Identification and absolute configuration of dihydroxy-arachidonic acids formed by oxygenation of 5S-HETE by native and aspirin-acetylated COX-2

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Abstract Biosynthesis of the prostaglandin endoperoxide by the cyclooxygenase (COX) enzymes is accompanied by formation of a small amount of 11R-hydroxyeicosatetraenoic acid (HETE), 15R-HETE, and 15S-HETE as by-products. Acetylation of COX-2 by aspirin abrogates prostaglandin synthesis and triggers formation of 15R-HETE as the sole product of oxygenation of arachidonic acid. Here, we investigated the formation of by-products of the transformation of 5S-HETE by native COX-2 and acetylsalicylic acid (aspirin)-acetylated COX-2 using HPLC-ultraviolet, GC-MS, and LC-MS analysis. 5S,15S- dihydroxy (di)HETE, 5S,15R-diHETE, and 5S,11R-diHETE were identified as by-products of native COX-2, in addition to the previously described di-endoperoxide (5S,15S-dihydroxy-9S,11R,8S,12S-diperoxy-6E,13E-eicosadienoic acid) as the major oxygenation product. 5S,15R-diHETE was the only product formed by aspirin-acetylated COX-2. Both 5,15-diHETE and 5,11-diHETE were detected in CT26 mouse colon carcinoma cells as well as in lipopolysaccharide-activated RAW264.7 cells incubated with 5S-HETE, and their formation was attenuated in the presence of the COX-2 specific inhibitor, NS-398. Aspirin-treated CT26 cells gave 5,15-diHETE as the most prominent product formed from 5S-HETE. 5S,15S-diHETE has been described as a product of the cross-over of 5-lipoxygenase (5-LOX) and 15-LOX activities in elicited rat mononuclear cells and human leukocytes, and our studies implicate crossover of the 5-LOX and COX-2 pathways as an additional biosynthetic route.—Mulugeta, S., T. Suzuki, N. T. Hernandez, M. Griesser, W. E. Boeglin, and C. Schneider. Identification and absolute configuration of dihydroxy-arachidonic acids formed by oxygenation of 5S-HETE by native and aspirin-acetylated COX-2. J. Lipid Res. 2010. 51: 575–585.

Supplementary key words cyclooxygenase-2 • hydroxy-eicosatetraenoic acid • lipoxygenase • macrophage • circular dichroism

Oxygenation of arachidonic acid by either of the two cyclooxygenase (COX) isozymes yields the prostaglandin endoperoxide PGH₂ as the major product and the monohydroxylated 11-hydroxyeicosatetraenoic acid (HETE) and 15-HETE as by-products of about 2–5% abundance (1). 11-HETE is exclusively of the 11R configuration, similar in configuration to the first oxygenation of arachidonic acid to the 11R-peroxyl radical that will form the 9,11-endoperoxide of PGH₂, (2, 3). 15-HETE is formed as a mixture of the 15S- and 15R-enantiomers, in contrast to the configuration of C₁₅ in PGH₂, which is strictly S (4). The HETE by-products are thought to arise from a slightly different alignment of substrate in the active site compared with when PGH₂ is formed (5), rather than resulting from incomplete or otherwise faulty catalysis. Neither 15-hydroperoxy-eicosatetraenoic acid (HPETE) nor 11-HPETE can serve as substrates for formation of PGH₂ in the cyclooxygenase reaction as demonstrated for the COX-1 enzyme (6, 7). It has not been established whether formation of the HETE by-products follows a particular biological rationale.

Acetylation by aspirin (acetylsalicylic acid) of a serine residue in the oxygenase active site channel of both COX isozymes has discrete effects on the catalytic activities of the two enzymes (8, 9). Whereas COX-1 loses all oxygenase

Abbreviations: CD, circular dichroism; CE, Cotton effect; CID, collision-induced dissociation; COX, cyclooxygenase; diHETE, dihydroxy-eicosatetraenoic acid; HETE, hydroxy-eicosatetraenoic acid; HPETE, hydroperoxy-eicosatetraenoic acid; 5-LOX, 5-lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; OTMS, O-trimethylsilyl; PG, prostaglandin; SRM, selected reaction monitoring; TPP, triphenylphosphine.

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activity following treatment with aspirin, acetylated COX-2 gains a novel catalytic activity and forms 15R-HETE as the sole product (10, 11).

The major product formed by oxygenation of the 5-lipoxygenase product, 5S-HETE, with COX-2 is a bicyclic di-endoperoxide with structural similarities to the arachidonic acid derived PGH$_2$ (12). The most significant difference between the two endoperoxides is that the typical cyclopentenyl ring of PGH$_2$, comprised of carbons 8 through 12, is extended to a seven-membered ring by insertion of a peroxide bridge from C8 to C12 in the 5-HETE derived di-endoperoxide. In addition, the ring by insertion of a peroxide bridge from C8 to C12 in 8 through 12, is extended to a seven-membered

Here, we report the structural identification and absolute configuration of two by-products of the COX-2 reaction with 5S-HETE. In addition, we analyzed the reaction of acetylated recombinant COX-2 with 5S-HETE. Finally, formation of diHETEs from exogenous 5S-HETE was confirmed to be dependent on COX-2 in two mouse cell lines, RAW264.7 and CT26.

EXPERIMENTAL PROCEDURES

Materials

Arachidonic acid was purchased from NuChek Prep, Inc. (Elysian, MN), lipopolysaccharide (LPS) (serotype 0111:B4) was from Calbiochem, and RAW264.7 and CT26 cells were obtained from ATCC (Manassas, VA). 5S-HETE was prepared by chemical synthesis from arachidonic acid as described (13). 15S-HETE and 11R-HETE were prepared through vitamin E-controlled autoxidation of arachidonic acid methyl ester and purified by consecutive RP-, straight phase-, and chiral phase HPLC [Chiralpak AD (14)], and a final step of mild hydrolysis of the methyl ester using KOH.

Cell culture

RAW264.7 cells were cultured in DMEM and grown at 37°C in an atmosphere of 5% CO$_2$. Cells of passages 5 and 6 only were used. Cells were stimulated by treatment with 100 ng/mL LPS and 10 units/mL of IFN-γ for 6 h to induce expression of COX-2. CT26 cells were cultured in RPMI 1640 medium. 5S-HETE, 5 µg dissolved in 1 µl of ethanol, was added to ~70% confluent cells in 100 mm dishes, and after 10 min at 37°C, the culture medium was removed, acidified to pH 4, and extracted using a 30 µg of triphenylphosphine (TPP) for 15 min at room temperature. 5S,15S-DiHETE and 5R,15S-diHETE were isolated by RP-HPLC using a Waters Symmetry C18 column (4.6 × 250 mm) eluted with a solvent of methanol/water/acetic acid (80/20/0.01, by vol) at 1 ml/min flow rate and UV detection at 235 nm. 5S,15R-DiHETE was synthesized by reaction of 15R-HETE with recombinant human 5-LOX. For the enzymatic transformation, a pellet of 5/9 insect cells expressing 5-LOX (~500 µl) was sonicated and transferred to 1 ml of PBS containing 2 mM CaCl$_2$ and 1 mM ATP. 15R-HETE (50 µg) was added, and the reaction was allowed to proceed for 15 min at room temperature. After 3 min reaction time, the solution was acidified to pH 4 and extracted with methylene chloride. The organic extract was evaporated, dissolved in methanol, and treated with 200 µg of triphenylphosphine (TPP) for 15 min at room temperature. 5S,15S-DiHETE and 5R,15S-diHETE were isolated by RP-HPLC using a Waters Symmetry C18 column (4.6 × 250 mm) eluted with a solvent of methanol/water/acetic acid (80/20/0.01, by vol) at 1 ml/min flow rate and UV detection at 235 nm.

Reaction of recombinant COX-2 with 5S-HETE

The reaction of 5S-HETE (120 µg total; containing 300,000 cpm of [1-14C]5S-HETE) with recombinant human COX-2 was performed in four separate 2 ml reactions with 30 µg substrate each as described (12). The products were extracted using a Waters HLB cartridge and analyzed by RP-HPLC using a Waters Symmetry C18 5-µm column (4.6 × 250 mm) eluted with a gradient of acetonitrile/water/acetic acid programmed from 20/80/0.01 (by vol) to 70/30/0.01 (by vol) within 20 min at 1 ml/min flow rate. The elution profile was monitored using an Agilent 1200 diode array detector coupled on-line to a Packard Radiomatic A100 Flo-one radioactive detector. The by-products eluting at 19.8 and 20.5 min retention time were collected, extracted from HPLC solvent, and stored in methanol at −20°C until further analysis.

Reaction of aspirin-acetylated COX-2 with 5S-HETE

Recombinant human COX-2 (0.5 µM final concentration) was diluted in 1 ml of 100 mM Tris-HCl buffer pH 8.0 and treated with 2 mM aspirin in a 37°C water bath for 30 min (15). A control reaction incubated with arachidonic acid and analyzed by LC-MS before and after treatment showed >95% inhibition of PG formation. The buffer was supplemented with hematin (1 µM) and phenol (500 µM), and 30 µg of 5S-HETE were added. After 5 min at room temperature, 25 µl of methanol were added, the mixture was acidified to pH 4 with glacial acetic acid, and loaded onto a preconditioned Waters Oasis HLB cartridge. After washing with water, the products were eluted with methanol. The 5,15-diHETE product was isolated using RP-HPLC as described above for the native enzyme.
5,11-R-diHETE was isolated using RP-HPLC conditions as described above for 5,15-S-diHETE. HPLC-purified 5,11-R-diHETE was dissolved in CDCl3 for NMR analysis using a Bruker AV-II 600 MHz spectrometer equipped with a cryoprobe. Chemical shifts are reported relative to the signal for residual CHCl3 at δ 7.25 ppm.

A mixture of 5,11-diHETE diastereomers was synthesized by autoxidation of racemic 11-HETE. Three 200 μg aliquots of 11-HETE were evaporated in small plastic tubes and placed in an oven at 37°C. After 2 h, the samples were dissolved in 50 μl of methanol, treated with triphenylphosphine (TPP), and isolated using RP-HPLC. The diastereomers eluted as a single peak and purification was performed as described for 5,15-S-diHETE.

### SP-HPLC analysis of diHETEs

The 5,15-diHETE diastereomers were resolved using an Agilent Zorbax RX-SIL 5-μm column (4.6 x 250 mm) eluted with hexane/isopropanol/acetic acid (95/5/0.1, by vol) at 1 ml/min flow rate. The 5,11-diHETEs were analyzed using the same HPLC conditions after conversion to the methyl ester derivatives with diazomethane. Eluting peaks were monitored using an Agilent 1200 series diode array detector.

### CD spectroscopy

Aliquots of ~20 μg each of 5-S-HETE, 15-S-HETE, 15-R-HETE, 11-S-HETE, 11-R-HETE, and the enzymatically synthesized standards of 5,15-diHETE, 5R,15S-diHETE, and 5S,11R-diHETE were treated with ethereal diazomethane for 30 s, evaporated, and dissolved in 50 μl of methanol, treated with triphenylphosphine (TPP), and isolated using RP-HPLC. The diastereomers eluted as a single peak and purification was performed as described for 5,15-S-diHETE.

### GC-MS and LC-MS analysis

For GC-MS analysis, 5,15-diHETE and 5,11-diHETE formed by reaction of COX-2 with 5,15-HETE were purified using RP- and SP-HPLC and methylated using ethereal diazomethane. Hydrogenation was performed in 100 μl of ethanol in the presence of palladium/carbon and bubbling with hydrogen gas for 5 min. Trimethylsilyl ethers were prepared using bis(trimethylsilyl)trifluoroacetamidate at room temperature for 1 h. The reagents were evaporated and the samples were dissolved in hexane. GC-MS analysis was carried out in the EI mode (70 eV) using a ThermoFinnigan DSQ mass spectrometer equipped with a 5 m SPB-1 column (0.1 mm i.d., film thickness 0.25 μm) and a temperature program from 100°C, hold 2 min, then increased to 260°C at 20°C/min.

LC-MS was performed using a ThermoFinnigan Quantum Access instrument equipped with an electrospray interface and operated in the negative ion mode. User modified parameters of sheath and auxiliary gas pressures, temperature, and voltage settings were optimized using direct infusion of a solution of PGD2. A Waters Symmetry Shield C18 3.5 μm-column (2 × 150 mm) was eluted with a linear gradient of acetonitrile/water, 10 mM NH4OAc (5/95, by vol; solvent A) to acetonitrile/water, 10 mM NH4OAc (95/5, by vol) at a flow rate of 0.2 ml/min within 10 min. Negative ion collision-induced dissociation (CID) mass spectra of the standards of PGD2, 5S-HETE, 5,15-S-diHETE, and 5,11-R-diHETE were obtained. The fragmentation patterns were used to establish ion transitions for analyses in the selected reaction monitoring (SRM) mode. The following transitions were monitored: for PGD2 and PGE2: m/z 351 → 271; 5-HETE: m/z 319 → 115; 5,15-diHETE: m/z 335 → 201; and 5,11-diHETE: m/z 335 → 183. Relative levels of prostaglandins and diHETEs between treatments were calculated using peak areas of the signals in the SRM chromatograms.

### RESULTS

#### Reaction of native and acetylated COX-2 with 5-S-HETE

RP-HPLC analysis of the transformation of [1-14C]5-SHETE by recombinant COX-2 shows one main product that was identified previously as a highly oxygenated diendoperoxide (12), in addition to two minor, less polar peaks designated I and II representing by-products of the reaction (Fig. 1A). When COX-2 was treated with aspirin prior to incubation with 5-SHETE, one major product (III) was formed with retention time similar to peak I in the untreated enzyme (Fig. 1B). Both I and III had a characteristic UV spectrum with a λmax at 243 nm that was readily identified as 5,15-diHETE (Fig. 1C) (18). The UV spectrum of peak II had a maximum at 238 nm with shoulders around 228 nm and 247 nm (Fig. 1C). The retention time and UV spectrum of II implicated that the product also contained two hydroxy groups and conjugated diene moieties.

The products I and II were isolated using RP-HPLC and further purified as the methyl ester derivatives using SP-HPLC. GC-MS analysis in the EI mode (70 eV) of the hydrogenated, TMSether derivatives confirmed the identification of the first peak I as 5,15-diHETE. Characteristic α-cleavage fragments were found at m/z 203 (55% relative intensity) and m/z 311 [after loss of O-trimethylsilyl (OTMS); 9%] for the 5-hydroxy, and at m/z 173 and 341 (after loss of OTMS) (56% and 7%, respectively) for the 15-hydroxy group; the base peak was at m/z 73. Peak III from the aspirin-acetylated COX-2 reaction was identified as 5,15-diHETE based on identical UV spectra and retention times on RP-HPLC, and in addition to subsequent experimental evidence as described below.

Product II gave a very weak [M']+ (m/z 502) and [M-CH3]+ (m/z 487) ion, with characteristic α-cleavage fragments at m/z 203 (42%) and m/z 311 (after loss of OTMS; 4%) indicating a 5-hydroxy group, and at m/z 229 (38% relative intensity) and m/z 285 (after loss of OTMS) (5%) indicative of a 11-hydroxy group. The LC-ESI mass spectrum confirmed the molecular weight as 336 and also gave a major fragment at m/z 183 and a minor fragment at m/z 115, compatible with two hydroxyls at carbons 5 and 11.
Based on UV, GC-MS, and LC-MS analyses, product II was identified as 5,11-diHETE.

$^1$H NMR and H,H COSY data for product II were recorded using a chromatographically and spectroscopically (UV, LC-MS/MS) identical standard of 5S,11R-diHETE that was prepared as described below. The $^1$H NMR spectrum showed eight signals in the double bond region that appeared as a pair of two similar motives of four protons each comprised of the two conjugated cis,trans-dienes (H7: $\delta$ 6.57 ppm, dd, $J = 15.1$ Hz/11.0 Hz; H8: $\delta$ 6.13, dd, $J = 11.0$ Hz; H6: $\delta$ 5.70, dd, $J = 14.9$ Hz/6.3 Hz; H9: $\delta$ 5.55, m; and H13: $\delta$ 6.51, dd, $J = 14.9$ Hz/11.4 Hz; H14: $\delta$ 5.96, dd, $J = 11.0$ Hz; H12: $\delta$ 5.67, m; H15: $\delta$ 5.46, m). Two protons attached to carbons bearing a hydroxyl group were located at 4.25 ppm (H11: $\delta$ 4.25, dt, $J = 6.3$ Hz/6.1 Hz) and 4.17 ppm (H5: $\delta$ 4.17, dt, $J = 6.2$ Hz/6.0Hz). H4 was detected as a cross-peak from H5 in the H,H-COSY spectrum at 1.57 ppm, H3 was a multiplet (1.70 ppm) and was coupled to the triplet signal of H2 at 2.34 ppm ($J = 7.4$ Hz). Both protons of H10 were detected as a multiplet at 2.47 ppm, and H16 was a dt signal at 2.17 ppm ($J = 7.6$ Hz/7.2 Hz).

The configuration of C-15 in the 5,15-diHETE products (I and III) and of C-11 in the 5,11-diHETE (II) was established by coelution with corresponding diHETE diastereomers of known configuration. The configuration of the 5-hydroxy group in all diHETE products was expected to be unchanged from the starting substrate, 5S-HETE.

Synthesis of standards of diastereomeric diHETEs

Table 1 gives an overview of the diHETE standards prepared as reference compounds. Authentic 5S,15S-diHETE was prepared by reaction of soybean LOX-1 with 5S-HETE. Synthesis of 5S,15R-diHETE by reaction of 15R-HETE with the recombinant human 5-LOX gave only a minor yield of product, albeit it was sufficient to determine the retention times on RP- and SP-HPLC. In addition, the enantiomer 5R,15S-diHETE was prepared by reaction of 5R-HETE with the lipoxygenase from soybean seeds. 5S,15R-diHETE and 5R,15S-diHETE have indistinguishable retention times on RP- and SP-HPLC.

An authentic standard of 5S,11R-diHETE was prepared by reaction of 5S-HETE with the recombinant 9R-LOX from Anabaena sp PCC7120. A mixture of the 5,11-diHETE diastereomers was prepared by thin-film autodissociation of racemic 11-HETE. Initial attempts to prepare 5S,11S- and 5S,11R-diHETEs by reaction of 11S-HETE and 11R-HETE, respectively, with the recombinant human 5-LOX did not yield a significant amount of either 5,11-diHETE diastereomer. The assignment of the absolute configuration of the hydroxy groups in the diHETE standards was confirmed using CD spectroscopy (see below).
TABLE 1. Overview of the standards of 5,15-diHETEs and 5,11-diHETEs; their method of synthesis and HPLC retention times

| diHETE | Method of Preparation | Retention Time (min) |
|--------|-----------------------|----------------------|
| 5S,15S (I) | 5S-HETE + soybean LOX | 6.8 12.3 |
| 5S,15R (II, III) | 15R-HETE + hum. 5-LOX | 6.8 12.8 |
| 5R,15S (a) | 5R-HETE + soybean LOX | 6.8 12.8 |
| 5S,11R (II) | 5S-HETE + Anabaena LOX | 7.3 18.3 |
| 5R,11S (a) | 11R-SHETE autoxidation | 7.3 18.3 |
| 5R,11S (a) | 11R-SHETE autoxidation | 7.3 18.3 |
| 5R,11R (a) | 11R-SHETE autoxidation | 7.3 18.3 |

- The roman numerals in parentheses refer to the numbering of the peaks in Fig. 1A, B.
- a-c. diHETEs with the same superscript letter are enantiomers.
- This reaction gave a very low yield.
- Although 5R-HETE was readily converted by the Anabaena LOX formation of 5R,11R-diHETE was not observed.
- Waters Symmetry C18 column (250 x 4.6 mm) eluted with methanol/water/acetic acid 80/20/0.01 at 1 ml/min flow rate.
- Agilent Zorbax RX-SIL column (250 x 4.6 mm) eluted with hexane/isopropanol/acetic acid 95/5/0.1 at 1 ml/min flow rate. Retention times for the 5,11-diHETEs are for the methyl ester derivatives.

Absolute configuration of 5,15-diHETEs from native andacyetylated COX-2

The diastereomers of 5,15-diHETE do not resolve on RP-HPLC (18), but there is adequate separation on SP-HPLC to allow for secure assignment of the absolute configuration at C-15. Therefore, the 5,15-diHETEs were first isolated as a single peak using RP-HPLC and then resolved using SP-HPLC (Fig. 2). The 5,15-diHETE (peak I) isolated from the reaction of human COX-2 gave a %-ratio for 5S,15S-diHETE to 5S,15R-diHETE of 77:33, 80:20, and 75:25 in three separate experiments (Fig. 2A). The authentic standards of 5S,15S-diHETE and 5S,15R-diHETE eluted at 12.3 min and 12.8 min retention times, respectively (Fig. 2B, 2C). Peak identification was further confirmed by cochromatography with the authentic standards (Fig. 2D). Aspirin-treatment of human COX-2 resulted in a shift of the chiral distribution of 5,15-diHETE, and now the product (peak III) was 95% 5S,15R-diHETE (Fig. 3).

Absolute configuration of 5,11-diHETEs

Standards for the 5S,11R and 5S,11S-diHETE diastereomers were prepared by thin-film autoxidation of racemic 11-HETE followed by reduction with triphenylphosphine. 5,11-DiHETE was the almost exclusive diHETE formed, and the diastereomers eluted as a single peak when analyzed by RP-HPLC. Using SP-HPLC, satisfactory resolution of the methyl ester derivatives was achieved (Fig. 4). The first peak comprised of the 5S,11S and 5R,11R-diastereomers eluted at 17.8 min and the second peak (5S,11R and 5R,11S-diastereomers) eluted at 18.3 min. The authentic standard of 5S,11R-diHETE prepared using the Anabaena LOX coeluted with the second peak on SP-HPLC and established the elution order. SP-HPLC analysis of 5,11-diHETE from recombinant human COX-2 showed that the configuration was >98% 5S,11R-diHETE.

CD-spectroscopy of diHETE standards

We used the exciton-coupled circular dichroism method in order to confirm assignment of the absolute configuration of the hydroxy groups in the diHETE standards. This method uses the coupling of two chromophores attached to the chiral center in circular polarized light in order to determine the absolute configuration from the sign of the.

DiHETEs from cross-over of the 5-LOX and COX-2 pathways 579
The configuration of C11 in 5S,11R-diHETE formed by reaction of the *Anabaena* 9-LOX with 5S-HETE was confirmed using the same approach. The individual CD spectra of 2-naphthoate-derivatized 5S-HETE and 11R-HETE are mirror images of each other, but unexpectedly, the CD spectrum of 5S,11R-diHETE was not a flat line (Fig. 6). The spectrum showed CEs at 245 nm (Δε +7.6) and 228
DiHETEs from cross-over of the 5-LOX and COX-2 pathways can be taken as a measure for the alignment of the chromophores within the conformer, however, gave essentially equivalent values, i.e., 6.7 Hz and 6.8 Hz, respectively. Because SP-HPLC has confirmed the relative configuration of the 5\textsuperscript{S},11\textsuperscript{R}-diHETE (Fig. 4D), the question why the corresponding CD spectrum showed slight predominance of the \text{S}-configured chiral center remains unexplained.

Formation of diHETEs in RAW264.7 and CT26 cells

RAW264.7 were treated in four different ways and incubated with 4 \mu M 5\textsuperscript{S}-HETE. We used nonstimulated cells, cells stimulated with LPS only, and LPS-stimulated cells treated with NS-398 or aspirin, respectively. Formation of diHETEs was analyzed using negative ion LC-ESI-MS in the SRM mode (Fig. 7A). Both 5,15-diHETE and 5,11-diHETE were detected in RAW264.7 cells activated with LPS and IFN-\gamma (Fig. 7A, upper panel), and their concentration was reduced to 0.5\% and 3\%, respectively, by incubation...
respectively (Fig. 7A, lower panel). Levels of PGD$_2$ and PGE$_2$ were reduced to 4% and 30% by NS-398 and aspirin, respectively. PGD$_2$ and PGE$_2$ are not completely absent in the inhibitor treated cells because a fraction was formed of the cells with the COX-2 inhibitor NS-398 (10 μM) prior to the addition of 5SHETE (Fig. 7A, middle panel). Treatment of RAW264.7 cells with 2 mM aspirin led to about 60% and 50% reduction in 5,15-diHETE and 5,11-diHETE, respectively (Fig. 7A, lower panel). Levels of PGD$_2$ and PGE$_2$ were reduced to 4% and 30% by NS-398 and aspirin, respectively. PGD$_2$ and PGE$_2$ are not completely absent in the inhibitor treated cells because a fraction was formed.
from (endogenous) arachidonic substrate already before addition of the drugs. Furthermore, aspirin showed only modest efficacy in reducing eicosanoid formation, consistent with previous findings that a high cellular redox state in activated RAW264.7 cells impedes aspirin’s ability to covalently modify COX enzymes (20).

Formation of diHETEs was also analyzed in CT26 mouse colon carcinoma cells incubated with 5SHETE. CT26 cells incubated with 5SHETE showed robust formation of 5,15-diHETE and 5,11-diHETE, and the levels of both were reduced >100-fold by preincubation with 10 μM NS398 (Fig. 7B). Pretreatment with 2 mM aspirin inhibited the formation of 5,11-diHETE by about 90%, and 5,15-diHETE was reduced to only 25% compared with the cells not treated with aspirin, reflecting enhanced formation of 5S,15R-diHETE.

**DISCUSSION**

After the initial oxygenation of arachidonic acid to form a conjugated diene hydro(pero)xide (H(P)ETE), additional sites remain in the molecule for subsequent reaction with molecular oxygen (21). This opens the possibility for formation of di-hydroxylated (diHETEs) and tri-hydroxylated derivatives of arachidonic acid. Enzymatic synthesis of diHETEs can be catalyzed via several distinct routes, all of which involve one or more LOX reactions: i) The consecutive reaction of two separate LOX enzymes is involved in the biosynthesis of 5S,15S-diHETE in elicited rat mononuclear cells and human leukocytes (18). In this case, arachidonic acid is first oxygenated by 5-LOX followed by 15-LOX or vice versa. ii) An alternative route to 5S,15S-diHETE is through double oxygenation of arachidonic acid catalyzed by a single LOX enzyme. This possibility is best recognized for the LOX-1 isozyme from soybean seeds in the formation of 5S,15S-diHETE in elicited rat mononuclear cells and human leukocytes (20). ii) An alternative route to 5S,15S-diHETE is through double oxygenation of arachidonic acid catalyzed by a single LOX enzyme. This possibility is best recognized for the LOX-1 isozyme from soybean seeds in the formation of 5S,15S-diHETE in elicited rat mononuclear cells and human leukocytes (18). In this case, arachidonic acid is first oxygenated by 5-LOX followed by 15-LOX or vice versa. In this case, arachidonic acid is first oxygenated by 5-LOX followed by 15-LOX or vice versa. iii) The third possibility is exemplified by the biosynthesis of leukotriene (LT)B4 and 12-epi-LTB4 (i.e., 5S,12R-diHETE and 5S,12S-diHETE, respectively). In this case, the diHETEs are hydrolysis products of the unstable LTA4 epoxide. The LTA4 epoxide is formed by 5-LOX catalyzing a second hydrogen abstraction (at C-10) of its initial 5SHETE product. But rather than inserting a second molecule of oxygen the reaction is completed by dehydration of the existing hydroperoxide to give the epoxide (24, 25). iv) Finally, a fourth distinct route to diHETEs is implicated by the findings presented in this report. This route involves a cross-over of the activities of the 5-LOX and COX-2 enzymes, with the 5-LOX product 5SHETE being oxygenated by COX-2 to form, as by-products, a mixture of 5S,15S-diHETE, 5S,15R-diHETE, and 5S,11R-diHETE.

The formation of 5,15-diHETE and 5,11-diHETE as by-products of the COX-2 catalyzed transformation of 5SHETE bears strong resemblance to the formation of 15-HETE and 11-HETE as by-products of the COX-catalyzed transformation of arachidonic acid to PGH2 (Fig. 8) (5, 26). In contrast to the reaction with arachidonic acid, 5SHETE reacts only with COX-2; the COX-1 isozyme is inactive with 5SHETE (12). The configuration of C-15 of 5,15-diHETE was a ∼3:5:1 mixture of 15S and 15R, and a similar mixture of 15S and 15R configuration is found in the 15-HETE formed by COX-1 and COX-2 (4). The configuration of C-11 in 5,11-diHETE is >98% 11R, identical to the near exclusive 11R-configuration of 11-HETE formed by COX-1 and COX-2 (2, 3, 27). We can conclude that the modes of binding of arachidonic acid and of 5SHETE in the cyclooxygenase active site must be very similar. There is precise control over the C-11 and C-15 oxygenations in the formation of the prostaglandin endoperoxide and the di-endoperoxide as well as in the formation of the 11R-HETE and 5S,11R-diHETE by-products, respectively (28). There is less control of the oxygen insertion and/or less tight binding of the α-tail of the fatty acid substrate in the case of the formation of the 15-HETE or 5,15-diHETE by-products. The major difference in catalytic outcome with 5SHETE, however, is the insertion of another molecule of oxygen in place of the C8-C12 carbon bond resulting in the formation of two endoperoxide rings.
Acetylation of Ser-516 in the COX-2 active site by aspirin has a remarkable effect on its catalytic activity (10, 11). Formation of the prostaglandin endoperoxide is prevented, and instead, a novel catalytic activity is gained, forming 15R-HETE as the sole enzymatic product. The basis for the complete inversion of the stereochemistry of C15 from 15S in PGH₂ to 15R in 15R-HETE has not been definitely elucidated but it likely involves a change in the binding of the ω-end of arachidonic acid beyond C-13 in the channel above Ser-516 (28–32). When 5S-HETE was incubated with acetylated COX-2, formation of the di-endoperoxide was inhibited and 5,15-diHETE was the only product detected. Not too surprisingly, the configuration of C-15 of the 5,15-diHETE was found to be >95% 15R.

The formation of diHETEs was analyzed using the RAW264.7 mouse macrophage cells and CT26 mouse colon carcinoma cells. Neither cell type produced detectable amounts of 5S-HETE, and we were unable to detect 5-LOX protein by Western blotting (data not shown). 5,15-DiHETE and 5,11-diHETE were detected in both cell types upon incubation with exogenous 5S-HETE. Formation of the diHETEs was dependent on COX-2 because they were absent in nonstimulated cells and in cells treated with the COX-2 inhibitor NS-398. Treatment of CT26 cells with aspirin prior to incubation with 5S-HETE enhanced the biosynthesis of 5,15-diHETE, consistent with the findings using recombinant COX-2 enzyme. Aspirin, even at a high concentration, did not show this effect in RAW264.7 cells, most likely due to lesser efficacy for covalent modification of the COX enzyme in cells with a highly oxidative tone (20, 33). 5S,11R-diHETE and 5S,15R-diHETE have not been described as metabolites of arachidonic acid before, although there are two reports published more than 20 years ago that implicate the possibility of COX-dependent biosynthesis of diHETEs in human umbilical arteries (34, 35). Unfortunately, the presumed diHETE metabolites were left uncharacterized, and it is difficult to estimate whether the products described could be similar or identical to 5,13-diHETE or 5,11-diHETE.

5S,15S-diHETE as well as 8S,15S-diHETE and 15S-HETE were reported to enhance the degranulation of human neutrophils elicited by platelet-activating factor, whereas they had no such effect when the neutrophils were stimulated with the tripeptide formyl-met-leu-phe, phorbol ester, LTB₄, or calcium ionophore (36). 5S,15S-diHETE and 8S,15S-diHETE were also identified as eosinophil-derivered eosinophil chemotactic lipids, invoking their participation in a self-sustaining mechanism of eosinophil accumulation (37). An additional, more potent eosinophil chemotactic eicosanoid was noted at the time and later identified as 5-oxo-15-hydroxy-eicosatetraenoic acid, an oxidation product of 5,15-diHETE (38).

Our studies invoke the possibility of a previously unrecognized biosynthetic route to 5S,15S-diHETE, and therefore, additional experiments are required to distinguish whether, if detected in vivo, 5,15-diHETE is formed by cross-over of the 5-LOX and 15-LOX pathways, or by cross-over of the 5-LOX and COX-2 pathways. Involve-ment of COX-2 can be implicated if the formation of 5,15-diHETE is attenuated upon application of a COX-2 specific inhibitor. Alternatively, a minor amount of 5,11-diHETE, in addition to 5,15-diHETE, could be indicative of COX-2 involvement. Formation of 5S,15R-diHETE as an alternative specific marker of COX-2 involvement is difficult to establish because the 5S,15S- and 5S,15R-diastereomers do not resolve using standard RP-HPLC conditions.

Formation of 5S,15R-diHETE is somewhat reminiscent of formation of the so-called aspirin-triggered lipoxins (39). Both products involve cross-over of the activities of aspirin-acetylated COX-2 and 5-LOX. Aspirin-triggered lipoxins are formed by the reaction of 15R-HETE (the metabolite of acetylated COX-2) with the 5-LOX enzyme initially forming the 5S-hydroperoxide of 15R-HETE followed by dehydration to the 5S,6S-epoxy-15R-hydroxy derivative, analogous to the biosynthesis of the leukotriene epoxide LT₄. Hydrolysis of the epoxy-tetraene at carbons 6 or 14 affords 5S,6R,15R-trihydroxy-eicosatetraenoic acid (15-epi-lipoxin A₄) and 5S,14R,15R-trihydroxy-eicosatetraenoic acid (15-epi-lipoxin B₄), respectively (40, 41). In contrast, the enzymatic activities are coupled "the other way round" for formation of 5S,15R-diHETE, i.e., 5-LOX first produces 5S-HETE, which is subsequently converted by acetylated COX-2 to 5S,15R-diHETE.

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