Quantification of Isoprostanes as Indices of Oxidant Stress and the Risk of Atherosclerosis in Humans

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Abstract—Enhanced oxidant stress occurring either locally in the vessel wall or systemically is implicated in the pathogenesis of atherosclerosis in humans. Nonetheless, evidence that oxidant stress is increased in vivo in association with this disease and that it can be quantified in living human beings has been lacking because of the unavailability of biomarkers to assess oxidant stress in humans. Recently, the development of methods to quantify the F₂-isoprostanes (IsoPs), prostaglandin (PG)-like compounds derived from the free radical-catalyzed peroxidation of arachidonic acid, has allowed, for the first time to the author’s knowledge, a facile and accurate assessment of oxidant stress in vivo. The purpose of this brief review is to discuss the usefulness of quantifying IsoPs as an index of oxidative injury in association with atherosclerosis. F₂-IsoPs can be measured in human biological fluids, such as plasma and urine, using highly precise assays. They have been shown to be increased in association in with a number of atherosclerotic risk factors, including cigarette smoking, hypercholesterolemia, diabetes mellitus, and obesity, among others. In addition, recent evidence suggests their quantification may represent an independent marker of atherosclerotic risk. A reduction in cardiovascular risk factors is associated with a decrease in IsoP formation in humans. Despite the fact that the role of oxidant stress in the pathogenesis of atherosclerosis is a hotly debated issue, current evidence suggests that the IsoPs represent a biomarker that has the potential to be of great importance in the assessment of human atherosclerotic cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2005;25:279-286.)

Key Words: lipid oxidation, peroxidation, isoprostane, oxidized low-density lipoprotein.

Oxidation of biomolecules including lipids, proteins, and nucleic acids has been implicated in the pathogenesis of many diseases, including atherosclerosis. Enhanced oxidant stress, occurring either locally in the arterial wall or systemically, is a hypothesis to explain the development and progression of atherosclerosis in humans. In particular, oxidative modification of the lipid components of low-density lipoprotein (LDL) is postulated to be causative. LDL is deposited in the vascular wall early in the course of atherosclerotic lesion development, where it is oxidized. There is evidence in in vitro and in animal models of human atherosclerosis that oxidized lipids derived from LDL contribute to many of the stages of atherosclerotic development. Mechanisms that have been proposed to result in enhanced lipoprotein oxidation, either locally or systemically, and the development and progression of atherosclerosis include increased production of tumor necrosis factor and other cytokines, upregulation of NADPH oxidase(s) and other oxidative enzymes in vascular tissue, and increases in the renin-angiotensin system.

Additional support for a role of lipid peroxidation and oxidant stress in the pathogenesis of atherosclerosis came from animal and human epidemiological studies performed in the 1980s and 1990s, which suggested that antioxidants decrease atherosclerosis presumably by reducing oxidative stress. However, prospective clinical trials of antioxidant supplementation using vitamin E and other agents have been disappointing. Three large trials, ATBC, GISSI, and HOPE, involving thousands of subjects failed to show a reduction of cardiovascular events when vitamin E was used at doses ranging from 50 to 400 IU per day. Two smaller trials, CHAOS and SPACE, involving far fewer patients, reported a significant reduction, by almost 50%, in the incidence of cardiovascular events. In these latter trials, doses of vitamin E of up to 800 IU were used.

Various reasons have been given to explain the lack of benefit of vitamin E in the prevention of atherosclerosis, but a critical assumption of the prospective clinical trials reported was that antioxidants decrease atherosclerosis by inhibiting oxidant stress. In no trial reported, however, was an assessment of oxidant stress undertaken in study participants, nor was the ability of vitamin E to inhibit oxidative injury determined. Thus, it is impossible to determine whether vitamin E inhibited oxidative injury in the populations studied.

A major difficulty with assessing the role of lipid peroxidation in any disease process is the lack of methods to quantify this entity in vivo in humans. Assays that exist to
measure lipid peroxidation include the quantification of primary peroxidation products such as conjugated dienes and lipid hydroperoxides. Secondary peroxidation products that can be measured include thiobarbituric acid reactive substances, malondialdehyde, and exhaled alkanes. Despite the fact that there are a number of assays, they are primarily of use to measure oxidation in vitro and are inaccurate when applied to the in vivo assessment of oxidant stress in humans. This has been shown in numerous studies. In addition, recent reviews have clearly shown that available markers of protein and nucleic acid oxidation do not accurately mirror the level of in vivo oxidant stress in animals or humans. Thus, there has been a clear need to develop accurate measures of oxidative injury in vivo. Over the past decade, one marker, the F2-isoprostanes (IsoPs), has emerged as the “gold standard” assessment of oxidative stress in vivo and has been used extensively to quantify lipid peroxidation in association with risk factors for atherosclerosis and other diseases. A recently completed study, termed the Biomarkers of Oxidative Stress Study (BOSS), which was sponsored by the National Institutes of Health, has shown that the IsoPs are the best index of oxidative injury in a well-accepted animal model of oxidant stress, the administration of carbon tetrachloride to rats. This trial examined the usefulness of quantifying a number of oxidative stress biomarkers and was performed by leading investigators in the field. It concluded that the IsoPs offered the best approach to an accurate quantification of oxidative injury in vivo. Interestingly, also, this study suggested that certain assays for malondialdehyde in biological fluids might be of use. This trial is particularly important because it represents the first time, to this author’s knowledge, that various biomarkers have been tested head-to-head in a controlled manner.

This brief review critically evaluates the available information regarding the usefulness of quantifying IsoPs in humans in association with atherosclerotic cardiovascular disease and provides future directions to determine the role that oxidative injury plays in the development and progression of this disorder.

**Novel Aspects of IsoP Formation**

IsoPs are prostaglandin (PG)-like compounds formed from the peroxidation of arachidonic acid, a ubiquitous polyunsaturated fatty acid. Unlike PGs, which are formed via the action of the cyclooxygenase enzymes, F2-IsoPs are generated as a result of the free radical-mediated peroxidation of arachidonic acid independent of this enzyme. The sources of free radicals that contribute to IsoP formation in vivo are likely multiple. These include the generation and leakage of reactive oxygen species such as superoxide and the hydroxyl radical from the mitochondrial electron transport system and the P450 family of drug-metabolizing enzymes, the generation of superoxide by the NADPH oxidases, hydroperoxy radicals from the lipooxygenases, and transition metal-catalyzed formation of reactive oxygen species, among others. Figure 1 outlines the mechanism by which IsoPs are formed. After abstraction of a bis-allylic hydrogen atom and the addition of a molecule of oxygen to arachidonic acid to form a peroxyl radical, endocyclization occurs and an additional molecule of oxygen is added to form PGG2-like compounds. These unstable bicycloendoperoxide intermediates are then reduced to the F2-IsoPs. Based on this mechanism of formation, 4 F2-IsoP regioisomers are generated. Compounds are denoted as 5-, 12-, 8-, or 15-series regioisomers, depending on the carbon atom to which the side chain hydroxyl is attached, and a nomenclature system has been proposed based on this feature. For purposes of discussion herein, compounds are denoted using this nomenclature primarily. An alternative naming system for the IsoPs has been proposed by FitzGerald et al in which the abbreviation iP is used for isoprostane, and regioisomers are denoted as III to VI based on their structure. IsoPs that contain F-type prostane rings are isomeric to PGF2α and are thus referred to as F2-IsoPs. The fact that 2 nomenclature systems exist has made an understanding of IsoPs more complicated for those less familiar with this field.

It should be noted that IsoPs containing alternative ring structures (such as those resembling PGD2/E2 and PGA2/J2) can also be formed by this mechanism. F2-IsoPs, however, have been the most studied class of IsoPs and, because of their stability, afford the most accurate measure of oxidant stress.

An important structural distinction between IsoPs and cyclooxygenase-derived PGs, and which affords marked differences in biological activities, is that the former contain side chains that are predominantly oriented cis to the prostanate ring, whereas the latter possess exclusively trans side chains. 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), whereas PGs are generated only from free arachidonic acid. The phospholipase(s) responsible for the hydrolysis of IsoPs from phospholipids is unknown. Previously, we have reported that various secretory phospholipase A2s from lower animal sources are capable of releasing IsoPs, although we do not know whether analogous mammalian enzymes possess this activity. In addition, preliminary studies performed by us do not suggest that the mammalian cytoplasmic phospholipase A2 responsible for hydrolyzing unoxidized arachidonic acid from tissue phospholipids is active on esterified IsoPs.
Methods to Quantify the F₂-IsoPs

Over the past decade, several methods have been developed to quantify the F₂-IsoPs. Our laboratory and others use a gas chromatographic (GC)/negative ion chemical ionization mass spectrometric (MS) approach using stable isotope dilution techniques. For quantification purposes, we measure the F₂-IsoPs, 15-F₂-IsoP, and other F₂-IsoPs that co-elute on GC with this compound (Figure 2). Other investigators quantify different F₂-IsoP isomers, as discussed later. Several internal standards are available from commercial sources to quantify the IsoPs. These include [²H₆]15-F₂-IsoP and [⁶D₆]PGF₂α. 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 et al. Like the assay for 15-F₂-IsoP, these methods require solid phase extraction using a C18 column, TLC purification, and chemical derivitization. Further, IsoPs are quantified using stable isotope dilution techniques using GC/negative ion chemical ionization MS, but the assays measure F₂-IsoP isomers other than 15-F₂-IsoP, including iPF₂α-IV (α 8-F₂-IsoP) or iPF₂β-VI (α 5-F₂-IsoP) (Figure 2). Standards are also commercially available for these compounds. In general, these various methods are comparable. In addition, several liquid chromatographic MS methods for F₂-IsoPs have been recently reported that require less sample preparation, but the sensitivity and reliability of these for the analysis of IsoPs in complex biological samples are unknown.

Alternative approaches have also been developed to quantify IsoPs using immunologic techniques. Antibodies have been generated against 15-F₂-IsoP and at least 3 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 immunnoassay to mass spectrometry. Analogous to immunologic methods to quantify cyclooxygenase-derived PGs, it might be predicted that immunnoassays for IsoPs suffer from a lack of specificity. Furthermore, the sensitivity and/or specificity of these kits may vary substantially between manufacturers. However, although mass spectrometric methods of IsoP quantification are considered the "gold standard," immunoassays have expanded research in this area because of their low cost and relative ease of use.

In addition to commercial immunoassays, several investigators have generated polyclonal antibodies and have developed assays for 15-F₂-IsoP. When compared with mass spectrometric approaches, it appears that unlike commercial kits, there is good correlation between these methods and mass spectrometry. Interestingly, this may, in part, be because of the rather extensive purification procedures of biological fluids that these investigators use before immunoassay.

Quantification of IsoPs in Biological Fluids

It is important to note that IsoPs can be quantified in tissues and in biological fluids such as blood and urine. To date, IsoPs have been detected in all human tissues and fluids examined, which is particularly important in that it allows for an assessment of the effects of diseases such as atherosclerosis on endogenous oxidant tone. In addition, defining levels of IsoPs in vivo is important because it allows for the determination of the extent to which various therapeutic interventions affect levels of oxidant stress.

For human studies, the quantification of IsoPs in body fluids such as urine or plasma is significantly more convenient and less invasive that measuring these compounds in organ tissue. Based on available data, quantification of F₂-IsoPs in either plasma or urine gives a highly precise and accurate index of oxidant stress. Further, to assess the integrated production of IsoPs in vivo, pooled urine samples are likely preferable to spot urine tests, because some diurnal variation in IsoP excretion occurs within individual humans, although this variation is not present when human populations are evaluated as a group.

The most accurate assessment of cyclooxygenase-derived PG production in vivo is the quantification of excreted metabolites in urine as opposed to the measurement of intact parent PGs. This stems from the fact that parent PGs in urine are largely derived from the kidney. Analogously, we have previously examined the metabolic fate of one F₂-IsoP, 15-F₂-IsoP, formed in abundance in vivo to identify the primary urinary metabolites. We have determined that the major urinary metabolite of this compound is 2,3-dinor-5,6-dihydro-15-F₂-IsoP, and we have developed a GC/MS assay to accurately measure it. Although it appears to provide an accurate index of oxidant stress in vivo as do plasma and urinary parent F₂-IsoPs, we have no evidence that quantification of this metabolite offers any advantages over measuring intact F₂-IsoPs, in contradistinction to the case with cyclooxygenase-derived PGs.

Another important point regarding the quantification of IsoPs in biological fluids is that levels in a particular tissue likely represent a steady-state concentration that is dependent on production (degree of oxidative stress) versus metabolism and excretion. We have previously examined the extent to which renal and hepatic dysfunction contribute to increases in circulating IsoP levels and have determined that even in situations of severe organ dysfunction, rapid metabolism of parent IsoPs occurs. Therefore, evidence to date suggests that
circulating F$_2$-IsoP concentrations are largely dependent on production rather than metabolism and excretion, suggesting that they truly are indicative of the level of oxidant stress in vivo.$^{23,30}$

**F$_2$-Isoprostanes as Index of Oxidant Stress Status In Vivo**

Numerous studies have been performed by various investigators showing that the quantification of F$_2$-IsoPs is the most accurate measure of oxidative stress in vitro and in animal models of oxidant injury.$^{23,27,28}$

Much of the work examining the formation of F$_2$-IsoPs in settings of oxidative injury has used animal models of atherosclerosis because oxidant stress has been pathophysically linked to this disease. The results of these studies, in general, have shown that rodent models of atherosclerotic cardiovascular disease in which hyperlipidemia has been induced are associated with enhanced oxidant stress, as quantified by measuring F$_2$-IsoP formation.$^{23,27,28,40}$ Further, interventions that decrease oxidant stress, such as administration of antioxidants including vitamin E and butylated hydroxytoluene, or the manipulation of various enzymatic sources of free radicals, such as target deletion of the 12/15-lipoxygenase and the inducible nitric oxide synthase or overexpression of catalase and catalase plus copper/zinc superoxide dismutase, decrease oxidant stress and the development of atherosclerosis in genetically altered mice.$^{28,41-43}$ In addition, inhibition of copper/zinc superoxide dismutase is also associated with vascular dysfunction that may be linked to an enhanced incidence of cardiovascular disease.$^{51}$ Taken together, these data support a role for enhanced oxidant stress in the pathogenesis of atherosclerosis in animals.

Although outside the scope of this review, in addition to excessive formation of F$_2$-IsoPs in association with atherosclerosis, there has been significant interest in these compounds as mediators of the pathophysiology of this disorder.$^{28}$ This stems from the fact that a number of F$_2$-IsoPs possess potent bioactivities that are of relevance to atherosclerosis, including vasoconstriction in many vascular beds, modulation of platelet aggregation, and proliferation of vascular smooth muscle cells.$^{25,28}$ The extent to which these compounds mediate either the development or the progression of atherosclerosis is unknown.

Because of the usefulness of quantifying IsoPs in animal models of atherosclerosis, a number of investigators have sought to determine the extent to which humans manifest excessive oxidant stress associated with risk factors for atherosclerosis. Using the quantification of F$_2$-IsoPs in this regard offers the opportunity to accurately explore, for the first time, the extent to which humans undergo enhanced oxidant stress under pathophysiological situations associated with the development of atherosclerotic cardiovascular disease. Surprisingly, as in in vitro and animal models, most risk factors for atherosclerosis are associated with significant increases in circulating and/or urinary F$_2$-IsoP levels, suggesting, at a minimum, that oxidant stress is enhanced in association with factors that contribute to this disorder in humans.$^{28,40}$ In addition, pharmacological or other manipulations that attenuate these risk factors are associated with a significant reduction in IsoP formation.$^{40}$ Although this does not prove a cause-and-effect relationship between oxidant stress and atherosclerosis, it does suggest that ongoing efforts should focus on determining the role that excessive oxidative injury plays in the pathogenesis of this disease. Further, additional efforts should be made to assess the extent to which oxidant stress is modulated by therapeutic interventions that reduce risk factors for atherosclerosis and also whether modulation of oxidative injury influences the progression of atherosclerotic cardiovascular disease. As noted, this is an issue for trials that failed to show a benefit of vitamin E or, for that matter, any other putative antioxidant including vitamin C or beta carotene, in the prevention of atherosclerotic events.$^{44}$ Whereas it is unlikely, in my opinion, that vitamin E is beneficial in the prevention of advanced disease, the extent to which the agent reduced oxidant stress in the reported trials is unknown. As has been reported by others, alpha-tocopherol is likely a relatively weak antioxidant, and it is conceivable that the dosages of the agent used in the clinical trials were subtherapeutic.$^{45}$

The Table shows the risk factors associated with atherosclerosis in which circulating or urinary F$_2$-IsoP formation is increased. This brief review highlights the evidence that these factors contribute to elevated oxidant stress. In addition, mention is made, when data are available, of therapeutic interventions aimed at decreasing these risk factors and their effects on F$_2$-IsoP generation.

**Risk Factors for Atherosclerotic Cardiovascular Disease in Which F$_2$-Isoprostane Formation is Increased**

<table>
<thead>
<tr>
<th>Cigarette smoking</th>
<th>Hypercholesterolemia</th>
<th>Diabetes mellitus</th>
<th>Overweight and obesity</th>
<th>Hyperhomocysteinemia</th>
<th>Renovascular hypertension</th>
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**Cigarette Smoking**

A link between cigarette smoking and risk of cardiovascular disease is well-established.$^{46}$ 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 lipoproteins to the gaseous phase of cigarette smoke in vitro induces oxidation of the lipids.$^{47}$ Thus, we explored the hypothesis that smoking induces an oxidative stress and specifically determined whether circulating lipoproteins contain higher levels of F$_2$-IsoPs, indicative of a greater degree of oxidative modification, in individuals who smoke. Individuals who smoked heavily (>30 cigarettes per day) and age- and sex-matched nonsmoking normal volunteers were studied.$^{48}$ Plasma concentrations of free and esterified F$_2$-IsoPs were significantly elevated by >2-fold in the smokers compared with the nonsmokers ($P=0.02$ and $P=0.03$, respectively). Confirmation that these differences in levels of F$_2$-IsoPs between smokers and nonsmokers were caused by cigarette smoking was obtained by measuring levels of F$_2$-IsoPs after 2 weeks of abstinence from smoking.
in smokers. In all subjects, levels of F$_2$-IsoPs free in the circulation and esterified to plasma lipoproteins were significantly lower after 2 weeks of abstinence from smoking ($P=0.03$ and $P=0.02$, respectively). The occurrence of enhanced formation of IsoPs in smokers has subsequently been confirmed in studies by FitzGerald, Pilz, and others, as has been the effect of smoking cessation on a decrease in plasma and urinary F$_2$-IsoP levels, and that it is progressive over time after cessation. Interestingly, resumption of smoking leads to a recurrent increase in levels of IsoPs. In addition, increases in urinary F$_2$-IsoP excretion are dependent on the number of cigarettes smoked. Collectively, these findings suggest strongly that smoking causes an oxidative stress and the observation that smokers have elevated levels of F$_2$-IsoPs in plasma lipids and urine also supports the hypothesis that the link between smoking and risk of cardiovascular disease may be attributed to enhanced oxidation of lipids.

**Hypercholesterolemia**

It has been well-established that patients with hypercholesterolemia have an increased risk for atherosclerosis. Thus, it was of interest to determine whether levels of F$_2$-IsoPs are increased in patients with hypercholesterolemia. Different populations of hypercholesterolemic subjects have been examined by various investigators, but the general conclusion of these studies is that F$_2$-IsoP formation is significantly increased in this disorder. Davi et al showed that urinary F$_2$-IsoP levels are significantly higher in type IIa hypercholesterolemic subjects by 2- to 3-fold. Similar observations were made by Reilly et al in individuals with homozygous familial hypercholesterolemia. Further, in one report, it has been shown that treatment of hypercholesterolemia with pravastatin markedly decreases both serum cholesterol levels and urinary F$_2$-IsoP excretion.

We examined levels of F$_2$-IsoPs in hypercholesterolemic subjects, and although they were found to be significantly increased a mean of 3.4-fold (range, 1.7- to 7.5-fold) above levels measured in normal controls ($P<0.001$), there was no correlation between levels of F$_2$-IsoPs and serum cholesterol, triglycerides, or LDL cholesterol. In addition, there was no correlation between IsoP and arachidonate levels. Thus, these data suggest that the finding of high levels of F$_2$-IsoPs in patients with hypercholesterolemia may not be simply because of the presence of more lipid, ie, arachidonic acid substrate. Rather, it is argued that hypercholesterolemia is associated with enhanced oxidative stress. The underlying basis for this observation, however, remains unclear.

**Diabetes Mellitus**

Patients with diabetes mellitus 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. Thus, a number of investigators have explored whether there is evidence for enhanced oxidative stress in vivo in patients with diabetes. F$_2$-IsoPs have been found to be increased in type I and type II diabetes, although the reason for enhanced lipid peroxidation is not clear. Various hypotheses have been proposed, including enhanced oxidizability of lipoproteins by metal ions caused by hyperglycemia and protein glycation and lower antioxidant protective capacity in plasma and tissues of diabetic subjects. In the case of type I disease in children and adolescents, increases in IsoP formation represent an early event in the disease and decrease as the disease progresses.

A number of reports have noted increases in plasma and urine F$_2$-IsoPs in humans with type II diabetes mellitus. Davi et al have reported that urinary IsoP excretion is markedly enhanced in the majority of a large group of patients with diabetes who were carefully characterized for other variables that affect F$_2$-IsoP formation. They also found a highly significant correlation between blood glucose and urinary IsoP levels, suggesting that lipid peroxidation is related to glycemic control. Further, that impaired glycemic control rather than some other factor is responsible for enhanced formation of F$_2$-IsoPs in type II disease is also supported by the fact that intensive antidiabetic treatment induced reductions in blood glucose levels and in urinary IsoP levels. In addition, increases in platelet activation induced by hyperglycemia paralleled increases in oxidant stress. This is of interest because 15-F$_2$t-IsoP is a ligand for the thromboxane receptor. In another report, levels of F$_2$-IsoPs esterified in plasma lipids were quantified in 61 patients who underwent coronary angiography. In this group were 15 patients with diabetes. The extent of coronary atherosclerosis in the diabetic patients was similar to that in the 46 nondiabetic individuals. Plasma levels of F$_2$-IsoPs measured in the diabetic patients (33.4±4.8 pg/mL, mean ±SEM) were found to be significantly increased compared with levels measured in the nondiabetic patients (22.2±1.9 pg/mL) ($P<0.02$). Similar findings have also been reported by Gopaul et al in which they found a mean 3.3-fold increase in free F$_2$-IsoP concentrations in plasma of diabetic patients compared with nondiabetic healthy control subjects. In addition, it has been reported that urinary IsoP levels in diabetics are suppressed by vitamin E.

**Overweight and Obesity**

The marked increase in the incidence of overweight and obese persons is recognized as perhaps the most serious public health issue in the United States. It is estimated that currently >60% of American adults are overweight and 20% are obese. Overweight and obesity are associated with a significantly increased mortality from atherosclerotic cardiovascular disease and other causes. Although obesity itself appears to augment the incidence of cardiovascular events, it is also associated with major risk factors for atherosclerosis, including hyperlipidemia, diabetes mellitus, hypertension, and the metabolic syndrome. How obesity and each of these risk factors are involved mechanistically in atherosclerosis have been areas of intense research but are poorly understood. Nonetheless, at least 3 recent reports provide evidence that elevated systemic oxidant stress may be an important mechanism by which obesity increases the incidence of atherosclerotic cardiovascular disease.

In a study by Keane et al, it is reported that an association exists between increasing body mass index and increasing...
systemic oxidant stress. Using the quantification of urinary F$_2$-IsoPs, the authors show in nearly 3000 patients involved in the Framingham Heart Study that enhanced IsoP formation in men and women is strongly associated with increasing body mass index. These findings add support to 2 smaller studies in which overweight/obesity was associated with enhanced oxidant stress and IsoP formation. In addition to obesity, smoking and diabetes were independently associated with increased IsoP excretion. The importance of the work by Keaney is that the study population was not a smaller targeted one but rather involved a large community-based cohort of otherwise healthy individuals. A particularly relevant aspect of Keaney’s work with respect to determining the role that obesity-associated oxidant stress plays in atherosclerotic cardiovascular disease is the fact that participants in the trial will be followed-up over time so that clinical outcomes such as cardiovascular events can be correlated with excessive oxidant stress. In this respect, this study allows for a more direct assessment of the extent to which oxidative injury contributes to atherosclerotic sequelae in humans than do previously reported intervention trials that used, for example, antioxidants. It should also be noted that Patrono et al have reported that weight loss in obese women is associated with a significant reduction in IsoP formation.

**Other Risk Factors for Atherosclerotic Cardiovascular Disease**

High plasma levels of homocysteine may be an independent risk factor for cardiovascular disease. 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. The mean plasma total homocysteine and F$_2$-IsoP concentrations were 11.1 μmol/L and 29.6 ng/L, 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 contrast, Hirsch et al did not find an association between hyperhomocysteinemia and increased IsoP formation in men with normal serum folate levels.

Hypertension has been variably associated with increases in IsoP formation. Cracowski, Croft, and others have reported that that hypertensive subjects are variably, but often not, undergoing increased oxidative stress, whereas Patrono has found that patients with hypertension and renovascular disease have elevated excretion of both F$_2$-IsoPs and thromboxane, suggesting that enhanced oxidative stress and platelet activation are increased in this population. They propose that activation of renin-angiotensin system and excessive production of free radicals may be responsible for this increase in IsoP formation. Further, Minuz et al found a significant positive correlation between urinary IsoP levels, renal vein renin ratios, and angiotensin II levels in individuals with renal artery stenosis who have elevation of the renin-angiotensin system. Finally, at least one report notes that in certain hypertensive populations, infusion of angiotensin II significantly increases plasma F$_2$-IsoP levels.

**F$_2$-IsoPs as an Independent Marker for the Risk of Atherosclerosis**

We and others have previously shown that F$_2$-IsoP levels are significantly increased in atherosclerotic plaques compared with normal vascular tissue, suggesting that these compounds may play a role in the pathogenesis of the disease. In addition, this finding suggests that if atherosclerotic lesions are a source of circulating IsoPs, levels may be increased in humans with an extensive burden of the disease compared with nondiseased individuals and could prove to be a useful biomarker of disease risk. Systemic IsoP levels increase significantly after angioplasty, suggesting that local formation of these compounds contributes to total body production in this population. Recently, Schwedhelm et al tested the hypothesis that IsoPs are increased in persons with atherosclerosis independent of other risk factors in a matched case control. They recruited 93 patients with established coronary artery disease and 93 matched control subjects without disease. Compared with control individuals, body mass index, systolic blood pressure, and C-reactive protein were elevated in coronary heart disease patients. High-density lipoprotein cholesterol was significant decreased in patients with coronary artery disease. Urinary F$_2$-IsoP levels were elevated by a mean of 1.9-fold in diseased patients. All of these characteristics predicted coronary atherosclerosis in a univariate analysis. In a multivariate analysis, however, the odds ratios of atherosclerosis were increased only for urinary F$_2$-IsoPs ($≥131$ pmol/mmol; $P<0.001$) and C-reactive protein ($≥3$ mg/L; $P<0.01$) by 30.8 and 7.2, respectively. The conclusion of this study is that the F$_2$-IsoPs represent a novel marker in addition to known risk factors of coronary heart disease and that urinary excretion of the compounds correlate with the number of risk factors for all subjects ($P<0.001$ for trend). This report is particularly exciting because it suggests the quantification of circulating or urinary IsoPs may prove to be a sensitive and independent risk marker of coronary heart disease. Obviously, these observations will need to be confirmed in larger studies, although another recent report by Vassalle et al also suggest that increases in F$_2$-IsoP formation predicts the extent and severity of coronary artery disease.

**Conclusions**

The F$_2$-IsoPs represent, in 2005, the most established index of oxidative stress status in vivo in humans. They can be measured in human body fluids such as plasma and urine using highly precise methods. Over the past 15 years, their quantification has provided important insights into the role of oxidant injury in human pathophysiology and has highlighted the potential importance of this entity in a number of human diseases. Of these, enhanced oxidant stress has been strongly implicated in atherosclerotic cardiovascular disease and we now know that most major risk factors for the disorder are
associated with elevated systemic F₂-IsoP formation. In addition, a reduction in risk factors is associated with a decrease in IsoP formation in humans. Despite the fact that the role of oxidant stress in the pathogenesis of atherosclerosis is a hotly debated issue, in part because of a presumed failure of antioxidants to prevent the disease, current evidence suggests that the IsoPs represent a biomarker that has the potential to be of great importance in the assessment of human atherosclerotic cardiovascular disease.

Acknowledgments

Supported by National Institutes of Health grants GM15431, CA77839, DK48831, and RR00095. Supported by National Institutes of Health grants GM15431, CA77839, DK48831, and RR00095.

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Arterioscler Thromb Vasc Biol. 2005;25:279-286; originally published online December 9, 2004;
doi: 10.1161/01.ATV.0000152605.64964.e0
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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