Protein Thiol Modification and Thiol Proteomics

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1. Introduction

Cysteine plays an important role in the regulation of redox chemistry and gene expression and is essential in the structural and macromolecular organisation of proteins. Thiol oxidation leads to misfolding and the influencing of the protein function (Buczek et al., 2007). In our experiments, we have identified an oxidised cytoskeletal protein actin involved in the rearrangement of filament in the cells, leading to cellular apoptosis (Wang et al., 2010). Redox signalling can be relayed through intramolecular or intermolecular disulphide formation (Li et al., 2005).

Redox proteomics is an emerging branch of proteomics aimed at detecting and analysing redox-based changes within the proteome in different redox statuses (D’Alessandro et al., 2011). For this reason, several experimental approaches have been developed for the systematic characterisation of thiol proteome. One major limit in such an analysis is the chemical labile nature of Cys redox modifications; thus - basically - two critical steps are needed in analysing the thiol proteome, which consists of a temporary trapping of free thiols and their subsequent reduction (Avellini et al., 2007; Butterfield and Sultana, 2007).

2. Analysis of redox-sensing and signalling thiols

The cysteine proteome includes 214000 Cys with thiols and other forms. A relatively small subset functions in cell signalling, while a large number functions in response to the redox state. The former are redox-signalling thiols and the latter are defined as redox-sensing thiols (Dietz, 2003; Jones and Go, 2011; Sen, 1998; Sen, 2000). Some proteins contain Cys residues that are regulatory: their oxidation leads to misfolding and the influencing of protein activity. Several cytoskeletal proteins have been identified to be oxidative sensitive. Specific Cys residues’ oxidation in these proteins has been identified. Among them, actin is the main component of the microfilament cytoskeleton and exists as monomeric G-actin, which can polymerise into filamentous F-actin upon extracellular stimuli. The constant and rapid reorganisation of the actin microfilament system is highly regulated (Carlier, 1991). A growing body of evidence indicates that the actin system is one of the most sensitive constituents of the cytoskeleton to oxidant attack. Recent redox proteomics studies have detected actin as the most prominent protein oxidised in response to the exposure of cells to oxidants (Fiaschi et al., 2006). The direct redox regulation of actin in vivo is one of the most important processes regulating the dynamics of the microfilament system. Trx1 was identified as interacting with actin and protecting the actin cytoskeleton from oxidative
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Moreover, actin can be kept at a reduced status, even at a higher concentration of H$_2$O$_2$ stimulation, under the protection of Trx1. Trx1 is expressed ubiquitously in mammalian cells and contains a conserved Cys-Gly-Pro-Cys active site (Cys 32 and Cys 35) that is essential for the redox regulatory function (Carlier, 1991). In addition to the conserved cysteine residues in the active site, three additional structural cysteine residues (Cys 62, Cys 69, and Cys 73) are present in the structure of the human Trx1 (Nishiyama et al., 2001). Trx1 is S-nitrosylated on Cys 69, which is required for scavenging ROS and preserving the redox regulatory activity, and contribute to the protein’s anti-apoptotic functions (Haendeler et al., 2002). Cys 73 residue is involved in dimerisation of Trx1 via an intermolecular disulphide bond formation between Cys 73 of each monomer in the oxidised state. The biological function of the Cys 62 and Cys 69 residues in the non-active domain remains to be fully elucidated. Some studies suggest that the formation of a disulphide bond between Cys 62 and Cys 69 created a way to transiently inhibit Trx1 activity for redox signalling under oxidative stress (Watson et al., 2003). A new role for Cys 62 - although it is not a key site that is involved in cellular redox regulation - plays an important role in mediating its interaction with actin. This interaction disappeared with the increasing concentration of H$_2$O$_2$ stimulation. One possible reason is that the intramolecular disulphide bond formation inhibits the activity of Trx1. Different H$_2$O$_2$ concentrations have different oxidative effects of functional relevance, leading to dimer formation, glutathionylation and depolymerisation of the actin system, depending on the location of the actin molecules, the source of the oxidant and the availability of surrounding reducing systems (Lassing et al., 2007). Many studies on oxidative stress have shown that both Cys 374 and Cys 272 of $\beta$-actin are highly reactive to oxidising agents. Chemical modification of Cys 374 affects polymerization ability and profilin binding (Dalle-Donne et al., 2007). The intracellular thiol homeostasis is maintained by the thioredoxin and glutaredoxin systems, which utilise NADPH as reducing equivalents in order to reduce proteins (Kalinina et al., 2008). Thus, oxidative modification may be restored by these redoxins and glutaredoxins. In vivo, the direct redox control of actin by Trx1 could be one of the most important processes regulating the dynamics of the microfilament system. It has been demonstrated that Trx1 could protect cells from apoptosis by the thiol oxidoreductase activity (Damdimopoulos et al., 2002; Poerschke and Moos, 2011; Smeets et al., 2005). Moreover, reduced Trx1 forms a complex with the apoptosis signalling regulating kinase-1 (ASK1) and protects cells from apoptosis by inhibiting ASK1 (Saitoh et al., 1998). Cys 62 in Trx1 plays an important role in protecting cells from apoptosis, independently of its role in the enzyme active site. Trx1, by binding to actin and regulating its dynamics, could protect cells from apoptosis (Wang et al., 2010). The results of oxidative stress on protein thiols and disulphides in Mytilus edulis revealed by proteomics also suggest that actin and protein disulphide isomerase are redox targets (McDonagh and Sheehan, 2008). Actin was also identified by affinity chromatography assay to be a Trx1 target in eukaryotic unicellular green algae (Saitoh et al., 1998). Both actin and Trx1 are evolutionarily conserved proteins, the protection of actin from oxidative insult by the TRX system could be a universal regulatory mechanism. Many crucial signalling pathways utilise the reversible oxidation and reduction of cysteine thiols as a molecular switch. Redox-based regulation of gene expression has emerged as a fundamental regulatory mechanism in cell biology. Some proteins have apparent redox-sensing activity: electron flow through side-chain functional CH2-SH groups of conserved cysteiny1 residues in these proteins accounts for their redox-sensing properties. Protein thiol groups with high thiol-disulphide oxidation potentials are likely to be redox-sensitive.
A class of signalling factors has been identified which uses cysteine residues in the conserved motifs (such as CXXC or CXXS) as redox-sensitive sulphhydryl switches to modulate specific signal transduction cascades that have similar redox-sensitive sites (Lemaire et al., 2005). The identity of the amino acids separating the two cysteines in the CXXC motif and protein location influences the redox properties of CXXC-containing proteins - these proteins may serve as reductants or oxidants. TRX is a key molecule in the maintenance of the cellular redox balance. In addition to the cytoprotective action against oxidative stresses, it is involved in various cellular processes, including gene expression, signal transduction, proliferation and apoptosis (Klemke et al., 2008). Hepatopoietin (HPO) is a novel hepatotrophic growth factor, which is involved in the process of liver regeneration in rats, mice and humans (Francavilla et al., 1994; Hagiya et al., 1994). It belongs to the family essential for respiration and vegetative growth (ERV) 1/augmenter of liver regeneration (ALR). Both HPO and TRX have conserved CXXC motifs as their enzymatic active site. These cysteines in the redox regulatory domain are reactive and can be covalently linked to other proteins by forming disulphide bridges. It has been known that the family of FAD-dependent sulphhydryl oxidase/quiescin-Q6-related genes contains thioredoxin (TRX) and yeast ERV1 domains (Hoober et al., 1999). If a composite protein is uniquely similar to two component proteins, no matter whether they are in the same species or not, the component proteins are most likely to interact or be involved in the same signal transduction (Marcotte et al., 1999a; Marcotte et al., 1999b). HPO is identified as functioning in conjunction with TRX by which it plays an important role in sensing the extracellular redox signals. Homologs of this family have been found in a large number of lower and higher eukaryotes and in some viruses. Recently, ALR was identified as a sulphhydryl oxidase by its ability to oxidise thiol groups of protein substrates and the presence of an FAD moiety in the carboxyl-terminal domain and the formation of dimer in vivo (Hofhaus et al., 2003; Lisowsky et al., 2001). It has also been shown that the effect of HPO on the activator protein-1 (AP-1) is dependent on its sulphhydryl oxidase activity. ERV2, a member of ERV1/ALR in yeast, is an essential element of the pathway for the formation of disulphide bonds within the endoplasmic reticulum. E10R, a viral member of the ERV1/ALR protein family, participates in a cytoplasmic pathway of disulphide bond formation (Senkevich et al., 2000) and is responsible for the oxidation of the viral G4L gene product, which is homologous to glutaredoxin. A common characteristic of the proteins in this family is that they are involved in the redox reaction by the regulation of disulphide bond formation. Two major mechanisms involving the reversible modification of amino acid side chains to modulate protein activity are phosphorylation/dephosphorylation by kinase and phosphatase systems and reduction/oxidation by thiol-dependent enzymes (Nakashima et al., 2002). Whereas many signalling processes involving phosphorylation are well-understood in terms of the mechanisms and identities of participating enzymes, redox regulation of cellular processes remains a poorly characterised area. HPO directly interacted with TRX, by which the redox state of TRX was changed, and then its effects on the activity of AP-1 and NF-κB were potentiated. The transcription factors NF-κB and AP-1 have been implicated in the inducible expression of a variety of genes involved in responses to oxidative stress and cellular defence mechanisms (Xanthoudakis et al., 1992). A feature of NF-κB is that both oxidants as well as reductants are known to activate it (Byun et al., 2002; Jeon et al., 2003). Activation of NF-κB by TRX could be attributed to its reduced form for the overexpression of TRX caused activation of NF-κB and the degradation of IκB in the cytosol;
at the same time, the c-Jun NH2-terminal kinase (JNK) signalling cascade was also activated. However, some investigations have shown that the transient expression of TRX resulted in a pronounced inhibition of NF-κB-dependent transactivation in CAT assays. Our studies showed that when the COS7 cells were cotransfected with HPO and TRX, a part of TRX is oxidised in cells expressing TRX, but the DNA binding activity of NF-κB and its transactivation were increased. The results of yeast two-hybrid analysis demonstrate that the binding ability of HPO with HPO is higher than that of HPO with TRX, implying that under the stimulation of an oxidative signal HPO tended to be assembled into dimers. The direct transfer of oxidising equivalents by dithiol/disulphide exchange reactions can be demonstrated by the oxidation of TRX by HPO in vivo and in vitro. We could infer that the oxidising equivalents’ flow might be from HPO to TRX and then to substrate proteins, leading to the change of the redox state of substrate protein and finally to affecting the activity of AP-1 or NF-κB. Recently, we have demonstrated that HPO can exist as a homodimer via disulphide bonds and that HPO has the capacity to form both homodimers and heterodimers with its alternatively spliced forms, which might contribute to the existence of various HPO compounds in hepatic cells. The HPO dimer still has sulphydryl oxidase activity and serves as an oxidant under oxidative conditions. These results imply that under oxidative stress conditions, intermolecular disulphide bonds formed within HPO could be transferred by a dithiol/disulphide exchange reaction to the active site of TRX and then to substrate proteins. In this sense, HPO links the redox chemistry of the cell to the formation of disulphide bonds within cytoplasm, while TRX acts as a mobile carrier of oxidising equivalents inducing the latter into nucleus to activate the expression of related genes. Two members of the ERV/ALR protein family (ERV2 and E10R) could use molecular oxygen directly to contribute oxidising equivalents for disulphide bond formation. Here, we found that another member of this family (HPO, as a FAD-linked sulphydryl oxidase) could also use reactive oxygen to generate disulphide bridges in protein substrate.

HPO is assembled into a dimer under the stimulation of oxidants, such as H2O2 and diamide, and the HPO dimer could be dissociated into monomer by DTT both in vivo and in vitro. TRX, another component protein in Q6, is also sensitive to the alteration of the redox state by the change of its free thiols. These results support the assumption that both HPO and TRX are sensitive to the cellular redox state and involved in the modulation to it.

TRX has been shown to interact directly with the apoptosis signal-regulating kinase 1 (ASK1) by forming disulphide bridges and acting as a physiological inhibitor of ASK1 in stress-free cells (Saitoh et al., 1998). The interaction is dependent on the redox status of TRX and can be regulated by intracellular reactive oxygen species (ROS) levels. In particular, an increase in ROS concentration causes the dissociation of TRX from ASK. As a result, ASK1 can undergo polymerisation, which corresponds to the active form of the enzyme. ASK1 has been suggested to activate the p38 and JNK upstream kinases, MKK3/6 and MKK4/7, respectively. We therefore speculate that the interaction between HPO and TRX might disrupt the interaction between TRX and ASK1. It is reduced but not oxidised thioredoxin that acts as a high affinity inhibitor of ASK1. When thioredoxin is oxidised by HPO in cytoplasm, it leads to the dissociation of TRX from ASK1 and polymerisation of ASK, it activates the stress-activated protein kinase pathway, and it promotes JNK activation and increases the activity of AP-1 and NF-κB.

A comparison of HPO-specific redox components with those of other known pathways for disulphide bond formation suggests some interesting analogies. The upstream components of the three known pathways - namely E. coli DsbB, yeast ERO1p and ERV2p - are proteins
having two pairs of active cysteines each. In each case, the catalytic pair of cysteines interacts with ubiquinone, oxygen or another nonthiol electron acceptor forming a CXXC motif. The oxidative equivalents are then transferred to the second pair of cysteines on the same polypeptide chain, in the case of ERV2p, to the second subunit of a homodimer, and so is HPO. The second protein in the cascade of disulphide bond formation invariably is a thioredoxin-like protein - namely DsbA in E. coli, protein disulphide isomerase or its homologs in the yeast ER and the G4L thioredoxin-like protein in poxviruses. The HPO pathway therefore represents the first eukaryotic pathway for disulphide transmission in cytoplasm.

The importance of these findings is that the redox signalling transduction is conducted by the thiol-disulphide cascade in cytoplasm of mammalian cells. Thus, the pathways of disulphide bond formation in such diverse systems appear to use the same general principles of thiol-disulphide transfer between protein components. In this sense, HPO serves as a signal factor in the regulation of AP-1 and NF-κB activity via its cysteine. Early in the course of liver regeneration initiation, the expression of HPO increases quickly so that the cellular milieu becomes highly oxidising and these conditions shift the thiol-disulphide equilibrium of cellular proteins, which may play an important role in the stimulation of signalling transduction for promoting hepatocyte proliferation. This issue also explains the important role of HPO in liver regeneration and the mechanisms found in the calcium-dependent oxidation of TRX during cellular growth initiation. The rise in intracellular calcium induced by a growth factor binding to their receptor resulted in a marked conversion of reduced thioredoxin to the oxidised disulphide form. This apparent inhibition of thioredoxin reductase, coupled with the burst of H$_2$O$_2$ formation, leads to transient redox changes in cellular thiol proteins that may play an essential role in mitogen signal transduction. Thus, the relationship between HPO and TRX demonstrated by our results might shed new light on the signal transduction that oxidoreductase is involved in the processes of cell proliferation, apoptosis and organogenesis (Li et al., 2005).

3. Proteomics studies to analyse the oxidation state of proteins

3.1 Covalent modification to identify oxidation/nitrosylation of cysteine thiol groups
To date, 2DE coupled with mass spectrometry (MS) is still the best separation tool for analysing redox-based protein changes. ROS/RNS caused covalent modifications to proteins, which makes it possible to reveal these changes by applying specific labelling. Among the many kinds of amino acid residues susceptible to oxidative stress, cysteine is one of the most sensitive. Its free thiol groups play an important role in regulating protein functions and are often the target of oxidative stress. So far, several approaches have been developed to analyse the thiol proteome. The main limit is the chemical labile nature of Cys redox modifications. So, there are two critical steps needed in analysing the thiol proteome, which consists in trapping and reducing of free thiols. TCA (trichloroacetic acid)-based acidification was often used to quench the thiol groups, and then cell permeable Cys-specific reagents such as the alkylating agents iodoacetamide (IAA) or N-ethylmaleimide (NEM) were used to label the free thiols. Some specific reducing agents can be used to detect specific forms of oxidation. For example, cysteine residues in the form of sulphenic acid are difficult to identify because of their unstable chemical nature; however, this has been achieved by the exclusive reduction of sulphenic acid by sodium arsenite or through its reaction with specific chemicals, such as dimedone. S-nitrosothiols are reduced by ascorbate,
whereas stronger reductants such as DTT reduce both nitrosothiols and disulphides (Jones and Go, 2011).

3.2 Quantification of redox proteomics
Several thiol-reactive reagents have been used to reveal the extent of Cys oxidation by 2DE gels, which include the IAM-derivatives 5-iodoacetamidofluorescein and Cys-specific fluorescent reagent monobromobimane. Differentials in the gel electrophoresis (DIGE) technique have been used to analyse the “redoxome” (Sethuraman et al., 2004). In this method, a set of fluorophores of similar molecular weights and chemical structures that differ according to their spectral features (absorption and emission wavelengths) were applied. NEM or IAM derivatives of cyanine (Cy3, Cy5) and DY-dyes were used in Redox-DIGE. The limitation of the above method is that only abundant proteins are detected, often missing low amplitude proteins such as transcription proteins and regulatory proteins. One way solving this problem is to perform an upstream enrichment step for the oxidised protein-thiol fraction of the proteome using the biotin-switch method originally developed by Jaffrey et al. (Salsbury et al., 2008). Biotin-based strategies are largely used for the detection of S-glutathionylation and S-nitrosylation - two Cys modifications which occur extensively in diseases characterised by oxidative stress.

Sethuraman et al. described the shotgun proteomic approach: isotope-coded affinity tag (ICAT) reagents were applied to quantify oxidant-sensitive protein thiols. This technique uses a certain type of marker which consists of three different parts: (i) a thiol-reactive compound (an iodoacetamide analogue), (ii) a linker containing either heavy or light isotopes, and (iii) a biotin tag for separation by avidin-coupled affinity chromatography. The principle of the ICAT approach in redox proteomics is that only free thiols are modified by the IAM moiety of the ICAT reagent. After equivalent samples were exposed to either control or oxidant conditions in a non-reducing environment, they are differentially labelled with the heavy or light form of the ICAT. The protein samples are mixed and then, with tryptic digestion, the labelled peptides are separated by affinity chromatography. Finally, the captured peptides are analysed by LC-MS/MS for the identification of the oxidant-sensitive cysteine thiols.

3.3 Shotgun proteomics
At present, 2DE-based methods are gradually substituted by gel-free technologies, such as shotgun-proteomics strategies. Shotgun-proteomics refers to the direct analysis by MS/MS of proteolysed protein mixtures so as to rapidly generate a global profile of the protein complement within the mixture itself. This mixture is highly complex. A solution to overcome this is represented by alternative sample preparation strategies, which could be suitable for performing a preliminary enrichment of peptides containing redox-modified cysteines. Several methods have been developed for isolating peptides containing oxidised cysteines. One of these approaches designed for the specific enrichment of sulpho peptides in tryptic digests is based on anionic affinity capture using poly-arginine-coated nanodiamonds as high affinity probes (Aggarwal et al., 2006; Barrios-Llerena et al., 2006; Haas et al., 2006).

4. Human diseases and early hints from redox proteomics
Redox biology is key to the life sciences because an increasing number of cellular functions and impairments are found to be linked to redox processes. Accumulating evidence
suggests that a large number of diseases are closely related to oxidative stress. There is a growing need for the assessment of metabolic/oxidative stress and its modulation by the administration of pharmaceutical products. From a therapeutic point of view, drugs need to be designed to target oxidative stress sensitive biomarkers. In this context, redox proteomics might be pivotal in highlighting the main targets of protein oxidations and the biological pathways involved or compromised by these phenomena. Although the application of proteomics to drug design and development is in its earliest phase, preliminary redox proteomics results help to pave the way for further research in this field (D’Alessandro et al., 2011).

4.1 Neurodegenerative diseases

Neurodegenerative diseases such as AD, PD and HD each have distinct clinical symptoms and pathologies: they all share common mechanisms, such as protein aggregation, oxidative injury, inflammation, apoptosis, and mitochondrial injury which all contribute to neuronal loss. In neurodegenerative diseases, ROS generated by dysfunctional mitochondria are known to have a strong impact on the cellular proteome. Redox proteomic analysis of the post-mortem brains of AD patients revealed the presence of oxidative modifications of various protein substrates. Among them, some are relevant mitochondrial proteins, such as ATP synthase $\alpha$- and $\beta$-chain and VDAC (Robinson et al., 2011). These mitochondrial resident proteins were found to be oxidised in the hippocampus and the observed modifications could play a role in the mitochondrial dysfunction and cell death observed in AD. Extensive oxidative stress has also been detected in PD. It has been reported that $\alpha$-SYN was oxidised in the substantial nigra at the early stages of the disease. In addition, DJ-1 has been found to be modified by carbonylation and parkin to be S-nitrosylated, which results in a decrease of its E3 ligase activity. Several subunits of the respiratory complex I are subjected to oxidative damage, resulting in misassembling and the functional impairment of the complex. Redox proteomic analysis of HD R6/2 transgenic mice striatum revealed increased carbonyl levels in six proteins, including aconitase, creatine kinase and VDAC. Aconitase is an iron-sulphur protein that catalyses the isomerisation of citrate to isocitrate via cis-aconitate, and its inactivation may lead to an accumulation of reduced metabolites, such as NADH. The increased carbonyl levels, associated with the decreased activity of creatine kinase, could be relevant to the energetic impairments observed in HD (Sorolla et al., 2008). The role played by oxidative stress in ALS pathogenesis seems to be more relevant than in other neurodegenerative diseases. Oxidative damage induced oxidative modification of SOD1, UCHL-1 and Hsp70 proteins, which leads to the formation of SOD1 aggregation (Sorolla et al., 2008; Sussmuth et al., 2008).

Therefore, in the treatment of neurodegenerative diseases, neuroprotective agents which target ROS sources and aim at preventing their generation represent one class of drug therapeutics of great interest in pharmaceutical endeavours. Among them, the inhibitors of type B monoamine oxidase (such as selegiline and rasagiline) are the most promising neuroprotective agents to date, in that they prevent ROS generation. These inhibitors protect neuronal cells against cell death induced in cellular and animal models. The neuroprotective functions are ascribed to the stabilisation of mitochondria, the prevention of the death signalling process and the induction of the pro-survival anti-apoptotic Bcl-2 protein family and neurotrophic factors, thus counteracting mitochondria-mediated apoptotic pathways (Jones and Go, 2011).
4.2 Cardiovascular aging under oxidative stress

Reactive oxygen species (ROS) play an important role in the pathologic genesis of cardiovascular disease. Vascular enzymes such as NADPH oxidases, xanthine oxidase and uncoupled endothelial nitric oxide synthase, are involved in the production of ROS. NO· is produced in endothelial cells by the activation of eNOS during the normal functioning of the vessel wall. Vasodilator hormones raise intracellular Ca²⁺, leading to an increase in eNOS activity and NO· release. Physical forces such as fluid shear stress activate eNOS via protein kinase A- or Akt-dependent phosphorylation. The pathophysiological expression of inducible NOS in both macrophages and VSMCs elevates cytokine levels, resulting in localised inflammation. This, in turn, results in the production of NO· in the absence of further stimuli. Moreover, under some circumstances, eNOS becomes uncoupled and O₂ and is made instead of NO. The NOS enzymes are thus potentially important sources of both NO and O₂, depending on the surrounding environment. Virtually all types of vascular cells produce O₂ and H₂O₂. In addition to mitochondrial sources of ROS, O₂ and/or H₂O₂ can be made by many enzymes. Two of the most important sources in the normal vessel are thought to be cytochrome P450 and the membrane-associated NAD(P)H oxidase(s). A cytochrome P450 isoform homologous to CYP 2C9 has been identified in coronary arteries and has been shown to produce O₂ in response to bradykinin. NAD(P)H oxidases that are similar in structure to the neutrophil respiratory burst NADPH oxidase, but which produce less O₂ for a longer time, have been identified in vascular cells. The endothelial, VSMC and fibroblast enzymes are not identical but have unique subunit structures and mechanisms of regulation. One important aspect of ROS production by at least the VSMC NAD(P)H oxidase is that it occurs largely intracellularly, making it ideally suited to modify signalling pathways and gene expression. The activity of the NAD(P)H oxidases can be modulated by vasoactive hormones and the small molecular weight G-protein rac-1. Angiotensin II, tumour necrosis factor-α, thrombin and platelet-derived growth factor all increase oxidase activity and raise intracellular levels of O₂ and H₂O₂ in VSMCs. Angiotensin II and lactosylceramide activate the endothelial cell enzyme, whereas fibroblasts increase O₂ production in response to angiotensin II, tumour necrosis factor-α, interleukin-1 and the platelet-activating factor. Physical forces, including cell stretch, laminar shear stress and the disturbed oscillatory flow that occurs at branch points, are also potent activators of O₂ production in endothelial cells. There are two major mechanisms by which hormones and physical forces activate the NAD(P)H oxidase: (1) acutely, whereby the expressed enzyme is activated by phosphorylation, GTPase activity and production of the relevant lipid second messenger, and (2) chronically, when the expression of rate-limiting subunits of the enzyme is induced, thereby providing higher levels of enzyme susceptible to activation. Macrophages are perhaps the major vascular source of O₂ in disease states. They oxidise LDL via the activation of diverse enzymes. Neutrophils and monocytes may also secrete myeloperoxidase, which appears to initiate lipid peroxidation. Two potential diffusible candidates to initiate myeloperoxidase dependent lipid peroxidation are the tyrosyl radical and nitrogen dioxide (NO2) (Elahi et al., 2009; Fearon and Faux, 2009; Lakshmi et al., 2009; Strobel et al., 2011).

4.3 Aging and metabolism

Early attempts at antioxidant intervention as a means to delay aging were initiated soon after the free radical theory of aging was proposed. These attempts stemmed from the postulation of the free radical theory of ageing which posits that the accumulation of
oxidative damage underlies the increased cellular, tissue and organ dysfunction and failure associated with advanced age.

However, these antioxidant interventions have so far failed to extend life spans in most cases. At present, a series of encouraging - albeit preliminary - results have been reported in C. elegans and drosophila through the use of enzymatic synthetic drugs miming SOD and CAT activities, such as EUK-8 and EUK-134. However, while increasing antioxidant defences in these organisms, the drugs have not produced any significant increase in lifespan. Transgenic mice that constitutively over-express human CuZn-SOD did not live longer than control animals, while heterozygous mice with reduced MnSOD activity have a life expectancy that is similar to wild-type mice (although these animals have increased oxidative damage to their DNA). If free radicals are actually correlated to aging, a winning strategy should be targeted at preventing their production rather than increasing defences and repairing mechanisms against ROS-induced damages. The Mitochondrial Free Radical Theory of Aging (MFRTA) proposes that mitochondrial free radicals are the major source of oxidative damage. According to MFRTA, the accumulation of these oxidative phenomena is the main driving force in the aging process (Sanz and Stefanatos, 2008).

Recent findings shed further light on the strong linkage between aging and metabolism and have opened brand new scenarios in the field of drug discovery. Insulin-like signalling in C. elegans activates the transcription factor SNK-1, which is known to defend against oxidative stress by mobilising the conserved phase 2 detoxification responses and it is thus referred to as the longevity-promoting factor. While aging remains a controversial issue, good results have been obtained in the field of cosmesis, as far as skin-aging is concerned. Antioxidant drug developments against skin aging have been extensively developed. A role has been proposed for ascorbic acid, alpha-tocopherol, carotenoids, polyphenols and other substances, such as ergothioneine, Zn(II)-glycine and CoQ10 in the treatment of skin-aging. In particular, the topical application of CoQ10 and antioxidants like alpha-glucosylrutin diminished resistance in the keratinocytes of old donors against UV irradiation, both in in vitro and in vivo studies (D’Alessandro et al., 2011).

5. Challenges to mapping the thiol proteome

At present, mass spectrometry based proteomics makes rapid progress in mapping the Cys proteome (Chiappetta et al., 2011). These methods were also used to develop quantitative Cys proteomic databases and maps of redox systems biology. The full spectrum of Cys reactivity, such as glutathionylation, nitrosylation and other Cys modifications, needs to be analysed in order to address multiple modifications of the same Cys. Multiple modifications (e.g., products of benzene or acetaminophen oxidation) of a single Cys (e.g., C34 in albumin or Cb93 in haemoglobin) are used to identify chemical exposures. Links to chemical reactivity data, such as that provided by systematic comparisons of maleimide and iodoacetamide reactivity, would support an important chemical-biology interface which is currently lacking (Marino and Gladyshev, 2011).

To address the entirety of the Cys proteome, there is a need to understand the fractional contribution of Cys with high and low reactivities. Considerable attention has been given to oxidation of the Cys proteome by H$_2$O$_2$. However, protein thiols can be oxidised by many other chemicals, including hydroperoxides, endoperoxides and quinones. CySS reacts slowly with GSH, but many protein Cys residues are much more reactive. Cys/CySS shuttle functions in the regulation of extracellular thiol/disulphide pools (Mannery et al., 2011).
An alternative possibility explaining the maintenance of cellular proteins under a non-equilibrium, kinetically-controlled steady-state oxidation involves the pseudo-oxidase and/or pseudo-peroxidase activities of proteins. Very slow oxidative and peroxidative activities can be considered to be pseudo-oxidase and pseudo-peroxidase activities because the reaction rates and specificity for reactants are more similar to chemical reactions than to enzyme-catalysed reactions. Such reactions can depend upon low levels of associated metals, such as Cu2+ and Fe3+. For instance, Cu2+ can catalyse the oxidation of thiols in the presence of O2, resulting in thiol oxidation to a sulphenic acid or disulphide. Reduction back to a thiol by TRX or GSH would complete a pseudo-oxidase cycle. At low rates of oxidation of the Cys proteome where ongoing cellular H2O2 generation occurs by other mechanisms, such a reaction sequence is difficult to verify. Earlier studies showed that H2O2 production in cellular fractions increases in proportion to O2 partial pressure, and that protein oxidation occurs as a function of cellular iron and copper. Consequently, for the development of redox maps of the Cys proteome, additional information will be needed regarding the contribution of reactions of Cys at relevant, slow biologic reaction rates so that the system descriptions will adequately interpret reaction rates in systems biology models (Jones and Go, 2011).

6. Conclusion

Redox regulation is a fundamental physiological process which plays an important role in pathophysiological events. It is via reversible thiol modification that transcriptional and posttranslational responses are triggered. At present, an increasing number of techniques have been developed that make it possible to investigate, either qualitatively or quantitatively, modifications to specific amino acids (cysteines, tyrosines, etc.) or specific groups (carbonylations, nitrosylations, etc.). Redox proteomics is a powerful tool for monitoring physiological changes under oxidative stress. The identification of redox regulated proteins will provide great help in directing drug design and administration, new therapeutic targets and their validation. Currently, an accurate quantification of oxidised proteins remains difficult. A major task for future proteomics studies will be to develop tools to identify the different types of oxidation forms and establish the means to quantify the extent of such modification.

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8. References


Proteomics was thought to be a natural extension after the field of genomics has deposited significant amount of data. However, simply taking a straight verbatim approach to catalog all proteins in all tissues of different organisms is not viable. Researchers may need to focus on the perspectives of proteomics that are essential to the functional outcome of the cells. In Integrative Proteomics, expert researchers contribute both historical perspectives, new developments in sample preparation, gel-based and non-gel-based protein separation and identification using mass spectrometry. Substantial chapters are describing studies of the sub-proteomes such as phosphoproteome or glycoproteomes which are directly related to functional outcomes of the cells. Structural proteomics related to pharmaceutics development is also a perspective of the essence. Bioinformatics tools that can mine proteomics data and lead to pathway analyses become an integral part of proteomics. Integrative proteomics covers both look-backs and look-outs of proteomics. It is an ideal reference for students, new researchers, and experienced scientists who want to get an overview or insights into new development of the proteomics field.

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