Formation of Prostaglandins E₂ and D₂ via the Isoprostane Pathway

A MECHANISM FOR THE GENERATION OF BIOACTIVE PROSTAGLANDINS INDEPENDENT OF CYCLOOXYGENASE

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It has heretofore been assumed that the cyclooxygenases (COXs) are solely responsible for peostaglandin (PG) synthesis in vivo. An important structural feature of PGH₂ formed by COX is the trans-configuration of side chains relative to the prostane ring. Previously, we reported that a series of PG-like compounds termed isoprostanes (IsoPs) are formed in vivo in humans from the free radical-catalyzed peroxidation of arachidonate independent of COX. A major difference between these compounds and PGs is that IsoPs are formed from endoperoxide intermediates, the vast majority of which contain side chains that are cis relative to the prostane ring. In addition, unlike the formation of eicosanoids from COX, IsoPs are formed as racemic mixtures because they are generated nonenzymatically. IsoPs containing E- and D-type prostane rings (E₂/D₂-IsoPs) are one class of IsoPs formed, and we have reported previously that one of the major IsoPs generated is 15-E₂c-IsoP (8-iso-PGE₂). Unlike PGE₂, 15-E₂c-IsoP is significantly more unstable in buffered solutions in vitro and undergoes epimerization to PGE₂. Analogously, the D-ring IsoP (15-D₂c-IsoP) was predicted to rearrange to PGD₂. We now report that compounds identical in all respects to PGE₂ and PGD₂ and their respective enantiomers are generated in vivo via the IsoP pathway, presumably by epimerization of racemic 15-E₂c-IsoP and 15-D₂c-IsoP, respectively. Racemic PGE₂ and PGD₂ were present esterified in phospholipids derived from liver tissue from rats exposed to oxidant stress. In this setting, PGD₂ and its enantiomer generated by the IsoP pathway represented ~30% of the total PG detected. Levels of racemic PGD₂ increased 35-fold after treatment of rats with carbon tetrachloride to induce oxidant stress. In this setting, PGD₂ and its enantiomer generated by the IsoP pathway represented ~30% of the total PG detected. These findings strongly support the contention that a second pathway exists for the formation of bioactive PGs in vivo that is independent of COX.

Cylooxygenase (COX)¹-1 and COX-2 catalyze the committed steps in formation of prostaglandins (PGs) by generating the unstable bicycloendoperoxide intermediate PGH₂ (1–3). PGH₂ is subsequently metabolized to the parent eicosanoids PGE₂, PGD₂, PGE₃, PGI₂, and thromboxane A₂, which exert a plethora of biological activities (1). The formation of PGH₂ is stereospecific in that, among other structural aspects, the side chains of PGH₂ are oriented in the trans-configuration relative to the prostane ring (Fig. 1A). This conformation is highly favored thermodynamically (4, 5).

We have previously reported that a series of PG-like compounds termed isoprostanes (IsoPs) are formed in vivo from the free radical-catalyzed peroxidation of arachidonate independent of COX (6). Analogous to PGs, we have determined that IsoPs contain E/D-, F-, and thromboxane-type prostane rings (7). Although the structures of these compounds are very similar to COX-derived PGs, an important distinction between IsoPs and PGs is that IsoP bicycloendoperoxide intermediates contain side chains that are predominantly (>90%) oriented cis in relation to the prostane ring because the generation of these intermediates is favored kinetically (4, 7, 8). Indeed, we have previously reported that two IsoPs that are formed in abundance in vivo are 15-F₂α-IsoP (8-iso-PGF₂α) and 15-E₂α-IsoP (8-iso-PGE₂), which are generated from the endoperoxide intermediate 15-H₂α-IsoP (8-iso-PGH₂) (Fig. 1B) (9, 10). Although not reported, it would also be predicted that 15-H₂α-IsoP (12-iso-PGH₂) is formed in abundance and can rearrange to the analogous D-ring IsoP 15-D₂c-IsoP (12-iso-PGD₂) (Fig. 1C).

In contrast to other types of prostanoinds, E₃/D₃-IsoPs are β-hydroxyketone-containing compounds that can undergo reversible keto-enol tautomerization under both acidic and basic conditions, allowing rearrangement of the side chains that are initially cis to the more stable trans-configuration. That the trans-configuration is highly favored has been demonstrated by the finding that, when PGE₂ is subjected to conditions that induce keto-enol tautomerism, <10% of the compound rearranges to the cis-side chain isomer 15-E₂α-IsoP (11). In addition, attempts to synthesize 15-D₂c-IsoP have been unsuccessful because epimerization at C-12 readily occurs during synthesis to yield PGD₂ (12). Furthermore, facile epimerization of a number of other PG-like compounds containing side chains cis to the prostane ring has been reported (13, 14).

In the course of studies to characterize various E/D/ring IsoPs formed in vitro and in vivo from the peroxidation of

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arachidonic acid, analysis of oxidation products by gas chromatography (GC)/mass spectrometry (MS) disclosed the generation of significant amounts of compounds that had retention times and molecular weights identical to those of PGE₂ and PGD₂. Because IsoPs are formed nonenzymatically, compounds generated by this pathway would be predicted to be racemic (6, 7). Using a variety of chromatographic and mass spectrometric approaches, we present evidence that compounds identical in all respects to COX-derived PGE₂ and PGD₂ and their respective enantiomers are formed in vitro and in vivo via the IsoP pathway. A proposed mechanism by which the formation of PGE₂ and PGD₂ occurs from 15-E₂t-IsoP and 15-D₂c-IsoP, respectively, via base-catalyzed isomerization is shown in Fig. 2 (A and B). Generation of PGE₂ and PGD₂ from 15-E₂t-IsoP and 15-D₂c-IsoP, respectively, would also be predicted to occur via acid catalysis (11).

These findings strongly support the contention that a second pathway exists for the formation of bioactive PGs in vitro that is independent of COX. This finding is of potential physiological and pharmacological importance because it would be predicted that the generation of PGs via this mechanism would not be inhibited by aspirin or other COX inhibitors. For purposes of discussion hereafter, PGs possessing a structure identical to those generated by COX are referred to as PGE₂ and PGD₂. Compounds that are enantiomeric to COX-derived PGs are referred to as ent-PGE₂ and ent-PGD₂. The racemic mixtures are termed rac-PGE₂ and rac-PGD₂ (Fig. 3).

EXPERIMENTAL PROCEDURES

Materials—Arachidonic acid, dimethylformamide, CCl₄, and undecane were purchased from Aldrich. Pentfluorobenzyl (PFB) bromide, methoxyamine HCl, diisopropylethylamine, and Apis mellifera venom phospholipase A₂ were from Sigma. [H₃]Methoxyamine HCl was from Cambridge Isotope Laboratories, Inc. (Andover, MA). N-O-Bis(trimethylsilyl)trifluoroacetamide was from Supelco Inc. (Bellefonte, PA). N-O-[H₃]Bis(trimethylsilyl)acetamide was from CDN Isotopes (Pointe-Claire, Quebec, Canada). Organic solvents were from EM Science (Darmstadt, Germany). C₁₈ and silica Sep-Pak cartridges were from Waters Associates (Milford, MA). 60ALK6D TLC plates were from Whatman (Maidstone, UK). [H₃]PGE₂, [H₃]PGD₂, and [H₃]PGB₂ were from Cambridge Isotope Laboratories, Inc. (Andover, MA). [2H₄]PGE₂, [2H₄]PGD₂, and [2H₄]PGB₂ were from Cambridge Isotope Laboratories, Inc. (Andover, MA). [2H₉]Bis(trimethylsilyl)acetamide was from CDN Isotopes (Pointe-Claire, Quebec, Canada). [2H₃]Methoxyamine HCl was from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Acid-Catalyzed Epimerization of 15-E₂t-IsoP to PGE₂—A mixture of edt-RPGs and PGs from rat liver was incubated with rat liver microsomal ATP-regenerating system and CCl₄ (2 mg/kg) in corn oil (16, 17). The animals were anesthetized with pentobarbital (60 mg/kg) intraperitoneally and killed, and the livers were removed. Depending on the experiment, 1–4 g of tissue was immediately extracted to obtain a crude phospholipid extract containing IsoPs and PGs esterified in phospholipids. The lipid extract was then subjected to hydrolysis (30 min) in borate buffer with A. mellifera venom containing phospholipase A₂ as described (16, 17). In control experiments, complete hydrolysis of 2-[H₃]arachidonoylphosphatidylethanolamine was effected during this incubation. In addition, these hydrolysis conditions resulted in <5% epimerization of 15-E₂t-IsoP to PGE₂. Subsequently, free Isops and PGs were extracted, partially purified using C₁₈ and silica Sep-Pak cartridges, and subjected to HPLC. For selected experiments, liver tissue was also obtained from day 19 COX₁⁻/⁻/COX₂⁻/⁻ mouse pups harvested in utero as described (18).

In some experiments, 24-h urine samples were collected from rats treated with CCl₄ or from normal humans. Unesterified IsoPs and PGs were extracted using Sep-Pak columns as described (6).

HPLC Separation of Racemic EDT-RPGs and Edt-IsoPs—Depending on the experiment, incubations of oxidized arachidonic acid, partially purified tissue extracts, or urine samples were analyzed for rac-PGE₂, rac-PGD₂, or rac-15-E₂t-IsoP (9, 10). To the biological sample was added ~0.5–3 μCi of [H₃]PGE₂, [H₃]PGD₂, or 15-E₂t-[H₃]IsoP. The mixture was then subjected to four successive HPLC purification steps. To

**Fig. 1.** Structure of the bicycloendoperoxide intermediate PGH₂ (A) derived from COX and structures of the IsoP endoperoxides 15-H₂c-IsoP (B) and 15-H₂c-IsoP (C).

**Fig. 2.** Base-catalyzed mechanism of epimerization of 15-E₂t-IsoP to PGE₂ (A) and 15-D₂c-IsoP to PGD₂ (B) involving keto-enol tautomerization. Acid-catalyzed epimerization can also occur. R = either hydrogen or phospholipids depending on whether the IsoPs epimerize as free acids or esterified in phospholipids.
maximize purification and resolution of each compound, we used HPLC procedures that yielded relatively long retention volumes for each compound (~15–40 ml), and each solvent was run isocratically. In pilot experiments, it was also shown that the three compounds readily separated from one another under the HPLC conditions utilized. In addition, radiolabeled PGE₂, PGD₂, and 15-E₂t-IsoP were separated to a significant extent (~2.5 ml) from unlabeled compounds due to the fact that the radiolabeled compounds contained either six or seven tritium atoms. Thus, for each HPLC step, fractions corresponding to those containing both labeled and unlabeled PGs or IsoPs were collected and pooled for further purification. For rac-PGE₂ and rac-15-E₂t-IsoP, the first HPLC step was normal-phase using a Econosil SI column (25 cm × 4.6 mm, 5-μm particles; Alltech Associates Inc., Deerfield, IL). The solvent system was 98:12:0.1 (v/v/v) hexane/isopropyl alcohol/hexane/isopropyl alcohol/acetic acid at a flow rate of 1 ml/min. The second HPLC step was reversed-phase using an Econosil C18 column (25 cm × 4.6 mm, 5 μm; Alltech Associates Inc.). The solvent system was 30:70:0.1 (v/v/v) acetonitrile/water/acetic acid at a flow rate of 1 ml/min. For the third and fourth HPLC steps, IsoPs or PGs were converted to PFB esters and rechromatographed on normal- and reversed-phase HPLC columns. A solvent system of 92:8 (v/v) hexane/isopropyl alcohol was used for the third HPLC step, and 51:49 (v/v) acetonitrile/water was used for the fourth HPLC step, both at a flow rate of 1 ml/min. For the purification of rac-PGD₂, the same columns were utilized, but the solvent systems varied. For the first HPLC step, the solvent system was 93:7:0.1 (v/v/v) hexane/isopropyl alcohol/acetic acid; the second HPLC solvent system was 33:67:0.1 (v/v/v) acetonitrile/water/acetic acid; the third HPLC solvent system was 95:5 (v/v) hexane/isopropyl alcohol; and the fourth HPLC solvent system was 58:42 (v/v) acetonitrile/water.

Chiral HPLC Separation of rac-PGE₂ and rac-PGD₂—Racemic PGs purified by the methods described above were subsequently subjected to chiral HPLC to separate enantiomers using a Chiralpak AD column (25 cm × 4.6 mm, 5 μm; Chiral Technologies, Exton, PA). To separate rac-PGE₂, a solvent system of 93.7:0.1 (v/v/v) hexane/isopropyl alcohol was utilized; and for rac-PGD₂, a solvent system of 95.5 (v/v) hexane/isopropyl alcohol was employed.

Analysis of PGs and IsoPs by GC/MS—Quantification of E/D-ring PGs and IsoPs in partially purified biological extracts and throughout subsequent HPLC purification procedures was performed by analyzing aliquots by selected ion monitoring GC/negative ion chemical ionization MS using either [3H₉]PGE₂ or [3H₇]PGD₂ as an internal standard. Compounds were quantified as O-methyloxime, PFB ester, trimethylsilyl ether derivatives by monitoring the M-PPB (M = 181) ions at m/z 528 for endogenous compounds and at m/z 524 for the deuterated standards (10, 19).

RESULTS

Epimerization of 15-E₂t-IsoP in Phosphate Buffer—We initially determined the extent to which 15-E₂t-IsoP undergoes epimerization to PGE₂ in a buffered solution at physiological pH (50 mM KPO₂, pH 7.4). Products that were quantified included the starting material 15-E₂t-IsoP and PGE₂ and their respective dehydration products, 15-A₂t-IsoP and PGA₂. Amounts are expressed as percent of total PG at a particular time point. The identification of PGE₂ was also confirmed by NMR comparison with a chemically pure PGE₂ standard. The results are shown in Fig. 4. As shown, 15-E₂t-IsoP epimerized in a time-dependent manner to PGE₂. The half-life for this conversion under the conditions noted was ~2 h. In addition, small amounts of 15-A₂t-IsoP and PGA₂ were formed, presumably as a result of dehydration of 15-E₂t-IsoP and PGE₂, respectively. These findings support the hypothesis that E₂/D₂-IsoPs can readily rearrange to E/D-ring PGs in aqueous environments.

Analysis of E₂/D₂-IsoPs from the Oxidation of Arachidonic Acid in Vitro and in Vivo—Fig. 5A shows the selected ion current chromatograms for E₂/D₂-IsoPs obtained from the analysis of arachidonic acid with iron/ADP/ascorbate for 2 h. Compounds were analyzed as O-methyloxime, PFB ester, trimethylsilyl ether derivatives. In the lower m/z 528 chromatogram are two peaks representing the syn- and anti-O-methyloxime isomers of the [2H₄]PGE₂ internal standard. In the upper m/z 524 chromatogram are a series of peaks representing various E₂/D₂-IsoPs. The peaks indicated by asterisks represent compounds that co-chromatographed upon GC with the O-methyloxime isomers of chemically synthesized PGE₂. In addition, the peaks denoted by plus signs co-chromatographed with the O-methyloxime isomers of chemically pure PGA₂. The total E₂/D₂-IsoPs present were ~1500 ng/g of arachidonic acid. The materials designated by the peaks denoted by asterisks and plus signs each represent ~20% of the total E₂/D₂-IsoPs in the mixture.

In addition to the analysis of oxidized arachidonic acid in vitro, Fig. 5B shows the selected ion current chromatograms obtained from the hydrolysis of rat liver phospholipids after administration of CCl₄ to animals to induce oxidant stress. As shown, a similar pattern of peaks was present as shown in Fig. 5A. The analyses in Fig. 5 (A and B) were performed on separate days, accounting for differences in GC retention time.
Again, the peaks indicated by asterisks represent compounds that co-chromatographed upon GC with chemically synthesized PGE₂, whereas those denoted by plus signs co-chromatographed with PGD₂. The total E₂/D₂-IsoPs present in this sample were ~400 ng/g of liver tissue. The materials designated by the peaks denoted by the asterisks and plus signs each repre-
sent 15–20% of the total E₂/D₂-IsoPs in the mixture. A very similar pattern of peaks representing E₂/D₂-IsoPs was obtained from the livers of both COX-1−/−/COX-2−/− and control fetal mice without induction of oxidant stress, although total E₂/D₂-IsoP levels were ~5–10 ng/g of liver tissue (data not shown). In addition, a pattern of peaks virtually identical to those shown in the chromatograms in Fig. 5 was obtained after hydrolysis of rat liver phospholipids that had been treated with methyloxime HCl prior to hydrolysis. These latter findings suggest that epimerization of E₂/D₂-IsoPs occurs while compounds are esterified in phospholipids. Taken together, these data support the contention that significant amounts of compounds that coelute upon GC with PGE₂ and PGD₂ are generated from the peroxidation of arachidonic acid in vitro and in vivo.

Purification of Putative rac-PGE₂ from Rat Liver Hydrolysates by HPLC—We subsequently sought to determine whether the compounds from rat liver hydrolysates that coeluted upon GC with PGE₂ and PGD₂ were, in fact, structurally identical to PGE₂ and PGD₂ and their respective enantiomers. If PGE₂ and PGD₂ are formed via the IsoP pathway, it would be predicted that they would be racemic mixtures because they would be formed from the epimerization of rac-15-E₂-IsoP and rac-15-D₂-IsoP, respectively (6, 7). Of note, enantiomers of PGE₂ and PGD₂ would not be expected to separate using standard non-chiral HPLC methods.

The formation of rac-PGE₂ was assessed initially. For these studies, ~2000 ng of E₂/D₂-IsoPs from rat liver containing 3 μCi of [³H]PGE₂ was subjected to four successive HPLC purification steps. The first HPLC step was normal-phase using a solvent system of 88:12.0:1 (v/v/v) hexane/isopropyl alcohol/acetic acid. Aliquots of fractions that eluted from the HPLC column were then analyzed for E₂/D₂-IsoPs by GC/MS and for radioactivity (Fig. 6A). Radiolabeled PGE₂ eluted in this system between 16 and 18.5 min. Compounds representing endogenous E₂/D₂-IsoPs were present that had the same retention time upon GC as PGE₂, but that eluted with different retention volumes compared with PGE₂ upon HPLC (10–12, 18–21, and 22.5–23.5 ml). Radiolabeled PGE₂ eluted at a volume of ~1.0–1.5 ml after unlabeled PGE₂ using this HPLC solvent system. Significantly, as shown in Fig. 6A, an endogenous E₂/D₂-IsoP peak (indicated by the plus sign) was detected that coeluted with unlabeled PGE₂, suggesting that this compound is endogenously derived rac-PGE₂.

The material that eluted from the HPLC column between 14.5 and 18.5 ml in Fig. 6A was subsequently subjected to reversed-phase HPLC using an isocratic solvent system of 30: 70:0.1 (v/v/v) acetonitrile/water/acetic acid. Aliquots of fractions collected were again analyzed for endogenous E₂/D₂-IsoPs by GC/MS and for radioactivity (Fig. 6B). Radiolabeled PGE₂ eluted from the HPLC column with a retention volume of 28.5–32.5 ml. Analysis of aliquots of the eluted fractions by GC/MS showed that almost all of the unlabeled E₂/D₂-IsoP material detected in the chromatogram eluted at the retention volume of unlabeled PGE₂ (29.5–33 ml), except for a small amount of additional material that eluted at 37–39 ml.

Altering the polarity of a compound by derivatization and rechromatography of the compound can provide a powerful approach for purification and separation of biomolecules (9). Thus, the material that eluted from the HPLC column between 28.5 and 33 ml in Fig. 6B was converted to a PFB ester and rechromatographed on a normal-phase HPLC column using a solvent system of 92:8 (v/v) hexane/isopropyl alcohol. Fig. 6C shows the result of this HPLC step. Radiolabeled PGE₂ eluted between 29.5 and 33.5 ml. A large peak representing endoge-

![Fig. 7. Selected ion current chromatogram obtained from the GC/MS analysis of the material that eluted at a retention volume between 40.5 and 44.5 ml in Fig. 6D. Only a single set of m/z 524 peaks representing the syn- and anti-O-methyloxime isomers of endogenous putative rac-PGE₂, remained after the four HPLC purification procedures shown in Fig. 6. The peaks in the m/z 528 chromatogram represent the syn- and anti-O-methyloxime isomers of the deuterated PGE₂ internal standard. The amount of putative rac-PGE₂ in the fraction analyzed was ~35 ng/1000 ng of total E₂/D₂-IsoP.](image-url)
group. Second, analysis as a deuterated trimethylsilyl ether derivative revealed that the compound had two hydroxyl groups. Third, catalytic hydrogenation showed two double bonds (16). Finally, treatment of putative rac-PGE2 with 15% methanolic KOH for 30 min converted it to a compound with a molecular weight and retention time identical to those of PGB2 when analyzed by GC/MS (Fig. 8) (20). Taken together, these findings strongly support the contention that the material represented in the m/z 524 chromatogram in Fig. 7 is rac-PGE2.

Analysis of Putative rac-PGE2 by Chiral HPLC—As noted, it is predicted that PGE2 generated by the IsoP pathway should be racemic. The HPLC steps utilized above to purify putative
Prostaglandin Formation via the Isoprostane Pathway

Fig. 9. Chiral HPLC separation of rac-PGE₂. The putative rac-PGE₂, purified as described in the legend to Fig. 6 was subjected to chiral column chromatography using the solvent system 93:7 (v/v) hexane/isopropanol alcohol. Aliquots of fractions that eluted from the HPLC column were then analyzed for PGE₂ by GC/MS. The peak indicated by the asterisk co-chromatographed in the HPLC solvent system with chemically pure PGE₂, whereas the peak denoted by the plus sign co-eluted with ent-PGE₂.

rac-PGE₂ will not separate enantiomers. Thus, the compounds represented in the chromatogram in Fig. 7 were subjected to chiral column chromatography, and fractions that eluted from the HPLC column were analyzed by GC/MS. Fig. 9 shows the results of the analysis. The peaks indicated by the asterisk and plus sign co-chromatographed with chemically synthesized PGE₂, whereas the peak denoted by the plus sign co-chromatographed with chemically synthesized ent-PGE₂. The material from each peak co-chromatographed perfectly with both PGE₂ and ent-PGE₂ upon GC and was indistinguishable upon MS analysis. Approximately equal amounts of the compounds were present, as would be expected. Furthermore, the ratio of methyloxime isomers of ent-PGE₂ was essentially identical to that of PGE₂. Taken together, these studies provide compelling evidence that PGE₂ and ent-PGE₂ are generated in vivo in significant quantities from the Isop pathway. Essentially identical results were obtained from the analysis of putative rac-PGE₂ formed from the peroxidation of arachidonate in vitro.

Purification of Putative rac-PGD₂ from Rat Liver Hydrolysates by HPLC—As noted in Fig. 5 (A and B), chromatographic peaks were present that coeluted not only with PGE₂, but also with PGD₂. We thus employed similar approaches as those used to obtain evidence for the formation of rac-PGE₂ in vitro and in vivo to determine whether rac-PGD₂ is also generated. Table I shows the HPLC conditions utilized to purify putative rac-PGD₂ and the retention time of the compound at each step. Fig. 10 illustrates the results from GC/MS analysis of the material that eluted between 21 and 24 ml, where PGD₂ eluted, upon the fourth HPLC step. As shown, two E₃/D₃-Isop peaks were present in the m/z 524 chromatogram, representing the syn- and anti-O-methyloxime isomers of putative rac-PGD₂. The amount of putative rac-PGD₂ present in the rat liver hydrolysate from this analysis was ~55 ng/1000 ng of total E₃/D₃-Isop, based upon losses of [⁴H]PGD₂ that occurred with the four HPLC purification steps. When the material denoted by the peaks in the m/z 524 chromatogram in Fig. 10 was mixed with an equivalent amount of derivatized synthetic PGD₂, the two compounds co-chromatographed perfectly upon capillary GC without any suggestion of a shoulder on the GC peaks (data not shown). Additional experiments confirmed the identification of the compound in Fig. 10 as PGD₂. First, analysis of the material as a deuterated O-methyloxime derivative disclosed the presence of one carbonyl group. Second, analysis as a deuterated trimethylsilyl ether derivative revealed that the compound had two hydroxyl groups. Third, catalytic hydrogenation showed two double bonds.

Analysis of Putative rac-PGD₂ by Chiral HPLC—As with PGE₂ generated by the Isop pathway, it is predicted that PGD₂ should be racemic. Thus, the compounds represented in the m/z 524 chromatogram in Fig. 10 were subjected to chiral column chromatography, and fractions that eluted from the HPLC column were analyzed by GC/MS (Table I). Fig. 11 shows the results of the analysis. The peak indicated by the asterisk co-chromatographed under these HPLC conditions with chem.-
Physically synthesized PGD₂, whereas the peak denoted by the plus sign represents ent-PGD₂. The material from each peak co-chromatographed perfectly with PGD₂ upon GC and was indistinguishable upon MS analysis. Approximately equal amounts of compounds were present, as would be expected; and the relative amounts of methyloxime isomers of PGD₂ and ent-PGD₂ were very similar. Taken together, these studies provide strong evidence that PGD₂ and ent-PGD₂, in addition to PGE₂ and ent-PGE₂, are generated in vivo in significant quantities from the IsoP pathway. Again, essentially identical results were obtained from the analysis of putative rac-PGD₂ formed from the peroxidation of arachidonate in vitro.

Quantitative Analysis of rac-PGE₂ and rac-PGD₂ Generated in Vitro and in Rat Liver—The above studies provide substantial support for the hypothesis that PGE₂ and PGD₂ and their respective enantiomers can be generated via the IsoP pathway. We subsequently undertook experiments to determine the total amounts of rac-PGD₂ and rac-PGE₂ generated from the oxidation of arachidonate in vitro and in vivo in comparison with other E₂/D₂-IsoPs. These determinations are highly important because, based on previous reports (4), the vast majority (>90%) of endoperoxide intermediates generated by the autoxidation of polyunsaturated fatty acids have side chains that are cis in relation to the prostane ring. Indeed, we have recently confirmed that endoperoxides with cis-side chains predominate over trans-side chain compounds when arachidonate is oxidized in vitro (5). Thus, it would be predicted that the IsoP endoperoxide with a structure identical to PGH₂ generated from the peroxidation of arachidonate in vitro would produce a trivial fraction of the total endoperoxides that are formed. Therefore, the amounts of rac-PGE₂ and rac-PGD₂ that are subsequently generated from this endoperoxide intermediate would be present at no more than a few nanograms/1000 ng of total E₂/D₂-IsoPs (4). Employing the HPLC protocols utilized for the studies described above, we quantified rac-PGE₂, rac-PGD₂, and rac-15-E₂t-IsoP in vitro and in vivo and also assessed the relative formation of each enantiomer in the racemic mixture. Losses of endogenous material during the chromatographic procedures were accounted for by determining the percent loss of the respective radiolabeled PG added to the samples prior to purification. As noted in Table II, the amounts of rac-PGE₂ and rac-PGD₂ far exceeded those predicted based upon the observations of O’Connor et al. (4), and the quantities of rac-PGE₂ were at least as great as, if not greater than, those of rac-15-E₂t-IsoP both in vitro and in vivo.

In summary, these quantitative data provide support that PGE₂ and PGD₂ and their respective enantiomers are generated in significant amounts via the IsoP pathway.

Excretion of Unesterified rac-PGE₂ and rac-PGD₂ in Rat and Human Urine in Vivo—In addition to detecting the in vivo formation of rac-PGE₂ and rac-PGD₂ esterified in rat liver tissue, we also sought to determine whether these compounds are present unesterified in human and rodent urine at base line and whether they increase in association with oxidant stress. Table III shows the results of studies performed to determine the relative amounts of these eicosanoids under these conditions. As shown, at baseline, the relative levels of both ent-PGE₂ and ent-PGD₂ in humans and rats were low and composed no more than 10% of the total rac-PGE₂ and rac-PGD₂ generated. In addition, in several of the urine samples obtained from normal humans and rats at baseline, the levels of rac-PGE₂ were below the limits of assay detection. On the other hand, after treatment of rats with CCl₄, the levels of both ent-PGE₂ and ent-PGD₂ increased significantly. This was particularly the case for ent-PGD₂. If one assumes that an amount of PGD₂ equivalent to that of ent-PGD₂ is generated via the IsoP pathway after administration of CCl₄, then ~15% of PGD₂ present in rat urine under these conditions is formed by a mechanism independent of COX. Analogously, ~30% of rac-PGD₂ (total of PGD₂ and ent-PGD₂) would thus be predicted to be generated by this mechanism. Fig. 12 (A and B) shows the results from chiral analysis of rat urine for PGD₂ and ent-PGD₂ at base line and after treatment with CCl₄. As shown, at base line, the chromatographic peak comprising PGD₂ (*) in Fig. 12A greatly exceeded the enantiomer (+), suggesting that COX contributes to the vast majority of PGD₂ production at base line. After CCl₄ administration (Fig. 12B), the levels of ent-PGD₂ (+) increased dramatically in relation to PGD₂ (*), supporting the contention that CCl₄ has induced PG formation via the IsoP pathway.

To provide further evidence that PGs can be generated via the IsoP pathway, we pretreated rats with indomethacin (10 mg/kg intraperitoneally for 24, 12, and 1 h) prior to CCl₄ administration and collected urine for 24 h after the oxidant was given (6, 21). Fig. 12C shows the results from the chiral analysis of rac-PGD₂. As shown, the chromatographic peaks representing PGD₂ and ent-PGD₂ are very similar. The levels of PGD₂ and ent-PGD₂ were ~30% of those present in CCl₄-treated rats not given indomethacin (Table III) and support the contention that significant amounts of unesterified PGD₂ (and to a lesser extent, PGE₂) can be formed by a mechanism independent of COX in settings of oxidant stress.
Prostaglandin Formation via the Isoprostane Pathway

### DISCUSSION

This study describes the formation of PGE$_2$ and PGD$_2$ independent of COX and involving the free radical-catalyzed peroxidation of arachidonate. We have reported that significant amounts of rac-PGE$_2$ and rac-PGD$_2$ are generated in vitro and in vivo in settings of oxidant stress. Unlike PGs formed via COX, generation of eicosanoids by this mechanism results in the formation of compounds as racemic mixtures because the oxygenation of arachidonic acid does not occur stereospecifically (2, 3, 6). Our initial interest in determining whether PGs are generated via the IsoP pathway emerged from the observation that compounds with the same molecular weights and GC retention times as PGE$_2$ and PGD$_2$ are present when analyzed by GC/MS in mixtures of arachidonate oxidized in vitro and in rat liver hydroxylases. Utilizing a variety of high-resolving chromatographic, chemical, and mass spectrometric approaches, we have found that substantial quantities of these racemic PGs can be generated. Analysis of putative rac-PGE$_2$ and rac-PGD$_2$ by chiral HPLC revealed that each compound is composed of two enantiomers generated in equal amounts in vitro and in liver tissue from rats exposed to oxidant stress. rac-PGE$_2$ and rac-PGD$_2$ were also present in the unesterified form in significant amounts in urine from rats treated with CCl$_4$, and their formation was unaffected by COX inhibition. That COX is not involved in the formation of these compounds is also supported by the findings that these PGs could be generated in vitro without COX and were present in vivo esterified in phospholipids. COX is not active on arachidonate esterified in phospholipids (1). Finally, compounds with retention times and molecular weights identical to those of PGE$_2$ and PGD$_2$ were present when liver tissue from COX-1$^{-/-}$/COX-2$^{-/-}$ mice was analyzed for E$_2$D$_2$-Isop-Isos.

We propose that the formation of PGs independent of COX involves the generation of two IsoP endoperoxide intermediates (rac-15-H$_2$-IsoP and rac-15-H$_2$-IsoP) that isomerize to rac-15-E$_2t$-IsoP and rac-15-D$_2c$-IsoP, respectively. These eicosanoids subsequently undergo rapid epimerization to compounds identical in all respects to racemic PGE$_2$ and PGD$_2$, respectively (Fig. 2). A number of lines of evidence that we and others have obtained support this proposed mechanism of formation. As noted, we previously showed that IsoPs contain E/D-, F-, and thromboxane-type prostanate rings (7). However, an important distinction between Isop-Isos and PGs is that IsoP bicycloendoperoxide intermediates contain side chains that are predominantly (>90%) oriented cis in relation to the prostanate ring (4). Indeed, we have recently confirmed that endoperoxides with cis-side chains predominate over trans-side chain compounds when arachidonate is oxidized (5). One IsoP that is formed in abundance in vivo is 15-E$_2$-IsoP, which is generated from the endoperoxide intermediate 15-H$_2$-IsoP (10). It would also be predicted that the endoperoxide 15-H$_2$-IsoP can rearrange to form the analogous D-ring Iso 15-D$_2$-IsoP. In contrast to other types of prostanoids, E$_2$D$_2$-Isop-Isos are $\beta$-hydroxyketone-containing compounds that can undergo reversible keto-enol tautomeration under both acidic and basic conditions, allowing rearrangement of the side chains that are initially cis to the more stable trans-configuration. That the trans-configuration is highly favored has been demonstrated by the finding that, when PGE$_2$ is subjected to conditions that induce keto-enol tautomerism, <10% of the compound rearranges to the cis-side chain isomer 15-E$_{2c}$-IsoP (11). Also, attempts to synthesize 15-D$_{2c}$-IsoP have been unsuccessful because epimerization at C-12 readily occurs during synthesis to yield PGD$_2$ (12).

In this study, we have shown that chemically synthesized 15-E$_{2c}$-IsoP is unstable and rapidly epimerizes nonenzymatically to PGE$_2$ in phosphate buffer at physiological pH. It is likely that the isomerization is further enhanced in the presence of protein-containing biological solutions, which have been shown to facilitate epimerization and dehydration of other eicosanoids (22). Whether the isomerization can be catalyzed enzymatically is unknown. That epimerization of IsoP endoperoxides occurred in the in vitro and in vivo studies reported herein is strongly supported by the fact that comparable amounts of rac-PGE$_2$ and rac-15-E$_{2c}$-IsoP were generated from the peroxidation of arachidonate. In addition, the abundance of rac-PGD$_2$ lends credence to the hypothesis that epimerization occurs readily. As noted by our findings, the formation of rac-PGD$_2$ predominates over that of rac-PGE$_2$ both at base line and after oxidant stress, perhaps because this compound would be predicted to form more readily from the epimerization of rac-15-D$_{2c}$-IsoP compared with PGE$_2$ from 15-E$_{2c}$-IsoP. In this study, the lack of a chemically synthesized 15-D$_{2c}$-IsoP standard precludes our detection of this compound in vitro and in vivo, although it would be predicted that it would not be present in significant amounts.

Our results also suggest that epimerization of 15-E$_{2c}$-IsoP and 15-D$_{2c}$-IsoP to PGE$_2$ and PGD$_2$, respectively, occurs to a significant extent while these compounds are esterified in phospholipids based on two lines of evidence. First, a pattern of peaks virtually identical to that shown in the chromatograms in Fig. 5 was obtained after hydrolysis of rat liver phospholipids that had been treated with methyloxime HCl prior to hydrolysis. Second, in control experiments, the conversion of exogenously added 15-E$_{2c}$-IsoP to PGE$_2$ occurred to a negligible extent during sample workup.

A number of important physiological and pharmacological issues emerge from the this study. The first relates to the fact that formation of bioactive PGs occurs in vivo to a significant extent via the IsoP pathway in settings of oxidative stress and potentially in other inflammatory situations. Although levels of PGs derived via this mechanism are low at base line in normal humans and animals, they represent up to 15% of PGD$_2$ present in the urine of rats treated with CCl$_4$, and these PGs are formed independent of COX inhibition. 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 adverse sequelae of oxidative injury.

Although the biological properties of PGE$_2$ and PGD$_2$ have been well characterized (1), our studies suggest that equal amounts of the enantiomers of these PGs are also produced. It will thus be of interest to explore the bioactivity of ent-PGE$_2$ and ent-PGD$_2$. In this respect, the former compound was syn-
Prostaglandin Formation via the Isoprostane Pathway

The metabolism of PGE$_2$ and PGD$_2$ has been extensively studied in animals and humans. The metabolism of parent PGs via the formation of C-13,14-dihydro-15-keto derivatives and subsequent COX-2 or COX-1 inhibition generally renders them inactive. However, this is not the case for the one IsoP whose metabolism has been studied in detail, 15-F$_2$-IsoP. The major metabolite of this compound is 2,3-dinor-5,6-dihydro-15-F$_2$t-IsoP, which results from one step of β-oxidation and an unusual C-5–C-6 double bond reduction (26). Interestingly, this metabolite displays bioactivity as a vasoconstrictor similar to that of 15-F$_2$-IsoP (27). Thus, studying the metabolism of ent-PGs, in addition to their biological activities, may provide important insights into their role as mediators of oxidant stress. In this regard, we have recently found that, unlike PGE$_2$, ent-PGE$_2$ is a poor substrate for 15-hydroxyprostaglandin dehydrogenase, suggesting that the metabolism of this eicosanoid is significantly different from that of PGE$_2$.²

The studies reported herein are highly relevant with regard to human pharmacology in that they suggest that a second pathway operates in vivo to generate PGs and is independent of COX. That this pathway contributes to the formation of PGs in settings of oxidant stress has been discussed above. On the other hand, the extent to which it contributes to PG production in other disease states or at base line has not been elucidated. Administration of nonsteroidal anti-inflammatory agents to humans has been shown to significantly decrease production of PGs and PG metabolites, although the degree of suppression varies depending on the eicosanoid measured. For example, administration of high doses of nonsteroids (e.g., 1.5 g of aspirin or more or the equivalent) to normal human volunteers is associated with a 90% reduction in thromboxane formation and a >80% reduction in PGI$_2$ (28–30). In contrast, the same doses of these agents have been reported to be associated with no greater than a 60% decrease in PGE$_2$ excretion (28). In this regard, we have made similar observations (31). The reasons for this discrepancy are unknown; but in light of our findings that PGs, particularly PGE$_2$, are formed via a non-COX mechanism, it is intriguing to postulate that part of the reason that aspirin-like drugs fail to inhibit PGE$_2$ production compared with other PGs in certain settings is that the former compound can be produced from IsoP intermediates.

In summary, we report that a second pathway exists for the formation of bioactive PGs in vivo that is independent of COX. This finding is likely of physiological and pharmacological importance because it would be predicted that the generation of PGs via this mechanism would not be inhibited by aspirin or other COX inhibitors. The extent to which formation of PGs independent of COX contributes to human physiology and pathophysiology remains to be elucidated.

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