Chapter 7

Biomarkers in ROS and Role of Isoprostanes in Oxidative Stress

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Abstract

Biomarkers of reactive oxygen species serve as indicators of oxidative stress in the pathology of cardiovascular diseases. This chapter presents an overview of the various biomarkers available to quantify oxidative stress to advance the understanding of the pathophysiology of cardiovascular diseases as well as to serve as an adjunct in their diagnosis and prognosis. The plasma levels of reactive oxygen species themselves are unstable and unreliable markers of oxidative stress. The commonly used stable biomarkers are derivatives of oxygen radicals such as products of lipid peroxidation and protein oxidation, with isoprostanes and malondialdehyde (MDA) being the most widely used biomarkers due to higher specificity and ease of measurement. Recently, micro-RNA is emerging as stable and specific biomarkers for detection of heart failure. Other biomarkers have a role in certain conditions; for example, advanced oxidation protein products indicate acute inflammation, whereas advanced glycation end products serve as indicators of chronic disease.

Keywords: biomarkers, reactive oxygen species, isoprostanes, lipid peroxidation, cardiovascular diseases

1. Introduction

Reactive oxygen species (ROS) are formed as by-products of cellular activity or cellular metabolism or cellular respiration. They have useful function-serving roles in cell signaling, cell differentiation, cell immunity, etc., when present in low concentrations, all of which are important in maintaining the body's physiological functions known as redox signaling [1].

Their concentration is controlled by the various antioxidants produced in the body such as superoxide dismutases, catalase, and glutathione peroxidase, with the goal to keep ROS concentration low [2]. Oxidative stress is a condition resulting from excessive reactive oxygen species due to either increased production or inadequacy of antioxidants to eliminate them. This increase in ROS results in damage to the cell which includes oxidizing lipids, nucleic acids, and proteins, thus leading to a change or loss in their function and ultimately causes cell death by apoptosis or necrosis. Due to this effect on the cells, oxidative stress has been implicated in aging [3] as well as many diseases including but not limited to cardiovascular disease [4], neurodegenerative diseases [5, 6], cancer [7], and diabetes [8].

Biomarkers are measurable characteristics of a biological condition; in this case, biomarkers of ROS serve as indicators of oxidative stress and how it influences a given disease. Hallmarks of a good biomarker are sensitivity, specificity, ease of obtaining and measuring samples, and cost-effectiveness. The quantification of oxidative stress with biomarkers is important not only in understanding the pathophysiology of cardiovascular disease but also in the diagnosis, prognosis as well as in designing new therapeutic measures for individual intervention. Oxidative stress plays a major role in the pathology of cardiovascular disease. In the heart, oxidative stress results in the inhibition of Na+-K- pump [9]. The mitochondrial electron transport chain and enzymes xanthine oxidase and nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase are the main producers of ROS. Risk factors and conditions which predispose to a cardiovascular event such as smoking, hypertension, atherosclerosis, hypercholesterolemia, diabetes, and obesity increase the effect of these enzymes which results in an increased production of ROS [10, 11].

2. ROS as biomarkers

ROS themselves act as biomarkers, their plasma levels being indicators of ROS production. A method known as the reactive oxygen metabolites (ROM) kit is used to measure total oxidative stress and measures superoxides (02-) and hydroxyl radical (HO) as well as hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2) [12] among others. This kit measures reactive oxidants in biological fluids and has been used to assess oxidative stress in animals such as in a study on ewes done by Rizzo et al. [13]. Cytochrome C reduction has been used in many studies to measure the production of superoxide (O_2^-) in the atrium [14], mouse aorta [15], and vessels [16]. Chemiluminescent probes which release photon when in contact with ROS can be detected and used for various ROS measurements with the lucigenin-enhanced chemiluminescence being the most commonly used to understand the way superoxide and diseases related to the cardiovascular system are affected in tissues [10]. Electron spin resonance detects free radical by the presence of its unpaired electron. Reactive radicals are detected by addition of probes [10]. As of 2003, the spin traps were not fit to be used in humans due to the potential for toxicity, but they can be used on tissues and body fluids; for example, PBN was used to show free radicals in coronary sinus blood during bypass surgery [17]. Aromatic traps for free radicals such as salicylate have been used in studies to detect superoxide in myocardial infarction [18]. High levels of dityrosine, an oxidation product of ROS, have been used to

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demonstrate the role of oxidative damage in atherosclerotic plaques [19]. Even though each reactive oxidant can be measured individually, they have drawbacks of being too costly and time-consuming. Their property of being inherently unstable with short half-lives of merely seconds, both of which combined with the antioxidants in the circulation, results in very low intracellular concentration of ROS thus making them unreliable markers of ROS.

The derivatives of oxygen radicals such as products of lipid peroxidation and protein oxidation on the other hand are stable and thus are more commonly used to measure the presence of ROS. The serum derivatives are new biomarkers of ROS which are mainly indicators of hydroperoxide levels produced by lipid peroxidation and have been shown to be high in atrial fibrillation [20]. The diacron reactive oxygen metabolites (dROM) test is an inexpensive analysis which measures ROS in both serum and plasma.

3. Peroxidation of lipids biomarkers

Lipids, especially polyunsaturated lipids, are more susceptible to oxidative damage due to the presence of many double bonds in their molecular structure [21], and thus, the indicators of lipid peroxidation are important indicators of free radicals. The presence of biomarkers in cardiovascular disease confirms the hypothesis that lipid peroxidation contributes to the development of cardiovascular diseases. There are many biomarkers of lipid peroxidation—MDA and isoprostanes being the most widely used. Others are lipid hydroperoxides, oxysterols, and oxidation resistance assays.

4. Isoprostanes

More accurate biomarkers of lipid peroxidation are isoprostanes along with its metabolites as stated in a study done by the National Institute of Health (NIH) [22]. In 1990, Roberts and Morrow discovered F2-isoprostane formed by the peroxidation of arachidonic acid [23] which is polyunsaturated fatty acid found in the cell membrane phospholipids and is one of the many targets of ROS. They are specific indicators of lipid peroxidation both *in vitro* and *in vivo* [24] and are stable compounds which are formed in large quantities *in vivo* following oxidative damage such as with CCl4 which is a producer of free radicals [25] as well as having detectable amounts present even in non-injured tissue making them reliable as a biomarker of ROS [26]. Isoprostanes are excellent biomarkers and have numerous advantages over other biomarkers of oxidative stress; they are chemically stable markers and are formed *in vivo.* They are specific to lipid peroxidation and are not affected by the dietary lipid content [27]. Since they are detectable in biological fluids, they have the significant advantage over other oxidative stress markers due to the ease of measuring them since they can be measured by noninvasive methods extracellularly in the urine, plasma, and tissue [27, 28]. High levels of F2-isoprostanes are found in many human diseases such as coronary heart disease [29], obesity, cancer, and even genetic disorders [30]. They are shown to be increased in risk factors such as smoking,

obesity, hypercholesterolemia, and myocardial ischemia reperfusion [31–33] as well as in atherosclerosis [34]. They are also observed in bypass [35] and angioplasty. The extent of lipid peroxidation can be measured by calculating the level of F2-IsoPs which is esterified in phospholipids due to them being initially formed esterified and subsequently gain their free form [27]. In humans, the two major metabolites of isoprostane which are detectable in the urine are 2,3-dinor-15-F2t-IsoP and 2,3 dinor-5,6-dihydro-15-F2t-IsoP [36]. Elevated levels of 8-isoprostane in the plasma and urine are also observed in cardiovascular disease [37]. There are many other derivatives identified [38]. The method for detecting isoprostanes is gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), but drawbacks in using mass spectrometry is the expense and time required for measurement [22]. ELISA-based isoprostane detection is currently most reliable indicator of lipid oxidation [39]. There are commercially available kits to measure them using the sandwich ELISA method where the isoprostanes in biological samples compete with isoprostane conjugated with an enzyme to bind to an isoprostane-specific antibody present in the microplate. The activity of the enzyme results in increased intensity of color development with increased amount of conjugated isoprostane bound. Isoprostanes are biomarkers of choice, but since the results obtained by these two methods do not corroborate, the result of the immune assays is yet to be given clinical validation [40]. There have been studies showing the variation in isoprostane levels according to the time of day, and thus, this needs to be taken into account when further research is conducted [41, 42].

5. MDA

Malondialdehyde (MDA) is a ketoaldehyde which is produced as an end product of polyunsaturated fatty acid and is found in increased concentration in tissue injury. It forms a red pigmentation when it reacts with thiobarbituric acid. This thiobarbituric acid-reactive substance (TBARS) assay can be used to measure lipid peroxidation using spectrophotometry. In relation to cardiovascular disease, elevated levels of MDA are associated with smoking [43]. They are seen to be elevated with the progression of atherosclerosis [44] and are predictors of future cardiovascular events in patients with coronary artery disease [45]. Limitations of this method include low specificity since TBARS includes many other products of lipid peroxidation other than MDA [46], tendency for inaccurate results due to the varying results generated with different assay conditions used as well as the production of artifacts due to the fact that the MDA measured is mostly generated *in vitro* [47] with most of the MDA formed due to the high temperature used in the procedure [48]. Now, high-pressure liquid chromatography (HPLC) is preferred over TBARS due to higher specificity [49]. Commercially available ELISA kits are also available for MDA detection which show better specificity [50].

6. Isolevuglandins

Isolevuglandins (IsoLGs) are also produced due to oxidation of arachidonic acid, but unlike isoprostanes, they are highly reactive and react with primary amines for example phosphati-

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dylethanolamine to form lactam and hydroxylactam. The unreacted isolevuglandins are not detected in the tissues or cells. They have been implicated in many disease processes such as atherosclerosis and neurodegenerative diseases [51]. Though methods such as mass spectrometry and immunohistochemical studies have shown increased levels of IsoLG, there is still not enough evidence connecting them with severity of a disease or of their use in predicting the onset of disease. Further studies need to be conducted to determine the utility of IsoLG as clinical biomarkers.

7. Oxidation of proteins biomarkers

7.1. Myeloperoxidase (MPO)

MPO is an enzyme found in inflammatory cells such as macrophages and neutrophils. It generates ROS by the conversion of hydrogen peroxide to hydroxy radical (OH), nitric oxide (ONOO-NO2), and hypochlorous acid (HOCl) and is a proinflammatory agent responsible for the oxidation of low-density lipoprotein (LDL) [52]. It is found in abundance in the atherosclerosis plaques [53] and coronary artery disease where it can serve as an inflammatory marker for both the risk of CAD and its existence [54]. MPO concentration is measured in biological samples by ELISA which is commercially available. Its function is measured by spectrophotometry by peroxidase activity assays such as measuring the formation of guaiacol oxidation products [55]. Its levels in the serum can predict risk for acute coronary syndromes [56], for risk of cardiovascular event in patients with chest pain [57] and increased risk of coronary artery disease in seemingly healthy population [58]. It is prone to varying and unreliable results due to the fact that the values are altered during the process of collection and handling as seen in a study done in 2008 by Shih et al. where it was determined that the concentration of MPO varied depending on the collection tube used and the presence of heparin in the patient serum [59]. A concern in the measurement of MPO is the artificial release of MPO from the neutrophils leading to false results showing an increase in MPO. In their study, Shih et al. used nine different types of tubes containing EDTA, citrate plasma, and heparin samples and serum samples. The level of MPO varied in all these tubes, with EDTA and citrate samples showing the lowest concentration and heparin and serum samples showing 10 and 100% higher values, respectively. This suggests that the serum levels of MPO are higher due to their release from leukocytes during coagulation. It has previously been shown by Li et al. that heparin leads to release of MPO from neutrophils during neutrophil activation [60].

8. Growth differentiation factor-15

It is a cytokine expressed in many cells including cardiomyocytes [61]. It increases in many cardiovascular diseases such as atherosclerosis [62] and heart failure [63]. It has been studied with respect to the progress and outcome of disease since it has a protective role in the heart

[64] such as against ischemia reperfusion injury [61] and acute myocardial infarction [65] making it a useful biomarker in clinical settings though more research still needs to be conducted.

9. Oxidized low-density lipoprotein (OxLDL)

The use of OxLDL as a biomarker of oxidative stress in cardiovascular diseases has been reported due to its ability to promote lipid deposition. The oxidation of LDL is linked to the pathology of atherosclerosis by immunohistochemical staining apolipoprotein B-100 [66]. It is thought to be formed by activated platelets [67]. High-density lipoproteins (HDL) lead to decreased activation of platelets since it competes with them to bind to oxidized LDL protecting against the development of atherosclerosis [68]. Circulating OxLDL is already proven to be able to predict the presence of atherosclerosis [69] and coronary artery disease [70]. OxLDL is detected by immune assays in plasma. According to Trpkovic et al., there are currently three ELISA assays namely 4E6, E06, and DLH3 developed to detect OxLDL in the blood [71]. Out of these, 4E6 binds to LDL but also detects native LDL and the other two, DLH3 which measures LDL and E06 are used for oxidized phosphatidylcholine [72]. A drawback of E06 method is its non-specificity to oxidized lipids. In 2001, Holvoet et al. measured circulating LDL levels by ELISA using monoclonal antibody 4E6 which detected higher number of circulating OxLDL in patients with coronary artery disease [73].

10. Allantoin

Over the years, allantoin has emerged as a reliable biomarker of oxidative damage both *in vivo* and *in vitro*. It is formed by the ROS-induced oxidation of uric acid [74], where uric acid is converted to allantoin due to overproduction of ROS [75]. In relation to cardiovascular diseases, increased levels of allantoin in the plasma have been shown in people with type 2 diabetes [75] as well as in heavy smokers [76] both of which are risk factors for developing cardiovascular disease. It has been shown to be increased in the plasma in oxidative stress-related chronic heart failure [77]. The use of allantoin as a widely applied biomarker is limited due to the difficulty of measuring allantoin in the body fluids [78]. The most specific and sensitive method for its measurement is liquid chromatography [79].

11. Protein carbonyls

Oxidation of protein amino acid residues leads to the formation of protein carbonyls by different means such as deamination of glutamic acid and lysine or due to the resulting breakage of protein backbone [80]. They are stable compounds formed early and are usually higher in concentration due to their multiple sources, making them good biomarkers due to the ease of detection as well as no need of expensive equipment. It is a commonly used pro-

tein oxidation marker, and there are various assays for its detection. They have been shown to increase with age implicating them in the process of aging [81]. They have even been reported in the human heart following coronary surgery [82]. Assay has been done to observe them in dilated cardiomyopathy [83]. In 1990, Levine et al. were the first ones to determine various methods to measure carbonyls in oxidized protein [84].

A highly sensitive assay is protein carbonyl content (PCC) which has various modifications but in all of them 2,4-dinitrophenylhydrazine (DNPH) reacts with the protein carbonyls and forms its 2,4-dinitrophenyl (DNP) hydrazone which is stable and can then be optically measured by immunohistochemistry or by radioactive counting [85]. Spectrophotometric assay can be employed due the ability of this hydrazone product to absorb ultraviolet light which when coupled with high-performance liquid chromatography, in short HPLC, makes the measurement more specific and sensitive [86]. One sensitive method is to detect carbonyls by first labeling them with tritiated sodium borohydride then separating with SDS-PAGE [87] or by reducing with tritiated sodium borohydride in solution [88]. An important limitation of carbonyl measurement is that there are different protocols used by researchers leading to variable levels of carbonyls in tissues.

12. Advanced oxidation protein products (AOPPS)

Advanced oxidation protein products (AOPPs) are the end products of free radical affected proteins. They have been shown to be linked to many human diseases such as diabetes mellitus [89], coronary artery disease [90], and chronic renal disease [91] among others, and since they have been shown to produce oxidative stress in inflammatory conditions [92], they serve to indicate acute inflammation.

13. Advanced glycation end products (AGES)

They are molecules which are formed as a result of the reaction between reducing sugars and amino groups. Their concentration tends to increase in conditions of oxidative stress. The two main advanced glycation end products are pentosidine and carboxymethyl valine which result from a process known as glycoxidation where the amino acids lysine and arginine react with carbohydrates as well as the oxidizing effect of ROS on polyunsaturated fatty acids. A precursor of carboxymethyl valine known as glyoxal is formed when RNase incubates with arachidonate [93]. The presence of AGES has been shown in diseases such as diabetes mellitus and obesity among others [94]. They also have a role in diabetic heart failure as shown by Brouwers et al., where they overexpressed glyoxalase-I, a glycation precursor detoxifying enzyme in order to reduce AGES, and found that it leads to prevention of diabetes-induced oxidative damage in the heart [95]. They are detected after derivatization with 2, 4-dinitrophenylhydrazine (DNP). The hydrazone formed is then detected using a spectrophotometer or by using anti-DNP antibodies with along with ELISA [96] or by high-performance liquid chromatography (HPLC) or by Western blot or immunohistocytochemistry

[97]. Out of these methods, HPLC is more specific and can measure carboxymethyl lysine CML [98] and pentosidine [99]. They mainly serve as indicators of chronic diseases [100].

14. Glutathione and glutathione disulfide

Reduced glutathione (GSH) is present in large quantities in the cells and acts as an inhibitor of lipid peroxidase. Glutathione disulfide (GSSG) is the oxidized form of glutathione. Protein glutathionylation regulates cardiovascular function [101]. Its values have been shown to increase in ischemia reperfusion injury [102], atherosclerosis [103], and cardiac hypertrophy [104]. In patients with atherosclerosis obliterans, increased glutathionylation is shown to be related to the progression of the disease proving to be a biomarker at early stage [105]. The ratio of GSH and GSSG is used as an indicator of ROS due to the fact that there occurs a decrease in GSH and increase in GSSG concentration in oxidative stress [106]. There are a number of methods to detect protein s-glutathionylation. Quantifying the total amount of s-glutathionylated proteins is by measuring fluorescence [107]. Labeling glutathione is a method for glutathionylation analysis such as 35s radiolabeling [108], though it is not very sensitive and can only be used in cell culture; furthermore, it cannot detect proteins which have already undergone glutathionylation. Biotinylated glutathione either reduced or oxidized is superior to the 35s labeling methods, it detects only glutathionylated proteins thus is specific plus it can be analyzed by multiple methods such as fluorescence microscopy [109] or immunoblotting using biotin antibodies [110]. One drawback of this method is that the presence of biotin tag on glutathione may have an effect on the protein function. Anti-glutathione antibodies allow the detection of glutathionylated proteins in physiological conditions. Studies done with antibodies are by using mouse monoclonal antibodies [111–113]. Drawback of antibody method is the lack of specificity, and it can only detect a few proteins in total extract [109] limiting its utility in detecting glutathionylated proteins on a large scale. Recently, the use of liquid chromatography-couple mass spectrometry using whole proteins is found to be a good method to identify proteins in larger numbers [114].

Recently, the role of micro-RNA (miRNA) in the generation of ROS and its consequences such as inflammation, angiogenesis, cell proliferation, and apoptosis has been a subject of research. They are found intracellularly and outside cells in body fluids [115]. They are stable and specific such as miR-499 miRNA for the heart. Another advantage of miRNA as a biomarker is that they are not affected by posttranslational modifications. They can also be easily assessed by methods like polymerase chain reaction (PCR) and microarrays. PCR is an expensive method which detects small quantities of miRNA, but the results are affected by the primer used. Microarray measurements require the development of probes and can thus be useful in that many RNAs can be detected at the same time [116]. Other less used methods are direct sequencing by next-generation sequencing [117], which eliminates the influence of primers as in the case of PCR but is still not used widely because of expense. Stem loop probe ligation [118] and Northern blotting are other methods which may be used to measure concentration of miRNA. The miRNA found most abundantly in the heart is mR-1 which is heart specific

and can be used as a sensitive and specific marker for diagnosis of acute myocardial infarction [119, 120].

Elevated miRNAs specifically miR423-5p has also been observed in heart failure patients making them important clinical biomarkers in the diagnosis of heart failure [121]. Although miRNA measurement has shown promise, there are still various issues that need to be addressed. The concentration of miRNA in body fluids is low making its isolation rather difficult. The values obtained also tend to be different in different body fluids which need to be normalized. Therefore, it is necessary to develop a method to obtain accurate results with miRNA measurement across the various samples [116]. The product of DNA damage, 8 dydroxy-2'-deoxyguanosine urinary levels, seems to be elevated in dilated cardiomyopathy [122]. It is also evidently a predictor of future events following myocardial infarction [123]. Other specific biomarkers have also been studied but are not yet studied as extensively as the above biomarkers. Ascorbic acid is an endogenous antioxidant which has been linked to unstable coronary syndrome where it is thought to have an effect on the lesion [124]. Glutathione peroxidase-I is evidently decreased in coronary artery disease patients [125] which is an antioxidant enzyme. Low levels of bilirubin have been linked to cigarette smoking and increased levels of triglycerides and cholesterol making it a potential biomarker of cardiovascular disease [126]. Oxidative bilirubin metabolites called biopyrrins are elevated in the urine in patients with heart failure [127] and are thought to be predictors of future cardiac events in acute myocardial infarction [128].

Several biomarkers of oxidative stress have been studied over the years in an effort to understand the mechanism of cardiovascular generation with the intention to use the information by targeting oxidative stress with cardio-protective drugs. Further research into understanding the mechanism of ROS generation and their role in therapeutic intervention will be beneficial for the management of cardiovascular diseases.

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