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Review

Protein oxidation and cellular homeostasis: Emphasis on metabolism

Valentina Cecarini [a], Jillian Gee [b], Evandro Fioretti [a], Manila Amici [a], Mauro Angeletti [a], Anna Maria Eleuteri [a], Jeffrey N. Keller [b,*]

[a] Post Graduate School of Clinical Biochemistry, Departments of Molecular and Cellular and Animal Biology, University of Camerino, Camerino, Italy
[b] Anatomy and Neurobiology, 205 Sanders-Brown Center on Aging, University of Kentucky Lexington KY 40536-0230, USA

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Abstract

Reactive oxygen species (ROS) are generated as the result of a number of physiological and pathological processes. Once formed ROS can promote multiple forms of oxidative damage, including protein oxidation, and thereby influence the function of a diverse array of cellular processes. This review summarizes the mechanisms by which ROS are generated in a variety of cell types, outlines the mechanisms which control the levels of ROS, and describes specific proteins which are common targets of ROS. Additionally, this review outlines cellular processes which can degrade or repair oxidized proteins, and ultimately describes the potential outcomes of protein oxidation on cellular homeostasis. In particular, this review focuses on the relationship between elevations in protein oxidation and multiple aspects of cellular metabolism. Together, this review describes a potential role for elevated levels of protein oxidation contributing to cellular dysfunction and oxidative stress via impacts on cellular metabolism.

Keywords: Aging; Lysosome; Neuron; Oxidative stress; Proteasome; Reactive oxygen specie

1. Oxidative stress

Oxidative stress is correlated with a plethora of cellular alterations including the accumulation of oxidized-damaged molecules, increased levels of dysfunctional macromolecules, and multiple compromises in cellular homeostasis. Oxidative stress occurs when there are insufficient levels of antioxidants to prevent reactive oxygen species (ROS) from promoting deleterious levels of oxidative damage. Examples of ROS include superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) [1]. Each of these ROS is highly reactive and unstable due to the fact that they contain an unpaired electron in their outer electron shell. This conformation promotes their ability to rapidly interact with cellular macromolecules such as proteins, lipids and nucleic acids [1]. Thus, when cells are unable to sufficiently regulate the levels of ROS, or are unable to adequately remove or replace oxidized macromolecules, cellular dysfunction can occur via oxidative stress. Interestingly, the propensity or sensitivity of cells to undergo oxidative stress appears to be cell type specific, with cells exhibiting dramatic differences with regards to their sensitivity to accumulate oxidized molecules and undergo toxicity during periods of high ROS exposure. The basis for this cell type specificity is poorly understood but is clearly an important topic for aging, hepatic, cardiovascular, cancer, and neuroscience research [2–4].

As mentioned previously there are several mechanisms by which ROS may be generated including aerobic respiration, nitric oxide synthesis, and NADPH oxidase pathways during inflammation. In aerobic respiration, the mitochondrial respiratory chain produces ROS as it transfers electrons during the reduction of molecular oxygen to water. During this process some electrons escape the electron transport chain and interact with oxygen to generate $O_2^{\bullet -}$, $OH^{\bullet}$, or H_2O_2 [5]. The nitric oxide synthase (NOS) enzymes produce nitric oxide (NO) via the deamination of L-arginine to L-citrulline. NO is not highly reactive per se but can interact with other intermediates like oxygen, $O_2^{\bullet -}$ and transition metal generating products that affect the functionality of macromolecules. For example, the

* Corresponding author. Tel.: +1 859 257 1412x333; fax: +1 859 323 2866.
E-mail address: Jnkell0@uky.edu (J.N. Keller).

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combination of NO with $O_2^{\bullet -}$ forms peroxynitrite ($ONOO^-$), a very reactive species that can cause several alterations especially the inactivation of proteins [6]. Finally, inflammatory cells can utilize the NADPH oxidase complex to generate several oxygen radicals including $O_2^{\bullet -}$ and H_2O_2 in order to kill bacteria and other fungal pathogens. Activated neutrophils can also release the enzyme myeloperoxidase which produces the highly active oxidant hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions.

In addition to these biological mechanisms of ROS generation there are also exogenous sources of free radicals including drugs, man-made pollutants like ozone, environmental toxins like rotenone and 3-nitropropionic acid (3-NP), cigarette smoke, radiation, and various fungal and bacteria toxins such as ochratoxin A and aflatoxin B1 [6–8]. Ozone (O_3) is one of the major oxidants present in air and is the cause of several pathologies for epithelial cells and the respiratory tract [9]. At physiological pH ozone reacts with water, producing free radicals like $OH^{\bullet}$ which can promote the oxidation of amino acids and lipids. DNA can also be altered by ozone to cause the formation of 8-oxodeoxyguanosine [10]. Ozone has also been shown to inactivate antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. Such effects further disrupt the balance between oxidation and antioxidant pathways [11], and thereby promotes oxidative stress.

The environmental pollutants rotenone and 3-NP are established inhibitors of mitochondria function and have been demonstrated to be pro-apoptotic. Rotenone is a lipophylic compound often used as a component of insecticides, or maintenance of fish populations, and generates ROS through the inhibition of mitochondrial complex I leading to an increase in H_2O_2 [12]. Interestingly, rotenone has been identified to cause some forms of Parkinsonism [13]. Similarly 3-NP has toxic effects towards the brain, causing symptoms similar to those in Huntington disease (HD) [14]. Much like rotenone, 3-NP generates ROS through the inhibition of the mitochondria enzyme, succinate dehydrogenase [15,16]. These studies provide experimental support for impairments in metabolism (mitochondria), serving as a promoter of oxidative stress.

Cigarette smoke is linked to several human diseases including bronchitis, emphysema, vascular disease, and a large number of cancers [17]. Cigarette tar is the major component responsible for the carcinogenic activity of cigarette smoke. It consists of quinone and hydroquinone molecules that promote a very reactive deleterious redox system involving $O_2^{\bullet -}$, $OH^{\bullet}$, NO and H_2O_2 [18–21]. These species cooperatively promote DNA damage, protein oxidation, and lipid peroxidation. Cigarette smoke is also capable of converting tyrosine to 3-nitrotyrosine and dityrosine, promoting further protein oxidation.

Ionizing radiations, like X- and λ-rays, also have important roles in the production of free radicals. Radiation-induced ROS generation requires the presence of oxygen and water. The highest reactive species formed from ionizing radiation is the hydroxyl radical, which is formed from the radiolysis of water [22]. Once formed, these reactive species can rapidly cause DNA and protein damage. Germ cells in the gonads, bone marrow cells and embryonic stem cells are extremely sensitive to the action of radiation because of their active and continuous proliferation. Proteins high in cysteine and methionine residues, such as crystallin, are particularly vulnerable to X-ray oxidation. Oxidation of these proteins may result in the unfolding of the proteins and formation of disulfide-linked dimers and oligomers [23]. Radiation may also cause oxidation of specific amino acid residues in the active site of enzymes, thus reducing their activity. For example, creatine kinase activity is greatly reduced following X-ray exposure, possibly due to the oxidation of an essential active site tryptophan [24].

Ultraviolet radiation (UVA or UVB) is a potent means of producing ROS and also decreases the levels of antioxidants enzymes [25]. The energy produced by UV-radiation is transferred to molecular oxygen from endogenous chromophores, like riboflavin, $NADH^-$/NADPH and tryptophan. This process leads to the production of O_2^- which is rapidly converted to H_2O_2. The final product is the highly reactive free radical $^{\bullet}OH$. The main consequences of these ROS appears to be alterations to DNA strands, and the generation of 8-hydroxydeoxyguanosine (8-OHdG) [26,27]. Furthermore, these species can interfere with the metabolism of collagen, down regulating collagen synthesis and contribute to potentially deleterious collagen oxidation and degradation [28]. UVA protein oxidation may also lead to the stabilization of various cell cycle proteins, such as p53, and cause an increase in the amount of pro-apoptotic proteins, such as FAS [29,30]. Additionally, UV may alter the activity of other proteins such as transcription factors which could lead to deleterious alterations in gene expression and cytotoxicity [31].

The toxic action of different fungal and bacteria metabolites is often mediated by oxidative stress. It has been shown that fungal toxins like ochratoxin A and aflatoxin B1, noted for their carcinogenic effects, can lead to the formation of ROS. Indeed, treatment of HepG2 and CaCo-2 cell lines with these two toxins induces a dose-dependent increase in ROS generation including O_2^- and $^{\bullet}OH$ [32]. Similar effects have been observed in different animal models. Rat hepatocytes and proximal tubular cells treated with these same toxins show an increase in the levels of ROS [33,34]. Although the majority of studies with these two compounds have focused on their ability to induce lipid peroxidation, more recent reports have shown that both ochratoxin A and aflatoxin B1 are capable of oxidizing proteins. For example, ochratoxin A was shown to induce protein carbonyls in a dose-dependent manner in rats [35]. Additionally, aflatoxin B1 was shown to oxidize the lysine residues of proteins [36].

2. Antioxidants

To protect organelles and cellular components against ROS-associated damage cells have developed several antioxidant defense systems. These can be divided into enzymatic and non-enzymatic mechanisms of ROS detoxification. The non-enzymatic antioxidants include lipid-soluble compounds like vitamin E (α-tocopherol) and vitamin A, and water-soluble compounds like vitamin C (ascorbic acid), ceruloplasmin, uric

acid and glutathione (GSH). Vitamin E is considered as the major lipid-soluble anti-oxidant compound due to its unique localization to cell membranes. In contrast, GSH is the major water-soluble compound which is crucial for the maintenance of enzymes and other cellular components in a reduced state [37].

Additional enzymes and proteins can also act as antioxidants including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). SOD is responsible for the reaction that reduces the radical superoxide to form hydrogen peroxide and oxygen (Fig. 1A). The SOD family includes three different enzymes: cytosolic Cu/Zn-SOD (SOD-1), mitochondrial Mn-SOD (SOD-2) and extracellular SOD (SOD-3) [38,39]. SOD-1 consists of two identical subunits and an active site containing copper and zinc atoms linked by a histidine residue. SOD-1 is constitutively expressed by all mammalian cells and is localized in the cell cytosol and nucleus [40]. Interestingly, several stressors such as ozone, radiation, drugs, NO and H_2O_2 can induce an increase in SOD-1 mRNA expression. SOD-2 is a homotetramer with each subunit containing a manganese atom. It is present in the inner mitochondrial space, and like SOD-1 is expressed by different cell types and tissues. Particularly high levels of SOD2 are found in organs with high respiration rates such as the kidney, liver and myocardium [41]. It has been shown that the activity of SOD2 is correlated with cell proliferation. SOD-3 is a tetrameric glycoprotein containing both copper and zinc [42]. The expression of SOD-3, unlike SOD-1 and SOD-2, is cell and tissue specific. High levels of SOD-3 are present in alveolar type II cells, proximal renal tubular cells, lung macrophages, vascular smooth muscle cells, some fibroblast lines, glial cells, and endothelial cells [42]. SOD-3 is able to detoxify superoxide specifically in the extracellular spaces and fluids, such as plasma and lymph. The final product of the activity of each of the aforementioned SOD types is the production of is H_2O_2. H_2O_2 is extremely dangerous for cells and is therefore immediately removed by two enzymes: catalase (CAT) and glutathione peroxidase (GSH-Px).

CAT is present in the peroxisomes and leads to the neutralization of H_2O_2 resulting in the formation of water and molecular oxygen (Fig. 1B). It is particularly active in liver, lung and kidney, and erythrocytes in humans [43]. Like other antioxidants enzymes, catalase activity is altered following exposure to oxidative stressors like UV-radiation [44], cigarette smoke [45] and ozone [46].

A. $O_2^- + O_2^- \rightarrow H_2O_2 + O_2$

B. $2H_2O_2 \rightarrow H_2O + O_2$

C. $H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$

Fig. 1. Anti-oxidation reactions catalyzed by specific cellular antioxidant enzymes. Superoxide dismutase reduces the superoxide radical in order to form hydrogen peroxide and oxygen (A) which can then undergo further detoxification. Catalase neutralizes reactive hydrogen peroxide by forming non-toxic water and molecular oxygen (B). Glutathione peroxidase also aids in the clearance of hydrogen peroxide by catalyzing the production of water and GSSG from hydrogen peroxide (C).

GSH-Px is an antioxidant enzyme which, in contrast to CAT, works in the cytosol and mitochondria. GSH-Px removes hydrogen peroxide by oxidizing reduced glutathione to oxidized glutathione (GSSG) (Fig. 1C). The GSH-Px family consists of four selenoproteins characterized by differences in localization and molecular structure [47]. The first enzyme to be identified was the cytosolic glutathione peroxidase (cGPx) which is ubiquitously expressed. cGPx is a tetramer of cGPx and each subunit contains a selenocysteine residue that is essential for all catalytic activity. cGPx is able to only react with soluble hydroperoxides like H_2O_2. Indeed, without the presence of phospholipase A_2 activity, that releases fatty acids, cGPx cannot metabolize fatty acid hydroperoxides present in phospholipids. The second form of GSH-Px is the plasma glutathione peroxidase (pGPx). Studies in rats have revealed that the majority of pGPx is generated in the kidney, although expression has also been shown in the liver, lung, heart, breast and placenta [48]. Once formed, pGPx is secreted into the extracellular space and can be found not only in the plasma but also in the lung lavage, breast milk and amniotic fluid. Lung cells, like epithelial cells and macrophages, can produce and secrete the pGPx into epithelial lining fluid [49]. This property makes the enzyme of particular interest as a defense against exposure to exogenous oxidants such as cigarette smoke and ozone. The third type of GSH-Px is the gastrointestinal glutathione peroxidase (GI-GPx) which has been detected in human liver and colon [50]. This highly selective distribution suggests that the main function of this enzyme subtype is to detoxify ingested lipid hydroperoxides and H_2O_2 formed as a consequence of liver effects on alimentary xenobiotics [50]. Lastly, GSH-Px is a phospholipid hydroperoxide glutathione peroxidase (PH-GPx). Testis presents the highest activity of this enzyme, suggesting it plays a role in sexual maturation and differentiation [47]. The main characteristic of this enzyme is its ability to detoxify a wide range of lipid hydroperoxides, including phosphatidylcholine hydroperoxide and those derived from cholesterol. Unlike the other forms of GSH-Px, PH-GPx is a monomer and this small size helps it to interact with hydroperoxides integrated in membranes [47]. Indeed, PH-GPx has been studied for its ability to confer protection against the UV irradiation and ROS-mediated peroxidation of phospholipids in cellular membranes, thereby preventing cellular damage and tissue breakdown [51].

Under normal conditions cells succeed in limiting the amount of damage from ROS attack thanks to the activity of these various antioxidant defense mechanisms. Unfortunately if cells undergo extremely high levels of oxidative stress, or if reduction of antioxidant activities occurs, macromolecules may become damaged and lead to loss in cellular function and oxidative stress. Oxidation of DNA can cause single and double strand breaks and alter DNA–protein and DNA–DNA interactions. Additionally, oxidation of proteins may cause changes in amino acids structure, increases in protein hydrophobicity and a loss of enzymatic activity. Oxidized proteins are normally recognized and degraded by intracellular proteases, such as the proteasome complex. Unfortunately, there is accumulating evidence that free radicals can react with these proteolytic

complexes inducing a decrease in their functionality. If oxidized proteins are not efficiently removed, oxidized proteins may accumulate and alter cell function and promote toxicity [52,53].

3. Protein oxidation

While there are many types of oxidative damage, increasing evidence suggest an important role for protein oxidation in aging and multiple diseases [37,54–56]. The importance of protein oxidation towards cellular homeostasis derives from the fact that proteins serve vital roles in regulating cell structure, cell signaling, and the various enzymatic processes of the cell. Protein oxidation can therefore rapidly contribute to oxidative stress by directly affecting cell signaling, cell structure, and enzymatic processes such as metabolism. There are many different modes of inducing protein oxidation including metal catalyzed oxidation, oxidation induced cleavage, amino acid oxidation, and the conjugation of lipid peroxidation products.

There is evidence that the metal-catalyzed oxidation (MCO) of proteins is one the most common mechanisms for inducing protein oxidation, especially for the introduction of carbonyl groups. This process requires the generation of H_2O_2 and the presence of ions such as Fe (III) or Cu (II). NADH and NADPH oxidase and other oxidation systems catalyze the formation of H_2O_2 and Fe (II) or Cu (I). This derives from oxygen and from Fe (III) or Cu (II) [56–58]. Fe (II) and Cu (I) ions then bind to a specific metal binding site within the protein and react with H_2O_2 to generate $^{\bullet}OH$ that then attacks the amino acid residues near the metal binding site [56–58].

ROS can also induce the cleavage of peptide bonds in proteins via two separate pathways: the diamide pathway and α-amidation pathway [58]. Hydroxyl radical, generated from radiolysis of water or generated from H_2O_2, reacts with proteins to form water and a carbon-centered radical (alkyl-radical). This radical can then cross-link with other alkyl-radicals and form protein aggregates or react with O_2 to generate an alkyl-peroxide radical. This can then be converted, through the action of Fe (II) or $HO_2^{\bullet}$, to alkyl peroxide [56,58]. Alkyl peroxide can then react with Fe (II), $HO_2^{\bullet}$ or by a dismutation reaction, to produce an alkoxyl radical [56,58]. The cleavage of the peptide bond can also be obtained by the reaction of the free radical $^{\bullet}OH$ with the glutamyl, prolyl and aspartyl residues of the protein chain [58,59].

Several amino acids can be directly modified via side chain reactions with ROS. The most sensitive amino acids are those with aromatic side chain groups and those containing sulfhydryl groups. In terms of aromatic side-chain amino acids, ROS-induced oxidation can occur through a variety of intermediates. For example, the oxidation of phenylalanine residues leads to the formation of mono- and di-hydroxy derivatives whereas tryptophan residues are converted to several hydroxy-derivatives, to formylkynurenine and to nitrotryptophan [56–58]. Furthermore, histidine residues can be oxidized to 2-oxohistidine and 4-OH-glutamate, while tyrosine residues are converted to a dihydroxy-derivative (dopa), nitrotyrosine, chlorotyrosine and a dityrosine derivative [56–58]. Interesting, in the case of nitrotryosine, the interaction between the two tyrosine radicals can be intra- or inter-molecular, promoting the production of potentially harmful protein aggregates [56–58]. Furthermore, the presence of the dityrosine derivatives can be used as a marker to determine the amount of cellular oxidative damage induced by free radicals [56].

In contrast to aromatic amino acids, methionine and cysteine resides are oxidized via reactions at the site of sulfhydryl residues. Both cysteine and methionine residues can interact with ROS resulting in the production of sulfoxide, sulfenic acids, and disulfide bridges [56,57,60]. Cysteine is also vulnerable to oxidant induced cross-linking. Oxidative modifications of sulfur-containing amino-acids can be reversible. For example, in the cytosol and the mitochondria of eukaryotic cells there are the thioredoxin and glutaredoxin systems which are responsible for the reduction of cysteine residues [61]. Once thioredoxin and glutaredoxin are oxidized, they can then be reduced via the actions of thioredoxin reductase and a glutathione reductase, respectively, in an NADPH dependent manner. A similar mechanism of reduction, called the methionine sulfoxide reductase system, has been demonstrated for the amino acid methionine. This system consists of two major proteins, MsrA and MsrB, which can reduce *S*-MetO and *R*-MetO, respectively. MsrA is the product of single gene and it is present both in the cytosol and the mitochondria. MsrA protein is highly expressed in human liver, kidney, and brain [62]. In contrast, there are three different MrsB enzymes, encoded by three genes, with different localizations in cellular compartments. MsrB1 is a selenoprotein and is present in the cytosol and in the nucleus. MsrB2 is associated with the mitochondria while MsrB3, which through alternative splicing originates two different mRNAs, has been found both in the mitochondria and in the endoplasmic reticulum. The highest levels of MsrB are in the human bladder, heart and skeletal muscle, while the brain, liver and kidney present low levels of the enzyme [62].

It has been demonstrated that both the enzymes, especially the MsrA form, have a protective role toward protein oxidation and premature death. Indeed, the overexpression of MsrA in yeast, human T-cells [63], human lens epithelial cells [64] and *Drosophila* [65] induces resistance to oxidative stress and increases cell survival. Additional experiments utilizing mice that lack the *MsrA* gene show an enhanced sensitivity to hyperoxia and have reduced life span compared to wild type controls [66]. Finally, senescent cells show a decrease in the activity of these enzymes suggesting it may be the basis for the accumulation of oxidized proteins observed during normal aging [67].

The major product of protein oxidation, such as peptide bond cleavage and amino acid oxidation, is the production of a protein carbonyl. Carbonyl groups can be formed by secondary reactions with the lipid peroxidation product 4-hydroxynonenal (HNE), or with reducing sugars or their oxidation products [6,56,57]. One of the most relevant consequences of the presence of carbonyls is that they can further react with the α-amino groups of lysine residues. This reaction leads to the formation of intra- or inter-molecular cross-links which can promote the formation of protein aggregates. These aggregates

are unable to be degraded via normal protein degradation mechanisms, leading to the inhibition of proteolytic processes. This lack of degradation can further induce the accumulation of oxidized proteins and enhances cellular dysfunction [57].

Studies conducted on housefly and *D. melanogaster* show that during normal aging or after exposure to hyperoxia there is an increase in the amount of carbonyl groups on aconitase, an important enzyme for mitochondrial metabolism. This increase in carbonyl groups was correlated with a loss in enzymatic activity [69,70]. Because of its central role in the citric acid cycle, a decrease in aconitase activity can lead to an increase in cellular oxidative status. In similar experiments the mitochondrial enzyme, adenine nucleotide translocase, was also showed particular susceptibility to oxidation [71]. Furthermore, mammalian models show a selective age-related increase in carbonyls levels in plasma proteins, particularly as part of albumin, transferrin, and α1-macroglubulin [72]. Unfortunately, the majority of oxidized proteins cannot be repaired and are eliminated through various degradation processes, including both proteasome-dependent and proteasome-independent pathways. This promotes their accumulation and ability to contribute to cellular dysfunction.

4. Degradation of oxidized proteins

One of the most important processes for maintaining homeostasis is the ability of proteolytic systems to eliminate oxidized and damaged proteins. Cells degrade oxidized proteins via the proteasome pathway and the lysosomal pathway. The proteasome is considered the major pathway responsible for the degradation and elimination of "mildly" oxidized proteins [53,56–73] and is involved in regulating proteins involved in several cellular activities like cell cycle. It is a multicatalytic protease located in both the cytosol and the nucleus of eukaryotic cells [74–76]. The catalytic core of the proteasome, known as the 20S proteasome, is a cylinder-shaped structure with a molecular weight of ~700 kDa [74–76]. It is made of four rings, each containing seven subunits. The two outer rings are constituted of α-subunits while the inner ones of β-subunits. In the constitutive proteasome the α-subunits are responsible for regulating the function and stability of the proteasome complex. In contrast, the β-subunits, which are characterized by an N-terminal threonine residue, mediate all the catalytic activity of the proteasome [74–76].

There are three main catalytic activities of the proteasome which exhibit different substrate specificity. The chymotrypsin-like activity (CT-like) cleaves substrates after hydrophobic residues whereas the trypsin-like activity (T-like), cleaves the substrate after basic residues. Finally, the peptidylglutamyl-peptide hydrolase activity (PGPH) is responsible for cleaving proteins after acidic residues [76]. In addition, two other activities have been found in the proteasome: the BrAAP activity (which cleaves the substrate after branched-chain amino acids) and the SNAAP (which cleaves the substrate after small neutral amino acids) [77].

In addition to the normal activities of the α and β subunits, different proteins may bind the proteasome and regulate its functions. The best characterized among them are the 19S cap (or PA700) and the 11S cap (or PA28) [74]. The 19S cap consists of 20 subunits and is able to bind to the external rings. Upon binding, the newly formed structure is named the 26S proteasome and requires ATP for its proteolytic activity. The formation of the 26S proteasome promotes degradation of ubiquitinated proteins as the 19S cap has a central role in unfolding target proteins and in the recognition of the ubiquitin chain [74]. The role of 20S versus 26S in degrading oxidized proteins remains to be fully established, although it appears the 20S proteasome is responsible for most oxidized protein degradation.

Ubiquitin is a small protein of 76 amino acids which can be attached to damaged proteins in order to target them for degradation. The ATP-dependent attachment of ubiquitin to proteins is called polyubiquitination and involves three different enzymes named E1, E2, and E3. E1 activates ubiquitin and transfers it to E2, a ubiquitin-conjugating enzyme. Then E3 interacts with both E2 and the target protein, facilitating the transfer of ubiquitin from E2 to the target protein. The reaction is repeated several times in order to label the protein with an ubiquitin chain which allows the target protein to be recognized by the proteasome. Once the target protein is degraded, the ubiquitin chain can then be disassembled via an isopeptidase activity inherent in the 19S cap. This allows ubiquitin to be effectively recycled [74]. It appears that ubiquitin plays a minimal role in targeting oxidized proteins for degradation, although it has been reported that oxidant-induced increases in hydrophobicity may be sufficient target for degradation. Clarification of this issue remains to be elucidated.

Although the proteasome is responsible for degradation of ubiquitinated proteins, it has also been shown that the degradation of oxidized proteins can be carried out by the 20S proteasome in an ATP-independent manner and without the presence of ubiquitin [53,56–78]. Indeed, experiments conducted on erythrocytes or reticulocytes, using intact cells and extracts, show that ATP is not necessary for the degradation of oxidized proteins and that ATP may even induce a partial inhibition of the reaction [79–81]. Nevertheless there are other studies which report involvement of the ubiquitin system in this process. They demonstrate that both the ATP-independent and ATP/ubiquitin-dependent proteolytic pathways are responsible for the degradation of native and oxidized α-crystallin in bovine lens epithelial cells [82,83]. Thus, some controversy still exits within the literature as to whether the proteasome may operate in an ATP-independent as well as an ATP-dependent manner.

As described previously, mildly oxidized proteins may undergo several alterations which increase their susceptibility to proteolysis. One of the most important changes is the rearrangement of their secondary and tertiary structure. This unfolding process induces them to expose hydrophobic residues otherwise hidden in the normal conformation. It has been shown that the exposure of these hydrophobic residues allows the proteasome to recognize and bind its substrates, and start the degradation process [53,56–73]. The result of proteasome activity is the production of short peptides which

can be further hydrolyzed by other cellular peptidases. The function of these peptidases is poorly understood, and deserves further attention.

The proteasome is not the only cellular structure able to degrade oxidized proteins. The lysosomal system contains different proteases which can contribute to protein turnover. Lysosome-mediated proteolysis is mainly non-selective and has as substrates long-lived proteins. These enzymes are able to process both extracellular and intracellular substrates thanks to two different mechanisms: autophagy and endocytosis. Through autophagy lysosomes can degrade intracellular components. There are three different types of autophagy including macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) [84,85]. Macroautophagy is activated upon multiple stimuli including starvation, and allows cells to recycle cellular components in order to maintain cellular homeostasis. Organelles, such as mitochondria or peroxisomes, and entire regions of cytosol are internalized in an intracellular double membrane, probably originated from endoplasmic reticulum [84,85]. This structure, named an autophagosome, then fuses with lysosomes which facilitate the degradation of those protein present [84,85]. In contrast, microautophagy is a continuous process and occurs under normal nutritional conditions. In this case it is the lysosomal membrane which is responsible for the sequestration of cytosol regions. Once the target cytosolic region is sequestered and the outer membrane disrupted, the trapped proteins are then degraded by lysosomal enzymes. The third lysosomal mechanism of degradation is called CMA and is mainly activated upon oxidative stress. This process acts in a selective way since it is specific for substrates carrying a KFERQ motif. This motif is recognized by an intracellular chaperone, the heat shock cognate protein of 73 kDa (hsc73), which guides it to the lysosome. Once at the lysosome, hsc73 interacts with its receptor, the lysosomal membrane protein (LAMP-2A) and allows the targeted protein to interact with a second lysosome-specific chaperone, hsc73 (lys-hsc73). This chaperone then helps the target protein to be translocated into the lysosomal lumen and subsequently degraded [84–86]. Recent studies indicate lysosomes and autophagy contribute to oxidized protein degradation [85], although the significance of this contribution remains to be elucidated.

Oxidative stress can contribute to cellular dysfunction by directly damaging the lysosomal membrane. This leads to the release of the lysosomal enzymes into the cytosol with serious consequences for cytosolic components and an increased risk in cell death. Nevertheless, there is also evidence for a protective role of lysosomes during stressful periods. They can contribute to the elimination of oxidized and damaged proteins, especially through the chaperone-mediated autophagy pathway. Studies on cultured mouse fibroblasts and rat liver show that in conditions of mild oxidation there is a constitutive activation of CMA with an increased degradation of its substrates [86]. Furthermore, there is a more efficient binding and translocation of oxidized proteins to the lysosome, with a concomitant increase in the synthesis of lysosome-associated chaperones and of the membrane receptor LAMP-2A [86].

Removal of oxidized proteins is crucial for cell survival. Indeed, if oxidized proteins are not eliminated either through proteasomal or lysosomal pathways, they are able to begin to accumulate and potentially aggregate. These aggregates can alter cell functions and lead to necrosis or apoptosis. Furthermore, these aggregates are extremely resistant to proteolysis and can act as inhibitory compounds towards both the proteasome and lysosome degradation pathways [52,53]. For example, lysosome hydrolase activity has been shown to be impaired by protein aggregates resulting from prolonged exposure to free radicals. Furthermore, treatment of mouse peritoneal macrophages with oxidized low density lipoprotein induces a decrease in the degradation activity of the lysosomal enzyme, cathepsin B [87]. Exposure of murine macrophage-like cells to protein hydroperoxides inhibits cathepsin L and B in a time- and dose-dependent manner [88]. All of these findings implicate oxidative stress as a contributing factor in the impairment of proteolytic systems responsible for the removal of oxidized and damaged macromolecules. This impairment, in turn, induces an increase in the cytoplasmic levels of oxidized proteins, leading to further elevations in protein oxidation. Oxidatively modified proteins that are not completely removed can potentially interact with other cellular components like lipids, carbohydrates and metals giving rise to an autofluorescent, brown-yellow pigment, termed lipofuscin [89,90]. Lipofuscin is mainly generated in the lysosomal compartment. It has been shown that lipofuscin production is increased by the presence of H_2O_2 derived from the mitochondrial respiration. Iron originating from the degradation of metalloproteins may also contribute to lipofuscin production. Once lipofuscin begins to accumulate, it may reduce lysosomal activity, thus increasing cellular susceptibility to oxidative stress. Interestingly, much like protein aggregates, lipofuscin is highly resistant to proteolysis and is considered a hallmark of aging [89,90]. Interestingly, recent studies indicate that proteasome inhibition is sufficient to increase lipofuscin, with elevations in lipofuscin were associated with a decline in mitochondrial respiration. This is in line with additional studies demonstrating that proteasome inhibitors have deleterious effects on mitochondrial homeostasis. These findings provide further linkages between proteolysis, protein oxidation, and metabolism.

5. Protein oxidation and cellular dysfunction

The reactions of glycolysis involve a variety of enzymes, including hexose kinase, phosphoglucose isomerase, phosphofructokinase, aldolase, gluceradlehdye-3-phosophate dehydrogenase (GAPDH), phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase. The byproducts of these enzymatic reactions are ATP, NADH, and pyruvate which are fed into the citric acid cycle for further production of ATP. Numerous lines of investigation suggest that oxidation of metabolic enzymes, such as those involved in glycolysis and the citric acid cycle, may contribute to oxidative stress (Table 1).

The most-well studied enzyme of the glycolytic pathway is GAPDH since it is one of the first enzymes capable of creating a high-energy bond capable of ATP production. Inhibition of

Table 1
Effect of oxidative stressors on metabolic enzymes in various tissues

Enzyme altered by oxidant	Type of oxidant	Cell/tissue effected	Change in enzymatic function	Reference
GAPDH	H_2O_2	Chondrocytes	↓	[93]
		Lens epithelial cell line	↓	[94]
		Myocardium	↓	[96,97,130]
		Human red blood cells	↓	[95]
	Acrylamide	Extensor muscles	↓	[99]
		Liver	↓	
		Peroneal and tibial nerve	↑	[100]
		Schwann cells	↓	
		Sciatic nerve	↓	[99]
Aconitase	Peroxynitrite	Yeast	↓	[98]
		Astrocytes	↓	
Alpha-ketoglutarate dehydrogenase	H_2O_2	Nerve terminal	↓	[108]
		Cardiac mitochondria	↓	[110]
Succinate dehydrogenase	H_2O_2	Nerve terminal	↓	[108]
		Cardiac mitochondria	↓	[110]
	3-nitropropionic acid	Cerebellar neurons	↓	[112]
		Cerebellar glia	↓	
		GABA neurons	↓	[111]
		Murine Glia	No Effect	
Isocitrate dehydrogenase	H_2O_2	Porcine Mitochondria	↓	[109]
		Nerve terminal	No Effect	[108]
		Cardiac mitochondria	No Effect	[110]
	Potassium superoxide	Porcine Mitochondria	↓	[109]
	Cupric chloride	Porcine Mitochondria	↓	
	Ascorbate	Porcine Mitochondria	↓	
	Visible light	Porcine Mitochondria	↓	
Creatine kinase	Peroxynitrite	Mitochondria	↓	[121]
	H_2O_2	Rat muscle	↓	[116]
		Rat heart	↓	
		Rat brain	↓	
	Acrylamide	Brain	↓	[125]
		Plasma	↓	

GADPH generates an acceleration of glycolytic processes which culminate in a loss of overall ATP production [91]. Many studies have identified an inhibition of GAPDH function with increased amounts of hydrogen peroxide exposure. For instance, isolated plant cells, chondrocytes, human red blood cells, and a mouse lens epithelial cell line all display a reduction of GAPDH following increasing concentrations of H_2O_2 [92–95]. In vivo studies have also shown a reduction in GAPDH activity in isolated rat myocardium, that implicate $H_2O_2^-$ mediated inhibition of GAPDH in vivo [96,97]. It is interesting to note that the sensitivity of GAPDH to oxidative inactivation may vary between cell types and vary in response to specific oxidants. For example, GAPDH isolated from yeast cells was found to be less sensitive to peroxynitrite oxidation compared to GAPDH derived from rat astrocytes [98]. Similarly, acrylamide administration to rats more potently inhibited GAPDH activity in the extensor muscles and liver compared to the medulla, pons, cerebellum, and cortex [99]. Also, GAPDH activity was reported to increase in denervated peroneal and tibial nerves of cats following acrylamide exposure although the activity in Schwann cells was shown to decrease [100]. GAPDH inhibition was also found to be greater in primary rat neurons following H_2O_2 exposure as compared to neurons exposed to diamide [101]. Finally, acrylamide administered to rats was more potent in inducing GAPDH inhibition in sciatic and tibial nerves as compared to glycidamide [102]. Together, these data indicate a cell type and oxidant type specificity with regards to the ability of oxidants to inhibit GADPH.

Inhibition of GAPDH via oxidative stress may also play a vital role in modulation of cellular apoptosis and cell signaling, independent of its effects on regulating ATP. GAPDH expression has been reported to increase during apoptosis and treatment of cells with anti-sense GAPDH was able to block apoptosis completely [103]. More recent reports indicate that GAPDH may translocate into the nucleus of stressed cells and alter gene transcription related to cell survival [103]. Indeed, following addition of H_2O_2 to neuroblastoma or embryo fibroblast cell lines high amounts of GAPDH appeared translocated into the nucleus [104]. An additional study has shown that oxidized GAPDH may have enhanced binding capabilities towards both tRNA and DNA [105]. GAPDH may also promote apoptosis during oxidative stress via undergoing self-aggregation and altering the functionality of other proteins [98,101,103]. In contrast, other groups have suggested an anti-apoptotic role for GAPDH under conditions of oxidative stress, possibly due to GADPH binding to other proteins [106]. For example, binding of GAPDH to phospholipase D2 in PC12 cells exposed to H_2O_2 regulates the activity of phospholipase D2 and leads to a reduction in apoptosis [106]. Together, these

data indicate the ability of oxidation to alter function or expression of GADPH (via oxidation of GADPH) may be a direct mechanism by which protein oxidation impinges upon metabolism and viability.

Enzymes in the citric acid cycle may also be important for cellular dysfunction caused by oxidative stress. Following glycolysis, pyruvate is transported into the mitochondria via a pyruvate-H+symportor. Pyruvate is then fed into the citric acid cycle where it is acted upon by various enzymes including pryuvate dehydrogenase, citrate synthase, aconitase, isocitrate dehydrogenase, alpha ketoglutarate dehydrogenase, succinyl-coa synthetase, succinate dehydrogenase, fumarase, and malate dehydrogenase [107]. Under various oxidative conditions, the activities of several of these enzymes have been shown to be reduced, including alpha-ketoglutarate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase, and aconitase [108–112]. The sensitivity of these enzymes to oxidative stress, though, appears to be enzyme specific. For example, in isolated nerve terminals citrate synthase, isocitrate dehydrogenase, and malate dehydrogenase were unaffected by H_2O_2 exposure although it induced a dramatic reduction in aconitase, alpha-ketogluarate dehydrogenase, and succinate dehydrogenase (Table 1). Interestingly, aconitase was shown to be most sensitive to H_2O_2 in this paradigm [108]. Similar results where obtained by exposure of isolated cardiac mitochondria to H_2O_2 [110]. Unfortunately, due to differences in the amount of H_2O_2 used in the different experimental conditions it is difficult to determine whether nerve synaptosomes or cardiac mitochondria are more sensitive to H_2O_2 exposure [108,110]. Similar to the effects of oxidative stress on glycolysis, differences may occur in the inhibition of these enzymes depending on the oxidative paradigm utilized. For example, 3-NP was shown to inhibit succinate dehydrogenase activity in cultured rat cerebellar neurons and astrocytes and also murine GABA neurons but shown to have no effect on succinate dehydrogenase activity in murine glia [111,112]. Also, isolated isocitrate dehydrogenase was shown to be inhibited by H_2O_2 whereas other groups reported no effect [109,113].

Another aspect of energy metabolism as it relates to oxidative stress is the functionality of creatine kinase, the enzyme which catalyzes the formation of phosphocreatine which can rapidly generate ATP under conditions of high energy demand. This enzyme is especially important in cell types which require large amounts of ATP for their function, including muscle and brain cells, and is not expressed in other less energy demanding cell types such as hepatocytes [107].

Functional creatine kinase is a dimeric protein that can be formed by two different types of monomers: M and B. The MM form of creatine kinase resides mostly in muscle tissue whereas the BB form is found mainly in the brain [107]. There is also a MB isoform which resides in cardiac tissue. In addition, there are intracellular pools of CK, one residing in the mitochondria and the other in the cytosol [114]. Though the exact mechanism underlying the inactivation of creatine kinase remains to elucidated, most groups concur that the enzyme is highly susceptible to H_2O_2 oxidation [113,115,116] and O_2^- oxidation [117,118]. The active site of all isoforms of the creatine kinase enzyme contain an essential sulfhydryl oxidation which may be susceptible to oxidative modification [115,118]. Indeed, reductions in mitochondrial creatine kinase activity has been shown to correlate with increases in protein sulfhydryl groups induced by xanthine and xanthine oxidase [118]. Furthermore, treatment of isolated CK with a variety of known oxidants, including peroxynitrite, H_2O_2, S-nitroso-N-acetylcysteine, and S-nitroso-glutathione, has shown inhibition of CK activity [119–121]. Also, oxidized CK regains activity following treatment with the sulfhydryl reducing agent DTT. The inhibition of CK by oxidants has also been reported in functional human kerotinocytes and rat monocytes and rat midbrain [120,122,123]. It should be noted, however, that tissue-specific CK may display differences in its response to oxidative stress. For example, CK activities in the rat heart were reduced more so than in the muscle or brain following treatment with xanthine and xanthine oxidase [116]. However, this effect may be oxidant specific as greater reduction of CK activity was seen in the brain compared to muscle and heart following treatment with H_2O_2 [116]. Other studies have also seen tissue-specific regulation of CK activity. Wang et al. found a significant reduction in CK activity following exposure to 1-bromopropane, with cerebrum and brainstem specific-CK having greater inhibition than CK located in the spinal cord or cerebellum [124]. Additionally, CK inhibition by acrylamide was most significant in the brain of mice but in rats was greatest in the plasma [125]. Finally, much like the enzymes of glycolysis and the Krebs cycle, alteration of CK may be oxidant specific. For example, glycidamide inhibited CK in rats whereas acrylamide had no significant effect [102]. Interestingly, aflatoxicosis and ochratoxin were shown to actually increase CK activity in broiler chicks and turkeys [7,8].

The inactivation of CK may have a variety of complications for cells, especially when energy demands are high. For instance, isolated rat hearts were shown to maintain cardiac performance when administered idoacetaminde, a CK inhibitor, under normal conditions, however, when energy demands were high, CK inhibition decreased ventricular performance and overall systolic function [126]. Similarly isolated muscle cells have been shown to have reduced titanic peak tension during exercise due to inhibition of muscle CK [127]. It is also interesting to note that systolic and diastolic function failed more rapidly in rat hearts undergoing CK inhibition, indicating that disruption of CK function via oxidative stress could lead to cardiac muscle failure [126]. CK inhibition may also disrupt neuronal activity as CK activity has been shown to correlate with neuronal firing [128,129]. There is some evidence that inhibition of CK may underscore neuronal damage incurred by toxins, leading to electrophysiological alterations in peripheral nerve, axon swelling, and reductions in cerebral volume [124].

6. Summary

It is clear that elevations in oxidative damage can promote oxidative stress in a variety of cell types. The ability of oxidative

damage to induce cellular dysfunction is very dependent on its ability to affect specific cellular processes, including metabolic function. The ability of oxidants to promote dysfunction and toxicity is cell type specific, which is likely mediated by the ability of cells to successfully respond to oxidants by removing, repairing, or replacing oxidized molecules. Thus the ability of cells to respond favorably to oxidant exposure is the ultimate predictor of whether a cell will exhibit oxidative stress. Understanding the basis for this cell type specificity in favorably responding to oxidant exposure is only beginning to be understood, and represents one of the cutting edges of oxidative stress research. The goal of such studies should be to define why the amount of oxidative damage does not predict the presence or severity of disease or dysfunction. Clearly, one of the principle cellular targets of protein oxidation is cellular metabolism. Oxidation of a limited number of metabolic proteins can dramatically and deleteriously impact the overall energy status of the cell, and thereby rapidly induce cellular dysfunction through a limited number of protein oxidation events. The ability of cells to prevent the oxidation of metabolic proteins, or to repair or replace metabolic proteins following oxidation, is likely to be an important predictor of how well cells are able to successfully respond to oxidative stressors. Studies which are designed to explore this important aspect of protein oxidation research in a cell type specific manner will likely yield data that is important in aging, diabetes, cancer, cardiovascular, and neuroscience research [131].

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