Oxidation Proteomics: The Role of Thiol Modifications

Beat M. Riederer*

Centre for Psychiatric Neurosciences, Proteomics Unit, Hospital Centre and University Hospital (CHUV), Lausanne, Switzerland

Abstract: Identification of thiol modifications has gained significant importance. It is increasingly recognized that cysteines play an important role in protein function under both physiological and patho-physiological conditions. Here we reviewed different approaches that are used to identify oxidized proteins and discuss different fluorescent labeling techniques, differential two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization – time of flight identification, in short MALDI-TOF. We illuminate processes that depend on protein oxidation of cysteines and we look into consequences of thiol oxidation during aging and in a variety of diseases, with a special reference to Alzheimer's disease. There is an urgent need for methods that detect specifically oxidized proteins and are able to distinguish different oxidation types.

Key Words: Aging, cysteine, oxidation, thiol, DIGE, MALDI-TOF, Western blot, dye labeling, disease.

1. INTRODUCTION

During lifetime, organisms are constantly exposed to one or more conditions that generate reactive oxygen and nitrogen species (ROS/RNS) that either serve as second messengers in signal transduction or may damage proteins, nucleic acids and lipids. A free radical is, by definition, a chemical species containing unpaired electrons and is therefore paramagnetic (Finkelstein et al., 1980). Most of the oxygen derived free radicals that are relevant to cell biology are unstable and are of short life (Florence, 1991). Factors that influence the formation of ROS/RNS include a number of environmental conditions such as irradiation and pollutants in the atmosphere such as ozon, N₂O₂ and NO₂; however, many are derived free radicals that are relevant to cell biology are unstable and are of short life (Florence, 1991). Factors that influence the formation of ROS/RNS include a number of environmental conditions such as irradiation and pollutants in the atmosphere such as ozon, N₂O₂ and NO₂; however, many are simple by-products of normal metabolic processes, such as auto-oxidation of reduced forms of electron carriers (Moller et al., 2007) i.e. NAD(P)H, flavins and cytochrome P450s, inflammatory reactions, nitric oxide synthesis, oxidase catalyzed reactions, lipid peroxidation, glycation/glycoxidation and metal catalyzed reactions (for review see: Stadtman and Levine, 2000; Stadtman, 2006). To avoid cellular damage by these processes, most biological systems have developed a battery of anti-oxidants that can convert ROS and RNS to unreactive derivatives. They include i) a number of enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, thiol-specific peroxidase, methionine sulfoxide reductase, thio redoxin peroxidase, glutathione reductase and glutaredoxins; ii) various metal-binding proteins such as ceruloplasmin, ferritin, transferrin, various metabolites and cofactors (NADs, lipoic acid, uric acid, bilirubin etc); iii) a number of dietary components (vitamin A, C and E, quercetin etc.) and iv) metal ions (Mg²⁺, Mn²⁺, Zn²⁺). It is evident that the amount of protein oxidation occurring under a given set of conditions may reflect the balance between pro-oxidant and anti-oxidant activities and is dictated by prevailing environmental, genetic and dietary factors. Aromatic and sulfur-containing amino acid residues such as tyrosine, phenylalanine, tryptophane, histidine, cysteine and methionine are particularly susceptible to oxidative transformation i.e. formation of L-DOPA from tyrosines, ortho-tyrosine from phenylalanine, disulfides from methionine or cysteine, and kynurenines from tryptophane (Gianazza et al., 2007; Ptolemy et al., 2007). Chronic oxidative stress can induce irreversible changes in normal cellular metabolism, such as in the pathogenesis of diabetes or cardiovascular and neurodegenerative disorders (Brennan and Hazen, 2003; Ceriello and Motz, 2004; Dalle-Donne et al., 2006; Markesbery and Lovell, 2006; Sultana et al., 2006). It is impossible to give an integral overview on protein oxidation and its diversified facets. Here we will focus in particular on the proteomics part, how to identify proteins with oxidized cysteine residues. We will discuss the role of such oxidation in the regular metabolism and various diseases.

2. TYPES OF MODIFICATIONS

Reactive thiolate anions are the site of the most abundant cysteine modifications and an accurate detection, identification and quantification become of growing importance (Berlett and Stadtman, 1997; Sullivan et al., 2000; Ying et al., 2007). The pKₐ of most protein thiols is approximately 8.5 and therefore they are less reactive at intracellular pH, while a subset of thiols has a lower pKₐ, depending on the local charge of the environment and so may exist as thiolate anions (cysteine-S⁻) at physiological pH (Ying et al., 2007). These thiolates are more readily oxidized and apt to react with reactive oxygen and nitrogen species such as nitric oxide, hydrogen peroxide, nitrogen dioxide, peroxinitrite and hydroxyl radicals (Winterbourn and Metodiewa, 1999). Among modifications are S-nitrosothiols (-SNO), sulfenic, sulfonic or sulfonic acids (-SOₓH), glutathione intermediates or combinations of glutathione and sulfenic, sulfonic or nitrrosyl forms (Fig. 1). Consequences of such redox modifica-
tions at thiols are changes in physiological activity of a significant subset of proteins (Berlett and Stadtman, 1997; Stroher and Dietz, 2006; Sullivan et al., 2000; Ying et al., 2007). In addition, protein carbonylation was identified as a novel mechanism in redox signaling (Wong et al., 2008). Protein carbonyls are biomarkers for the presence and action of hydroxyl radicals. In the literature, "protein oxidation" appears to be used as a synonym for "protein carbonylation" and a commercial kit termed "OxiBlot" is widely used to detect gamma-glutamyl semialdehyde and 2-amino-adipic semialdehyde from arginine, proline and lysine as main targets of such oxidation (Gianazza et al., 2007). Disulfides are generally viewed as structurally stabilizing elements in proteins, however, some bonds contribute little to stabilization but act as reversible redox switches during aging and disease (Wouters et al., 2007). Such “forbidden” disulfide switches involve cysteines and may affect cellular function, cell cycle, protein carriers, entry of proteins into cells, blood coagulation or may influence cell immunity via cytokines.

3. DIFFERENT OXIDATION MECHANISMS

In several reviews, various mechanisms and enzymes involved in the redox control of neural function were discussed (Maher, 2006; Niwa, 2007; Poole et al., 2004; Townsend, 2007) and are briefly addressed here.

Glutathione (GSH) provides the major line of defense to protect cells from oxidative and other forms of stress, since it can scavenge free radicals, reduce peroxides or bind electrophilic compounds. However, cysteine is the limiting factor for GSH biosynthesis. Glutathione disulfide is harmful for cells and either is transported outside the cell or converted by glutathione disulfide reductase back to GSH. Glutathione peroxidases catalyze the reduction of hydrogen peroxide and organic hydroperoxides at the expense of GSH. In the brain, only little catalase is present. Glutathione transferases play a critical role in defending cells against reactive chemicals formed both from the breakdown of endogenously produced compounds and from biotransformation via conjugation with GSH. The addition of GSH to the cysteine sulfhydryl groups of proteins is defined as glutathionylation. Two basic mechanisms are involved in protein glutathionylation: GSH addition can occur either by a thiol-disulfide exchange reaction, wherein the thiolute anion (S-H) reacts with activated GSH (GS-SG, GS-OH or GS-NO) or with partially oxidized cysteine sulfhydryl group, sulfenic acid intermediates or S-nitroso intermediates.

Glutaredoxins are the enzymes that are considered to be responsible for the removal of GSH from glutathionylated proteins and include glutathione disulfide reductase and NADPH (Shelton et al., 2005).

Thioredoxin, Thioredoxin reductases and NADPH form one of the main redox couples in mammalian cells. Their function is to reduce disulfide bonds in a variety of proteins, including methionine sulfoxide reductases and peroxiredoxins (Kondo et al., 2006).

Peroxiredoxins are a family of thioredoxin-dependent peroxidases and thiol-specific anti-oxidant enzymes and are involved in the degradation of hydrogen peroxide. Among its members, the sulfiredoxins are important for reducing oxidized cysteine residues and also represent a signaling switch (Jacob et al., 2004; Jeong et al., 2006).

Fig. (1). A multitude of factors affect the formation of reactive oxygen species and modification of protein thiol oxidation and consequently influence the organism in different ways. Different modification types such as nitrosylation, sulfoxidation and glutathionylation modulate protein function and/or result in reversible or irreversible protein damage. Because many different proteins are modified, it is evident that many metabolic pathways and signaling cascades are influenced and modulate many physiological and patho-physiological cellular processes. This figure was adapted from various sources (Berk et al., 2008; Bonaventura et al., 2002; Hess et al., 2005; Hipkiss, 2006; Humphries et al., 2006; Klaunig and Kamendulis, 2004; Lamprecht et al., 2004; Passos and Von Zglinicki, 2006; Petropoulos and Friguet, 2006; Poppek and Grune, 2004; Stadtman and Levine, 2000; Wouters et al., 2007; Ying et al., 2007). Second messengers influence protein expression and kinases and phosphatases in signaling cascades. Among environmental factors are radiation, ozone and pollutants like smoke, fine dust particles etc. Diets include caloric restriction, vitamins, anti-oxidants and protein supplements. For diseases where oxidation plays a role are: neurodegenerations such as Parkinson’s and Alzheimer’s disease, cardiovascular diseases, atherosclerosis, diabetes, cancer and inflammation.
**Methionine sulfoxide reductase** is especially important to reduce the oxidation sensitive methionine; while a reduction of sulfoxide is reversible, but sulfones are no longer reducible (Petropoulos and Friguet, 2006).

Redox regulation of protein phosphatases and kinases is involved in a variety of signaling pathways. By changes of the intracellular redox state, specific phosphatases are inhibited while oxidants tend to increase activity of both, tyrosine and serine/threonine kinases.

Transcription factors that contain cysteine sulphydryl groups as the regulatory element are sensible to oxidation such as Nrf2, members of the AP-1 transcription factors containing Jun and Fos families or NF-xB (Townsend, 2007).

### 4. DETECTION TECHNIQUES

The identification, quantification and validation of putative biomarkers indicative of oxidative stress remain an ongoing challenge in analytical chemistry. There are several strategies for a comprehensive analysis of amino acid biomarkers for oxidative stress. Yet, despite a vast choice of detection methods, there is no ideal method (Bantscheff et al., 2007; Dalle-Donne et al., 2006; Kim et al., 2007; Ptolemy et al., 2007; Spickett et al., 2006; Stroher and Dietz, 2006; Ying et al., 2007). A variety of techniques, including radioisotopes or isotope coded affinity tags, or combined with fractional diagonal electrophoresis and protein labeling techniques using fluorophores, diagonal 2-DE and differential gel electrophoresis (DIGE) have been used to identify oxidized proteins. For an identification of modified amino acids, gas and liquid chromatography, tandem mass spectrometry, electrospray ionization and mass fingerprint spectrometry, ELISA and cyclic Voltammetry are essential to detect oxidative/nitrosative stress reporters in a variety of diseases (Palmieri and Sblendorio, 2007; Ptolemy et al., 2007); they are especially suitable for the detection of tyrosine residues. Different labeling methods that are used in mass spectrometry have been compared, including metabolic-, chemical-, enzymatic- protein or peptide labeling techniques or are label free. Despite this multitude of methods, not each technique can be meaningfully applied, especially for the quantitative analysis of post-translational modifications (Bantscheff et al., 2007).

Recent developments of differential covalent labeling techniques allow the detection of oxidant sensitive thiol proteins via fluorescence labeling and 2-DE. Maleimide dyes are specific for labeling of the cysteine thiol groups (Haugland, 1996) and may be used to label oxidation-sensitive cysteines (thiolate anions), as mentioned before.

By blocking reduced thiols first with N-ethyl-maleimide and by reducing oxidized thiol groups with dithiothreitol, Baty and colleagues (Baty et al., 2002) labeled the previously oxidized cysteine residues with 5-iodoacetamido-fluorescein of Jurkat T-lymphocytes and tested the influence of 2mM diamide resulting in an induction of oxidative stress. The protein composition was subsequently analyzed by 2-DE. Over 200 thiol proteins were detected. Unfortunately, it was difficult to detect any change in the oxidized thiol protein patterns. One should also realize that a reduction of proteins opens up di-sulfide bonds, essential for the structural conformation of proteins and thus the set of labeled proteins may not contain exclusively oxidized cysteines. Therefore, the use of the term "oxidized" cysteines should be cautioned, since the oxidation state of cysteines remains to be validated for each protein.

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**Fig. (2).** This figure was adapted from Riederer et al., (2008) and modified. Human brain sample from frontal cortex (68 year old male, 5 hours postmortem with mild Alzheimer's disease) was labeled with infrared maleimide dyes 680 (seen in red), and after reduction with 2-mercapto-ethylamine, the previously di-sulfhydryl-bound or oxidized proteins were labeled with the second infrared maleimide dye 780 (seen in green). Infrared labeled proteins together with unlabeled proteins were run on a 2-DE gel pH3-9 IEF NL isolectric focusing strip and separated on 12.5% polyacrylamid gel. The gel was scanned with the Odyssey Infrared Imaging System from LiCor and stained with colloidal Coomassie brilliant blue. Selected spots were identified by MALDI-TOF and peptide fingerprints revealed (represented by the letters that represent different amino acid residues at bottom of figure). Identified proteins are: (1) WD repeat protein 1 & succinate dehydrogenase flavoprotein subunit; (2) alpha-internexin & 60KD heat shock protein; (3) alpha-tubulins; (4) beta-tubulins; (5) GFAP; (6) actin cytoplasmic 1; (7) glyceraldehyde-3-phosphate dehydrogenase; (8) ubiquitin carboxyl-terminal hydrolase isozyme 1; (9) peroxiredoxin-1; (10) phosphatidylethanolamine-binding protein 1; (11) coflin-1; (12) peptidyl-prolyl cis-trans isomerase A; (13) cytochrome c oxidase; (14) Hemoglobin subunit alpha; (15) FK506-binding protein 1A; (16) hemoglobin subunit alpha; (17) macrophage migration inhibitory factor.
In an extension of this procedure, the N-ethylmaleimide blocking of accessible (reduced) thiol groups was replaced by a first labeling step with fluorescent dye maleimide, followed by a subsequent reduction with 2-mercapto-ethylaminal-HCl and a labeling of the rest of the cysteines with a second fluorescent maleimide dye of a different spectral wavelength emission than the first dye (Riederer et al., 2008). In that way, natively occurring reduced proteins could be distinguished from di-sulfhydryl or oxidized cysteines in a single 2-DE by a difference in the emission wavelength of 700nm (visualized in red color) and 800nm (seen in green color) respectively (Fig. 2). In Alzheimer brain tissue, it was shown that many proteins contain cysteines in reduced and in conjugated or oxidized form. Previously, several proteins were identified by DIGE with infrared maleimide dyes and a subsequent identification of oxidized cysteine residues by 2-DE (Riederer et al., 2008; Riederer, 2008). DIGE is a means to identify potential candidates; yet with a subsequent mass spectrometry, many peptide finger prints may escape detection since there are too many oxidation modification possibilities to consider. In addition, possibilities increase exponentially when two or more cysteine, methionine or other oxidation-prone amino acid residues may be present in a given peptide finger print. In addition, some modifications resist alkylation and reduction prior to mass spectrometry digestion (Granvogl et al., 2007). Considering these difficulties, to identify the variety of oxidized forms any tandem mass spectrometry identification of proteins should be verified with additional methods. It would be very helpful to have specific antibodies that detect nitrosylated or sulfonated cysteine or oxidized amino acid residues. Furthermore, the functional role and significance of the different oxidation forms need to be identified for each modified site. From a recent study (Riederer, 2008), several of the identified proteins that are susceptible to protein oxidation and their role are summarized in (Table 1). In particular, the glial fibrillary acidic protein (GFAP) contains a single cysteine residue, and the method shows that it is possible i) to label proteins with a single cysteine residue; and ii) that this thiol group is present partially in oxidized and also in reduced form. The same study indicated that much tubulin and strongly oxidized proteins are probably not labeled by maleimides, since they remain oxidized even after reduction with 2-mercaptoethylamine-HCl. Thus, a large proportion of cysteines may escape identification by mass fingerprinting (Riederer, 2008). Furthermore, mass spectrometry indicated that more methionines than cysteines are present in oxidized state. Yet, the specific oxidation modifications still need to be identified. One realizes that we see just the tip of the iceberg.

The "OxiBlot" was used to identify protein carbonyl groups by derivatization of oxidized proteins with 2,4-dinitrophenyl hydrazine (DNP), followed by a 2-DE separation, and a detection of proteins on polyvinylidenfluorid membranes with an anti-dinitrophenyl antibody (Kim et al., 2007). This method was applied to study effects of a dietary supplement (grape seed extract as anti-oxidant) on the aging rat brain. The same technique was applied to identify over 100 oxidized proteins in brain tissue from Alzheimer's patients, see part 7 (Korolainen et al., 2002). One needs to keep in mind that DNP is reactive with oxidized arginine, proline and lysine and not necessarily with cysteine (Gianazza et al., 2007).

Cysteine-labeling with iodoacetylated cyanine dyes and lysine–labeling with NHS-ester cyanine dyes were compared to monitor redox-dependent thiol-containing proteins in mammary luminal epithelial cells (Chan et al., 2005). Although, lysine is not a principal target in oxidation, it may be involved in cellular redox regulation, protein folding, proliferative suppression, glycolysis and cytoskeletal organization and add to complexity of the responses to oxidative stress.

In an approach, termed "focused proteomics", phosphoproteins were identified via Pro-Q Diamond staining in monoclonal antibody-based isolation of the oxidative phosphorylation machinery (Murray et al., 2004). Five complexes that are involved in oxidative phosphorylation were isolated: NADH dehydrogenase, succinate dehydrogenase, cytochrome c reductase, cytochrome c oxidase and F1F0 ATP synthase. Pro-Q Diamond stain was used to identify phosphoproteins in an oxidative stress-induced signaling pathway during inflammation. Macrophages and epithelial cultures were exposed to diesel exhaust particles. Several proteins of the cytokine proteome and mitogen-activated protein kinase signaling pathway were activated, including extracellular signal regulator kinases 1 & 2, c-Jun N-terminal kinases 1 & 2, protein phosphatase 2A, heatshock protein 27 and other proteins involved in oxidative stress (Wang et al., 2005a). Thus, redox- and phospho-proteomics have many common points in terms of identification of oxidized or phosphorylated sites and their involvement in signal transduction (Larsen et al., 2001; Morandell et al., 2006). However, while phosphorylated residues contain one single phosphate, oxidized amino acid residues may be present in a multitude of modification forms.

The principal shortcoming in using labeling methods in 1- or 2-DE gels and mass spectrometry is the misidentification of the labeled proteins and not knowing which one of the cysteine or methionine residue in an identified peptide is present in an oxidized form. In Fig. (3), a Coomassie 2-DE (A) and a Western blot stained for S-nitrosylated proteins (B) point to a common problem: A positive identification of the most abundant protein in a spot does not necessarily mean that it is the same protein that is present in oxidized form, because several S-nitrosylated spots were at sites where no spots were visible on the Coomassie gel. A careful comparison of the two gels is currently published (Riederer et al., 2009).

In 2-DE proteomics, usually proteins are reduced and then separated on a 2-DE; yet there are several protocols to investigate protein structure with a combination of steps and various redox conditions. The apparent size of proteins may therefore vary considerably with either covalent disulphide bonds or monomeric forms present (Wait et al., 2005). The most definitive detection of modified cysteines requires purification of a protein of interest. In matrix-assisted laser desorption ionization (MALDI) mass spectrometry, peptide masses are matched to a protein database, and potential mass increments representing adducts to cysteine mass (modification type) can be queried from a database – (often limited to one or two modification types). Reduction and
alkylation steps are included in most in-gel digestion procedures – if not performed before electrophoresis to reduce disulfide bridges. Upon alkylation with iodoacetamide, cysteines are transformed into carboxy-amido-methylcysteine with a mass change of 57.02 Da (Granvogl et al., 2007). When samples are not reduced, increments of 16, 32 or 64 Da indicative for oxygen or 305 Da for GSH and combinations with nitrosyl and sulfoxides have to be considered (Ying et al., 2007). Given that many amino acid side chains are subject to oxidation adducts (Stadtman, 2006; Hipkiss, 2006), a search can become cumbersome and the complexity may rapidly increase when different types of modifications per peptide or fingerprint have to be expected and need to be identified.

5. OXIDATION IN AGING AND DISEASE

A variety of proteomic changes characterize the aging tissue; firstly, age-related protein expression, and secondly, post-translational modifications such as phosphorylation, glycosylation and oxidation, leading to an alteration in the protein metabolism. The role of oxidation processes in brain aging (Barja, 2004; Berlett and Stadtman, 1997; Pakkenberg et al., 2003; Pamplona et al., 2005; Poon et al., 2004a;b; 2005a; b; 2006a;b; Stadtman, 2006) and in neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease have been extensively reviewed (Akterin et al., 2006; Aslan and Ozben, 2004; Canet-Aviles et al., 2004; Castegna et al., 2002; Choi et al., 2004; Chong et al., 2005a; 2005b; Cole et al., 2005; Jayakumar et al., 2003; Lee et al., 2006; Moore and O’Banion, 2002; Newman et al., 2007; Tezel et al., 2005; Widmer et al., 2006). It is apparent, that during aging, metabolic or functional oxidation results in an increased formation of ROS/RNS and as a consequence, induces various changes such as conformational changes in protein structure and protein dysfunction and/or results in cell and DNA damage. Such events may have even more profound consequences in a variety of diseases. However, oxidation may represent a form of cellular stress, therefore similar proteins may be identified in a variety of diseases. Among the different redox mechanisms, GSH plays a central role in psychiatric disorders (Berk et al., 2008), in particular in Schizophrenia, genetic and proteomic variations have been identified (Gysin et al., 2006; 2007, 2009). In aging, a disruption of redox regulation likely contributes to the exponential rise in the level of oxidized proteins, including a redox-dependent modulation of Krebs cycle enzymes, oxidative protein damage and protein degradation and age-dependent accumulation of oxidized proteins that are no longer degraded but rather accumulate (Humphries et al., 2006; Poppek and Grune,

Fig. (3). Human frontal cortex tissue (78 year old female, 13 hours postmortem delay without neurological disease) was focused on a pH3-pH11 NL 7cm isoelectrofocusing strip and separated on a 13% SDS-PAGE. Gels were either stained with colloidal Coomassie brilliant blue (A) or proteins were electrically transferred to nitrocellulose filters and immunostained with antibodies and revealed by electro-chemiluminescence, as previously described (Riederer and Riederer, 2007). The following antibodies were used: (B) a rabbit anti-S-Nitrocysteine "NOcys" (Sigma N5411), (C) a monoclonal anti-phospho-serine "ser-P" (Sigma P3430) and (D) tau-1 hybridoma supernatant (Riederer et al., 2001), all antibodies were used at a dilution of 1:1000. The molecular weights are indicated to the right. Selected proteins are indicated with arrows (tubulin, actin, GFAP and tau proteins). Note that S-nitrosylated cysteines are still detectable, despite a reduction of thiol-bonds with 130mM dithiothreitol during an equilibration of isoelectrofocusing strips prior to the electrophoresis separation in the second dimension.
Therefore, such an increase of free radicals and oxidation of molecules is promoting the aging and degeneration process (Maccioni et al., 2004; Petropoulos and Friguet, 2006). Carboxylation, as a result of oxidative stress, induces protein aggregation that may become cytotoxic and may be involved in a large number of age-related diseases such as Parkinson and Alzheimer's disease (Nystrom, 2005; Thomas and Beal, 2007). A result of oxidative stress is also carcinogenesis, i.e. an unregulated or prolonged production of cellular oxidants that can be linked to mutation (DNA damage), as well as including the gene expression of the AP-1 and NFKb genes (Klaunig and Kamendulis, 2004), or changes in proliferation and apoptosis (Giles, 2006). In the formation of cataracts, a selective oxidation of cysteines and methionines to disulfides, cysteic acid, sulfoxide, sulfones or mixed with GSH was observed and was followed by an aggregation of proteins (Garner and Spector, 1980). During aging, oxidative defense mechanisms are altered (Cao et al., 2004; Scaduto and Grottyohann, 1992) and glycation or irreversible oxidation seem the main causes for protein damage and dysfunction (Yin and Chen, 2005). It is well known that aging has an effect on mental decline (Gallagher et al., 2003; Jorissen and Riedel, 2002; Mattsson, 2002; Solfrizzi et al., 2003). An updated free radical theory of aging hypothesizes that oxidation has not only an important metabolic function but at the same time is a limiting mean and may control maximum functional life span (Harman, 2006), and furthermore, may represent an important risk factor for a pathological aging. The search for longevity genes still bears some discrepancies due to the habits of traveling and population dynamics. Therefore, large epidemiology studies will be necessary to identify longevity genes and their potential effect on oxidation (Salvioli et al., 2006). Oxygen free radicals may have an effect on telomeres and may be limiting factors in cellular senescence (Passos and Von Zglinicki, 2006). Oxidants can activate signaling pathways and modulate a variety of cellular activities. It may also result in metabolic changes and a functional impairment of the cytoskeleton (Dalle-Donne et al., 2002; Landino et al., 2004a-d). While most intracellular thiols are maintained in a reduced form, by various mechanisms such as GSH and thioredoxins (Baty et al., 2002), oxidation can result in transient formation of inter- and intermolecular disulfides and sulfenic acids. Plasma thiols are essential in rheumatoid arthritis (Rafter, 1994). Protein oxidation is also closely related to protein damage and to a loss of specific protein function, with abnormal protein clearing, depletion of the cellular redox balance, an interference with the cell cycle and ultimately cell death in aging and neurodegenerative diseases such as Alzheimer's disease (AD) (Akterin et al., 2006; Castegna et al., 2002; Tezel et al., 2005). Although, the use of autopsy tissue may raise criticism due to postmortem delay and modifications during storage, as well as medical treatment of the patient prior to death, however there is already much data published which validate that oxidized proteins may be involved in the pathogenesis of Alzheimer's disease and confirm that redox proteomics is essential to identify target proteins (Korolainen et al., 2006; Riederer et al., 2008; Sultana and Butterfield, 2004). The protein profiles of various aged rat astrocytes in response to oxidative stress were investigated by a 30 minutes exposure of 100µM H2O2 and subsequent 2-DE, silver nitrate protein staining and Pro-Q Diamond phosphoprotein staining (Miura et al., 2005). Among the down-regulated proteins were peroxiredoxin II and III, usually involved in anti-oxidant defense, increased calpactin I light chain, involved in calcium homeostasis. Peroxide-exposure induced the phosphorylation of heat shock protein 60kD and α-tubulin. Annexins may act as endogenous neuroprotective agents and seem also involved in pathological states of neurodegeneration (Eberhard et al., 1994).

6. OXIDATION SENSITIVE PROTEINS

Oxidative events also play an essential role in the modulation and control of cell differentiation, development and maturation, and participate in different cellular signaling processes (Allen et al., 1999; Finkel, 2000; Kamata and Hirata, 1999; Paget and Butner, 2003; Sauer et al., 2001). Oxidation may act on a variety of systems and it seems essential to set up a list of proteins that play a key role and that are susceptible to oxidation. Cysteines are essential in the structural and macromolecular organization of proteins and their oxidation, leading to misfolding and influencing protein function (Guan and Chance, 2005; Netto et al., 2007; Wanders and Waterham, 2006; Zhang and Kaufman, 2006), such as a reduced assembly capacity for tubulin (Landino et al., 2002), a changed estrogen receptor function (Atsriku et al., 2007), or leading to misfolding and aggregation of superoxide dismutase and eventually causing familial amyotrophic lateral sclerosis (Kabashi et al., 2007) as well as affecting mental functions (de Grey, 2006; Hu et al., 2007; Maier and Chan, 2002). Labeling techniques for cysteine thiol groups are therefore of special interest (Baty et al., 2002; Berlett and Stadman, 1997; Ying et al., 2007; Riederer et al., 2008).

In brain, several oxidized cytoskeletal proteins have been identified such as tubulin and neurofilament proteins, as well as the membrane-related spectrin (Landino et al., 2004b). Tubulins are partially oxidized (Riederer et al., 2008), and it was shown that oxidation affects microtubule assembly, since peroxynitrite oxidized tubulin is less stable despite being competent (Landino et al., 2002). One can speculate that the functional role of tubulin is affected by oxidation of cysteines and consequently by conformational and functional changes, thus explaining that microtubule elongation may depend on a correct tubulin conformation (Chrétién and Fuller, 2000). Consequently, modifications in the oxidation status of the cytoskeletal proteins may strongly influence microtubule function. It is tempting to speculate that oxidized tubulin decreases microtubule stability and influences transport functions, cell division or cell morphology.

7. ALZHEIMER PROTEOMICS

Especially in Alzheimer's disease, abnormal phosphorylated tau proteins accumulate in the form of paired helical filaments (also called tangles) that are no longer involved in microtubule dynamic control (Goedert et al., 1989; Mandelkow et al., 2007); tau proteins are also influenced by redox mechanisms (Korolainen et al., 2006; Landino et al., 2004c; McDonagh and Sheehan, 2007; Riederer et al., 2008). In Fig. (3) it is shown that β-tubulin, GFAP and many tau isoforms contain phosphorylated serine residues (panel C), and tau isoforms are nitrosylated at cysteine residues (arrows in panel B).
Over 100 oxidized proteins were detected in Alzheimer’s disease tissue by using DNP derivatization, 2-DE, electro-transfer to polyvinylidenfluoride membranes (Korolainen et al., 2002). Firstly, total proteins were stained with SYPRO Ruby and secondly oxidized proteins were detected with an anti-dinitrophenyl antibody. Interestingly, a number of proteins tended to be less oxidized in AD, while glutamate dehydrogenase and cytosolic malate dehydrogenase were increased in amount but significantly decreased in their degree of oxidation in AD brain samples. Apparently, also some compensatory changes may occur in relation to oxidative stress (Korolainen et al., 2006). A panel of proteins were identified in Alzheimer tissue (Riederer et al., 2008) and are summarized in Table 1. Cytoskeletal proteins, chaperone proteins, proteins involved in ubiquitination, and several redox proteins were found, suggesting that several pathways are modulated by oxidation. It is now of interest to study these proteins and their oxidation changes in a large cohort of control and Alzheimer cases.

8. AGE AND NUTRITION

Among the dietary manipulations known to retard aging is a caloric restriction by lowering sulfhydryl-containing amino acids, by restricting the concentration of methionines and by maintaining high blood levels of GSH, an essential anti-oxidant (Mattison et al., 2003; Poon et al., 2006a; Zimmerman et al., 2004). This underlines the importance of cysteines and a control of anti-oxidants. Many studies point to a benefic effect of anti-oxidants to reduce oxidative stress (Roussel and Ferry, 2002), inflammation (De la Fuente et al., 2005; Hirokawa and Utsuyama, 2002; Hu et al., 2000; Reimund, 2002), atherosclerosis (Cai et al., 2007; Cao et al.,

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<th>Protein</th>
<th>Function</th>
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<tr>
<td>Tubulins &amp; actin</td>
<td>Cytoskeleton, shape, motility, transport, mitosis</td>
<td>Landino et al., 2004a,c,d; McDonagh and Sheehan, 2007</td>
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<td>Cofilin</td>
<td>Cytoskeleton, spine shape</td>
<td>Ono, 2003, Zhou et al., 2004; Fass et al., 2004</td>
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<td>GFAP</td>
<td>Astroglia cytoskeleton</td>
<td>Korolainen et al., 2006; Porchet et al., 2003</td>
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<td>Alzheimer’s and Parkinson’s disease</td>
<td>Wright and Brown, 2008; Leuba, et al., 2008</td>
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<td>Chaperonin, protein folding and conformation</td>
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<td>Immunophilins and chaperone function</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Glycolysis, transcription, DNA repair, ubiquitination</td>
<td>Sirover, 1997; Ishitani et al., 2003; Duncan and Heales, 2005; Wang et al., 2005b; Tezel et al., 2005; Hara et al., 2006; Cueille et al., 2007</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>Mitochondrial &amp; age-related stress</td>
<td>Korolainen et al., 2002; Haripriya et al., 2004</td>
</tr>
<tr>
<td>Succinate Dehydrogenases (ubiquinone)</td>
<td>Mitochondrial disease &amp; age-related stress</td>
<td>Pithukpakorn, 2005; Samat and Marin-Garcia, 2005; Abd El Mohsen et al., 2005; Choksi et al., 2007; Wada et al., 2007</td>
</tr>
<tr>
<td>Ubiquitin carboxyl-terminal hydrolase isozyme L1</td>
<td>Alzheimer’s &amp; Parkinson’s ubiquitination</td>
<td>Liu et al., 2002; Choi et al., 2004</td>
</tr>
<tr>
<td>Phosphatidylethanolamine-binding proteins</td>
<td>Cell signaling in MAP kinase pathway</td>
<td>Odabaei et al., 2004; Hoffrogge et al., 2007; Li et al., 2007</td>
</tr>
<tr>
<td>Peroxiredoxins, thio- &amp; sulfiredoxins</td>
<td>Oxidative stress, biomarker in Alzheimer’s disease</td>
<td>Hol et al., 2005; Woo et al., 2005; Patenaude et al., 2005; Akterin et al., 2006; Butterfield et al., 2006; Grundke-Iqbal et al., 2006; Jeong et al., 2006; Sata, 2006; Graff-Radford and Woodruff, 2007</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>Oxidation defense, dysfunction in Alzheimer’s disease</td>
<td>Kakihana et al., 2003; Herold et al., 2004; Ohta and Ohsawa, 2006; Fukui et al., 2007; Opici et al., 2007; Newman et al., 2007; Trisdall et al., 2007; Weinreb et al., 2007</td>
</tr>
<tr>
<td>Macrophage migration inhibition factor</td>
<td>Migration inhibition, redox regulation</td>
<td>Thiele and Bernhagen, 2005</td>
</tr>
</tbody>
</table>

* Contains no cysteine residues.
2004; Guo et al., 2002; Rinaldi et al., 2006) and neurodegeneration (Cole et al., 2005; Desport and Couratier, 2002; Fraker and Lill-Elghanian, 2004). Moderate exercise is also known to reduce oxidative stress and inflammation (Asghar et al., 2007; Navarro-Arevalo et al., 1999; Radak et al., 2005). Furthermore, nitric oxide production has a beneficial effect on hypertensive rats and results in a reduced blood pressure via the regulation of endothelial and neuronal NO synthase isoforms (Kojsova et al., 2006). In a recent review, several dietary components have been discussed to affect cognitive abilities (Gómez-Pinilla, 2008), indicating that several anti-oxidants such as omega-3 fatty acids, curcumin, flavonoids and vitamins improve cognition and may reduce cognitive decline in the elderly. One must realize that the effects of anti-oxidants on the oxidation state of individual proteins and the involved pathways are not well characterized and may need many more detailed studies.

9. CONCLUSION AND OUTLOOK

Up to date, an accurate quantification of oxidized proteins remains difficult. As cysteines are often subject to modification by oxidation and are involved in various signaling cascades, it is of growing importance to locate the different amino acid residues and to identify the types of oxidation. A major task for future proteomics studies will be to develop tools to identify the different types of oxidation forms and establish means to quantify the extent of such modifications. Since not all types of modification are deleterious, one needs to determine its relation to functional impairment and define when oxidation becomes pathological. For this, enzymes and receptor proteins may provide assays to measure the influence of oxidation on structure and function and allow an estimation of when oxidation becomes deleterious and when a protein is transforming from a physiologically conformation into a pathological one, either as a reversible or irreversible oxidation form.

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ABBREVIATIONS

1-DE & 2-DE = One- and two-dimensional gel electrophoresis
AD = Alzheimer’s disease
DGE = Differential gel electrophoresis
DNP = 2,4-Dinitrophenyl hydrazine
GFAP = Glial fibrillary acidic protein
GSH = Glutathione
MALDI-TOF = Matrix-assisted laser desorption ionization- time of flight
NADPH = Nicotinamide adenine dinucleotide phosphate
ROS/RNS = Reactive oxygen/nitrogen species

REFERENCES


