Geriatr Gerontol Int 2010; **10 (Suppl. 1):** *S25–S31*

Proteomic approaches to oxidative protein modifications implicated in the mechanism of aging

Tosifusa Toda, Megumi Nakamura, Hiraku Morisawa, Mikako Hirota, Ryuichi Nishigaki and Yoji Yoshimi

Research Team for Mechanism of Aging, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

Accumulation of oxidatively modified proteins is widely observed in aged animal tissues. Protein carbonyls are mostly derived from lysine, arginine, proline and threonine residues under oxidative conditions. Many groups have investigated carbonylated proteins since a convenient immunochemical procedure was established for detecting dinitrophenyl derivatives of carbonyls and applied to proteomic research. An alternative method of tagging with biotin or fluorescent dyes has been also introduced to proteomic analysis of protein carbonyls. Nitrotyrosine was primarily identified as a biomarker of cellular damage and inflammation under nitrosative stress. Nitrated proteins have been subsequently detected in aged animal tissues and Alzheimer's disease affected brains by Western blotting, and identified by mass spectrometry. Protein s-thiolation, a mixed-derivatization of cysteine (Cys) by conjugation of low-molecular-weight thiol compounds, is recognized as protecting functional proteins from more serious damage. A method of biotin labeling has been used in proteomics for tracing protein s-thiolation. Among all kinds of amino acid residues, methionine (Met) is the most susceptible to reactive oxygen species, and Met oxidation seems to occur in ordinary cellular circumstances because most cells contain Met sulfoxide reductases, which might prevent serious cellular damage. In proteomic analysis, Met sulfoxide-containing peptides are generally observed as 16-Da-high mass peaks in peptide mass fingerprinting. A modified procedure of two-dimensional gel electrophoresis, in which proteins are kept under non-oxidative conditions throughout the procedure, is appropriate for the estimation of the Met sulfoxide level of each protein in aged animal tissues and cells to evaluate the pathophysiological significance of Met oxidation in the mechanism of aging. **Geriatr Gerontol Int 2010; 10 (Suppl. 1): S25–S31.**

Introduction

Biological aging is quite a complex process, in which various organ and cell functions decline with the passage of time at the late stage of the animal lifespan. Among many theories of aging, proposed as working hypotheses for carrying out research on mechanisms of aging, the "free radical theory of aging" developed by Denham Harman[1,2] has been adopted by many researchers as it is consistent with observations in aged cells and tissues. In the free radical theory, it has been hypothesized that the decline of cell functions with aging is a result of the accumulation of altered molecules generated by the effect of free radicals. The free radical theory was originally only concerned with typical free radicals, such as superoxide anion radical ($\cdot O_2^-$) and hydroxyl radical ($\cdot OH$), but it has since been expanded to encompass all reactive oxygen species (ROS). The ROS are inevitably generated in metabolic pathways in all cells, and some of them might play important roles in cell signaling.[3,4] However, excess ROS damages a wide range of biomolecules, including DNA and functional proteins (Fig. 1).

The pathophysiological role of ROS-induced DNA damage had been initially discussed in the mechanism

Accepted for publication 20 November 2009.

Correspondence: Dr Tosifusa Toda PhD, Research Team for Mechanism of Aging, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan. E-mail: ttoda@tmig.or.jp

doi: 10.1111/j.1447-0594.2010.00606.x

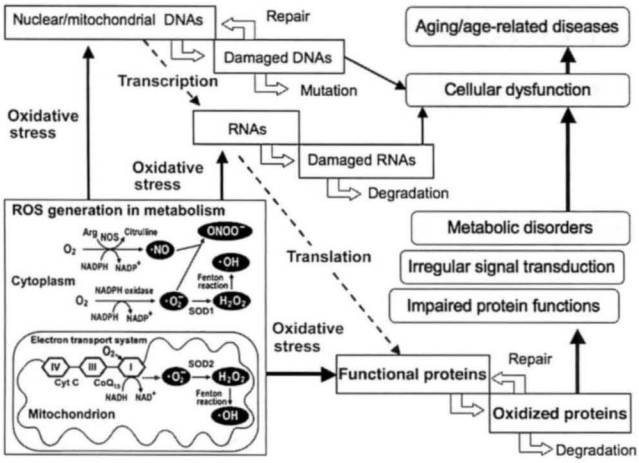

Figure 1 Oxidative modifications of biomolecules by reactive oxygen species implicated in aging and age-related diseases.

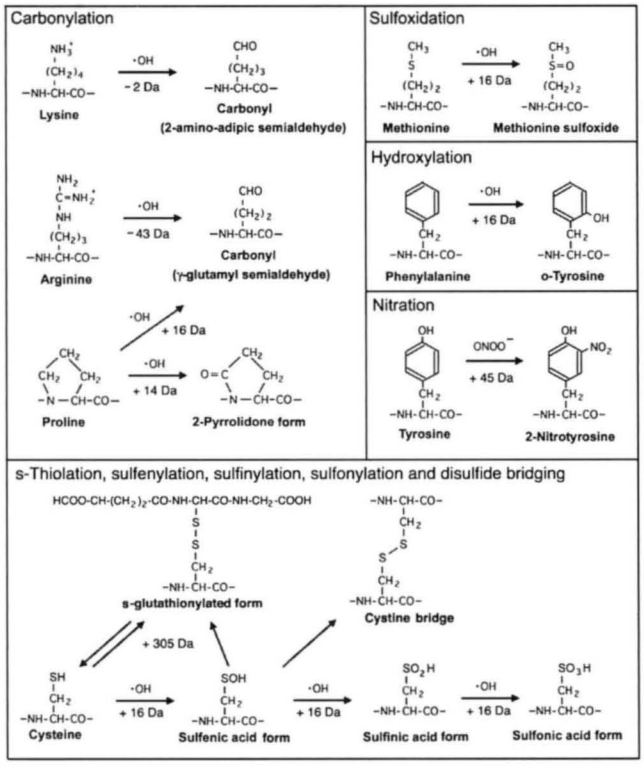

Figure 2 Oxidative modifications of amino acid residues in proteins under oxidative circumstance.

of chemical carcinogenesis.[5–7] The implication of oxidative DNA damage in the mechanism of aging was proposed by Ames.[8,9] Kaneko *et al.* at our institute also observed the significant increase of 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidation derivative of deoxyguanosine, in aged rat DNA at the late stage of the lifespan.[10] However, the common pathway and divergence of the two distinct phenomena, that is cellular immortalization and senescence, is still unclear.

In contrast, the apparent increase in oxidatively modified proteins has been observed in aged animal tissues and cells, suggesting protein oxidation is involved in the process of individual aging. Actually, proteins have many amino acid residues that are more susceptible to oxidative stress than deoxyguanosine in DNA (Fig. 2).

The pathophysiological meaning of variously oxidized protein molecules has been discussed in the physiological process of aging[11–13] and in the pathological process of age-related diseases such as Alzheimer's disease (AD),[14] cataracts[15,16] and atherosclerosis.[17,18]

Carbonylation of protein at lysine, arginine, proline and threonine residues

Most of the protein carbonyls observed in aged cells and oxidatively damaged cells are derived from lysine (Lys), arginine (Arg) and proline (Pro) as shown in Figure 2. 2-Amino-adipic semialdehyde (AAS) and g-glutamyl semialdehyde (GGS) are the most abundant carbonyls in aged cells. AAS might be derived from only peptidyl Lys, whereas g-glutamyl semialdehyde (GGS) is generated from both peptidyl Arg and Pro.[19] Ketone forms of carbonyls might be generated from threonine (Thr) residues (the structure of ketone form is not shown in Fig. 2).

After a convenient method for detecting protein carbonyls on PVDF membrane was developed,[20] electrophoretic and proteomic analyses of carbonylated proteins have been extensively carried out by many groups.[21–29]

In their proteomic analyses, the increase of protein carbonyls in AD brain has also been reported, however, more careful consideration should be taken before concluding that oxidative stress is implicated in AD, because alternative production of protein carbonyls through non-oxidative pathways has also been suggested.[30]

Nitration of protein at tyrosine residues

Nitrated proteins are also good target of proteomic analysis, because specific antibodies detecting 2-nitrotyrosine (Tyr) have been commercially available and the nitro-Tyr-containing peptides are easily identified by mass spectrometry as +45-Da mass shift (Fig. 2).

Since protein nitration was first found in cytochrome c,[31] the nitrosative protein modification has been studied as an alternative pathway of ROS-induced aging and diseases.[32–37] Many nitrated proteins were detected in AD brain by 2-D Western blotting and identified by mass spectrometry. The data obtained by proteomic approaches suggest the involvement of protein nitration in neurodegeneration. However, implication of the

nitrosative protein modification in physiological process of brain aging still remains to be investigated further.[34,38]

Protein s-thiolation at cysteine residues

Protein s-thiolation is a mixed-disulfide derivatization of cysteine (Cys) residues by the conjugation of low-molecular-weight thiols, such as glutathione. Detection of protein s-thiolation was first reported in cardiac cells treated with diamide, a thiol-specific oxidant.[39] Protein s-thiolation is known to cause inactivation or activation depending on the protein structure. Inactivation of creatine kinase by s-glutathiolation suggests the implication of the Cys modification in cardiac injury occurred under ischemic conditions.[40] The similar inactivation was observed in protein kinase C-alpha.[41] In contrast, it has been suggested that the activation of the small G protein Ras by s-glutathiolation plays an important role in myocardial remodeling after ischemic injury.[42]

However, protein s-glutathiolation has been generally recognized as a protective reaction for most proteins from more serious irreversible oxidation, because the glutathionyl mixed disulfide can be reversed by the action of thioltransferase (glutaredoxin),[43] or in the nicotinamide adenine dinucleotide (NADH)- and nicotinamaide adenine dinucleotide phosphate (NADPH)-dependent protein reducing system.[44] The implication of protein s-thiolation in the physiological aging process and in the anti-aging defense system still remains to be further investigated.

A thin-gel isoelectric focusing method was initially developed for the analysis of protein s-thiolation.[45] The method of isotope labeling by the incorporation of [S^{35}]-glutathione has been developed for tracing *in vitro* s-thiolated proteins.[46] Isotope labeling is the most sensitive method for detecting low levels of modification, however, the radioactive protein is not applicable to the general procedure in proteomic identification by mass spectrometric analysis. The non-radioactive biotin-labeling method has been also developed for concentrating and detecting *in vitro* s-cysteinylated proteins.[47] The biotin-labeled protein is suitable for proteomic analysis by mass spectrometry, however, it is not applicable to *in vivo* s-cysteinylated samples, such as human clinical specimens.

Thus, we developed another method for detecting free thiols and s-thiolated Cys by differential fluorescence labeling. By our post-labeling method, the conjugated counterparts of s-thiolation could not be directly detected by mass spectrometry, because mixed-disulfide was replaced with the thiol-specific fluorescent dyes. However, the level of s-thiolation and disulfide bridging in human specimens under oxidative stress could be easily quantified by 2-D gel-based fluorescence imaging. By using this method, increased level of s-thiolation and disulfide bridging in specific proteins were detected in the cerebrospinal fluid (CSF) of senile dementia patients.

Sulfoxidation of protein at methionine (Met) residue

Amongst the many kinds of amino acid residues, the methyl-thio-ether group of Met is particularly susceptible to ROS, and changes to the sulfoxide form of Met (MetO) as shown in Figure 2. Sulfoxidation of Met leads most proteins to conformational alteration, and in some cases, loss of function. To prevent serious consequence of Met sulphoxidation, most cells express methionine sulfoxide reductase (MsrA), which works to repair damaged protein by reducing MetO.[48,49] However, a high enough level of activity of MsrA appears to be essential for cells to survive in the presence of ROS[50], and it has been confirmed that *msrA* knockout mice have a significantly shorter lifespan than controls.[51] MsrA activity is significantly low in AD brain when compared with the normal control brain, suggesting the involvement of Met sulfoxidation in the process of hippocampal neurodegeneration in AD.[52] Furthermore, downregulation of *msrA* gene expression and the decrease in enzyme activity of MsrA with aging are observed in rat tissues.[53] These data suggested that the level of oxidized protein might increase, even in the physiological process of normal brain aging, and the situation is much worse in AD brain. Anyway, Met sulfoxidation might occur on almost all Met-containing proteins under oxidative conditions in cells, however, pathophysiological consequences might vary with site of MetO and degree of conformational alteration in each oxidized protein.

The proteomic method is a powerful tool for comprehensively analyzing alterations of proteins in both relative abundance and post-translational modifications. However, special care should be taken to avoid artificial Met sulphoxidation during analysis. The procedure of 2-D gel electrophoresis and MS analysis has been optimized for determining the level of MetO in each protein spot separated on a gel, and applied to the analysis of protein alterations with aging in the mouse hippocampus.[54]

A significant decrease in protein expression was detected in the spots on the 2-D gel corresponding to calmodulin (CaM), ubiquitin carboxyl-terminal esterase L1 (UCH-L1) and nm23-M1, in contrast to the increase in spots corresponding to molecular chaperons such as heat-shock protein (HSP) 60 and HSP70 (Fig. 3).

The decrease in CaM expression levels might be a result of downregulation in gene expression and/or the increase in protein degradation. However, downregulation of CaM gene expression in the hippocampus was not detected, even in a global survey of age-related changes in mRNA levels in the mouse hippocampus.[55,56] In contrast,

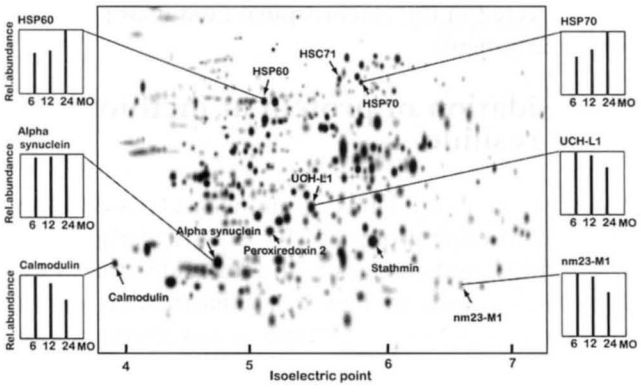

Figure 3 2-D gel electrophoretic observation of protein alterations in the mouse hippocampus with aging.

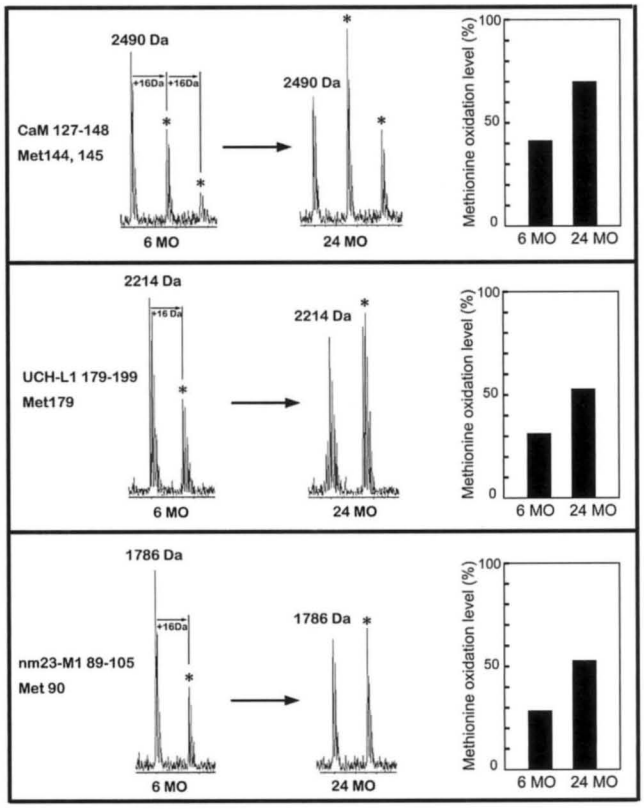

Figure 4 Quantitative determination of the level of Met sulfoxide in calmodulin (CaM), UCH-L1 and nm23-M1 by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. Asterisks indicate MetO-containing peptides.

the loss of conformational stability of CaM by oxidation[57] and the acquisition of a high susceptibility to proteolytic degradation on 20S proteasome[58] without polyubiquitination suggest that the decrease in the relative abundance of CaM might be the result of increased degradation in the aged mouse hippocampus.

The increase in MetO-levels on CaM, UCH-LA and nm23-M1 in the aged mouse hippocampus has been observed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of tryptic digests of these protein spots separated by 2-D gel electrophoresis.[54] MetO-containing peptide appears as a 16-Da-high mass peak on primary MS spectra, and the real Met oxidation can be confirmed by detecting the 64-Da "neutral loss", that is mass deduction by secondary MS/MS carried out in a Post-source-Decay (PSD) mode. Thus, obtained mass spectra indicate that the MetO level in these proteins increases in the mouse hippocampus with aging (Fig. 4).

Furthermore, it has been also confirmed that Met144 and Met145 located in the EF-hand 4 of CaM are more susceptible to oxidation when compared with Met36 in the EF-hand 1. The observation suggests that the Met sulfoxidation occurs in a site-specific manner in CaM under oxidative stress in aged animal tissues (Fig. 5).

CaM is a highly conserved Ca^{2+}-binding protein essential for various biological functions mediated by Ca^{2+} in a concentration-dependent manner. The reduction of CaM content in the AD brain (66% of control) was originally found by radioimmunoassay.[59] In that study, it was also reported that the CaM extracted from the temporal cortex of AD brain showed reduced efficacy as an activator of 3′,5′-cyclic-nucleotide phosphodiesterase. These data suggest that the impaired CaM function in AD brain might affect calcium homeostasis and calcium-mediated signal transduction in the process of neurodegeneration.

The decline in CaM function was already reported in the physiological aging of the rat brain.[60] Squier *et al.* at

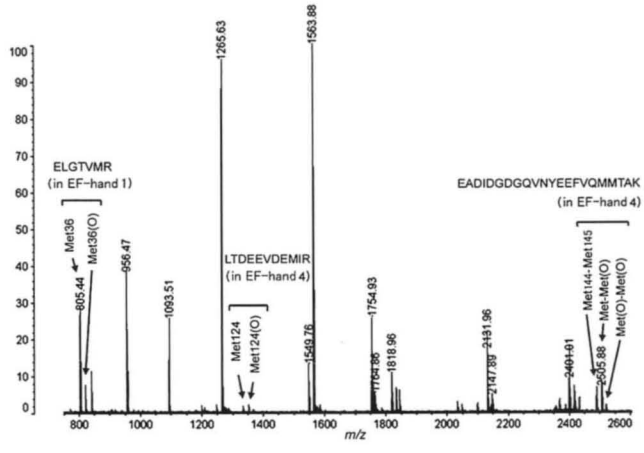

Figure 5 Mass spectrometric profiling of site-specific Met sulfoxidation in calmodulin. Met144 and Met 145 in EF-hand 4 are more preferentially oxidized than Met36 in EF-hand 1.

the University of Kansas have carried out further analyses and confirmed that Met sulfoxidation is responsible for the age-dependent decline in the ability of CaM to activate plasma membrane (PM) Ca-ATPase.[61,62]

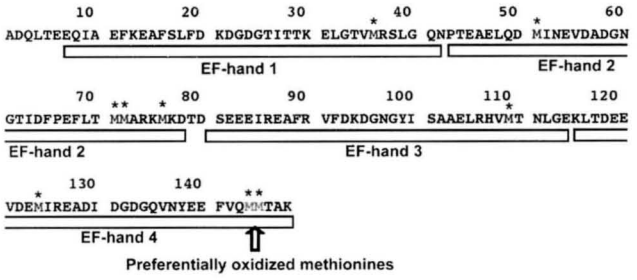

Figure 6 The highly conserved primary structure of calmodulin, which is comprised of 4 EF-hands containing functional Met. Met144 and Met145, located in the EF-hand 4, are preferentially oxidized in the aged mouse hippocampus.

Both the methylthio groups of Met and the thiol group of Cys are especially susceptible to oxidation by all kinds of ROS compared with other amino acid residues. However, CaM has no Cys in all of its highly conserved primary structure of 148 amino acids, though it contains nine Mets in the mature form of the small protein (Fig. 6).

The results of our 2-D gel-based proteomic analysis indicate that the total amount of CaM decreases and the level of Met-oxidized CaM increases in the mouse hippocampus during aging. From the data of our MS analysis, we concluded that not all of the nine Met residues are evenly oxidized, but Met144 and Met145 located at the Ca-binding site in the EF-hand 4 are preferentially oxidized in the aged mouse hippocampus.

It has been known that oxidation of Met144 and Met145 in CaM blocks CaM-dependent activation of the plasma membrane Ca-ATPase.[63] We carried out the analysis of the conformational response of native and Met-oxidized CaM to calcium binding by using the method of dual polarization interferometry (DPI)[64] to obtain evidence for probable direct effect of Met sulphoxidation on the calcium-binding affinity of CaM. The details of the DPI analysis will be reported in a separate paper.

The present data obtained by proteomic analysis indicated that the protein expression of CaM, UCH-L1 and nm23-M1 decrease, and the oxidized forms of CaM, UCH-L1 and nm23-M1 increase with aging in the mouse hippocampus. The increase in oxidation of CaM might disturb the CaM-dependent calcium signaling in brain function. Oxidation of UCH-L1 and nm23-M1 might also affect ubiquitin recycling in proteasome-dependent protein degradation and guanosine triphosphate-mediated signal transduction, respectively, in the aged mouse hippocampus.

Conflicts of interest

None declared.

References

1 Harman D. Aging theory based on free radical and radiation chemistry. *J Gerontol* 1956; **2**: 298–300.
2 Harman D. Prolongation of life: role of free radical reactions in aging. *J Am Geriatr Soc* 1969; **17**: 721–735.
3 Finkel T. Oxygen radicals and signaling. *Curr Opin Cell Biol* 1998; **10**: 248–253.
4 Yang JQ, Li S, Huang Y *et al.* V-Ha-Ras overexpression induces superoxide production and alters levels of primary antioxidant enzymes. *Antioxid Redox Signal* 2001; **3**: 697–709.
5 Swartz HM. Free radicals in cancer. *Ciba Found Symp* 1978; **67**: 107–130.
6 Troll W, Frenkel K, Teebor G. Free oxygen radicals: necessary contributors to tumor promotion and carcinogenesis. *Princess Takamatsu Symp* 1983; **14**: 207–218.
7 Breimer LH. Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. *Mol Carcinog* 1990; **3**: 188–197.
8 Ames BN. Measuring oxidative damage in humans: relation to cancer and ageing. *IARC Sci Publ* 1988; **89**: 407–416.
9 Adelman R, Saul RL, Ames BN. Oxidative damage to DNA: relation to species metabolic rate and life span. *Proc Natl Acad Sci USA* 1988; **85**: 2706–2708.
10 Kaneko T, Tahara S, Matsuo M. Non-linear accumulation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidized DNA damage, during aging. *Mutat Res* 1996; **316**: 277–285.
11 Fucci L, Oliver CN, Coon MJ, Stadtman ER. Inactivation of key metabolic enzymes by mixed-function oxidation reactions: possible implication in protein turnover and ageing. *Proc Natl Acad Sci USA* 1983; **80**: 1521–1525.
12 Oliver CN, Ahn BW, Moerman EJ, Goldstein S, Stadtman ER. Age-related changes in oxidized proteins. *J Biol Chem* 1987; **262**: 5488–5491.
13 Stadtman ER. Protein oxidation and aging. *Science* 1992; **257**: 1220–1224.
14 Smith CD, Carney JM, Starke-Reed PE *et al.* Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proc Natl Acad Sci USA* 1991; **88**: 10540–10543.
15 Garner MH, Spector A. Selective oxidation of cysteine and methionine in normal and senile cataractous lenses. *Proc Natl Acad Sci USA* 1980; **77**: 1274–1277.
16 Taylor A, Davies KJ. Protein oxidation and loss of protease activity may lead to cataract formation in the aged lens. *Free Radic Biol Med* 1987; **3**: 371–377.
17 Ylä-Herttuala S. Macrophages and oxidized low density lipoproteins in the pathogenesis of atherosclerosis. *Ann Med* 1991; **23**: 561–567.
18 Lyons TJ. Glycation and oxidation: a role in the pathogenesis of atherosclerosis. *Am J Cardiol* 1993; **71**: 26B–31.
19 Requena JR, Chao CC, Levine RL, Stadtman ER. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc Natl Acad Sci USA* 2001; **98**: 69–74.
20 Nakamura A, Goto S. Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis. *J Biochem* 1996; **119**: 768–774.
21 Yan LJ, Orr WC, Sohal RS. Identification of oxidized proteins based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunochemical detection, isoelectric focusing, and microsequencing. *Anal Biochem* 1998; **263**: 67–71.

22 Goto S, Nakamura A, Radak Z *et al.* Carbonylated proteins in aging and exercise: immunoblot approaches. *Mech Ageing Dev* 1999; **107**: 245–253.
23 Nagai M, Takahashi R, Goto S. Dietary restriction initiated late in life can reduce mitochondrial protein carbonyls in rat livers: western blot studies. *Biogerontology* 2000; **1**: 321–328.
24 Aksenov M, Aksenova M, Butterfield DA, Markesbery WR. Oxidative modification of creatine kinase BB in Alzheimer's disease brain. *J Neurochem* 2000; **74**: 2520–2527.
25 Aksenov MY, Aksenova MV, Butterfield DA, Geddes JW, Markesbery WR. Protein oxidation in the brain in Alzheimer's disease. *Neuroscience* 2001; **103**: 373–383.
26 Korolainen MA, Goldsteins G, Alafuzoff I, Koistinaho J, Pirttilä T. Proteomic analysis of protein oxidation in Alzheimer's disease brain. *Electrophoresis* 2002; **23**: 3428–3433.
27 Castegna A, Aksenov M, Thongboonkerd V *et al.* Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrimidinase-related protein 2, alpha-enolase and heat shock cognate 71. *J Neurochem* 2002; **82**: 1524–1532.
28 Mirzaei H, Regnier F. Affinity chromatographic selection of carbonylated proteins followed by identification of oxidation sites using tandem mass spectrometry. *Anal Chem* 2005; **77**: 2386–2392.
29 Chaudhuri AR, de Waal EM, Pierce A, Van Remmen H, Ward WF, Richardson A. Detection of protein carbonyls in aging liver tissue: A fluorescence-based proteomic approach. *Mech Ageing Dev* 2006; **127**: 849–861.
30 Adams S, Green P, Claxton R *et al.* Reactive carbonyl formation by oxidative and non-oxidative pathways. *Front Biosci* 2001; **6**: A17–A24.
31 Skov K, Hofmann T, Williams GR. The nitration of cytochrome c. *Can J Biochem* 1969; **47**: 750–752.
32 Castegna A, Thongboonkerd V, Klein JB, Lynn B, Markesbery WR, Butterfield DA. Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem* 2003; **85**: 1394–1401.
33 Sacksteder CA, Qian WJ, Knyushko TV *et al.* Endogenously nitrated proteins in mouse brain: links to neurodegenerative disease. *Biochem* 2006; **45**: 8009–8022.
34 Gokulrangan G, Zaidi A, Michaelis ML, Schöneich C. Proteomic analysis of protein nitration in rat cerebellum: effect of biological aging. *J Neurochem* 2007; **100**: 1494–1504.
35 Sultana R, Reed T, Perluigi M, Coccia R, Pierce WM, Butterfield DA. Proteomic identification of nitrated brain proteins in amnestic mild cognitive impairment: a regional study. *J Cell Mol Med* 2007; **11**: 839–851.
36 Butterfield DA, Reed TT, Perluigi M *et al.* Elevated levels of 3-nitrotyrosine in brain from subjects with amnestic mild cognitive impairment: implications for the role of nitration in the progression of Alzheimer's disease. *Brain Res* 2007; **1148**: 243–248.
37 Reed TT, Pierce WM Jr, Turner DM, Markesbery WR, Butterfield DA. Proteomic identification of nitrated brain proteins in early Alzheimer's disease inferior parietal lobule. *J Cell Mol Med* 2009; **18**: 2019–2029.
38 Suzuki Y, Tanaka M, Sohmiya M, Ichinose S, Omori A, Okamoto K. Identification of nitrated proteins in the normal rat brain using a proteomics approach. *Neurol Res* 2005; **27**: 630–633.
39 Grimm LM, Collison MW, Fisher RA, Thomas JA. Protein mixed-disulfides in cardiac cells. S-thiolation of soluble proteins in response to diamide. *Biochim Biophys Acta* 1985; **844**: 50–54.
40 Reddy S, Jones AD, Cross CE, Wong PS, Van Der Vliet A. Inactivation of creatine kinase by S-glutathionylation of the active-site cysteine residue. *Biochem J* 2000; **347**: 821–827.
41 Ward NE, Stewart JR, Ioannides CG, O'Brian CA. Oxidant-induced S-glutathiolation inactivates protein kinase C-alpha (PKC-alpha): a potential mechanism of PKC isozyme regulation. *Biochem* 2000; **39**: 10319–10329.
42 Kuster GM, Siwik DA, Pimentel DR, Colucci WS. Role of reversible, thioredoxin-sensitive oxidative protein modifications in cardiac myocytes. *Antioxid Redox Signal* 2006; **8**: 2153–2159.
43 Gravina SA, Mieyal JJ. Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase. *Biochem* 1993; **32**: 3368–3376.
44 Schuppe-Koistinen I, Gerdes R, Moldéus P, Cotgreave IA. Studies on the reversibility of protein S-thiolation in human endothelial cells. *Arch Biochem Biophys* 1994; **315**: 226–234.
45 Thomas JA, Beidler D. A thin-gel isoelectric focusing method for quantitation of protein S-thiolation. *Anal Biochem* 1986; **157**: 32–38.
46 Ward NE, Stewart JR, Ioannides CG, O'Brian CA. Oxidant-induced S-glutathiolation inactivates protein kinase C-alpha (PKC-alpha): a potential mechanism of PKC isozyme regulation. *Biochem* 2000; **39**: 10319–10329.
47 Eaton P, Byers HL, Leeds N, Ward MA, Shattock MJ. Detection, quantitation, purification, and identification of cardiac proteins S-thiolated during ischemia and reperfusion. *J Biol Chem* 2002; **277**: 9806–9811.
48 Sun H, Gao J, Ferrington DA, Biesiada H, Williams TD, Squier TC. Repair of oxidized calmodulin by methionine sulfoxide reductase restores ability to activate the plasma membrane Ca-ATPase. *Biochem* 1999; **38**: 105–112.
49 Sharov VS, Ferrington DA, Squier TC, Schöneich C. Diastereoselective reduction of protein-bound methionine sulfoxide by methionine sulfoxide reductase. *FEBS Lett* 1999; **455**: 247–250.
50 Moskovitz J, Flescher E, Berlett BS, Azare J, Poston JM, Stadtman ER. Overexpression of peptide-methionine sulfoxide reductase in Saccharomyces cerevisiae and human T cells provides them with high resistance to oxidative stress. *Proc Natl Acad Sci USA* 1998; **95**: 14071–14075.
51 Moskovitz J, Bar-Noy S, Williams WM, Requena J, Berlett BS, Stadtman ER. Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. *Proc Natl Acad Sci USA* 2001; **98**: 12920–12925.
52 Gabbita SP, Aksenov MY, Lovell MA, Markesbery WR. Decrease in peptide methionine sulfoxide reductase in Alzheimer's disease brain. *J Neurochem* 1999; **73**: 1660–1666.
53 Petropoulos I, Mary J, Perichon M, Friguet B. Rat peptide methionine sulphoxide reductase: cloning of the cDNA, and down-regulation of gene expression and enzyme activity during aging. *Biochem J* 2001; **355** (Pt 3): 819–825.
54 Toda T, Morimasa T, Kobayashi S, Nomura K, Hatozaki T, Hirota M. Proteomic approach to determination of the significance of protein oxidation in the ageing of mouse hippocampus. *Appl Genomics Proteomics* 2003; **2**: 43–50.
55 Verbitsky M, Yonan AL, Malleret G, Kandel ER, Gilliam TC, Pavlidis P. Altered hippocampal transcript profile accompanies an age-related spatial memory deficit in mice. *Learn Mem* 2004; **11**: 253–260.

56 Blalock EM, Chen KC, Sharrow K *et al.* Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. *J Neurosci* 2003; **23**: 3807–3819.
57 Gao J, Yin DH, Yao Y *et al.* Loss of conformational stability in calmodulin upon methionine oxidation. *Biophys J* 1998; **74**: 1115–1134.
58 Strosova M, Voss P, Engels M, Horakova L, Grune T. Limited degradation of oxidized calmodulin by proteasome: formation of peptides. *Arch Biochem Biophys* 2008; **475**: 50–54.
59 McLachlan DR, Wong L, Bergeron C, Baimbridge KG. Calmodulin and calbindin D28K in Alzheimer disease. *Alzheimer Dis Assoc Disord* 1987; **1**: 171–179.
60 Michaelis ML, Bigelow DJ, Schöneich C *et al.* Decreased plasma membrane calcium transport activity in aging brain. *Life Sci* 1996; **59**: 405–412.
61 Yao Y, Yin D, Jas GS *et al.* Oxidative modification of a carboxyl-terminal vicinal methionine in calmodulin by hydrogen peroxide inhibits calmodulin-dependent activation of the plasma membrane Ca-ATPase. *Biochem* 1996; **35**: 2767–2787.
62 Gao J, Yin D, Yao Y, Williams TD, Squier TC. Progressive decline in the ability of calmodulin isolated from aged brain to activate the plasma membrane Ca-ATPase. *Biochemistry* 1998; **37**: 9536–9548.
63 Bartlett RK, Bieber Urbauer RJ, Anbanandam A, Smallwood HS, Urbauer JL, Squier TC. Oxidation of Met144 and Met145 in calmodulin blocks calmodulin dependent activation of the plasma membrane Ca-ATPase. *Biochemistry* 2003; **42**: 3231–3238.
64 Swann MJ, Peel LL, Carrington S, Freeman NJ. Dual-polarization interferometry: an analytical technique to measure changes in protein structure in real time, to determine the stoichiometry of binding events, and to differentiate between specific and nonspecific interactions. *Anal Biochem* 2004; **329**: 190–198.