Urinary Prostaglandin F$_{2\alpha}$ Is Generated from the Isoprostane Pathway and Not the Cyclooxygenase in Humans*

Received for publication, September 20, 2006, and in revised form, November 8, 2006. Published, JBC Papers in Press, November 15, 2006, DOI 10.1074/jbc.M608975200

Huiyong Yin$^{1,5}$, Ling Gao$^1$, Hsin-Hsiung Tai$^1$, Laine J. Murphey$^1$, Ned A. Porter$^6$, and Jason D. Morrow$^{11}$

From the $^1$Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, Department of Chemistry, Center in Molecular Toxicology, and the $^2$Vanderbilt Institute of Chemical Biology, Vanderbilt University, Nashville, Tennessee 37232 and the Department of Pharmaceutical Sciences, $^3$University of Kentucky, Lexington, Kentucky 40536

Prostaglandins (PGs) derived from the enzymatic oxidation of arachidonic acid by the cyclooxygenases (COXs) are potent lipid mediators involved in human physiology and pathophysiology. Structurally similar compounds, the isoprostanes (IsoPs), are generated from the free radical-catalyzed oxidation of arachidonic acid independent of COX. IsoPs exhibit significant bioactivity and play a role in the pathogenesis of diseases associated with oxidant injury. As one of the major PGs, prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) is present in human urine in significant concentrations and is presumed to be derived from COX activity. We determined, however, that levels of putative PGF$_{2\alpha}$ in urine cannot be suppressed by nonsteroidal anti-inflammatory agents, suggesting that it is generated via another mechanism(s). An important difference between COX-derived PGF$_{2\alpha}$ and the IsoPs is that the former is an optically pure compound, whereas IsoPs are racemic. Utilizing a rodent model of oxidative stress, we now show that significant amounts of compounds identical in all respects to PGF$_{2\alpha}$ and its enantiomer, ent- PGF$_{2\alpha}$, are formed in equal amounts esterified in tissue phospholipids, suggesting that these compounds are derived via the IsoP pathway. Further, employing liquid chromatography/mass spectrometry, the vast majority of putative PGF$_{2\alpha}$ in human urine is derived from the free radical-initiated peroxidation of arachidonate independent of COX and is composed of PGF$_{2\alpha}$ and its enantiomer, although the latter compound is ~2-fold more abundant. Thus, quantification of urinary PGF$_{2\alpha}$ actually reflects oxidative stress status as opposed to COX activity. Indeed, levels of this compound are elevated in urine from cigarette smokers and in humans with hypercholesterolemia, two conditions associated with oxidative stress. The elucidation that urinary PGF$_{2\alpha}$ in humans is derived from the IsoP pathway has implications regarding PG formation and inhibition in vivo.

*This work was supported by National Institutes of Health Grants DK48831, RR00095, CA77839, GM15431, ES00267, P30 ES000267, and ES13125. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† To whom correspondence should be addressed: Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, 1313 23rd and Pierce Aves., Nashville, TN 37232-6602. Tel.: 615-343-1124; Fax: 615-322-3669; E-mail: Jason.morrow@vanderbilt.edu.

‡‡ The abbreviations used are: PG, prostaglandin; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; PGE-M, 11α-hydroxy-9,15-dio-2,3,4,5-tetranor-prostan-1,2-dioic acid; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; COX, cyclooxygenase; APCI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; ESI, electrospray ionization; GC, gas chromatography; HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; SRM, selective reaction monitoring; IsoP, isoprostane; LPS, lipopolysaccharide; NSAID, nonsteroidal anti-inflammatory drug; PFB, pentafluorobenzyl; SAPC, 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine.
Generation of PGF$_{2\alpha}$ via the Isoprostane Pathway

cally. Further, certain lipid oxidation products such as lipid hydroperoxides generated from free radical-mediated lipid peroxidation can activate the COXs (13, 14).

PGF$_{2\alpha}$ exerts a wide variety of biological activities and is a potent constrictor of smooth muscle in various organs and vasculature (15). It has been noted previously that human urine contains large amounts of PGF$_{2\alpha}$ when analyzed by immunoassay and mass spectrometry (MS). It has been assumed that in human urine, PGF$_{2\alpha}$ is derived from the COX pathway (16–18). In preliminary studies, however, we determined that unlike other PGs, levels of this compound could not be suppressed in humans by NSAIDs, suggesting that this compound was generated independently of COX. Herein, we report studies to clarify the origin of this compound in human urine. As noted above, the generation of PGF$_{2\alpha}$ via the COX results in the formation of a single stereoisomer, whereas that formed via the IsoP pathway consists of PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$. Utilizing an animal model of oxidative stress and human clinical studies, we have determined that that urinary PGF$_{2\alpha}$ is derived largely, if not entirely, via the IsoP pathway and not from COX. The elucidation that urinary PGF$_{2\alpha}$ in humans is formed via the IsoP pathway has significant implications regarding PG formation and inhibition in diseases associated with oxidative stress including atherosclerosis.

EXPERIMENTAL PROCEDURES

Reagents and Animals—Lipids were purchased from Nu Chek Prep (Elysian, MI) and were of the highest purity (>99.4%). PGF$_{2\alpha}$ [{$^{3}$H$_{4}$}]PGF$_{2\alpha}$ and 13,14-dihydro-15-keto-PGF$_{2\alpha}$ were purchased from Cayman Chemicals (Ann Arbor, MI). Male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). Human recombinant 15-hydroxyprostaglandin dehydrogenase (15-PGDH) was partially purified by blue agarose affinity chromatography as described previously (19). The standard ent-PGF$_{2\alpha}$ was prepared from ent-PGE$_{2}$ by sodium boron hydride reduction and separated from the 1,3-trans-diols on the cyclopentane ring by HPLC (20). Compound structure was confirmed by various mass spectrometric and chemical approaches and compared with commercially available (Cayman Chemical) chemically pure ent-PGF$_{2\alpha}$.

Oxidation of lipids in Vitro—Phospholipids were oxidized as a thin film under atmospheric oxygen at 37 °C for 24 h as described (30). Lipid autoxidation mixtures were reduced with triphenylphosphine and hydrolyzed with methanolic KOH or phospholipase A$_{2}$ prior to analysis. Other arachidonate oxidation mixtures were obtained using 15(S)-hydroperoxyeicosatetraenoic acid as a precursor. 15(S)-hydroperoxyeicosatetraenoic acid was generated using soybean 15-lipoxygenase as described (21).

Extraction of Esterified IsoPs from the Rat Liver—The extraction and hydrolysis of IsoPs from livers of rats treated with COC$_{4}$ was performed as reported previously (22). IsoPs were then purified by C18 and Si Sep-Pak extraction. The mixture was reconstituted in the liquid chromatography (LC) mobile phase for LC-electrospray ionization (ESI)-MS analysis as IsoP-free acids. For atmospheric pressure chemical ionization (APCI)-MS analysis, IsoP-free acids were esterified to the pentafluorobenzyl (PFB) ester followed by TLC separation. For gas chromatography (GC)/MS analysis, the PFB ester was further derivatized to trimethylsilyl derivatives.

Urine Sample Preparation—An internal standard of [{$^{3}$H$_{4}$}]PGF$_{2\alpha}$ (1.0 ng) in ethyl alcohol was added to 1 ml of urine, and the mixture was allowed to equilibrate for 15 min. The urine sample was purified by the Sep-Pak methods above. APCI-MS was carried out using PFB esters, and GC-MS analysis was done using PFB and trimethylsilyl derivatives. The levels of F$_{2}$-IsoPs and PGs were expressed as ng per mg of creatinine. In some studies, either larger amounts of urine were analyzed for PGF$_{2\alpha}$ or urine was also quantified for the major urinary metabolites of thromboxane, prostacyclin, or PGE$_{2}$ as described (23).

LC-MS Analysis of F$_{2}$-IsoPs and PGs—F$_{2}$-IsoPs were analyzed by normal phase HPLC using 12% isopropyl alcohol in hexanes. Chiral LC separation was carried out on a ChiralPak AD column (25 × 0.46 cm, Chiral Technologies, Exton, PA) with 7% ethyl alcohol and 7% isopropyl alcohol in hexane. For reverse phase HPLC, a Phenomenex Luna ODS 5 μm 2.1 mm × 25 cm column (Torrence, CA) was utilized at 0.2 ml/min flow rate with a gradient starting with 80% solvent A (2.0 mm NH$_{4}$Ac) and 20% solvent B (5.95, MeOH:CH$_{3}$CN), holding for 2 min and increasing to 65% B in 30 min and holding for 10 min. LC/MS was carried out using a ThermoFinnigan TSQ Quantum 1.0 SR 1 mass spectrometer in negative ion mode. The instrument conditions were reported previously (20). Data acquisition and analysis were performed using Xcaliber software, version 1.3.

15-PGDH Assay—15-PGDH activity was determined by following the formation of NADH fluorometrically as described previously (19).

Clinical Studies—All volunteers in clinical studies gave written informed consent, and the Vanderbilt University Institutional Review Board approved all studies. Human protocols utilizing the administration of ibuprofen (800 mg orally four times daily for 4 days) and lipopolysaccharide (LPS) were reported previously (14, 23). Urine was collected for 24 h before and during treatment. In these studies, platelet thromboxane production was also measured in serum ex vivo as described (14). For studies utilizing LPS, 11 male and 11 female volunteers, hospitalized at Vanderbilt University, received a bolus injection of 2 ng/kg of reference Escherichia coli endotoxin purified LPS (U. S. Reference E. coli endotoxin lot E.C.-5) with a specific activity of 5 units/ng.

The protocol for cigarette smoking was published previously (24, 25). Four male and four female smokers were selected who were consuming at least a pack (20 cigarettes) per day. 24-hour urine samples were measured for urinary PGF$_{2\alpha}$. Urine samples were also analyzed from eight hypercholesterolemia patients (total cholesterol >200 mg/dl). Importantly, for these studies, several experiments were performed to show that PGF$_{2\alpha}$ was stable during the 24-h urine collection. First, levels of endogenous PGF$_{2\alpha}$ did not vary significantly when human urine samples were incubated at room temperature for up to 5 days. Secondly, when synthetic PGF$_{2\alpha}$ (5 ng/ml) was added to urine and incubated at room temperature for 24 h, measured recovery was 98 ± 7% when quantified by mass spectrometry (n = 5).

Statistical Analysis—Data are presented as the mean ± S.E. Statistical analysis was performed using analysis of variance fol-
RESULTS

The IsoP Pathway Generates Racemic Mixtures of F$_2$-IsoPs with Structures Identical to PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$ in Vitro and in Vivo—Free radical-induced autoxidation of polyunsaturated fatty acids has been associated with numerous human disorders, including atherosclerosis and neurodegenerative diseases (1, 26–28). F$_2$-IsoPs are one of the major oxidation products of arachidonic acid generated in vitro and in vivo. We have previously proposed a unified mechanism to explain the formation of these compounds (Fig. 1). According to this mechanism, four regioisomers can be generated from the oxidation of arachidonic acid (29). Among these four possible regioisomers, 5- and 15-series compounds are formed in significantly greater amounts than 8- and 12-series molecules (10). Within each regioisomeric group, eight racemic diastereomers are generated. Previous studies have shown that diastereomers with cis-alkyl side chains are produced in greater amounts when compared with those with trans-side chains although the latter are readily detectable (11). We developed an LC-MS method to study the regioisomeric and diasteromeric distribution of F$_2$-IsoPs from the in vitro oxidation mixture of the arachidonic acid-containing phospholipid 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphatidylcholine (SAPC), which is the biologically relevant form of this fatty acid. The results are shown in Fig. 2A. The 5- and 15-series of F$_2$-IsoPs are indeed formed in much greater amounts than those of 8- and 12-series. Interestingly, however, a peak in the 15-series IsoP chromatogram that co-elutes with PGF$_{2\alpha}$ is readily detectable, although it is not the major diastereomeric product. Total quantities of F$_2$-IsoPs generated are 500 ± 30 ng/mg of SAPC. Of these, material co-eluting with PGF$_{2\alpha}$ is ∼10% of the total F$_2$-IsoPs.

After further purification of material that co-elutes with PGF$_{2\alpha}$ by HPLC, a single peak was obtained when analyzed by APCI-MS (Fig. 2B). Subsequently, this material was subjected to chiral LC, and two peaks with nearly equal amounts are present (Fig. 2C). Moreover, the first perfectly co-elutes with chemically synthesized ent-PGF$_{2\alpha}$ and the latter with PGF$_{2\alpha}$. These two peaks were then collected and derivatized to trimethylsilyl ethers; GC-MS analysis showed that these two enantiomers have exactly the same retention time as that of a PGF$_{2\alpha}$ standard (data not shown). These experimental results support the contention that free radical-mediated peroxidation of phospholipid-bound arachidonate generates racemic mixture of isoprostanes, and the isomer with structures identical to PGF$_{2\alpha}$ is formed in significant amounts.

FIGURE 1. Free radical-induced oxidation of arachidonic acid forms four series of F$_2$-IsoPs. A, a mechanism of formation of F$_2$-IsoPs from the oxidation of arachidonic acid. B, chemical structures of the four F$_2$-IsoP regioisomers.
Generation of PGF$_{2\alpha}$ via the Isoprostane Pathway

We next sought to determine the extent to which PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$ are generated in vivo esterified to phospholipids. For these studies, oxidant stress was induced in rats by the intragastric administration of carbon tetrachloride (30). Esterified F$_{2}$-IsoPs in liver tissue were analyzed using the same approaches as noted for the in vitro oxidation above. A virtually identical pattern of F$_{2}$-IsoP regioisomers was observed as in the in vitro oxidation of arachidonate-containing phospholipids (Fig. 2D). The total amount of F$_{2}$-IsoPs generated is ~630 ± 50 ng/g of liver tissue, and material co-eluting with PGF$_{2\alpha}$ represents ~5–10% of the total. When material co-eluting with PGF$_{2\alpha}$ was then analyzed by chiral LC, two peaks were obtained representing a racemic mixture of equal amounts of PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$ (Fig. 2E). These results demonstrate that similar to the generation of PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$ in vitro, racemic PGF$_{2\alpha}$ can be formed in vivo from the peroxidation of arachidonate esterified in tissue phospholipids.

Human Urine Contains PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$—Having found that significant amounts of PGF$_{2\alpha}$ are formed in vitro and in vivo esterified to phospholipids via the IsoP pathway, we next sought to determine whether PGF$_{2\alpha}$ present in human urine is enantiomerically pure or consists of a mixture of PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$. This determination is of significance since it is generally assumed that PGF$_{2\alpha}$ is exclusively derived in vivo via COX (16, 17). Thus, urine was obtained from healthy volunteers, and material representing putative PGF$_{2\alpha}$, as well as F$_{2}$-IsoPs, was subjected to purification and analysis by LC/MS. The results are shown in Fig. 3. The pattern of F$_{2}$-IsoPs found in human urine is different from that observed in phospholipids from an in vitro oxidation or from liver extract from rats after CCl$_{4}$ treatment primarily because of the different relative abundance of IsoP isomers. Interestingly, material representing putative PGF$_{2\alpha}$ is one of the major 15-series IsoP peaks in human urine (Fig. 3A). Material co-eluting with PGF$_{2\alpha}$ was then purified (Fig. 3B) and subjected to chiral LC analysis (Fig. 3C). The first peak in the upper chromatogram of Fig. 3C perfectly co-elutes with ent-PGF$_{2\alpha}$, and the second perfectly co-elutes with PGF$_{2\alpha}$. CID experiments were carried out on the two enantiomers, and identical spectra were obtained and were essentially the same as that of chemically pure PGF$_{2\alpha}$ (Fig. 3D). In addition, using various chemical approaches, these compounds were found to have three hydroxyls and two double bonds consistent with their structure as PGs containing F-type prostane rings (8). Interestingly, there is significantly more ent-PGF$_{2\alpha}$ (~0.62 ng/mg of creatinine) than PGF$_{2\alpha}$ (~0.39 ng/mg of creatinine) present in urine from this human. Although PGF$_{2\alpha}$ in human urine is not racemic as observed when analyzed from liver phospholipids extracts, these results suggest that the vast majority of material co-eluting with PGF$_{2\alpha}$ is likely derived from the IsoP pathway because ent-PGF$_{2\alpha}$ can only be generated via the IsoP pathway.

The reason(s) that levels of ent-PGF$_{2\alpha}$ are significantly greater than PGF$_{2\alpha}$ is not readily apparent. One might predict...
that levels of PGF$_{2\alpha}$ would exceed those of ent-PGF$_{2\alpha}$, because of the fact that the former compound can be generated not only via the IsoP pathway but also via COX. One potential explanation for our observations, however, is that PGF$_{2\alpha}$ is preferentially metabolized in vivo when compared with ent-PGF$_{2\alpha}$. It is proposed that urinary PGF$_{2\alpha}$ is derived from production in tissues such as the kidney and is excreted into the urine (16, 31). In addition to excretion of parent PGs, eicosanoids, including PGF$_{2\alpha}$, are extensively metabolized, and the metabolites are also excreted into urine. The metabolism of PGF$_{2\alpha}$ has been well studied, and several enzymes have been shown to be involved in its catabolism. The first and key regulatory step in this metabolic pathway is the oxidation of 15-hydroxyl group of PGF$_{2\alpha}$ to a ketone by 15-PGDH (32, 33). We tested the ability of 15-PGDH to metabolize PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$ in vitro using the recombinant human enzyme. Interestingly, we found that PGF$_{2\alpha}$, as reported previously, is a substrate for this enzyme with a $V_{\text{max}} = 11.61 \pm 0.79 \mu M/min/\mu g$ of protein and $K_m = 12.76 \pm 3.2 \mu M$ but that 15-PGDH did not metabolize ent-PGF$_{2\alpha}$. These results are consistent with the notion that there is more ent-PGF$_{2\alpha}$ in human urine than PGF$_{2\alpha}$, due to the fact that PGF$_{2\alpha}$ is readily metabolized, whereas the enantiomer is not. To provide further evidence for this hypothesis, we also examined the optical purity of the initial metabolite of PGF$_{2\alpha}$, generated in vivo, 13,14-dihydro-15-keto-PGF$_{2\alpha}$, in human urine and plasma. Again, our result support the notion that PGF$_{2\alpha}$, but not ent-PGF$_{2\alpha}$, is a substrate for 15-PGDH since 13,14-dihydro-15-keto-PGF$_{2\alpha}$, in human urine and plasma is an optically pure compound and co-elutes perfectly on GC and LC with chemically pure 13,14-dihydro-15-keto-PGF$_{2\alpha}$ (data not shown). Together, these observations are consistent with the hypothesis that PGF$_{2\alpha}$, but not its enantiomer, is a substrate for 15-PGDH.

We next established the levels of PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$ in human urine of healthy subjects (11 men and 11 women). Healthy female subjects excrete 0.87 ± 0.06 ng of PGF$_{2\alpha}$ + ent-PGF$_{2\alpha}$/mg of creatinine, whereas levels in urine from males are 1.15 ± 0.06 ng of PGF$_{2\alpha}$ + ent-PGF$_{2\alpha}$/mg of creatinine ($p < 0.01$, male > female). The ratio of PGF$_{2\alpha}$/ent-PGF$_{2\alpha}$ in urine from the different sexes is not statistically different in that the ratio in males is 0.56 ± 0.16 and the ratio in females is 0.50 ± 0.13.

The Cyclooxygenases Contribute Insignificantly to Levels of PGF$_{2\alpha}$ in Urine from Healthy Humans—To examine the contribution of COXs to the generation and excretion of PGF$_{2\alpha}$ in vivo, we quantified levels of the compound in healthy humans administered the nonselective COX inhibitor ibuprofen (3200 mg/d for 4 days; n = 8). 24-h urine samples were collected during the final day of drug treatment, and the levels of PGF$_{2\alpha}$ + ent-PGF$_{2\alpha}$ and the ratio of PGF$_{2\alpha}$/ent-PGF$_{2\alpha}$ were quantified. The results are shown in Fig. 4, A and B. Ibuprofen treatment caused no significant decrease in levels of PGF$_{2\alpha}$ + ent-PGF$_{2\alpha}$, (1.04 ± 0.14 ng/g of creatinine versus 0.81 ± 0.17 ng/g of creatinine; $p > 0.05$), and it did not affect the ratio of PGF$_{2\alpha}$/ent-PGF$_{2\alpha}$ (0.65 ± 0.13 versus 0.58 ± 0.15; $p > 0.05$). It is noteworthy that in the same volunteers, there was a significant decrease in the excretion of the major urinary metabolite of COX-derived PGF$_2$, 11α-hydroxy-9,15-dioxy-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGF-M), with ibuprofen administration (66 ± 13% $p < 0.01$) (23). In addition, ex vivo platelet thromboxane formation was decreased >95% in serum from humans treated with ibuprofen when compared with controls (14). These data suggest that the vast majority of PGF$_{2\alpha}$ in healthy humans is derived from the IsoP pathway independent of COX.

We also studied the effect of COX activation on PGF$_{2\alpha}$ excretion in healthy humans following the intravenous administration of LPS. LPS infusion has been shown to markedly increase urinary PG excretion (14). Indeed, following infusion of LPS, PGF$_{2\alpha}$ + ent-PGF$_{2\alpha}$ levels in these volunteers increased to 1.72 ± 0.33 ng/mg of creatinine from a basal level of 1.04 ± 0.12 (p < 0.04) (Fig. 4C). Further, the ratio of PGF$_{2\alpha}$/ent-PGF$_{2\alpha}$ increased in this population from 0.53 ± 0.08 to 0.82 ± 0.12 ($p < 0.01$) (Fig. 4D), suggesting that the majority of this increase was due to increased urinary levels of enantio-merically pure PGF$_{2\alpha}$ derived from COX. In these studies, levels of ent-PGF$_{2\alpha}$ did not increase significantly. Further, the increase in PGF$_{2\alpha}$ could be significantly reduced by up to 70% ($p < 0.01$) after LPS infusion by pretreatment of volunteers with ibuprofen, but levels of ent-PGF$_{2\alpha}$ were not affected. It is noteworthy that LPS infusion also caused significant increases ($p < 0.001$) in the excretion of other eicosanoids including thromboxane (as quantified by measuring its major urinary metabolite 11-dehydro-thromboxane B$_2$) and prostacyclin (as

**FIGURE 4. Analysis of putative PGF$_{2\alpha}$ in human urine after ibuprofen and LPS treatment by LC-MS.** A, levels of PGF$_{2\alpha}$ + ent-PGF$_{2\alpha}$ (ng/mg of creatinine (Cr)) in human urine treated with ibuprofen. B, ratio of PGF$_{2\alpha}$/ent-PGF$_{2\alpha}$. C, levels of PGF$_{2\alpha}$ + ent-PGF$_{2\alpha}$ (ng/mg of creatinine) in human urine treated with LPS. D, ratio of PGF$_{2\alpha}$/ent-PGF$_{2\alpha}$. Bars are mean ± S.E. (*, $p < 0.05$ with respect to the control).
quantified by measuring 2,3-dinor-6-keto-PGF₁α (14). These data suggest that levels of PGF₂α, but not ent-PGF₂α, in human urine increase with LPS infusion and that the increased PGF₂α is COX-derived.

PGF₂α and ent-PGF₂α in Human Urine Are Markers of Oxidative Stress—The above findings support the hypothesis that the vast majority of PGF₂α, and ent-PGF₂α, in urine from normal humans is derived from the IsoP pathway. Therefore, levels of these compounds may more appropriately reflect oxidative stress status rather than be a marker of COX activity. Indeed, F₂-IsoPs, such as 8-iso-PGF₂α (15-F₂t-IsoP), have been used as markers of oxidative stress, and elevated levels of these compounds have been linked to a number of human diseases and conditions that are believed to be associated with oxidative damage (30, 34). We have previously reported that F₂-IsoP production is markedly increased in humans who smoke cigarettes heavily and in hypercholesterolemic individuals, two risk factors for atherosclerosis. We therefore analyzed levels of PGF₂α and ent-PGF₂α in urine of cigarette smokers and patients with hypercholesterolemia. In both of these populations, levels of 8-iso-PGF₂α (15-F₂t-IsoP) were increased >2-fold (p < 0.05) when compared with controls. The results are summarized in Fig. 5. In both cases, markedly elevated levels PGF₂α and ent-PGF₂α were observed when compared with control individuals. Further, interestingly, the ratio of ent-PGF₂α to PGF₂α was also significantly higher in both smokers and hypercholesterolemic than those of their respective control groups. These latter findings are consistent with our earlier observation that ent-PGF₂α is present in human urine at concentrations higher than PGF₂α, because of the fact that the latter compound may be more readily metabolized than the former.

Formation of Significant Amounts of PGF₂α via the IsoP Pathway Is Theoretically Feasible and Is Increased at Elevated Temperatures—Extensive research has led to an understanding of free radical mechanisms involved in the formation of IsoPs (Fig. 1) (12, 35). As mentioned previously, elegant studies by a number of investigators have shown that IsoP diastereomers with cis-alkyl side chains are produced in predominant amounts when compared with those with trans-side chains (11, 36). It is therefore surprising to us that significant amounts of compounds with structures identical to PGF₂α, and ent-PGF₂α, are generated from the peroxidation of arachidonate in vitro, are esterified in tissue phospholipids in vivo, and are present in human urine. Several reasons could explain these discrepancies. First, we quantify stable F-ring IsoPs using deuterated standards employing LC/MS and GC/MS, which is considerably more accurate than previously reported methods (37, 38). A second important difference relates to the temperature at which the autoxidations were performed. O’Connor et al. (36) performed experiments at 20 °C, and we have performed our studies at 37 °C, which is a more biologically relevant temperature. The radical cyclization reactions that lead to F₂-IsoPs typically operate under kinetically controlled conditions, and the product distribution should therefore be temperature-dependent. To confirm this, we carried out experiments to establish the product selectivity temperature dependence of IsoP formation. Fig. 6 shows a representative LC/ESI/MS chromatogram of 15-series F₂-IsoPs formed at 37 °C. At this temperature, the sum of all trans-side chain IsoPs makes up 19.8 ± 0.5% of the products with PGF₂α comprising 8.0 ± 0.3% of the mixture. By comparison, at 0 °C, the sum of all trans-side chain IsoPs is 9.5 ± 0.4% of the products and PGF₂α comprises 4.1 ± 0.2% of the mixture. It is also of note that the total amounts of F₂-IsoPs generated at 37 °C are 3.2-fold higher (p < 0.05) when compared with that of 0 °C. In accord with theoretical expectations, a plot of the natural log of the cis/trans product ratio versus 1/T (Eyring or Arrhenius analysis) gives a straight line (data not shown) (39). Analysis of the data by the Eyring equation shows that the enthalpy of the transition states leading to the cis-IsoPs are some 3.4 kcal/mol lower than those leading to the trans-
PGF$_{2\alpha}$-like IsoPs. This difference in enthalpies is relatively small, and at elevated temperatures, formation of the F$_2$-IsoPs having the PGF$_{2\alpha}$ side chain orientation, whereas not having the favored pathway, is significant as noted. These findings, therefore, support the contention that significantly greater amounts of trans-side chain-containing IsoPs can be generated in vivo at higher temperatures.

**DISCUSSION**

We report herein that the majority of PGF$_{2\alpha}$ present in human urine is derived from the IsoP pathway rather than via COX (16). Support for this conclusion is based on the fact that administration of NSAIDs does not suppress urinary levels of PGF$_{2\alpha}$ in humans and that oxidation of arachidonate in vitro and in vivo yields material that co-elutes with chemically pure PGF$_{2\alpha}$ and its enantiomer under various chromatographic conditions. Further, these compounds are indistinguishable from chemically pure PGF$_{2\alpha}$ and its enantiomer using various chemical and mass spectrometric approaches.

The fact that urinary levels of PGF$_{2\alpha}$ are not suppressed by COX inhibitors, and yet platelet thromboxane production and the major urinary metabolites of eicosanoids such as PGE$_2$ are significantly reduced, suggests that PGF$_{2\alpha}$ is derived from sources other than COX. PGs are potent lipid mediators that are involved in a number of homeostatic and inflammatory processes in humans. As one of the major PGs, PGF$_{2\alpha}$ possesses important biological activities including constriction of smooth muscle in various organs and the vasculature (15). It has been reported that body fluids such as urine from humans and animals contains significant amounts of unmetabolized PGF$_{2\alpha}$. The source of PGF$_{2\alpha}$ in urine has been presumed to be the kidney, although other organs may contribute (16). Despite the fact that urinary PGF$_{2\alpha}$ has been presumed to be derived from COX, there is little evidence to support this. We determined in this study that the levels of putative PGF$_{2\alpha}$ are unaffected, whereas the major urinary metabolite of PGE$_2$ was suppressed nearly 70% by NSAIDs. Further, platelet thromboxane production in serum in the same subjects was suppressed >95%. Stud-

**FIGURE 6. Identification of eight diastereomers of 15-series F2-IsoPs by ESI-MS.** A, representative SRM spectrum of 15-series F$_2$-IsoP-free acid derived from 15-hydroperoxyoctadecatrienoic acid oxidation at 37°C analyzed by reverse phase LC-ESI-MS. SRM, m/z 353 to 193 at 30 eV, PGF$_{2\alpha}, d_9$ m/z 357 to 197 at 30 eV. B, chemical structure of the eight diastereomers of 15-series F$_2$-IsoPs.

**Generation of PGF$_{2\alpha}$ via the Isoprostane Pathway**

Urinary metabolites of PGF$_{2\alpha}$, analogues to other IsoPs, is presumed to be derived from tissue phospholipid-bound PGF$_{2\alpha}$ since we can easily detect esterified racemic PGF$_{2\alpha}$ in organs from animals. As with other IsoPs, once formed, racemic PGF$_{2\alpha}$ is hydrolyzed from tissue phospholipids by the action of various phospholipase A$_{2\alpha}$, such as platelet-activating factor acetylhydrolase, and excreted in the urine (40).

Further support for our hypothesis that PGF$_{2\alpha}$ in humans is generated via the IsoP pathway is based on the fact that we readily detect in urine not only PGF$_{2\alpha}$ but its enantiomer ent-PGF$_{2\alpha}$, a compound that can only be generated from free radical processes. In fact, we are able to measure significantly more ent-PGF$_{2\alpha}$ in human urine than PGF$_{2\alpha}$. This is somewhat surprising in that it would be predicted that PGF$_{2\alpha}$ would be more abundant since it can be generated via both the IsoP and the COX pathways. On the other hand, we propose that levels of PGF$_{2\alpha}$ are significantly lower than its enantiomer since PGF$_{2\alpha}$ is readily metabolized by 15-PGDH, whereas ent-PGF$_{2\alpha}$ is not a substrate for this enzyme. Further, we determined that a major circulating and urinary metabolite of PGF$_{2\alpha}$ in humans, 13,14-dihydro-15-keto-PGF$_{2\alpha}$, is enantiomerically pure and is derived in vivo only from PGF$_{2\alpha}$ and not ent-PGF$_{2\alpha}$. Additional support for a mechanism of formation of PGF$_{2\alpha}$ via the IsoP pathway is provided by evidence that levels of PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$ are significantly increased in human disorders associated with oxidant stress. Of note, however, we can selectively increase urinary PGF$_{2\alpha}$ levels with activation of COX in vivo such as after infusion of LPS, although levels of ent-PGF$_{2\alpha}$ are not elevated. Finally, it should be noted that levels of PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$ in human urine that are derived via the IsoP pathway are not trivial and either exceed, or are similar, to those of COX-derived eicosanoid metabolites, suggesting that the IsoP pathway contributes significantly to the total body metabolism of arachidonate in vivo.

We have proposed a mechanism to explain the formation of large amounts of PGF$_{2\alpha}$ and ent-PGF$_{2\alpha}$ both in vitro and in vivo. Although O’Connor _et al._ (36) have previously reported that the autoxidation of polyunsaturated fatty acids leads to the preferential formation of cis-side-chain IsoPs when compared with trans-side-chain products such as PGF$_{2\alpha}$, the transition state energy leading to trans-products is only 3.4 kcal/mol higher than that leading to the favored cis-compounds. Thus, increasing temperatures should favor enhanced formation of trans-side chain containing IsoPs, and our _in vitro_ studies show that oxidation of arachidonate at 37°C does indeed give significantly more of the trans-side-chain products than are formed at lower temperatures.
Generation of PGF$_{2\alpha}$ via the Isoprostane Pathway

We have previously reported that compounds identical in all respect to PGE$_2$ and PGD$_2$ and their respective enantiomers are generated via the IsoP pathway by a mechanism involving the keto-enol tautomerization of 15-E$_2t$-IsoP and 15-D$_2t$-IsoP (22). This pathway, however, cannot account for the generation of PGF$_{2\alpha}$ via the IsoP pathway since it involves the isomerization of E/D-ring-containing IsoPs. Whether PGE$_2$ and PGD$_2$ can be formed in significant amounts via the mechanism proposed herein for PGF$_{2\alpha}$ remains to be determined, although our previous studies have disclosed that the majority of PGE$_2$ and PGD$_2$ in baseline urine from rodents is enantiomerically pure and that little ent-PGE$_2$ or ent-PGD$_2$ is present.

A number of important physiological and pathophysiological issues emerge from this study. The first relates to the fact that formation of bioactive PGF$_{2\alpha}$ occurs in vivo to a significant extent via the IsoP pathway at baseline and is markedly increased in settings of oxidative stress and, potentially, in other inflammatory situations. Second, IsoPs have been implicated as mediators of oxidant stress (23–25). Thus, it will be important to investigate the extent to which not only IsoPs, but PGs, contribute to the adverse sequelae of oxidative injury. Further, although the biological properties of PGF$_{2\alpha}$ have been well characterized, our studies suggest that equal amounts of the enantiomers of this PG are produced in significant amounts. It will thus be of interest to explore the bioactivity of ent-PGF$_{2\alpha}$. In this respect, this compound has been synthesized, and experiments to determine its biological relevance will likely yield important insights into its role in oxidative injury.

In summary, we report that a second pathway exists for the formation of bioactive PGF$_{2\alpha}$ in vivo that is independent of COX. Further, the majority of PGF$_{2\alpha}$ present in urine from normal humans is derived not via COX but via the IsoP pathway. This finding is likely of physiological and pharmacological importance since the generation of PGF$_{2\alpha}$ via this mechanism would not be inhibited by aspirin or other NSAIDs. The extent to which formation of PGF$_{2\alpha}$ independent of COX contributes to human physiology and pathophysiology remains to be elucidated.

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