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Review

Protein carbonyl groups as biomarkers of oxidative stress

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Abstract

Oxidative stress, an imbalance toward the pro-oxidant side of the pro-oxidant/antioxidant homeostasis, occurs in several human diseases. Among these diseases are those in which high levels of protein carbonyl (CO) groups have been observed, including Alzheimer's disease (AD), rheumatoid arthritis, diabetes, sepsis, chronic renal failure, and respiratory distress syndrome.

What relationships might be among high level of protein CO groups, oxidative stress, and diseases remain uncertain.

The usage of protein CO groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins. Most of the assays for detection of protein CO groups involve derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl (DNP) hydrazone product. This then can be detected by various means, such as spectrophotometric assay, enzyme-linked immunosorbent assay (ELISA), and one-dimensional or two-dimensional electrophoresis followed by Western blot immunoassay.

At present, the measurement of protein CO groups after their derivatisation with DNPH is the most widely utilized measure of protein oxidation.

Keywords: Reactive oxygen species; 2,4-Dinitrophenylhydrazine; Carbonyl assay; Human diseases; Oxyblot

1. Introduction

There now exists convincing evidence that oxidative stress and reactive oxygen species (ROS) play an important role in the aetiology and/or progression of a number of human diseases [1]. Actually, the medical significance of oxidative stress has become increasingly recognised to the point that it is now considered to be a component of virtually every disease process.

The generation of ROS may occur by a large number of physiological and nonphysiological pro-

Abbreviations: CO, carbonyl; DNPH, 2,4-dinitrophenylhydrazine; DNP, 2,4-dinitrophenyl; ELISA, enzyme-linked immunosorbent assay; GSSG, glutathione disulfide; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; RNS, reactive nitrogen species; ROS, reactive oxygen species; ARDS, adult (acute) respiratory distress syndrome; BAL, bronchoalveolar lavage; IBD, inflammatory bowel disease; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 1D-PAGE, one-dimensional polyacrylamide gel electrophoresis; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; AD, Alzheimer's disease.

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cesses, which include their generation as by-products of normal cellular metabolism, primarily in the mitochondria. ROS may damage all types of biological molecules. Oxidative damages to proteins, lipids, or DNA may all be seriously deleterious and may be concomitant. However, proteins are possibly the most immediate vehicle for inflicting oxidative damage on cells because they are often catalysts rather than stoichiometric mediators; hence, the effect of damage to one molecule is greater than stoichiometric.

ROS leading to protein oxidation include radical species such as superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), alkoxyl ($RO^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$), and nonradical species such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$) [2,3].

Carbonyl (CO) groups (aldehydes and ketones) are produced on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidised (Fig. 1). These moieties are chemically stable, which is useful for both their detection and storage. Protein carbonyl derivatives can also be generated through oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains, leading to formation of a peptide in which the N-terminal amino acid is blocked by an α-ketoacyl derivative [3]. In addition, CO groups may be introduced into proteins by secondary reaction of the nucleophilic side chains of Cys, His, and Lys residues, with aldehydes (4-hydroxy-2-nonenal, malondialdehyde, 2-propenal [acrolein]) produced during lipid peroxidation or with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a consequence of the reaction of reducing sugars, or their oxidation products with lysine residues of proteins (glycation and glycoxidation reactions), with the eventual formation of the advanced glycation/lipoxidation end products (AGEs/ALEs), that is, glycoxidation products, such as carboxymethyllysine and pentosidine, and lipoxidation products, such as malondialdehyde-lysine and 4-hydroxy-nonenal-protein adduct (for exhaustive reviews showing detailed chemical structures, see Refs. [1,3–5]). Hence, the presence of carbonyls is not necessarily indicative of oxidation of amino acid residues in proteins. To establish whether the CO groups come from direct or indirect modification of the amino acid side chain, additional assay methods must be employed. For example, lipid peroxidation adducts can be detected using specific antibodies [6]. Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation [3,7–9], and accumulation of protein carbonyls has been observed in several human diseases including Alzheimer's disease (AD), diabetes, inflammatory bowel disease (IBD), and arthritis, just for citing a few (Refs. [1,7] and Table 1).

Most protein carbonyl assays offer the advantage that no special or expensive equipment is required. As a result, they can be performed in any normally equipped biochemistry laboratory. Actually, carbonyl assays have become widely used and many laboratories have developed individual protocols for them. Sometimes, the procedures used in a particular laboratory are not precisely specified in published papers. This point is important because there is a considerable variation in the basal levels of protein carbonyls in certain tissues, depending on how the carbonyl assay is performed. For example, reported carbonyl levels for human brain cortex range from 1.5 to 6.4 nmol/mg

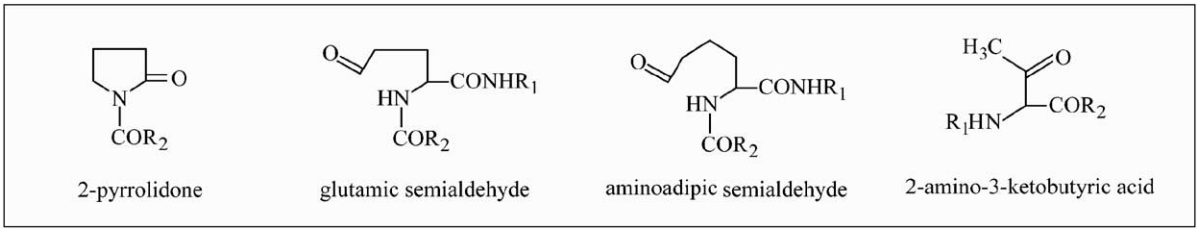

Fig. 1. The structure of carbonyl derivatives produced by direct oxidation of amino acid side chains: 2-pyrrolidone from prolyl residue, glutamic semialdehyde from arginyl and prolyl residue, α-aminoadipic semialdehyde from lysyl residue, and 2-amino-3-ketobutyric acid from threonyl residue.

Table 1
Methods used for detection of carbonylated proteins in human diseases

Spectrophotometric DNPH assay
Alzheimer's disease [24]
Amyotrophic lateral sclerosis [25,93]
Cataractogenesis [94]
Chronic hepatitis C [21]
Cystic fibrosis [32,95]
Diabetes [26–29,96]
Inflammatory bowel disease [67]
Juvenile chronic arthritis [31]
Newborn infants at risk of chronic lung disease [97]
Rheumatoid arthritis [30]
Systemic amyloidosis [98]
Uremia [99]
Varicocele [100]
Spectrophotometric DNPH assay coupled to protein fractionation by HPLC
Acute respiratory distress syndrome [33]
Enzyme-linked immunosorbent assay (ELISA)
Chronic renal failure/uremia [44,59]
Coronary heart surgery [43]
Pre-eclampsia [41,42]
Premature infants [38]
Preterm babies at risk of chronic lung disease [101]
Respiratory disease in newborn babies [39]
Sepsis [40,102]
One- or two-dimensional electrophoresis and Western blot immunoassay
Alzheimer's disease [46,54–57,60,61,103]
Atrial fibrillation [104]
Bronchopulmonary dysplasia [58]
Chronic renal failure/uremia [44,59]
Duchenne and Becker muscular dystrophy [105]
Helicobacter pylori infection and inflammation [106]
Parkinson's disease [107]
Psoriasis [108]

proteins [10]. By contrast, most groups seem to obtain broadly comparable values for protein carbonyls in human plasma, of 0.4–1.0 nmol/mg protein [11].

2. Methods for detection of protein carbonyl groups

Many assays are available for detection of protein carbonyls. Protein CO groups can be detected by labelling it with tritiated borohydride [12], either in solution [13] or before gel electrophoresis [14]. The radioactive hydrogen can then be detected by standard methods. This method is highly sensitive and specific when applied to samples of purified proteins, but high backgrounds and poor specificity (as tritiated borohydride also reacts with Schiff bases) can complicate its application to unfractionated tissue supernatants. However, oxidatively modified proteins in bronchoalveolar lavage (BAL) fluid, as measured by the reduction of protein carbonyl groups with tritiated borohydride, were observed in patients with acute respiratory distress syndrome [13].

Highly sensitive assays for detection of protein carbonyls involve derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product. The measurement of protein carbonyls following their covalent reaction with DNPH was pioneered by Levine et al. [12] and has become the most widely utilized measure of protein oxidation in several human diseases (Table 1).

Stable DNP adduct can be detected by various means (Fig. 2). The DNP group itself absorbs ultraviolet light so that the total carbonyl content of a protein or mixture of proteins can be quantified by a spectrophotometric assay [12], which can be coupled to protein fractionation by high-performance liquid chromatography (HPLC) to give greater sensitivity and specificity than measuring total carbonyls in a protein mixture [15].

More work needs to be done to identify the molecular nature of the carbonyls, that is, which amino acid residues have been damaged and on what proteins in human tissues and body fluids. In the last years, the identification of carbonylated proteins has been facilitated by the availability of commercial specific antibodies to anti-DNP that allow their detection by immunoblotting analysis in analogy, for example, with the protocols for the study of phosphorylated proteins. Immunoblotting assays based on the use of anti-DNP antibodies have been developed in an attempt to identify oxidatively damaged proteins in human tissues and body fluids [16–18]. The carbonyl content in individual proteins is assessed by one-dimensional (1D) or two-dimensional (2D) sodium dodecyl sulfate (SDS) gel electrophoresis followed by Western blot immunoassay (oxyblot). These two methods have significantly more sensitivity

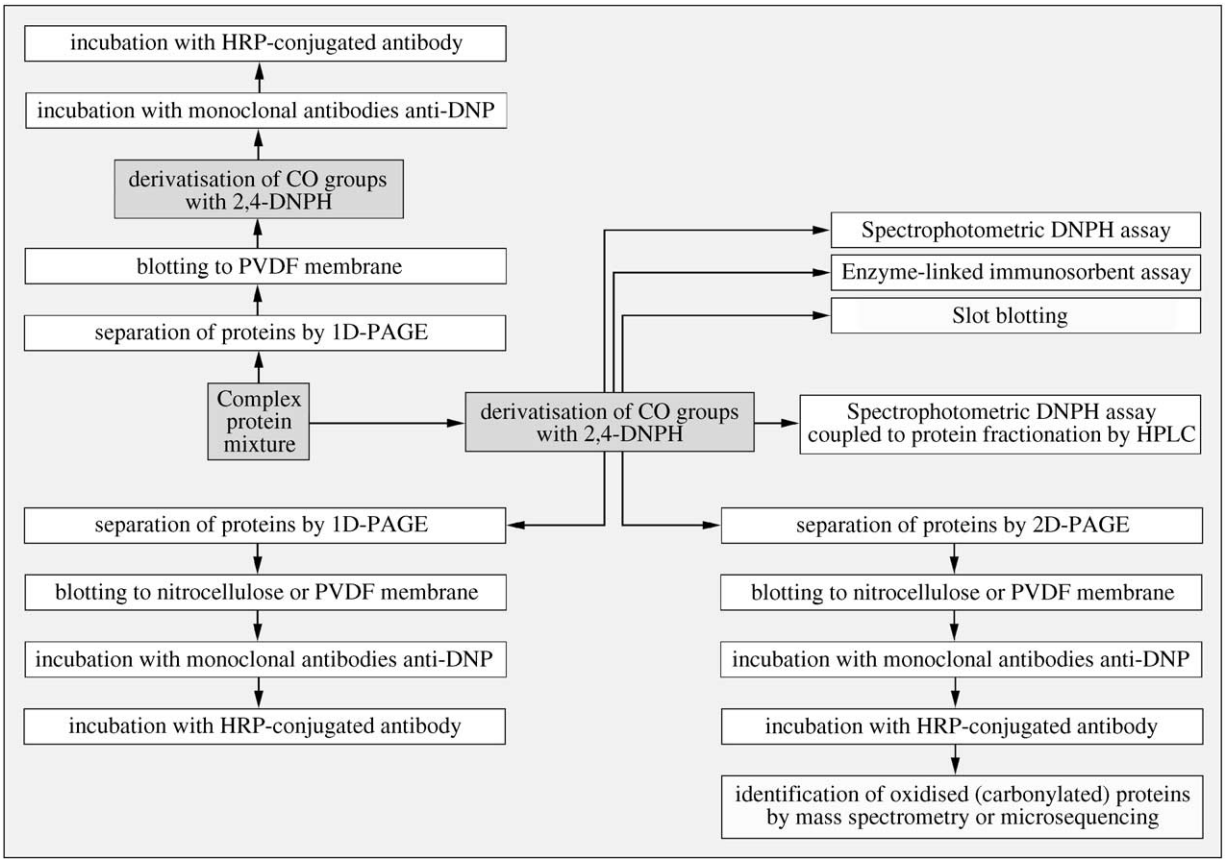

Fig. 2. Assays for detection of protein carbonyl (CO) groups involving derivatisation with 2,4-dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl (DNP) hydrazone product.

and specificity than all other total carbonyl assays, but they are only semiquantitative.

Many high-quality, both polyclonal and monoclonal, anti-DNP antibodies are available commercially, for example, from Sigma (St. Louis, MO, USA), Intergen (Purchase, NY, USA), and Oncor (Gaithersburg, MD, USA). In our laboratory, we employ an anti-DNP-KLH antibody, rabbit IgG fraction, marketed from Molecular Probes (Eugene, OR, USA).

2.1. Spectrophotometric DNPH assay

The classical approach to the detection of protein carbonyl groups involves their reaction with DNPH followed by the spectrophotometric quantification of the acid hydrazones at 370 nm [12,15]. Spectrophotometric DNPH method is useful to quantify carbonyl content in mixture of proteins, such as plasma, tissue homogenates, cellular extracts, or in isolated proteins. In general, protein concentration should be no greater than 5 mg/ml.

The quantitative derivatisation requires that a large excess of reagent be present, and that the reagent must be removed to allow spectrophotometric determination of the protein-bound hydrazone. This assay is unreliable for quantitating carbonyl content in protein extracts that contain high amounts of chromophore that absorbs at 370 nm (e.g., haemoglobin, myoglobin, retinoids). Several improvements to the spectrophotometric assay are described, which allow for the quantitative determination of carbonyl content in a variety of tissue types [19].

Nucleic acids also contain carbonyl groups and will react with DNPH. If not removed, contaminating

nucleic acids may thus cause an erroneously high estimate of protein-bound carbonyl, then tissue homogenates and cellular extracts should be treated to decrease nucleic acid contamination [12]. Precipitation of the nucleic acids with 1% streptomycin sulfate (stock solution, 10%, in 50 mM HEPES, pH 7.2) is usually effective in minimizing interference of nucleic acids. In general, interference will be minimized when the nucleic acid is reduced to the point at which the ratio of absorbance at 280 nm to that at 260 nm is greater than 1.

Spectrophotometric DNPH assay has been shown to be very sensitive for the determination of carbonyl content in purified proteins [20]. Here we describe the detailed procedure for the colorimetric assay used in our laboratory for the quantification of carbonyl content in human serum proteins [21] or in isolated proteins [20].

The serum is diluted 1:40 with phosphate-buffered saline (PBS) containing 10 mM sodium phosphate, pH 7.4, and 0.14 M NaCl, and this was centrifuged twice (5–10 min at 14,000 rpm in a tabletop microcentrifuge) to eliminate all particulate matter that might interfere with the reaction. The diluted proteins are precipitated with cold trichloroacetic acid (TCA, 20% final concentration) and then collected by centrifugation for 3–5 min. A solution of 10 mM DNPH in 2 N HCl is added to the protein pellet of each sample to give a final protein concentration of 1–2 mg/ml, with 2 N HCl only added to corresponding sample aliquot reagent blanks. Samples are allowed to stand in the dark at room temperature for 1 h with vortexing every 10 min; they are then precipitated with 10–20% TCA (final concentration) and centrifuged for 5 min. The supernatants are discarded, the protein pellets are washed once more with 10–20% TCA, and then washed three times with 1 ml portions of ethanol/ethyl acetate (1:1, v/v) to remove any free DNPH. Samples are then resuspended in 6 M guanidine hydrochloride (GdmCl, dissolved in 2 N HCl or in 20 mM phosphate buffer, pH 2.3) at 37 °C for 15 min with vortex mixing. Carbonyl contents are determined from the absorbance at 366 nm using a molar absorption coefficient of 22,000 M^{-1} cm^{-1} [15]. As an alternative to acid precipitation in some cases, we lyophilise protein samples in a vacuum centrifuge (Savant).

Nonspecific absorbance, mainly due to free DNPH retained after washing, can be estimated by measuring the absorbance of a sample fully reduced with 20 mM sodium borohydride ($NaBH_4$). Protein loss during washing also needs to be taken into account [22]. Standard curves can be constructed by mixing HOCl-oxidised BSA and BSA fully reduced with $NaBH_4$ in differing proportions to maintain a constant total protein concentration [23].

The spectrophotometric DNPH assay was used for quantification of protein CO groups, as marker of oxidative damage, in postmortem brain tissue of patients with Alzheimer's disease [24] or with amyotrophic lateral sclerosis [25], in plasma and in the subretinal fluid of diabetic patients [26–29], in plasma and in synovial fluid of patients with different forms of arthritis [30,31], and in blood samples of patients with cystic fibrosis [32].

While this method has provided much valuable data on the correlation of carbonyl formation with oxidative stress and does not require any expensive or specialized equipment, it has the disadvantages that does not provide any information on the extent of oxidative damage to a particular protein in a complex mixture like plasma, tissue homogenates, or cellular extracts; it requires more protein than may be available from clinical samples; it is labor intensive; and washing steps can give rise to variability [10,22].

2.2. Spectrophotometric DNPH assay coupled to protein fractionation by HPLC

The spectrophotometric detection of DNP-carbonyl derivatives after separation of the proteins by gel-filtration HPLC [15,33] could provide information on the extent of oxidative damage to a particular protein in a complex mixture like tissue homogenate or cellular extracts. The reaction with DNP followed by HPLC fractionation has been applied to quantify the oxidative modification of proteins in pulmonary fluid obtained during routine suctioning of neonates receiving ventilation [33]. The amount of oxidatively modified protein(s) may provide a quantitative assessment of oxygen toxicity and of pulmonary antioxidant defences.

Gel-filtration by HPLC has proved to be a convenient and efficient technique in which DNP-carbonyl derivatised proteins are separated by molecular

weight, allowing a more specific analysis of carbonyl content. HPLC spectrophotometric detectors are also far more sensitive than stand-alone spectrophotometers; so much less sample is required for quantitation of carbonyl content. HPLC method has sufficient sensitivity for analysis of cellular extracts from a single tissue culture dish or for analysis of the small amounts of tissue available from biopsy and autopsy samples. As most HPLC detectors provide chromatograms at two or more wavelengths, protein CO content can be followed at 366–370 nm, and protein content by absorbance either at 280 nm, which is suitable for proteins with aromatic amino acids, or at ~210–215 nm, which detects the peptide bond, obviating the need for a separate protein assay. The total carbonyl content of a protein may be calculated following integration of the chromatograms to obtain the area of the protein peaks at 276–280 and 366–370 nm and using molar extinction coefficient for the hydrazone of 9460 and 22,000 M^{-1} cm^{-1} at 276 and 370 nm, respectively (for a detailed step-by-step procedure, see Ref. [15]). If a diode-array detector is available in the HPLC detection system, then one also obtains full spectra of the peaks. These can be useful in checking for contaminants, which might artifactually affect either CO group or protein determination. For example, it should be possible to simultaneously monitor both at 366 and at 260 nm, thereby providing a relative measurement of both the protein-bound hydrazone and the interference of nucleic acid, respectively.

Derivatisation with DNPH is typically developed in solutions of 2 M HCl. There are two disadvantages to use of 2 M HCl in preparing samples for HPLC analysis. First, very few HPLC columns can tolerate the HCl. Second, many proteins are insoluble in the HCl and must be solubilised before injection into the HPLC system. These problems are dealt with by derivatisation in 6 M GdmCl at pH 2.5. GdmCl effectively solubilises most proteins, while pH 2.5 is compatible with most HPLC columns.

In conclusion, spectrophotometric DNPH assay coupled to protein fractionation by HPLC provides more quantitative information than the simple spectrophotometric assay, but it has the disadvantage that protein mixture fractionation by HPLC cannot completely separate proteins of close molecular weights. On the other hand, it is a highly sensitive technique for the quantitation of protein oxidative damage, especially for investigating purified proteins, but is less useful in protein mixtures due to problems with resolution [34].

2.3. Enzyme-linked immunosorbent assay

Buss et al. [23,35] and Winterbourn and Buss [36] have developed an enzyme-linked immunosorbent assay (ELISA) method using an anti-DNP antibody for measuring total protein CO groups that is highly sensitive, reproducible, and correlates directly with the classical colorimetric assay. Protein samples are reacted with 10 mM DNPH (in 6 M GdmCl, 0.5 M potassium phosphate buffer) to give a final protein concentration of ~ 1 mg/ml. After a 45-min incubation, with vortexing every 10 min, samples are nonspecifically adsorbed to wells of an ELISA plate. The adsorbed protein is reacted with a biotinylated anti-DNP antibody followed by streptavidin-biotinylated horseradish peroxidase. Absorbances are related to a standard curve prepared from a mixture of HOCl-oxidised and reduced serum albumin containing varying amounts of carbonyls, which are measured colorimetrically. Free DNPH and nonprotein constituents are easily washed away and give minimal interference. This allows much greater sensitivity and accuracy at the lower end of the range than for the spectrophotometric DNPH assay. In addition, the ELISA test is easier to use, less labor-intensive, and handles more samples per day than the colorimetric assay.

The ELISA test has also the important advantage that requires only microgram amounts (about 60 μg) of protein, which is similar to the requirement for the HPLC method [15], compared with the 10 mg required for optimal results with clinical samples using the colorimetric assay [37]. Therefore, ELISA should have wide application for measuring protein oxidation both experimentally and clinically in situations where only limited amounts of protein are available for analysis (e.g., with lung aspirates, where often there would be too little protein to perform the spectrophotometric assay).

Analyses performed for protein carbonyls by the ELISA method indicate that protein CO groups are elevated in tracheal aspirates from very low birth weight (<1500 g) infants [38], in BAL fluid from

newborn babies [39], and in plasma as well as bronchial aspirates in patients with severe sepsis [40]. In addition, by using the ELISA method to measure plasma protein CO groups in pre-eclamptic women, it was suggested that oxygen-free radical damage occurs in normal pregnancy and to a much higher extent in pre-eclampsia [41,42]. The ELISA test was also used to evaluate serum protein carbonyl content as a parameter of oxidative stress in patients during coronary heart surgery [43] and plasma protein targets of oxidative stress in uremic subjects with chronic renal failure [44].

An excellent kit for ELISA analysis (Zentech PC test) is available from Zenith Technology (Dunedin, New Zealand).

The drawback of the ELISA test is that it requires somewhat expensive and specialized equipment and, like the spectrophotometric assay, does not provide any information on the extent of oxidative damage to a particular protein in a complex mixture.

2.4. Slot blotting

Taking advantage of the potential sensitivity and specificity afforded by the immunochemical approach, Robinson et al. [18] published a sensitive and quantitative solid-phase immunoassay for the determination of total protein carbonyls in small tissue samples and cell culture extracts. The method is based on a combination of DNPH derivatisation, the preparation of blanks by treatment with 20 mM $NaBH_4$, and immunological detection. Because $NaBH_4$ reduces carbonyls to alcohols, such a treatment eliminates any immunostaining due to carbonyl groups. Thus, the difference between the staining intensities of a $NaBH_4$-treated and an untreated aliquot of the same sample constitutes a specific measure of the carbonyl content of the sample. Five-microgram protein samples are slot-blotted onto a polyvinylidene difluoride (PVDF) membrane using the slot blotter (Bio-Dot SF apparatus) from Bio-Rad Laboratories. Satisfactory results are also obtained with protein loading as low as 0.5 μg per slot, thus permitting an analysis to be carried out using the cells from a single well of a 96-well culture plate. Differently, protein loadings greater than 10 μg/slot give a definite plateau in immunostaining. PVDF membrane is then incubated with 0.1 mg/ml DNPH in 2 N HCl for 5 min, washed extensively in 2 N HCl (3 × 5 min) and 100% methanol (7 × 5 min) to remove free, unreacted DNPH, blocked with 5% (w/v) nonfat dry milk, and then treated with a primary anti-DNP antibody and a peroxidase-conjugated secondary antibody. Slot blot detection may be performed with chromogenic or chemiluminescent method.

The slot blot immunoassay has a theoretical minimum limit of detection of 60 pmol carbonyl/mg protein, being therefore approximately 1700-fold more sensitive than the standard DNPH-based spectrophotometric technique. Results by both methods are anyway highly correlated ($r = 0.932$, $p < 0.0001$). In addition, the minimum detection limit of this method is approximately 500-fold lower than that for carbonyl determinations by the HPLC method (based on the data reported in Ref. [15]) and of the same order of magnitude as that claimed for the detection of carbonyls by Western blotting with chemiluminescent detection reagents. Thus, the slot blotting method is well suited for measuring protein oxidation both experimentally and clinically, where only small amounts of protein can be obtained (e.g., tissue biopsies and cultured cells).

Other advantages of the slot blot immunoassay over the colorimetric technique are that the former does not require any expensive equipment and there is no loss of TCA-soluble proteins as it occurs during the washing of unbound DNPH from the protein samples in the spectrophotometric assay. In the immunochemical method, the total protein sample is blotted under vacuum and TCA-soluble proteins (usually low molecular weight) are included in the analysis. It should be noted that $NaBH_4$ treatment does not eliminate immunostaining by DNA, because DNA carbonyl groups are likely to be generated by incubation in HCl before and during the DNPH derivatisation step (i.e., after $NaBH_4$ treatment). However, the use of a detergent-free, hypotonic buffer in the preparation of supernatants from tissue and cellular samples (a condition known to limit the rupture of the nuclear envelop), as well as a 5-μg protein loading, eliminate the possibility of DNA interference with protein carbonyl determination. As a consequence, another advantage of this method over the colorimetric one is that the former does not require any pretreatment with streptomycin sulphate, thus avoiding the concomitant loss of protein.

The only disadvantage of this method is that it does not provide any information on the extent of oxidative damage to a particular protein in a complex mixture.

2.5. One-dimensional or two-dimensional electrophoresis and Western blot immunoassay

DNPH-derivatised proteins can be separated by molecular weight using polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), that is, one-dimensional electrophoresis (1-DE or 1D-PAGE), blotted to an adsorbent porous membrane (usually nitrocellulose or PVDF) and visualized by immunostaining with antibodies that recognise the DNP portion of the hydrazone.

It is obvious that the choice of gel to be used depends on the size of the proteins being studied. If proteins covering a wide range of molecular weight values need to be separated, then the use of a gradient gel is more appropriate. For example, plasma proteins can be conveniently separated on 4–12% gradient gels [17]. Because SDS-PAGE is based on the separation of proteins according to size, the method can also be used to determine the relative molecular mass of carbonylated proteins.

Nitrocellulose is widely used as adsorbent porous membrane. This is an efficient protein binder and does not require activation, but it is fragile and need careful handling. In the assay for the determination of protein carbonyl content, nitrocellulose membranes cannot be used when oxidised proteins are derivatised with DNPH after electroblotting, as they do not stand incubation in strong acids [2 N HCl or 5–10% trifluoroacetic acid (TFA)]. Therefore, nitrocellulose membranes may be used only when oxidised proteins are derivatised before SDS-PAGE and Western blotting [15,45–47]. Differently, PVDF membranes such as Immobilon-P (Millipore) are more robust and are used when DNPH-derivatisation is performed after Western blotting [48–51].

The Western blot assay can detect as little as 1 pmol carbonyl in a protein sample and requires as little as 50 ng protein oxidised to the extent of 0.3–0.5 mol carbonyl/mol protein. The sensitivity of the method for detecting minor oxidised proteins in a cell can be enhanced greatly by preparing subcellular fractions before derivatisation and performance of the Western blot immunoassay.

The Western blot immunoassay has the advantage of avoiding complications such as incomplete removal of the free DNPH before spectrophotometric measurement, as it detects only DNP groups conjugated to proteins. Small amounts of free DNPH, which may remain in a sample for electrophoresis, do not react with the anti-DNP antibodies even if they bind to transfer membranes. Furthermore, the carbohydrate groups in glycoproteins have no apparent effect in the assay.

If reaction of derivatisation is performed before the SDS-PAGE, proteins are reacted with DNPH in a solution containing 6% SDS/5% TFA/5 mM DNPH [DNPH/TFA stock solution: 20 mM DNPH in 20% (v/v) TFA]. Following incubation for 5–30 min at room temperature, the reaction mixture is neutralised with 2 M Tris base containing 30% (v/v) glycerol ± β-mercaptoethanol [17,52]. The sample should turn from yellow to orange on neutralisation. Samples are then subjected to SDS-PAGE, electroblotted to nitrocellulose or PVDF membranes, and immunoassayed for carbonyl content with anti-DNP antibody. Alternatively, the reaction of protein carbonyls with DNPH can be performed after SDS-PAGE and Western transfer. As an example, we report here the detailed procedure for the 1D Western blot immunoassay used in our laboratory to investigate the carbonyl content in protein mixtures or isolated proteins [51]. Protein samples are run in 1D-PAGE, blotted to a PVDF membrane, and sequentially incubated in 2 N HCl and DNPH (0.1 mg/ml in 2 N HCl) for 5 min each. The membrane is then washed three times in 2 N HCl and seven times in 100% methanol for 5 min each, followed by one wash in PBST (10 mM Na-phosphate, pH 7.2, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20) and blocking for 1 h in 5% (w/v) nonfat dried milk in PBST. After washing 3 × with PBST for 5 min each, immunological evaluation of carbonyl formation is performed for 2 h in 5% milk/PBST containing anti-DNP. As additional control, replicate blots can be incubated in 5% milk/PBST without the primary antibody to DNP, which is expected to prevent the appearance of carbonyl bands in the immunoblots. After three washes with PBST for 5 min each, the membrane is incubated with the secondary antibody linked to horseradish peroxidase (HRP) in PBST containing 5% milk for 1 h. After washing 3 × with PBST for 5 min each, development is performed with a chromogenic kit. To perform a blank analysis, a parallel set of oxidised samples could be prepared in buffer containing 20 mM $NaBH_4$ and incubated for 1 h.

One-dimensional electrophoresis (1D-PAGE) can resolve many protein mixtures, but complex biological samples require high-resolution two-dimensional electrophoresis (2D-PAGE) for critical protein identification [53–55]. However, although high-resolution 2D-PAGE can resolve virtually all proteins present in a complex protein mixture, like tissue homogenate or cellular extracts, it has been difficult to determine the carbonylated proteins because the DNP-derivatisation process alters the isoelectric points of proteins, and additional procedures must be utilized to remove reaction by-products. These additional procedures can lead to loss of sample, and poor isoelectric resolution on immobilized pH gradient (IPG) strips. Recently, Conrad et al. [56] and Choi et al. [57] have developed a method that allows the IPG strips to be derivatised with DNP directly following isoelectric focusing of the proteins. This procedure allows the visualization of oxidised proteins by 2D-PAGE with high reproducibility.

A commercial kit for the Western blot method (OxyBlot, Oxidised Protein Detection Kit) published by Shacter et al. [17] is currently marketed by Intergen.

1D-PAGE or 2D-PAGE, followed by Western immunoblotting, are actually widely used methods for the study of protein carbonylation in human disorders associated with oxidative stress. For instance, 1D-PAGE and Western blot immunoassay allowed to evidence the specific oxidation (carbonylation) of Clara cell secretory protein in the tracheal aspirate fluid of premature infants who develop bronchopulmonary dysplasia [58] as well as that albumin is the major plasma protein target of oxidant stress in patients with uremia [59].

2D-PAGE and subsequent Western blot immunoassay (2D immunoblotting) is actually the most commonly used method for the identification of oxidised proteins in Alzheimer's disease. Total CO groups in plasma proteins are increased and a 78-kDa protein appears to be specifically oxidised in subjects with AD. In addition, when plasma was subjected to increased oxidative stress in vitro, this protein from AD patients was more susceptible to increased oxidation than the corresponding protein from non-AD controls [46]. By Western blotting with anti-DNP antibody, followed by matrix-assisted laser desorption mass spectroscopy (MALDI-TOF/MS), it was showed that these carbonylated plasma proteins were isoforms of fibrinogen γ-chain precursor protein and of α-1-antitrypsin precursor [57]. Using 2D immunoblot, Aksenov et al. [55] demonstrated that specific protein carbonyl levels in β-actin and creatine kinase BB were significantly higher in brain extracts from AD patients than in control brain extract. Very recently, the same authors have presented the first proteomics approach to identify specifically carbonylated proteins in brain extracts from AD subjects, by coupling 2D fingerprinting with immunological detection of carbonyls and identification of proteins by mass spectrometry. This method has revealed the presence of other specific targets of protein oxidation in AD brain: glutamine syntase, ubiquitin carboxy-terminal hydrolase L-1, dihydropyrimidinase-related protein 2, α-enolase, and heat shock cognate 71 [60,61].

In conclusion, 1D and 2D oxyblots (term that refers to immunoblots of oxidised proteins) appear to provide better information than the spectrophotometric assay, slot immunoblotting, or ELISA about the oxidative status of proteins in a complex mixture like plasma, tissue homogenates, or cellular extracts, especially when 1D and 2D Western blot analyses are followed by microsequencing [62] or mass spectrometry [57,60,61] for the identification of carbonylated proteins.

3. Patho-physiological aspects of protein carbonylation

In the last decade, there has been a considerable growth in the number of papers reporting increased levels of protein carbonyls in various human diseases (Table 1) often correlating well with the progression of the disease, as well as of reviews summarising such results [1–3,7,9,63].

Two examples of human pathologies, in which patho-physiological aspects of protein carbonylation have been extensively investigated, are adult (or acute) respiratory distress syndrome (ARDS) and IBDs. In the plasma of patients with ARDS and patients at-risk for ARDS because they are undergoing cardiopulmonary bypass surgery, oxidative stress was concluded from the formation of lipid peroxidation products [64] and protein oxidation, measured as an increase in protein carbonyls and a decrease in protein thiol groups [37,64]. However, the situation in plasma does not necessarily reflect the situation in the lungs. Therefore,

a study investigated whether a pulmonary oxidant–antioxidant imbalance (i.e., an increased ROS production exceeding antioxidant defence capacities) is indicated by substantial oxidative modification of proteins in epithelial lining fluid recovered as BAL fluid [65]. Oxidatively modified proteins in BAL fluid, as measured by the reduction of protein carbonyl groups with tritiated borohydride, were studied in control subjects, patients with clinically established ARDS, and patients considered at-risk for ARDS because they had had coronary bypass surgery. Subsets of these at-risk patients were pretreated either with methylprednisolone (MP) or *N*-acetylcysteine (NAC). A great and moderate increase in the CO content of BAL fluid proteins was detected in ARDS patients and untreated patients at-risk for ARDS, respectively. Furthermore, the oxidative injury index indicating the extent of the oxidative modification of proteins on a mole/mole basis revealed that ARDS patients incurred, on average, five times the level of oxidation of the proteins of control subjects. The two other at-risk groups pretreated either with MP or NAC showed carbonyl values that were statistically not different from the controls. These results show that oxidatively modified proteins, functionally altered, either activated or crippled, clearly accumulated in BAL fluid of ARDS patients and to a minor extent in untreated at-risk patients. Consequently, relevant systems and functions in the respiratory tract such as the protease/antiprotease balance, surfactant, or mucus may become dysfunctional. On the basis of these results, it is proposed that the modulation of pulmonary protein functions by oxidation may be an important event in the pathogenesis of ARDS [65].

The IBDs (Crohn's disease, ulcerative colitis) are a collection of chronic idiopathic inflammatory disorders of the intestine and/or colon, characterized histologically by the presence of large numbers of leukocytes such as neutrophils, monocytes, and lymphocytes in the intestinal and/or colonic interstitium. Although the primary aetiology of IBD is multifactorial, gut leakiness and diffusion of intraluminal proinflammatory molecules (e.g., bacterial products) are considered to be reasonable initial steps for subsequent intestinal inflammation. The mechanisms of inflammatory intestinal injury, leading to mucosal barrier dysfunction, are not completely clear; however, it is known that chronic gut inflammation is associated with enhanced production of ROS/reactive nitrogen species (RNS) and protein oxidation, which may be responsible for mucosal barrier dysfunction [66]. Increased levels of ROS and protein carbonyls were found in the inflamed mucosal lesions of Crohn's disease and ulcerative colitis biopsies [67]. Using oxidant-induced hyperpermeability of monolayers of intestinal (Caco-2) cells as a model for the pathophysiology of IBD, Banan et al. [49,68] have shown that ROS cause inducible nitric oxide synthase up-regulation, NO overproduction, as well as carbonylation and nitration of the actin cytoskeleton, resulting in the disruption of the F-actin filaments. These data suggest that the underlying mechanism of intestinal barrier dysfunction induced by oxidants seen in disorders such as IBD may be due to the carbonylation, nitration, disassembly, and disruption of the actin cytoskeletal network. The suggestion that cytoskeletal instability in general and actin filament disruption in particular could be major contributing factors to the loss of barrier integrity is consistent with other studies on animal models showing the importance of cytoskeletal stability in gastrointestinal healing in vivo [69,70].

4. Conclusions

To investigate the role of oxidative/nitrosative stress and ROS/RNS in the pathogenesis and/or progression of human diseases, the use of appropriate biomarkers is necessary (Table 2). In determining whether to use lipids, DNA, or proteins as a marker of oxidative stress, the nature of the ROS will play a significant role. For example, HOCl, a major endogenous oxidising species derived from myeloperoxidase [71], induces formation of CO groups in proteins but causes little or no modification of DNA or lipids. Hence, when HOCl is the predominant ROS, proteins must be used as the marker. On the other hand, other ROS are more effective at inducing oxidative DNA damage or lipid peroxidation than they are at modifying proteins. However, products of protein side chain oxidative modification are relatively stable, and there are sensitive assays available for their detection; thus, from a purely technical perspective, they serve as suitable markers for oxidative stress.

Many mechanisms may induce protein oxidation, and potentially, all of the amino acyl side chains can

Table 2
Common biomarkers of oxidative stress used in the study of human diseases

DNA
Aldehyde/other base adducts
Nitrated/deaminated bases
Oxidised bases
Lipid
Chlorinated/nitrated lipids (isoprostanes, isoleukotrienes)
Oxysterols (aldehyde)
Peroxides (malondialdehyde, 4-hydroxy-2-nonenal, acrolein)
Protein
Aldehyde adducts
Carbonyl group formation
Nitrated/chlorinated Tyr, Trp, Phe
Oxidised Tyr, Trp, His, Met, Lys, Leu, Ileu, Val
Protein peroxides/hydroxides
SH (thiol) oxidation

become oxidatively modified. Therefore, there are numerous different types of protein oxidative modification and there is no single universal marker for protein oxidation. Some oxidative modifications are quite specific, both in the residue oxidised and the product generated; others can alter multiple residues and may give rise to several products. In the former case, the highly specific nature of protein oxidation constitutes one of the advantages of using protein oxidative modifications as markers of oxidative stress because it gives significant information as to the type of ROS or RNS involved in the oxidation process. For example, chlorotyrosyl moieties and amino acyl adducts on lysine residues (both of these oxidation products have been found in human atherosclerotic lesions) are specific markers of oxidation by HOCl, and hence reflect neutrophil and/or monocyte involvement in the oxidative stress. Similarly, the presence of 3-nitrotyrosine in proteins is thought to be a relatively specific marker of oxidative damage mediated by peroxynitrite [9]. Interestingly, increased concentrations of both protein carbonyls and 3-nitrotyrosine, two protein oxidative modifications frequently occurring concurrently, have been documented in normal ageing as well as in AD, Parkinson's disease, amyotrophic lateral sclerosis, IBD, and ARDS [9,68,72–75]. Another example of specific oxidation is the conversion of tyrosine to dityrosine. Whereas, carbonyl group introduction into side chain of Pro, Arg, Lys, and Thr is an example of a global modification, which can also arise from secondary reaction with the primary oxidation products such as 4-hydroxy-2-nonenal. Because so many different protein oxidation products can be formed, it may be necessary to set up several different assays to find the most appropriate assay for the type of oxidative stress involved. The choice of a specific or global assay may also depend on the purpose of the study being undertaken, and in some cases, it may be a useful marker for oxidative stress or damage. However, it should be considered that the specific modifications reported to date affect only a very little fraction of the "at-risk" residues or proteins, while the global modifications often affect a substantial fraction of the proteins or of the residues of a specific protein in the sample. For example, dityrosine is clearly increased in atherosclerotic regions of human aorta, with the highest level reported in early fatty streaks [76]. However, the actual content of dityrosine was 1 residue for each 3300 Tyr residues, and measurement of dityrosine levels required the development of a highly sensitive and quantitative method using stable isotope dilution gas chromatography–mass spectrometry [76]. In advanced human atherosclerotic plaques, dityrosine content was 150 pmol/mg protein, that is, 5 diTyr/10,000 Tyr [77]. In contrast, the protein CO content increases drastically under various pathological conditions of oxidative stress. For example, up to 8 nmol protein carbonyls/mg protein have been found in diseased brain samples [78]. Renke et al. [31] reported that the CO content in plasma proteins of children with different forms of juvenile chronic arthritis was significantly higher than in healthy group (1.36 ± 0.68 vs. 0.807 ± 0.16 nmol carbonyl/mg of protein, $p < 0.01$). It was also observed the important correlation between the analysed parameter level and the activity of the disease: the carbonyls increased parallel with the activity of the disease. Significant differences have been found in plasma protein CO groups between normal volunteers (0.76 ± 0.51 μmol/l), patients with chronic renal failure (13.73 ± 4.45 μmol/l, $p = 0.015$), and patients on chronic maintenance haemodialysis (16.95 ± 2.62 μmol/l, $p = 0.0001$) [44]. The carbonyl content of BAL fluid proteins was greatly increased in patients with clinically established ARDS (5.0 ± 1.3 nmol carbonyl/ml BAL fluid, $p = 0.0004$) and moderately increased in patients at-risk for ARDS because they had had coronary bypass surgery (1.3 ± 0.2 nmol/

ml, $p = 0.027$) compared with controls (0.8 ± 0.2 nmol/ml) [65]. Moreover, although there is a large number of potential oxidative modifications, only a few have been systematically studied, protein carbonyl groups being the most frequently studied.

The usage of protein CO groups as a marker may have some advantages in comparison with lipid peroxidation products because the formation of protein-bound CO groups seems to be a common phenomenon of protein oxidation and because of the relatively early formation and relative stability of oxidised proteins. It is known that cells degrade oxidised proteins within hours and days [79,80], whereas lipid peroxidation products are detoxified within minutes [81]. Interestingly, protein CO groups form early and are circulating for longer periods in the blood of patients compared with other parameters of oxidative stress, such as glutathione disulfide (GSSG) or the lipid peroxidation product malondialdehyde; their elevation in serum is stable for at least 4 h [43]. The chemical stability of protein carbonyls makes them suitable targets for laboratory measurement and is also useful for their storage: their stability on storage has been demonstrated for 3 months at -80 °C [82].

On the other hand, protein CO groups can be induced by almost all types of ROS, and hence, these do not shed significant light on the source of the oxidative stress. Furthermore, CO groups are relatively difficult to induce compared to Met sulfoxide and cysteinyl derivatives, and thus, these may be reflective of more severe cases of oxidative stress. Indeed, detection of elevated levels of protein carbonyls is generally a sign not only of oxidative stress but also of a protein dysfunction. An example of protein carbonylation followed by protein dysfunction is reported in our studies on isolated actin. A significant elevation of actin carbonyl content in neurons in the brain regions most severely affected by AD pathology ($442 \pm 23\%$ of control, $p < 0.01$, determined from the intensities of anti-DNP immunostaining—normalised to the specific protein content—of 2D oxyblots) [54], as well as in post-ischemic isolated rat hearts [83], has been reported. Actin carbonylation has been also determined in a human intestinal cell line following incubation with HOCl used as a model of oxidant insult [49]. Our studies on purified actin showed that oxidative modifications of the actin polypeptide chain can lead to protein functional alterations, including impaired interaction with other cytoskeletal proteins [84–86]. In particular, actin carbonylation is not only a simple marker of protein oxidation, but it is a sign of severe functional impairment associated with filament disruption [20].

One of the greatest challenges in oxidative stress research today is the identification of specific oxidised proteins in human diseases. Oxidative damage often leads to loss in specific protein function. Because proteins often have unique biological functions, there are often unique functional consequences resulting from their modification, for example, loss of clotting from oxidation of fibrinogen [87]. The relationship among protein oxidation, protein dysfunction, and diseases remain largely unclear; however, it is known that oxidative modification of enzymes and structural proteins may play a significant role in the aetiology of diseases. For instance, glutamine synthetase, which plays a crucial role in balancing the potentially excitotoxic effect of glutamate, and the activity of which is decreased in AD brain [88,89], has been identified as specifically oxidised enzyme in AD compared with control brain [60]. Furthermore, creatine kinase BB, which has decreased activity in AD brain [88], is specifically oxidised in AD brain as indicated by increased carbonyl content [54,60]. Oxidatively modified low-density lipoprotein (LDL) has been found in atherosclerotic tissues by several groups, lending strong support for the idea that oxidation of LDL has a significant role in the aetiology of atherosclerosis [76,90–92].

The current rapid progress in identification of specified oxidised proteins and elucidation of mechanisms of protein oxidation should provide further insight into the importance of oxidative stress in human diseases.

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