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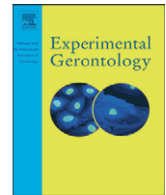

Mini review

Oxidative proteome modifications target specific cellular pathways during oxidative stress, cellular senescence and aging

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ABSTRACT

Oxidatively modified proteins build-up with age results, at least in part, from the increase of reactive oxygen species and other toxic compounds originating from both cellular metabolism and external factors. Experimental evidence has also indicated that failure of protein maintenance is a major contributor to the age-associated accumulation of damaged proteins. We have previously shown that oxidized proteins as well as proteins modified by lipid peroxidation and glycoxidation adducts are accumulating in senescent human WI-38 fibroblasts and reported that proteins targeted by these modifications are mainly involved in protein maintenance, energy metabolism and cytoskeleton. Alterations in the proteome of human muscle adult stem cells upon oxidative stress have also been recently analyzed. The carbonylated proteins identified were also found to be involved in key cellular functions, such as carbohydrate metabolism, protein maintenance, cellular motility and protein homeostasis. More recently, we have built a database of proteins modified by carbonylation, glycation and lipid peroxidation products during aging and age-related diseases, such as neurodegenerative diseases. Common pathways evidenced by enzymes involved in intermediate metabolism were found targeted by these modifications, although different tissues have been examined. These results underscore the implication of potential deleterious effects of protein irreversible oxidative modifications in key cellular pathways during aging and in the pathogenesis of age-related diseases.

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1. Introduction

Accumulation of damaged macromolecules, including oxidatively damaged proteins, is a hallmark of cellular and organismal aging. This accumulation has been viewed as the combined result of increased production of reactive oxygen species (ROS) and other toxic compounds coming from both cellular metabolism and external factors as well as the failure of protein maintenance (i.e. degradation and repair) with age. Protein oxidation is particularly detrimental as the resulting damages can render oxidized proteins inactive and lead to cellular functional abnormalities (Berlett and Stadtman, 1997). Various types of protein oxidative damage are induced either directly by ROS or indirectly by reactions with secondary products of oxidative stress such as reactive aldehydes as 4-hydroxy-2-nonenal and malondialdehyde. These aldehydes are produced by the peroxidation of polyunsaturated fatty acids of membrane lipids and can react with protein to form covalent Michael adducts with the side chains of cysteine, histidine and lysine residues (Esterbauer et al., 1991). Proteins can also be modified through the reaction of arginine and lysine side chains with reducing sugars or reactive dicarbonyl compounds such as glyoxal and methylglyoxal, based on the Maillard reaction (Thornalley et al., 2003). Formation of these lipid peroxidation and glycation adducts are found in many tissues and believed to contribute to a variety of age-associated diseases.

Although an increased load of oxidatively-modified proteins has been clearly associated with normal and pathological aging, in most cases the target proteins have not been identified and, only recently significant advances have been made towards the identification of proteins targeted by these modifications. Indeed, identification of these proteins, the modified forms of which are accumulating during aging or upon the development of an age-related disease, would be expected to give some insights into the mechanisms by which these damaged proteins would build-up and potentially affect protein function. Moreover, although the causative role of protein oxidative modifications has not yet been determined, the accumulation of oxidatively damaged proteins during aging and their particular increase in organs and tissues affected by age-related diseases imply that the restricted set of proteins targeted by damage may be a potential substratum for many of the observed cellular dysfunction.

In this mini-review, we will first briefly describe the most relevant protein oxidative and related modifications that have been previously documented to accumulate in senescent cells and aged tissues, and then address the intracellular fate of these modified proteins. Then, the recent identification of proteins preferentially targeted by oxidation within human myoblasts upon acute oxidative stress and of proteins

Abbreviations: ROS, Reactive Oxygen Species; SNO, s-nitrosothiol; ALE, advanced lipoperoxidation end products; AGE, advanced glycoxidation end products; Msr, methionine sulfoxide reductase; 2D-DIGE, 2 dimensional differential in gel electrophoresis; pI, isoelectric point; HNE, 4-hydroxy-2-nonenal; DNPH, 2,4-dinitrophenylhydrazine.

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accumulating as oxidized, glycated and conjugated with lipid peroxidation products in senescent human embryonic fibroblasts will be presented. Finally, we will elaborate on a newly created database aimed at inventorying all the mammalian proteins, mostly from brain origin, that have been shown to accumulate as oxidatively modified during normal and pathological aging. Indeed, over 180 proteins have been reported in the literature as increasingly modified. Interestingly, these proteins are involved in key cellular pathways such as inflammatory response, energy metabolism, protein homeostasis and antioxidant response, hence indicating the particular susceptibility of certain proteins for being prone for detrimental modifications and suggesting their likely implication in the underlying mechanisms of aging and age-related diseases.

2. Modification of proteins by oxidation and related pathways

Proteins represent the main targets for ROS mediated damage that occurs either directly or indirectly through their reaction with lipids and carbohydrates and the subsequent generation of oxidized products that can react with proteins. Protein oxidation by free radicals can be classified into those that oxidize and cleave the peptide bond and those that oxidize the side chains. Almost all amino acid side chains can react with the hydroxyl radical (OH^*), but certain amino acids are more sensitive to oxidation with such reactive oxygen species as hydrogen peroxide and superoxide. Indeed, the sulfur-containing amino acids methionine and cysteine are readily oxidized by all sorts of ROS, while aromatic amino acids and histidine are also prone to oxidation.

Oxidation products of cysteine include disulfide bridges and sulfenic acid that can be converted to disulfide bridges or further oxidized to sulfinic and then sulfonic acids. Both disulfide bridges and sulfenic acid can be enzymatically reduced while sulfinic acid reduction has so far been limited to oxidized cysteines within the active site of peroxiredoxins (Biteau et al., 2003). Cystein can also react with nitric oxide to produce S-nitrosothiol (SNO). Methionine oxidation leads to the formation of methionine sulfoxide and further oxidation of methionine sulfoxide leads to the irreversible formation of methionine sulfone. Some oxidative modifications are quite specific in terms of oxidized residues and products generated such as the oxidation of phenylalanine to tyrosine, which can be further converted to di-tyrosine (Giulivi et al., 2003). Moreover, tyrosine residues represent preferred targets for nitration by nitrogen dioxide and peroxynitrite and can be converted to nitrotyrosine.

Oxidation of several amino acid residues such as lysine, arginine, proline and threonine results in the formation of carbonyl groups (Berlett and Stadtman, 1997). Carbonyl derivatives can also originate from the fragmentation products of the peptide bond oxidative cleavage (Stadtman and Levine, 2003). Aminoadipic and glutamic semialdehydes resulting from the oxidation of lysine and arginine, respectively, are quantitatively important products of the carbonylation reaction. Protein carbonyls are the most commonly used marker of protein oxidation and different methods have been developed for the detection and quantification of carbonylated proteins. Most of these methods are based on immunochemical and/or spectrophotometric assays of protein carbonyls previously derivatized by 2-4-dinitrophenylhydrazine to form 2-4-dinitrophenylhydrazone protein adducts (Levine, 2002). Protein carbonylation has been considered as an indicator of severe oxidative damage as well as age- and disease-derived protein dysfunction since this modification often leads to a loss of protein function, as well as an increased thermosensitivity and hydrophobicity (Berlett and Stadtman, 1997).

In addition to direct oxidation of certain amino acid side chains, protein carbonyl derivatives can originate from the conjugation on cysteine, lysine and histidine residues of such aldehydes as malondialdehyde and 4-hydroxy-2-nonenal. Indeed, oxygen free radicals can attack cellular membranes and induce lipid peroxidation resulting in the production of these reactive aldehydes which are precursors of advanced lipid peroxidation end products (ALE) that have been found to accumulate on proteins during aging and certain age-related diseases (Sayre et al., 1997; Szweda et al., 2003). Moreover, sugar aldehydes or ketones can also react spontaneously with the amino groups of lysine and arginine through a Schiff base which is slowly rearranged to form an Amadori product (e.g. fructosamine when the reacting sugar is glucose). These products are referred as to early stage glycation adducts that are further modified to form stable end-stage products also called advanced glycation end products (AGE) through either rearrangement, oxidation, dehydration, fragmentation and/or cyclization. Deleterious effect on protein function is observed when the modification affects critical amino acids within the protein and many proteins, including intracellular proteins, accumulate with age as AGE-modified in vivo (Horiuchi and Araki, 1994).

3. Elimination of modified proteins by degradation and repair

In contrast to DNA, for which many repair enzymes and pathways have been described for oxidative and other insults, oxidized protein repair is limited to the reduction of certain oxidation products of the sulfur-containing amino acids, cysteine and methionine. Indeed, damaged intracellular proteins are mainly eliminated be degradation by the proteasomal and the lysosomal pathways. Major systems that have been implicated in oxidized protein repair include thiredoxin/thioredoxin reductase and the glutathione/glutathione reductase systems for the reduction of sulfenic acid and disulfide bridges, the sulfiredoxin and sestrin for the reduction of sulfinic acid when formed on the catalytic cysteine of peroxiredoxins, and the methionine sulfoxide reductases (Msr) for the reduction of methionine sulfoxide within proteins (Petropoulos and Friguet, 2005).

The Msr system is found in almost all organisms, from bacteria to mammals, and is composed of two enzyme families, MsrA and MsrB, that catalytically reverse the oxidation of the S-sulfoxide and R-sulfoxide diastereoisomeric forms of methionine sulfoxide, respectively (Boschi-Muller et al., 2008). Oxidized methionine sulfoxide reductases are then reduced by the thiredoxin/thioredoxin reductase system. Oxidation of methionine has been implicated in the impairment of protein structure and/or function while the reduction of methionine sulfoxide has been associated with the recovery of protein function. Hence, oxidation/reduction of methionine has been involved in redox regulation of protein function and protein–protein interactions. In combination with protein surface-exposed methionine residues, the Msr system has also been shown to be efficient as a built-in ROS scavenging system preventing further irreversible protein oxidation (Picot et al., 2005). Another protein repair system that has been described and that relates to modification of proteins by glycation, is fructosamine-3-kinase, an enzyme that has been involved in glycated protein repair since it phosphorylates fructosamines on proteins, making them unstable and causing them to detach from proteins, hence acting as a deglycating enzyme (Van Schaftingen et al., 2010).

Non-repairable protein alterations, which represent the majority of protein damage, are removed through degradation by the proteasomal or the lysosomal systems in the cytosol while oxidized proteins are degraded by the Lon protease in the mitochondrion (Ugarte et al., 2010). These proteolytic systems as well as the oxidized protein repair Msr system have been documented to decline with age and during replicative senescence, hence implicating protein maintenance failure in the age-associated build-up of damaged proteins (Baraibar and Friguet, 2012).

The proteasomal and lysosomal pathways are the two main proteolytic machineries by which intracellular proteins are degraded. Protein degradation by the proteasome is a key process for the maintenance of cellular protein homeostasis. In the cytosol and in the nucleus, the proteasome plays a key role in the elimination of altered proteins since moderately oxidized proteins are good substrates of

the proteasome in vitro and they have also been shown not to require both ubiquitin and ATP to be eliminated in vivo. However, some studies have shown that the ubiquitin–proteasome system could be implicated in the degradation of certain oxidized proteins. The increased susceptibility of oxidized proteins to degradation by the proteasome has been associated to an increased exposure of hydrophobic amino acids at the protein surface and an increased flexibility of their C- and N-terminus extremity, making them more prone to their degradation by either the 20S or 26S proteasomes (Grune et al., 2003). However, when altered proteins are highly oxidized or modified by glycation or conjugation by lipid peroxidation products, intra- and/or inter-molecular cross-links are formed, hence rendering these heavily modified proteins resistant to proteolysis by the proteasome. Moreover, although proteins modified by either glycoxidation or conjugation with lipid peroxidation products, have been evidenced as ubiquitinated, suggesting that they might be substrates of the 26S proteasome, these modified proteins have also been found to be targeted to and degraded by the lysosomes (Bulteau et al., 2001; Marques et al., 2004).

4. Oxidized protein pattern generated upon oxidative stress

ROS are generated during normal physiological processes, including aerobic metabolism, and are known to play a dual role in biological systems resulting in either beneficial or harmful effects. Low concentrations or transient exposure to ROS induce cell proliferation and regulate the activation of several cellular pathways by redox signaling mechanisms (Thannickal and Fanburg, 2000). However, in conditions where cellular antioxidant responses are overwhelmed, ROS are important mediators of macromolecular and cellular damage. A serious imbalance between ROS production and the antioxidant capacity of the cell toward a more oxidized state is generally referred to as oxidative stress. Indeed, protein oxidation, lipid peroxidation or DNA damage has been observed following the induction of oxidative stress. Although the primary cellular target of oxidative stress can vary depending on the cell type, the nature of stress (e.g., radical vs. non-radical), its site of generation (e.g., intra- vs. extra-cellular), oxidative stress gives rise to: increased protein oxidation, disassembly of the cytoskeleton, depletion of pyridine nucleotide and ATP pools, increased plasma-membrane peroxidation and permeability, release of cytosolic components, elevation of free $Ca2^{+}$, and breaking of DNA strands (Halliwell and Gutteridge, 1986).

Of particular interest in recent years are the effects of oxidative stress on human stem cells and the consequences on their differentiation capacity impacting on decreased tissue regeneration. We have recently characterized the proteome changes of adult human muscle stem cells in response to oxidative stress (Baraibar et al., 2011). Using a dual proteomic approach, we intended to unravel the mechanism involved in human myoblast dysfunction upon oxidative stress. Selective proteins either modulated at the expression level or those targeted by oxidation (carbonylated) were identified after a sub-toxic insult of hydrogen peroxide, which induced intermediate cellular damage without suppressing the myoblasts anti-oxidant response mechanisms. Although cell viability was not compromised at least in the 24 h after the insult, increased carbonylation of proteins was observed (Baraibar et al., 2011). Since intracellular accumulation of carbonylated proteins results in deleterious effects on cell function and survival, the identification of those proteins specifically targeted by oxidation is of valuable interest. For this purpose, a 2D gel electrophoresis-based proteomic approach coupled with immunodetection of carbonylated proteins, after their derivatization with DNPH, and identification of the spots of interest by mass spectrometry has been used. Twenty-one protein spots were evidenced, as increasingly carbonylated upon oxidative stress, indicating that only a restricted set of proteins is prone to accumulation upon oxidative stress. Most of the carbonylated proteins identified belong not only from the cytosol but also proteins from the nucleus, endoplasmic reticulum as well as the plasma membrane were identified (Fig. 1). Major functional categories include energy metabolism, cellular assembly, protein synthesis, cell morphology and protein degradation. Since protein carbonylation is an irreversible modification, the cell must degrade those damaged proteins and make de novo synthesis in order to maintain a healthy protein pool and reduce the accumulation of potentially toxic proteins. However, proteins involved either in protein degradation such as the proteasome regulatory subunit 10B, and in protein synthesis such as elongation factor 2 were found to be carbonylated. Previous studies have shown that protein synthesis is inhibited by oxidative stress (Shenton et al., 2006). Furthermore, carbonylation of proteasome subunits may explain, at least in part, the decreased proteasome activity observed, suggesting that oxidative stress does not only induce the modification of proteins, but also compromise their degradation by affecting proteasome function.

Another relevant outcome of this study was the structural changes observed in proteins involved in the antioxidant response and energy metabolism, such as peroxiredoxins, GAPDH and alpha-enolase. 2D differential in gel electrophoresis (2D-DIGE) analyses showed a mobility shift of the protein to a more acidic pI, with a concomitant decrease in levels of the non-modified protein and an increase in two other forms with a reduced pI upon oxidative stress. Previous studies in Jurkat T-cells showed that the increase in acidic spots of peroxiredoxins correspond to inactive forms of the protein due to oxidation of the active site cystein into cysteic acid (Rabilloud et al., 2002). Strikingly, hyperoxidized GAPDH has been associated with both the inhibition of glycolysis and the decreased ability to reduce hydrogen peroxide (Maller et al., 2011), suggesting that carbohydrate metabolism is impaired in human myoblasts upon oxidative stress.

Taken together, our results indicate that proteins involved in several cellular pathways are affected upon oxidative stress and that the impairment of these pathways may be implicated in oxidative stress-induced cellular dysfunction. Furthermore, this study underscores the importance of performing proteomic analyses looking at different aspects, such as the expression level and specific post-translational modifications, in order to have a broader view of changes affecting the cellular proteome.

5. Protein damage is restricted to specific protein targets during cellular senescence

The occurrence of increased oxidative damage at the total proteome level during aging is well documented and is believed to have a causative role in cellular aging (Berlett and Stadtman, 1997). However, the identification of the specific protein targets has been usually performed approaching only a single type of modification (e.g. carbonylation or

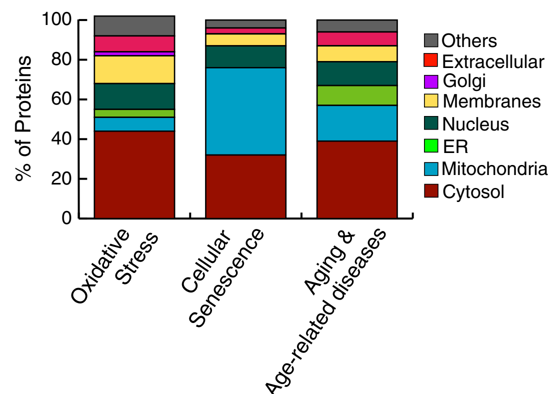

Fig. 1. Damaged protein sub-cellular localization. Primary sub-cellular location of modified proteins (carbonylated, AGE- and HNE-modified) previously reported in human myoblasts upon oxidative stress (oxidative stress), during replicative senescence of WI-38 fibroblasts (senescence) and during aging and age-related diseases. Rare primary localization terms were grouped as *Other* representing less than 5% of total identifications.

conjugation with HNE) in different aging model systems and tissues from aged animals (Kapphahn et al., 2006).

Pioneer studies showed an increase in proteins modified with HNE, AGE and carbonylation in senescent WI-38 human embryonic fibroblasts cells (Ahmed et al., 2007). The identification of proteins targeted by these modifications showed that they represent a restricted set within the total cellular proteome that fall in key functional categories, such as protein quality control, energy metabolism and cellular morphology (Ahmed et al., 2010). Since impairment of these systems has been previously documented in senescent cells, the reported protein modifications may play a role in the development of the senescent phenotype. Almost half of the modified proteins identified upon replicative senescence of human WI-38 fibroblasts were from mitochondria, which indicate a highly oxidative environment within this organelle during cellular aging (Fig. 1). In addition, the detoxification systems such as glyoxalase 1 and Msr were found to be impaired, pointing out a potential role of detoxification systems in the age-related build-up of damaged proteins (Ahmed et al., 2010).

The cytoskeletal proteins vimentin, actin and tubulin were found among the proteins identified as HNE-modified. Cytoskeletal proteins are involved in key cellular processes such as cell division, signal transduction, cell motility and protein synthesis. Follow-up studies showed several structural changes of the intermediate filament protein vimentin during cellular senescence. Vimentin filaments form thick, long dense bundles in senescent cells while irregular and small fur-like networks in young or early-passage fibroblasts (Ahmed et al., 2010).

Cellular senescence is also accompanied by alterations in energy metabolism. Increased oxidative damage causes impairment of mitochondrial respiration affecting mainly the activity of complexes I, III and IV of the respiratory chain. In senescent WI-38 fibroblasts, the iron–sulfur subunit of complex I and subunit α of ATP synthase, the subunit 1 of complex III, and FAD subunit of complex II have been identified as increasingly modified by HNE-, AGE-, and carbonylation, respectively. In addition, iron–sulfur subunit of complex I and FAD subunit of complex II were previously found to be HNE-modified in kidney mitochondria of aged rat. Among the modified proteins identified in senescent fibroblasts, the citric acid cycle enzymes malate dehydrogenase and 2-oxoglutarate dehydrogenase E1 component, glycerol-3-phosphate dehydrogenase, glycerol kinase and glutaminase appear to be specifically targeted by oxidation (Ahmed et al., 2010). These results suggest that modification of proteins responsible for energy metabolism may participate in the impairment of mitochondrial function observed in senescent cells.

Proteins directly linked with the regulation of protein homeostasis, such as protein folding and degradation were also identified as increasingly modified in senescent cells. Proteins with chaperone function, such as Hsc70, calreticulin, endoplasmic reticulum protein ERp29, as well as proteasome subunits linked to a decreased proteasome activity underscore this issue.

6. Accumulation of damaged proteins during aging and age-related diseases

In order to further characterize the role of modified proteins we addressed the occurrence of protein oxidative and related modifications in human or mammalian animal models of aging and age-related diseases (neurodegenerative diseases, cancer, and diabetes), where increased protein modifications have been consistently evidenced. A specific search was performed for proteins identified as increasingly carbonylated or modified by AGE or HNE in articles published in peer-reviewed journals. A total of 183 proteins were listed (Baraibar et al., in press). Modified proteins were identified in brain, cerebellum, spinal cord, skeletal muscle, liver, eye, and cerebrospinal and bronchoalveolar fluids. Of note is that most of the proteins are from the brain due to the high number of studies addressing the importance of protein carbonylation in the pathogenesis of several neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's diseases (Butterfield et al., 2006). However, several modified proteins have been consistently identified in other organs such as liver and eye, indicating that the spectrum of proteins targeted by these modifications may be conserved. Among them, cytoplasmic proteins were predominant, followed by proteins from mitochondria, nucleus, endoplasmic reticulum and plasma membrane (Fig. 1). However, most of the studies were performed in total tissue soluble extracts, where mitochondrial and membrane proteins are clearly underrepresented when compared to cytosolic proteins.

Functional annotation indicated that proteins were mainly distributed within biological processes such as inflammatory response, cellular metabolism, free radical scavenging, protein synthesis and folding (Fig. 2). Concerning proteins involved in the inflammatory response, physiological aging is associated with a chronic sub-clinical systemic inflammatory response, also referred as to "inflamm-aging", characterized by elevated levels of serum pro-inflammatory cytokines such as interleukin 6 (IL-6), TNFα and acute phase proteins such as C-reactive protein (CRP) (Franceschi et al., 2000). Inflammation is now accepted as a key pathogenic factor in the development of several age-related pathologies including cardiovascular disease, type 2 diabetes and neurodegenerative diseases. Importantly, the inflammatory environment is highly oxidative, and increased protein oxidation has been described, generating a positive feedback process.

Proteins involved in energy metabolism were also evidenced in the modified proteins referenced. The most significant canonical pathways across the entire dataset included: glycolysis/gluconeogenesis, citrate cycle, pyruvate metabolism, amino acids degradation, mitochondrial dysfunction, cell death, butanoate metabolism, nrf-2 oxidative stress response and cellular function and maintenance.

7. Concluding remarks

Twelve proteins (heat shock cognate 71 kDa protein, vimentin, actin, tubulin, glyceraldehyde-3-phosphate dehydrogenase, ATP synthase, proteasome subunit 11, elongation factor Tu, cytochrome b-c1 complex, annexin A5, proteasome subunit alpha type-2 and malate dehydrogenase), which correspond to about one third of the proteins identified during replicative senescence of WI38 fibroblasts in vitro have been also identified as increasingly modified in other models of aging and age-related diseases. Interestingly, comparison of these two data sets indicates a similarity in the proteins targeted by these modifications to a certain extent. However, the majority of the studies have been achieved in brain, where the protein expression profile could differ significantly from the one of fibroblasts cultured in vitro. Further studies should address this issue by using senescent cells from different tissues, including human senescent stem cells since they develop both chronological and replicative aging.

Important cellular functions, like energy production, carbohydrate metabolism, protein synthesis, folding and degradation may be implicated and since they are directly targeted by modification of proteins belonging to those cellular pathways (Fig. 2). Common processes are suggested to be causally involved or at least contribute to aging and age-related diseases, including increased oxidative stress, accumulation of protein damage and general metabolic dysfunction. However, these processes have been mostly seen as independent events. An important outcome of the present study is that several enzymes that catalyze intermediate metabolism, such as glycolysis and gluconeogenesis, the citrate cycle and fatty acid metabolism have been found to be oxidatively modified (Fig. 2). These results indicate a potential effect of protein modification on the impairment of cellular energy metabolism. Future studies should address this important issue for instance by combining metabolomics and targeted modification proteomic analysis during cellular and organismal aging.

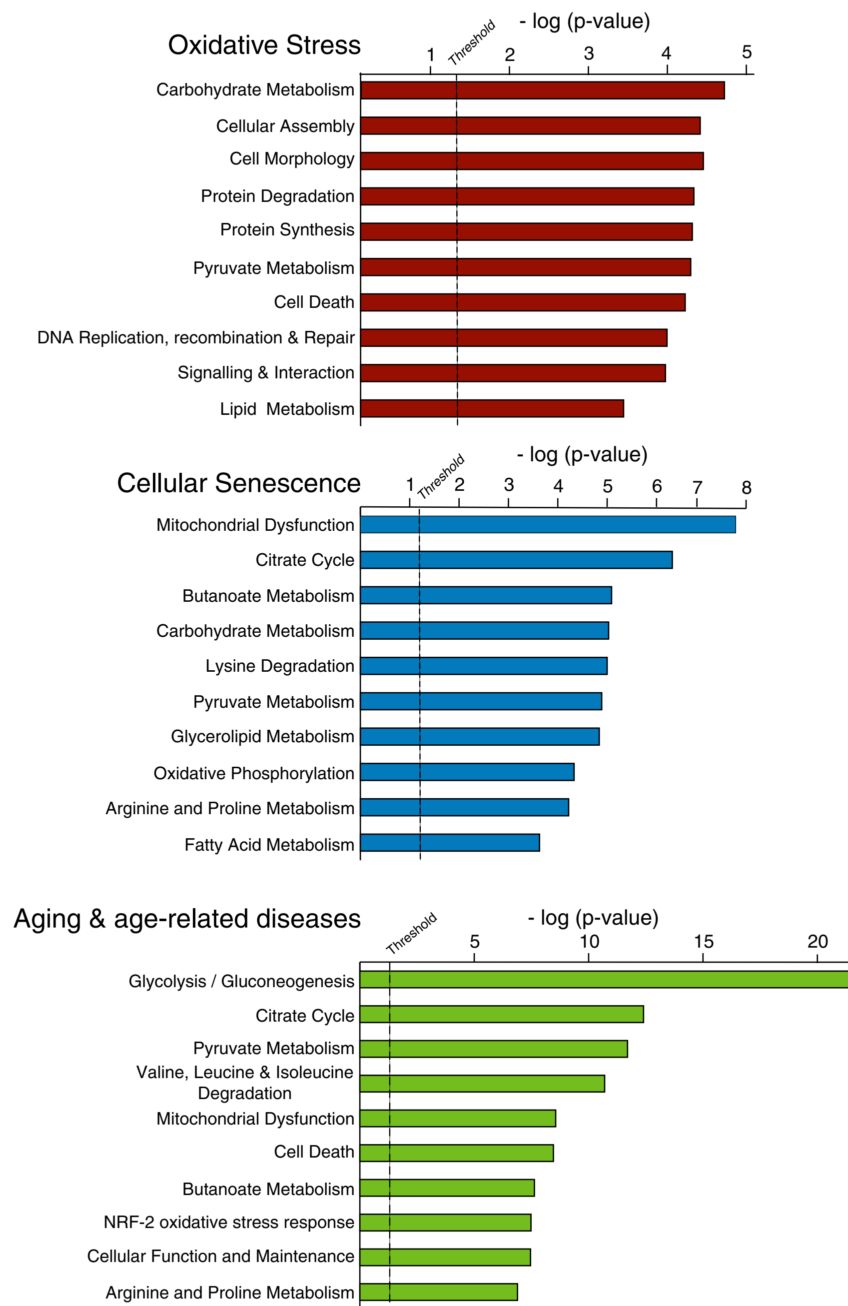

Fig. 2. Functional grouping of modified proteins. Proteins modified upon oxidative stress, cellular senescence and during aging and age related diseases were grouped in functional categories through the use of Ingenuity Pathways Analysis. The bars represent the biological functions identified, named in the x-axis. The dotted line represents the threshold above which there are statistically significantly more genes in a biological function than expected by chance.

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