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Keiki Ogino* Da-Hong Wang†

*Okayama University,

†Okayama University,

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Abstract

Oxidative/nitrosative stress is responsible for a variety of degenerative processes in some human diseases. Measurement of oxidatively/nitrosatively modified DNA, proteins, lipids, and sugars in biological samples has been expected to detect appropriate biomarkers for diseases in which reactive oxygen/nitrogen species are involved. Recently, the application of these biomarkers to epidemiological studies has resulted in a new discipline, molecular epidemiology, which provides the opportunity for better understanding of their causal relation with disease outcomes in a population level. In this brief review, we cover some specific biomarkers of oxidative/nitrosative stress with regard to the commonly used analytical methods for these biomarkers, their integration with epidemiology, and their application in antioxidant intervention trials, with an emphasis on those applicable to human studies and their potentialities for disease prevention.

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Review

Biomarkers of Oxidative/Nitrosative Stress: An Approach to Disease Prevention

Keiki Ogino* and Da-Hong Wang

Department of Public Health, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

Oxidative/nitrosative stress is responsible for a variety of degenerative processes in some human diseases. Measurement of oxidatively/nitrosatively modified DNA, proteins, lipids, and sugars in biological samples has been expected to detect appropriate biomarkers for diseases in which reactive oxygen/nitrogen species are involved. Recently, the application of these biomarkers to epidemiological studies has resulted in a new discipline, molecular epidemiology, which provides the opportunity for better understanding of their causal relation with disease outcomes in a population level. In this brief review, we cover some specific biomarkers of oxidative/nitrosative stress with regard to the commonly used analytical methods for these biomarkers, their integration with epidemiology, and their application in antioxidant intervention trials, with an emphasis on those applicable to human studies and their potentialities for disease prevention.

Key words: biomarker, oxidative/nitrosative stress, molecular epidemiology, disease prevention

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced as by-products of normal metabolic processes in all aerobic organisms. In physiological conditions, the antioxidant defense systems in the body protect the cells and tissues against these species [1]. When the generation of ROS/RNS exceeds the ability of antioxidant defense systems to remove them, such an imbalance can cause oxidative/nitrosative damage to cellular constituents (DNA, proteins, lipids, and sugars), which is defined as oxidative/nitrosative stress [1, 2]. Many studies have shown that oxidative/nitrosative stress is responsible for a variety of the degenerative processes of some human diseases [1, 3]. Since ROS/RNS themselves are very reactive and have an extremely short half-life, direct determination of them in tissue or body fluids is generally impracticable. Therefore, measurement of oxidatively/nitrosatively modified DNA, proteins, lipids, and sugars in biological samples has been expected to detect appropriate biomarkers for diseases in which ROS/RNS are involved.

The National Academy of Sciences in the United States defines biomarkers as "indicators, signaling events in biological systems or samples" [4]. The biomarkers can be used as "intermediate endpoints or early-outcome predictors" of disease development for preventive purposes [5]. Recently, there has been a great improvement in assay methods and measurement accuracy for biomarkers of oxidative/nitrosative

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*Corresponding author. Phone: +81-86-235-7184; Fax: +81-86-226-0715
E-mail: kogino@md.okayama-u.ac.jp (K. Ogino)

stress [6, 7], and the incorporation of biomarkers into epidemiological studies provides a promising field for better understanding the role of ROS/RNS in the pathogenesis and progression of diseases. In this brief review we cover some specific biomarkers of oxidative/nitrosative stress, with an emphasis on those applicable to human studies and their potentialities for disease prevention.

Biomarkers of Protein Oxidation/Nitration

Protein carbonyls. Protein carbonyl groups are generated by direct oxidation of amino acid residues, particularly lysine, arginine, threonine, and proline (Fig. 1), or by secondary reaction with the primary oxidation products of sugars and lipids [7–9]. Such oxidative modifications of proteins result in important changes in the proteins' structure and function. Several studies have proved that proteins are major initial cell targets of ROS, leading to earlier formation of the protein carbonyls in biological systems [10–12], and detection of increased levels of protein carbonyls has been proposed as "a sign of disease-associated dysfunction" [13]. Patients with neurodegenerative illnesses [14], diabetes and hypercholesterolemia [15], and children with juvenile chronic arthritis [16] were found to have elevated levels of total protein carbonyls, suggesting the potentiality of carbonated proteins serving as biomarkers for early diagnosis of these diseases.

Protein carbonyls are widely used and chemically stable biomarkers of oxidative stress. They circulate for longer periods in the blood compared to other oxidized products [17], and the assay sample can be kept at −80 °C for at least 10 years [18]. For detection of carbonylated proteins in human diseases, the commonly employed methods are spectrophotometric 2,4-dinitrophenylhydrazine (DNPH) assay, spectrophotometric DNPH assay coupled to protein fractionation by HPLC, and one- or two-dimensional electrophoresis and Western blot immunoassay [7, 19]. However, these methods cannot identify which amino-acid residues are oxidatively attacked and which protein has been modified [1]. Recently, the proteomics technique, which allows one to identify specific carbonated proteins in the plasma and hippocampus of subjects with Alzheimer's disease, has thrown new light on this issue [20, 21]. The proteomics technique mainly consists of two-dimensional gel electrophoresis for protein separation and mass spectrometry for protein identification; this technique may allow researchers to develop a specific intervention strategy for this disease.

Nitration of tyrosine. The 3-nitrotyrosine (Fig. 1) generated by nitration of the amino acid tyrosine and protein-bound tyrosine is another biomarker for studying the *in vivo* oxidation/nitration of protein [22]. There is considerable evidence in the literature that elevated levels of 3-nitrotyrosine occur in diseases associated with ROS/RNS. Mean plasma levels

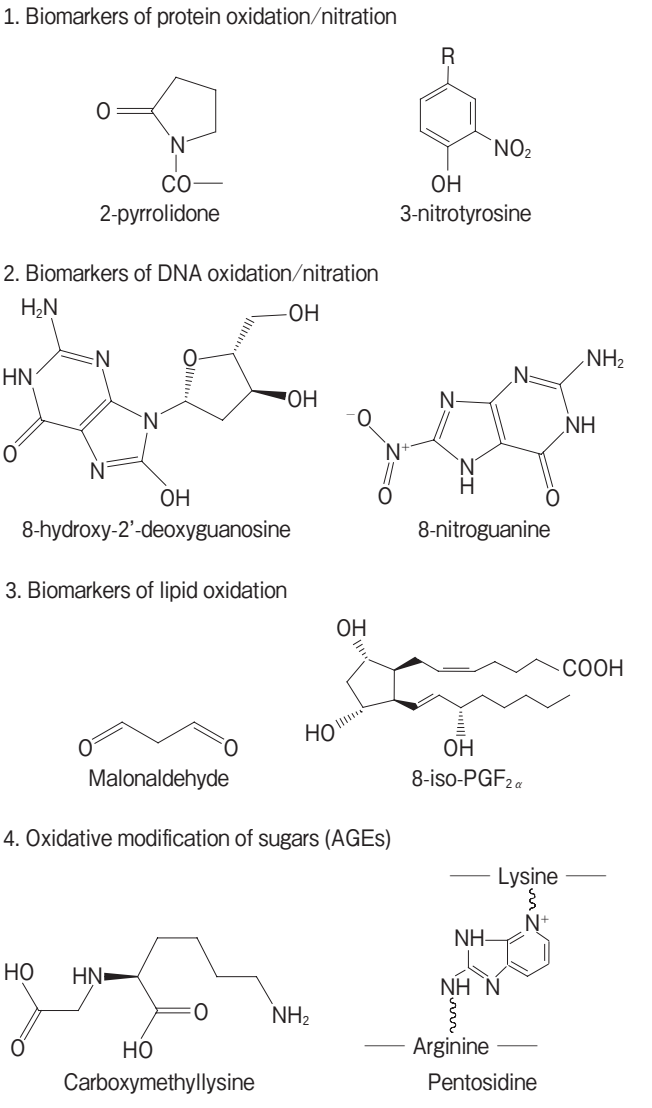

Fig. 1 Chemical structures of some examples of biomarkers.

of nitrotyrosine are significantly higher in diabetic patients with lower intake of some antioxidant vitamins (vitamin A, C) and positively correlated with serum fasting glucose [23], and are also higher in patients with coronary artery disease and modulated by statin therapy [24]. Taken together, these results imply that plasma nitrotyrosine measurement in humans is possibly a useful tool for monitoring the effect of antioxidant intervention. In a controlled weight loss trial, weight reduction was strongly associated with a decrease in serum protein 3-nitrotyrosine levels in Caucasian women but not in African-American women [25].

The 3-nitrotyrosine in biological samples has been detected and quantified by a variety of methods. Antibody-based methods {enzyme-linked immunosorbent assay (ELISA)} are considered to be semiquantitative because there is no strict assay validation and it is difficult to assess the tests' reliability [7]. HPLC with electrochemical detection (ECD), mass spectrometry-based assays {gas chromatograph-mass spectrometry (GC-MS) and gas chromatograph-tandem mass spectrometry (GC-MS/MS), liquid chromatograph-mass spectrometry (LC-MS) and liquid chromatograph- tandem mass spectrometry (LC-MS/MS)} are proposed to have adequate sensitivity for quantification of 3-nitrotyrosine, especially the use of MS/MS technique can remove interference caused by the coelution of substances in GC-MS [7]. However, recent reviews have raised concerns about the quantification of circulating 3-nitrotyrosine in human plasma because of the varied plasma levels of both free and protein-bound 3-nitrotyrosine in healthy subjects in reported findings [26, 27]. More efforts are needed to improve the methodology for measurement of 3-nitrotyrosine *in vivo*, particularly at low concentrations, and standardization of nitrotyrosine measurements is probably needed to make comparisons possible among studies [26, 27]. Shishehbor *et al.* found that protein-bound nitrotyrosine values in plasma determined by isotope-dilution LC-tandem MS are reproducibly greater than that in serum and are stable over time in healthy subjects [24]. In addition, great care should be taken during sample preparation and analysis because the artifactual formation of nitrotyrosine during sample processing, especially under acidic conditions, can confound nitrotyrosine determinations [26].

The results of several studies have demonstrated the successful detection of 3-nitrotyrosine-containing proteins *in vivo* using a qualitative proteomics approach [28–30], by which a total of 48 and 11 putative proteins containing nitrotyrosine in heart and skeletal muscle of aged rats were identified, respectively [31, 32], and 40 nitrotyrosine-immunopositive proteins were also identified in both rat tissue extract and cell culture inflammatory disease models [28]. This promising approach may offer an early diagnostic tool for disease by defining patterns of abnormal proteins [33].

Biomarkers of DNA Oxidation/Nitration

8-hydroxy-2'-deoxyguanosine. Elevated levels of oxidatively modified DNA lesions are considered responsible for an increased risk of cancer development later in life [34]. The most representative product that may reflect oxidative damage to DNA in the cells is 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Fig. 1), a product of oxidatively modified DNA base guanine [1]. Elevated levels of 8-OHdG have been found in the serum and myocardium of patients with heart failure [35] and in the urine of patients with Parkinson's disease [36]. Many methods such as HPLC-ECD, GC-MS, LC-MS, and immunoassay have been established to measure 8-OHdG in biological specimens and are reviewed in detail in several articles [6, 37, 38].

HPLC-ECD is a frequently used method with high accuracy and sensitivity, but the procedures are complex and time-consuming [6]. Measurement of low levels of oxidative DNA damage is still an issue for GC-MS and HPLC-tandem MS [38]. Isotope-dilution LC-tandem MS has been proposed as a highly specific and sensitive analytical method for urinary 8-OHdG in human subjects [39]. Immunohistochemistry is also a popular method with good sensitivity and simplicity, but it can only semiquantitatively measure 8-OHdG [37]. Two commercially available kits are used; the one using monoclonal antibody N45.1 is from the Japan Institute for the Control of Aging (Fukuroi, Shizuoka, Japan), and the other using monoclonal antibody clone 1F7 is from Trevigen (Gaithersburg, MD, USA) [40]. The 2 showed a strong correlation ($r = 0.9$), although the latter demonstrated 3 times higher urinary values, in which not only 8-OHdG but

also 8-hydroxyguanosine (8-OHG: analogue of 8-OHdG derived from RNA) and 8-hydroxyguanine (8-OHGua: an oxidatively modified free guanine base) were included [40]. None of the available methods for measuring 8-OHdG formation can locate the original site of oxidative DNA damage [37].

The measurement of urinary 8-OHdG has been considered to reflect the whole-body oxidative DNA damage [37], and the correlation coefficient of 8-OHdG measurements between spot and 24 h urine samples is 0.50 (by HPLC) and 0.87 (by ELISA), respcetively [41]. The level of urinary 8-OHdG was found to be independent of dietary influence in humans [42], although that was not the case in rats [43].

8-nitroguanine. It is known that RNS such as oxides of nitrogen (NO_X) and peroxynitrite ($ONOO^-$) generated in various pathophysiological conditions can nitrate guanine and its related nucleosides and nucleotides in the free form or in DNA/RNA [44]. The 8-nitroguanine (Fig. 1) is a representative DNA nucleobase product of nitrative lesion by RNS. Several studies have demonstrated that 8-nitroguanine is not detected in normal tissues but is mostly found in the nucleus of inflammatory cells and/or epithelial cells in inflamed tissues, indicating that 8-nitroguanine may serve as a potential biomarker for nitrative DNA damage induced by RNS in inflamed tissues [45–47]. Recent findings of 8-nitroguanine at the sites of carcinogenesis under various inflammatory conditions in animals and humans imply that the excess generation of RNS may be a risk factor for cancer development in patients suffering from inflammation-related diseases [46, 48, 49].

Several methods have been developed for measurement of 8-nitroguanine *in vivo*, such as HPLC with electrochemical detection, HPLC with a UV detector, GC-MS, and immunohistochemistry. However, their reproducibility and validity have not been well verified [37, 44]. Recently, Sawa *et al.* first reported a sensitive method to quantitate 8-nitroguanine in human urine using immunoaffinity columns with an anti-8-nitroguanine antibody, followed by HPLC-ECD [50]. They found that cigarette smoking is associated with elevated urinary levels of 8-nitroguanine.

Biomarkers of Lipid Oxidation

Malonaldehyde. Malonaldehyde (MDA) (Fig. 1) is one of the end products of lipid peroxidation in the cell membranes or in low-density lipoproteins (LDL) [1]. Levels of MDA are often measured spectrophotometrically by the thiobarbituric acid-reacting substance (TBARS) assay. This simple assay is the most frequently used method in lipid peroxidation research, but some scientists question its clinical utility. Because some aldehydes other than MDA can also be generated in peroxidizing lipid and have the same range of absorbance as MDA, the TBARS assay can be confounded by these chromogens [1]. The HPLC-based TBARS assay can separate MDA from other aldehydes and is suggested as a useful method for examining large numbers of biological samples for lipid peroxidation [1]. The GC-MS method has been used to analyze the end-products of peroxide breakdown such as MDA in human plasma [51]. Peroxides and aldehydes from food can be absorbed via the gut and can affect the determination of the MDA, especially in urine [52].

The results of a recent three-year longitudinal study suggest that serum levels of TBARS (measured by reverse-phase HPLC and spectrophotometric approaches) are strongly predictive of cardiovascular events in patients with stable coronary artery disease, independent of some risk factors (age, low-density lipoprotein, high-density lipoprotein, total cholesterol, triglyceride, BMI, and blood pressure) and inflammatory markers (C-reactive protein, soluble intercellular adhesion molecule-1, interleukin-6) [53].

F_2-Isoprostanes. F_2-Isoprostanes, especially 8-iso-$PGF_{2\alpha}$ (Fig.1), have been proposed as specific, reliable, and non-invasive markers of lipid peroxidation *in vivo* [52, 54]. F_2-Isoprostanes are a group of bioactive prostaglandin-like compounds generated via a non-enzymatic mechanism involving the free radical-initiated peroxidation of arachidonic acid *in vivo*, and they can be measured in most of the biological fluids, among which plasma and urine are the most commonly used samples. The short half-life of F_2-Isoprostanes in plasma limits their practical use, but this is not the case in urine [6]. F_2-Isoprostanes can also be detected in exhaled breath condensate and induced sputum. The MS techniques (GC-MS, GC- MS/MS, LC-MS, LC-MS/MS) can accurately and sensitively measure F_2-Isoprostanes in biological samples [52]. Immunoassays {ELISA, radio immuno assays (RIA)} are also frequently used techniques to quantify

F_2-Isoprostanes because of their low cost and ease of use, although there is limited information on their precision and accuracy [54]. F_2-Isoprostanes are stable in isolated samples, and the samples should be stored at −70 °C to prevent artifactual formation of isoprostanes products [6]. Unlike MDA, the levels of F_2-Isoprostanes are not influenced by lipid content in the diet [55].

F_2-Isoprostanes can also be detected in normal human biological fluids and tissues [54]. The increased levels of 8-iso-$PGF_{2\alpha}$ in plasma, tissue, and urine have been found in many human disorders and have been suggested to play a causative role in oxidative damage in diseases like cardiovascular disease, allergic asthma, hepatic cirrhosis, scleroderma, and Alzheimer's disease [54, 56].

Oxidative Modification of Sugars

Advanced glycation end-products (AGEs) are products of non-enzymatic glycation of proteins by reducing sugars (the Maillard reaction). It has been reported that AGEs accumulate in plasma and tissues with age, diabetes, renal failure, and Alzheimer's disease [1, 57, 58], and they have been considered potentially useful biomarkers for monitoring glycemic control, predicting the risk of diabetes-associated clinical complications, and monitoring the treatment effect of diabetic patients with retinopathy, nephropathy, and neuropathy [57].

Carboxymethyllysine (CML) and pentosidine (Fig. 1) are products of oxidation-accompanied glycation and have been regarded as representative biomarkers of AGEs [56]. In comparison with healthy subjects, serum levels of CML and pentosidine are about 3- and 10-fold higher in diabetic patients with decreased renal function, respectively [60, 61], and the serum levels of AGEs increase with the severity of glomerular lesions in patients with diabetic nephropathy [62] and with the severity of diabetic retinopathy [63]. These findings suggest that AGEs may be a clinically useful tool for assessing diabetic complications. A recent population-based 18-year follow-up study also showed that serum levels of AGEs could predict mortality from cardiovascular disease and coronary heart disease in nondiabetic women [64].

HPLC, GC-MS, ELISA, and immunohistochemistry are commonly used methods for analysis of AGEs. The accuracy and reproducibility of these techniques have not been well examined because there is no universally established unit of measurement for comparing study findings from different laboratories [59, 65]. In addition, confounding factors such as food and tobacco smoke can affect the level of AGE precursors in the body [66, 67].

The Integration of Biomarkers with Epidemiology

Many biomarkers have been developed for the identification of oxidative/nitrosative damage to DNA, proteins, lipids, and sugars in biological samples. Whether these biomarkers are truly useful in the early detection and prevention of diseases requires further validation via human field investigations. There has been a growth in application of biomarkers to epidemiological studies. Such an integrated approach has resulted in a new discipline, molecular epidemiology, which may provide specific information concerning the causal relation of biomarkers with disease outcomes in a population level, ultimately contributing to the development of strategies for health risk assessment and disease prevention [5, 68, 69].

In the literature, a cross-sectional study design has been commonly employed to examine the relation between biomarkers of oxidative/nitrosative stress and diseases. Cross-sectional studies can be easily and rapidly accomplished, but they can only provide information on the association between the biomarkers and some diseases. Whether there are any causal relations between biomarkers and the diseases should be examined by a prospective cohort study [68, 70]. This approach is also the optimum epidemiological study design for biomarker validation, through which the alteration of biomarker values on the course of diseases and the response of biomarkers to the intervention trials could be directly observed, although such a study will require large numbers of participants, an appropriate follow-up period, and high cost. Recently, the use of meta-analysis to re-evaluate published data from many small clinical studies has been considered an efficient approach to obtain information on the effectiveness of intervention trials [5], and a checklist containing specifications for reporting meta-analyses of observational studies (cohort, cross-sectional, and case-control studies, *etc.*) in epidemiology has been

proposed [71].

Application of Biomarkers of Oxidative/Nitrosative Stress to Intervention Trials

The ultimate goal of developing ideal biomarkers for oxidative/nitrosative damage is to find better tools for the prevention of diseases (Fig. 2). In intervention trials, close monitoring of the alteration of biomarker levels in biological samples may provide important information on which antioxidants and at what dose(s) oxidative/nitrosative damage can be reduced in study subjects with the aim of finding a safe and reliable antioxidant treatment [37, 72]. Many antioxidants from natural products are known to be capable of decreasing oxidative/nitrosative damage *in vitro*, but determining whether they will act the same way *in vivo* will require more convincing evidence from animal and human studies [72–74]. Some studies have demonstrated short-term antioxidant effects against oxidatively/nitrosatively damaged DNA, protein, and lipid peroxidation *in vivo* [41, 74–76], but long-term effects remain to be elucidated.

Several antioxidant interventional trials in humans have showed controversial results, such as the effects of vitamin E on cardiovascular outcomes [77, 78] and of beta-carotene and vitamin A on lung cancer [79], and the reduction by vitamin C supplementation of certain types of oxidative protein damage in subjects with low basal antioxidant but not in those with normal basal level [75]. Moller *et al.* recently have reviewed 139 cross-sectional and intervention studies regarding the effect of antioxidants on oxidatively damaged DNA and have found that many of the studies were "of mediocre value because of problems with design or high baseline DNA damage values" [41]. They also found it impossible to analyze such studies by a meta-analysis because of the great differences in design, biomarkers, and antioxidant supplementation among them. Clearly, the protective effects of antioxidants against human disease still need evidence-based confirmation by well-designed, randomized, controlled epidemiological studies in various study populations.

Conclusions

In the last 2 decades, there has been great progress in the development of biomarkers of oxidative/nitrosative stress that may eventually be useful in disease prevention. The challenges for the future are (1) to validate available biomarkers for oxidative/nitrosative damage in animal and human studies based on their specificity, stability for storage, reproducibility, causal relation with disease, and response to antioxidant intervention; (2) to examine the basal levels of oxidative/nitrosative damage in healthy subjects; and (3) to assess the long-term effect of antioxidants on oxidative/nitrosative damage by well-designed, randomized, controlled trials in humans and as well as to examine the consistency of the findings among various studies. The biomarkers of oxidative/nitrosative damage, if validated, may open the way for

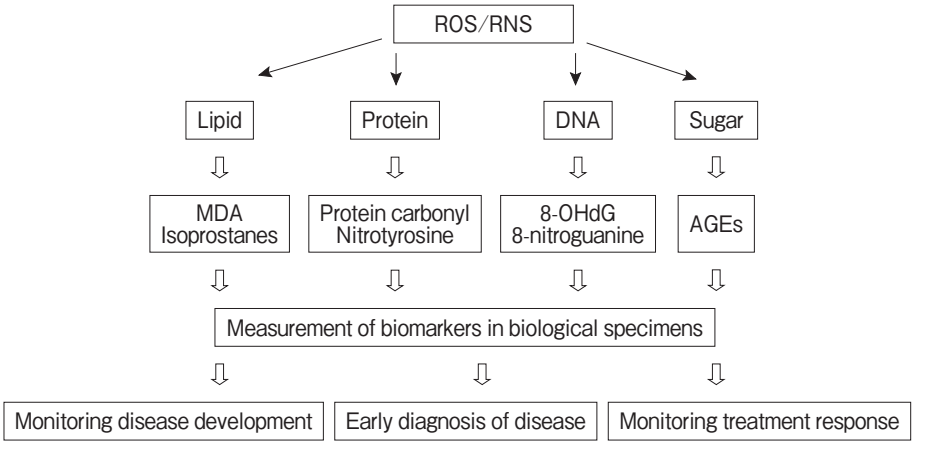

Fig. 2 Potentialities of oxidative/nitrosative stress-related biomarkers for disease prevention.

the development of early detection and prevention strategies for oxidative/nitrosative stress-associated human diseases.

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