Review

Oxidized proteins and their contribution to redox homeostasis

Gregorio Martínez-Sánchez[1], Attilia Giuliani[2], Gema Pérez-Davison[1], Olga Sonia León-Fernández[1]

[1]*Centre for Research and Biological Evaluations, Institute of Pharmacy and Food Sciences, Havana University, Cuba*
[2]*Department of Chemistry and Medical Biochemistry, University of Milan, Milan, Italy*

Proteins are major target for radicals and other oxidants when these are formed in both intra- and extracellular environments *in vivo*. Formation of lesions on proteins may be highly sensitive protein-based biomarkers for oxidative damage in mammalian systems. Oxidized proteins are often functionally inactive and their unfolding is associated with enhanced susceptibility to proteinases. ROS scavenging activities of intact proteins are weaker than those of misfolded proteins or equivalent concentrations of their constituent amino acids. Protein oxidation and enhanced proteolytic degradation, therefore, have been suggested to cause a net increase in ROS scavenging capacity. However, certain oxidized proteins are poorly handled by cells, and together with possible alterations in the rate of production of oxidized proteins, may contribute to the observed accumulation and damaging actions of oxidized proteins during ageing and in pathologies such as diabetes, arteriosclerosis and neurodegenerative diseases. Protein oxidation may play a controlling role in cellular remodelling and cell growth. There is some evidence that antioxidant supplementation may protect against protein oxidation, but additional controlled studies of antioxidant intake to evaluate the significance of dietary/pharmacological antioxidants in preventing physiological/pathological oxidative changes are needed.

Keywords: Oxidized proteins, redox homeostasis

INTRODUCTION

The earliest work on oxidation of a biologically important protein was on the effects of selected radicals generated by radiolysis on the enzyme, lysozyme.[1] Both the thiocyanate radical (a selective modifier of tryptophan) and the hydroxyl radical (HO$^{\bullet}$) were found to inactivate the enzyme, implying that tryptophan residues are essential for biological activity, now well established from classical enzymology. Similarly, an α_1-antitrypsin, modification of a single methionine residue at position 358 rendered the protein inactive.[2] Subsequent hydrolysis and amino acid analysis revealed the presence of methionine sulphoxide. This was one of the first pieces of evidence linking amino acid oxidation to denaturation of proteins and loss of function. The importance of protein oxidation in respect to altered function is exemplified by oxidative modifications to histidine and lysine in low density lipoproteins (LDLs), which cause altered receptor recognition; LDL modified in this way is preferentially taken up by scavenger receptors in a non-regulated process.[3] The oxidative modification of proteins by reactive oxygen species (ROS) is implicated in the aetiology or progression of a panoply of disorders and diseases.[4] Some of the major oxidation pathways, and products thereof, have been reviewed extensively.[5] The formation of radicals on peptides and proteins and how radical damage may be propagated and transferred within protein structures also were reviewed recently.[6–8]

The process of protein oxidation frequently introduces new functional groups, such as hydroxyls and carbonyls, which contribute to altered function and turnover.

Received 25 February 2005
Revised 20 May 2005
Accepted 18 June 2005

Correspondence to: Gregorio Martínez-Sánchez PhD, Centre for Research and Biological Evaluations, Institute of Pharmacy and Food Sciences, Havana University, Cuba (CEIEB-IFAL), PO Box 4301, Ciudad de La Habana 4, Cuba
Tel: +53 7 2719531/38; Fax: +53 7 336811;
E-mail: gregorio@cieb.sld.cu, gema@infomed.sld.cu

DOI 10.1179/135100005X57382

Improved characterisation of the effects of protein oxidation has identified a spectrum of secondary effects including fragmentation, cross-linking and unfolding, which may accelerate or hinder proteolytic and proteosome-mediated turnover, according to the severity of oxidative damage.[9]

Proteolytic degradation is executed mainly by proteasomes.[10] In one study, proteolysis was estimated to increase more than 11-fold after exposure to superoxide or hydrogen peroxide.[11] Proteolysis is enhanced by 20–400 μM hydrogen peroxide, whereas millimolar concentrations inhibit proteolysis and may lead to the intracellular accumulation of oxidized proteins.[12,13] In line with these results, several authors assessed the proteolytic susceptibility of oxidized protein, and demonstrated biphasic effects, whereby limited oxidation leads to enhanced susceptibility, while more extensive oxidation may be associated with increasing resistance.[9]

The proteins may differ strongly in their susceptibility to oxidative damage. The redox-sensitive amino acids of bovine serum albumin, for example, were shown to be oxidized about twice as fast as those of glutamine synthase,[14] and intact proteins are less sensitive to oxidation than misfolded proteins.[15] These findings imply that: (i) phylogenetic evolution has selected for protein structures that are relatively well-protected against oxidation; and (ii) ROS scavenging activities of intact proteins are weaker than those of misfolded proteins or equivalent concentrations of their constituent amino acids.[16] Protein oxidation and enhanced proteolytic degradation[17] may cause, therefore, a net increase in ROS scavenging capacity[16] as schematically illustrated in Figure 1. More

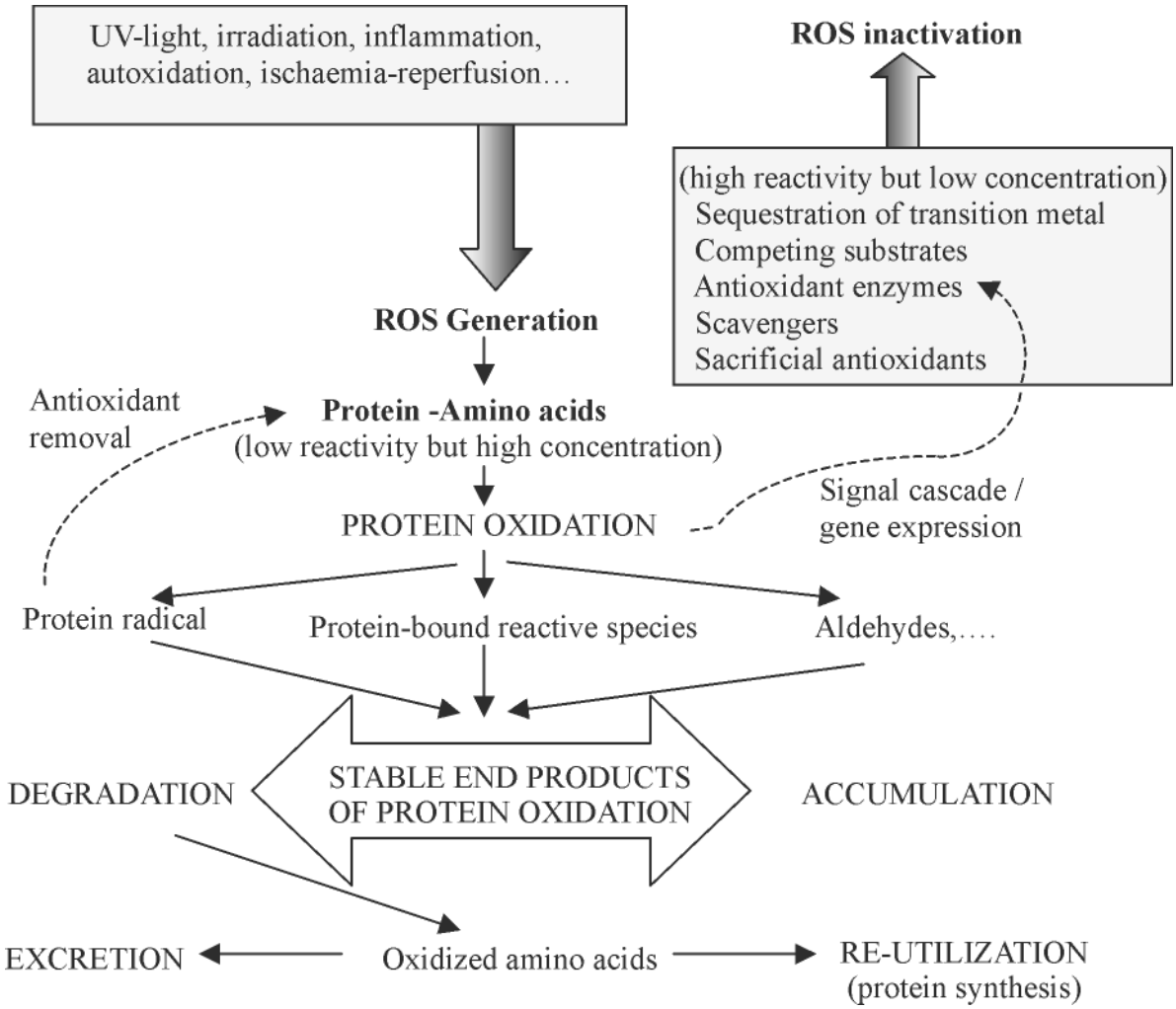

Fig. 1. Mechanisms of protein redox homeostasis. Balance between reactive oxygen species (ROS) production and various types of scavengers. The steady-state levels of ROS are determined by the rate of ROS production and their clearance by scavenging mechanisms. Certain antioxidative enzymes including superoxide dismutase (SOD), glutathione peroxidase, catalase, and thioredoxin are potent ROS scavengers but occur in cells only at relatively low concentrations. The same is true for non-enzymic antioxidants. Amino acids and proteins are also ROS scavengers. Amino acids are less effective than the classical antioxidants on a molar basis, but their cumulative intracellular concentration is > 0.1 M.

systematic studies are needed to determine the relative contribution of proteins, free amino acids, and classical antioxidant compounds and enzymes to the total ROS scavenging capacity of different cells and tissues.

BIOMARKERS OF PROTEIN OXIDATION

The complexity of protein structure, arising from the primary sequence and involvement of carbohydrate moieties in structure stabilisation, together with a lack of specific and sensitive methodologies, has hindered the identification of oxidative biomarkers.[18,19] Generally, those amino acids capable of delocalising charge, such as amino acids containing aromatic and thiol side chains, are more susceptible to oxidative attack. However, a large number of aliphatic residues are subject to oxidation with the generation of protein carbonyl moieties. Interest also has focused on the analysis of specific protein-bound oxidised amino acids (*i.e.* dityrosine-containing protein cross-linking products, which were designated as advanced oxidation protein products – AOPP).[20] Of the 22 amino acids, aromatic and sulphydryl containing residues have been regarded as being particularly susceptible to oxidative modification, forming L-DOPA from tyrosine, *ortho*-tyrosine from phenylalanine, sulphoxides and disulphides from methionine and cysteine, respectively, and kynurenines from tryptophan.[21]

In addition, the identification of valine and leucine hydroxides, reduced from hydroperoxide intermediates, has been described. Latterly, based on the phenomenon that oxidatively modified proteins acquire antiradical capacity,[22] a novel parameter was developed, the antiradical ability of blood plasma protein (ARAP). The following sections will examine these potential biomarkers against the criteria laid out above, through examination of methodology and applications in antioxidant studies (Table 1).

Protein carbonyl biomarkers

Carbonyls are ubiquitous products of oxidation arising on amino acid side chains as well as sugars and lipids.[23] Whilst glucose addition is a simple condensation reaction between the carbohydrate aldehyde group and protein amino group, the resultant Schiff's base can undergo further oxidative reactions, which are collectively referred to as glycoxidation reactions. Both oxo-acids and aldehydes are formed following oxidative attack, having either the same number of carbon atoms or one less than the original amino acid.[24] Carbonyls can be generated in response to a wide variety of oxidising stimuli including alkoxy and peroxy radicals. Different oxidants give rise to variable yields of bound and released carbonyl products.[25]

The methods applied to the measurement of physiological protein oxidation vary from immunodetection by ELISA or Western blot to analytical HPLC. A specific reaction between protein carbonyls and dinitrophenyl hydrazine generates the hydrazone chromophore with an absorbance (A_{max}) at 360 nm, with a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$. This requires a large sample size for spectrophotometric determination, and gives little information on the protein affected.

Products can be separated by HPLC. Several antibodies are available for the detection of the dinitrophenyl hydrazine carbonyl product, which has been used in a semi-quantitative manner in Western blot analysis of

Table 1. Recommendations for appropriate protein oxidation biomarkers

	Specific	Stable	Validity	Disease	Dose response
Carbonyls	–	++	++	++	++
Thiols	+/–	++	++	?	++
Nitrated amino acid	++	+	?	?	++
Protein Tyr oxidation products	++	+	?	?	?
Protein Trp oxidation products	++	++	?	++	++
Methionine sulphoxide	++	++	?	?	++
Hydroxides/hydroperoxides	++	–	?	++	++
Protein 2–adipic semi-aldehyde	?	++	?	?	?
Neoepitopes	?	++	?	++	++
AOPP	+	+	+	++	+
ARAP	++	+	++	++	+

++ Criterion met with confidence; + criterion met; ? criterion not previously fully evaluated; – criterion not met.
Strengths and weaknesses of protein oxidation biomarker are defined in priority order as: (i) specificity (both biological in terms of source, and chemical in terms of analysis); (ii) stability (–80°C for at least 4 weeks); (iii) reproducibility (CV < 10%); (iv) association with disease; and (v) responsiveness (*i.e.* dose relationship between oxidant and formation, and antioxidant and protection).
AOPP, advanced oxidation protein products; ARAP, antiradical ability of blood plasma protein.

both SDS-PAGE and 2-D proteomic gels. These methods have been reviewed elsewhere.[26,27] An ELISA procedure has been developed for quantitative analysis of the carbonyl content of specific proteins.[28,29] This offers the advantages of sensitivity, small sample volume, reproducibility and large sample throughput. Although protein carbonyls are a generic marker of oxidation, they appear to be yielding useful information.

Protein thiol biomarkers

Examination of the percentage native amino acid remaining following oxidation *in vitro* shows that, for many proteins, cysteine/cystine is a highly susceptible moiety. The free thiol group of cysteine readily undergoes reversible oxidation to form a disulphide, which can be 'repaired' in the presence of a thiol donor such as glutathione. Parallel thiol oxidation leads irreversibly to cysteic acid.[30] Oxidation of the single amino acid cysteine is complicated by the observation that the product may be a composite mixed disulphide. Indeed, in biological systems, the levels of such a product are also indirectly under the influence of inducible enzymes of the glutathione cycle, and thus inherently related to changes in gene expression, conditioning and priming.

Another sulphur-containing amino acid, methionine, is also considered to be highly susceptible to oxidative change; end products include the sulphoxide and the sulphone. Hypochlorous acid (HOCl), nitric oxide and singlet oxygen are all capable of eliciting this change. It is also subject to an enzymic repair process via methionine sulphoxide reductase (PMSR), the activity of which declines with age.[31] Recently, Biewenga *et al*.[32] have shown that dihydrolipoate can function as a co-factor for PMSR in the repair of α_1-antitrypsin. This enzyme restores the oxidised residue to its native form on the protein backbone, without any detectable product, and so methionine oxidation may give misleading information on the degree of oxidation if analysed alone.

Cysteine residues are notoriously labile in an isolated system, which explains the inactivation of proteins during isolation. Great care must be taken to minimise artefactual oxidation through incorporation of chelating agents (*e.g*. EDTA) in buffers, maintenance of ice-cold temperatures throughout and addition of dithiothreitol.

Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid, DTNB) readily forms a mixed disulphide with thiols, liberating the chromophore 5-mercapto-2-nitrobenzoic acid (A_{max} 410 nm, ~13,600 $M^{-1}cm^{-1}$).[33] Only protein thiols that are accessible to this water-soluble reagent are modified. Carrying out the titration in the presence of 6 M guanidinium chloride usually enables quantification of inaccessible thiols. DTNB conjugates of glutathione and other thiols can be separated by HPLC.

Ultrasensitive colorimetric quantitation of both protein and non-protein thiols is now possible using a method reported by Singh *et al*.[34] Thiols or sulphides reduce a disulphide-inhibited derivative of papain, stoichiometrically releasing the active enzyme. Activity of the enzyme is then measured using the chromogenic papain substrate L-3-N-alpha-benzoyl-DL-arginine *p*-nitroanilide (BAPNA). The enzymic amplification step has a sensitivity for detection of thiols or sulphides of approximately 0.2 nanomoles or about 100-fold better than that obtained with DTNB. Thiols in proteins can be detected indirectly by incorporating the disulphide cystamine into the reaction mixture. Cystamine undergoes an exchange reaction with protein thiols, yielding 2-mercaptoethylamine (cysteamine), which then releases active papain. The applicability of the Singh *et al*.[34] methods requires that the thiols be able to interact with the disulphide-inhibited enzyme. Some thiols may not be able to do this for steric/electronic reasons and hence the values obtained will be erroneous.

The major limitation of this approach is that only changes in reduced thiol content can be measured. Without any measure of oxidised product, thiol analysis should be considered a poor marker. At present, there are no adequate procedures available for assaying disulphide formation, and it is likely that the only adequate methodology may arise from the development of immunological reagents.

Aliphatic amino acid biomarkers

One of the longer-lived reactive species produced by ROS attack on proteins is hydroperoxide. In time, these oxidising species decay to form stable, long lived hydroxides, and both can be measured by HPLC.

The amino acids valine, leucine, isoleucine, proline, glutamic acid, arginine and lysine are susceptible to hydroperoxide formation.[7] Within proteins, however, the hydrophobic residues are protected from bulk aqueous radicals, and lysine hydroperoxides are the more sensitive indicators. Hydroperoxides are rapidly broken down in the presence of metal ions,[35,36] and the presence of reduced glutathione (GSH) rapidly removes peptide, but not protein, peroxides, consistent with substrate size being a key factor. Protein thiols, GSH, other low-molecular-weight thiols, and the seleno-compound ebselen react, in a non-stoichiometric manner, with both peptide and protein peroxides.[37] Hydroperoxides can be effectively reduced to hydroxides post-extraction, which are poorly susceptible to further oxidative modification.

The formation of hydroxides, by the addition of $HO^{\bullet}$ in the presence of oxygen, across the side chains of amino acids is relatively specific to aliphatic amino acid side chains. Extensive characterisation of the three isomeric

forms of valine and leucine hydroxides has been undertaken by Fu and Dean[38] and Morin *et al.*[39]

Oxidation products of sugars, lipids, and amino acids can lead to the conversion of some lysine residues to lysine carboxymethylation derivatives. This conversion increases their metal binding capacity. Post-translational modifications that enhance their metal binding capacity should also increase their susceptibility to metal-catalyzed oxidation leading to increased formation of carbonyls and of the specific oxidation products glutamic and adipic semi-aldehydes.[40] α-Semi-adipic aldehyde is also formed enzymatically, and this may confound some determinations of this product. Nevertheless, researchers in Denmark have adopted this biomarker.[41]

Histidine is frequently involved in the co-ordination of metal ions, and thus is a target for metal-catalysed oxidation.[42] Little is known about the chemistry of oxidation of histidine by the range of biologically relevant reactive species, and hence it is too early to determine whether the oxidation product (2-oxohistidine) is an useful biomarker.

Hydroperoxides can be detected following HPLC separation of amino acids by chemiluminescence, following post-column reaction between microperoxidase and hydrogen peroxide. Hydroperoxides in plasma are degraded by either enzymic or acid hydrolysis, and can only be successfully measured in low molecular weight plasma filtrates, where the quenching effect of larger proteins is removed.[26] Following reduction of hydroperoxides by borohydride, subsequent analysis of hydroxides has largely been restricted to measurement of leucine, valine and more recently the hydrophilic surface amino acid, lysine.[38,39] Observations that these analytes are not stable on storage or hydrolysis mean that hydroperoxides do not fulfil the criteria required for biomarkers.

Lysine modification by lipid peroxidation products (linoleic hydroperoxide) can yield neo-antigenic determinants such as N-epsilon-hexanoyl lysine (HEL). An antibody has been successfully raised to this epitope.[43]

Oxidised tryptophan biomarkers

After cysteine loss, aromatic amino acids are the next most sensitive residues, owing to their capacity to delocalise radical intermediates around their ring structures.[44] The most frequent changes are ring addition reactions and introduction of oxygen, which in the case of tryptophan causes ring cleavage to yield N-formyl kynurenine.[45] Many of these changes are also associated with generation of a novel autofluorescence of a longer wavelength, which confers increased sensitivity and specificity in analysis.[46]

The light energy emitted by UV radiation can be directly absorbed by tryptophan, producing an excited intermediate.[45] The tryptophanyl radical can be dissipated though addition reaction with oxygen to give a peroxyl radical (main reaction), transfer to dissolved oxygen (to yield $O_2^{\bullet-}$) by antioxidants or through charge transfer, *i.e*. the movement of an unpaired electron along a peptide or protein backbone to a susceptible amino acid of lower redox potential.[20]

Thus, in any given protein, the target of attack may not be the ultimate site of damage. In the absence of charge transfer or repair, tryptophan undergoes a ring opening following $HO^{\bullet}$ attack to yield predominantly N-formyl kynurenine and kynurenine.[47] Kynurenines are also synthesised enzymically, and whilst these compounds are not incorporated during protein synthesis, which makes them potential markers in proteins, they are excreted in urine; therefore, it is not possible to use these analytes in urine analysis.

All aromatic amino acids are inherently light-sensitive, and so should be stored in light-tight containers. Urinary analysis of kynurenines as oxidative products cannot be undertaken, since these moieties can also arise directly through enzymic processes.

Measurement of oxidised tryptophan metabolites has utilised reverse-phase chromatography,[48] eluting the oxidised residues with increasing acetonitrile concentration. Eluent is monitored with native UV absorption and autofluorescence, and peak identity confirmed by spiking with authentic standard, and UV and fluorescence spectra.

Oxidised tyrosine biomarkers

Tyrosine dimerisation has been proposed to contribute to the aggregation of proteins frequently observed on oxidation. However, since oxygen and antioxidants rapidly repair the phenoxyl radical intermediate, it is only likely to be a significant product in metalloproteins, where the intermediate is stabilised through change in redox status of the associated transition metal.[49] L-DOPA is formed from the electrophilic addition of $HO^{\bullet}$ to the unsaturated/ring of tyrosine, where its presence within a protein structure is indicative of post-synthetic oxidation.[50]

Dityrosine-containing protein cross-linking products, designated as advanced oxidation protein products (AOPP), is a novel marker of oxidative stress correlating tightly with the degree of monocyte activation, dityrosine, advanced glycation end products, neopterin and inflammatory cytokines (*i.e*. TNF-α and its soluble receptor) levels.[19,51] The chlorinated oxidants are closely related with the mechanism of generation of AOPP. Moreover, AOPP-induced ROS production in endothelial cells is partially mediated by NADPH oxidase activation.[52] In addition, very high plasma levels of AOPP were observed in HIV patients,[51] in uraemia,[51,53] inflammatory disease (Bechcet's disease)[54] and Type 2 diabetes.[55] AOPP appeared to act as true inflammatory

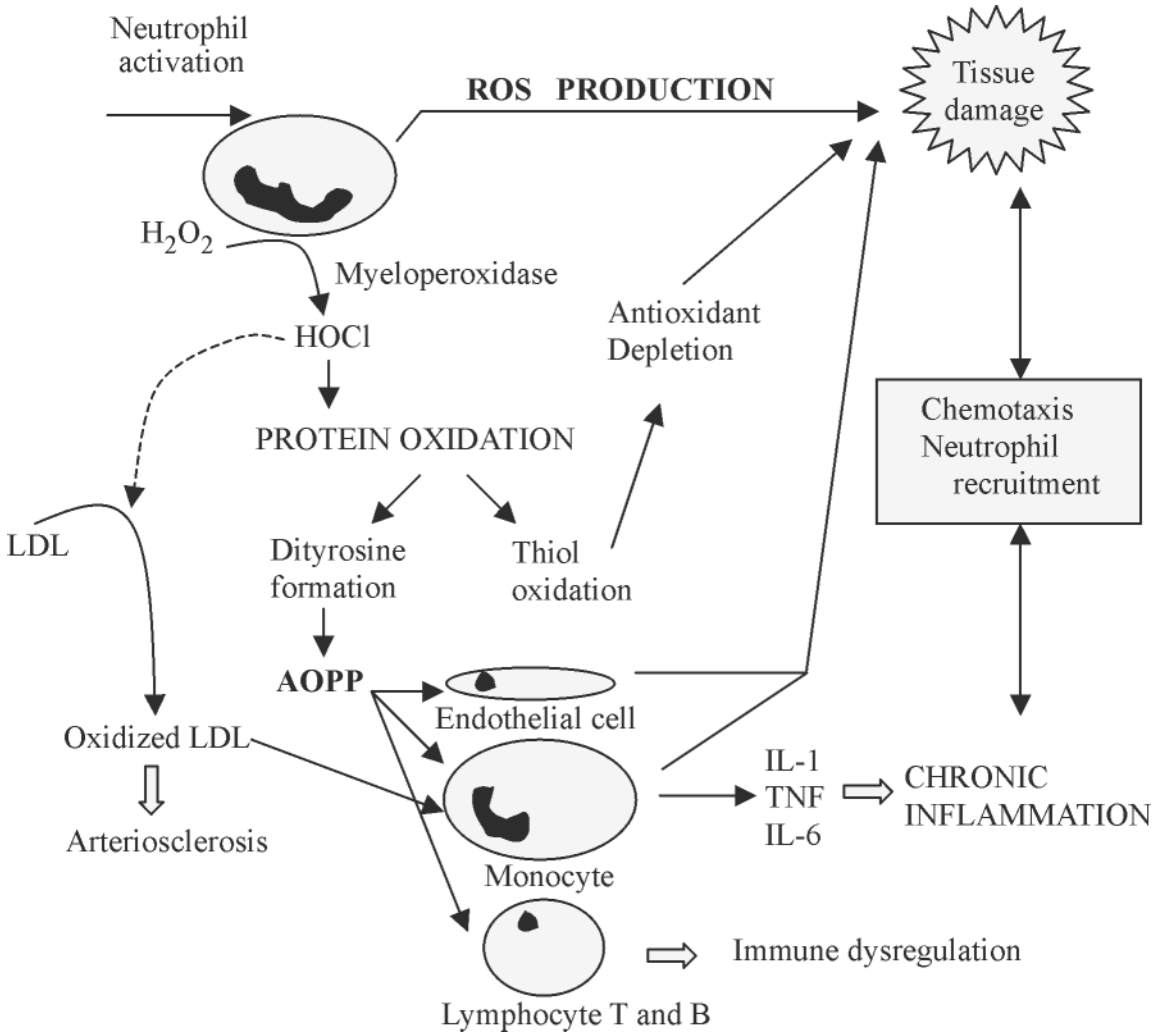

Fig. 2. Schematic diagram showing the concomitant contribution of neutrophils, monocytes and endothelial cell to production of reactive oxygen species (ROS) and inflammation via formation of advanced oxidation protein products (AOPP).

mediators since they are able to trigger the oxidative burst and the synthesis of cytokines (Fig. 2).

Hydroxylation of phenylalanine is a typical reaction of $HO^{\bullet}$, in which the resultant tyrosine is hydroxylated at the *ortho*- or *meta*-position and is, therefore, distinct from native tyrosine. The debate as to the physiological relevance of dityrosine is set to rage with a number of contradictory reports in the literature.[56] However, this may reflect the dependence of dityrosine formation on the structure of the protein under attack.

Hypochlorous acid is the major strong oxidant generated by neutrophils. Levels of 3-chlorotyrosine and 3,5-dichlorotyrosine are increased in proteins after exposure to low concentrations of hypochlorous acid.[57,58] Their analysis by gas chromatography and mass spectrometry is currently the best method available for probing the involvement of oxidation by myeloperoxidase in the pathology of particular diseases.[57] Furthermore, bromotyrosine may also be generated under the action of eosinophil peroxidase. However, it has been reported that both chloro-and bromotyrosine can undergo a second halogenation step, which interferes with their measurement. A further ring addition product of tyrosine, nitrotyrosine, is formed from attack by nitrosylating species including nitric oxide, peroxynitrite and reactions between hypochlorite and nitrogen-containing species.[59]

HPLC has been used to analyse phenylalanine oxidation products. However, caution must be employed in the detection system used following HPLC separation, as co-elution of an unknown compound that interferes with the determination of *m*-tyrosine has been reported.[60] As with any of these detection systems, the use of diode array or colorimetric detection can overcome these problems. Detection of the oxidised tyrosine moiety, L-DOPA, following HPLC also relies on native UV absorption (280 nm) and autofluorescence (excitation wavelength [Ex] 280 nm, emission wavelength [Em]

320 nm). The formation of *o*-and *m*-tyrosine from phenylalanine can be observed under the same analytical conditions. In contrast, dityrosine is followed using longer wavelength fluorescence emission at 410 nm. 3-Nitrotyrosine can be quantified by ELISA or HPLC using either UV or electrochemical detection.[61,62]

L-DOPA is a relatively long-lived species that can confer reducing activity in its environment. Whiteman and Halliwell have demonstrated the reduction of nitrotyrosine by hypochlorous acid, thus questioning the validity of nitrotyrosine as a biomarker.[62] This possibility for destruction by other oxidants may account for some of the ambiguities existing in the determination of nitrotyrosine in arteriosclerotic plaques.

Antiradical capacity of protein

In contrast to lipid peroxidation, the products which typically appear after a lag time, protein damage by ROS take place directly and immediately. Being sensitive indices of oxidative stress, protein degradation assays registering alterations in amino acids (formation of certain characteristic products, interconversions, changes in secondary, tertiary, and quaternary structure, overall charge, folding, hydrophobicity, fragmentation, covalent inter- and intramolecular cross-links) or increased susceptibility to proteolysis, provide many advantages over other techniques. Their most common drawback is the complexity of the applied methods, such as radioactive or fluorescent labelling, gel electrophoresis, Western blots, or immunoprecipitation.[63]

Detection of antiradical capacity of amino acid and protein by means of two independent assays based on the thermal homolysis of an azo-initiator and photo-sensitised chemiluminescence suggest that the oxidized products of some amino acids and proteins acquire antiradical properties at the same time that they are being produced.[21] The antiradical capacity of proteins (ACP) is thought to be an important component of the total antioxidant capacity of blood plasma, being not a characteristic of antioxidant defence but reflecting the degree of oxidative stress. Investigation of donor blood samples showed that H_2O_2-initiated chemiluminescence of plasma protein was higher in smokers compared to non-smokers.[64] In addition, the ACP of human blood plasma after removal of low-molecular-weight antioxidants by means of gel filtration was positive and showed a clear difference between the results in healthy donors and cancer patients.[65]

Formation of neo-epitopes on oxidised proteins

Following the structural alterations introduced by an oxidant insult, proteins can acquire new antigenic properties, owing to the formation of new epitopes on the polypeptide chain. This is primarily the case with reactive aldehydes derived from lipid peroxidation, which are able to bind to several amino acid residues. By means of specific polyclonal or monoclonal antibodies, the occurrence of malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE) bound to cellular protein has been documented under a number of experimental and clinical conditions.[66–69]

Specific epitopes are also present in oxidised LDL (ox-LDL), a distinctive class of oxidised proteins probably involved in the pathogenesis of arteriosclerosis. The exact nature of such epitopes is a matter of debate, although it seems certain that the antigenicity of ox-LDL can be at least partially accounted for by the binding of lipid peroxide-derived aldehydes, such as MDA and 4-HNE, to the LDL apoprotein moiety.[69]

Relationship of protein oxidation to disease

Protein carbonyls have been most strongly linked with ageing and associated phenomena, as reviewed by Chevion *et al*.[70] In a study of low birth weight babies, Winterbourn *et al*.[71] observed raised levels of protein carbonyls in cord blood compared to full-term babies; there was no relation to chronic lung disease and retinopathy. In addition, the carbonyl content of plasma protein was higher in Type 2 diabetes mellitus than in controls.[72] Systemic markers are less sensitive and may not reflect the changes at target tissues. In light of this, Chen *et al*.[73] examined levels of protein carbonyls in seminal fluid from men with varicocele and observed higher levels in the disease group and subclinical varicocele compared to controls.

Analysis of thiol status has been undertaken immunohistochemically in a semiquantitative manner in cancer cells.[74] Thiol status of plasma decreases with ageing but this effect may be explained, in part, through reduced albumin production from ageing liver. However, if the focus is directed to target tissue, such as seminal fluid in patients with varicocele,[73] a clear reduction in thiols is observed, which supports the hypothesis of increased oxidative stress in this tissue. Protein thiols were significantly decreased in patients with chronic renal failure (CRF) and with CRF on haemodialysis.[75]

Both 3-hydroxy valine and 5-hydroxy leucine have been detected in normal tissues, including plasma, intimal artery and lenses, and can increase 7–10-fold in diseased tissue, depending on the severity of disease.[76] Elevated levels of N-formyl kynurenine have been observed in cataractous lenses, IgG from patients with rheumatoid arthritis and in LDL from arteriosclerosis patients.[43] Limited analysis of kynurenines has been done in normal healthy subjects, and they are detectable in plasma IgG but not albumin. Porphyrin-enhanced

photo-oxidation of bovine serum albumin *in vitro* causes the formation of oxidised tryptophan metabolites.[77]

There are a number of reports demonstrating the generation of protein-bound L-DOPA, from tyrosine, in a dose-dependent fashion following $HO^{\bullet}$ attack.[5,76] There is confusion in the literature, however, as to the presence of nitrotyrosine in atheromatous plaques, with some workers describing elevated levels and others not observing any difference between normal and diseased tissue. Nonetheless, L-DOPA, dityrosine, 3-chlorotyrosine and 3-nitrotyrosine are all detectable in normal intima, but only L-DOPA and dityrosine are present in normal lenses, demonstrating the lack of inflammatory processes in oxidation of lens tissue. In contrast, studies on the vascular intima suggest that levels of dityrosine present are greater than would be predicted from $HO^{\bullet}$ alone, which would suggest the involvement of myeloperoxidase-catalysed oxidation reactions.[76]

Modulation of protein oxidation biomarkers by antioxidants

Supplementation of the rat diet with the flavonoid rutin for 18 days caused a reduction in protein carbonyl content.[78] Supplementation with α-tocopherol or a combination of α-tocopherol (40 mg/day) and ascorbic acid (24 mg/day) for 15 days protected against protein carbonyl formation in iron-deficient rats during iron repletion.[79] Tocotrienols reduced the protein carbonyl levels in the ageing nematode, *Caenorhabditis elegans*.[80] These limited studies are consistent in demonstrating a protective effect against plasma protein oxidation by dietary intervention.

Long-term vitamin C supplementation has been observed to protect against protein oxidation in human subjects.[28] In subjects with low baseline antioxidant status, vitamin C supplementation significantly reduced the degree of protein oxidation after 10, but not 5, weeks. An overall negative correlation between ascorbate and IgG carbonyl content was observed ($r = –0.145$; $P = 0.019$).

Partial analysis of samples from a study of long-term supplementation[81] demonstrated that individuals receiving both vitamins C and E show a 30% decrease in protein carbonyl levels after 1 year. Again, there is a significant inverse correlation between vitamin C and protein carbonyl status ($r = –0.575$; $P < 0.001$).

Wander and Du[82] have examined the antioxidant effect of vitamin E in supplementation with eicosapentaenoic and docosahexaenoic acids. The fish oil had no effect on protein carbonyl content, nor was the presence of 400 mg α-tocopherol in the diet a modulator of plasma protein carbonyl formation. Studies *in vitro* of HDL oxidation have confirmed the early formation of methionine sulphoxide, which is enhanced by α-tocopherol.[83] This lesion is also subject to active repair by methionine sulphoxide reductase, where reduced activity of the enzyme is associated with ageing.[30]

Analysis of thiols has been undertaken in antioxidant supplementation studies in humans.[28] In plasma, measurements are confounded by the high thiol content of albumin and its associated function in transport of small molecules, including drugs such as D-penicillamine. Generation of 2-oxohistidine is observed during LDL oxidation *in vitro*, which can be inhibited by vitamin C.[84] Nitrogen-centred radicals of lysine, formed by HOCl, are protected by the presence of ascorbic acid, glutathione and Trolox *in vitro*.[46] A recent study in Watanabe heritable hyperlipidaemic rabbits, using an antibody against lysine-malondialdehyde, examined uptake of radiolabelled antibody into plaques in animals fed diets supplemented with dietary vitamins C and E, for 6 months. Those on normal diets developed lesions rich in modified lysine, whereas those fed diets supplemented with vitamins C and E showed fewer oxidation-specific epitopes.[85]

Grape seed extract rich in catechins and phenolics was given as a supplement for 1 week to 15 subjects; no effect was seen on protein oxidation.[86] A second study looked at fruit juice in 5 subjects, again for 1 week, with three doses. In this study, plasma 2-amino-adipic semialdehyde increased with time and dose, indicating a pro-oxidant effect.[39] A third study examined parsley, rich in flavone, for 1 week in 14 subjects. Again, no significant changes were observed in plasma protein 2-adipic semialdehyde residues.[87] The rather inconclusive nature of these studies may be simply a reflection of the lack of statistical power, combined with short duration of supplementation. In contrast, a *Mangifera indica* extract rich in polyphenols was given as a supplement for 6 months in HIV subjects; the supplemented group ($n = 36$) had an increase in plasma thiol groups ($P < 0.05$) when compared to placebo ($n = 32$).[88] There are no published reports describing the effects of dietary antioxidants on tyrosine oxidation products.

FUTURE PERSPECTIVES

There are several techniques and markers with the requisite sensitivity and specificity to be applied in the evaluation of effects of dietary antioxidants on protein oxidation. It is important, however, that any protein selected should be of relevance to the outcome of an altered function. A recent study in SOD2 knockout mice clearly showed increased oxidation and inactivation of aconitase and NADPH oxidase, and adverse effects on mitochondrial energy production.[89]

Other issues that should be considered are: (i) is there a difference between the ability of different classes of antioxidant to protect plasma proteins, compared to cellular proteins? Is this a direct function of their hydrophobicity indices? (ii) What is the subcellular distribution of

protein oxidation, and how is the breakdown of oxidised proteins via the proteosome affected by antioxidants? Protein-L-isoaspartate O-methyl transferase (PCMT) catalyses methylation of oxidised aspartate residues, and thereby signals repair or degradation of age-damaged proteins. Antioxidants can inhibit appropriate methylation,[90] and this may impair the removal of oxidised proteins, suggesting a negative benefit of antioxidants. This merits further study.

Conclusions

It is clear from the body of evidence presented that a 'fingerprint' of the ROS present *in vivo* should be attainable by adopting a broad spectrum of protein-based biomarkers. Based on the criteria outlined in the introduction of this report, and the observations of unequivocal elevation in disease, the likely candidates for biomarkers are isolated protein carbonyls (accepting that these may arise from secondary processes), dityrosine, L-DOPA, and *ortho*-tyrosine. In addition, AOPP and ARAP are promising biomarkers due to simplicity, low cost and high association with disease.

It is also possible to examine LDL in plasma and its propensity for uptake by monocytes and endothelial cells, where modified LDL is taken up by the scavenger receptor. Specific modification to one lysine residue is sufficient to dramatically increase uptake of LDL.[24] This validated procedure could be applied to evaluate the functional consequences of antioxidant supplementation in a human intervention study.

This paper has highlighted the advantages of adopting several biomarkers for studying *in vivo* oxidation of proteins, and these methods should now be carefully applied, using quality assurance material, to controlled studies of antioxidant intake to evaluate the significance of dietary/pharmacological antioxidants in preventing physiological/pathological oxidative changes.

Acknowledgements

The authors thank Dr Igor Popov for critically reviewing this contribution prior to submission. They gratefully acknowledge the support from the Department of Chemistry and Medical Biochemistry, University of Milan. This work was funded partially by the Alexander von Humboldt Foundation, Germany. Clive Prestt kindly checked the English of the manuscript.

References

1. Adams GE, Willson RL, Aldrich JE, Cundall RB. On the mechanism of radiation induced inactivation of lysozyme in dilute aqueous solutions. *Int J Radiat Biol* 1969; **16**: 333–342.
2. Carp H, Janoff A. *In vitro* suppression of serum elastase-inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes. *J Clin Invest* 1979; **63**: 793–797.
3. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 1989; **320**: 915–924.
4. Stadtman ER, Levine RL. Protein oxidation. *Ann NY Acad Sci* 2000; **899**: 191–208.
5. Davies MJ. The oxidative environment and protein damage. *Biochim Biophys Acta* 2005; **1703**: 93–109.
6. Hawkins CL, Davies MJ. Generation and propagation of radical reactions on proteins. *Biochim Biophys Acta* 2001; **1504**: 196–219.
7. Stadtman ER, Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* 2003; **25**: 207–218.
8. Alvarez B, Radi R. Peroxynitrite reactivity with amino acids and proteins. *Amino Acids* 2003; **25**: 295–311.
9. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 1997; **324**: 1–18.
10. Grune T, Reinheckel T, Davies KJA. Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. *J Biol Chem* 1996; **271**: 15504–15509.
11. Davies KJA, Goldberg AL. Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J Biol Chem* 1987; **262**: 8220–8226.
12. Grune T, Reinheckel T, Davies KJA. Degradation of oxidized proteins in mammalian cells. *FASEB J* 1997; **11**: 526–534.
13. Stadtman ER. Protein oxidation and aging. *Science* 1992; **257**: 1220–1224.
14. Berlett BS, Levine RL, Stadtman ER. Comparison of the effects of ozone on the modification of amino acid residues in glutamine synthetase and bovine serum albumin. *J Biol Chem* 1996; **271**: 4177–4182.
15. Dukan S, Farewell A, Ballesteros M, Taddei F, Radman M, Nystro MT. Protein oxidation in response to increased transcriptional errors. *Proc Natl Acad Sci USA* 2000; **97**: 5746–5749.
16. Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002; **82**: 47–95.
17. Grune T, Merker K, Sandig G, Davies KJA. Selective degradation of oxidatively modified protein substrates by the proteasome. *Biochem Biophys Res Commun* 2003; **305**: 709–718.
18. Davies MJ, Fu S, Wang H, Dean RT. Stable markers of oxidant damage to proteins and their application in the study of human disease. *Free Radic Biol Med* 1999; **27**: 1151–1163.
19. Griffiths HR. Antioxidants and protein oxidation. *Free Radic Res* 2000; **33**: S47–S58.
20. Witko-Sarsat V, Friedlander M, Khoa TN *et al.* Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure. *J Immunol* 1998; **161**: 2524–2532.
21. Griffiths HR, Moller L, Bartosz G *et al.* Biomarkers. *Mol Aspect Med* 2002; **23**: 101–208.
22. Popov I, Lewin G. Photochemiluminescent detection of antiradical activity. VI. Antioxidant characteristics of human blood plasma, low density lipoprotein, serum albumin and aminoacids during *in vitro* oxidation. *Luminescence* 1999; **14**: 169–174.
23. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 1997; **272**: 20313–20316.
24. Stadtman ER, Berlett BS. Fenton chemistry. Amino acid oxidation. *J Biol Chem* 1991; **266**: 17201–17211.
25. Headlam HA, Davies MJ. Markers of protein oxidation: different oxidants give rise to variable yields of bound and released carbonyl products. *Free Radic Biol Med* 2004; **36**: 1175–1184.

26. Griffiths HR, Bevan R, Lunec J. Measurement of oxidised amino acids by HPLC. In: Lunec J, Griffiths HR. (eds) *Handbook of Oxidative Methods*. London: Wiley, 2000: 27–50.
27. Schacter E, Williams JA, Stadtman ER, Levine RL. Determination of carbonyl groups in oxidised proteins. In: Punchard A, Kelly FJ. (eds) *Free Radicals. A Practical Approach*. Oxford: IRL Press, 1996; 159–170.
28. Buss H, Chan TP, Sluis KB, Domigan NM, Winterbourn CC. Protein carbonyl measurement by a sensitive ELISA method. *Free Radic Biol Med* 1997; **23**: 361–366.
29. Carty JL, Bevan R, Waller H *et al.* The effects of vitamin C supplementation on protein oxidation in healthy volunteers. *Biochem Biophys Res Commun* 2000; **273**: 729–735.
30. Creed D. The photochemistry and photophysics of the near UV absorbing amino acids. III Cystine and its simple derivatives. *Photochem Photobiol* 1984; **39**: 577–584.
31. 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**: 819–825.
32. Biewenga GP, Veening-Griffioenen DH, Nicastia AJ, Haenen GRMM. Effects of dihydrolipoic acid on peptide methionine sulfoxide reductase implications for antioxidant drugs. *Arzneimittelforschung* 1998; **48**: 144–148.
33. Ellman GL. Tissue sulphydryl groups. *Arch Biochem Biophys* 1959; **82**: 70–77.
34. Singh R, Blattler WA, Collinson AR. An amplified assay for thiols based on reactivation of papain. *Anal Biochem* 1993; **213**: 49–56.
35. Fu S, Gebicki S, Jessup W, Gebicki JM, Dean RT. Biological fate of amino acid, peptide and protein hydroperoxides. *Biochem J* 1995; **311**: 821–827.
36. Robinson S, Bevan R, Lunec J, Griffiths H. Chemiluminescence determination of hydroperoxides following radiolysis and photolysis of free amino acids. *FEBS Lett* 1998; **430**: 297–300.
37. Morgan PE, Dean RT, Davies MJ. Protective mechanisms against peptide and protein peroxides generated by singlet oxygen. *Free Radic Biol Med* 2004; **36**: 484–496.
38. Fu SL, Dean RT. Structural characterization of the products of hydroxyl-radical damage to leucine and their detection on proteins. *Biochem J* 1997; **324**: 41–48.
39. Morin B, Bubb WA, Davies MJ, Dean RT, Fu S. 3-Hydroxylysine, a potential marker for studying radical-induced protein oxidation. *Chem Res Toxicol* 1998; **11**: 1265–1273.
40. Requena JR, Stadtman ER. Conversion of lysine to N(epsilon)-(carboxymethyl)lysine increases susceptibility of proteins to metal-catalyzed oxidation. *Biochem Biophys Res Commun* 1999; **264**: 207–211.
41. Young JF, Nielsen SE, Haraldsdottir J *et al.* Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. *Am J Clin Nutr* 1999; **69**: 87–94.
42. Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu Rev Biochem* 1993; **62**: 797–821.
43. Kato Y, Miyake Y, Yamamoto K *et al.* Preparation of a monoclonal antibody to N(epsilon)-(hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. *Biochem Biophys Res Commun* 2000; **274**: 389–393.
44. Guptasarma P, Balasubramania D, Matsugo S, Saito I. Hydroxyl radical mediated damage to proteins, with special reference to the crystallins. *Biochemistry* 1992; **31**: 4296–4303.
45. Maskos Z, Rush JD, Koppenol WH. The hydroxylation of tryptophan. *Arch Biochem Biophys* 1992; **262**: 521–529.
46. Hawkins CL, Davies MJ. Hypochlorite-induced oxidation of proteins in plasma: formation of chloramines and nitrogen-centred radicals and their role in protein fragmentation. *Biochem J* 1999; **340**: 539–548.
47. Griffiths HR, Lunec J, Blake DR. Oxygen radical induced fluorescein proteins; identification of the fluorescent tryptophan kynurenine, as a biological index of radical damage. *Amino Acids* 1992; **3**: 183–194.
48. Giessauf A, van Wickern B, Simat T, Steinhart H, Esterbauer H. Formation of N-formylkynurenine suggests the involvement of apolipoprotein B-100 centered tryptophan radicals in the initiation of LDL lipid peroxidation. *FEBS Lett* 1996; **389**: 136–140.
49. Wilks A, Ortiz de Montellano PR. Intramolecular translocation of the protein radical formed in the reaction of recombinant sperm whale myoglobin with H_2O_2. *J Biol Chem* 1992; **267**: 8827–8833.
50. Rodgers KJ, Hume PM, Dunlop RA, Dean RT. Biosynthesis and turnover of DOPA-containing proteins by human cells. *Free Radic Biol Med* 2004; **37**: 1756–1764.
51. Witko-Sarsat V, Nguyen-Khoa T, Jungers P, Drueke TB, Descamps-Latscha B. Advanced oxidation protein products as a novel molecular basis of oxidative stress in uraemia. *Nephrol Dial Transplant* 1999; **14**: 76–78.
52. Yuan F, Liu SX, Tian JW. Advanced oxidation protein products induced reactive oxygen species production in endothelial cells. *Di Yi Jun Da Xue Xue Bao* 2004; **24**: 1350–1352.
53. Witko-Sarsat V, Gausson V, Descamps-Latscha B. Are advanced oxidation protein products potential uremic toxins?. *Kidney Int Suppl* 2003; **84**: S11–S14.
54. Yazici C, Kose K, Calis M, Delmlr M, Kirnap M, Ates F. Increased advanced oxidation protein products in Bechcet's disease: a new activity marker? *Br J Dermatol* 2004; **151**: 105–111.
55. Martínes-Sánchez G, Al-Dalain SM, Popov I, Menendez S, Guiliani A, León OS. Contribution to characterization of oxidative stress in diabetes patients with macroangiopathic complications. *Acta Farm Bonaerese* 2005; **24**: 197–203.
56. Kato Y, Maruyama W, Naoi M, Hashizume Y, Osawa T. Immunohistochemical detection of dityrosine in lipofuscin pigments in the aged human brain. *FEBS Lett* 1998; **439**: 231–234.
57. Winterbourn CC, Kettle AJ. Biomarkers of myeloperoxidase-derived hypochlorous acid. *Free Radic Biol Med* 2000; **29**: 403–409.
58. Hazen SL, d'Avignon A, Anderson MM, Hsu FF, Heinecke JW. Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to oxidize alpha-amino acids to a family of reactive aldehydes. Mechanistic studies identifying labile intermediates along the reaction pathway. *J Biol Chem* 1998; **273**: 4997–5005.
59. Crow JP. Measurement and significance of free and protein-bound 3-nitrotyrosine, 3-chlorotyrosine, and free 3-nitro-4-hydroxy-phenylacetic acid in biologic samples: a high-performance liquid chromatography method using electrochemical detection. *Methods Enzymol* 1999; **301**: 151–160.
60. Reddy S, Halliwell B, Jones AD, Longhurst JC. The use of phenylalanine to detect hydroxyl radical production *in vivo*: a cautionary note. *Free Radic Biol Med* 1999; **27**: 1465.
61. Shigenaga MK. Quantitation of protein-bound 3-nitrotyrosine by high-performance liquid chromatography with electrochemical detection. *Methods Enzymol* 1999; **301**: 27–40.
62. Whiteman M, Halliwell B. Loss of 3-nitrotyrosine on exposure to hypochlorous acid: implications for the use of 3-nitrotyrosine as a bio-marker *in vivo*. *Biochem Biophys Res Commun* 1999; **258**: 168–172.
63. Pacifici RE, Devis KJA. Protein degradation as an index of oxidative stress. *Methods Enzymol* 1990; **186 B**: 485–502.
64. Popov I, Volker H, Lewin G. Photochemiluminescent detection of antiradical activity. V. application in combination with the

hydrogen peroxide-initiated chemiluminescence of blood plasma proteins to evaluate antioxidant homeostasis in human. *Redox Report* 2000; **6**: 42–48.
65. Lewin G, Kuhn G, Deuse U, Buhring M, Ramsauer B, Popov I. Paradox of the antiradical capacity of blood plasma protein. In. SFRR Europe Summer Meeting, July 2–5, 1999, Dresden, Germany. Abstract Book, p 029.
66. Bedossa P, Houglum K, Trautwein C, Holstege A, Chojkier M. Stimulation of collagen alpha 1(I) gene expression is associated with lipid peroxidation in hepatocellular injury: a link to tissue fibrosis? *Hepatology* 1994; **19**: 1262–1271.
67. Paradis V, Mathurin P, Kollinger M *et al. In situ* detection of lipid peroxidation in chronic hepatitis C: correlation with pathological features. *J Clin Pathol* 1997; **50**: 401–406.
68. Cambiaggi C, Dominici S, Comporti M, Pompella A. Modulation of human T lymphocyte proliferation by 4-hydroxynonenal, the bioactive product of neutrophil-dependent lipid peroxidation. *Life Sci* 1997; **61**: 777–785.
69. Requena JR, Fu MX, Ahmed MU *et al.* Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. *Biochem J* 1997; **322**: 317–325.
70. Chevion M, Berenshtein E, Stadtman ER. Human studies related to protein oxidation: protein carbonyl content as a marker of damage. *Free Radic Res* 2000; **33**: S99–S108.
71. Winterbourn CC, Chan T, Buss IH, Inder TE, Mogridge N, Darlow BA. Protein carbonyls and lipid peroxidation products as oxidation markers in preterm infant plasma: associations with chronic lung disease and retinopathy and effects of selenium supplementation. *Pediatr Res* 2000; **48**: 84–90.
72. De Cristofano R, Rocca B, Vitacolonna E *et al.* Lipid and protein oxidation contribute to a prothrombotic state in patients with type 2 diabetes mellitus. *J Thromb Haemost* 2003; **1**: 250–256.
73. Chen S, Chang LS, Wei Y. Oxidative damage to proteins and decrease of antioxidant capacity in patients with varicocele. *Free Radic Biol Med* 2001; **30**: 1328–1334.
74. Wardman P, von Sonntag C. Kinetic factors that control the fate of thiyl radicals in cells. *Methods Enzymol* 1995; **251**: 31–45.
75. Prakash M, Upadhya S, Prabhu R. Protein thiol oxidation and lipid peroxidation in patients with uraemia. *Scan J Clin Lab Invest* 2004; **64**: 599–604.
76. Fu S, Davies MJ, Stocker R, Dean RT. Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque. *Biochem J* 1998; **333**: 519–525.
77. Silvester JA, Timmins GS, Davies MJ. Photodynamically generated bovine serum albumin radicals: evidence for damage transfer and oxidation at cysteine and tryptophan residues. *Free Radic Biol Med* 1998; **24**: 754–766.
78. Funabiki R, Takeshita K, Miura Y, Shibasato M, Nagasawa T. Dietary supplement of G-rutin reduces oxidative damage in the rodent model. *J Agric Food Chem* 1999; **47**: 1078–1082.
79. Srigiridhar K, Nair KM. Supplementation with alpha-tocopherol or a combination of alphatocopherol and ascorbic acid protects the gastrointestinal tract of iron-deficient rats against iron-induced oxidative damage during iron repletion. *Br J Nutr* 2000; **84**: 165–173.
80. Adachi H, Ishii N. Effects of tocotrienols on life span and protein carbonylation in *Caenorhabditis elegans*. *J Gerontol A Biol Sci Med Sci* 2000; **55**: B280–B285.
81. Porkkala-Sarataho E, Salonen JT, Nyyssonen K *et al.* Long-term effects of vitamin E, vitamin C, and combined supplementation on urinary 7-hydro-8-oxo-2′-deoxyguanosine, serum cholesterol oxidation products, and oxidation resistance of lipids in non-depleted men. *Arterioscler Thromb Vasc Biol* 2000; **20**: 2087–2093.
82. Wander RC, Du SH. Oxidation of plasma proteins is not increased after supplementation with eicosapentaenoic and docosahexaenoic acids. *Am J Clin Nutr* 2000; **72**: 731–737.
83. Garner B, Witting PK, Waldeck AR, Christison JK, Raftery M, Stocker R. Oxidation of high density lipoproteins. I. Formation of methionine sulfoxide in apolipoproteins AI and AII is an early event that accompanies lipid peroxidation and can be enhanced by alpha-tocopherol. *J Biol Chem* 1998; **273**: 6080–6087.
84. Retsky KL, Chen K, Zeind J, Frei B. Inhibition of copper-induced LDL oxidation by vitamin C is associated with decreased copper-binding to LDL and 2-oxo-histidine formation. *Free Radic Biol Med* 1999; **26**: 90–98.
85. Tsimikas S, Shortal BP, Witztum JL, Palinski W. *In vivo* uptake of radiolabeled MDA2, an oxidation-specific monoclonal antibody, provides an accurate measure of atherosclerotic lesions rich in oxidized LDL and is highly sensitive to their regression. *Arterioscler Thromb Vasc Biol* 2000; **20**: 689–697.
86. Young JF, Dragsted LO, Daneshvar B, Lauridsen ST, Hansen M, Sandstrom B. The effect of grape-skin extract on oxidative status. *Br J Nutr* 2000; **84**: 505–513.
87. Nielsen SE, Young JF, Daneshvar B *et al.* Effect of parsley (*Petroselinum crispum*) intake on urinary apigenin excretion, blood antioxidant enzymes and biomarkers for oxidative stress in human subjects. *Br J Nutr* 1999; **81**: 447–455.
88. Gil L, Martínez G, González I *et al.* Effects of VIMANG on oxidative stress and marker of disease progression in HIV/AIDS patients. *Free Radic Res* 2002; **36**: 107–109.
89. Williams MD, Remmen H, Conrad CC, Huang TT, Epstein CJ, Richardson A. Increased oxidative damage is correlated to altered mitochondrial function in heterozygous manganese superoxide dismutase knockout mice. *J Biol Chem* 1998; **273**: 28510–28515.
90. Ingrosso D, D'angelo S, Carlo E, Perna AF, Zappia V, Galletti P. Increased methyl esterification of altered aspartyl residues in erythrocyte membrane proteins in response to oxidative stress. *Eur J Biochem* 2000; **267**: 4397–4405.