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Abstract

In physiological conditions intracellular radical formation is mostly due to mitochondrial activity. This is in contrast to clinical and pathophysiological situations, where the oxidant formation is additionally driven by xenobiotics and inflammation. Oxidative damage accumulation in macromolecules especially in proteins has been considered as a cause of cellular damage and pathology impairing the clinical outcome of patients. However, up to now strategies to measure oxidative stress in clinical settings are limited. A lot of parameters and techniques are available for the determination of oxidized proteins in biological systems. Unfortunately, most of them are no reliable markers in clinical settings due to their unknown clinical relevance or the lack in clinical feasibility. Major problems are the sample availability, sample stability and cost-, time- and man-power intensive methods. The present review focuses on the measurement of protein oxidation products from a chemical, biological, and medical point of view.

Formation of free radicals and oxidants: pros and cons

An inevitable byproduct of aerobic cellular metabolism is the continuous formation of free radicals and other highly reactive oxidants. Intracellular radical formation is mostly due to mitochondrial activity, releasing large amounts of superoxide anion, resulting from electron leakage of the respiratory chain (for review see Grune et al., 2002). But there are several other cellular systems able to release reactive species. Among them are the peroxisomes, generating hydrogen peroxide as a byproduct, the xanthine oxidase, releasing superoxide anions, and the oxidative burst of macrophages while responding to infections. Additionally, the three enzymes monoamine oxidase, tyrosine hydrolase, and L-amino acid oxidase release hydrogen peroxide during their normal activity. Further, the four nitric oxide synthetases (NOS) are able to produce the nitric oxide radical, which might react with superoxide forming the highly reactive peroxynitrite.

Free radicals and oxidants are often highly reactive and short-lived. Extensive damage is caused by the formation of secondary reactive radicals. For example hydroxyl radicals are generated in the Haber Weiss reaction by the reaction of hydrogen peroxide and superoxide. This reaction can be accelerated by the presence of metals, whereas the metals serve as catalysts. This reaction is known as the Fenton reaction. In subsequent oxidative reactions the target molecules like lipids, proteins, nucleic acids and carbohydrates will be modified, probably leading to a loss or impairment of their biological function. An overproduction of ROS has the potential to disturb the redox balance of a cell leading to the activation of redox sensitive signal pathways and inducing changes in gene expression (Suzuki et al., 1997).

Besides their “damaging” potential ROS play an important role in living organisms. A continuous “natural” production of ROS by some systems within the body is

considered to be physiological and has beneficial effects (Valko et al., 2007). As an example, ROS are generated by cells of the immune system to be involved in the defense of penetrating microorganisms. On the other hand it is known that superoxide anion, hydrogen peroxide and nitric oxide formation are essential for normal cellular function since they constitute a second messenger system involved in the modulation of cellular redox poise, gene expression and cell differentiation (Linnane et al., 2007).

Under physiological conditions the antioxidant defense system within the body can easily handle the amount of free radicals produced. There are three cellular antioxidative defense lines. The first one contains low molecular antioxidants like glutathione, vitamin C or uric acid. These molecules are able to scavenge free radicals/reactive species and thus less reactive compounds are formed. Enzymatic antioxidants, building the second line, are proteins that convert highly reactive particles into less reactive ones. Superoxide dismutase, catalase, transferrin, and glutathione peroxidase belong to the second defense line. Iron and iron-binding proteins play a special role due to the potential of iron (like all transition metals) to be involved in the Fenton-reaction.

Enzymes involved in DNA repair, in membrane repair, and in degradation of lipid peroxidation products belong to the third line of defense. These enzymes are able to repair oxidative damage inflicted to cellular structures. During times of increased oxygen flux or in individual exogenous conditions (diet, environment, smoking and others) free radical production may exceed that of removal ultimately resulting in the oxidation of cellular structures. This situation, where the amount of reactive species in a particular system overwhelms the antioxidative repair capacities, is called oxidative stress (Sies et al., 1986). During periods of oxidative stress oxidative damage may be potentiated increasing the amount of irreducible oxidized molecules.

Oxidative stress has been implicated as a major causative agent of cellular dysfunction and cell death. On the other hand, repeated or permanent exposure to sublethal doses of oxidative stress leads to cellular adaption (Davies et al., 1995; Siems et al., 1994; Vile et al., 1994). In this context, transient adaption depends upon transcription and translation shown by an altered expression of genes during the adaptive response. Interestingly, the development of a resistance against the used oxidant is limited if either transcription or translation is inhibited during the adaptive response (Davies et al., 2005). It seems possible that an `oversupply` with antioxidants can block the adaptive response of the organism abrogating health-promoting effects. Ristow et al. (2009) have shown that ROS are required for the insulin-sensitizing capabilities of physical exercise, whereas this effect was blocked by a supplementation with vitamin C and E.

Measurement of oxidative stress and resulting problems

In theory, the detection of free radicals seems to be a reliable method for the quantification of oxidative stress. A direct determination of free radicals can be achieved by using the Electron Spin Resonance (ESR) method, allowing a sensitive detection of unpaired electrons and paramagnetic substances. Oxidative stress can also be quantified by the degradation or the consumption of substrates, which are potentially involved in radical reactions. For example a declining concentration of non-oxidized membrane lipids might be used as a measure for an ongoing lipid peroxidation. A decrease in the concentrations of antioxidants as well as antioxidative enzymes in cells or tissues might be a further consequence (or cause) of oxidative stress. Therefore, oxidative stress can be assessed by the analysis of the activity and the function of antioxidative defense systems (f.e. antioxidants, antioxidant enzymes, and essential mineral nutrients). Although a large battery of

highly sensitive, specific and reproducible methods for the determination of antioxidants are available, a few methodological disadvantages exist. The primary problem is the stability of antioxidants from the time point of the sample collection over sample storage to the time point of the analysis. The stabilization of samples, which are aimed for vitamin C analysis with metaphosphoric acid, is a possible prevention.

The determination of oxidized products in biological systems exhibits by far the most commonly used method for the measurement of oxidative stress. Depending on the site of generation of oxidative stress, a number of endogenous formed substances are considered to be markers or indicators of oxidative stress. A multitude of potential biomarkers including lipid peroxidation products, protein oxidation products, and DNA oxidation products have been pronounced in the literature [Irshad et al., 2002]. The present review article is aimed to focus on the measurement of protein oxidation products from a chemical, biological, and medical point of view.

Protein oxidation products from a chemical point of view

Proteins can be oxidatively modified by a variety of free radicals and oxidants. As a result, many different amino acid modifications can occur [Stadtman, 1993; Naskalski and Bartosz, 2000] leading to damaged proteins with changed, reduced, or vanished catalytic activity. It can be distinguished between changes that concern single amino acid residues and those leading to fragmentation of polypeptide chains or covalent cross-linking of two amino acids either of the same or of two different proteins (Voss and Grune, 2006). Oxidative protein modifications might be differentiated into three stages (Jung et al., 2009): proteins in stage I are characterized by a slight oxidation rate, whereas proteins in stage II are additionally defolded. The third stage is the result of long term accumulation of oxidatively modified protein and further chronic

oxidation. At this stage proteins might be covalently cross-linked to other proteins in similar stages of modification. Further, by the presence of lipid peroxidation products like malondialdehyde or hydroxynonenal the formation of these aggregates, termed lipofuscin, can be driven.

The sulfur-containing amino acids methionine and cysteine are most susceptible to oxidative modifications (Levine et al., 1999). Inter- and intramolecular disulfide bonds as well as the formation of sulfenic acid, sulfinic acid and sulfonic acid are possible implications of an interaction between ROS and cysteine (Voss and Grune, 2006). The ROS action with methionine results in methionine sulfoxide, which can further be oxidized to methionine sulfone. Additionally, primary oxidation products of aliphatic amino acids are for example 2-oxo-histidine (from histidine), glyoxylic acid (from aspartate), glutamic semialdehyde (from arginine or proline) and 3- hydroxyleucin, 4- hydroxyleucin and 5- hydroxyleucin (from leucine). Protein-bound carbonyls are the remaining structures if aliphatic side chains of amino acid residues are removed via β -scission. Therefore, protein carbonyls are suited to be a reliable biomarker of an ongoing protein oxidation.

Aromatic side chains are further important targets of oxidative damage, resulting in primary products like 2-oxo-histidine (from histidine), 3,4-dihydroxyphenylalanine (from tyrosine) or further modifications like dityrosine and kynurenine (Voss and Grune, 2006). Besides these ROS reactions on the amino acid residues, peptide backbone might be another target of ROS resulting either in fragmentation of the protein or aggregation due to polymerization. It has to be mentioned that the described modifications are mainly due to the action of ROS and that a large number of further amino acid derivatives might also be formed by the action of reactive species derived from nitrogen monoxide or hypochloric acid (for details see Voss and Grune, 2006).

Considering the multiple adducts of possible protein damage it is a major challenge to establish techniques, especially for the quantification of small amounts of oxidized proteins. The literature provides us with a large number of examples of which methods are useful for the detection of oxidation-induced protein modifications. Among them, fluorescence detection of peroxynitrite-induced modifications of glyceraldehydes-3-phosphate (Buchczyk et al., 2003), electrophoresis of irradiated GAPDH (Voss et al., 2007) and the quantification of reduced SH-groups by DTNB (Voss et al., 2008) may serve as selected examples. More specific methods to detect several amino acid modifications are chromatographic and mass spectrometric methods (for review see Tsikas and Caidahl, 2005; Requena et al. 2003). Most of these methods have the additional potential to determine protein glycation and nitration adduct residues (Thornalley, 2006). Proteomics is another technical approach to investigate the extent of oxidative damage occurring at the protein level offering a rapid means of identifying oxidatively modified proteins without the limitations of immunochemical detection methods. Recent advances in proteomics research made possible the identification of novel biomarkers and therapeutic targets in several human diseases (for review on proteomics see Dalle-Donne et al., 2006).

Protein oxidation products from a biological point of view

The nature of the oxidant in the used stress model or individual pathophysiological situation determines the types (the diversity) of oxidative protein products and modifications arising. An accumulation of oxidized proteins and resulting secondary products like protein aggregates and protein cross-links is reported in a multitude of diseases, like atherosclerosis, diabetes, Alzheimer's disease, Parkinson's disease, hepatitis, and rheumatic arthritis. Oxidative damage to a protein can result in a loss of function. But this effect does not automatically have a deleterious consequence for

the whole cell. It has been shown that key enzymes of the glycolytic pathway are inactivated under oxidative stress, whereas antioxidant responses are driven by the preferential supply with NADPH from the pentose phosphate pathway (Cabiscol and Ros, 2006).

Two factors are crucial for the qualification of oxidized proteins or oxidative stress-induced protein modifications as reliable biomarkers of oxidative protein oxidation: biological significance and technical or methodological feasibility. Regarding the biological significance it is important that the biomarker is specific, which means that the formation of the biomarker is mainly due to ongoing oxidative processes and not to other endogenous processes or to an oral uptake. Therefore, the use of a biomarker in biological systems should require the exact knowledge of the routes of formation. Further the biomarker should be formed in the organism in high yields to allow a successful analytical detection and should show a strong correlation/dependence to the extent of (induced) oxidative stress. Despite the usefulness of ortho-tyrosine and dityrosine as indicators of oxidative damage to model proteins and the evidence for age-dependent oxidative changes, it was pointed out that both substances are not useful as indicators for assessing oxidative damage to lens proteins with age (Wells-Knecht et al., 1993).

Although a certain endogenous degradation or repair of oxidative protein modifications is inevitable, it is pivotal for the presence/existence of high circulating levels that the degradation as well as the repair is much lower than the formation of the oxidative protein modification. Since most compounds suitable as biomarkers are not metabolically stable, a successful determination is dependent on the quality of the sampling process. A time-dependent decline in the protein carbonyl content was demonstrated by Jung et al. (2006) after inducing protein oxidation by a H_2O_2 -treatment. This emphasizes the relevance of choosing a meaningful sampling time. It

was recently shown that an overexpression of the repair enzyme methionine sulfoxide reductase 2 noticeably prevents the formation of irreversibly oxidized proteins that accumulate upon oxidative stress by maintaining a low level of intracellular ROS (Cabreiro et al., 2008).

Methods for the determination of oxidative proteins should be specific and sensitive. A useful marker of protein oxidation should also be characterized by a high reproducibility. Biological and chemical sample stability is one important factor in this context. Ideally the concentration of the marker is stable over the time from sample drawing until the measurement and freezing and thawing are not significantly changing the readout. Unfortunately, protein oxidation can also take place as an experimental artifact during the analytical process as well as by oxygen in the air, so that it may be difficult to distinguish between the actual and the artificial level of oxidation. Besides methodological influence, the formation of artefacts during sample preparation, sample analysis, and sample storage is discussed to be the main reason for the existence of a low reproducibility.

The formation of protein carbonyls is orders of magnitude greater than other oxidative modifications making them to be the most widely used marker of protein oxidation (Stadtman and Levine, 2006). Furthermore, a wide spectrum of methods for the determination of protein carbonyls like colorimetry, ELISA, immunoblotting and immunohistochemistry are available, making the determination of this marker widespread and easy to establish in laboratories. However, since these methods are not very specific and protein carbonyls are partially degraded, its use as a marker of protein oxidation is derogated from a biological point of view. 3-nitrotyrosin is another specific marker of oxidative protein damage which is caused by peroxynitrite or other reactive nitrogen species interacting with protein structures (Jung et al. 2007). In contrast to protein carbonyls, only a few methods are available for its determination.

Protein oxidation products from a medical point of view

In physiological conditions mitochondria produce a large part of the cellular oxidants. This is in contrast to clinical and pathophysiological situations, where also other pathways like a xenobiotic and inflammation-driven oxidant production may significantly contribute (Grune and Berger, 2007). Since it is clearly shown that oxidative stress is deleterious in critically ill patients and associated with poor outcome (Motoyama et al., 2003), the measurement of oxidative stress is of high clinical relevance. Although numerous parameters have been proposed and used for the determination of oxidative stress in clinical situations, their use is still limited, mostly due to a lack of clinical feasibility. As a consequence, studies on oxidative stress in acute and chronic diseases are still performed in tight collaboration with research-oriented laboratories.

It is discussed that every third protein in a cell of older animals is dysfunctional as enzyme or structural protein due to oxidative damage (Poon et al., 2004). Therefore, for the prediction of the aging process and age-related diseases the measurement of the protein oxidation is clinically an important tool. To study the contribution of oxidative stress to the clinical outcome, strategies have to be found to measure markers of oxidative stress, especially protein oxidation in clinical routine analysis.

There are several factors necessary to consider a parameter of protein oxidation as a useful biomarker in clinical settings. First, the biomarker should be able to estimate the individual profile of protein oxidation and should have clinical relevance. The latter is difficult to realize since the functional relevance of protein oxidation in most pathophysiological situations but also in aging processes is only marginally or not known.

Further, it has to be recognized that the determination of a biomarker is mostly a snap-shot. Even so, by means of this singular event the biomarker should fulfil the above mentioned criteria. A reliable and clinically useful biomarker should further be distinguished by a high specificity, accuracy and reproducibility. These factors are mostly influenced by the quality of the sample preparation and stability. The assessment of protein oxidation products is especially hampered by factors like the sample stability since compounds of protein oxidation are very sensitive against autoxidation. Avoiding autoxidation in routine analysis is difficult since a quick stabilization of the samples does mostly not fit with the clinical 'reality' and feasibility. Another problem is that the determination of a lot of biological relevant biomarkers may only be managed by cost-, time- and man power intensive methods. Especially methods based on high-performance liquid chromatography (HPLC) separation and gas-chromatography based methods are time consuming and expensive and therefore hardly applicable in clinical routine analysis. Though, these methods are characterized by a high specificity and sensitivity, especially in the measurement of protein oxidation, making them to the method of choice in research-based laboratories. The measurement of protein carbonyls by antibody-based and spectroscopic methods seems to be an acceptable compromise in clinical settings, although it has limited relevance so far.

Besides the problems resulting from the methods itself (specificity, standardization, accuracy, reproducibility), another problem is the lack of available reference values for protein oxidation parameters. In contrast, reference values for plasma concentrations of HNE in healthy humans are available (Gil et al., 2006). By the use of validated reference values it would be possible to characterize the individual risk as well as to make inter-study comparisons. The lack of available reference values results mainly from the multitude of available methods for the determination of one

parameter (for example protein carbonyls) facing huge differences in data reproduction. Further, the neglected use of those methods in clinical studies so far slows the establishment of reference values additionally.

Sample availability is a further important factor which may limit the classification of a biomarker as reliable. Whereas the collection of blood is mostly unproblematic in adults, even small blood volumes of newborns and children may limit the sample availability. Therefore non-invasive methods are appreciated and deliver an advantage over more invasive methods. Examples for non-invasive methods for the measurement of protein oxidation are the determination of modified amino acids in urine and the determination of the skin fluorescence. The collection of buccal mucosa cells is another non-invasive method eligible to be used for the assessment of protein oxidation in a tissue with a relatively short turnover time.

A lot of parameters eligible from a biological point of view, are only limitedly applicable in clinical settings. A few parameters and techniques are only suitable in experimental models due to highly complex techniques (spin traps, mass spectrometry based methods), to a very short half-life or to their impact by the cellular metabolism. For instance, the blood glutathione status is strongly determined by metabolic influences of the red blood cell and by transport processes of reduced and oxidized glutathione within different tissues (Pantke et al., 1999).

It has been demonstrated in a few clinical settings that protein carbonyls reflect the degree of oxidative stress in a sufficient way. Furthermore, protein carbonyl concentrations in healthy humans related to the individual age have been reported (Gil et al., 2006). From further studies it was concluded that the extent of protein oxidation, assessed by protein carbonyls, correlates well with other markers for oxidative stress. Pantke et al. (1999) have shown that a 2- to 3-fold increase of

protein carbonyls in the reperfusion period during cardiovascular surgery correlates strongly with other markers of oxidative stress.

It can be summarized that there are some good parameters of protein oxidation available from a biological point of view. Biological significance of a biomarker results from its chemical stability, its formation in high yields, its biological stability and its specificity for protein oxidation (Fig. 1). Biological significance is one of the main requirements a biomarker should have for its use in clinical settings. Furthermore, a good biomarker should be characterized by a high sample stability, methodological feasibility, clinical relevance, availability of reference values and a non-invasive sample collection method (Fig. 1). Unfortunately, up to now a few of these demands are not achieved by the available biomarkers explaining the limited use of protein oxidation markers in clinical settings. Therefore, future research should focus on strategies to solve the problem in order (i) to understand the role of oxidative stress in clinical situations and (ii) to improve the clinical outcome of the patients.

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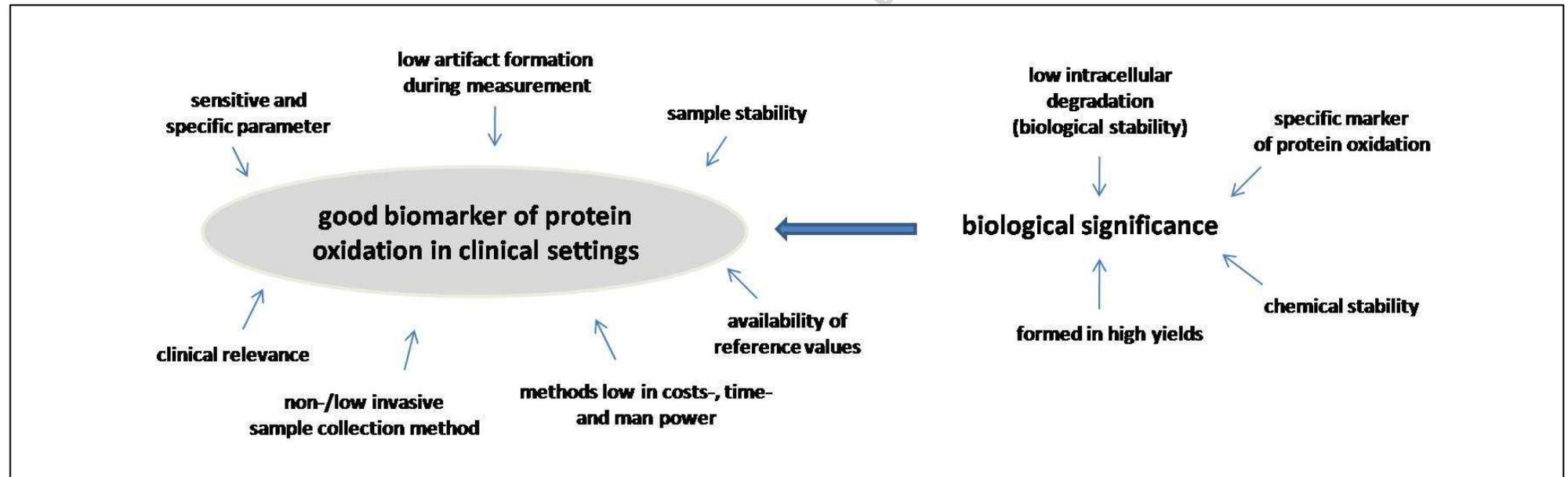


Fig. 1 Demands for biomarkers of protein oxidation in clinical settings