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Oxidative stress assays for disease risk stratification

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Despite the fact that oxidative stress is a significant aetiological factor in several degenerative diseases, its measurement is rarely a part of »routine analyses« performed in hospital clinical chemistry laboratories. This situation is likely to change, as interest in this topic is increasing rapidly. Here we review the pertinent literature, assess assays for oxidative stress, and categorize them under: *(i)* assays for monitoring lipid peroxidation, *(ii)* assays for measuring oxidized amino acids, *(iii)* assays for measuring oxidized nucleic acids, *(iv)* assays based on physicochemical and immunological properties of oxidized low-density lipoprotein, and *(v)* assays for measuring the antioxidant capacity of body fluids and tissues. Our overview should be of help when choosing appropriate laboratory assays for oxidative stress and for routine disease risk stratification.

Keywords: oxidative stress, assay, disease risk stratification

INTRODUCTION

Oxidative stress is a term denoting imbalance between the production of oxidants and the related defence systems of an organism (1). Oxygen, though essential to life, is the major source of oxidants (2). Due to its electronic structure, it favours its reduction by stepwise addition of one electron at a time, leading to generation of the highly reactive oxygen radicals, superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$). A radical is any chemical species with one or more unpaired electrons in outer orbital, which can initiate chain reactions by removal of an electron from another biological molecule, such as unsaturated lipids, carbohydrates, amino acids or nucleic acids, in order to complete its own orbital. The third molecule generated from stepwise reduction of O_2 to H_2O is hydrogen peroxide (H_2O_2), a relatively unreactive compound that diffuses easily across cell membranes. However, in the presence of reduced metal ions (Fe^{2+}), it is decomposed, in the Fenton reaction, to $^{\bullet}OH$, one of the most aggressive oxidants that can cause damage to all major classes of macromolecules in cells. Free transition metal ions ($Fe^{2+/3+}$, $Cu^{1+/2+}$) have been claimed to play a crucial role in transforming various non-radical,

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rather unreactive oxidant species into radicals. Thus, hydroperoxides (ROOH), generated for example in lipid peroxidation, are decomposed to peroxyl ($ROO^{\bullet}$) or alkoxyl ($RO^{\bullet}$) radicals. In addition, oxidant species may interact with each other, yielding more potent oxidants. For instance, nitric oxide ($NO^{\bullet}$), a rather weak reducing agent, in the presence of $O_2^{\bullet -}$ forms the strongly oxidizing peroxynitrite ($ONOO^-$), further decomposing at acid pH to release small amounts of $^{\bullet}OH$ independently of metal catalysis. Hypochlorous acid (HOCl), a powerful oxidant by itself, may react with $O_2^{\bullet -}$ or can be reduced by Fe^{2+} yielding $^{\bullet}OH$. In the Haber-Weiss reaction, H_2O_2 reacts with $O_2^{\bullet -}$ yielding $^{\bullet}OH$. Last but not least, oxidants are not necessarily oxygen derivatives. Thiols, for instance, are readily reduced to thiyl radicals, which may react with various biomolecules, at the same time yielding also $O_2^{\bullet -}$ and reduced transition metal ions.

Major intracellular and extracellular sources of reactive oxidant species are: *(i)* leakage of electrons from the mitochondrial electron transport chain, *e.g.*, from ubiquinol to O_2, *(ii)* peroxisomal oxidation of fatty acids and other compounds by transfer of two electrons from reduced flavine adenine dinucleotide ($FADH_2$) to O_2, *(iii)* cytochrome P450 mediated oxidation of various compounds, chemicals, xenobiotics and drugs in endoplasmic reticulum, *(iv)* host defence systems mediated by reduced nicotinamide adenine dinucleotide (NADPH) oxidase that produce $O_2^{\bullet -}$, and myeloperoxidase, leading to the formation of HOCl, *(v)* several other enzymes, *e.g.*, 15-lipooxygenase, prostaglandin synthase and xanthine oxidase, *(vi)* nitric oxide synthase yielding nitric oxide, *(vii)* thiols, *e.g.*, homocysteine and cysteine, *(viii)* hyperglycaemia caused by high glucose-driven superoxide formation and glycoxidation, and *(ix)* cosmic radiation.

Oxidants are highly reactive species with a half-life of only seconds. The only method for direct detection of radicals is the electron spin resonance (3). Alternatively, short-lived radicals are allowed to react with a trap molecule to produce a stable radical (4) or with a detector molecule to yield a stable product, which can be further evaluated (5). These methods require rigorous sampling procedures and reflect current exposure to oxidative stress species. In contrast, oxidation of lipids, carbohydrates, amino acids or nucleic acids yield profuse oxidation and degradation products with lifetimes ranging from hours to years (Fig. 1). Quantification of these species allows assessment of the prior existence of oxidative stress over short or long periods that have left their footprints in various biological molecules. Alternatively, oxidative stress can be assessed indirectly by measuring the antioxidant status in various body fluids and tissues, since extensive oxidative stress can be the cause of their deterioration.

Current evidence strongly suggests that oxidative stress constitutes a significant aetiological factor in ageing and in several degenerative diseases such as atherosclerosis, cancer, diabetes, chronic renal failure, ischaemia reperfusion injury and certain nervous system diseases. Despite this, the measurement of oxidative stress is rarely part of »routine analyses« performed in hospital clinical chemistry laboratories (6). The situation is likely to change in the future, as interest in this topic is increasing rapidly (7). Our aim is to provide a critical overview of the assays currently available for oxidative stress assessment. This should provide a useful basis for choosing appropriate oxidative stress assays for routine disease risk stratification.

ASSAYS FOR MONITORING LIPID PEROXIDATION

Oxidation products formed by the radical reaction with various biological molecules are frequently themselves radicals, which are in many cases able to propagate the reaction, leading to extensive damage. Polyunsaturated fatty acid peroxidation is an example of such a proliferation reaction where one radical can induce the oxidation of a comparatively large number of polyunsaturated fatty acids (PUFAs) in low density lipoprotein (LDL) and cell membranes, particularly in phospholipids and cholesteryl esters, which contain the bulk of PUFAs (8) (Fig. 1). Products formed during this process may serve as a direct and accurate index of the oxidative status of a biological system. Conjugated diene formation occurs first, due to hydrogen abstraction and molecular rearrangement, and then peroxyl radicals are formed after oxygen uptake, initiating an autocatalytic reaction that leads to the formation of hydroperoxides. In a metal-catalysed reaction, the fatty acid hydroperoxides subsequently form aldehydes, alkenals and hydroalkenals, which react with the nitrogen groups of lysine, arginine and histidine on numerous proteins, forming Schiff bases (9). In the case of LDL, these reactions increase the negative charge on apolipoprotein B (apo B). Concurrently with PUFAs, peroxidation of cholesterol yields oxidation products, which may also serve as an index of oxidative stress (Fig. 1).

Continuous monitoring of PUFA peroxidation in isolated LDL and body fluids in vitro

Continuous monitoring of copper-induced formation of conjugated dienes in isolated LDL by UV radiation absorption at 234 nm is widely accepted (10) (Fig. 1). The kinetic curve shows three phases: the lag phase (weak diene formation, linearly related to antioxidant content), the propagation phase (rapid diene formation, a measure of lipid oxidizability), and finally the decomposition phase. An assay measuring the baseline level of conjugated dienes in lipids extracted from precipitated LDL has been also proposed (11). The main drawbacks of these assays are the time-consuming isolation of LDL from plasma and/or the demanding extraction procedure, which increases the possibility of PUFA autoperoxidation *in vitro*. To overcome these drawbacks, assays involving continuous monitoring of dienes in plasma (12) or serum (13) have been proposed, but they lack specificity, even when a multi-wavelength approach is used for the differential diene monitoring.

PUFA peroxidation yields several chemiluminescent species, such as triplet carbonyl compounds and singlet oxygen. This has formed the basis for an assay involving continuous monitoring of the low-level chemiluminescence from oxidation of isolated LDL *in vitro* (14) (Fig. 1). The so-called lag time correlates very well with that from UV monitoring of dienes and the method avoids interference from UV-absorbing compounds. However, it again requires the time-consuming isolation of LDL from plasma, which can increase the possibility of PUFA autoperoxidation *in vitro*. Measurement of low-level chemiluminescence in whole blood, plasma, cells, tissues, isolated organs or human breath has therefore been proposed (15), but its specificity is questionable since the radiation appears to arise from several sources.

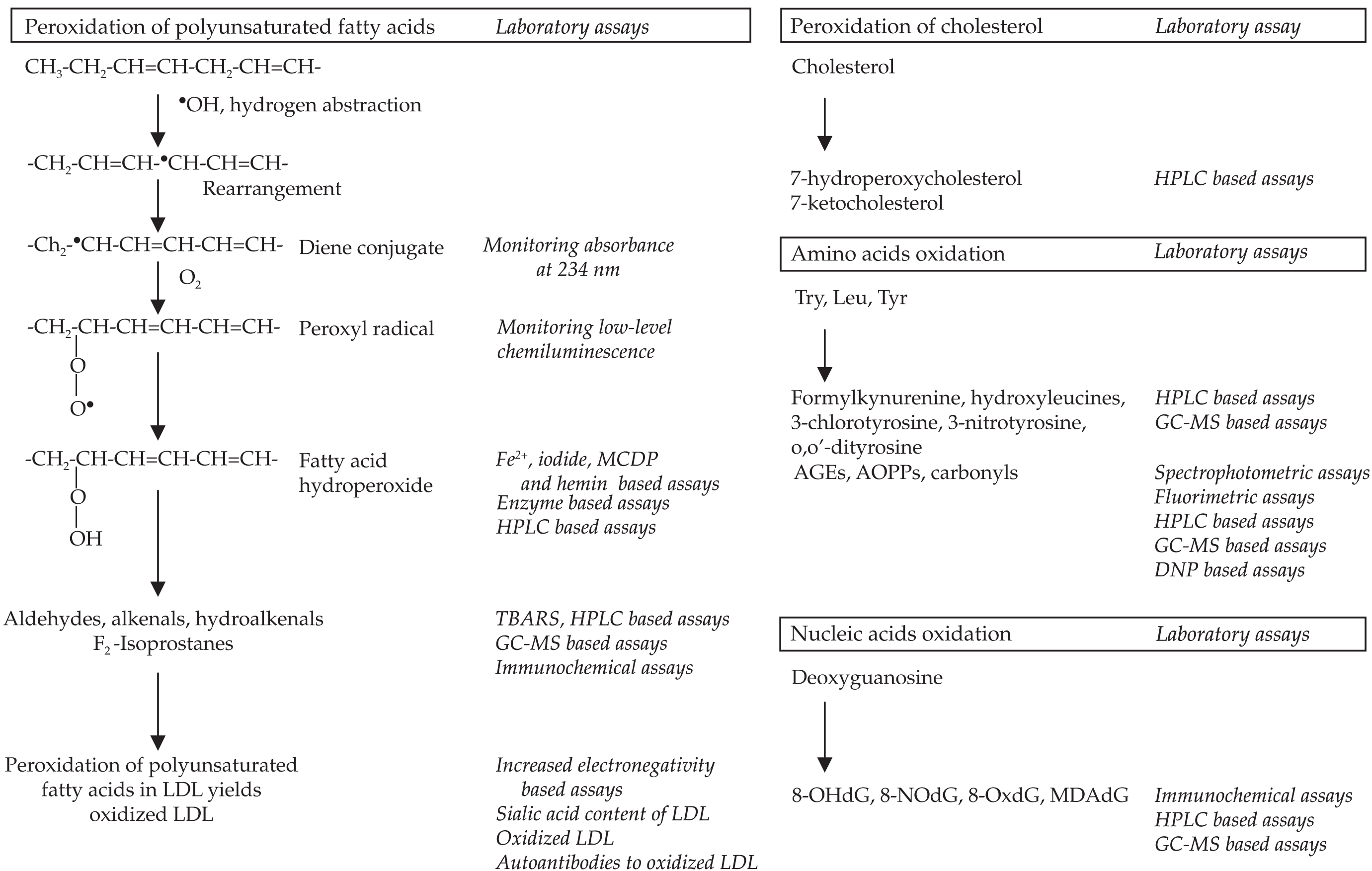

Fig. 1. Oxidative modification of lipids, amino acids and nucleic acids, and the corresponding oxidative stress assays (for detailed description see the text).

Lipid hydroperoxides

Numerous methods for determining lipid hydroperoxides can be roughly clustered into two groups – methods for measuring total hydroperoxides and those for a specific hydroperoxide, with the latter indicating the fatty acid undergoing the peroxidation burst.

In the former group, plasma total lipid hydroperoxides are used to oxidize Fe^{2+} (16), iodide (17) or 10-(*N*-methylcarbamoyl)-3,7-(dimethylamino)-phenothiazine (MCDP) (18), yielding oxidation products further quantified spectrophotometrically, or they may be decomposed by hemin, giving chemiluminescence in the presence of luminol (19) (Fig. 1). In isolated LDL, an easy iodide oxidation-based assay is frequently used (20). Alternatively, plasma total lipid hydroperoxides can be decomposed by specific enzymes whose activity is assessed by a specific detection system (Fig. 1). For instance, in the glutathione peroxidase-based assay, the formation of oxidized glutathione or consumption of NADPH (after reduction of oxidized glutathione with glutathione disulphide reductase) is followed by UV-spectroscopy (21). In the cyclooxygenase-based assay, the consumption of O_2 is usually monitored (22). Some of the methods for determining total lipid hydroperoxides can be readily automated, but nearly all lack specificity and/or sensitivity. To overcome the problem, various extraction procedures have been introduced, but these lead to extensive pretreatment of biological samples and to artefacts *in vitro*. In enzyme based assays, the activity of the enzyme used varies to some extent with the type of lipid peroxide present in the sample. Despite this, these assays yield results comparable to the colorimetric assays cited above, when applied to human plasma (6).

On the other hand, specific lipid peroxides, indicating the fatty acid undergoing the peroxidation process, can be determined by using a number of high pressure liquid chromatography (HPLC) assays coupled with appropriate detection systems (Fig. 1). It should be noted that nearly all of them require extensive isolation of lipid peroxides before application to the column and/or demanding post-column manipulations of the eluate before injection into the detector. Such assays have been used for determining phospholipid hydroperoxides isolated from LDL (23), phospholipid and cholesteryl ester hydroperoxides isolated from plasma (24), phosphatidylcholine and cholesteryl ester hydroperoxides extracted from LDL (25), cholesteryl ester hydroperoxides in plasma (26), cholesteryl linoleate hydroperoxides extracted from LDL (27), cholesteryl, phosphatidylcholine and triacylglycerol ester hydroperoxides extracted from plasma (28), fatty acid hydroperoxides in intact native or copper ion oxidized LDL (29) and four major lipid hydroperoxides, their hydroxy derivatives and PUFAs precursors on a single run (30), to name a few from the list of assays expanding daily. Along with PUFAs, cholesterol may oxidize to various hydroperoxides (Fig. 1). Thus, determination of 7-hydroperoxycholesterol and its products by the normal-phase HPLC separation and UV detection at 205 nm has been also proposed (32).

The evaluation of PUFAs and cholesterol hydroperoxides in plasma, LDL and other biological matrices is a debated issue, and the concentration obtained is largely dependent on the assay and the instrumentation used (for a critical review see reference 6).

Lipid hydroperoxide degradation products

Fatty acid hydroperoxides undergo fragmentation, giving rise to numerous short and long chain aldehydes, alkenals and hydroxyalkenals, including phospholipid and

cholesterol ester core aldehydes, which can be used in oxidative stress assessment (Fig. 1). In an assay measuring thiobarbituric acid reactive substances (TBARS), malondialdehyde is the main reactant (32). Although this test is widely used to assess lipid peroxidation, it lacks specificity and is prone to artefacts during the sample work-up, despite the various modifications that have been proposed (33). Several more specific HPLC assays for determining individual lipid hydroperoxide degradation products, *e.g.*, 9-oxononanoyl cholesterol (a cholesterol core-aldehyde formed from cholesteryl linoleate during lipid peroxidation) in isolated LDL or atherosclerotic lesion (34), or simultaneous quantification of different *n*-alkenals, hydroxyalkenals and aldehydes in various biological fluids in one run (9, 35) have been proposed, to name only a few. 7-Ketocholesterol was identified as the main cholesterol peroxidation degradation product, allowing an HPLC assay with UV detection (36) (Fig. 1).

Recently, a series of bioactive prostaglandin F_2-like compounds (F_2-isoprostanes) were discovered to be produced by free radical peroxidation of arachidonic acid, independently of cyclooxygenase activity (Fig. 1). In contrast to the lipid hydroperoxides and lipid hydroperoxide degradation products mentioned previously, F_2-isoprostanes are chemically stable end-products, raising the possibility of using urine samples to monitor oxidative stress. A highly sophisticated gas chromatography-mass spectrometry (GC-MS) (37) and a much simpler immunoassay (38) have been proposed, but there might be differences in what these two basic analytical approaches measure (39).

ASSAYS FOR MEASURING OXIDIZED AMINO ACIDS

Determination of oxidized amino acids for monitoring oxidative stress is an alternative approach to measuring lipid hydroperoxides or lipid hydroperoxide degradation products. Chemical evidence was reported recently for the oxidation of amino acids in LDL and of other proteins during the early phase of oxidative stress, possibly concurrently with lipid peroxidation, reflecting oxidative stress over an extended period of life span (Fig. 1).

Several heterogeneous families of stable oxidized protein moieties have been characterized, such as advanced glycation end products (AGEs), advanced oxidation protein products (AOPPs) and protein carbonyls (Fig. 1). Their chemical basis, known and unknown chemically reactive compounds are still under intensive investigation. AGE formation involves oxidative modification of fructolysine and creation of carboxymethyl-lysine and pentosidine. Serum or tissue AGEs can be detected fluorimetrically (40) or measured by more specific chromatographic methods (41, 42). AOPPs, which share several homologies with AGEs, are rich in di-tyrosine content and have high molecular mass, limiting their clearance. A spectrophotometric assay was proposed for their quantification (43). The close relationship between AGEs and AOPPs led to the concept of carbonyl stress. Carbonyls are produced by oxidant species driven oxidation of amino acids residues in proteins, particularly lysine, arginine, threonine and proline. Dinitrophenylhydrazine (DNP) based assays with spectrophotometric or immunochemical detection have been proposed (44).

The other general approach is to determine specific oxidized amino acids. HPLC based assays for determining *N*-formylkynurenine in LDL, an oxidation product of tryp-

tophan (Try) (45), and hydroxyleucines, degradation products of the corresponding hydroperoxyleucines generated during leucine oxidation (Leu) (46), have been proposed. Oxidation products of tyrosine (Tyr) may be used to identify biologically relevant sources of oxidative stress that mediate damage. Thus, 3-chlorotyrosine, a specific marker of the myeloperoxidase oxidative stress pathway, can be determined by GC-MS analysis in isolated LDL or in atherosclerotic tissue after delipidation and hydrolysis of proteins (47). The assay for 3-nitrotyrosine, an indicator of the reactive nitrogen oxidative stress pathway, involves acid hydrolysis of tissue proteins, isolation of 3-nitrotyrosine by ion exchange chromatography, reduction to 3-aminotyrosine, and derivatization to per-heptafluorobutyryl, which is finally quantified by GC-MS (48). Another tyrosine oxidation product is *o,o'*-dityrosine, which may serve as a specific indicator of the NADPH oxidase oxidative stress pathway (49). However, it again requires sophisticated techniques as do the two assays above.

One further alternative is to determine alterations in the functional properties of oxidatively modified amino acids. An interesting example is the decreased binding of transition metals (Cu, Co, Ni) to the N-terminal region of albumin as a result of its damage due to exposure to reactive oxidant species (50). Consequently, the commercial Albumin Cobalt Binding Assay for assessing reduced binding of Co^{2+} to oxidatively modified albumin was proposed as a promising biochemical marker of ischaemia-reperfusion injury disease (51).

ASSAYS FOR MEASURING OXIDIZED NUCLEIC ACIDS

The most devastating oxidant in terms of deoxyribonucleic acid (DNA) damage is $^{\bullet}OH$, which can attack guanine at its C-8 position, yielding the oxidation product 8-hydroxydeoxyguanosine (8-OHdG), and $ONOO^-$, which reacts at the same position generating 8-nitrodeoxyguanosine (8-NOdG) and 8-oxodeoxyguanosine (8-OxdG) (Fig. 1). After digestion of DNA, 8-OHdG levels in tissue can be quantified by an electrochemical HPLC assay (52), GC-MS assay (53) or with the aid of specific antibodies (54). Each of these methods has advantages and disadvantages, and results cannot be compared due to lack of standardization, differences in specificity and sensitivity, variations in DNA extraction, DNA digestion and storage conditions. Similarly, 8-NOdG can be determined by an electrochemical HPLC assay after enzymatic or acidic digestion of DNA (or ribonucleic acid, RNA) and its reduction to 8-aminoguanine (55). Among other oxidized derivatives, deoxyguanosine-malondialdehyde adducts (MDAdG) are frequently analyzed, either by GC-MS (56) or immunochemically (57). Some of the above-mentioned methods could easily be adopted to measure oxidized nucleic acids in serum, urine and circulating leukocytes. Devastating oxidants may also react with other bases, yielding a number of oxidation products (5-hydroxymethyldeoxyuridine, 8-hydroxyadenine), some of which have found analytical applications (58, 59). Besides modification of DNA (or RNA) bases, several other types of nucleic acid damage have been recognized, such as DNA fragmentation (60) (which is not necessarily related to oxidative damage) or loss of purines, damage to the deoxyribose sugar, DNA-protein cross linking and damage to the DNA repair system (61), offering a further range of possibilities of oxidative stress assessment, which are still awaiting thorough critical evaluation.

ASSAYS BASED ON PHYSICOCHEMICAL AND IMMUNOLOGICAL PROPERTIES OF OXIDIZED LDL

Attention was initially drawn to oxidative stress in atherosclerosis and its acute complications. Oxidatively modified LDL is powerfully atherogenic. Oxidation of lipids and proteins substantially modifies the physical, chemical and immunological properties of LDL. These changes are indicators of the extent of LDL oxidation and may serve as the basis for separating oxidized from non-oxidized LDL and for determining oxidized LDL concentration in plasma as a marker of oxidative stress *in vivo* (Fig. 1). Increased electronegativity, characteristic of oxidized LDL, is frequently used in analytical techniques. Hodis *et al.* (62) attempted to separate a negatively charged LDL from total LDL, using anion-exchange HPLC with UV detection at 280 nm. An improved anion-exchange HPLC assay includes complete separation of HDL, LDL (divided into three subfractions) and VLDL from plasma in 20 min, using a column packed with DEAE-glucomannan gel and post-column enzymatic oxidation of cholesterol by a two-step reaction to a fluorogenic product (63). Alternatively, increased LDL electronegativity may be assessed by increased electrophoretic mobility in agarose gel electrophoresis (64). After isolation by ultracentrifugation, the electrophoretic mobilities of the native and oxidized LDL are compared. This very reliable way of quantifying LDL oxidation *in vitro* unfortunately lacks the sensitivity of an *in vivo* test. A procedure has been developed that uses high performance capillary electrophoresis to monitor changes in the electrophoretic mobility of isolated LDL and to separate oxidized from non-oxidized LDL (65). Since protein migration is monitored by UV absorbance at 234 nm, changes occurring in the absorption spectra of lipoproteins, for instance because of conjugated diene formation, can be simultaneously measured. Capillary isotachophoresis was suggested for separating whole plasma lipoproteins, isolated by ultracentrifugation and pre-stained with a lipophilic dye, into 11 subfractions (66). The advantages of capillary electrophoresis include easy automation, on-line monitoring and rapid separation.

Most recently, desialylation of LDL, *e.g.*, loss of sialic acid residues on apo B and glycolipids, was shown to be the initial step in atherosclerotic modification that finally yields oxidized LDL. Therefore, the decreased sialic acid content of isolated LDL might be a marker of the extent of LDL oxidation. We developed an assay for determining the sialic acid content of LDL (67) however, in an assessment of the presence and extent of atherosclerosis in various arterial beds, the sialic acid content of LDL was found to be only marginally important (68).

Several immunoassays for determining oxidized LDL concentration have been proposed. The specificity of these assays relies on the primary (capturing) monoclonal antibody used. Holvoet *et al.* raised a monoclonal antibody (mAb) specifically recognizing malondialdehyde-modified LDL (MDA-LDL) (69) or copper-oxidized LDL (70). Shoji *et al.* (71) used a mAb specifically recognizing oxidized phosphatidylcholine. In our assay, we used mAb OB/04, which specifically recognizes copper-oxidized LDL and LDL modified with arachidonic acid oxidation products (72). In contrast to the first two assays, in which oxidized LDL was determined directly in plasma, we proposed determination of oxidized LDL in isolated LDL. This minimizes the artefactual influence of autoantibodies to oxidized LDL, but the extensive isolation procedure increases the possibility of LDL oxidation *in vitro* by potentiating »pre-seeded« lipid hydroperoxides.

Physicochemical changes in oxidized LDL trigger synthesis of autoantibodies to oxidized LDL (aAb). This can also be used as a measure of oxidative stress *in vivo*. Several assays for determining plasma concentration of aAb have been proposed by Salonen *et al.* (73), followed by other authors (*e.g.* refs. 68, 74–76). Most of the assays are based on comparison of the reactivities of a sample with immobilized oxidized LDL and with immobilized native LDL, and the results are expressed in arbitrary units, either as a difference or as a ratio that reflects the increased binding to oxidized LDL. Craig *et al.* (77) suggested the use of human serum albumin as blocking buffer, to minimize non-specific binding of human immunoglobulins to oxidized and native LDL. Koskinen *et al.* (78) proposed a competitive assay that allows calculation of specific binding, *e.g.*, the difference in binding between unabsorbed and oxidized LDL-absorbed aliquots. Furthermore, they suggest preparation of a standard with a known antibody concentration, allowing expression of antibody concentration in standard mass units rather than in arbitrary units. Further attempts to standardize the assay will probably help clarify the pathogenic significance of aAb in the atherosclerotic process and the usefulness of their determination in oxidative stress assessment for atherosclerotic risk stratification, which is currently uncertain.

ASSAYS FOR MEASURING ANTIOXIDANT CAPACITY OF BODY FLUIDS AND TISSUES

Multilevel defence systems (antioxidants, Table I) counteract oxidative stress driven alteration of numerous biological molecules and their involvement in various diseases. An antioxidant is any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (79). According to their action (Table I), antioxidants can be classified into: *(i)* free transition metal ions chelating proteins, *e.g.*, ferritin, transferrin, ceruloplasmin and metallothionein, *(ii)* haem binding proteins, *e.g.*, hemopexin, *(iii)* nonessential, endogenous antioxidants, *e.g.*, glutathione, uric acid, bilirubin and albumin, *(iv)* essential, exogenous antioxidants, *e.g.*, vitamin C, vitamin E and carotenoids, *(v)* enzymes, *e.g.*, superoxide dismutase, catalase and glutathione peroxidase, and *(vi)* systems restoring damaged biological molecules, *e.g.*, DNA repair enzymes. Many antioxidants are capable of multiple mechanisms of action.

Any deterioration of the antioxidant status of body fluids and tissues could be an indirect indicator of oxidative stress of an organism. Depletion of essential antioxidants consumed during oxidative stress is frequently assessed by numerous analytical approaches. For instance, simultaneous measurement of α-tocopherol, retinol and five carotenoids (lutein, cryptoxanthin, lycopene, and α- and β-carotene) in plasma or isolated LDL can be performed using a reverse-phase HPLC assay (80). According to Levine *et al.* (81), there is a reliable method for determining vitamin C. The main drawback of this approach is the great dependence of vitamin concentrations on their dietary supply. For this reason, determination of nonessential endogenous antioxidants, such as glutathione, may be as effective. Several methods for glutathione assessment have been proposed, including an enzymatic assay for its content in tissue (82). Evaluation of the ratio of reduced-to-oxidized forms of an antioxidant, such as reduced-to-oxidized glutathione (GSH/GSSG), may better reflect the exposure to oxidative stress than assays of an indi-

Table I. Antioxidants and their principal mechanisms of action (for detailed description see the text)

Antioxidant	Mechanism of action
Ferritin	Binds ferric ions
Transferrin	Binds ferric ions
Ceruloplasmin	Binds copper ions, utilizes H_2O_2
Metallothionein	Binds transition metal and heavy metal ions
Hemopexin	Binds haem
Glutathione	Reductive capacity of sulphydryl groups
Uric acid	Radical scavenger and binds metal ions
Bilirubin	Scavenges peroxyl radicals
Albumin	Binds copper, haem, and scavenges HOCl
Flavonoids and other polyphenols	Scavenge radicals
Vitamin C	Scavenges $^{\bullet}OH$
Vitamin E	Chain-breaking antioxidant
Carotenoids	Scavenge radicals
Superoxide dismutase	Removes $O_2^{\bullet -}$
Catalase	Removes H_2O_2
Glutathione peroxidase	Removes H_2O_2 and hydroperoxides

vidual antioxidant form. In the case of GSH/GSSG ratio, the method requires careful differentiation of GSH from GSSG (6), especially when analyzed in plasma, where concentrations are very low. A fast, sensitive and selective HPLC method with simultaneous UV detection of picomole quantities of GSH and GSSG in plasma, which does not require prior derivatization, was recently described (83). Determinations of ubiquinol/ubiquinone ratio (84) and vitamin C/dehydroascorbate ratio (81) were also proposed, although the latter is questionable due to dehydroascorbate instability (81).

An alternative approach to the antioxidant status assessment is measuring antioxidant enzyme activities. Commercially available kits for determining superoxide dismutase (85), glutathione peroxidase (86) and catalase (87) in erythrocytes are available. These enzymes are not consumed while performing their protective functions, but may be damaged and inactivated by oxidant species, similarly to any other protein. On the other hand, oxidant species may induce enzyme gene expression leading to increase rather than decrease in their activity.

Body fluids contain a variety of low-molecular-mass antioxidants (LMWA), such as uric acid, bilirubin, albumin, flavonoids and other polyphenols. The fact that these LMWAs work in concert with vitamins, glutathione and enzymes, led to the concept of measuring total reductive capacity of body fluids. Some antioxidants are still unknown or rarely assayed and measuring only a few out of many could be misleading. The principle of this approach is to face body fluids (*e.g.*, plasma) with a constant challenge of oxidants generated *ex vivo* and indicate the time when the sample can no longer scavenge them or withstand ensuing oxidative damage. The lag phase of oxidation, as determined by continuous monitoring of conjugated dienes or by low-level chemiluminescence, is directly proportional to the antioxidant content of the sample, usually isolated LDL. The assays have already been described above. Alternatively, lipophilic 2,2'-azobis (2,4-di-

methylvaleronitrile) (AMVN) or water soluble 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2-amidopropane) (ABAP) radical generators may be used, and the total peroxyl radical-trapping potential (TRAP) then measured, as originally proposed by Wayner *et al*. (88) and by numerous modifications (89). Alternatively, trolox equivalent antioxidant capacity (TEAC), which is based on the oxidation-induced decolorization of the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation, may be measured (90). The capacity of a sample to reduce ferric tripyridyltriazine to a coloured ferrous tripyridyltriazine measures the ferric reducing antioxidant power (FRAP) (91). Although all these methods measure the total antioxidant capacity of body fluids, the assay results intercorrelate weakly and rarely. As reviewed by Dotan *et al.* (92), there are differences in what these assays measure and in the contributions of individual antioxidants to each of these assays.

CONCLUSIONS

Basic research has demonstrated the important aetiological role of oxidative stress in various acute, life-threatening, and chronic degenerative diseases. We have described five basic approaches to assessing oxidative stress and numerous assays for monitoring various lipid, amino acid and nucleic acid oxidation products, modified LDL and antioxidant capacity. The current problems are that the majority of proposed assays have not yet been tested in relevant clinical trials, and that the optimal choice of an analytical approach appears to be at least partly dependent on the pathology of the population being tested. What is urgently needed is a small assembly of simple and precise assays for oxidative stress assessment and disease risk stratification. Experts from different fields (medical biochemists, chemists, pharmacists, medical doctors and immunologists) should work together to solve this challenging problem as soon as possible.

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Abbreviations. – aAb – autoantibodies to oxidized low density lipoprotein; AAPH – 2,2'-azobis (2-amidinopropane) dihydrochloride; ABAP – 2,2'-azobis(2-amidopropane); ABTS – 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); AGEs – advanced glycation end products; AMVN – 2,2'-azobis (2,4-dimethylvaleronitrile); AOPPs – advanced oxidation protein products; apo B – apolipoprotein B; DNA – deoxyribonucleic acid; DNP – dinitrophenylhydrazine; $FADH_2$ – reduced flavine adenine dinucleotide; FRAP – ferric reducing antioxidant power; GC-MS – gas chromatography-mass spectrometry; GSH – reduced glutathione (glutamic acid-cysteine-glycine); GSSG – oxidized glutathione; HPLC – high pressure liquid chromatography; LDL – low density lipoprotein; Leu – leucine; LMWA – low-molecular-weight antioxidants; mAb – monoclonal antibody; MCDP – 10-(*N*-methylcarbamoyl)-3,7-(dimethylamino)-phenothiazine; MDAdG – deoxyguanosine-malondialdehyde adducts; MDA-LDL – malondialdehyde-modified low density lipoprotein; NADPH – reduced nicotinamide adenine dinucleotide; $NO^{\bullet}$ – nitric oxide radical; $O_2^{\bullet -}$ – superoxide anion; $^{\bullet}OH$ – hydroxyl radical; $ONOO^-$ – peroxynitrite; PUFA – polyunsaturated fatty acid; RNA – ribonucleic acid; $RO^{\bullet}$ – alkoxyl radical; $ROO^{\bullet}$ – peroxyl radical; ROOH – hydroperoxide; TBARS – thiobarbituric acid reactive substances; TEAC – trolox equivalent antioxidant capacity; TRAP – total peroxyl radical-trapping potential; Try – tryptophan; Tyr – tyrosine; 8-NodG – 8-nitrodeoxyguanosine; 8-OhdG – 8-hydroxydeoxyguanosine; 8-OxdG: 8-oxodeoxyguanosine.

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P O V Z E T E K

Merjenje oksidacijskega stresa pri ocenjevanju bolezenskega tveganja

DARKO ČERNE in JANA LUKAČ-BAJALO

Oksidacijski stres, pomemben etiološki vzrok nastanka mnogih bolezni, se v vsakdanji bolnišnični laboratorijski praksi praviloma ne meri, kljub večkrat dokazanemu diagnostičnemu in prognostičnemu pomenu. Razmere se bodo verjetno kmalu spremenile, saj zanimanje za problematiko narašča. Članek je pregled analiznih pristopov merjenja oksidacijskega stresa in glede na proučevani analit so postopki razdeljeni v pet skupin: *(i)* postopki merjenja lipidne peroksidacije, *(ii)* postopki merjenja oksidacije aminokislin, *(iii)* postopki merjenja oksidacije nukleinskih kislin, *(iv)* postopki osnovani na fizikalno-kemičnih in imunoloških lastnostih oksidiranega lipoproteina nizke gostote in *(v)* postopki merjenja antioksidantne sposobnosti telesnih tekočin in tkiv. Namen prispevka je pomagati pri izbiri in uporabi primernih preiskav merjenja oksidacijskega stresa za vsakdanje ocenjevanje bolezenskega tveganja.

Ključne besede: oksidacijski stres, analizni postopki, ocenjevanje bolezenskega tveganja

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