Biosynthetic Crossover of 5-Lipoxygenase and Cyclooxygenase-2 Yields 5-Hydroxy-PGE$_2$ and 5-Hydroxy-PGД$_2$

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Running title: Discovery of 5-hydroxy-prostaglandins

Abbreviations:
COX        cyclooxygenase
ESI        electrospray ionization
HETE       hydroxy-eicosatetraenoic acid
HHT        hydroxy-heptadecatrienoic acid
4-HNE      4-hydroxy-nonenal
H-PGDS     hematopoietic prostaglandin D synthase
LC-MS      liquid chromatography-mass spectrometry
LOX        lipoxygenase
LPS        lipopolysaccharide
MDA        malondialdehyde
PG          prostaglandin
Abstract

Biosynthetic crossover of 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) enzymatic activities is a productive pathway to convert arachidonic acid into unique eicosanoids. Here we show that COX-2 catalysis with 5-LOX derived 5-hydroxyeicosatetraenoic acid yields the endoperoxide 5-hydroxy-PGH$_2$ that spontaneously rearranges to 5-OH-PGE$_2$ and 5-OH-PGD$_2$, the 5-hydroxy analogs of arachidonic acid derived PGE$_2$ and PGD$_2$. The endoperoxide was identified via its predicted degradation product, 5,12-dihydroxy-heptadecatri-6E,8E,10E-enoic acid, and by SnCl$_2$-mediated reduction to 5-OH-PGF$_{2\alpha}$. Both 5-OH-PGE$_2$ and 5-OH-PGD$_2$ were unstable and degraded rapidly upon treatment with weak base. The instability hampered detection in biologic samples which was overcome by in situ reduction using NaBH$_4$ to yield the corresponding stable 5-OH-PGF$_2$ diastereomers and enabled detection of 5-OH-PGF$_{2\alpha}$ in activated primary human leukocytes. 5-OH-PGE$_2$ and 5-OH-PGD$_2$ were unable to activate EP and DP prostanoid receptors suggesting their bioactivity is distinct from PGE$_2$ and PGD$_2$. 
1. Introduction

The inducible form of cyclooxygenase (COX), COX-2, has a larger active site (1) resulting in a wider substrate specificity than COX-1, the isoform that reacts preferably with arachidonic acid (2,3). Among the unique substrates for COX-2 is 5-hydroxyeicosatetraenoic acid (5-HETE), a product of the reaction of 5-lipoxygenase (5-LOX) with arachidonic acid (4). The reaction of COX-2 with 5-HETE yields the highly oxygenated hemiketal (HK) eicosanoids HKE_2 and HKD_2 that result from triple oxygenation of the substrate (5) while arachidonic acid is reacted with two molecules of oxygen when forming prostaglandins (Scheme 1) (6,7). Reaction of COX-2 with 5-HETE and other isoform-specific substrates like fatty acid amides (anandamide) (8) and esters (2-arachidonoyl-glycerol) (9) suggests that the resulting products serve specific biologic functions that are distinct from those of traditional prostaglandins (3,10).

Scheme 1

**Biosynthesis and transformation of endoperoxides in the reaction of cyclooxygenases with arachidonic acid and 5-HETE**

The main catalytic product of the reaction of COX-2 with 5-HETE is a diendoperoxide as a precursor to the two HK eicosanoids (Scheme 1) (4,5). The diendoperoxide features two fused 5- and 7-membered rings with peroxides linking carbons 9 and 11 as well as 8 and 12, respectively. Spontaneous opening of both peroxides in a reaction equivalent to the non-enzymatic rearrangement of PGH_2 to PGE_2 and PGD_2 (11) followed by forming the hemiketal ring moiety yields HKE_2 and HKD_2 (Scheme 1) (5). Alternatively, the diendoperoxide degrades to the aldehyde fragments malondialdehyde (MDA), 4-hydroxy-2E-nonenal (4-HNE), and 8-oxo-5-hydroxy-6E-octenoic acid in the presence of...
ferrous iron or excess heme (12), again in a reaction equivalent to the degradation of PGH$_2$ to MDA and 12-hydroxy-heptadecatrienoic acid (12-HHT) (11). Other, less abundant products resulting from the COX-2 reaction with 5-HETE are 5,11- and 5,15-diHETE (13), the 5-hydroxy analogues of the minor AA/COX products, 11- and 15-HETE (11,14).

A critical intermediate in COX-2 transformation of 5-HETE is the C-8 carbon radical of the 9,11-endoperoxide 1 (Scheme 2). During prostaglandin formation from arachidonic acid the C-8 radical reacts with C-12 to yield the five-membered prostanoid ring. During formation of the diendoperoxide from 5-HETE, the C-8 radical reacts with O$_2$ followed by reaction of the peroxyl radical with C-12, thus expanding the prostanoid ring to include a peroxide linking carbons 8 and 12 (4). The final product in either case is formed by stereospecific oxygenation and reduction at C-15. Direct addition of C-8 to C-12 would predict the formation of 5-hydroxy-analogue 2 of PGH$_2$ as a precursor to novel prostanoids (Scheme 2). Here, we describe the conversion of 5-HETE to 5-OH-PG$_2$H by COX-2 yielding 5-OH-PGE$_2$ and -PGD$_2$ as novel products of the biosynthetic crossover of 5-LOX and COX-2 and their formation in human leukocytes that have been activated to express both enzymes.

**Scheme 2**

Reaction of the C-8 carbonyl radical intermediate 1 in COX-2 catalysis with 5-HETE determines the formation of hemiketals (HK) or 5-hydroxy-prostaglandins as enzymatic products.
2. Results and Discussion

Novel products in the reaction of COX-2 with 5-HETE
Negative ion LC-MS analysis of products formed by the reaction of recombinant human COX-2 with 5-HETE revealed two products 3 and 4 with \( m/z \) 367.3, equivalent to a molecular weight of 368.5 g/mol (Fig. 1). The increase of 16 mass units compared to prostaglandins like PGD\(_2\) and PGE\(_2\) (MW 352.5 g/mol) suggested that the novel products represent 5-hydroxy analogs of PGD\(_2\) and PGE\(_2\). Products 3 and 4 eluted at a retention time similar to the hemiketal products HKD\(_2\) and HKE\(_2\) (\( m/z \) 399.3; MW 400.5 g/mol) while unreacted 5-HETE substrate eluted at 5.1 min retention time (Fig. 1).

![Figure 1. LC-MS detection of HKE\(_2\) and HKD\(_2\) (\( m/z \) 399 ion trace) and novel products 3 and 4 (\( m/z \) 367) in a reaction of recombinant human COX-2 with 5-HETE substrate (\( m/z \) 319).](image)

Comparison of the ion intensities in negative ion mode MS1 analyses, albeit an imperfect measure of relative intensities, suggested that 3 and 4 were about 20\% of the abundance of HKE\(_2\) and HKD\(_2\), the major products formed in the reaction of COX-2 with 5-HETE in vitro as determined using radiolabeled [\( 1^{-14}C\)]5-HETE substrate (5).

Identification of 5-OH-PGD\(_2\) and 5-OH-PGE\(_2\)
Product 4 was isolated from a large-scale reaction of 5-HETE with COX-2 conducted in the presence of hematopoietic prostaglandin D synthase (H-PGDS). H-PGDS was included in the enzymatic reaction to facilitate transformation of the predicted COX-2 catalytic product, 5-OH-PGH\(_2\) 2, to 5-OH-PGD\(_2\). Homo- and heteronuclear 1D- and 2D-NMR analyses of product 4 showed signals for a 5-membered prostanoid ring with 9-hydroxyl and 11-keto moieties and the side chains at C-8 and C-12 in trans configuration. The presence of two allylic hydroxy groups, representing the 5-OH-6E-ene and 15-OH-13\( E\)-ene moieties, confirmed the identification of 4 as 5-OH-PGD\(_2\). Incubations of 5-HETE with COX-2 did not afford a sufficient amount for NMR analysis of 3 which was presumed to be 5-OH-PGE\(_2\). As an alternative approach, a standard of 5-OH-PGE\(_2\) was independently prepared by chemical synthesis starting from PGE\(_2\) as described below. Comparison to the synthetic standard confirmed identification of 3 as 5-OH-PGE\(_2\).

Synthesis of 5-OH-PGD\(_2\) and 5-OH-PGE\(_2\)
5-OH-PGD\(_2\) and 5-OH-PGE\(_2\) were synthesized by singlet oxidation of PGD\(_2\) and PGE\(_2\), respectively. Reaction of PGD\(_2\) in methanol with methylene blue and visible light followed by reduction (PPh\(_3\)) yielded \( \delta \)-5-OH-PGD\(_2\) 4 and \( \delta \)-5-OH-PGD\(_2\) 5 as well as the 6-OH-4\( E\)-isomers (6, 7) (Supporting Information, Fig. S1A). Singlet oxidation of PGE\(_2\) gave two major products 3 and 8, identified as \( \delta \)-5 and \( \delta \)-5-OH-PGE\(_2\), respectively, with
insignificant formation of the 6-OH isomers (Supporting Information, Fig. S1B). The absolute configuration of C-5 of the synthesized 5-OH-PGs (3, 4, 5, and 8) was determined by LC-MS analysis of diastereomeric cleavage products formed by methylation (CH₂N₂), derivatization with (-)-menthyl chloroformate, and oxidative ozonolysis (15) (Supporting Information, Fig. S2). Authentic standards for the assignment of the configuration of (-)-menthoxycarbonyl 2S- and 2R-hydroxy-1,6-hexanedioc acid-6-methyl ester diastereomers were obtained starting with synthetic 5S- and 5R-HETE, respectively, for which the absolute configuration was known (16).

**Reduction and degradation of 5-OH-PGH₂**

The structural analogy of 5-OH-PGD₂ and 5-OH-PGE₂ with PGD₂ and PGE₂ suggested the former were formed by spontaneous rearrangement of an endoperoxide precursor, predicted to be 5-OH-PGH₂ 2, as the actual product of the COX-2 reaction with 5-HETE. 5-OH-PGH₂ was predicted to be too unstable to attempt isolation and direct structural identification. In order to test whether endoperoxide 2 was the product of COX-2 catalysis with 5-HETE, the enzymatic reaction was treated with SnCl₂ as a mild reducing agent for the endoperoxide group (17). LC-MS analysis showed formation of 5-OH-PGF₂α 9 as a reduction product with a molecular ion at m/z 369 that was absent without SnCl₂ treatment (Fig. 2A). Product 9 co-eluted with 5-OH-PGF₂α that was formed by NaBH₄ reduction of synthetic 5-OH-PGD₂ as described below.

**Figure 2.** Indirect identification of 5-OH-PGH₂. (A) Treatment with SnCl₂ of a 5-min reaction of COX-2 with 5-HETE generated product 9 with m/z 369 that had the same retention time as a standard of 5-OH-PGF₂α formed by reduction (NaBH₄) of 5-OH-PGD₂ (inset). (B) Treatment of the enzymatic reaction with hematin yielded product 10...
identified as 5,12-dihydroxy-heptadecatri-6E,8E,10E-enoic acid (5,12-diHHT) due to its UV spectrum characteristic of a conjugated triene in E,E,E configuration (inset) and (C) fragment ions derived from m/z 295.4 (10) in the MS2 spectrum.

Further evidence for 5-OH-PGH$_2$ as the product of COX-2 catalysis with 5-HETE was obtained from its degradation in the presence of hematin, similar to the transformation of PGH$_2$ to 12-HHT (18). Recombinant human COX-2 was incubated with 5-HETE for 5 min followed by addition of hematin to induce degradation. The products were extracted and analyzed by RP-HPLC with diode array detection and by LC-MS. The RP-HPLC chromatogram showed elution of product 10 with a UV chromophore typical of a conjugated triene with λ$_{\text{max}}$ at 268 nm and diagnostic shoulders at 260 and 280 nm, with the deeply defined bathochromic shoulder indicating E,E,E-configuration of the conjugated triene (Fig. 2B) (19,20). Product 10 was isolated using RP-HPLC and analyzed by LC-MS operated in the negative ion mode which gave a molecular ion at m/z 295.4, indicating an MW of 296.4 g/mol (Fig. 2C). UV spectrum, MW, and the MS fragment ions indicated that product 10 was 5,12-dihydroxy-heptadecatri-6E,8E,10E-enoic acid (5,12-diHHT).

**Stability of 5-OH-PGD$_2$ and 5-OH-PGE$_2$**

Treatment of PGE$_2$ and PGD$_2$ with base induces dehydration of the hydroxyl groups at C-11 or C-9, respectively, resulting in formation of the respective enone prostanoids (21,22). Base treatment dehydrates PGE$_2$ (λ$_{\text{max}}$ 205 nm) to the enone PGA$_2$ (λ$_{\text{max}}$ 217 nm) (23) that further rearranges to the dienone PGB$_2$ with λ$_{\text{max}}$ at 278 nm (Fig. 3A) (24). Likewise, PGD$_2$ dehydrates to PGJ$_2$ that rearranges to Δ$^{12}$-PGJ$_2$ and undergoes a second dehydration to yield 15-deoxy-Δ$^{12,14}$-PGJ$_2$ (22,25).

When base treatment is conducted in a UV/Vis cuvette, the dehydration can be followed by observing the change of the chromophores over time. For example, treatment of PGE$_2$ with NaOH (200 mM) in Fig. 3B illustrates the time course of formation of PGB$_2$. To test the stability of 5-OH-PGE$_2$ a 10-fold lower concentration of base was used (20 mM NaOH) and the scan frequency was doubled to 1/min. Under these conditions, the first scan recorded immediately after addition of 5-OH-PGE$_2$ to the cuvette indicated complete conversion to a product with a dienone-like chromophore at UV295 nm (Fig. 3C). The dienone-like chromophore decreased rapidly to yield products with less distinct absorbance. This indicated that 5-OH-PGE$_2$ was much more sensitive to base-induced dehydration than PGE$_2$, and that further transformation yielded several undefined products. When 5-OH-PGE$_2$ was incubated in a buffer of pH 8 the time course of formation of the UV295 nm chromophore, taken as a measure of the initial dehydration, indicated a half-life of ≈12 min (Fig. 3D). When 5-OH-PGD$_2$ was treated with base (20 mM NaOH, scan rate 1/min) a similar change of chromophores was observed (Fig. 3E), suggesting a comparable half-life.
**Figure 3.** Base-catalyzed dehydration of prostaglandins. (A) Dehydration of PGE$_2$ yields PGA$_2$ that rearranges to PGB$_2$. (B) Time-course of dehydration of PGE$_2$ in NaOH (200 mM) illustrates formation of PGA$_2$ ($\lambda_{\text{max}}$ 217 nm) and PGB$_2$ ($\lambda_{\text{max}}$ at 278 nm). UV/Vis spectra were acquired every 2 min. (C) Degradation of 5-OH-PGE$_2$ in 10-times dilute NaOH (20 mM) indicating complete conversion to a product with $\lambda_{\text{max}}$ 295 nm at the time of the first scan. (D) Degradation of 5-OH-PGD$_2$ in NaOH (20 mM), with t = 0 min indicating conversion to a product with $\lambda_{\text{max}}$ 295 nm. The UV/Vis scanning rate was 1/min in panels C-E.

**In situ reduction of 5-OH-PGs**

The chemical instability was predicted to impede analysis of 5-OH-PG biosynthesis in a biologic environment due to their rapid transformation to uncharacterized products. To minimize loss of 5-OH-PGs during cell incubations, extraction, and analysis a method was developed for in situ reduction to the 5-OH-PGF$_2$ analogues. Reduction of the dehydration-prone cyclopentanones and remaining endoperoxide was predicted to increase stability as is observed for PGF$_{2\alpha}$ and F$_2$-isoprostanes relative to cyclopentanone PGs (26). Recombinant COX-2 was incubated with 5-HETE for 5 min followed by addition of NaBH$_4$ in order to achieve reduction of the immediate products to 5-OH-PGF$_2$ diastereomers. Analysis using LC-MS gave two products with the expected m/z 369 molecular ion, eluting at 1.81 min (11) and 1.90 min (9) retention time, respectively (Fig. 4A). It was assumed that 11 and 9 were diastereomers of 5-OH-PGF$_2$ but the configuration of the hydroxy groups on the prostanoid ring was unclear, i.e., whether it was $\alpha$ or $\beta$. Reduction of synthetic 5-OH-PGE$_2$ gave 11 and 9 in a ratio of $\approx$75:25 (Fig. 4B). Reduction of synthetic 5-OH-PGD$_2$ gave 9 as the overwhelming product together with a second isomer 12 (Fig. 4C) (27). These analyses suggested that the $\alpha$ and $\beta$
diastereomers were not formed in a 1:1 ratio in the reduction of the carbonyl by NaBH₄. It should be noted that reduction of 5-OH-PGE₂ and 5-OH-PGD₂ will only yield three diastereomers since both precursors will form the F₂α diastereomer whereas only the other diastereomer is unique. Comparison to the PGF₂ diastereomers formed by reduction of PGD₂ and PGE₂ (Supporting Information, Fig. S3) enabled to identify product 11 as 5-OH-PGF₂β and 9 as 5-OH-PGF₂α. The third diastereomer, 5-OH-11β-PGF₂α 12, was formed only at low abundance by reduction of 5-OH-PGD₂.

Figure 4. 5-OH-PGF₂ diastereomers formed by NaBH₄ reduction of (A) a 5-min reaction of 5-HETE with COX-2, (B) synthetic 5-OH-PGD₂, and (C) synthetic 5-OH-PGE₂. The ion trace for m/z 369 from LC-MS analysis of the reactions is shown. Products in (A) are expected to be mainly derived from reduction of 5-OH-PGH₂.

Reduction of the diendoperoxide and hemiketals in a crude reaction of 5-HETE with COX-2 as well as reduction of HKE₂ gave a single peak (RT 1.69 min) with m/z 403, representing 5,8,9,11,12,15-hexahydroxy-eicosadienoic acid 13 (Supporting Information, Fig. S4). Detection of a single peak indicated that fewer than the expected diastereomers were formed and/or that the diastereomers were not resolved chromatographically. This matter was not further investigated.

**Formation of 5-OH-PGs by human leukocytes**

Biosynthetic cross-over of 5-LOX and COX-2 activities in leukocyte mixtures isolated from normal human volunteers and stimulated ex vivo with LPS and A23187 yielded HKE₂, HKD₂, as well as 5,11- and 5,15-diHETEs (28,29). The use of inhibitors confirmed the role of the two enzymes in biosynthesis (28,29). To test whether human leukocytes also form 5-OH-PGs the cells were activated and then treated in situ using NaBH₄ to
reduce unstable 5-OH-PGs to the 5-OH-PGF₂ diastereomers prior to extraction and LC-MS analysis.

Freshly isolated human leukocytes stimulated with LPS and A23187 showed formation of 5-OH-PGF₂α, along with 5,8,9,11,12,15-hexahydroxy-eicosadienoic acid 13, the reduction product of HKE₂, HKD₂, and their diendoperoxide precursor, respectively (Fig. 5). 5-OH-PGE₂ and 5-OH-PGD₂ could not be detected, even when samples were not treated with NaBH₄ (Fig. 5). The absence of 5-OH-PGF₂β indicated that the leukocyte mixtures preferentially formed 5-OH-PGD₂ over 5-OH-PGE₂ or that 5-OH-PGH₂ was the most abundant product at the time when NaBH₄ was added. As expected, HKE₂ and HKD₂ were absent or markedly decreased after NaBH₄ reduction.

Figure 5. Formation of 5-OH-PGs by activated human leukocytes. Cells were treated with LPS for 45 min and then with A23187 for 15 min, followed by reduction or not with NaBH₄, extracted, derivatized with AMPP, and analyzed using LC-MS in the positive ion SRM mode. Ion traces for the detection of 5-OH-PGF₂α, 5-OH-PGD₂ and 5-OH-PGE₂, hexahydroxy-eicosadienoic acid 13, HKE₂, and HKD₂ are shown.

Receptor activation
The understanding of the biological role of the 5-LOX/COX-2 crossover eicosanoids is still in its infancy. A factor holding back progress is the difficulty to prepare HKs by chemical or enzymatic synthesis (30,31). The ready synthesis, albeit on a small scale, of 5-OH-PGE₂ and 5-OH-PGD₂ starting from PGE₂ and PGD₂, respectively, enabled to test whether they activate EP and DP prostanoid receptors. Prostanoid receptors appeared a logical target due to the close structural similarity between prostaglandins and their 5-OH-analogues. However, neither 5-OH-PGE₂ nor 5-OH-PGD₂ activated the EP (EP1-EP4), DP1, FP, IP, or TP prostanoid receptors (Supporting Information, Figs. S5 and S6). This outcome was consistent with the absence of a 5-hydroxy modification in the structures of the known prostanoid analogues that are agonists of these receptors (https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=58; accessed March 2021).

3. Conclusion

Biosynthesis of 5-OH-PGH₂ from 5-HETE follows the known radical and oxygenation reactions catalyzed in the COX reaction with arachidonic acid (6). The 9,11-endoperoxide-8-carbinyl radical is a crucial catalytic intermediate (Scheme 2). Reaction of the carbon radical with oxygen or not prior to addition to the 12E double bond determines the outcome of the enzymatic reaction, i.e., formation of the diendoperoxide versus 5-OH-PGH₂ as the precursors to HKs and 5-OH-PGs, respectively. Using the purified enzyme in vitro, HKs are about 10-fold more abundant than 5-OH-PGs, indicating that oxygenation of the C-8 radical is favored over direct reaction with C-12. Nevertheless, it is interesting to speculate on a mechanism how the enzyme might control the ratio between the products. Control may be accomplished, for example, via regulating the concentration of O₂ or through an allosteric effect of the non-catalytic on the catalytic subunit of the functional COX-2 heterodimer (33,34). In the latter scenario, binding of non-substrate fatty acids like palmitate to the allosteric subunit of COX-2 increases efficiency of the catalytic subunit with arachidonic acid by more than 2-fold (35,36). Subunit allosteric effects also account for substrate-selective inhibitor potency. For example, the NSAIDs ibuprofen and mefenamic acid inhibited COX-2 oxygenation of 2-AG with orders of magnitude greater potency than oxygenation of arachidonic acid (37,38). This testifies to regulation of catalysis through interaction between the subunits of the COX dimer, and similar regulatory events may control the reactivity of the C-8 radical and thus the ratio of HKs versus 5-OH-PGs formed in COX-2 catalysis with 5-HETE.

Formation of 5-OH-PGs is biologically relevant beyond the mechanistic insight into COX-2 catalysis since it occurred in response to ex vivo stimulation of primary leukocytes isolated from normal human volunteers. Biological effects of the novel prostanoids await to be uncovered but the lack of activation of EP and DP prostanoid receptors by 5-OH-PGs suggests they exhibit bioactivity that is unique and different from traditional prostaglandins.
Associated Content
The Supporting Information is available free of charge at.

Experimental procedures, SI figures, and NMR data for new compounds

Notes
The authors declare no competing financial interest exists.

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