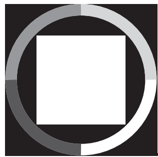
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REVIEW

MOLECULAR SIGNATURES OF OXIDATIVE STRESS: RELEVANCE TO CLINIC AND RESEARCH

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Abstract
Study on the redox biology of health and disease has come of age. Redox imbalance also defined as redox stress constitutes one of the main cellular lesion associated with many types of pathologies including cardiometabolic, neurodegenerative, oncological, rheumatic and autoimmune diseases. Reactive species (either centered on oxygen or on nitrogen) can induce many damaging cellular processes, such as DNA oxidative lesions and loss of membrane integrity due to lipid peroxidation. Thus the evaluation of the redox imbalance of biological samples is extremely important in clinical practice as well as in research settings. This may constitute an important tool to evaluate the level of cellular lesions associated with specific pathological mechanisms, or the response to pharmacological and nonpharmacological treatment. The present review focuses on the molecular signatures of redox imbalance in various diseased processes.

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Introduction
The oxidative stress is the outcome of intense generation of reactive oxygen species (ROS) and of reactive nitrogen species (RNS), which are not counteracted by endogenous antioxidant molecules, therefore being the result of imbalance between the prooxidant and antioxidant systems. Currently, oxidative damage is linked to many types of pathologies including cardiometabolic diseases (atherosclerosis, hypertension, obesity, type 2 diabetes mellitus, and the metabolic syndrome), also neurodegenerative, cancer and rheumatic diseases (1,2). Free radicals are chemical species containing unpaired electrons, which are highly reactive and unstable, due to their structure; they can react locally, accepting or donating electrons, in order to become more stable. The reaction between a radical and a non-radical compound leads to the propagation of the radical chain reaction, therefore generating a cascade of new free radicals. Many types of free radicals are generated during normal biochemical processes; among most reactive are the oxygen-, sulfur-, bromide- and chloride-centered species (3). The most commonly reported cel-

lular free radicals are centered on oxygen and are named ROS; among them most studied are singlet oxygen ($^1\Sigma g^+ O_2$), hydroxyl (-OH), superoxide (O_2^-) radicals. The free radicals centered on nitrogen are named reactive nitrogen species; most cited is the nitric monoxide (NO). Also, some other molecules, like hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (which are not free radicals from the chemical point of view, having all-paired electrons) are reported to generate free radicals in living organisms through various chemical reactions (3).

In order to counteract the damaging action of the physiologically generated ROS, the living organisms developed antioxidant systems. They act through different mechanisms: the transport of metal ions (e.g. iron, copper) by specific or non-specific proteins (ferritin, transferrin, albumin). Thus these ions can no longer participate in (*i*) redox reactions, (*ii*) arrest the ROS formation chain reaction, generally *via* antioxidants with small molecular mass, such as reduced glutathione (GSH), vitamins E and C, and uric acid, (*iii*) scavenging ROS with antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione-peroxidase (GPx), and glutathione-reductatse (GR), and (*iv*) the reparation of lesions caused by ROS *via* specific enzymes such as endonucleases, peroxidases, and lipases.

The present review focuses on the molecular signatures of redox imbalance that can be used both in clinical and research practice.

Adipose-derived ROS

Reactive species (ROS and RNS) can induce many damaging cellular processes, such as DNA oxidative lesions, loss of membrane integrity due to lipid peroxidation and protein and carbohydrate structural changes. All these changes modify the normal metabolic functions, thus resulting in pathological phenomena associated with the increase of capillary permeability, impairment of the blood cell function and also the acceleration of aging process. ROS-induced damages are frequently associated with cancers (3,4), diabetes mellitus (5), rheumatoid arthritis (6,7), and obesity and related diseases (1,2,8-10). Of note, in obese mice, oxidative stress is enhanced in adipose tissue before diabetes development, suggesting that in obesity, adipose tissue may be a major source of ROS (1,8). Moreover, adipose-derived ROS is involved the decrease of circulating level of adiponectin, an essential event in the pathogenesis of metabolic syndrome and related disorders (8). Accordingly, treatment of obese mice with anti-oxidative agents improves insulin resistance and restores adiponectin secretion (1,8).

Redox status of biological samples

Since the redox imbalance is associated with several pathologies, the evaluation of the redox status of biological samples is extremely important in clinical practice as well as in research settings. Several methods for determining biomarkers of cellular oxidative stress have been developed, and some have been proposed for sensitive assessment of antioxidant defense and oxidative damage for example in diabetes and its complications (10-13).

The assessment of redox imbalance is focused on the type of cellular biomolecules that might be affected by the reactive species - lipid peroxidation, protein modification, DNA oxidative changes. Generally, these processes do not occur individually – there is an overlap of the pathological changes induced by the redox imbalance.

The term "biomarker" has been defined by The American National Institute of Health as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention". Applications include diagnosis, prognosis and individualization of the therapy. The most promising biomarkers are the ones that closely correlate with the pathophysiological process of the disease (14-16).

The biochemical markers of the redox imbalance are difficult to classify. One criterion might be the type of molecules that are the target of the reactive species; depending on this, the markers might be specific for lipid peroxidation (malondialdehyde, hydroxy-trans-2-nonenal, thiobarbituric acid reactive substances - TBARS), isoprostanes, oxidized LDL particles), for protein oxidation (advances oxidation protein products – AOPP, protein carbonyls, nitrotyrosine), or for DNA oxidative damage (such as 8-hydroxydeoxyguanosine-8-OHdG).

Another very important issue is related to the specific function of the molecules that are assayed; for example, important markers of redox homeostasis are some important enzymes (such as catalase, superoxide dismutase, peroxidases, enzymes involved in the metabolism of glutathione – glutathione peroxidase, glutathione reductase, DNA repair enzymes, etc). On the other hand, important molecules involved in the redox equilibrium are some vitamins – ascorbic acid, tocopherols, ubiquinones, retinols, carotenes, or other small molecules (lipoic acid, uric acid, bilirubin) or proteins (albumin). Technically speaking, some assays show the global effect induced by increased reactive species production on biological samples ferric reducing ability of plasma (FRAP), oxygen radical absorbance capacity (ORAC), total antioxidant capacity (TAC). Enzymatic activities, on vitamin assays or on genetic markers of oxidative stress are reviewed elsewhere (17,18).

Markers of protein oxidation: AGE come of age

Studies show that radical-mediated oxidation of proteins leads

to fragmentation of the polypeptide chain, oxidation of amino acid side chains, and generation of protein–protein cross linkages. It was subsequently established that all of these protein modifications can also be mediated by metal-catalyzed oxidation (MCO) systems. The attack of reactive species on protein generates carbonyl derivatives as markers of magnitude of protein oxidation; the carbonyl content of proteins has become the most generally used method for estimation of oxidative-stress-mediated protein oxidation. Glycation and glycooxidation products also accumulate during exposure to oxidative stress (19).

The advanced glycation end products (AGE) formation process, or the Maillard reaction, begins from Schiff bases and the Amadori product, a 1-amino-1-deoxyketose, produced by the reaction of the carbonyl group of a reducing sugar, like glucose, with proteins, lipids, and nucleic acid amino groups. During Amadori reorganization, these highly reactive intermediate carbonyl groups, known as α-dicarbonyls or oxoaldehydes, products of which include 3-deoxyglucosone and methylglyoxal, accumulate. Further glycation of proteins and lipids causes molecular rearrangements that lead to the generation of AGE. The α-dicarbonyls have the ability to react with amino, sulfhydryl, and guanidine functional groups in proteins. The reaction results in denaturation, browning, and cross-linking of the targeted proteins. In addition, the α-dicarbonyls can react with lysine and arginine functional groups on proteins, leading to the formation of stable AGE compounds, such as Nε-(carboxymethyl) lysine (CML), which are nonfluorescent AGE. Further, AGE form *in vivo* in hyperglycemic environments and during aging and contribute to the pathophysiology of cardiovascular disease in diabetes (20-23). Noteworthy, the research group of Hiroshi Yamamoto demonstrated that the expression of receptor for advanced glycation end products (RAGE) is found in adipocytes, immune cells, endothelial cells, and pancreatic β-cells, and implicated RAGE in adipocyte hypertrophy and insulin resistance, thus in the development of obesity and type 2 diabetes (24, also see 14).

The plasma level of AGE can be evaluated using a fluorimetric method, since AGE are fluorescent markers (25,26). Plasma samples are excited at 350 nm; the emission was registered between 350nm and 550nm, with the maximum at 450nm.

Protein tyrosine nitration is mediated by reactive nitrogen species such as peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2), and results in a nitro group adduct on susceptible tyrosine residues. Myeloperoxidase (MPO), with its transition metal centre, can react with $ONOO^-$ to yield oxo-metal complexes and NO_2 thus facilitating the nitration reaction. Although the precise intermediates and mechanism for nitration in vivo have been a matter of controversy, measures of tyrosine nitration have been used as indicators of oxidative stress. Free nitrotyrosine can be measured by tandem mass spectrometry (MS/MS) coupled with GC or HPLC. Further studies are required to establish a normal basal range of circulating free nitrotyrosine in healthy individuals Other ways of quantifying protein nitration are immunocytochemical and immu- nohistochemical assays based on either monoclonal or polyclonal nitrotyrosine antibodies.

The carbonylation of protein represents the introduction of carbonyl groups (aldehyde or ketone) in the protein structure, through several mechanisms: by direct oxidation of the residues of lysine, arginine, proline and threonine residues from the protein chain, by interaction with lipid peroxidation products with aldehyde groups (such as 4-hydroxy-2-nonenal, malondialdehyde, 2-propenal), or by the interaction with the compounds with the carbonyl groups resulting from the degradation of the lipid or glycoxidation. All of these molecular changes occur under oxidative stress conditions. The protein carbonylation may constitute a marker for oxidative stress since it indicates in early stages and with accuracy the level of the oxidative damage. The assay of protein carbonyls may be based on spectrophotometrical data (after derivatisation of the carbonyl groups with 2,4-dinitrophenylhydrazine - DNPH), high-performance liquid chromatography (HLPC) method with diode-array detector (at 370 nm) after an initial separation of the protein fractions by gel-filtration, gas chromatography (GC) or the immunoassay using specific antibodies (27-29).

Markers of lipid peroxidation

Isoprostanes (IsoPs) are a family of stable, prostaglandin-like compounds gen- erated from the peroxidation of arachidonic acid, a polyunsaturated fatty acid present in phospholipids of cell membranes. The generation of IsoPs from arachidonic acid is independent of the cyclooxygenase enzyme that catalyzes the formation of prostaglandins from arachidonic acid. Isoprostanes are generated as a result of the action of ROS on membrane lipids; the ROS may be generated from mitochondrial electron transport chain, P450, lipoxygenase or transition-metal catalyzed formation of free radicals. IsoPs are subsequently released from the cell membrane into circulation by phospholipases, and can then be quantified in tissues, blood and urine. Most frequenly used methods for tha assay of IsoPs in plasma and urine samples are gas chromatography–mass spectrometry (GC/MS), liquid chromatography–mass spectrometry (LC/MS), enzyme-linked immunosorbance assays (ELISA) and radioimmunoassay.

MDA is generated in vivo via peroxidation of polyunsaturated fatty acids. MDA interacts with proteins; for example MDA's reaction with lysine residues generates lysine– lysine cross-links

which have been identified in apolipoproteinB (apoB) fractions of oxidized low density lipoprotein (OxLDL). MDA is typically quantified from plasma samples with the most popular method being a colorimetric assay based on the reaction between MDA and thiobarbituric acid (TBA). However, although suitable for high throughput analysis, this TBA reacting substances (TBARS) assay lacks specificity for MDA,with aldehydes other than MDA reacting with TBA to produce compounds that absorb in the same rangeas MDA. Several ELISA kits to detect MDA but also high-performance liquidc hromatography (HPLC) methods are available (16).

Monitoring the induced lipid peroxidation may also constitute an important marker in the assessement of redox imbalance, with great relevance on cardio-vascular risk evaluation. The susceptibility of lipoprotein particles to lipid peroxidation can be assessed, after the isolation of LDL, either by treatment with copper salts, with mixtures of ferric compounds and ascorbic acid, or other prooxidant systems. We previously published data regarding the assessment of induced lipid peroxidation markers in biological samples (30).

Global tests for the assay of redox status

The oxygen radical absorbance capacity (ORAC) assay measures the oxidative degradation of the fluorescent molecule (fluorescein) after exposure to free radical generators (azo-initiator compounds, such as (2,2'-azobis-2-amidino-propane-dihydrochloride-AAPH). Antioxidants are considered to protect the fluorescent molecule from the oxidative degeneration. The fluorescent intensity decreases as the oxidative degeneration develops, and this intensity is typically recorded as time function after the addition of the free radical generator. The presence of the antioxidant slows the fluorescence decay. Decay curves (fluorescence intensity *vs.* time) are recorded. Subsequently, the degree of antioxidant-mediated protection is quantified using the antioxidant Trolox (a vitamin E analogue) as a standard. The method is especially used when measuring foods and supplements that contain complex ingredients with various slow- and fast-acting antioxidants, as well as ingredients with combined effects that cannot be pre-calculated.

The ferric reducing ability of plasma (FRAP) is a simple, automated test measuring the ferric reducing ability of plasma, and is used for assessing "antioxidant power." Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form. There is no apparent interaction between antioxidants. The FRAP assay offers a putative index of antioxidant, or reducing, potential of biological fluids within the technological reach of every laboratory and researcher interested in oxidative stress and its effects (31).

Total antioxidant capacity, uses 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation (ABTS+), which is decolorized by antioxidants according to their concentrations and antioxidant capacities. This change in color is measured as a change in absorbance at 660 nm and the process is calibrated with Trolox. The assay has no interference from hemoglobin, bilirubin, EDTA or citrate (32).

Recent literature data mentions the use of fluorescent probes for the assessment of reactive species in biological samples, especially when speaking about non-specific processes. To deal with the issue of non-specificity to ROS species, newer probes that measure the nucleophilic nature of the superoxide anion rather than its oxidative properties have been developed. These probes incorporate leaving groups that when cleaved yield the free fluorescent molecule. In addition fluorophores such as aminophenyl fluorescein and hydroxyphenyl fluorescein are more specific to downstream oxidants such as HOCl and the hydroxyl radical. AmplexRed is a highly sensitive fluorophore that specifically measures H_2O_2 (in picomolar range) in the presence of a catalyst (33).

Conclusion

Assessing the redox imbalances in biological samples may constitute an important tool to evaluate the level of cellular lesions associated with specific pathological mechanisms, or the response to pharmacological and nonpharmacological treatment. This may be important in "reading" of the redox signatures, particularly that "written" in the adipose tissue, as implicated in the pathogenesis and therapy of cardiometabolic diseases. Steps are yet to be made on this path of redox (patho)biology since most of the redox stress markers are not properly validated from the analytical point of view.

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