Biomarkers, November 2005; 10(Supplement 1): S24–S29

Taylor & Francis
Taylor & Francis Group

Isoprostanes and other markers of peroxidation in atherosclerosis

PAOLA PATRIGNANI & STEFANIA TACCONELLI

Department of Medicine and Center of Excellence on Aging, 'G. d'Annunzio' University and 'G. d'Annunzio' University Foundation, Chieti, Italy

Abstract
Several lines of evidence suggest that reactive oxygen species play a role in the development of vasculopathies, including those that define atherosclerosis, hypertension and restenosis after angioplasty. Confused picture emerging from prospective clinical trials of anti-oxidants may reflect inadequacy of traditional indices of lipid peroxidation in the recruitment of appropriate patients and in guiding the selection of the appropriate dose of anti-oxidant to be tested. *Ex vivo* indices of oxidant stress could have questionable veracity in assessing the actual rate of lipid peroxidation *in vivo*. The measurement of F_2-isoprostanes (F_2-iPs), formed non-enzimatically through free radical catalysed attack on esterified arachidonate, provides a reliable tool for identifyng populations with enhanced rates of lipid peroxidation. Enhanced formation of F_2-iPs, together with increased *in vivo* platelet activation, has been reported in association with several cardiovascular risk factors. Thus, it has been suggested that F_2-iPs may transduce oxidant stress-dependent platelet activation. Measurements of 8-iso-$PGF_{2\alpha}$, an abundant F_2-iP formed *in vivo*, in urine may provide sensitive biochemical end-points for the assessment of the oxidant status of the patient and the true efficacy of anti-oxidant therapies. The incorporation of such biochemical end-points in clinical trials may help to verify the reliability of the oxidative modification hypothesis in the development of atherosclerosis.

Keywords: *Reactive oxygen species, isoprostanes, lipid peroxidation, atherosclerosis*

Introduction

Several lines of evidence suggest that oxidative stress may promote endothelial dysfunction through increased production of reactive oxygen species (ROS). Increased levels of diverse ROS are produced in the vessel wall and they individually or in combination may contribute to the pathogenesis of vascular disease.

The family of ROS includes highly bioactive, short-living molecules that are derived from reduction of molecular oxygen. Multiple enzyme systems use different substrates as sources of electrons to produce a variety of ROS, the most important of which are nitric oxide ($NO\cdot$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). Many enzyme systems, including NAD(P)H oxidase, xanthine oxidase and uncoupled nitric oxide synthase (NOS) among others, contribute to production as well as to degradation of ROS (Wassmann et al. 2004). Superoxide results from one

Correspondence: Paola Patrignani, PhD, Sezione di Farmacologia, Dipartimento di Medicina e Scienze dell'Invecchiamento, Università di Chieti 'G. d'Annunzio', c/o Palazzina delle Scuole di Specializzazione, Via dei Vestini 31, 66013 Chieti, Italy. Tel: +39-0871-3556775. Fax: +39-0871-3556718. Email: ppatrignani@unich.it

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500500215084

electron reduction of oxygen by a variety of oxidases and acts either as an oxidizing agent, where it is reduced to H_2O_2 by superoxide dismutase (SOD) or as a reducing agent, where it donates its extra electron (e^-) to form $ONOO^-$ with NO. $ONOO^-$ is an important mediator of lipid peroxidation and protein nitration, including oxidation of low density lipoprotein (LDL), which has dramatic proatherogenic effects. Hydrogen peroxide H_2O_2 can be scavenged by catalase, glutathione and thioredoxin systems and can also be reduced to generate $\cdot OH$ in the presence of Fe^{2+}. $NO\cdot$ is a crucial mediator of endothelium-dependent vasodilation and may also play a role in platelet aggregation and in maintaining the balance between smooth muscle cell growth and differentiation (Griendling and FitzGerald 2003a). Oxidative stress may lead to many cellular events, such as inactivation of NO, oxidative modifications of DNA and proteins, lipid oxidation, enhanced mitogenicity and apoptosis of vascular cells and increased expression and activation of redox-sensitive genes, such as the receptor for oxidized LDL, adhesion molecules, chemotaxis factors, proinflammatory cytokines, regulators of cell cycle progression and matrix metalloproteinases (Griendling and FitzGerald 2003b, Touyz and Schiffrin 2004). The biological effects of ROS and impaired NO bioactivity in vascular cells contribute to the development and progression of atherosclerosis at all stages of the disease.

Monitoring ROS formation

In vitro studies unequivocally demonstrate that all vascular cells produce ROS and that ROS mediate diverse physiological functions in these cells (Griendling et al. 2000). The short half-life of these species makes them ideal signalling molecules, but it also confounds their measurement in complex biological systems. Traditional approaches to the assessment of oxidative stress in humans relied on *ex vivo* indices such as the lag time to oxidation of LDL and *in vivo* indices of questionable veracity such as thiobarbituric acid reactive substances and in plasma malondialdehyde (Table I). Recently, attention has focused on the development of *in vivo* biomarkers of ROS generation. Oxidative stress can result in modification of lipids, protein and DNA (Lorch et al. 2002, Roberts and Morrow 2002). Mass spectrometry has also been used to identify oxidized amino acids in inflammatory lesions (Heinecke 2002) and in plasma and urine (Greenacre and Ischiropoulos 2001, Heinecke 2002). Quantitative methods for assessing oxidative modifications of DNA are developing. The compound 8-hydroxy-2′-deoxyguanosine is one of the most common markers for evaluating oxidative DNA damage and is a product formed by the specific attack of a hydroxyl radical on DNA (Nakae et al. 1997, Helbock et al. 1998). Several studies have suggested that oxidative DNA damage is increased in non-insulin-dependent diabetes mellitus (Fraga et al. 1990), renovascular hypertension (Higashi et al. 2002) and ageing (Leinonen et al. 1997). However, these methods continue to be refined and new biomarkers of DNA modification, such as 1,N-etheno-2′-deoxyadenosine and 1,N2-etheno-2′-deoxyguanosine, have begun to emerge (Griendling et al. 2000). Because plasma or urine measurement of F_2-isoprostanes, chemically stable, free radical–catalysed products of arachidonic acid, appears to provide a sensitive and specific index of non-enzymatic lipid peroxidation, it has been used extensively to assess *in vivo* lipid peroxidation in several human disease states (Patrono and FitzGerald 1997). Recently, a range of antibodies directed against oxidation-dependent epitopes in LDL (anti-oxLDL) have been developed for quantification of

Table I. Main characteristics of some laboratory methods for monitoring oxidative stress.

Product	Significance	Interpretation
Thiobarbituric acid reactive substance	Peroxidation of poliunsatured fatty acids are derivatized with thiobarbituric acid to yield a red compound measured colourimetrically	Low sensitivity and low specificity
Malondialdeide (MDA)	It is a byproduct of lipid peroxidation and is formed by β-scission of oxidied polyunsatured fatty acids. It is measured by HPLC or mass spectrometry	Low sensitivity; MDA is also a product of cyclooxygenase activity in platelets
Alkanes pentane and ethane gases	Intermediate or end products of lipid peroxidation. They are measured by gas-cromatography	They are also potential air pollutants and may be confounded by oxygen concentrations attained *in vivo*
Lag time to oxidation of LDL	It is the continuous measurement of the formation of conjugated diene at OD 234 nm. The concept of 'lag time', derived from such measurements, has been used to test the efficacy of various anti-oxidants for their ability to inhibit the oxidation of LDL	Conjugated dienes could be generated *ex vivo* following sampling
Oxidation products in isolated proteins	Proteins are major targets for radicals and other oxidants when these are formed *in vivo* either in intra- or extra-cellular environments. Oxidation products derive by a covalent modification of amino acids, determining a specific molecular mass variation in the products, easily detectable by mass spectrometric measurements	They are markers of oxidatively/ nitrosatively modified proteins
8-hydroxy-2′ deoxyguanosine	It is produced by a specific attack of a hydroxyl radical to DNA	It is a marker of oxidative DNA damage
F2-isoprostane	They are free radical-catalysed products of arachidonic acid	They are systemic and non-invasive marker of lipid peroxidation

lipid peroxidation in animal models and in humans (Tsimikas and Witztum 2001). The increased iPs generation in hypercholesterolemic mice correlates closely with titers of anti-oxLDL and both are depressed in a gene dose-dependent fashion by deletion of the 12/15-lipoxygenase, an animal model of atherosclerosis (Cyrus et al. 2001).

Isoprostanes

Since 1995, studies have been performed to investigate the formation of F_2-isoprostanes in clinical settings putatively associated with oxidant stress, supporting the concept that these compounds are reliable markers of *in vivo* lipid peroxidation (Roberts and Morrow 2002). Isoprostanes (iPs) are a family of prostaglandin (PG)-like compounds formed non-enzimatically through free radical catalysed attack on esterified arachidonate followed by enzymatic release from cellular or lipoprotein phospholipids. IPs are formed *in situ* in the phospholipid domain of cell membranes and circulating lipoproteins. Then, they are cleaved by phospholipases, released extra-cellularly, circulate and are excreted in urine. The measurement of F_2-isoprostanes (F_2-iPs), containing F-type ring analogous to prostaglandin (PG)$F_{2\alpha}$,

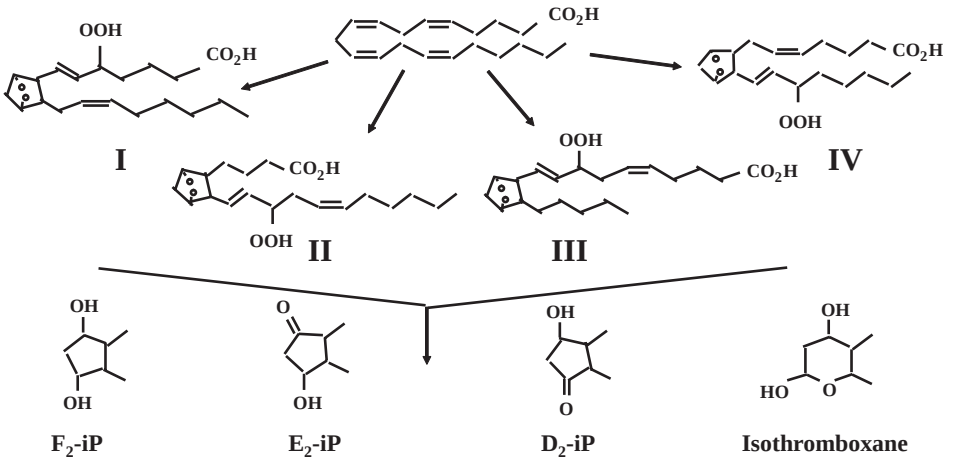

Figure 1. Products of isoprostane pathway of lipid peroxidation that are formed *in vivo*. Isoprostanes are formed in abundance *in vivo* by free radical catalysed peroxidation of arachidonic acid. This mechanism leads to the formation of bicyclic PGG_2-like endoperoxides that exist as four regioisomers (I–IV), each of which can be composed of a mixture of eight racemic diastereoisomers. Thus, the reduction of the bicyclic PGG_2-like endoperoxides undergo to rearrangement *in vivo* to form $PGF_{2\alpha}$-like compounds, that are isomeric to $PGF_{2\alpha}$, thus, they have been termed F_2-isoprostanes (F_2-IsoPs). Bicyclic PGG_2-like endoperoxides undergo also to rearrangement *in vivo* to form PGD_2-like compounds (D_2-IsoPs), PGE_2-like compounds (E_2-IsoPs) and thromboxane (TX)-like compounds (IsoTXs).

provides a reliable tool for identifyng populations with enhanced rates of lipid peroxidation. Levels of 8- iso-$PGF_{2\alpha}$ (also referred as $iPF_{2\alpha}$-III) (Rokach et al. 1997) are most frequently measured in human body fluids, such as plasma or urine. Both stable-isotope dilution assays using gas chromatography/mass spectrometry (GC/MS) and immunoassays for 8-iso-$PGF_{2\alpha}$ have been developed (Wang et al. 1995, Roberts and Morrow 2002). In addition to their potential usefulness as indexes of oxidant stress *in vivo*, 8-iso-$PGF_{2\alpha}$ is a vasoconstrictor and modulates platelet activation in response to other agonists. These biological effects on platelet function and vascular tone *in vivo* are mediated via interaction with receptor for TXA_2 (TP) in the mouse and do not depend on the existence of related but distinct iP receptors (Audoly et al. 2000). Altered generation of iPs has been reported in a variety of syndromes associated with oxidant stress: coronary ischemia-reperfusion syndromes, acute coronary syndromes, Alzheimer's disease, chronic obstructive pulmonary disease and cystic fibrosis; in association with several cardiovascular risk factors, including hypercholesterolemia, hyperhomocysteinemia, diabetes mellitus, hypertension and cigarette smoking (revised in Davì et al. 2004). Elevated levels of 8-iso-$PGF_{2\alpha}$ have been reported in pericardial fluid of patients with heart failure and some iPs, but not all, are increased in the urine of patients with severe heart failure (Mallat et al. 1998). Recently, urinary 8-iso-$PGF_{2\alpha}$ levels have been reported in conjunction with evidence of platelet activation in women, with android obesity (Davi et al. 2004) and in association with age and alcohol consumption (Griendling and FitzGerald 2003b). Lipid peroxidation is markedly increased in patients with renovascular hypertension as compared to patients with essential hypertension and comparable levels of blood pressure (Minuz et al. 2002, 2004), evidencing an important role of oxidative stress in

mediating the impact of angiotensin II on persistent abnormalities in endothelial and platelet function.

F_2-isoprostanes may be a signal transduction mechanism of oxidative stress-dependent platelet activation; in fact, enhanced 8-iso$PGF_{2\alpha}$ biosynthesis, shown in association with several cardiovascular risk factors, correlated with platelet activation as assessed by urinary excretion of 11-dehydro-TXB_2, an *in vivo* marker of TXA_2 biosynthesis (Davi et al. 2004). The finding of a linear correlation between the basal rate of 8-iso-$PGF_{2\alpha}$ excretion and the slope of changes in this index of lipid peroxidation as a function of changes in plasma vitamin E associated with short-term dosing with 600 mg d^{-1} in different clinical settings is consistent with the hypothesis that the basal rate of lipid peroxidation is a major determinant of the response to vitamin E (Patrignani et al. 2000). Thus, measurements of specific isoprostane in urine may provide sensitive biochemical end-points for the assessment of the oxidant status of patients and the true efficacy of anti-oxidant therapy.

Conclusion

Isoprostanes are emerging as a new class of biologically-active products of arachidonic acid metabolism of potential relevance to human disease. Their formation seems to reflect primarily, if not exclusively, a non-enzymatic process of lipid peroxidation *in vivo*. Besides providing a reliable, non-invasive index of lipid peroxidation, measurements of specific isoprostanes, such as 8-iso-$PGF_{2\alpha}$, in urine may provide sensitive biochemical end-points for dose-finding studies of natural and synthetic inhibitors of lipid peroxidation. The incorporation of such biochemical end-points in clinical trials may help to verify the reliability of the oxidative modification hypothesis in the development of atherosclerosis.

References

Audoly LP, Rocca B, Fabre JE, Koller BH, Thomas D, Loeb AL, Coffman TM, FitzGerald GA. 2000. Cardiovascular responses to the isoprostanes iPF(2alpha)-III and iPE(2)-III are mediated via the thromboxane A(2) receptor in vivo. Circulation 101:2833–2840.

Cyrus T, Pratico D, Zhao L, Witztum JL, Rader DJ, Rokach J, FitzGerald GA, Funk CD. 2001. Absence of 12/15-lipoxygenase expression decreases lipid peroxidation and atherogenesis in apolipoprotein E-deficient mice. Circulation 103:2277–2282.

Davi G, Falco A, Patrono C. 2004. Determinants of F2-isoprostane biosynthesis and inhibition in man. Chemistry & Physics of Lipids 128:149–163.

Fraga CG, Shigenaga MK, Park JW, Degan P, Ames BN. 1990. Oxidative damage to DNA during aging: 8-hydroxy-2′-deoxyguanosine in rat organ DNA and urine. Proceedings of the National Academy of Sciences (USA) 87:4533–4537.

Greenacre SA, Ischiropoulos H. 2001. Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. Free Radical Research 34:541–581.

Griendling KK, FitzGerald GA. 2003a. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. Circulation 108:1912–1916.

Griendling KK, FitzGerald GA. 2003b. Oxidative stress and cardiovascular injury: Part II: animal and human studies. Circulation 108:2034–2040.

Griendling KK, Sorescu D, Ushio-Fukai M. 2000. NAD(P)H oxidase: role in cardiovascular biology and disease. Circulation Research 86:494–501.

Heinecke JW. 2002. Oxidized amino acids: culprits in human atherosclerosis and indicators of oxidative stress. Free Radical Biology & Medicine 32:1090–1101.

Helbock HJ, Beckman KB, Shigenaga MK, Walter PB, Woodall AA, Yeo HC, Ames BN. 1998. DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. Proceedings of the National Academy of Sciences (USA) 95:288–293.

Higashi Y, Sasaki S, Nakagawa K, Matsuura H, Oshima T, Chayama K. 2002. Endothelial function and oxidative stress in renovascular hypertension. New England Journal of Medicine 346:1954–1962.

Leinonen J, Lehtimaki T, Toyokuni S, Okada K, Tanaka T, Hiai H, Ochi H, Laippala P, Rantalaiho V, Wirta O, Pasternack A, Alho H. 1997. New biomarker evidence of oxidative DNA damage in patients with non-insulin-dependent diabetes mellitus. FEBS Letters 417:150–152.

Lorch S, Lightfoot R, Ohshima H, Virag L, Chen Q, Hertkorn C, Weiss M, Souza J, Ischiropoulos H, Yermilov V, Pignatelli B, Masuda M, Szabo C. 2002. Detection of peroxynitrite-induced protein and DNA modifications. Methods in Molecular Biology 196:247–275.

Mallat Z, Philip I, Lebret M, Chatel D, Maclouf J, Tedgui A. 1998. Elevated levels of 8-iso-prostaglandin F2alpha in pericardial fluid of patients with heart failure: a potential role for *in vivo* oxidant stress in ventricular dilatation and progression to heart failure. Circulation 97:1536–1539.

Minuz P, Patrignani P, Gaino S, Degan M, Menapace L, Tommasoli R, Seta F, Capone ML, Tacconelli S, Palatresi S, Bencini C, Del Vecchio C, Mansueto G, Arosio E, Santonastaso CL, Lechi A, Morganti A, Patrono C. 2002. Increased oxidative stress and platelet activation in patients with hypertension and renovascular disease. Circulation 106:2800–2805.

Minuz P, Patrignani P, Gaino S, Seta F, Capone ML, Tacconelli S, Degan M, Faccini G, Fornasiero A, Salamini G, Tommasoli R, Arosio E, Santonastaso CL, Lechi A, Patrono C. 2004. Determinants of platelet activation in human essential hypertension. Hypertension 43:64–70.

Nakae D, Kobayashi Y, Akai H, Andoh N, Satoh H, Ohashi K, Tsutsumi M, Konishi Y. 1997. Involvement of 8-hydroxyguanine formation in the initiation of rat liver carcinogenesis by low dose levels of N-nitrosodiethylamine. Cancer Research 57:1281–1287.

Patrignani P, Panara MR, Tacconelli S, Seta F, Bucciarelli T, Ciabattoni G, Alessandrini P, Mezzetti A, Santini G, Scialli MG, Cipolline F, Davi G, Gallina P, Bon GB, Patrono C. 2000. Effects of vitamin E supplementation on F(2)-isoprostane and thromboxane biosynthesis in healthy cigarette smokers. Circulation 102:539–545.

Patrono C, FitzGerald GA. 1997. Isoprostanes: potential markers of oxidant stress in atherothrombotic disease. Arteriosclerosis Thrombosis Vascular Biology 17:2309–2315.

Roberts LJ, Morrow JD. 2002. Products of the isoprostane pathway: unique bioactive compounds and markers of lipid peroxidation. Cellular & Molecular Life Sciences 59:808–820.

Rokach J, Khanapure SP, Hwang SW, Adiyaman M, Lawson JA, FitzGerald GA. 1997. Nomenclature of isoprostanes: a proposal. Prostaglandins 54:853–873.

Touyz RM, Schiffrin EL. 2004. Reactive oxygen species in vascular biology: implications in hypertension. istochem Cell Biology 122:339–352.

Tsimikas S, Witztum JL. 2001. Measuring circulating oxidized low-density lipoprotein to evaluate coronary risk. Circulation 103:1930–1932.

Wang Z, Ciabattoni G, Creminon C, Lawson J, Fitzgerald GA, Patrono C, Maclouf J. 1995. Immunological characterization of urinary 8-epi-prostaglandin F2 alpha excretion in man. Journal of Pharmacology & Experimental Therapy 275:94–100.

Wassmann S, Wassmann K, Nickenig G. 2004. Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. Hypertension 44:381–386.