Biosynthetic Crossover of 5-Lipoxygenase and Cyclooxygenase-2 Yields 5-Hydroxy-PGE_2 and 5-Hydroxy-PGD_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

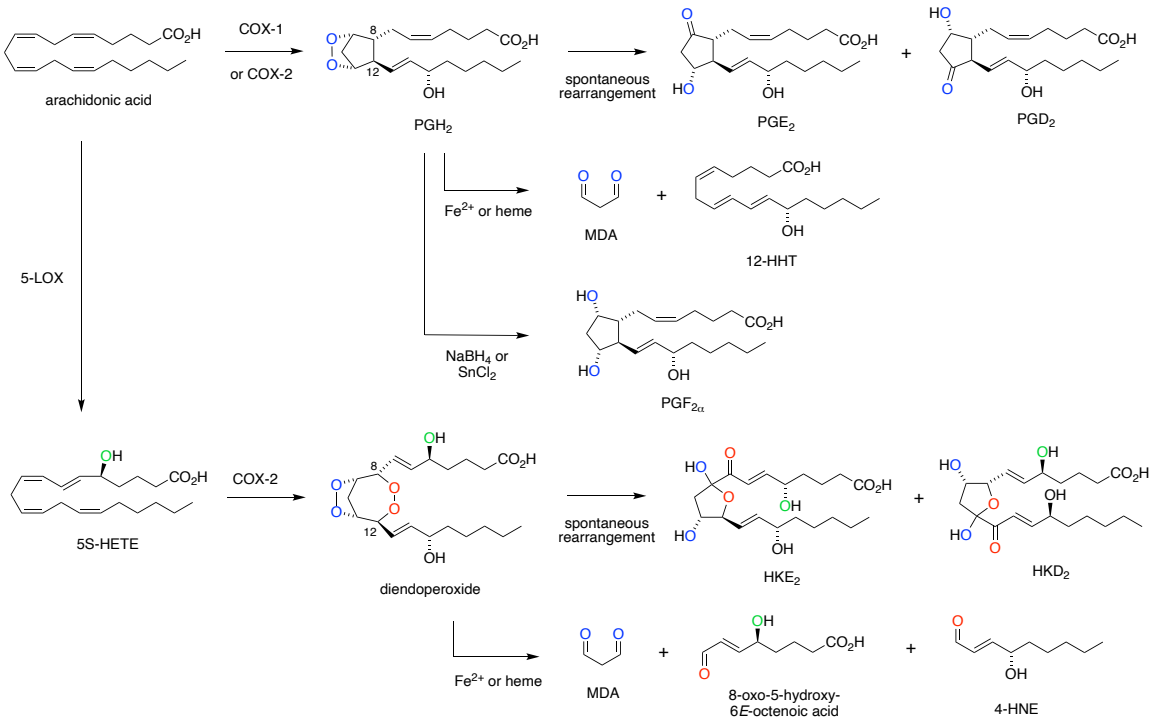
Abstract

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-hydroxy-eicosatetraenoic 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-6*E*,8*E*,10*E*-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-hydroxy-eicosatetraenoic 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-arachidonyl-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

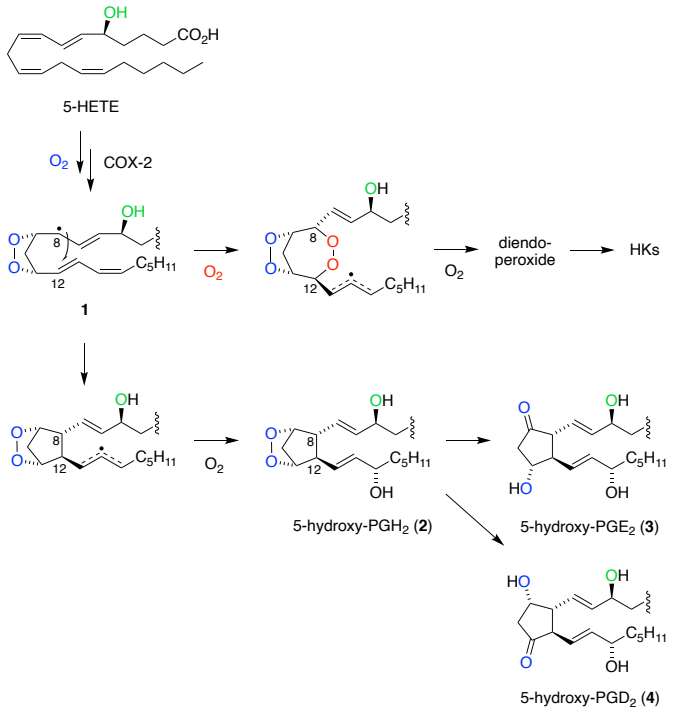


The main catalytic product of the reaction of COX-2 with 5-HETE is a diendoperoxide as a precursor to the two HK eicosanoids (Scheme 1) (4,5). The diendoperoxide features two fused 5- and 7-membered rings with peroxides linking carbons 9 and 11 as well as 8 and 12, respectively. Spontaneous opening of 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-2*E*-nonenal (4-HNE), and 8-oxo-5-hydroxy-6*E*-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-PGH_2 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 carbinyl 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).

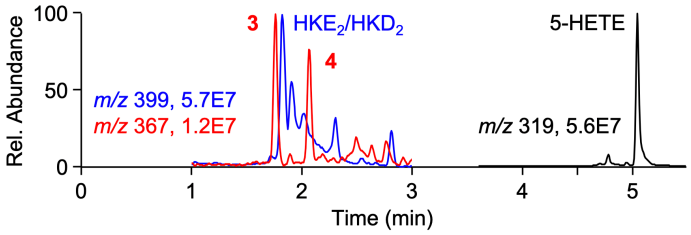


Figure 1. LC-MS detection of HKE_2 and HKD_2 (*m/z* 399 ion trace) and novel products **3** and **4** (*m/z* 367) in a reaction of recombinant human COX-2 with 5-HETE substrate (*m/z* 319).

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-6*E*-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 5*S*-OH-PGD_2 **4** and 5*R*-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 5*S*- and 5*R*-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 (CH_2N_2), derivatization with (-)-menthyl chloroformate, and oxidative ozonolysis (15) (Supporting Information, Fig. S2). Authentic standards for the assignment of the configuration of (-)-menthoxycarbonyl 2*S*- and 2*R*-hydroxy-1,6-hexanedioic acid-6-methyl ester diastereomers were obtained starting with synthetic 5*S*- and 5*R*-HETE, respectively, for which the absolute configuration was known (16).

Reduction and degradation of 5-OH-PGH$_2$

The structural analogy of 5-OH-PGD$_2$ and 5-OH-PGE$_2$ with PGD$_2$ and PGE$_2$ suggested the former were formed by spontaneous rearrangement of an endoperoxide precursor, predicted to be 5-OH-PGH$_2$ **2**, as the actual product of the COX-2 reaction with 5-HETE. 5-OH-PGH$_2$ 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 $SnCl_2$ as a mild reducing agent for the endoperoxide group (17). LC-MS analysis showed formation of 5-OH-PGF$_{2\alpha}$ **9** as a reduction product with a molecular ion at *m/z* 369 that was absent without $SnCl_2$ treatment (Fig. 2A). Product **9** co-eluted with 5-OH-PGF$_{2\alpha}$ that was formed by $NaBH_4$ reduction of synthetic 5-OH-PGD$_2$ as described below.

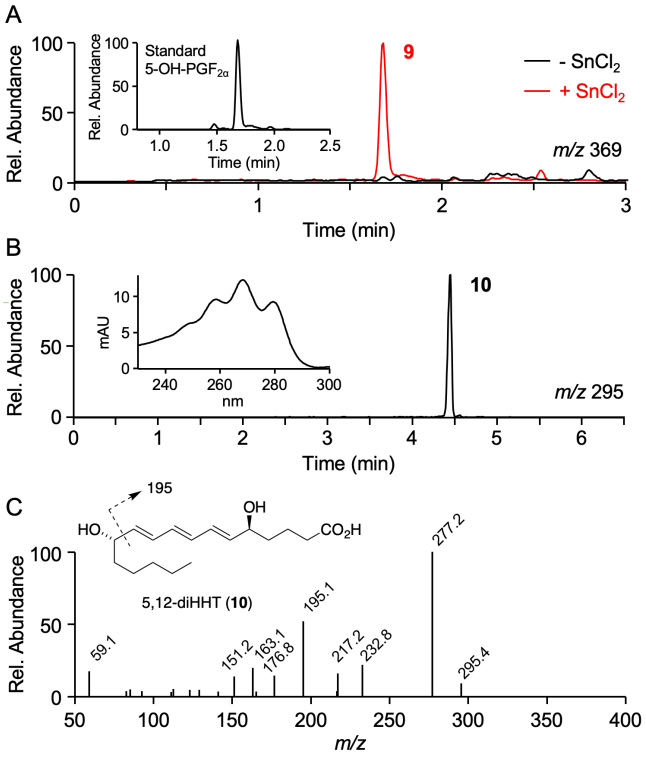


Figure 2. Indirect identification of 5-OH-PGH$_2$. (A) Treatment with $SnCl_2$ of a 5-min reaction of COX-2 with 5-HETE generated product **9** with *m/z* 369 that had the same retention time as a standard of 5-OH-PGF$_{2\alpha}$ formed by reduction ($NaBH_4$) of 5-OH-PGD$_2$ (inset). (B) Treatment of the enzymatic reaction with hematin yielded product **10**

identified as 5,12-dihydroxy-heptadecatri-6*E*,8*E*,10*E*-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 λmax 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-6*E*,8*E*,10*E*-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 (λmax 205 nm) to the enone PGA_2 (λmax 217 nm) (23) that further rearranges to the dienone PGB_2 with λmax 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-$\Delta^{12,14}$-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.

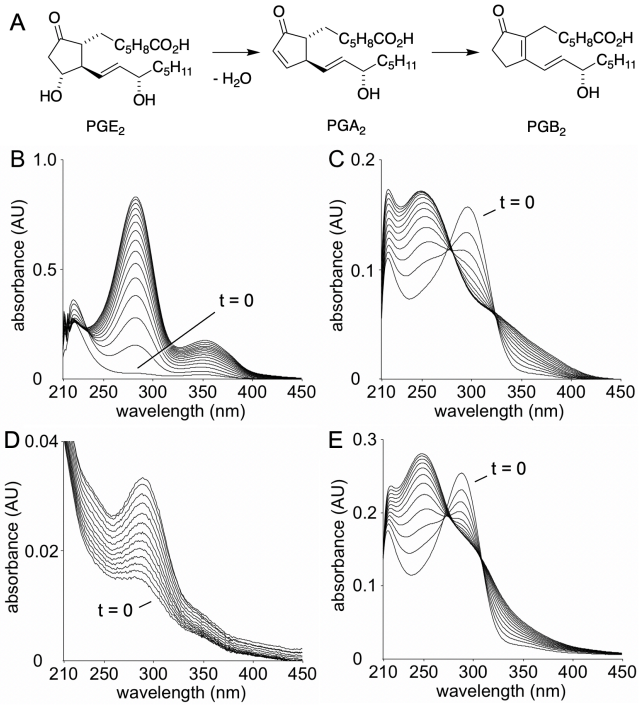


Figure 3. Base-catalyzed dehydration of prostaglandins. (A) Dehydration of PGE_2 yields PGA_2 that rearranges to PGB_2. (B) Time-course of dehydration of PGE_2 in NaOH (200 mM) illustrates formation of PGA_2 (λmax 217 nm) and PGB_2 (λmax at 278 nm). UV/Vis spectra were acquired every 2 min. (C) Degradation of 5-OH-PGE_2 in 10-times dilute NaOH (20 mM) indicating complete conversion to a product with λmax 295 nm at the time of the first scan. (D) Degradation of 5-OH-PGE_2 in Tris-HCl (50 mM, pH 8). (C) Degradation of 5-OH-PGD_2 in NaOH (20 mM), with t = 0 min indicating conversion to a product with λmax 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 α or β. Reduction of synthetic 5-OH-PGE_2 gave **11** and **9** in a ratio of ≈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 α and β

diastereomers were not formed in a 1:1 ratio in the reduction of the carbonyl by $NaBH_4$. It should be noted that reduction of 5-OH-PGE_2 and 5-OH-PGD_2 will only yield three diastereomers since both precursors will form the $F_{2\alpha}$ diastereomer whereas only the other diastereomer is unique. Comparison to the PGF_2 diastereomers formed by reduction of PGD_2 and PGE_2 (Supporting Information, Fig. S3) enabled to identify product **11** as 5-OH-$PGF_{2\beta}$ and **9** as 5-OH-$PGF_{2\alpha}$. The third diastereomer, 5-OH-11β-$PGF_{2\alpha}$ **12**, was formed only at low abundance by reduction of 5-OH-PGD_2.

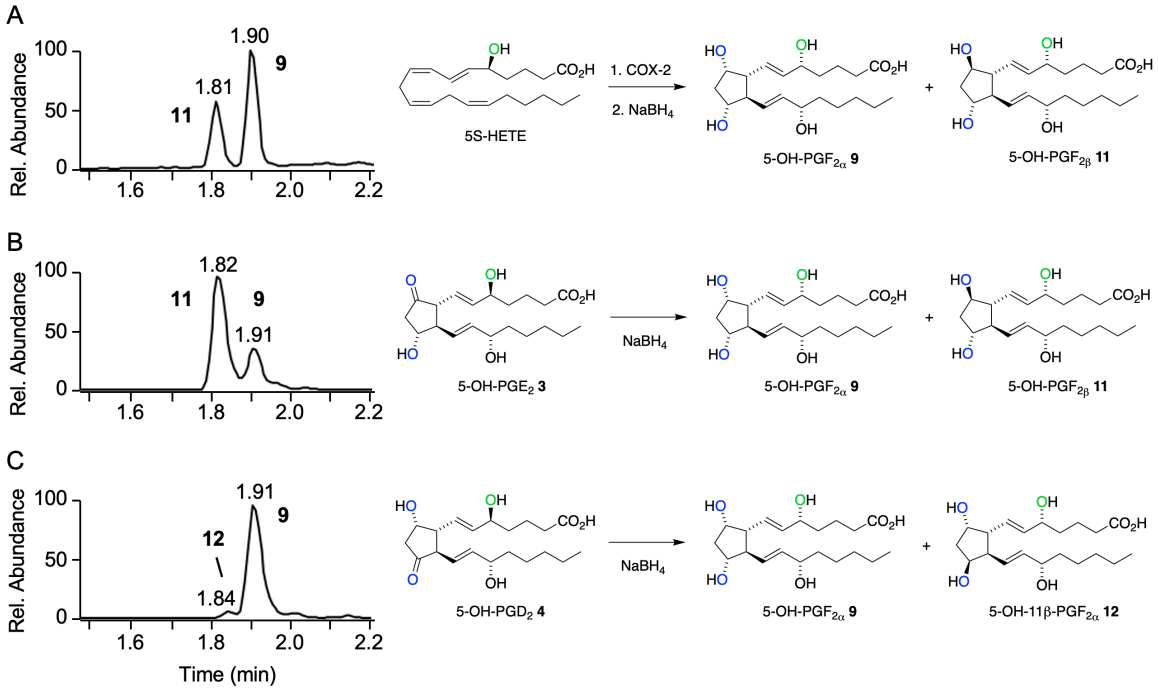


Figure 4. 5-OH-PGF_2 diastereomers formed by $NaBH_4$ reduction of (A) a 5-min reaction of 5-HETE with COX-2, (B) synthetic 5-OH-PGD_2, and (C) synthetic 5-OH-PGE_2. The ion trace for *m/z* 369 from LC-MS analysis of the reactions is shown. Products in (A) are expected to be mainly derived from reduction of 5-OH-PGH_2.

Reduction of the diendoperoxide and hemiketals in a crude reaction of 5-HETE with COX-2 as well as reduction of HKE_2 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 HKE_2, HKD_2, 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 $NaBH_4$ to

reduce unstable 5-OH-PGs to the 5-OH-PGF_2 diastereomers prior to extraction and LC-MS analysis.

Freshly isolated human leukocytes stimulated with LPS and A23187 showed formation of 5-OH-$PGF_{2\alpha}$ along with 5,8,9,11,12,15-hexahydroxy-eicosadienoic acid **13**, the reduction product of HKE_2, HKD_2, and their diendoperoxide precursor, respectively (Fig. 5). 5-OH-PGE_2 and 5-OH-PGD_2 could not be detected, even when samples were not treated with $NaBH_4$ (Fig. 5). The absence of 5-OH-$PGF_{2\beta}$ indicated that the leukocyte mixtures preferentially formed 5-OH-PGD_2 over 5-OH-PGE_2 or that 5-OH-PGH_2 was the most abundant product at the time when $NaBH_4$ was added. As expected, HKE_2 and HKD_2 were absent or markedly decreased after $NaBH_4$ reduction.

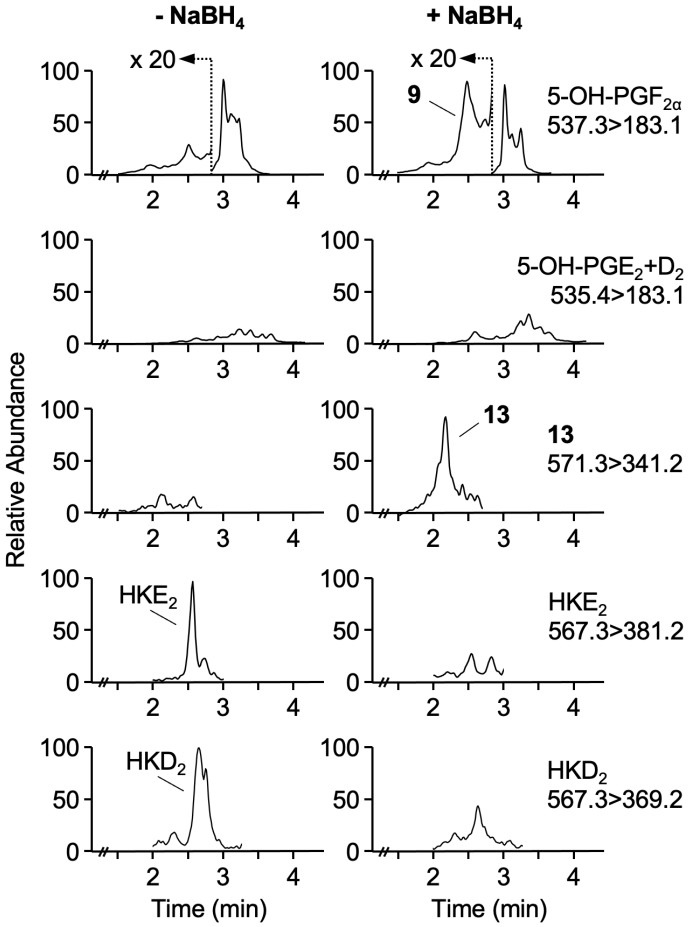


Figure 5. Formation of 5-OH-PGs by activated human leukocytes. Cells were treated with LPS for 45 min and then with A23187 for 15 min, followed by reduction or not with $NaBH_4$, extracted, derivatized with AMPP, and analyzed using LC-MS in the positive ion SRM mode. Ion traces for the detection of 5-OH-$PGF_{2\alpha}$, 5-OH-PGD_2 and 5-OH-PGE_2, hexahydroxy-eicosadienoic acid **13**, HKE_2, and HKD_2 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-PGE_2 and 5-OH-PGD_2 starting from PGE_2 and PGD_2, 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-PGE_2 nor 5-OH-PGD_2 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-PGH_2 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 12*E* double bond determines the outcome of the enzymatic reaction, i.e., formation of the diendoperoxide versus 5-OH-PGH_2 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 O_2 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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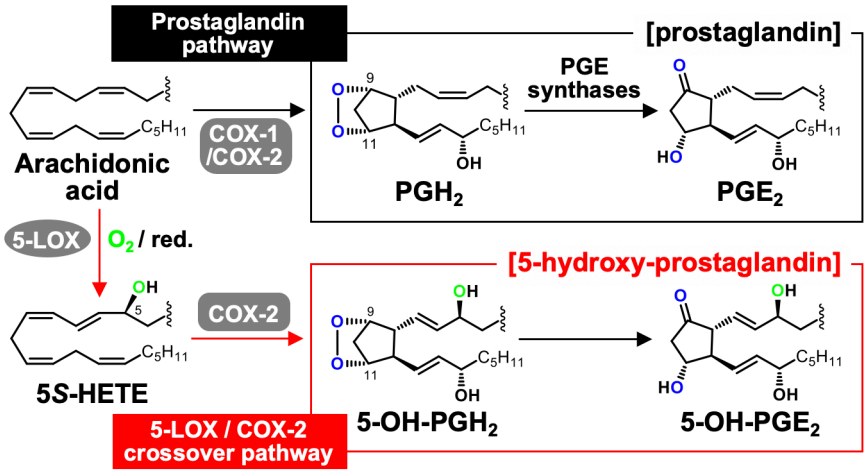
TOC Graphic



Synopsis

New prostaglandin analogues are formed in the reaction of cyclooxygenase-2 with 5-HETE, a 5-lipoxygenase metabolite of arachidonic acid, and identified in activated human leukocytes.