein complexes (Fig. 1).

3.2. ROS regulate ATG activity

The *ATG* genes in yeast and respective homologues in mammals are the primary initiators and regulators of autophagy. Seminal work in yeast has shown that nutrient depletion causes vacuoles to fill with autophagosomes, a process initiated by inactivation of Tor, and that mutations of the *ATG* gene family disrupted that process [61–63]. Nutrient depletion has not only been associated with increased autophagic signaling, but also with changes in redox homeostasis and oxidative stress. Eisler et al. showed that prolonged amino-acid depletion induces apoptosis, as well as high concentrations of ROS. The group also showed that 40% of starving cells survived despite having high ROS, indicating that ROS is not a consequence of apoptosis, but rather a signal to initiate this process [64].

ROS is also a signaling mechanism for autophagy during acute starvation. In *in vitro* models of acute starvation, Scherz-Shouval et al. demonstrated that autophagosome formation depended on increases in ROS. Specifically, the activity of Atg4, a cysteine protease, was shown to be inhibited by treatment with H_2O_2. Under ideal growth conditions, Atg4 removes an arginine residue of Atg8, allowing Atg8 to be activated by Atg7. With H_2O_2 treatment Atg4 becomes oxidized at specific cysteine residues, rendering it inactive and allowing Atg8 to conjugate with autophagosomes [65].

Autophagy and mitophagy share some similar proteins, but others, such as Atg32, a newly identified mitochondrial outer membrane protein which interacts with Atg11, promotes the autophagy machinery specifically for mitophagy [66,67]. Notably, no metazoan homologues of Atg32 have been identified, but other molecules that work with Atg homologues in eukaryotic organisms such as NIX (also called BNIP3L), integrate redox sensitivity with organelle degradation mediated by Atg8 [68,69].

3.3. Oxidative modification of proteins that alter mitochondrial bioenergetic function

Some of the earliest and most well characterized molecules that have been linked to oxidative stress signaling include the serine kinase p66[shc]. This protein is a member of the Shc family which plays important roles in oncogenic transformation and cell signaling. Unlike other Shc family members, p66[shc] has evolved a glycine and proline-rich second collagen homology domain (CH2), which confers a unique ability to sense redox stress. Phosphorylation at serines 36 and 54 within the CH2 domain in response to oxidative stress results in changes in protein structure required for p66[shc]'s ability to serve as a redox sensing signaling molecule. In addition, cysteine 59 in the CH2 domain is the target of reversible, specific amino acid oxidation that promotes the bioactivity of p66[shc] allowing the formation of tetramers [70]. Polymorphisms in the protein are linked with long life [71], and mice that lack p66[shc] live 30% longer than wildtype animals [72] and have a significantly decreased redox burden as they age.

Phosphorylation of Ser36 of p66[shc] is promoted by protein kinase Cβ. Once phosphorylated, this redox sensitive molecule binds to Pin1, a prolyl hydroxylase [73]. p66[shc] then relocalizes to the intermembrane space of the mitochondria. p66[shc] activation in response to oxidative stress is rapid; our group has found Ser36 phosphorylation of p66[shc] occurs within 30 min after sublethal oxygen and glucose deprivation in neurons and mitochondrial

localization is maximal 2–3 h after initiation of stress [2]. Once localized to mitochondria, p66[shc] oxidizes cytochrome c in cell free assays which is thought to promote ETC production of H_2O_2, and impair the ability of mitochondria to buffer calcium [74].

While these effects of p66[shc] activity have largely been associated with apoptosis, our group has identified p66[shc] as a discrete but essential mediator of neuronal metabolic tone and autophagosome formation as an adaptive mechanism to promote the removal of injured organelles following low level oxygen and glucose deprivation [2]. Indeed, interruption of p66[shc] activity during mild stress profoundly increased lipid and protein injury as well as the number of and autophagosomes that form. These events were all correlated with poor outcomes for neurons and a reduction in the ability to upregulate the expression of neuroprotective chaperone proteins [2]. This data supports a model in which we have come to appreciate that oxidative modification of proteins such as p66[shc] is not synonymous with cell death, but this modification serves as a rapid means to promote communication from the cytosol back to the mitochondria. Indeed, localization to mitochondria of activated p66[shc] suggests this protein may be one of several molecules that temporally and spatially mediate mitochondria homeostasis, as well as mitophagy.

3.4. Oxidatively-modified transcription factors alter activity to promote autophagy

3.4.1. SIRT1 is oxidatively modified to regulate autophagy—While cytosolic signaling molecules like Akt and p66[shc] promote unique redistribution in response to ROS, nuclear transcription factors also play essential roles in autophagic regulation. Notably, mammalian sirtuins function as important regulators of metabolism, cell survival, growth, and senescence. Sirtuins – mammalian homologues of yeast silent information regulator 2 (Sir2) – are Class III histone deacetylases that respond to a variety of stressors and are involved in protection against several diseases and aging. The most well-studied is Sirtuin 1 (SIRT1). Several seminal studies point to SIRT1's capability of attenuating age-related deficiencies and protecting against oxidative stress [75].

SIRT1 mRNA is especially high in the developing CNS and heart. While most sirtuins are expressed at high levels in the brain, the most extensively studied are SIRT1[76], which is primarily located in neurons, and SIRT2, which is primarily located in oligodendrocytes and Schwann cells [75]. SIRT1-deficient mice exhibit profound developmental abnormalities, including cardiac defects, exencephaly, and perturbed retinal histology [77]. Tissues cultured from these mice contain heavily damaged mitochondria and impaired metabolic function [78]. SIRT1 deacetylase also complexes with Atg5, Atg7, and Atg8 leading to comparisons between the SIRT1$^{-/-}$ phenotype with ATG5$^{-/-}$ cells and animals [78,79]. Transgenic animals deficient in either protein displayed similar deficiencies in autophagy, lending the idea that SIRT1 deacetylase activity may be important for Atg function.

SIRT1 also acts as an indirect regulator of autophagic processes via regulation of FoxO and p53 (Fig. 1). Sirt1 forms a complex with FoxO3 in response to oxidative stress, deacetylating FoxO3 to further promote cell survival via increased DNA binding and activation of FoxO3 target genes [80].

3.3.2. ROS can increase HIF1α-mediated autophagic signaling and mitophagy

—A cell's ability to sense intracellular oxygen levels is critical for cell survival and adaptation. The primary sensors of oxygen levels within the cell are prolyl hydroxylase domain-containing enzymes (PHDs) and hypoxia-inducible factors (HIFs) [81,82]. When oxygen supply is ideal, PHDs hydroxylate proline residues on HIFα subunits [83]. Hydroxylated HIF is then ubiquitinated by the E3 ubiquitin ligase von Hippel-Lindau protein (pVHL) and is subsequently degraded. In low oxygen conditions, however, PHDs decrease activity, stabilizing HIF and inducing transcription of target genes such as vascular endothelial growth factor, cyclooxygenase-2, and erythropoietin [84].

Several studies demonstrated that mitochondrial ROS regulate HIF proteins at the level of pVHL induced degradation and expression of genes regulated by HIF such as metallothionein. Using both pharmacological and genetic techniques, Brunelle and colleagues subjected HEK293 cells to hypoxia following inactivation of mitochondrial complex III and demonstrated a rapid increase in HIF1α degradation [85]. Although ROS is not required for HIF transcription, the authors of these studies posit that ROS generated by the mitochondria left shifted the dose response curve of oxygen to prolyl hydroxylase activity [86]. In addition, a separate study found that a functional ETC and mitochondria-derived ROS are necessary for hypoxia-induced HIF1α stabilization [87].

This model, however, is limited to mild hypoxia. During periods of anoxia, HIF can be stabilized in the absence ROS. Other studies provide evidence for HIF1α as an essential cofactor for metallothionein gene transcription in response to hypoxic stress [88]. Metallothionein proteins are potent oxidant scavengers which regulate heavy metal sequestration and metabolism. Triggers such as NO promote the release of zinc from metallothioneins which can be a potent means to induce apoptotic cell death in neurons. Furthermore, elevated levels of ROS following hypoxic stress can stimulate zinc release from metallothioneines, which inhibits further HIF1α activity.

HIFs are essential transcriptional regulators of genes with essential functions in energy metabolism and vascular remodeling which optimize available oxygen and glucose. HIF stabilization also aides in autophagic signaling via transcription of BNIP3 and BNIP3L, which are members of the so-called BH3-only subfamily of Bcl-2 family proteins that heterodimerize and antagonize the activity of the largely prosurvival proteins (Bcl-2 and Bcl-X$_L$), Semenza and colleagues presented convincing evidence that hypoxia-induced HIF1α stabilization and regulation of BNIP3 reduced the numberof mitochondria in hypoxic cells through mitophagy [89]. The mechanism proposed involves elevated BNIP3 in competition with Beclin-1 for binding with Bcl2. Bound Bcl2–BNIP3 increases levels of free Beclin-1, which then triggers formation of autophagosomes. Similar reports from other groups revealed that both BNIP3 and BNIP3L are crucial for hypoxia-induced autophagy through disruption of Bcl2-Beclin1 binding [90]. Additionally, rodent models of hypoxia tolerance demonstrate that BNIP3 expression is strongly correlated with mitochondrial clearance and mitophagic signaling in cardiac tissue depending on the degree of hypoxia [58].

Taken together, redox status of the cell is a key factor in determining HIF1α stability and thus regulation of downstream modulators of autophagy and mitophagy (Fig. 2).

Although these and other studies of hypoxia-induced signaling have been essential to understanding cellular response to oxygen levels and ROS, it is important to remember that neurons rely heavily on oxygen levels, which in turn promotes high levels of ROS within the CNS. This effect is compounded by the largely post-mitotic nature of these cells. Although hypoxic conditions disrupt neuronal metabolic and redox tone, not all neurons are equally vulnerable to hypoxia; for instance, CA1 hippocampal neurons are some of the most sensitive [91]. At sea level, atmospheric oxygen is approximately 20%, but oxygen present *in vivo* in tissue is lower than ambient oxygen [92]. Furthermore, levels of oxygen vary between tissue *in vivo* (20–40 mmHg) [93], cells *in vivo* (1–10 mmHg) and cells grown in culture (150 mmHg) [94], providing yet another caveat to studying the consequences of hypoxic stress.

Therefore, in attempting to capture the CNS responses to hypoxia, our group – among others – is particularly cautious in using immortalized cell lines. These mitotic cultures are primarily glycolytic, although they can be forced to rely on oxidative phosphorylation for ATP production by substituting glucose with galactose [95]. This discrepancy makes the use of primary cultures for the investigation of hypoxia-dependent signaling far more appealing from a physiological standpoint.

4. Conclusions

In summary, ROS-mediated signaling may occur independently of cell death mechanisms and may instead promote survival by activating specific regulators of mitophagy and autophagy. ROS working in concert with target redox-sensitive proteins is another facet of intracellular signaling that impacts the ways in which we study neuropathological diseases associated with high concentrations of ROS. In order to expand this area of study, better methods for differentiating between mitophagy and autophagy are needed. Klionsky and colleagues recently released guidelines for studying autophagy [96], yet the identification of methods specifically targeting mitophagy is limited. As for studying ROS-mediated signaling, many groups have taken advantage of redox sensors, such as dyes and fluorescent markers, as well as free radical spin traps to inhibit ROS [97]. Additionally, novel methods for isolating redox-sensitive proteins can be coupled with mass spectrometry to identify proteins oxidized at specific amino acids [98,99]. This method holds promise in pin-pointing modified protein targets that would affect the pathways involved following cellular stress. Furthermore, the connection between *in vivo* and *in vitro* disease models for oxidative stress and neuropathological disease must be re-evaluated and tuned more toward recapitulation of physiological conditions.

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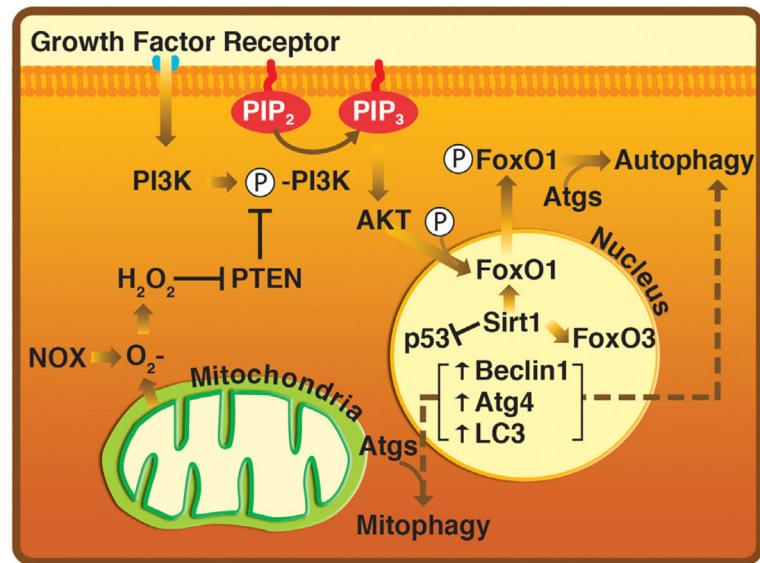
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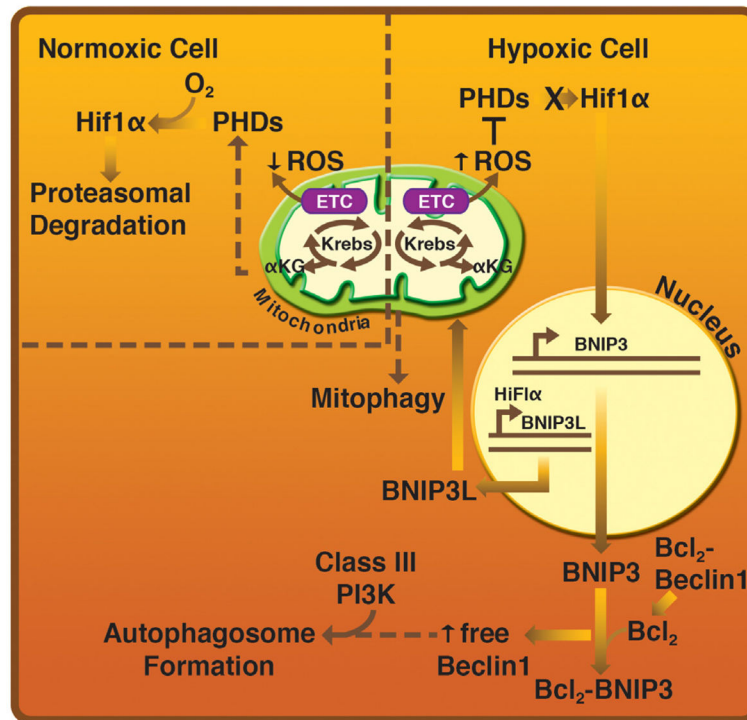
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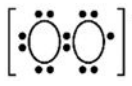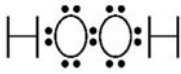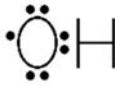
Fig. 1.
Oxidation of PTEN promotes autophagic and mitophagic signaling. ROS reversibly oxidize PTEN allowing for prolonged PI3K/Akt signaling in response to growth factor receptor stimulation. Downstream targets of Akt stimulation include pro-survival transcription factors, such as FoxO1 and FoxO3. Transport of FoxO1 out of the nucleus initiates autophagosome formation by Atg recruitment. FoxO3 is known to target pro-autophagic genes promoting autophagosome formation.

Fig. 2.
ROS inhibit PHD activity to promote autophagy and mitophagy via HIF-regulated transcription. Hypoxia and reperfusion increase production of ROS by perturbing oxidative phosphorylation. ROS inhibit the HIF1α degrading PHD complex, increasing expression of cytosolic HIF1α. HIF1α relocalizes to the nucleus when associated with HIF1β, targeting pro-survival genes, including BNIP3 and BNIP3L as well as pro-death molecules based on the extent and duration of oxygen deprivation. When BNIP3L is synthesized, it associates with mitochondria complexes with Bcl2 in competition with Beclin1. The liberation of Beclin1 promotes autophagosome formation.

Table 1

Primary types of reactive oxygen species produced in neurons. ROS are primarily produced in aerobically dependent organisms due to inefficient electron transfer in mitochondria. Reduction of the highly reactive superoxide radical results in the formation of hydrogen peroxide, a more stable signaling molecule. Reduction of hydrogen peroxide results in formation hydroxyl radicals. Each of these ROS can be detoxified by catalytic and non-catalytic antioxidants.

Reactive oxygen species	Structure	Reactivity	Sources	Detoxification
Superoxide radical		High	Mitochondria, ETC NOX isoforms NOS	SOD GSH Trx
Hydrogen peroxide		Low	Spontaneous reduction of $O_2^{-\bullet}$ SOD reduction of $O_2^{-\bullet}$	Catalase GPx
Hydroxyl radical		Very high	Breakdown of organic peroxides	GSH