Identification and absolute configuration of dihydroxy-arachidonic acids formed by oxygenation of 5*S*-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 11*R*-hydroxyeicosatetraenoic acid (HETE), 15*R*-HETE, and 15*S*-HETE as by-products. Acetylation of COX-2 by aspirin abrogates prostaglandin synthesis and triggers formation of 15*R*-HETE as the sole product of oxygenation of arachidonic acid. Here, we investigated the formation of by-products of the transformation of 5*S*-HETE by native COX-2 and by aspirin-acetylated COX-2 using HPLC-ultraviolet, GC-MS, and LC-MS analysis. 5*S*,15*S*- dihydroxy (di)HETE, 5*S*,15*R*-diHETE, and 5*S*,11*R*-diHETE were identified as by-products of native COX-2, in addition to the previously described di-endoperoxide (5*S*,15*S*-dihydroxy-9*S*,11*R*,8*S*,12*S*-diperoxy-6*E*,13*E*-eicosadienoic acid) as the major oxygenation product. 5*S*,15*R*-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 5*S*-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 5*S*-HETE. 5*S*,15*S*-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 cross-over 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.**

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Oxygenation of arachidonic acid by either of the two cyclooxygenase (COX) isozymes yields the prostaglandin endoperoxide PGH_2 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 11*R* configuration, similar in configuration to the first oxygenation of arachidonic acid to the 11*R*-peroxyl radical that will form the 9,11-endoperoxide of PGH_2 (2, 3). 15-HETE is formed as a mixture of the 15*S*- and 15*R*-enantiomers, in contrast to the configuration of C15 in PGH_2, 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 PGH_2 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 PGH_2 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

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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 15*R*-HETE as the sole product (10, 11).

The major product formed by oxygenation of the 5-lipoxygenase product, 5*S*-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 cyclopentyl 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 di-endoperoxide contains two hydroxy groups, one at carbon 5 stemming from the 5*S*-HETE substrate and the other at C15, equivalent to the 15-hydroxy in the prostaglandins. The relative and absolute stereochemistries of carbons 9, 11, and 15 are the same in PGH_2 and the di-endoperoxide, i.e., 9*S*, 11*R*, and 15*S* (13).

Here, we report the structural identification and absolute configuration of two by-products of the COX-2 reaction with 5*S*-HETE. In addition, we analyzed the reaction of acetylated recombinant COX-2 with 5*S*-HETE. Finally, formation of diHETEs from exogenous 5*S*-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). 5*S*-HETE was prepared by chemical synthesis from arachidonic acid as described (13). 15*R*-HETE and 11*R*-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. 5*S*-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 mg Waters HLB cartridge. Products were eluted from the cartridge with methanol, evaporated, and dissolved in 50 μl of LC-MS solvent A. CT26 and RAW264.7 control cells were not treated with LPS. In some experiments, CT26 and activated RAW264.7 cells were treated with 2 mM aspirin, respectively (from a 40 mM stock solution in DMSO), or with 10 μM NS-398 (from a 10 mM stock solution in ethanol) 30 min prior to incubation with 5*S*-HETE.

Reaction of recombinant COX-2 with 5*S*-HETE

The reaction of 5*S*-HETE (120 μg total; containing 300,000 cpm of [1-^{14}C]5*S*-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 5*S*-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 5*S*-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.

Synthesis and isolation of diHETE reference compounds

5*S*,15*S*-diHETE was synthesized by reaction of 5*S*-HETE with the LOX-1 isozyme from soybean seeds (16). To 3 ml of 100 mM K_2HPO_4 buffer pH 10 were added 100 μg of 5*S*-HETE and 1 μl of soybean lipoxygenase solution (~25,000 units; Sigma). 5*R*,15*S*-diHETE was synthesized using 5*R*-HETE (100 μg) as substrate and 3 μl of soybean lipoxygenase solution. After 1 min reaction time, the solution was acidified (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. 5*S*,15*S*-DiHETE and 5*R*,15*S*-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.

5*S*,15*R*-DiHETE was synthesized by reaction of 15*R*-HETE with recombinant human 5-LOX. For the enzymatic transformation, a pellet of *Sf9* insect cells expressing 5-LOX (~300 μl) was sonicated and transferred to 1 ml of PBS containing 2 mM $CaCl_2$ and 1 mM ATP. 15*R*-HETE (50 μg) was added and the reaction was allowed to proceed for 15 min at room temperature. The reaction was terminated by the addition of 250 μl of methanol and 10 mg of $NaBH_4$. After 15 min at room temperature, the pH was adjusted to 3 using 1 N HCl, and the products were extracted using methylene chloride. 5*S*,15*R*-DiHETE was purified by RP-HPLC as described above for the 5*S*,15*S*-diastereomer.

5*S*,11*R*-DiHETE was synthesized by reaction of 5*S*-HETE with the recombinant linoleic acid 9*R*-LOX from *Anabaena* sp. PCC7120 expressed in *Escherichia coli*, a gift from Alan R. Brash at Vanderbilt University (17). The substrate, 5*S*-HETE (100 μg), was added to 3 ml of 100 mM Tris-HCl pH 7.5 containing 150 mM NaCl and 0.01% CHAPS. The reaction was initiated by addition of 1 μl of the purified *Anabaena* 9*R*-LOX. After 3 min reaction time, the solution was acidified to pH 4 using 1 N HCl, and the products were extracted using a 30 mg Waters HLB cartridge and eluted with methanol. After evaporation of the solvent, the residue was dissolved in 100 μl of methanol, and the products were reduced with 150 μg TPP at room temperature for 15 min.

5*S*,11*R*-DiHETE was isolated using RP-HPLC conditions as described above for 5*S*,15*S*-diHETE. HPLC-purified 5*S*,11*R*-diHETE was dissolved in $CDCl_3$ 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 $CHCl_3$ 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 analyzed using RP-HPLC. The diastereomers eluted as a single peak and purification was performed as described for 5*S*,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 × 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*S*,15*S*-diHETE, 5*R*,15*S*-diHETE, and 5*S*,11*R*-diHETE were treated with ethereal diazomethane for 30 s, evaporated, and dissolved in 50 μl of dry acetonitrile. To the solution was added 1 μl of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and a few grains each of 4-dimethylaminopyridine (DMAP) and 2-naphthoylchloride. The reaction was carried out at room temperature overnight, the solvent was evaporated, and the residue was dissolved in methylene chloride and washed with water twice. Purification of the methyl ester, 2-naphthoyl derivatives of the HETEs and diHETEs was achieved by RP-HPLC using a Waters Symmetry C18 column (4.6 × 250 mm) eluted with a solvent of methanol/water/acetic acid (95/5/0.01, by vol) at 1 ml/min flow rate and UV detection at 235 nm. Samples were extracted from HPLC solvent using methylene chloride and dissolved in acetonitrile to an optical density (OD) of 0.75 absorbance unit (AU) for HETE derivatives and OD 1.5 AU for diHETE derivatives (when possible), respectively. Circular dichroism (CD) spectra were recorded using an Aviv Model 215 CD spectrometer at room temperature in a 1 cm pathlength cuvette scanning from 350–200 nm. The ^1H NMR spectrum (600 MHz) of the 2-naphthoate derivatized 5*S*,11*R*-diHETE methyl ester was recorded in CD_3CN, δ 1.93 ppm.

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*S*-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)trifluoroacetamide 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 Thermo-Finnigan 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, and 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 PGD_2. A Waters Symmetry Shield C18 3.5 μm-column (2.1 × 150 mm) was eluted with a linear gradient of acetonitrile/water, 10 mM NH_4OAc (5/95, by vol; solvent A) to acetonitrile/water, 10 mM NH_4OAc (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 PGD_2, 5*S*-HETE, 5*S*,15*S*-diHETE, and 5*S*,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 PGD_2 and PGE_2: *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-^{14}C]5*S*-HETE 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*S*-HETE, 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, TMS-ether 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-CH_3^+] (*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

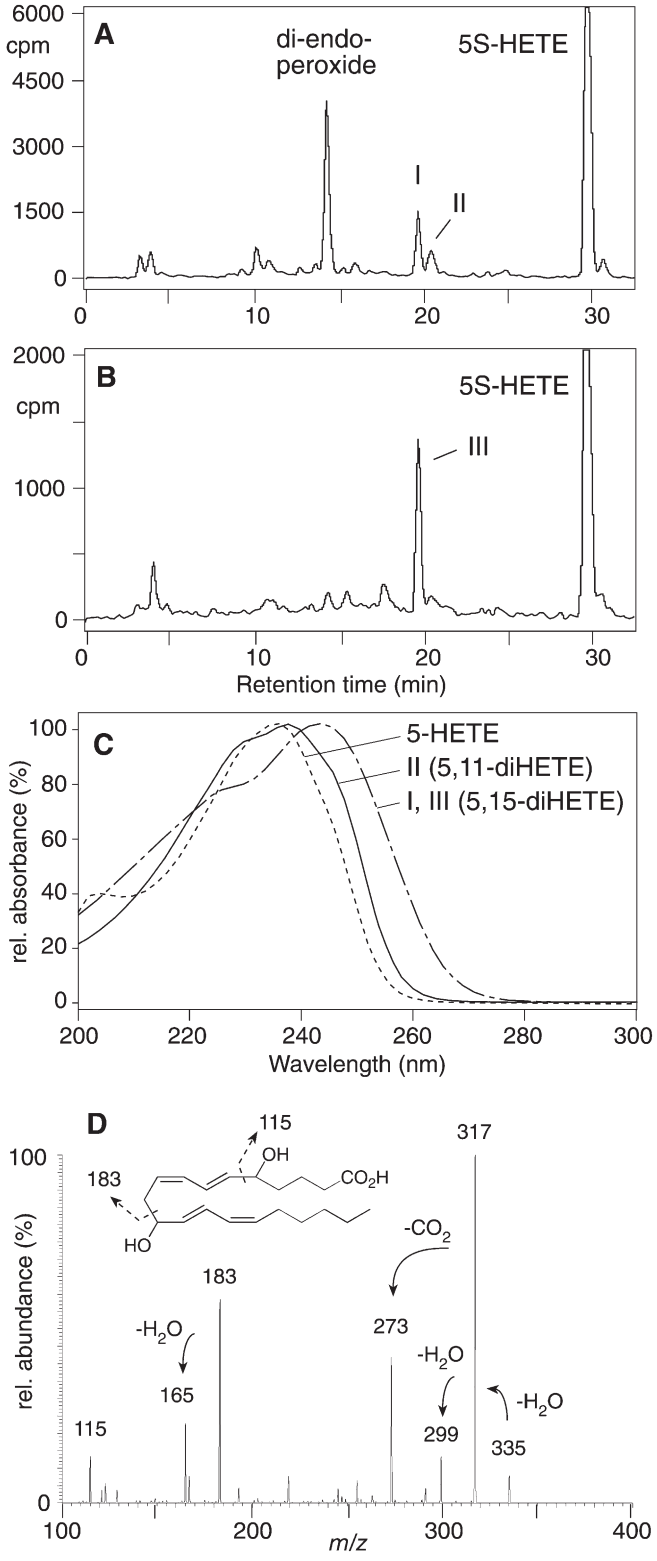

Fig. 1. Analysis of the reaction of native and aspirin-acetylated human COX-2 with 5*S*-HETE. A: [1-^{14}C]5*S*-HETE was reacted with human COX-2 and analyzed by RP-HPLC with detection of radioactive products as described in Experimental Procedures. The di-endoperoxide is the major product of native COX-2 in addition to the two by-products labeled as I and II. B: Recombinant human COX-2 was pretreated with 2 mM aspirin for 30 min before incubation with [1-^{14}C]5*S*-HETE to give one major product (III) with a similar retention time as peak I in panel A. C: UV spectra of 5-HETE, I, III (5,15-diHETE), and II (5,11-diHETE) recoded during RP-HPLC analysis using a diode array detector. D: LC-ESI mass spectrum of II (5,11-diHETE) obtained in the negative ion mode. Fragmentation of the precursor ion at *m/z* 335 was induced at 20 eV collision energy.

(Fig. 1D). 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 5*S*,11*R*-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: δ 6.57 ppm, dd, J = 15.1 Hz/11.0 Hz; H8: δ 6.13, dd, J = 11.0; H6: δ 5.70, dd, J = 14.9 Hz/6.3 Hz, H9: δ 5.55, m; and H13: δ 6.51, dd, J = 14.9 Hz/11.4 Hz; H14: 5.96, dd, J = 11.0 Hz; H12: δ 5.67, m; H15: δ 5.46, m). Two protons attached to carbons bearing a hydroxyl group were located at 4.25 ppm (H11: δ 4.25, dt, J = 6.3 Hz/6.1 Hz) and 4.17 ppm (H5: δ 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, 5*S*-HETE.

Synthesis of standards of diastereomeric diHETEs

Table 1 gives an overview of the diHETE standards prepared as reference compounds. Authentic 5*S*,15*S*-diHETE was prepared by reaction of soybean LOX-1 with 5*S*-HETE. Synthesis of 5*S*,15*R*-diHETE by reaction of 15*R*-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 5*R*,15*S*-diHETE was prepared by reaction of 5*R*-HETE with the lipoxygenase from soybean seeds. 5*S*,15*R*-diHETE and 5*R*,15*S*-diHETE have indistinguishable retention times on RP- and SP-HPLC.

An authentic standard of 5*S*,11*R*-diHETE was prepared by reaction of 5*S*-HETE with the recombinant 9*R*-LOX from *Anabaena* sp PCC7120. A mixture of the 5,11-diHETE diastereomers was prepared by thin-film autoxidation of racemic 11-HETE. Initial attempts to prepare 5*S*,11*S*- and 5*S*,11*R*-diHETEs by reaction of 11*S*-HETE and 11*R*-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) RP-HPLC[g]	SP-HPLC[h]
5*S*,15*S*- (I)[a]	5*S*-HETE + soybean LOX	6.8	12.3
5*S*,15*R*-[b] (I, III)	15*R*-HETE + hum. 5-LOX[e]	6.8	12.8
5*R*,15*S*-[b]	5*R*-HETE + soybean LOX	6.8	12.8
5*S*,11*R*-[c] (II)	5*S*-HETE + *Anabaena* LOX / 11*R*,*S*-HETE autoxidation	7.3	18.3
5*R*,11*S*-[c]	11*R*,*S*-HETE autoxidation	7.3	18.3
5*S*,11*S*-[d]	11*R*,*S*-HETE autoxidation	7.3	17.8
5*R*,11*R*-[d]	11*R*,*S*-HETE autoxidation[f]	7.3	17.8

[a] The roman numerals in parentheses refer to the numbering of the peaks in Fig. 1A, B.

[b, c, d] diHETEs with the same superscript letter are enantiomers.

[e] This reaction gave a very low yield.

[f] Although 5*R*-HETE was readily converted by the *Anabaena* LOX formation of 5*R*,11*R*-diHETE was not observed.

[g] 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.

[h] 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 and acetylated 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 form the reaction of human COX-2 gave a %-ratio for 5*S*,15*S*-diHETE to 5*S*,15*R*-diHETE of 77:33, 80:20, and 75:25 in three separate experiments (Fig. 2A). The authentic standards of 5*S*,15*S*-diHETE and 5*S*,15*R*-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% 5*S*,15*R*-diHETE (**Fig. 3**).

Absolute configuration of 5,11-diHETEs

Standards for the 5*S*,11*R*- and 5*S*,11*S*-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 5*S*,11*S*- and 5*R*,11*R*-diastereomers eluted at 17.8 min and the second peak (5*S*,11*R*- and 5*R*,11*S*-diastereomers) eluted at 18.3 min. The authentic standard of 5*S*,11*R*-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% 5*S*,11*R*-diHETE.

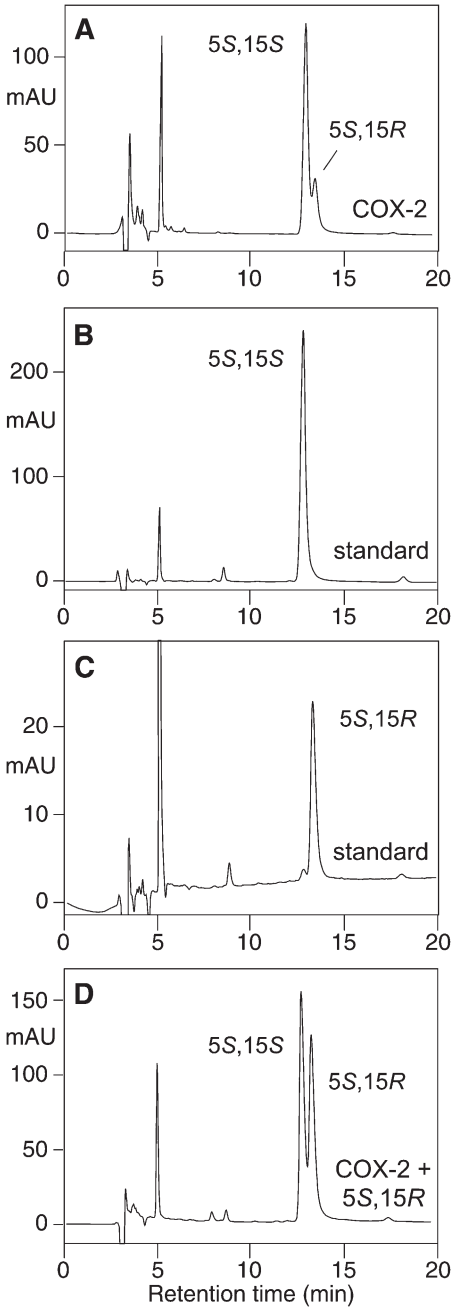

Fig. 2. Configurational analysis of 5,15-diHETE formed by the reaction of human COX-2 with 5*S*-HETE. Resolution of the 5,15-diHETE diastereomers was achieved using SP-HPLC as described in Experimental Procedures. A: Analysis of the 5,15-diHETE by-product from human COX-2 isolated by RP-HPLC. B, C: Elution of authentic standards of 5*S*,15*S*-diHETE and 5*S*,15*R*-diHETE, respectively. D: Cochromatography of the sample in A with the 5*S*,15*R*-diHETE standard (sample from C). All chromatograms were recorded at UV 235 nm using a diode array detector.

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

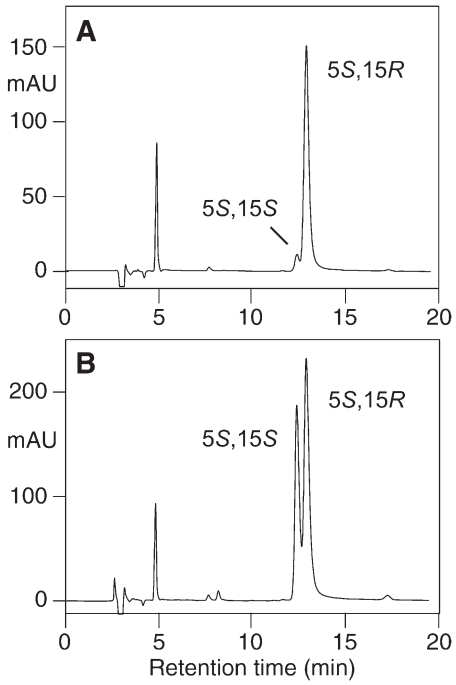

Fig. 3. Configurational analysis of 5,15-diHETE formed by the reaction of acetylated human COX-2 with 5*S*-HETE. A: SP-HPLC analysis of 5,15-diHETE formed by acetylated human COX-2 isolated by RP-HPLC. B: Cochromatography of the sample in A with an authentic standard of 5*S*,15*S*-diHETE. The same chromatographic conditions as in Fig. 2 were used. All chromatograms were recorded at UV 235 nm using a diode array detector.

Cotton effects (CEs). One of the chromophores in the (di)HETEs is present as the conjugated diene system, and in order to introduce the second chromophore, the hydroxy groups were derivatized to the 2-naphthoate ester (19). Mono- and di-2-naphthoate derivatives, of the following methyl ester fatty acids were prepared, respectively: 5*S*-HETE, 11*S*-HETE, 11*R*-HETE, 15*S*-HETE, 15*R*-HETE, 5*S*,15*S*-diHETE, 5*R*,15*S*-diHETE, and 5*S*,11*R*-diHETE.

As expected, pairs of derivatized enantiomers, e.g., 15*S*-HETE and 15*R*-HETE, gave mirror-image CD spectra (**Fig. 5**). Furthermore, the CD spectra of all *S*-configuration HETEs (5*S*-HETE, 11*S*-HETE, and 15*S*-HETE) gave a positive first Cotton effect (i.e., the CE at the higher wavelength) and a negative second CE. Due to the additive nature of the absorbance in the CD spectrum, the two chiral centers in the diHETEs were expected to result in a CD spectrum that represents the mathematical sum of the spectra obtained for the two individual chiral centers. The spectrum of 5*S*,15*S*-diHETE showed increased intensities for the two CEs, although the $\Delta\varepsilon$ intensities were not doubled when compared with 15*S*-HETE, which was likely due to saturation effects at the high concentration measured (1.5 AU in the UV) (Fig. 5). In contrast, for the 5*R*,15*S*-diHETE diastereomer, the CEs cancelled each other out and the resulting CD spectrum was an almost flat line. The CD spectra of the diastereomeric 5,15-diHETEs confirmed the assignment of the absolute configuration of the two chiral centers in the 5*S*,15*S*-diHETE standard.

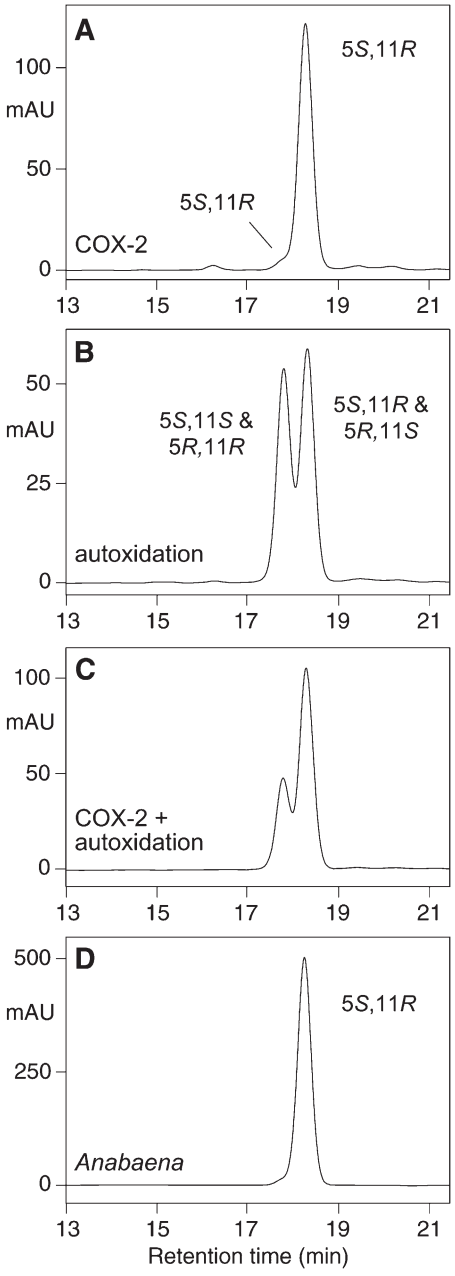

Fig. 4. SP-HPLC analysis of 5,11-diHETE formed by recombinant human COX-2. A: Analysis of 5,11-diHETE from the reaction of 5*S*-HETE with COX-2. B: Analysis of diastereomers of 5,11-diHETE formed by autoxidation of racemic 11-HETE. C: Cochromatographic elution of a mixture of the samples in A and B. D: Analysis of 5*S*,11*R*-diHETE from the reaction of 5*S*-HETE with recombinant 9*R*-LOX from *Anabaena*. The same chromatographic conditions as in Fig. 2 were used except that all samples were analyzed as the methyl ester derivatives. Only partial chromatograms (13–21.5 min) recorded at UV 235 nm are shown.

The configuration of C11 in 5*S*,11*R*-diHETE formed by reaction of the *Anabaena* 9-LOX with 5*S*-HETE was confirmed using the same approach. The individual CD spectra of 2-naphthoate-derivatized 5*S*-HETE and 11*R*-HETE are mirror images of each other, but unexpectedly, the CD spectrum of 5*S*,11*R*-diHETE was not a flat line (**Fig. 6**). The spectrum showed CEs at 245 nm ($\Delta\varepsilon$ +7.6) and 228

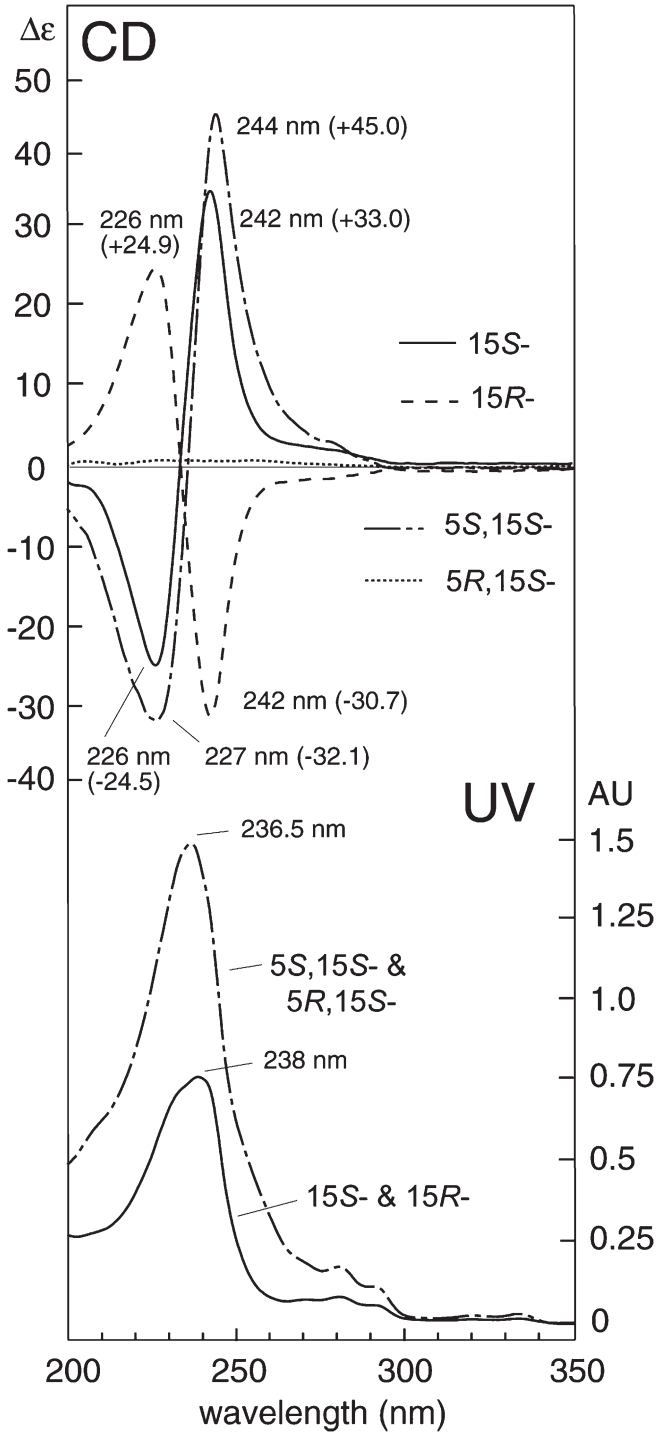

Fig. 5. CD and UV spectra of the 2-naphthoate derivatives of 15-HETEs and 5,15-diHETEs. The methyl esters of 15*S*-HETE, 15*R*-HETE, 5*S*,15*S*-diHETE, and 5*R*,15*S*-diHETE were reacted with 2-naphthoyl chloride, and the derivatives were dissolved in acetonitrile to the concentration shown in the UV spectra.

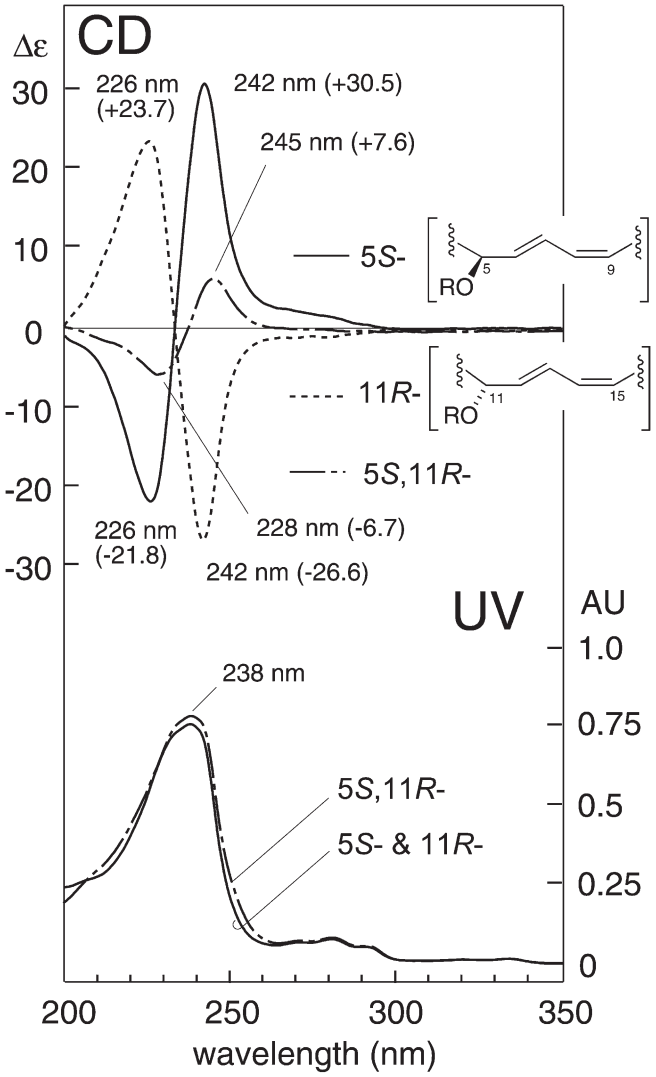

Fig. 6. CD and UV spectra of 2-naphthoate derivatives of 5*S*-HETE, 11*R*-HETE, and 5*S*,11*R*-diHETE. The 2-naphthoate, methyl ester derivatives of 5*S*-HETE, 11*R*-HETE, and 5*S*,11*R*-diHETE from the *Anabaena* reaction were dissolved in acetonitrile to the concentration shown in the UV spectra.

nm (Δε –6.7), resembling a weaker version of the CD spectrum of 5*S*-HETE with slightly shifted maxima. We hypothesized that the transition moment of the chromophores at C5 gave a stronger spectrum due to more optimal alignment of the two chromophores, thereby overcompensating the spectrum for the chiral center at C11. ^{1}H NMR determination of the $J_{5,6}$ and $J_{11,12}$ coupling constants that 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*S*,11*R*-diHETE (Fig. 4D), the question why the corresponding CD spectrum showed slight predominance of the *S*-configurated 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 μM 5*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-γ (Fig. 7A, upper panel), and their concentration was reduced to 0.5% and 3%, respectively, by incubation

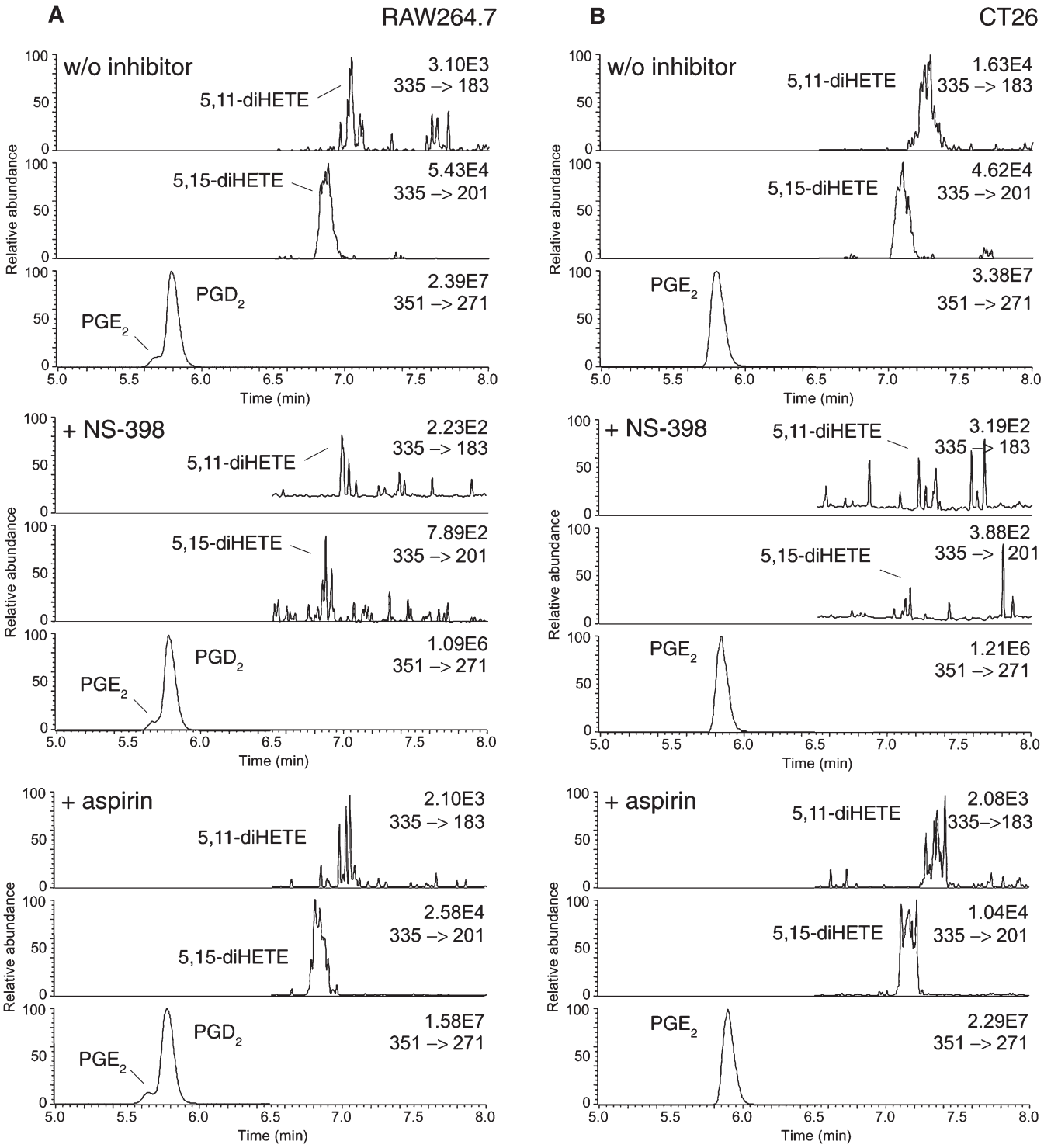

Fig. 7. LC-MS analysis of formation of diHETEs in RAW264.7 and CT26 cells incubated with 5*S*-HETE. A: RAW264.7 cells were stimulated with LPS and IFN-γ for 6 h prior to incubation with 4 μM 5*S*-HETE for 10 min, extracted, and analyzed by LC-ESI-MS operated in the negative ion SRM mode as described in Experimental Procedures (top panel). NS-398, 10 μM (middle panel) and aspirin (2 mM, bottom panel) were added to the cells 30 min prior to incubation with 5*S*-HETE. B: CT26 cells were incubated with 4 μM 5*S*-HETE in the absence (top panel) or after 30 min preincubation with 10 μM NS-398 (middle panel) or 2 mM aspirin (bottom panel). There was a slight shift in retention times between analyses of the samples in panels A and B.

of the cells with the COX-2 inhibitor NS-398 (10 μM) prior to the addition of 5*S*-HETE (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 5*S*-HETE. CT26 cells incubated with 5*S*-HETE 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 NS-398 (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 23% compared with the cells not treated with aspirin, reflecting enhanced formation of 5*S*,15*R*-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 5*S*,15*S*-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 5*S*,15*S*-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 5*S*,15*S*-diH(P)ETE and 5*S*,8*S*-diH(P)ETE by oxygenation of the primary product 15*S*-H(P)ETE (22, 23). *iii*) The third possibility is exemplified by the biosynthesis of leukotriene (LT)B_4 and 12-*epi*-LTB_4 (i.e., 5*S*,12*R*-diHETE and 5*S*,12*S*-diHETE, respectively). In this case, the diHETEs are hydrolysis products of the unstable LTA_4 epoxide. The LTA_4 epoxide is formed by 5-LOX catalyzing a second hydrogen abstraction (at C-10) of its initial 5*S*-HPETE 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 5*S*-HETE being oxygenated by COX-2 to form, as by-products, a mixture of 5*S*,15*S*-diHETE, 5*S*,15*R*-diHETE, and 5*S*,11*R*-diHETE.

The formation of 5,15-diHETE and 5,11-diHETE as by-products of the COX-2 catalyzed transformation of 5*S*-HETE bears strong resemblance to the formation of 15-HETE and 11-HETE as by-products of the COX-catalyzed transformation of arachidonic acid to PGH_2 (**Fig. 8**) (5, 26). In contrast to the reaction with arachidonic acid, 5*S*-HETE reacts only with COX-2; the COX-1 isozyme is inactive with 5*S*-HETE (12). The configuration of C-15 of 5,15-diHETE was a ~3.5:1 mixture of 15*S* and 15*R*, and a similar mixture of 15*S* and 15*R* 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% 11*R*, identical to the near exclusive 11*R*-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 5*S*-HETE 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 11*R*-HETE and 5*S*,11*R*-diHETE by-products, respectively (28). There is less control of the oxygen insertion and/or less tight binding of the ω-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 5*S*-HETE, 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.

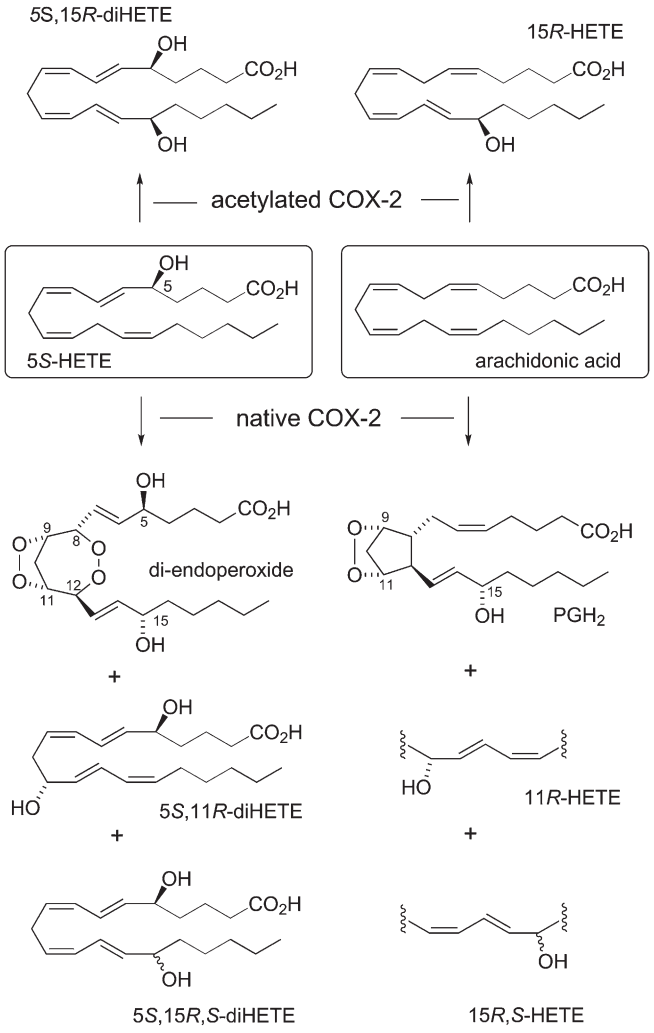

Fig. 8. Synopsis of the transformation of 5*S*-HETE and arachidonic acid by native and acetylated COX-2, respectively.

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 15*R*-HETE as the sole enzymatic product. The basis for the complete inversion of the stereochemistry of C15 from 15*S* in PGH_2 to 15*R* in 15*R*-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 5*S*-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% 15*R*.

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 5*S*-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 5*S*-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 5*S*-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). 5*S*,11*R*-diHETE and 5*S*,15*R*-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 5,15-diHETE or 5,11-diHETE.

5*S*,15*S*-DiHETE as well as 8*S*,15*S*-diHETE and 15*S*-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, LTB_4, or calcium ionophore (36). 5*S*,15*S*-DiHETE and 8*S*,15*S*-diHETE were also identified as eosinophil-derived 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 5*S*,15*S*-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. Involvement 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 5*S*,15*R*-diHETE as an alternative specific marker of COX-2 involvement is difficult to establish because the 5*S*,15*S*- and 5*S*,15*R*-diastereomers do not resolve using standard RP-HPLC conditions.

Formation of 5*S*,15*R*-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 15*R*-HETE (the metabolite of acetylated COX-2) with the 5-LOX enzyme initially forming the 5*S*-hydroperoxide of 15*R*-HETE followed by dehydration to the 5*S*,6*S*-epoxy-15*R*-hydroxy derivative, analogous to the biosynthesis of the leukotriene epoxide LTA_4. Hydrolysis of the epoxy-tetraene at carbons 6 or 14 affords 5*S*,6*R*,15*R*-trihydroxy-eicosatetraenoic acid (15-*epi*-lipoxin A_4) and 5*S*,14*R*,15*R*-trihydroxy-eicosatetraenoic acid (15-*epi*-lipoxin B_4), respectively (40, 41). In contrast, the enzymatic activities are coupled "the other way round" for formation of 5*S*,15*R*-diHETE, i.e., 5-LOX first produces 5*S*-HETE, which is subsequently converted by acetylated COX-2 to 5*S*,15*R*-diHETE.

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