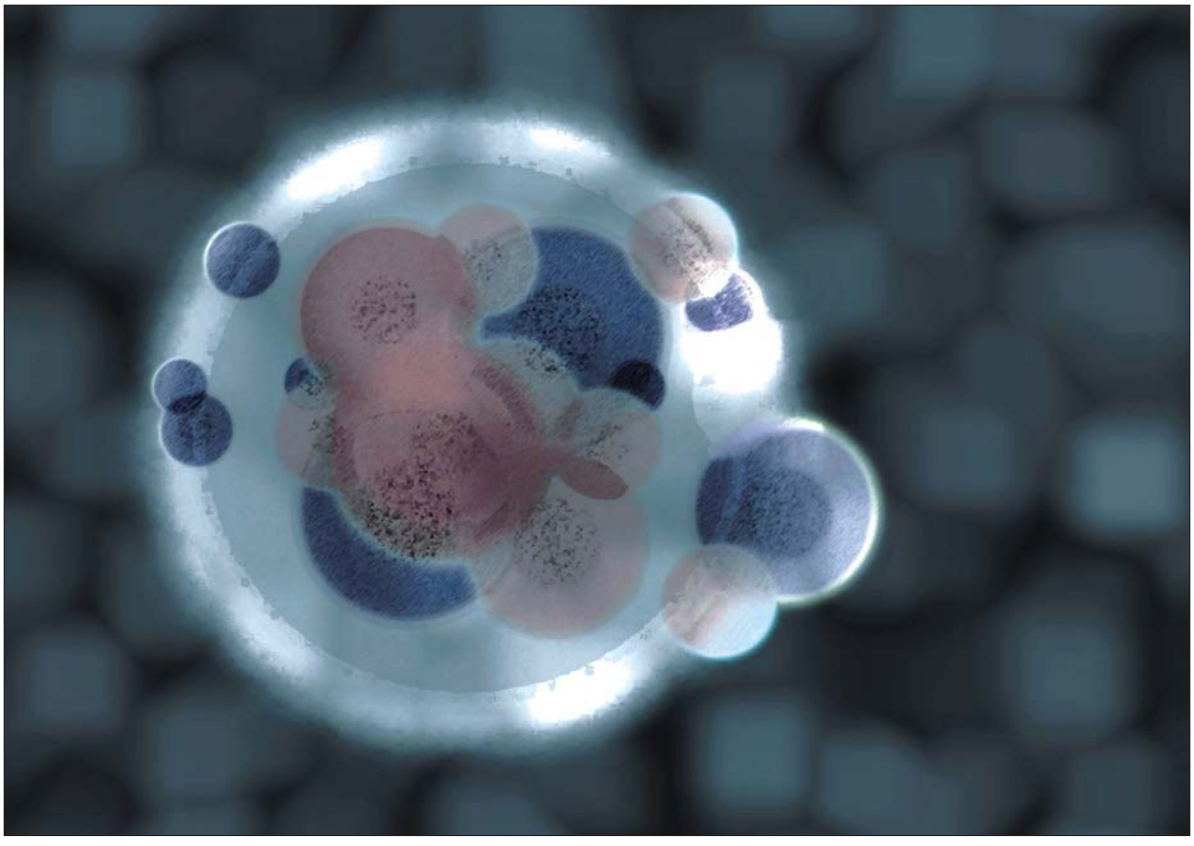

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Quantification of Isoprostanes as an Index of Oxidative Stress: A Update

Huiyong Yin, Erik S. Musiek and Jason D. Morrow
Division of Clinical Pharmcology, Departments of Medicine and Pharmacology,
Vanderbilt University School of Medicine, Nashville TN 37232-6602, USA

Abstract: Isoprostanes are prostaglandin-like compounds formed from the free radical-catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase enzymes. The isoprostanes possess potent biological activity and likely mediate certain aspects of the detrimental effects of oxidant stress. The purpose of this study was to summarize the current knowledge regarding novel aspects related to the biochemistry of isoprostane formation and analytical methods by which these compounds are analyzed. A considerable portion of this review deals with the utility of measuring isoprostanes as markers of oxidant injury both *in vitro* and *in vivo*. A number of studies have shown that these compounds are extremely accurate indices of lipid peroxidation in animal models of oxidative stress and in certain human diseases. Thus the isoprostanes may have an important role in the pathophysiology of oxidant injury associated with a number of human disorders.

Key words: Isoprostane, arachidonic acid, lipid peroxidation, free radical, mass spectrometry

INTRODUCTION

Lipid peroxidation, the oxidation of polyunsaturated fatty acids (PUFA), is a central feature of oxidant stress, a phenomenon that has been increasingly implicated as causative in numerous pathological conditions (Porter *et al*., 1995; Porter, 1986; Montine and Morrow, 2005). Lipid peroxidation products are frequently used to quantify oxidative injury and can be assessed by a number of methods that include the measurement of either primary or secondary peroxidation end products. The development of specific, reliable and non-invasive methods for measuring oxidative stress in humans is of fundamental importance for establishing the role of free radicals in human diseases (Morrow, 2005; Morrow *et al*., 1999). Primary end products of lipid peroxidation include conjugated dienes and lipid hydroperoxides (Kenar *et al*., 1996), while secondary end poducts include thiobarbituric reactive substances (TBARS) (Yin and Porter, 2003), gaseous alkanes and a group of prostaglandin (PG) F_2-like products termed F_2-isoprostanes (F_2-IsoPs) (Yin *et al*., 2002; Morrow *et al*., 1990a,b; Halliwell and Grootveld, 1987). Quantification of these various compounds has proven highly useful for the study of free radical-mediated lipid peroxidation in *in vitro* model systems. However, the F_2-IsoPs appear to be a significantly more accurate marker of oxidative stress *in vivo* in humans and animals than other compounds (Morrow *et al*., 1999; Fam and Morrow, 2003). The biochemistry of F_2-IsoPs, as well as considerations regarding their utility as markers of oxidative stress will be discussed herein.

MECHANISM OF FORMATION OF THE ISOPROSTANES

IsoPs are PG-like compounds formed from the peroxidation of arachidonic acid, a ubiquitous PUFA (Morrow *et al*., 1990a,b). Unlike PGs, which are formed via the action of the cyclooxygenase enzymes, F_2-IsoPs are formed non-enzymatically as a result of the free radical-mediated peroxidation of arachidonic acid. The mechanism of formation of IsoPs is outlined in Fig. 1 and is based on the generation of bicyclic endoperoxide intermediates resulting from the peroxidation of arachidonic acid (Morrow *et al*., 1990b; Yin and Porter, 2005). Precursor arachidonic acid initially undergoes abstraction of an allylic hydrogen atom to yield a delocalized pentadienyl carbon-centered radical. Subsequently, there is an insertion of oxygen to yield peroxyl radicals. The peroxyl radical undergoes further cyclization, followed by the addition of another molecule of oxygen to yield bicyclic endoperoxide (PGG_2-like). These intermediates are then reduced to F_2-IsoPs, so named because they possess F-type prostane rings. The F_2-IsoPs were the first class of

Corresponding Author: Dr. Jason D. Morrow, 526 RRB, 23rd and Pierce Aves.,
Vanderbilt University School of Medicine, Nashville, TN 37232-6602, USA
Tel: +615 343-1124 Fax: +615 343-9446

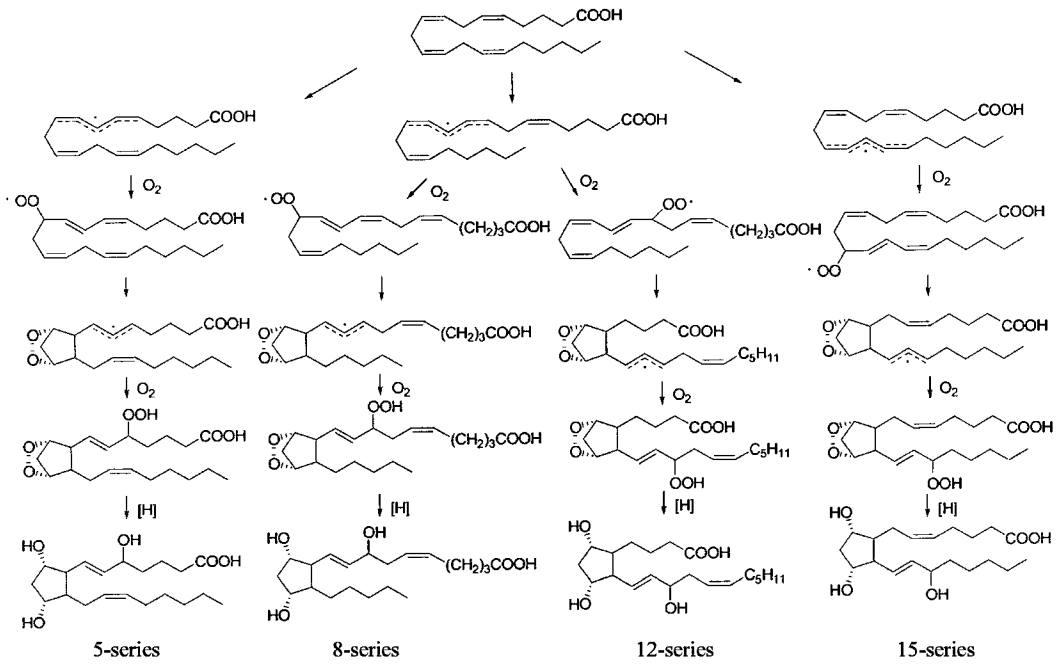

Fig. 1: Mechanism of formation of the F_2-isoprostanes. Four regioisomers are formed each consisting of 8 racemic diastereomers

IsoPs discovered and quantification of these compounds by MS is the one most frequently utilized to assess oxidant stress status (Morrow *et al*., 1990a). Depending on the site of hydrogen abstraction and oxygen insertion, four different regioisomers of IsoPs are formed. These regioisomers are denoted as either 5-, 12-, 8-, or 15-series compounds depending on the carbon atom to which the side chain hydroxyl is attached (Yin *et al*., 2002). Besides the isoprostanes, other highly oxidized products can also be formed from the oxidation of arachidonic acid (Yin and Porter, 2005). An alternative pathway for IsoP formation has been proposed involving a dioxetane/endoperoxide mechanism that would lead to the generation of the same compounds as the endoperoxide pathway (Lawson *et al*., 1999; Corey and Wang, 1994). However, this latter mechanism is recently proven to be less likely for formation of IsoPs *in vivo* (Yin *et al*., 2003).

An important structural distinction between IsoPs and cyclooxygenase-derived PGs is that the former contain side chains that are predominantly oriented *cis* to the prostane ring while the latter possess exclusively *trans* side chains (O'Connor *et al*., 1981, 1984). A second important difference between IsoPs and PGs is that IsoPs are formed *in situ* esterified to phospholipids and are subsequently released by a phospholipase(s), while PGs are generated only from free arachidonic acid (Morrow *et al*., 2005, 1999, 1992).

In addition to IsoPs containing F-type prostane rings, it has been determined that IsoP bicyclic endoperoxide intermediates can also undergo rearrangement to D_2/E_2-IsoPs containing ring structures analogous to PGD_2 and PGE_2 and to thromboxane-like compounds termed isothromboxanes (Fam and Morrow, 2003). D_2/E_2-IsoPs dehydrate *in vivo* resulting in the formation of cyclopentenone-containing compounds termed J_2-IsoPs and A_2-IsoPs, respectively. Further, it has been recently determined that IsoP-endoperoxide intermediates can undergo cleavage of the cyclopentane ring resulting in the generation of levulglandin-like molecules termed isoketals (Davies *et al*., 2004). Unlike other classes of compounds derived from IsoP endoperoxides, cyclopentenone IsoPs and isoketals contain reactive functional groups and readily covalently adduct relevant biomolecules. Cyclopentenone IsoPs have been shown to conjugate thiols such as glutathione *in vivo* and isoketals readily adduct proteins via interaction with lysine residues (Milne *et al*., 2005, 2004). Furthermore, the stereochemistry of D_2/E_2 and A_2/J_2 IsoPs can be complicated by the keto-enol tautomerization under physiological conditions. The thermodynatically favored *trans*-alkyl IsoPs can be formed via this pathway from the kinetically favored *cis*- analogues (Gao *et al*., 2003).

Other polyunsaturated fatty acids containing at least three interrupted carbon-carbon double bonds can theoretically undergo oxidation and cyclization resulting in the formation of IsoP-like compounds. Thus, it has been shown that IsoP-like substances can be generated *in vitro* and *in vivo* from linolenic acid, dihomo-γ-linolenic

acid and docosahexanoic acid (DHA). Because of the preponderance of docohexanoic acid in the central nervous system, it has been hypothesized that IsoP-like compounds (trivially termed neuroprostanes) derived from this fatty acid may be a specific marker of neuronal oxidative injury (Musiek *et al*., 2004; Roberts *et al*., 1998; Quan and Cha, 2004; Montine *et al*., 2004).

QUANTIFICATION OF F_2-ISOPROSTANES

Over the past 12 years, several methods have been developed to quantify the F_2-IsoPs. Our laboratory uses a gas chromatographic/negative ion chemical ionization mass spectrometric (GC/NICI MS) approach employing stable isotope dilution (Morrow *et al*., 1999a; Rokach *et al*., 1997). For quantification purposes, we measure the F_2-IsoP, 15-F_{2t}-IsoP and other F_2-IsoPs that co-elute with this compound. Other investigators quantify different F_2-IsoP isomers, as discussed subsequently (Rokach *et al*., 1997). Several internal standards are available from commercial sources to quantify the IsoPs. These include [$^2H_4$]15-F_{2t}-IsoP ([$^2H_4$]8-iso- $PGF_{2\alpha}$) and [$^2H_4$]-$PGF_{2\alpha}$. The advantages of mass spectrometry over other approaches include its high sensitivity and specificity, which yields quantitative results in the low picogram range. Its drawbacks are that it is labor intensive and requires considerable expenditures on equipment.

Several alternative mass spectrometric assays have been developed by different investigators including FitzGerald and colleagues (Rokach *et al*., 1997; Pratico *et al*., 1998a). Like our assay, these methods require solid phase extraction using a C_{18} column, TLC purification and chemical derivitization. Further, IsoPs are quantified using isotope dilution NICI GC/MS but the assay measures F_2-IsoP isomers other that 15-F_2-IsoP. These methods appear to be comparable to ours in terms of utility. In addition, a number of liquid chromatographic MS methods for F_2-IsoPs have been recently developed which require less sample preparation (Liang *et al*., 2003; Li *et al*., 1999), but the sensitivity and reliability of these for the analysis of IsoPs in complex biological samples is unknown.

Alternative methods have been developed to quantify IsoPs using immunological approaches (Basu, 2004). Antibodies have been generated against 15-F_{2t}-IsoP and at least three immunoassay kits are commercially available. A potential drawback of these methods is that limited information is currently available regarding their precision and accuracy. In addition, little data exist comparing IsoP levels determined by immunoassay to mass spectrometry. Analogous to immunological methods to quantify cyclooxygenase-derived PGs, it might be predicted that immunoassays for IsoPs will suffer from a lack of specificity (Roberts and Morrow, 2000). Furthermore, the sensitivity and/or specificity of these kits may vary substantially between manufacturers. However, while mass spectrometric methods of IsoP quantification are considered the "gold standard", immunoassays have expanded research in this area due to their low cost and relative ease of use.

F_2-ISOPROSTANES AS AN INDEX OF OXIDANT STRESS

Measurement of F_2-IsoPs *in vitro*: In order to demonstrate the utility of quantifying F_2-IsoPs as an index of oxidant stress, it is necessary to compare the formation of these compounds with other known indices of oxidant stress using established *in vitro* models of oxidant stress. The formation of F_2-IsoPs has been compared to malondialdehyde (MDA), one of the most commonly used measures of lipid peroxidation, utilizing Fe/ADP/ascorbate-induced peroxidation of rat liver microsomes (Longmire *et al*., 1994; Kadiiska *et al*., 2005a,b). Both F_2-IsoP and MDA (measured as thiobarbituric acid reacting substances) formation increased in parallel in a time dependent manner and correlated with the loss of arachidonic acid and with increasing oxygen concentrations up to 21%. Although the formation of F_2-IsoPs correlated with other measures of lipid peroxidation in this *in vitro* model, we have reported that quantification of F_2-IsoPs is far superior to measurements of MDA as an index of lipid peroxidation *in vivo*.

It is hypothesized that the oxidation of Low Density Lipoprotein (LDL) *in vivo* converts it to an atherogenic form which is taken up by macrophages in the vessel wall. Subsequent activation of these cells likely plays an important role in the development and progression of atherosclerotic lesions in humans (Steinberg *et al*., 1989; Steinberg, 2004, 2005). Thus, we have performed studies examining the formation of F_2-IsoPs in LDL that is oxidized to determine whether measurement of F_2-IsoPs esterified to lipoproteins may provide an approach to assess lipoprotein oxidation *in vivo* (Lynch *et al*., 1994). In these studies, either plasma lipids or purified LDL from humans was peroxidized with Cu^{2+} or the water soluble oxidizing agent 2,2-azo-bis (2-amidinopropane) (AAPH) and the formation of F_2-IsoPs was compared to other markers of lipid peroxidation including formation of cholesterol ester hydroperoxides, phospholipid hydroperoxides, loss of antioxidants and changes in the electrophoretic mobility of LDL (Kenar *et al*., 1996). In plasma oxidized with AAPH, increases in the formation of F_2-IsoP paralleled increases in lipid hydroperoxide formation and occurred only after depletion of the antioxidants ascorbate and ubiquinol-10. In purified LDL that was oxidized, formation of F_2-IsoPs

again correlated with increases in lipid hydroperoxides and increases in the electrophoretic mobility of LDL and occurred only after depletion of the antioxidants α-tocopherol and ubiquinol-10. Similar findings have been reported by others (Gopaul *et al*., 1994; Pratico and FitzGerald, 1996).

Taken together, these *in vitro* studies suggest that quantification of F_2-IsoP correlates with other established indices of oxidative stress and serves as a useful marker of lipid peroxidation.

Measurement of F_2-IsoPs *in vivo*: It has been previously recognized that one of the greatest needs in the field of free radical research is a reliable non-invasive method to assess lipid peroxidation *in vivo* in humans (Roberts and Morrow, 2000). In this respect, most methods available to assess oxidant stress, which are adequate for *in vitro* purposes, have suffered from a lack of sensitivity and/or specificity or are unreliable when applied to complex biological fluids and tissues. However, a substantial body of evidence indicates that measurement of F_2-IsoPs in body fluids such as plasma provides a reliable approach to assess lipid peroxidation *in vivo* and represents a major advance in our ability to assess oxidative stress status in animals and humans (Morrow and Roberts, 1997). We have defined normal levels of F_2-IsoPs in human biological fluids such as plasma and urine (Morrow *et al*., 1999; Roberts and Morrow, 1999; Tsan Liu *et al*., 2001). It is important to note that quantities of these compounds exceed those of cyclooxygenase-derived PGs by at least an order of magnitude, suggesting that IsoPs are a major pathway of arachidonic acid disposition. Further, levels of F_2-IsoPs are sufficient to be detected in every normal biological fluid that has been assayed including plasma, urine, bronchoalveolar lavage fluid, cerebrospinal fluid and bile (Morrow *et al*., 1999a).

This finding of significant levels of F_2-IsoPs in normal animal and human biological fluids and tissues indicates there is ongoing lipid peroxidation that is incompletely suppressed by antioxidant defenses, even in normal humans and animals, lending support to the hypothesis that the normal aging process is due to enhanced oxidant damage of relevant biological molecules over time. In this regard, previous studies have suggested that IsoP levels in normal mice and humans increase with age (Rokach *et al*., 1997; 2001), although another report refutes this (Feillet-Coudray, 1999).

An attractive possibility suggested by these findings is the measurement of F_2-IsoPs in urine as an index systemic or "whole body" oxidant stress integrated over time. However, the measurement of free F_2-IsoPs in urine can be confounded by the potential contribution of local IsoP production in the kidney although the extent to which this occurs is unclear (Morrow *et al*., 1999a; Roberts and Morrow, 2000; Roberts *et al*., 2001). In light of this issue, we have previously identified the primary urinary metabolite of 15-F_{2t}-IsoP to be 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP and have developed a highly sensitive and accurate mass spectrometric assay to quantify this molecule (Roberts *et al*., 1996; Morrow *et al*., 1999b; Morale *et al*., 2001). Thus, the quantification of 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP may represent a truly non-invasive, time-integrated measurement of systemic oxidation status that can be applied to living subjects.

Formation of isoprostanes in animal models of oxidant stress: F_2-IsoPs have also proven highly valuable in studying oxidative injury *in vivo* in many animal models of disease. Administration of carbon tetrachloride (CCl_4) intragastrically to rats is a well established model of oxidative injury, causing severe free-radical-induced damage to the liver and other organs. Esterified levels of F_2-IsoPs in liver tissue increase by 200-fold within 1 h of CCl_4 treatment and subsequently decline over 24 h, while plasma free and lipid esterified IsoP concentrations increased after liver levels up to 50-fold in a dose-dependent manner (Morrow *et al*., 1992). Administration of the antioxidant lazaroid U78517 to CCl_4-treated animals significantly blunted the enhanced formation of F_2-IsoPs in this model (Morrow and Roberts, 1997).

As a second example, F_2-IsoP formation has been employed to study the toxicity of diquat, a dipyridyl herbicide. Diaquat undergoes redox cycling *in vivo*, generating large amounts of the superoxide anion and causing hepatic and renal injury in rats. This effect is markedly augmented in animals deficient in selenium (Se), a trace element that is required for the enzymatic activities of glutathione peroxidase and other antioxidant proteins. To study whether lipid peroxidation occurs in this model, levels of F_2-IsoPs were quantified in plasma and tissues from Se-deficient rats following diquat administration. Se-deficient rats administered diquat showed 10- to 200-fold increases in plasma F_2-IsoPs, with the primary sites of IsoP generation being the kidney and liver. Further studies disclosed that the extent of tissue injury and IsoP formation directly correlated with the degree of Se depletion (Awad *et al*., 1994).

The measurement of F_2-IsoPs can also be employed to examine the oxidation status of transgenic animals. For instance, Pratico *et al*. (1998b) demonstrated that apolipoprotein E deficient mice, which develop severe atherosclerotic disease, also show marked increases in plasma F_2-IsoP levels. Dietary supplementation with the antioxidant α-tocopherol prevented the increase in plasma

F_2-IsoP levels and reduced atherosclerosis in there animals. Increases in F_2-IsoPs have been observed in animal models of disease in nearly every organ. Using the brain as an example, increased F_2-IsoP levels have been described in mice subjected to a vast array of neurological insults, including amyloid precursor protein overexpression (Pratico *et al*., 2001), intracerebroventricular lipopolysacharide injection (Montine *et al*., 2002), kainate-induce seizures (Patel *et al*., 2001) and cerebral ischemic injury (Marin *et al*., 2000). Taken together, these studies suggest that quantification of F_2-IsoPs in animal models of oxidant injury represents an accurate method to assess lipid peroxidation *in vivo*.

F_2-ISOPROSTANE FORMATION IN HUMAN DISEASES

From the above examples, measurement of IsoPs appears to be a reliable index of lipid peroxidation *in vivo* and thus potentially provides us with a tool to assess the role of oxidative stress in the pathophysiology of human disease. Elevations of IsoPs in human body fluids and tissues have been found in diverse array of human disorders (Table 1). For purposes of this brief review, we have chosen to discuss IsoP formation in several human diseases in which their generation has been examined in some detail, namely atherosclerosis and Alzheimer's disease.

Atherosclerotic cardiovascular disease: We and others have extensively explored the association between various risk factors for atherosclerosis and enhanced IsoP generation and have found that IsoP formation is increased in humans with these risk factors. These data suggest that enhanced oxidant stress may play a role in the development of atherosclerosis, although the mechanisms involved have not been elucidated. Although trials with antioxidants have generally failed to show benefit in the prevention of heart disease in humans, there is still considerable evidence to support the hypothesis that oxidative stress is intimately involved in the atherosclerosis.

A link between cigarette smoking and risk of cardiovascular disease is well established (Kannel, 1981). However, the underlying mechanism(s) for this effect is not fully understood. The gaseous phase of cigarette smoke contains a number of oxidants and exposure of LDL to the gaseous phase of cigarette smoke *in vitro* induces oxidation of the LDL lipids (Frei *et al*., 1991). Thus, we explored the hypothesis that smoking induces an oxidative stress by examining F_2-IsoP levels in plasma from smokers. Ten individuals who smoked heavily (>30 cigarettes d^{-1}) and ten age and sex matched non-smoking normal volunteers were studied (Morrow *et al*., 1995). Plasma concentrations of free and esterified F_2-IsoPs were significantly elevated in the smokers compared to the non-smokers ($p=0.02$ and $p=0.03$, respectively). In all subjects, levels of F_2-IsoPs both free in the circulation and esterified to plasma lipoproteins were significantly reduced following two weeks of abstinence from smoking ($p=0.03$ and $p=0.02$, respectively). The occurrence of enhanced formation of IsoPs in smokers has also subsequently been confirmed in studies by other groups (Reilly *et al*., 1996). Collectively, these findings suggest strongly that smoking causes an oxidative stress and the observation that smokers have elevated levels of F_2-IsoPs esterified in plasma lipids also supports the hypothesis that the link between smoking and risk of cardiovascular disease may be attributed to enhanced oxidation of lipoproteins.

It has been well established that patients with hypercholesterolemia have an increased risk for the development of atherosclerosis. Thus, it was of interest to determine whether levels of F_2-IsoPs are increased in patients with this condition.

Levels of F_2-IsoPs esterified in plasma lipids were determined in patients with polygenic hypercholesterolemia (Roberts and Morrow, 1999). Levels in patients with hypercholesterolemia were found to be significantly increased a mean of 3.4-fold (range 1.7-7.5-fold) above levels measured in normal controls ($p<0.001$). Interestingly, in these patients, there was no correlation between levels of F_2-IsoPs and serum cholesterol, triglycerides or LDL-cholesterol. In addition, plasma

Table 1: Disorders in which measurements of F_2-IsoPs has implicated a role for free radicals in the disease process

Smoking (Morrow *et al*., 1995)	Cystic fibrosis (Roberts and Morrow, 2000)
Atherosclerosis (Morrow, 2005)	Rhabdomyolysis renal injury (Holt *et al*., 1999)
Alzheimer's Disease (Montine *et al*., 1999a)	Acute cholestasis (Roberts and Morrow, 2000)
Huntington's Disease (Montine *et al*., 1999b)	Adult respiratory distress syndrome (Carpenter *et al*., 1998)
Hypercholesterolemia (Davi *et al*., 1997)	Halothane hepatotoxicity (Roberts and Morrow, 2000)
Hyperhomocysteinemia (Voutilainen *et al*., 1999)	Ischemia/reperfusion injury (Reilly *et al*., 1997)
Scleroderma (Stein *et al*., 1996)	Cr (IV) poisoning (Roberts and Morrow, 2000)
Se deficiency (Awad *et al*., 1994)	Cisplatin-induced renal dysfunction (Roberts and Morrow, 2000)
Vitamin E deficiency (Awad *et al*., 1994)	Transplant organ injury during cold preservation (Roberts and Morrow, 2000)
Retinopathy of prematurity	Chronic obstructive lung disease (Roberts and Morrow, 2000)
Alcohol-induced liver injury (Aleynik *et al*., 1998)	Interstitial lung disease (Roberts and Morrow, 2000)
Diabetes (Gopaul *et al*., 1995)	CCl_4-induced hepatotoxicity (Roberts and Morrow, 2000)
Heart failure (Roberts and Morrow, 2000)	Preeclampsia (Roberts and Morrow, 2000)

arachidonic acid content was measured in these patients and normal controls. Again, no correlation between IsoP and arachidonate levels was found. Thus, these data suggest that the finding of high levels of F_2-IsoPs in patients with hypercholesterolemia is not due simply to the presence of more lipid, i.e. arachidonic acid substrate. Rather, it is suggested that hypercholesterolemia is associated with enhanced oxidative stress. The underlying basis for this observation, however, remains unclear. Interestingly, a report also found that the urinary excretion of F_2-IsoPs was also increased in patients with Type II hypercholesterolemia by a mean of 2.5-fold which was suppressed by approximately 60% with vitamin E treatment (600 mg d^{-1}) (Davi *et al*., 1997).

Patients with diabetes are known to have an increased incidence of atherosclerotic vascular disease. Interestingly, the formation of F_2-IsoPs has been found to be induced in vascular smooth muscle cells *in vitro* by elevated glucose concentrations (Natarajan *et al*., 1996). Thus, we explored whether there was evidence for enhanced oxidative stress *in vivo* in patients with diabetes (Koulouris *et al*., 1995). In this study, levels of F_2-IsoPs esterified in plasma lipids were quantified in 61 patients who underwent coronary angiography, 15 of whom had diabetes. The extent of coronary atherosclerosis in the diabetic patients was similar to that in the 46 non-diabetic individuals. Plasma levels of F_2-IsoPs measured in the diabetic patients (33.4±4.8 pg mL^{-1}, mean±SEM) were found to be significantly increased compared with levels measured in the non-diabetic patients (22.2±1.9 pg mL^{-1}) ($p<0.02$). Similar findings have also been reported by Gopaul *et al*. (1995), in which they found a mean 3.3-fold increase in free F_2-IsoP concentrations in plasma of diabetic patients compared to non-diabetic healthy control subjects. In addition, it has been reported that urinary IsoP levels in diabetics are suppressed by vitamin E and by control of hyperglycemia (Lawson *et al*., 1999).

High plasma levels of homocysteine are an independent risk factor for cardiovascular disease (Boushey *et al*., 1995). The mechanism by which hyperhomocysteinemia induces atherosclerosis is not fully understood but promotion of LDL oxidation has been suggested. The relationship between total plasma concentrations of homocysteine and F_2-IsoPs in 100 Finnish male participants in the Antioxidant Supplementation in Atherosclerosis Prevention study has been explored (Voutilainen *et al*., 1999). The mean plasma total homocysteine and F_2-IsoP concentrations were 11.1 μmol L^{-1} and 29.6 ng L^{-1}, respectively. The simple correlation coefficient for association between plasma concentrations of homocysteine and F_2-IsoPs was 0.40 ($p<0.0001$). Plasma concentrations of F_2-IsoPs increased linearly across quintiles of homocysteine levels. The finding of a positive correlation between plasma concentrations of F_2-IsoPs and homocysteine supports the suggestion that the mechanism underlying the link between high homocysteine levels and risk for cardiovascular disease may be attributed to enhanced lipid peroxidation.

In accordance with the LDL oxidation hypothesis of atherosclerosis, levels of F_2-IsoPs should be higher in atherosclerotic plaques than in normal vascular tissue. To address this issue, levels of F_2-IsoPs were measured in fresh advanced atherosclerotic plaque tissue removed during arterial thrombarterectomy (n=10) and compared with levels measured in normal human umbilical veins removed from the placenta immediately after delivery (n=10) (Gniwotta *et al*., 1997). Levels of F_2-IsoPs esterified in vascular tissue normalized to both wet weight and dry weight were significantly higher in atherosclerotic plaques compared to normal vascular tissue. When the data was normalized to arachidonic acid content, the F_2-IsoP/arachidonic acid ratio was ~4-fold higher than the ratio in normal vascular tissue ($p=0.009$). This finding indicates that unsaturated fatty acids in atherosclerotic plaques are more extensively oxidized than lipids in normal vascular tissue. These observations are also in accord with data from FitzGerald and colleagues who have shown increased amounts of F_2-IsoPs in human atherosclerotic lesions including the localization of F_2-IsoPs in atherosclerotic plaque tissue to foam cells and vascular smooth muscle cells (Pratico *et al*., 1997).

Alzheimer's disease: Oxidative stress has been implicated in the pathogenesis of numerous neurodegenerative conditions, including Alzheimer's Disease (AD). Regional increases in oxidative damage and lipid peroxidation have been described in brain tissue obtained post mortem from patients with AD (Markesberry, 1997). Similarly, F_2-IsoP levels are significantly elevated in affected regions of post mortem brain samples from AD patients as compared to controls (Reich *et al*., 2001). However, an objective index of oxidative damage associated with AD that can be assessed in living patients is lacking. Such a biomarker could be vital for understanding the role oxidative damage in AD patients by permitting repeated evaluation of disease progression or responses to therapeutic interventions. Toward such a goal, we obtained post mortem ventricular fluid from 11 patients with a pathological diagnosis of AD and 11 control patients, in order to evaluate F_2-IsoP levels in cerebrospinal fluid

(CSF) (Montine *et al*., 1998). All subjects participated in a rapid autopsy protocol such that fluid was collected within three hours of death. F_2-IsoP levels were significantly increased in ventricular fluid from AD patients (72±7 pg mL^{-1}, mean±SEM) compared to CSF from control individuals (46±4 pg mL^{-1}, $p<0.01$) and correlations were identified between increases in IsoP levels and higher Braak stage and decreased brain weight, two indices of AD severity. In a larger study, we have shown that CSF F_2-IsoP level correlates with the extent of pathological neurodegeneration but not with density of neuritic plaques or neurofibrillary tangles (Montine *et al*., 1999a).

Subsequently, we undertook a study to examine CSF F_2-IsoP levels in living patients with probable AD (Montine *et al*., 1999a). CSF was obtained from the lumbar cistern in 27 patients with AD and 25 controls without neurodegenerative disorders matched for age and gender. In keeping with post mortem studies, lumbar CSF levels of F_2-IsoPs were significantly increased (31.0±2.6 pg mL^{-1}) in AD patients compared to control subjects (22.9±1.0 pg mL^{-1}, $p<0.05$). Pratico *et al*. (2000, 2002) have also observed increased F_2-IsoPs in CSF of patients with probable AD, as well as in patients with mild cognitive impairment (MCI), a condition which precedes symptomatic dementia in AD. However, this group also reports increased F_2-IsoPs in plasma and urine of both MCI and AD patients, though our laboratory and others have not been able to detect these changes in peripheral F_2-IsoPs (Mountine *et al*., 2002; Bohnstedt *et al*., 2003). Taken together, these studies suggest that quantification of IsoPs in cerebrospinal fluid of patients with Alzheimer's disease may be of use as an *intra vitum* index of disease progression or as a tool to monitor response to therapy.

CONCLUSIONS

The discovery of IsoPs as products of non-enzymatic lipid peroxidation has been a major breakthrough regarding the quantification of oxidant stress *in vivo*. The quantification of these molecules has opened up new areas of investigation regarding the role of free radicals in human physiology and pathophysiology and appears to be the most useful tool currently available to explore the role of free radicals in the pathogenesis of human disease. Although considerable information has been obtained since the initial discovery of IsoPs, much remains to be understood about the role of these molecules as markers of oxidant stress *in vivo*. It is anticipated that additional research in this area will continue to provide important insights into the role of oxidative stress in human disease.

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