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COMMENTARY

The Isoprostanes

CURRENT KNOWLEDGE AND DIRECTIONS FOR FUTURE RESEARCH

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ABSTRACT. The isoprostanes are a unique series of prostaglandin-like compounds formed *in vivo* from the free radical-catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme. The purpose of this commentary is to summarize the status of our current knowledge regarding the isoprostanes and discuss what we consider to be avenues for further research. Novel aspects related to the biochemistry of isoprostane formation and methods by which these compounds are analyzed, including potential pitfalls that may occur during analysis, are discussed first. The isoprostanes possess potent biological activity, and their potential role in mediating certain aspects of the detrimental effects of oxidant stress is then examined. In addition, evidence is presented that these biological effects may be mediated through interaction with a unique receptor. A considerable portion of this commentary deals with the utility of measuring isoprostanes as markers of oxidant injury both *in vitro* and *in vivo*. A number of studies have shown these compounds to be extremely accurate markers of lipid peroxidation in animal models of oxidative stress and have illuminated the role of oxidant injury in association with several human diseases. Finally, based upon our current knowledge of the isoprostanes, directions for future research are proposed. BIOCHEM PHARMACOL 51;1:1–9, 1996.

KEY WORDS. isoprostane; prostaglandin; eicosanoid; peroxidation; lipid; fatty acid

Free radicals, derived primarily from oxygen, have been increasingly implicated in the pathophysiology of a number of human diseases including cancer, atherosclerotic cardiovascular disease, neurodegenerative disorders, and even the normal aging process [1–4]. Definitive evidence for this association, however, is often lacking because of recognized shortcomings with methods previously available to assess oxidative stress status *in vivo* in humans [5].

A central feature of oxidant injury is peroxidation of lipids. Various methods have been developed to quantify products of free radical-induced lipid peroxidation as a potential means to assess oxidant injury. These include measurements of MDA,† lipid hydroperoxides, conjugated dienes, and short-chain alkanes, among others [5]. While many of these approaches appear to provide an accurate index of lipid peroxidation *in vitro*, shortcomings and inaccuracies have been recognized with most of these methods when used to assess oxidant stress *in vivo* [5]. In 1990, we reported the discovery that a series of PG-like compounds are produced *in vivo* in humans independent of the cyclooxygenase enzyme by the free radical-catalyzed peroxidation of arachidonic acid [6]. Since that discovery, we have accumulated a substantial body of evidence that suggests that measurement of these unique products of lipid peroxidation, now termed isoprostanes, can provide a reliable measure of oxidant injury not only *in vitro* but, more importantly, *in vivo* [7–10]. In addition, we have found that these compounds are capable of exerting potent biological activity [6, 11–13]. Thus, isoprostanes may also participate as mediators of oxidant injury. It is the purpose of this commentary to briefly summarize the status of our current knowledge of the isoprostanes and discuss what we consider to be avenues for potential fruitful research.

Discovery and Biochemistry of the Formation of Isoprostanes

Previous work by Pryor, Porter, and others elucidated that auto-oxidation of fatty acids *in vitro* leads to the formation of a variety of complex products, including PG-like bicycloendoperoxides [14, 15]. Along these same lines, utilizing mass spectrometry, we made the observation that arachidonyl-containing lipids in biological fluids, i.e. plasma, readily undergo auto-oxidation *in vitro*, resulting in the formation of a series of PGF_2-like compounds [16]. A mechanism to explain the formation of these compounds is outlined in Fig. 1. As noted, four bicycloendoperoxide PG-like intermediates are formed, which are reduced to four PGF_2-like regioisomers (I–IV). Each of these regioisomers can theoretically be comprised of 8 racemic diastereomers. Thus, 64 different compounds can potentially be produced by this mechanism. Because these compounds are structurally isomeric with $PGF_{2\alpha}$, these compounds are collectively referred to as F_2-isoprostanes.

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† Abbreviations: MDA, malondialdehyde; PG, prostaglandin; AAPH, 2,2′-azo-bis(2-amidinopropane) hydrochloride; LDL, low density lipoprotein; and TxA_2, thromboxane A_2.

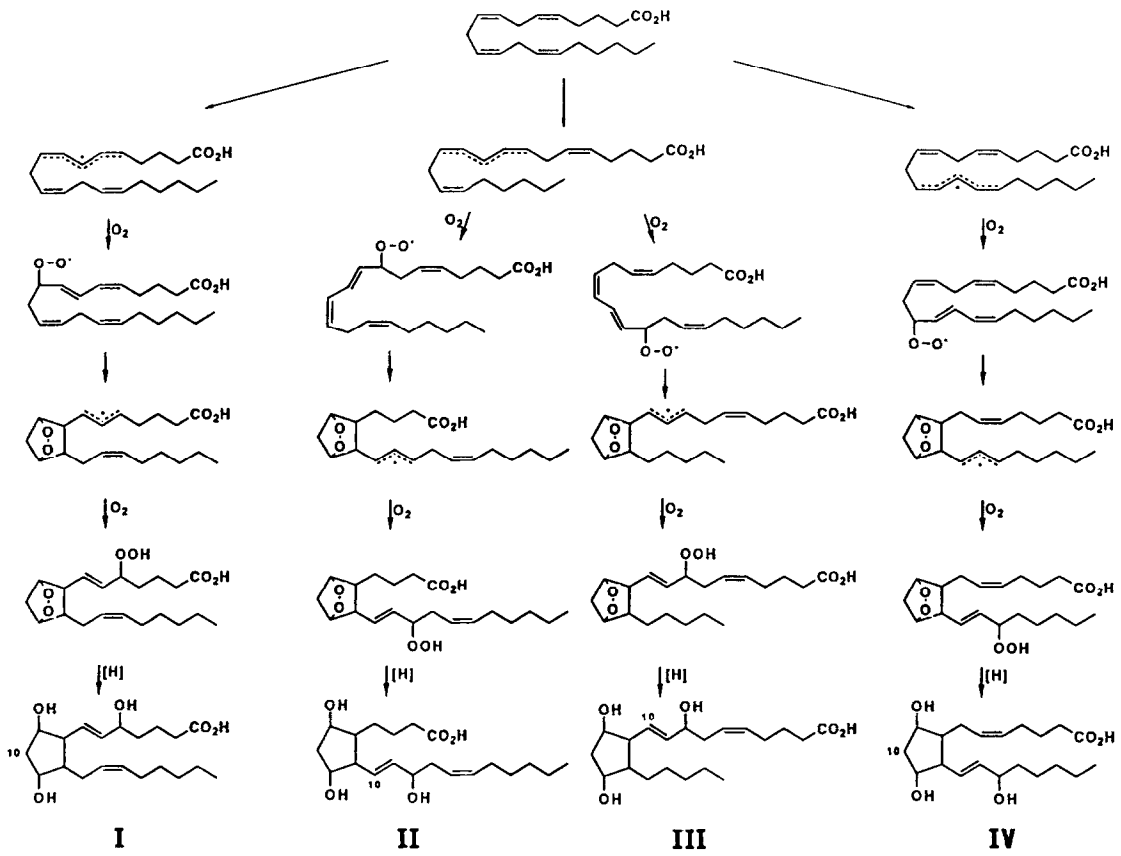

FIG. 1. Mechanism of the formation of the F_2-isoprostanes. The pathway leads to the formation of four F_2-isoprostane regioisomers (I–IV). For simplicity, stereochemistry is not indicated. Each regioisomer theoretically is composed of eight racemic diastereomers. Reprinted with permission from *Analyt Biochem* 184: 1–10, 1990. Copyright (1990) Academic Press, Inc., Orlando, FL. [Ref. 16].

We were impressed by how easily the formation of F_2-isoprostanes occurred *in vitro*. In fact, we initially found that large quantities of these compounds were formed by auto-oxidation of plasma during storage at −20° [16]. This prompted us to explore whether F_2-isoprostanes are also formed *in vivo* [6]. Added to initial observations suggesting that F_2-isoprostanes may be produced *in vivo* was the finding that they could be detected in measurable quantities in fresh human biological fluids that were analyzed immediately and not stored. The levels measured in fresh human plasma and urine from normal volunteers were 35 ± 6 pg/mL and 1600 ± 600 pg/mg creatinine (mean ± 1 SD), respectively. What was intriguing to us was that the levels of these compounds detected in human plasma and urine were at least an order of magnitude higher than levels of cyclooxygenase-derived prostaglandins. Levels measured were unaffected by administration of high doses of cyclooxygenase inhibitors, indicating that they were not derived from the cyclooxygenase, nor were they suppressed by the addition of antioxidants and reducing agents during sample processing, suggesting that they were not formed by auto-oxidation *ex vivo* [6]. Convincing evidence for the formation of F_2-isoprostanes *in vivo* was obtained by demonstrating that circulating levels of F_2-isoprostanes increased dramatically (up to 200-fold above baseline values) in two well-established models of free radical-induced lipid peroxidation, administration of diquat to selenium-deficient rats and CCl_4 to normal rats [6]. These latter observations were also enlightening in that they suggested that measurement of F_2-isoprostanes might provide a useful approach to assess oxidant injury *in vivo* in humans.

Knowing that only trivial amounts of arachidonic acid exist in cells in free form, the vast majority being esterified to tissue phospholipids, we examined the possibility that the biochemical process outlined in Fig. 1 occurs with esterified arachidonic acid. Indeed, we were able to isolate and identify, by liquid secondary ion mass spectrometry, F_2-isoprostane-containing phospholipids from the livers of rats treated with CCl_4 to induce lipid peroxidation [17]. Evidence was also obtained that following the formation of F_2-isoprostanes *in situ* on phospholipids *in vivo*, they are subsequently released in free form, presumably by phospholipases. The discovery of prostanoid-containing species of phospholipids was a novel finding in that prostaglandins derived from the cyclooxygenase do not exist esterified to phospholipids.

The discovery that isoprostanes are formed *in situ* esterified to tissue lipids has important ramifications from both biological and analytical perspectives. First, molecular modeling of isoprostane-containing phospholipids reveals them to be remarkably distorted molecules [17], which would be expected to alter profoundly membrane fluidity and integrity, a well-known sequela of oxidant injury [18]. However, as discussed subsequently, once released, isoprostanes can exert potent bi-

ological activity. Thus, how these two processes are regulated and balanced can influence the overall consequences of the formation of isoprostanes.

The endoperoxide, PGH_2, formed by the cyclooxygenase enzyme is an unstable molecule that readily undergoes rearrangement in aqueous solutions to form PGD_2 and PGE_2 [19]. Thus, we reasoned that if the isoprostane endoperoxide intermediates are not efficiently reduced to F-ring isoprostanes, rearrangement may occur, resulting in the formation of D-ring and E-ring isoprostanes. Studies undertaken to investigate this possibility convincingly identified the formation of D_2/E_2-isoprostanes *in vivo* [20]. The relative amounts of F_2- and D_2/E_2-isoprostanes formed vary somewhat in different tissues for reasons that remain to be determined; in some tissues, F_2-isoprostanes are the predominant isoprostane species present, whereas in other tissues approximately equivalent or greater amounts of D_2/E_2-isoprostanes are detected relative to F_2-isoprostanes.

Method of Analysis of the F_2-Isoprostanes

The method by which we initially detected and now quantify F_2-isoprostanes is a stable isotope dilution assay utilizing capillary gas chromatography/negative ion chemical ionization mass spectrometry [21]. This assay quantifies only free compounds. For measurement of levels of isoprostanes esterified to tissue lipids, the isoprostanes must be hydrolyzed from tissue lipids either enzymatically, i.e using bee venom phospholipase A_2, or chemically, i.e. using alkaline saponification, prior to quantification [21]. Alkaline hydrolysis can be used to hydrolyze F_2-isoprostanes from phospholipids, whereas phospholipase A_2 must be used for the hydrolysis of D_2/E_2-isoprostanes because these compounds are readily dehydrated in the presence of base. While the mass spectrometric method is very sensitive and accurate, several disadvantages are associated with it. First, mass spectrometry is not available to many investigators because of the large capital investment required to purchase a mass spectrometer and the high cost of maintaining the instrument. Second, the assay method is time-consuming and labor-intensive. To overcome these impediments, immunoassay methods for the measurement of isoprostanes are currently under development by commercial and academic investigators. In this regard, the mass spectrometric method for analysis of isoprostanes will be invaluable in validating the accuracy of immunoassay methods of analysis.

Biological Activity of the Isoprostanes

A key question that we have asked is whether the importance of the discovery of isoprostanes is limited to the value of measuring these compounds as a means to assess oxidative stress or whether they might also exert biological activity and thus participate as mediators in oxidant injury. Studies exploring the biological activity of isoprostanes have been limited by the availability of compounds in synthetic form. Previous studies in which PG-like compounds were observed to be formed by auto-oxidation *in vitro* determined that the side chains were predominantly oriented *cis* in relation to the cyclopentane ring, unlike cyclooxygenase-derived prostaglandins in which the side chains are exclusively oriented *trans* [6, 16]. With this information in hand, we hypothesized that one of the F_2-isoprostanes that would likely be produced would be 8-iso-$PGF_{2\alpha}$, and recently we provided evidence that 8-iso-$PGF_{2\alpha}$ is, in fact, one of the more abundant F_2-isoprostanes formed *in vivo* [22]. Fortuitously, Dr. Gordon Bundy at the Upjohn Co. had previously synthesized large quantities of 8-iso-$PGF_{2\alpha}$ and provided us with this compound to test for bioactivity. Because large quantities of F_2-isoprostanes are present in urine which, analogous to cyclooxygenase-derived prostaglandins, may derive in substantial part from local production in the kidney [23, 24], we initially examined the effect of infusions of 8-iso-$PGF_{2\alpha}$ on renal function in the rat. 8-iso-$PGF_{2\alpha}$ was found to be an extremely potent renal vasoconstrictor, at least an order of magnitude more potent than any other eicosanoid, reducing blood flow and glomerular filtration rate by 40–45% at low nanomolar concentrations [6, 11]. Subsequent studies also established that 8-iso-$PGF_{2\alpha}$ is a potent vasoconstrictor in the pulmonary vascular bed of rats and rabbits [12, 13].

Experiments were then undertaken to explore the mechanism by which 8-iso-$PGF_{2\alpha}$ exerts its vascular effects. Interestingly, we found that SQ29548, a TxA_2 receptor antagonist, completely abrogated the vascular effects of 8-iso-$PGF_{2\alpha}$, suggesting that it was interacting with thromboxane receptors [11]. To further explore the putative interaction of 8-iso-$PGF_{2\alpha}$ with thromboxane receptors, we examined the effects of 8-iso-$PGF_{2\alpha}$ on platelet aggregation. Surprisingly, 8-iso-$PGF_{2\alpha}$ was found to be only a weak partial agonist of platelet thromboxane receptors and acted primarily as an antagonist of platelet thromboxane receptors [25]. To explain this unexpected result, two possibilities were considered: (a) the platelet thromboxane receptor is different from that in the vasculature, or (b) the vasculature contains a unique "isoprostane" receptor that is absent in platelets.

At present, only a single thromboxane receptor gene has been cloned [26]. Splicing variants of the thromboxane receptor were identified recently, but these post-translational modifications were without obvious functional consequence [27]. In consideration of the latter hypothesis above, we and others have obtained indirect evidence suggesting that 8-iso-$PGF_{2\alpha}$ interacts with a receptor on vascular smooth muscle that may be distinct from the thromboxane receptor [28–30]. First, in rat vascular smooth muscle cells, there is a great discordance between the potency of 8-iso-$PGF_{2\alpha}$ to displace TxA_2 receptor ligand binding and its potency to induce a receptor-mediated response, i.e. induction of calcium flux, phosphoinositide turnover, and mitogenesis [28]. These latter effects occur at concentrations in the range of 10^{-9} M, whereas concentrations as high as 10^{-5} M are required to displace TxA_2 ligand binding. Further, very high concentrations of 8-iso-$PGF_{2\alpha}$ (approximately 10^{-5} M) are also required to displace TxA_2 ligand binding in COS-7 cells transfected with the human thromboxane receptor [28]. Further, whereas 8-iso-$PGF_{2\alpha}$ is significantly more potent than U-46619, a thromboxane receptor agonist, in causing renal vasoconstriction, it is two orders of magnitude less potent than U-46619 in inducing a functional

response, i.e. calcium mobilization, in thromboxane receptor transfected HEK 293 cells [30]. A recent preliminary report of results using radioligand binding approaches with radiolabeled 8-iso-$PGF_{2\alpha}$ is also consistent with the notion that 8-iso-$PGF_{2\alpha}$ interacts with a unique receptor distinct from the thromboxane receptor [29]. Although the above indirect evidence suggests that 8-iso-$PGF_{2\alpha}$ interacts with a receptor on vascular smooth muscle that is distinct from the thromboxane receptor, studies using molecular cloning strategies will be required to provide unequivocal proof for the existence of a unique receptor.

An additional interesting finding was made recently regarding the biological actions of isoprostanes. Following our discovery that D_2/E_2-isoprostanes are also produced in abundant quantities *in vivo*, we explored whether the E-ring counterpart to 8-iso-$PGF_{2\alpha}$, 8-iso-PGE_2, was also bioactive. Based on the fact that PGE_2 and $PGF_{2\alpha}$ often have opposing effects on vascular smooth muscle, i.e. $PGF_{2\alpha}$ is a vasoconstrictor whereas PGE_2 is a vasodilator, we were surprised to find that 8-iso-PGE_2 is also a potent renal vasoconstrictor, approximately equipotent compared with 8-iso-$PGF_{2\alpha}$ [20]. Evidence has also been obtained suggesting that 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ may interact with the same receptor on vascular smooth muscle [20, 31]. This is of interest in that it suggests that the spatial orientation of the side chains, rather than structural differences in the cyclopentane ring, is an important determinant of receptor interaction of these isoprostanes.

F_2-Isoprostanes as Markers of Oxidant Injury

As previously mentioned, it has long been recognized that one of the greatest needs in the field of free radical research is the availability of a reliable means to assess oxidant stress status *in vivo* [5]. There are several reasons that suggested to us that measurement of F_2-isoprostanes may represent an important advance in this area. First, F_2-isoprostanes are very stable molecules and can be detected in measurable quantities esterified in all body tissues and in free form in every biological fluid tested, which includes plasma, urine, bile, gastric juice, synovial fluid, and cerebrospinal fluid, thus allowing the definition of a normal range ([6, 21], and unpublished observations). In addition, levels increase dramatically in experimental animal models of oxidant injury and levels can be suppressed by administration of antioxidants [9, 32]. Further, levels are increased in animals rendered deficient in natural antioxidants, e.g. vitamin E, even in the absence of administering an agent to induce lipid peroxidation [8].

Salient features of some of the studies that support the notion that quantification of isoprostanes represents an important advance in our ability to assess oxidative stress status *in vivo* are presented below. The reader is referred to the cited references for more detailed discussions of particular studies.

Precautions Related to Measurements of F_2-Isoprostanes

It should be noted that our original discovery of the formation of F_2-isoprostanes occurred as a result of auto-oxidation of arachidonic acid in stored plasma [16]. Thus, appropriate precautions must be taken to prevent artifactual generation of isoprostanes by auto-oxidation *ex vivo* in lipid-containing biological samples. Whereas generation of isoprostanes by auto-oxidation can occur during storage of plasma at −20°, we have found that this does not occur during storage of plasma at −70° for up to 6 months [16, 21]. Thus, auto-oxidation can be prevented during storage of samples by rapidly freezing samples in liquid nitrogen and storing them at −70°. However, thawing and refreezing samples should be avoided. Alternatively, samples should be processed for analysis immediately after collection. Because of the additional handling and processing required for analysis of F_2-isoprostanes esterified to tissue lipids, the antioxidant butylated hydroxytoluene (0.005%) is added to the chloroform/methanol extraction solution [21]. As a further measure to prevent auto-oxidation *ex vivo* when analyzing levels of F_2-isoprostanes esterified to plasma lipids, which seems to undergo very facile auto-oxidation, 5 mg/mL triphenylphosphine is also added to rapidly reduce lipid hydroperoxides to hydroxy compounds, thus preventing the cyclization required to form isoprostanes [17]. When these precautions are taken, we have found that artifactual generation of isoprostanes *ex vivo* can be effectively prevented. Owing to the fact that urine contains only trace quantities of lipids, the concern of *ex vivo* formation of F_2-isoprostanes does not apply to the measurement of F_2-isoprostanes in urine.

A number of years ago it was demonstrated that the cyclooxygenase enzymes produces very small quantities of 8-iso-$PGF_{2\alpha}$ as a byproduct of its catalytic action converting arachidonic acid to PGH_2 [33]. This phenomenon was demonstrated again recently, following activation of platelets *in vitro* [34, 35]. This has raised a question as to the possibility that measurements of 8-iso-$PGF_{2\alpha}$ may not always be a reliable and specific marker of non-enzymatic lipid peroxidation [35]. Although this would not be a concern using the mass spectrometric method for analysis of F_2-isoprostanes that we have described, in which GC peaks representing F_2-isoprostanes other than 8-iso-$PGF_{2\alpha}$ can be measured, this theoretically might be a concern using immunoassays specific for 8-iso-$PGF_{2\alpha}$, which are under development. However, the amount of thromboxane formed by the cyclooxygenase in activated platelets is more than 1000-fold greater than the amount of 8-iso-$PGF_{2\alpha}$ generated, indicating that 8-iso-$PGF_{2\alpha}$ is a trivial byproduct of the cyclooxygenase. It should be kept in mind that the levels of F_2-isoprostanes in human biological fluids exceed levels of the major products of the cyclooxygenase by more than an order of magnitude. Thus, the amount of 8-iso-$PGF_{2\alpha}$ that might be formed via the cyclooxygenase enzyme would not be expected to contribute significantly to the amount of 8-iso-$PGF_{2\alpha}$ formed independent of the cyclooxygenase. This notion is supported by our previous demonstration that quantification of F_2-isoprostanes in plasma and urine from normal volunteers, using the intensity of the GC peak where 8-iso-$PGF_{2\alpha}$ elutes, is unaffected by treatment with high doses of inhibitors of the cyclooxygenase [6]. This was also confirmed recently by Catella and colleagues [36]. Although there was speculation by these investigators that a meaningful

contribution by the cyclooxygenase to levels of 8-iso-$PGF_{2\alpha}$ measured might occur in situations where platelets are activated *in vivo*, recent data from Ciabottoni and colleagues, using an RIA with an antibody specific for 8-iso-$PGF_{2\alpha}$, suggest that this is not the case [37]. Further, if a significant cyclooxygenase contribution to the formation of 8-iso-$PGF_{2\alpha}$ occurs *in vivo*, this should be detectable in any situation in which there is markedly increased cyclooxygenase activity. However, this was not found when we analyzed plasma from a patient with unusually severe systemic mastocytosis in whom there was an approximately 100-fold increase in endogenous production of PGD_2, assessed by quantification of the excretion of the major urinary metabolite of PGD_2 [38]. The level of F_2-isoprostanes measured in this patient was 26 ± 3 pg/mL plasma, and the level in normal individuals is 35 ± 6 pg/mL. Further, we have found that the levels measured in serum from whole blood allowed to clot at room temperature are virtually identical to levels measured in plasma (unpublished observations), indicating that quantities of 8-iso-$PGF_{2\alpha}$ that may be generated by the cyclooxygenase from platelets and leukocytes during blood sampling and plasma isolation are also insufficient to influence endogenous levels measured.

In vitro Studies

We have carried out several studies involving the measurement of F_2-isoprostanes in *in vitro* systems of lipid peroxidation, and in some of these we have compared F_2-isoprostane formation with other indicators of lipid peroxidation. This work has demonstrated the utility of measuring F_2-isoprostanes as an index of lipid peroxidation *in vitro*. In one study, we compared isoprostane formation with measurement of MDA by the thiobarbituric acid reacting substances test in Fe/ADP/ascorbate-induced peroxidation of rat liver microsomes [10]. Both MDA and F_2-isoprostane formation increased in a parallel fashion in a time-dependent manner and correlated with the loss of arachidonic acid and with increasing oxygen tensions up to 21%. Although the formation of F_2-isoprostanes correlated with measurements of MDA in this *in vitro* system, measurement of F_2-isoprostanes was found to be far superior to measurements of MDA as an index of lipid peroxidation *in vivo* (*vide infra*).

Because oxidation of LDL converts it to an atherogenic form that is taken up by macrophages, there has been considerable interest in the role of oxidation of LDL in atherogenesis [39, 40]. Thus, we have carried out studies examining the formation of F_2-isoprostanes in LDL exposed to a variety of oxidizing conditions to explore the possibility that measurement of isoprostanes esterified to lipoproteins may provide an approach to assess lipoprotein oxidation *in vivo* [41]. Such studies were also of interest in that the F_2-isoprostane, 8-iso-$PGF_{2\alpha}$, is a potent vasoconstrictor and induces mitogenesis in vascular smooth muscle cells, and these biological effects could be relevant to the pathophysiology of atherosclerosis [11]. In one study, isolated human LDL and plasma lipids were peroxidized with Cu^{2+} or AAPH, which is a source of peroxyl radicals, and the formation of F_2-isoprostanes was compared with that of other markers of lipid peroxidation, i.e. formation of lipid hydroperoxides, loss of antioxidants, and changes in the electrophoretic mobility of LDL [41]. In plasma exposed to AAPH, formation of F_2-isoprostanes and lipid hydroperoxides occurred following depletion of the antioxidants ascorbate and ubiquinol-10. In isolated LDL exposed to copper ions or AAPH, formation of F_2-isoprostanes and lipid hydroperoxides proceeded only after depletion of α-tocopherol and ubiquinol-10. In both plasma and LDL, the time–course of formation of F_2-isoprostanes closely parallel the formation of lipid hydroperoxides and correlated with relative changes in electrophoretic mobility of LDL. Similar results were also reported by Gopaul and colleagues [42] during oxidation of LDL by both endothelial cells and copper.

Recently, there has been considerable interest in the potential role of the oxidant peroxynitrite, a product formed by reaction of nitric oxide with superoxide, in LDL oxidation *in vivo* [43]. Therefore, we carried out a study examining the formation of F_2-isoprostanes in LDL exposed to peroxynitrite [44]. Peroxynitrite was found to catalyze the formation of F_2-isoprostanes in LDL in a concentration-dependent fashion, which again correlated with changes in electrophoretic mobility of LDL. These findings suggest that quantification of F_2-isoprostanes esterified to plasma lipoproteins may provide a useful approach to assessing oxidation of lipoproteins *in vivo*. This notion is supported by our recent finding of increased quantities of F_2-isoprostanes esterified to plasma lipids in smokers (*vide infra*) [45]. Important in this regard is that we can detect measurable quantities of F_2-isoprostanes esterified to plasma lipids even in normal young healthy volunteers, thus allowing the definition of a normal range [41]. Previous studies using other markers of lipid peroxidation have failed to convincingly demonstrate the presence of oxidized plasma lipids in the circulation under normal circumstances.

F_2-Isoprostanes as Markers of Oxidant Injury in vivo

We have accumulated a substantial body of evidence that suggests strongly that measurement of F_2-isoprostanes can provide a valuable approach to assessing oxidant injury *in vivo*. As previously mentioned, in animal models of oxidant injury, levels of F_2-isoprostanes increase dramatically both free in the circulation and esterified to tissue phospholipids in target organs that are damaged. In some studies, we have obtained data that illustrate the much greater utility of measuring F_2-isoprostanes compared with other markers of lipid peroxidation to assess oxidant injury *in vivo*. For example, following administration of CCl_4 to rats, the levels of F_2-isoprostanes esterified to lipids in the liver increased greater than 80-fold, whereas the levels of MDA in the liver increased only 2.7-fold [10]. In another study, we also found that measuring F_2-isoprostanes afforded a more sensitive indicator of CCl_4-induced lipid peroxidation compared with measurement of lipid hydroperoxides by mass spectrometry [32].

An important concept regarding the formation of isoprostanes is that they are initially formed *in situ* esterified to tissue phospholipids and are subsequently released preformed, pre-

sumably by phospholipases. Whereas measurement of free F_2-isoprostanes in the circulation can be utilized to assess endogenous isoprostane formation from all potential sources in the body, measurement of levels of isoprostanes esterified to tissue lipids can be employed to assess oxidant injury in key tissues of interest [8, 9]. This is particularly advantageous when oxidant injury may be localized to a particular organ. As an example, we have shown that administration of CCl_4 to rats leads to dramatic increases in levels of F_2-isoprostanes esterified to phospholipids in the liver and kidney but not the brain or heart, suggesting organ-specific injury from this agent [9]. This approach also allowed us to identify organ-selective injury, resulting from selenium deficiency and vitamin E deficiency in rats [8]. In humans, the sensitivity of the mass spectrometric assay for F_2-isoprostanes should allow measurements of levels of F_2-isoprostanes esterified to tissue phospholipids in biopsy specimens. Although we have not had extensive experience with this approach, preliminary results obtained from studies using biopsies from human gastric mucosa appear to support feasibility of this approach, at least in some tissues.

Application of the Isoprostane Assay to Explore the Role of Oxidant Injury in the Pathophysiology of Human Disease

GENERAL ASPECTS. As mentioned, quantification of non-esterified F_2-isoprostanes in plasma offers an approach to assess isoprostane production from all potential sources in the body. One disadvantage with this approach is that it provides information at only a single point in time. Thus, if there are wide fluctuations in the production of isoprostanes over time, levels of F_2-isoprostanes measured in a single sample of blood may not provide an accurate time-integrated assessment of isoprostane formation. However, our recent discovery of the presence of urinary metabolites of F_2-isoprostanes in human urine, which can be quantified by a mass spectrometric assay that we previously developed for quantification of the major urinary metabolite of prostaglandin D_2, may circumvent this problem [46]. Measurement of the level of excretion of these urinary F_2-isoprostane metabolites in urine collected over several hours can provide an integrated assessment of total endogenous isoprostane formation over prolonged periods of time.

Some discussion about current uncertainties related to the origin of unmetabolized F_2-isoprostanes in urine is relevant as it relates to the interpretation of data obtained regarding measurements of F_2-isoprostanes in urine. Previous studies have shown that unmetabolized urinary prostaglandins formed via the cyclooxygenase enzyme derive, to a large extent, from local production in the kidney rather than filtration from the circulation [23, 24]. This is important in that it indicates that measurements of urinary prostaglandins cannot be utilized to accurately assess systemic prostaglandin production. At present, we do not know the source of urinary F_2-isoprostanes, namely whether they derive exclusively from formation in the kidney, exclusively from filtration from the circulation, or a combination of both. Data that we have obtained recently suggest that urinary F_2-isoprostanes, at least in part, may derive from local production in the kidney. In individuals who smoke, we found a highly significant correlation between circulating concentrations of F_2-isoprostanes and the excretion of F_2-isoprostane urinary metabolites, suggesting that both accurately reflect systemic F_2-isoprostane production. However, in these individuals, there was a significant lack of correlation between levels of circulating and urinary unmetabolized F_2-isoprostanes ([45], and unpublished data). Thus, until definitive studies are undertaken to determine the source of urinary isoprostanes, it remains unclear whether quantification of urinary F_2-isoprostanes can be utilized as a reliable measure of isoprostane formation from extra-renal tissues.

SPECIFIC CLINICAL APPLICATIONS. One of our initial studies utilizing measurements of F_2-isoprostanes to explore the role of free radicals in the pathophysiology of human disease involved testing the hypothesis that oxidative stress may be involved in the pathogenesis of the hepatorenal syndrome [47]. The hepatorenal syndrome is an almost uniformly fatal disorder characterized by the occurrence of renal failure of unknown cause in patients with severe liver disease. The renal dysfunction is due to intense renal vasoconstriction, but the etiology of the vasoconstriction remains unclear. The basis for considering the hypothesis that oxidant stress may be involved in the pathogenesis of this disease is that these patients frequently exhibit chronic endotoxemia and tissue hypoxia, an environment that may be conducive to the generation of free radicals [48]. We found that circulating concentrations of F_2-isoprostanes were selectively increased by a mean of 7.8-fold above normal in patients with hepatorenal syndrome compared with appropriate control groups [47]. Moreover, infusion of superoxide dismutase in three patients resulted in a reduction by approximately 50% in circulating levels of F_2-isoprostanes between 30 and 60 min after initiating the infusion. Since the isoprostanes, 8-iso-$PGF_{2\alpha}$ and 8-iso-PGE_2, are potent renal vasoconstrictors, it is attractive to consider the possibility, although it remains to be proven, that isoprostanes may contribute to the intense renal vasoconstriction that characterizes this disease. Nonetheless, these findings do suggest that oxidant stress may play a role in the pathogenesis of the hepatorenal syndrome, which provides a basis for considering hypotheses regarding therapeutic intervention strategies in these patients.

We have also recently found markedly elevated levels of F_2-isoprostanes in the circulation of patients with renal failure and severe liver injury resulting from acetaminophen (paracetamol) overdose (unpublished data). Whether this occurs because this is a form of hepatorenal syndrome in these patients or is due to generation of free radicals by acetaminophen is not clear.

More recently, we explored the hypothesis that cigarette smoking causes an oxidant stress by determining whether the production of F_2-isoprostanes is increased in 10 heavy smokers, who smoked approximately 2 packs of cigarettes a day, compared with age- and gender-matched non-smokers [45]. Oxidant stress from smoking could arise because cigarette smoke is known to contain a large number of oxidants or from generation of free radicals by phagocytes [49]. This was an attractive hypothesis in that oxidation of LDL and DNA by cigarette

smoking could provide a mechanistic basis to explain the known association of smoking with accelerated atherosclerosis and cancer. Previous attempts to demonstrate that smoking is associated with an oxidative stress, using other methods to assess oxidant stress, have yielded results that are conflicting and difficult to interpret [50–52]. As a group, the smokers had significantly increased levels of F_2-isoprostanes, both free in the circulation and esterified to plasma lipids, and exhibited markedly increased urinary excretion of F_2-isoprostane metabolites. Further, we found that levels of circulating F_2-isoprostanes fell significantly (by approximately 35%) after only 2 weeks following cessation of smoking. The finding that smoking is associated with enhanced formation of isoprostanes provides compelling evidence that smoking causes oxidative damage, which may explain the causative link between smoking and the development of atherosclerosis and cancer.

Summary and Directions for Future Research

The importance of the discovery of isoprostanes encompasses two general areas. The first relates to the fact that, compared with other methods, measurement of isoprostanes appears to provide a far superior approach to assess oxidative stress status *in vivo*. At present, however, the lack of validated methods of assay for isoprostanes other than mass spectrometry has greatly curtailed the general use of this approach to assess oxidant injury. Thus, there is a great need to develop an inexpensive method of assay for isoprostanes, which can be performed by a large number of investigators. An immunoassay method of analysis would seem to fulfill these requirements, and such methods are currently under development. With the general availability of a reliable method for the measurement of isoprostanes, we can likely anticipate that new and exciting insights into the role of free radicals in human disease will be forthcoming in the near future.

Another area for important research that is related to the use of measurements of isoprostanes is the definition of the clinical pharmacology of antioxidant agents. Because of the shortcomings of methods previously available to quantify the level of oxidative stress *in vivo*, little information is currently known regarding the optimal doses and combinations of antioxidants that effectively suppress oxidant injury in humans. This applies not only to the natural antioxidants, e.g. vitamin C, vitamin E, and β-carotene, but to antioxidant drugs as well. Because large clinical trials either are planned or are underway examining the efficacy of antioxidants to suppress various disease processes, e.g. atherosclerosis, information regarding the optimal doses and combinations of these agents will be crucial in the interpretation of results from such studies and in designing future studies. In this regard, we have found that at least high doses of vitamin C, vitamin E, and β-carotene that are considered safe can suppress significantly the formation of F_2-isoprostanes in normal human volunteers (unpublished data). Thus, it seems plausible that it will be possible to gain insights into the clinical pharmacology of antioxidant agents using suppression of isoprostane formation as an endpoint.

The other important aspect of the discovery of isoprostanes, in which we currently have only a very limited amount of information, relates to the biological actions of these compounds. Interest in further pursuing the biological actions of isoprostanes is driven by the fact that the lead compound that was initially available to us for biological testing, 8-iso-$PGF_{2\alpha}$, was found to have very potent and unique biological actions. Subsequently, the second compound available for testing, 8-iso-PGE_2, was also found to exert potent bioactivity. Not only do we not know the full spectrum of biological activity of these two isoprostanes, but we currently know nothing regarding the biological activity of the additional plethora of compounds that are produced. Advances in this area are hampered by the unavailability of additional isoprostanes in synthetic form. However, several groups are actively pursuing the chemical synthesis of other isoprostanes. Thus, additional synthetic compounds should be forthcoming in the near future, which will allow us to explore further the biological ramifications of isoprostane formation.

The indirect evidence that has been obtained suggesting that 8-iso-$PGF_{2\alpha}$ and 8-iso-PGE_2 may interact with a unique receptor on vascular smooth muscle is certainly very intriguing. One wonders why there would be a unique receptor for compounds that are generated as products of a process thought to be injurious and that organisms have developed elaborate systems to suppress, namely oxidant injury. Might these compounds have a physiological role? Alternatively, the binding characteristics of these compounds may have allowed the recognition of a receptor for which the natural ligand remains unclear. Nonetheless, future studies aimed at the molecular cloning of this putative unique receptor will be of considerable value as would the development of specific antagonists of the receptor. The latter would allow a better definition of the role of these compounds as mediators in settings of oxidant injury. Although we found that SQ29548, a thromboxane receptor antagonist, can abrogate the vascular effects of these two isoprostanes, interpreting the results from the use of SQ29548 is clouded by the fact that it also inhibits the actions of TxA_2. Nonetheless, it is of interest that thromboxane receptor antagonists have been found to exert salutary effects that are difficult to explain by antagonism of the actions of TxA_2 in some pathological situations in which free radicals have been implicated [53, 54].

Thus, much remains to be learned about the isoprostanes. The initial discovery of the formation of these compounds by auto-oxidation *in vitro* in biological fluids was a biochemical curiosity that had potential analytical ramifications for the analysis of eicosanoids. However, the subsequent demonstration that they are also formed in abundant quantities *in vivo* and can exert biological activity has opened up new unexpected vistas for scientific inquiry and investigation. We are confident that many more new and exciting discoveries will emerge from continued research in this area.

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