

# Human CYP2S1 Metabolizes Cyclooxygenase- and Lipoxygenase-Derived Eicosanoids<sup>S</sup>

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## ABSTRACT:

CYP2S1 is a recently described dioxin-inducible cytochrome P450. We previously demonstrated that human CYP2S1 oxidizes a number of carcinogens but only via the peroxide shunt. In this article, we investigated whether human CYP2S1 can metabolize cyclooxygenase- and lipoxygenase-derived lipid peroxides in a NADPH-independent fashion. Human CYP2S1 metabolizes prostaglandin G<sub>2</sub> (PGG<sub>2</sub>) ( $K_m = 0.267 \pm 0.072 \mu\text{M}$ ) into several products including 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid (12-HHT). It also metabolizes prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) ( $K_m = 11.7 \pm 2.8 \mu\text{M}$ ) into malondialdehyde, 12-HHT, and thromboxane A<sub>2</sub> (TXA<sub>2</sub>). The turnover to 12-HHT by human CYP2S1 ( $1.59 \pm 0.04 \text{ min}^{-1}$ ) is 40-fold higher than that of TXA<sub>2</sub> ( $0.04 \text{ min}^{-1}$ ). In addition to PGG<sub>2</sub> and PGH<sub>2</sub> metabolism, human CYP2S1 efficiently metabolizes the hydroper-

oxyeicosatetraenoic acids (5S-, 12S-, and 15S-) and 13S-hydroperoxyoctadecadienoic acid into 5-oxo-eicosatetraenoic acid (turnover =  $16.7 \pm 0.3 \text{ min}^{-1}$ ), 12-oxo-eicosatetraenoic acid 1 ( $11.5 \pm 0.9 \text{ min}^{-1}$ ), 15-oxo-eicosatetraenoic acid ( $16.9 \pm 0.8 \text{ min}^{-1}$ ), and 13-octadecadienoic acid ( $20.2 \pm 0.9 \text{ min}^{-1}$ ), respectively. Other cytochromes P450 such as CYP1A1, 1A2, 1B1, and 3A4 underwent similar conversions but at slower rates. The fatty acid hydroperoxides were also converted by human CYP2S1 to several epoxyalcohols. Our data indicate that fatty acid endoperoxides and hydroperoxides represent endogenous substrates of CYP2S1 and suggest that the enzyme CYP2S1 may play an important role in the inflammatory process because some of the products that CYP2S1 produces play important roles in inflammation.

## Introduction

Eicosanoids are a family of molecules generated by oxygenation of arachidonic acid. They exert complex controls over many physiological and pathological processes, including inflammation and the ac-

tivity of the central nervous system (Haeggstrom et al., 2010). There are several classes of eicosanoids, including prostaglandins, prostacyclins, thromboxanes, leukotrienes, epoxyeicosatrienoic acids, hydroxyeicosatetraenoic acids (HETEs), hydroperoxyeicosatetraenoic acids (HpETEs), and lipoxins (Haeggstrom et al., 2010). Some eicosanoids have proinflammatory effects (e.g., prostaglandins and leukotrienes), whereas others have anti-inflammatory properties (e.g., lipoxins). Some have vasodilatory properties (e.g., prostacyclins), whereas others cause vasoconstriction (e.g., thromboxanes). In addition, some are involved in bronchiolar constriction and dilation, the recruitment of hematopoietic cells, and cancer (Haeggstrom et al., 2010).

Cytochromes P450 are a group of heme-thiolate enzymes, which play critical roles not only in the metabolism of a wide variety of

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**ABBREVIATIONS:** HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; P450, cytochrome P450; MDA, malondialdehyde; TXA<sub>2</sub>, thromboxane A<sub>2</sub>; PGH<sub>2</sub>, 9 $\alpha$ ,11 $\alpha$ -epidioxy-15S-hydroxy-prosta-5Z,13E-dien-1-oic acid; PGG<sub>2</sub>, 9 $\alpha$ ,11 $\alpha$ -epidioxy-15S-hydroperoxy-prosta-5Z,13E-dien-1-oic acid; PGE<sub>2</sub>, 9-oxo-11 $\alpha$ ,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid; PGD<sub>2</sub>, 9 $\alpha$ ,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid; TXB<sub>2</sub>, 9 $\alpha$ ,11,15S-trihydroxythromba-5Z,13E-dien-1-oic acid; 15(S)-HETE-d<sub>8</sub>, 15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-d<sub>8</sub> acid; 13(S)-HODE-d<sub>4</sub>, 13(S)-hydroxy-9Z,11E-octadecadienoic-9,10,12,13-d<sub>4</sub> acid; 5-HpETE, 5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 12-HpETE, 12S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; 15-HpETE, 15S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; 13-HpODE, 13S-hydroperoxy-9Z,11E-octadecadienoic acid; 5-oxoETE, 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid; 12-oxoETE, 12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid; 15-oxoETE, 15-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid; 12-oxoODE, 13-oxo-9Z,11E-octadecadienoic acid; 5-HETE, 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 12-HETE, 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; 15-HETE, 15S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; 13-HODE, 13S-hydroxy-9Z,11E-octadecadienoic acid; 12-HHT, 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid; HPLC, high-performance liquid chromatography; Kpi, potassium phosphate buffer; LC, liquid chromatography; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; SPE, solid-phase extraction; oxoETE, oxo-eicosatetraenoic acid; oxoODE, oxo-octadecadienoic acid; HpODE, hydroperoxy-octadecadienoic acid; MRM, multiple reaction monitoring; SIM, selected ion monitoring; COX, cyclooxygenase; 15-oxo-PGG<sub>2</sub>, 9 $\alpha$ ,11 $\alpha$ -epidioxy-15S-oxo-prosta-5Z,13E-dien-1-oic acid; 12-oxo-HT, 12-oxo-5Z,8E,10E-heptadecatrienoic acid; AQ4N, 1,4-bis[[2-(dimethylamino-N-oxide)ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione.

xenobiotic substances but also in the metabolism of a variety of endogenous compounds. Several cytochromes P450 are involved in the biosynthesis as well as the degradation of eicosanoids (Nebert and Karp, 2008). The reactions by which P450s are involved include monooxygenations,  $\omega$ - and  $\omega$ -1-hydroxylations, epoxidations, one-electron oxidations, peroxidations, and isomerizations (Capdevila et al., 2005). Depending on the nature of the oxygenated substrates, P450-catalyzed reactions can either be dependent on or independent of NADPH, cytochrome P450 reductase, and molecular oxygen. The first three of the above reaction types are NADPH-dependent, whereas the last three are not. Although the P450 NADPH-dependent metabolism of several bioactive eicosanoids is well established, P450 NADPH-independent metabolism is less well characterized but seems to be equally important in the generation of biologically significant oxygenated eicosanoids. Although cytochromes P450 in families 1 to 3 are mainly involved in the metabolism of exogenous compounds (Nebert and Karp, 2008), several also participate in eicosanoid synthesis and degradation (Nebert and Russell, 2002), and several have been shown to catalyze  $\omega$ - and  $\omega$ -1-hydroxylation of prostaglandins, arachidonic acid, epoxyeicosatrienoic acids, and HETEs in an NADPH-dependent fashion (Holm et al., 1989; Tanaka et al., 1990). CYP1A2, 2E1, and 3A4 have also been shown to break down prostaglandins into 12-hydroxyheptadecatrienoate and malondialdehyde (MDA), independently of cytochrome P450 reductase, NADPH, and O<sub>2</sub> (Plastaras et al., 2000). Furthermore, certain P450s catalyze the isomerization of 15-hydroperoxyeicosatetraenoic acid to biologically active epoxyalcohols, and isomerization of the prostaglandin H<sub>2</sub> endoperoxide to active prostanoids such as thromboxane A<sub>2</sub> (TXA<sub>2</sub>) and prostacyclin, independently of NADPH (Weiss et al., 1987; Hecker and Ullrich, 1989; Chang et al., 1996).

One of the most recently discovered human P450s is CYP2S1, which has not been well characterized in terms of its physiological role (Rylander et al., 2001; Rivera et al., 2002). Despite the efforts of many researchers, no substrate was identified (Smith et al., 2003; Wang et al., 2005; Wu et al., 2006; Bui and Hankinson, 2009), until we demonstrated that CYP2S1 cannot use NADPH for oxidative metabolism because of its inability to accept electrons from NADPH (Bui and Hankinson, 2009), but it has peroxxygenase activity and can efficiently use cumene hydroperoxide, hydrogen peroxide, and numerous fatty acid hydroperoxides to oxidize several carcinogens including benzo[*a*]pyrene-7,8-diol (Bui et al., 2009). However, no endogenous substrates for CYP2S1 have been identified. In our current report, we demonstrate peroxidase and isomerase activities of CYP2S1 with various oxygenated eicosanoids such as prostaglandin G<sub>2</sub>, prostaglandin H<sub>2</sub>, HpETEs (5-, 12-, and 15-), and 13-hydroperoxyoctadecadienoic acid and identify the products of the reactions. In addition, we tested certain other P450s for their ability to metabolize the same substrates. Our findings suggest a physiological role for CYP2S1 in the metabolism of eicosanoids and a potential role for the enzyme in inflammation, cancer, and other pathological processes.

## Materials and Methods

**Chemical and Reagents.** Microsomes containing recombinant human CYP1A1, CYP1A2, or CYP3A4, were purchased from Biocatalytics Inc. (Pasadena, CA) (all contain P450 reductase). Microsomes containing CYP1B1 including P450 reductase was purchased from BD Gentest (Woburn, MA). Purified CYP2S1 was prepared as described previously (Bui and Hankinson, 2009). 9 $\alpha$ ,11 $\alpha$ -Epidioxy-15S-hydroxy-prosta-5Z,13E-dien-1-oic acid (PGH<sub>2</sub>), 9 $\alpha$ ,11 $\alpha$ -epidioxy-15S-hydroperoxy-prosta-5Z,13E-dien-1-oic acid (PGG<sub>2</sub>), 9-oxo-11 $\alpha$ ,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid (PGE<sub>2</sub>), 9 $\alpha$ ,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid (PGD<sub>2</sub>), 9 $\alpha$ ,11,15S-trihydroxythromba-5Z,13E-dien-1-oic acid (TXB<sub>2</sub>), 15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-d<sub>8</sub> acid (15(S)-HETE-d<sub>8</sub>), 13(S)-hydroxy-9Z,11E-octadecadienoic-9,10,12,13-d<sub>4</sub> acid [13(S)-HODE-d<sub>4</sub>], 5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HpETE), 12S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HpETE), 15S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (15-HpETE), 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13-HpODE), 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-oxoETE), 12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-oxoETE), 15-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid (15-oxoETE), 13-oxo-9Z,11E-octadecadienoic acid (13-oxoODE), 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE), 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE), 15S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (15-HETE), 13S-hydroxy-9Z,11E-octadecadienoic acid (13-HODE), and 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid (12-HHT) were purchased from Cayman Chemical (Ann Arbor, MI). HPLC solvents (HPLC grade) were obtained from Sigma-Aldrich (St. Louis, MO). c33 cells were derived in our laboratory from Hepa-1 cells. c33 cells carrying an empty vector (c33-MSCV) or cells carrying the vector expressing human CYP2S1 (c33-h2S1) were constructed as described previously (Bui et al., 2009).

**Enzyme Assays.** All assays were conducted in 1.5-ml Eppendorf tubes. No NADPH was used in any assay. Substrate storage solvents were evaporated to dryness with a stream of argon. To determine whether human CYP2S1 can metabolize PGG<sub>2</sub> or PGH<sub>2</sub>, the 100- $\mu$ l reaction mixture containing 100 mM potassium phosphate buffer (Kpi), pH 7.5, and 0.25  $\mu$ M CYP2S1 was incubated with 133  $\mu$ M PGG<sub>2</sub> or 50  $\mu$ M PGH<sub>2</sub>. Incubations containing heat-treated CYP2S1 (20 min at 85°C) or no CYP2S1 were used as controls. The reactions with PGG<sub>2</sub> and PGH<sub>2</sub> were incubated at 37°C for 15 and 5 min, respectively. To determine whether human CYP2S1 can metabolize 5-HpETE, 12-HpETE, 15-HpETE, or 13-HpODE, 100- $\mu$ l reaction mixtures containing 100 mM Kpi, pH 7.5, 0.1 to 0.2  $\mu$ M purified human CYP2S1, and 50  $\mu$ M 5-HpETE, 12-HpETE, 15-HpETE, or 13-HpODE were incubated at 37°C for 5 to 15 min. Samples without CYP2S1 or with heat-treated CYP2S1 were used as controls. All reactions were stopped with 100  $\mu$ l of acetonitrile containing 2% acetic acid or 100  $\mu$ l of methanol containing an internal standard mixture [(15(S)-HETE-d<sub>8</sub>, 12(S)-HETE-d<sub>8</sub>, and 13(S)-HODE-d<sub>4</sub>, 10 ng/ml each)]. The samples stopped with acetonitrile were analyzed by HPLC, and the samples stopped by methanol were analyzed by LC-ESI-MS/MS. For the latter analysis, samples were diluted with 1800  $\mu$ l of water followed with purification using an Oasis HLB solid-phase extraction (SPE) cartridge before they were analyzed as described under *Sample Preparation for LC-MS/MS*. The products were identified by comparison with authentic standards and mass spectrometry.

To investigate whether human CYP2S1 overexpressed in mammalian c33 cells (c33-h2S1) metabolizes the above fatty acid peroxides, particularly PGH<sub>2</sub>, 500- $\mu$ l lysates derived from  $2.5 \times 10^6$  cells were incubated with 40  $\mu$ M

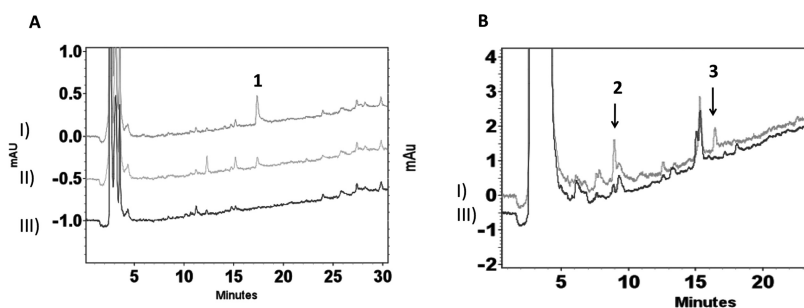


FIG. 1. Metabolism of PGG<sub>2</sub> by recombinant human CYP2S1. HPLC chromatograms of 133  $\mu$ M PGG<sub>2</sub> incubated with 0.1  $\mu$ M human CYP2S1 (line I) or without CYP2S1 (line III) or with heat-treated human CYP2S1 (line II) with detection set at 275 nm (A) or at 220 nm (B). 1, unknown, but probably either 12-oxo-HT or 15-oxo-PGG<sub>2</sub>; 2, unknown; and 3, 12-HHT, which was identified with an authentic standard. mAU, milli-arbitrary unit.

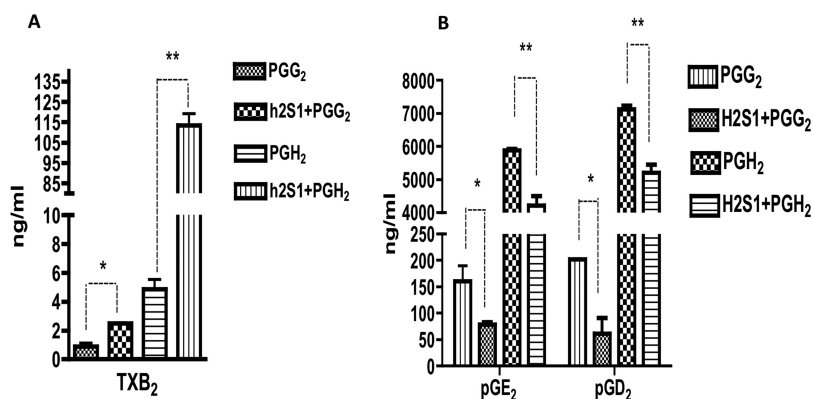


Fig. 2. Metabolism of PGG<sub>2</sub> and PGH<sub>2</sub> by recombinant human CYP2S1 (h2S1). PGG<sub>2</sub> and PGH<sub>2</sub> incubated without enzyme were used as controls. The concentrations of (A) TXB<sub>2</sub> and (B) PGE<sub>2</sub> and PGD<sub>2</sub> were determined by LC-ESI-MS/MS MRM in the negative ion mode. Data are presented as means ± S.D. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , respectively. Turnover of TXB<sub>2</sub> from metabolism of PGH<sub>2</sub> by human CYP2S1 was determined to be 0.04 min<sup>-1</sup>.

PGH<sub>2</sub> for 20 min at 37°C. The reactions were stopped with the addition of 1.5 ml of methanol containing internal standards (as described above), followed by the addition of 3 ml of chloroform. After 1 h at room temperature, 1.25 ml of H<sub>2</sub>O was added, and the samples then were left for 10 min to allow separation of the two phases. The samples were centrifuged for 10 min at 1000g, and the chloroform fraction containing lipophilic compounds was collected. The samples were evaporated to dryness under a stream of argon, followed by resuspension in 100 μl of methanol and 1.8 ml of H<sub>2</sub>O (pH 2–3). The resuspended samples were then purified with an Oasis HLB SPE cartridge as described under *Sample Preparation for LC-MS/MS*.

**K<sub>m</sub> and Turnover Numbers Determination for Various P450s.** To determine the K<sub>m</sub> values of CYP2S1 for PGG<sub>2</sub>, PGH<sub>2</sub>, 5-HpETE, 12-HpETE, 15-HpETE, and 13-HpODE, 50 μM and five progressive 1:3 dilutions of each substrate were incubated in 50-μl reaction mixtures containing 100 nM CYP2S1. The reactions were terminated after 120 s at room temperature by the addition of 50 μl of ice-cold acetonitrile containing 2% acetic acid. The 2-min incubation with 100 nM enzyme was chosen because its kinetics was predetermined to fall within the linear range with regard to both enzyme concentration and time. The K<sub>m</sub> values were determined on the basis of the rates of the selected product formation (area of product per minute) measured by HPLC. The 275 nm absorbing unknown product, 12-HHT, 5-oxoETE, 12-oxoETE, 15-oxoETE, and 13-oxoODE were the selected products for PGG<sub>2</sub>, PGH<sub>2</sub>, 5-HpETE, 12-HpETE, 15-HpETE, and 13-HpODE, respectively. The K<sub>m</sub> values were then calculated using Prism software with nonlinear regression and the Michaelis-Menten equation [ $V = V_{max} \cdot S / (K_m + S)$ ].

To determine the turnover numbers for 12-HHT, 5-oxoETE, 12-oxoETE, 15-oxoETE, and 13-oxoODE for CYP2S1, 1A1, 1A2, 1B1, and 3A4, 100-μl

reaction mixtures containing 100 mM Kpi, pH 7.5, and 100 nM enzyme were incubated with 50 μM PGH<sub>2</sub>, 5-HpETE, 12-HpETE, 15-HpETE, or 13-HpODE. The reactions were incubated for 5 min at 37°C before being stopped with 100 μl of acetonitrile containing 2% acetic acid. The amounts of product were determined by HPLC. Turnover numbers were calculated as nanomoles of product formed per nanomole of P450 per minute or expressed simply as minutes<sup>-1</sup>.

**Analytical Procedures and Product Identification.** The HPLC system consisted of the Shimadzu Prominence series, including the LC-20 AT Prominence LC pump, DGU-20A5 degasser, CBM-20 Prominence communications bus module, SPD-20A Prominence UV/VIS detector, and RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan). HPLC method 1 used a C18 reverse-phase column (Discovery C18, 2.2 mm × 150 mm, 5 μm; Supelco, Bellefonte, PA) plus a C18 guard column and a 20-μl injection volume. Solvents were held at 50% B from 0 to 3 min, increased to 80% B from 3 to 18 min, and then held for another 7 min before returning to the starting condition (solvent A, H<sub>2</sub>O; solvent B, 100% acetonitrile; flow rate, 0.4 ml/min). The diode array detector was set from 200 to 400 nm. HPLC method 2 used a C18 reverse-phase column (4.5 mm × 250 mm, 5 μm; Shimadzu) and a 50-μl injection volume. Solvents were held at 40% B from 0 to 3 min, increased to 95% B from 3 to 30 min, and then held for another 10 min before returning to the starting condition (solvent A, H<sub>2</sub>O; solvent B, 100% acetonitrile; flow rate, 1 ml/min). The diode array detector was set from 200 to 400 nm. The oxoETEs and oxoODE products of HpETEs and HpODE, respectively, were monitored at 279 nm. Their identities were confirmed using authentic product standards, and their concentrations were calculated on the basis of the standard curves of the latter. 12-HHT and other products of PGG<sub>2</sub> and PGH<sub>2</sub> metabolism were monitored at 220, 230, and 275 nm. 12-HHT was confirmed using an authentic product standard.

**Sample Preparation for LC-MS/MS.** The samples from enzyme assays and cell culture incubations were diluted with water to a final concentration of 95% water before they were loaded onto a preconditioned 1-ml Oasis HLB SPE cartridge on a vacuum manifold (Waters, Milford, MA). The SPE cartridge was conditioned with 1 ml of methanol and 1 ml of water before sample loading. The sample was slowly loaded onto the cartridge, and the cartridge was washed with 1 ml of 5% methanol in water. HETEs/hydroxy-octadecadienoic acids, prostaglandins, TXB<sub>2</sub>, and other lipid metabolites were subsequently eluted with 1 ml of methanol. The eluates were then evaporated to

TABLE 1

Turnover numbers of 12-HHT from metabolism of PGH<sub>2</sub> by CYP1A1, 1A2, 1B1, 3A4, and 2S1

Turnovers are expressed as nanomoles of 12-HHT formed per nanomole of P450 per minute. Data are presented as means ± S.D. (n = 3).

P450s	Turnover of 12-HHT from PGH <sub>2</sub> min <sup>-1</sup>
CYP1A1	2.25 ± 0.15
CYP1A2	1.56 ± 0.02
CYP1B1	2.46 ± 0.15
CYP3A4	2.40 ± 0.15
CYP2S1	1.59 ± 0.04

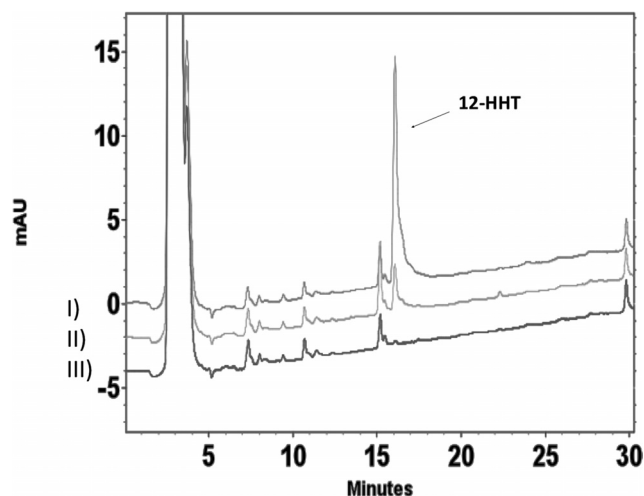


Fig. 3. Metabolism of PGH<sub>2</sub> by recombinant human CYP2S1. PGH<sub>2</sub> was incubated for 5 min with human CYP2S1 (line I) or heat-treated human CYP2S1 (line II) or no enzyme (line III). 12-HHT was detected at 230 nm and identified using an authentic standard. Samples were analyzed by HPLC method 2. mAU, milli-arbitrary unit.

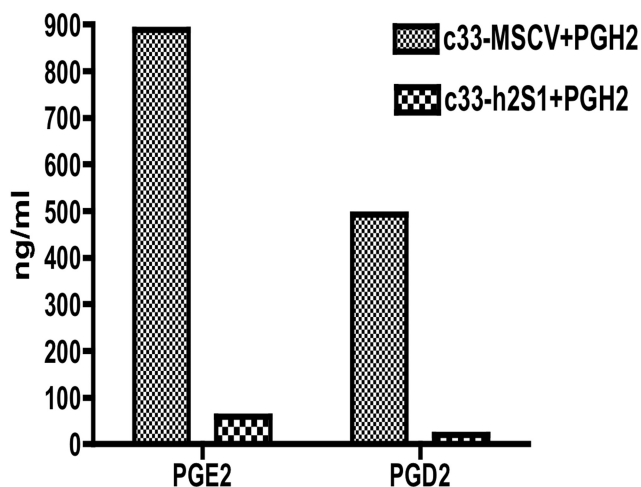


FIG. 4. Metabolism of PGH<sub>2</sub> by extracts of mammalian cells expressing CYP2S1. Lysates of c33-MSCV or c33-h2S1 cells incubated with PGH<sub>2</sub>. Concentrations of PGE<sub>2</sub> and PGD<sub>2</sub> were determined by LC-ESI-MS/MS using MRM negative ion mode. The data are representative of two separate experiments.

dryness under a stream of argon. Then 100  $\mu$ l of methanol was added to the dried extract and vortexed for 30 s, and the reconstituted extract was centrifuged at 13,200 rpm for 20 min at 4°C to remove any particulates that could clog the LC-MS/MS instrument. The resulting supernatants were transferred to autosampler vials and processed for LC-MS/MS analysis.

**LC-MS/MS Analysis.** LC-MS/MS was performed using a quadrupole mass spectrometer (4000 QTRAP; Applied Biosystems, Foster City, CA) equipped with ESI. The HPLC system used an Agilent 1200 series LC pump equipped with a thermostated autosampler (Agilent Technologies, Santa Clara, CA). Chromatography was performed using column A (Luna C18 column, 150  $\times$  3.0 mm, 3  $\mu$ m; Phenomenex, Torrance, CA) with a security guard cartridge (C-18; Phenomenex) or column B (C18 reverse-phase column, Discovery C18, 2.2 mm  $\times$  150 mm, 5  $\mu$ m; Supelco) plus a C18 guard column held at 40°C. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The autosampler was set at 4°C. The injection volume was 10  $\mu$ l, and the flow rate was controlled at 0.4 ml/min. Gradient program A using column A was as follows: 0 to 3 min, linear gradient from 50 to 60% B; 3 to 18 min, linear gradient from 60 to 65% B; 18 to 22 min, linear gradient from 65 to 100% B; 22 to 24 min 100% B; 24 to 26 min, linear gradient from 100 to 50% B; and 26 to 38 min, 50% B. The gradient program B using column B was as follows: 0 to 3 min, linear gradient from 40 to 60% B; 3 to 18 min, linear gradient from 60 to 65% B; 18 to 22 min, linear gradient from 65 to 100% B; 22 to 24 min, 100% B; 24 to 26 min, linear gradient from 100 to 50% B; and 26 to 38 min, 50% B. Data acquisition and instrument control were performed using Analyst 1.4.2 software (Applied Biosystems). Detection was performed by using the multiple reaction monitoring (MRM) mode with negative ion detection. The parameter settings used were as follows: ion spray voltage, -4500 V; curtain gas, 25 (nitrogen); ion source gas 1, 45; ion source gas 2, 55; and ion source gas 2 temperature, 450°C. Collision energy, declustering potential, and collision cell exit potential were optimized for each compound to obtain optimum sensitivity. The transitions monitored were *m/z*: 319.1  $\rightarrow$  219.0 for 15-HETE; 319.1  $\rightarrow$  115.0 for 5-HETE; 319.1  $\rightarrow$  179.0 for 12-HETE; 369.1  $\rightarrow$  169.1 for TXB<sub>2</sub>; 351.1  $\rightarrow$

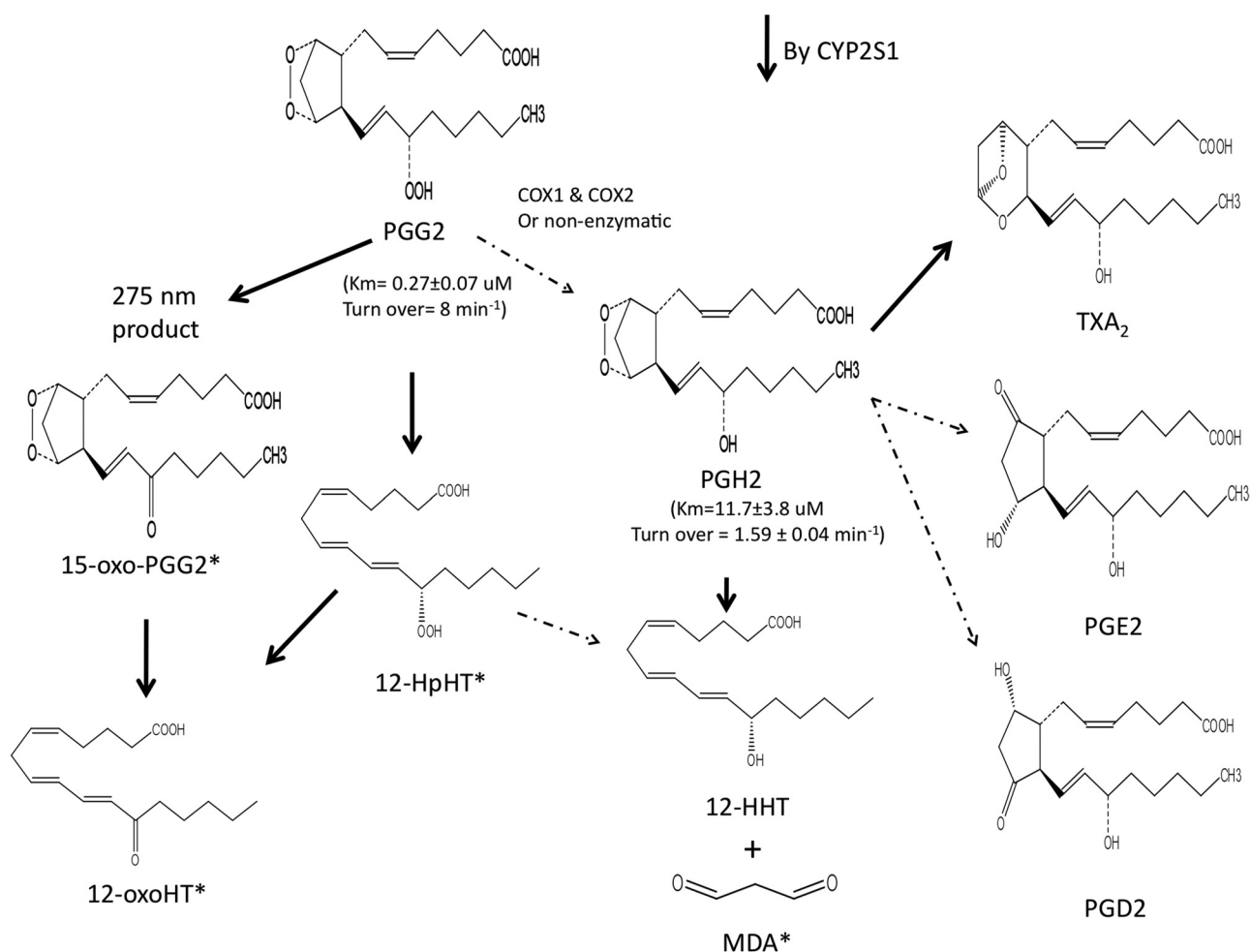


FIG. 5. Reaction scheme for PGG<sub>2</sub> metabolism by CYP2S1. \*, these products are speculative and have not been confirmed with authentic standards. Bold lines indicate reactions catalyzed by CYP2S1; dashed lines represent spontaneous reactions and/or reactions catalyzed by other enzymes.

271.2 for PGD<sub>2</sub>; 351.1 → 270.9 for PGE<sub>2</sub>; 327.1 → 226.1 for 15(*S*)-HETE-d<sub>8</sub>; 327.1 → 184.0 for 12(*S*)-HETE-d<sub>8</sub>; 299.0 → 197.9 for 13(*S*)-HODE-d<sub>4</sub>; 310.9 → 166.5 for 13(*S*)-HpODE; 335.3 → 139.0 for 15(*S*)-HPETE; 335.0 → 59.1 for 5(*S*)-HPETE; and 335.0 → 273.1 for 12(*S*)-HPETE. For each product standard, seven concentrations (1.25, 2.5, 5, 10, 20, 40, and 80 ng/ml) were used to create standard curve. The *R*<sup>2</sup> values for goodness of fit were greater than 0.990. The limit of detection for each standard was less than 0.5 ng/ml.

In several cases, some products were detected using the selected ion monitoring (SIM) mode with negative ion detection. In addition, the unknown metabolites were identified using MS/MS with parameter settings as follows: ion spray voltage, -4500 V; curtain gas, 25 (nitrogen); ion source gas 1, 45; ion source gas 2, 55; and ion source gas 2 temperature, 450°C. The MS/MS fragmentation data of the unknown metabolites are shown in the supplemental data. Each determination was repeated at least once to ensure reproducibility.

## Results

**Human CYP2S1 Converts Prostaglandin G<sub>2</sub> and H<sub>2</sub> into Several Products.** Prostaglandin G<sub>2</sub> is the initial product of the prostanoïd biosynthesis pathway and is generated from arachidonic acid by either the cyclooxygenase (COX) 1 or COX2 enzyme. PGG<sub>2</sub> contains both endoperoxy and hydroperoxy groups, suggesting that it might be metabolized by CYP2S1 because we established previously that CYP2S1 is capable of homolytic cleavage of the O–O bond (Bui et al., 2009). When human CYP2S1 was incubated with PGG<sub>2</sub>, several products were identified by UV detection (Fig. 1). The amounts of these products formed were reduced by 80% when human CYP2S1 was heat-treated, indicating that the reactions are enzymatic (Fig. 1A and data not shown). The residual activity could be due to incomplete inactivation of CYP2S1 or could be the result of a nonenzymatic reaction from the heme released from denatured CYP2S1, because high concentrations of heme [(hydroxo-(porphyrinato)iron(III)] have been shown to have some peroxxygenase-like activity (Dix et al., 1985). Figure 1 shows that there were at least three UV-absorbing products. Two products were detected at 220 nm (Fig. 1B) and one product at 275 nm (Fig. 1A). One of the 220 nm absorbing products is 12-HHT because it had the same retention time as the authentic standard. 12-HHT formation from PGG<sub>2</sub> probably occurs via 12-hydroperoxy-5*Z*,8*E*, 10*E*-heptadecatrienoic acid and PGH<sub>2</sub> intermediates (Fig. 5). The other 220 nm absorbing product is unknown. The

275 nm absorbing product is also unknown, but it could be either 9α,11α-epidioxy-15*S*-oxo-prosta-5*Z*,13*E*-dien-1-oic acid (15-oxo-PGG<sub>2</sub>) or 12-oxo-5*Z*,8*E*,10*E*-heptadecatrienoic acid (12-oxo-HT) on the basis of the mechanism of homolytic O–O cleavage by CYP2S1 (Fig. 5). These two products should absorb at approximately 275 nm because they contain a conjugated carbonyl group. The *K*<sub>m</sub> for PGG<sub>2</sub> based on the formation of the 275 nm absorbing products was calculated to be 0.27 ± 0.07 μM, and the turnover was estimated to be approximately 8 min<sup>-1</sup> on the basis of PGG<sub>2</sub> consumption as measured by mass spectrometry.

In addition to the above products, there was a significantly greater amount of TXB<sub>2</sub> detected in the sample containing PGG<sub>2</sub> plus human CYP2S1 sample compared with the control (Fig. 2). The TXB<sub>2</sub> in this case is probably generated via PGH<sub>2</sub> (Fig. 5). The presence of TXB<sub>2</sub> is indicative of TXA<sub>2</sub> formation because the former is a more stable nonenzymatically hydrolyzed product of the latter, and TXB<sub>2</sub> has been shown to accurately reflect TXA<sub>2</sub> synthesis (Patrono et al., 1983).

PGH<sub>2</sub> is an immediate product of PGG<sub>2</sub> that is either formed spontaneously or by the peroxidase activity of the COX enzymes (Fig. 5). Unlike PGG<sub>2</sub>, PGH<sub>2</sub> lacks the hydroperoxy group, but it still contains the endoperoxy group, which could be targeted by CYP2S1. To test this, CYP2S1 was incubated with PGH<sub>2</sub>. At least one product that exhibited UV absorbance at 220 nm was detected with the diode array detector and was identified as 12-HHT (Fig. 3). The formation of 12-HHT was reduced by 80% when CYP2S1 was heat-treated, indicating that its formation is enzymatic (Fig. 3). PGH<sub>2</sub> has been shown to be converted to 12-HHT and MDA by various P450s including P450<sub>CAM</sub>, CYP1A1, CYP2E1, CYP3A4, and CYP5A, probably via a radical fragmentation pathway (Hecker and Ullrich, 1989; Plataras et al., 2000). The rate of 12-HHT formation by CYP2S1 was slightly slower compared with that of CYP1A1, CYP1B1, and CYP3A4, but slightly faster compared with CYP1A2 (Table 1). It should be noted that although we did not directly measure MDA, we can assume it was formed in amounts equimolar to those of 12-HHT because of the nature of the chemical reaction (Plataras et al., 2000). The *K*<sub>m</sub> of CYP2S1 for PGH<sub>2</sub> was 11.7 ± 2.8 μM, which is approximately 40-fold higher than its *K*<sub>m</sub> for

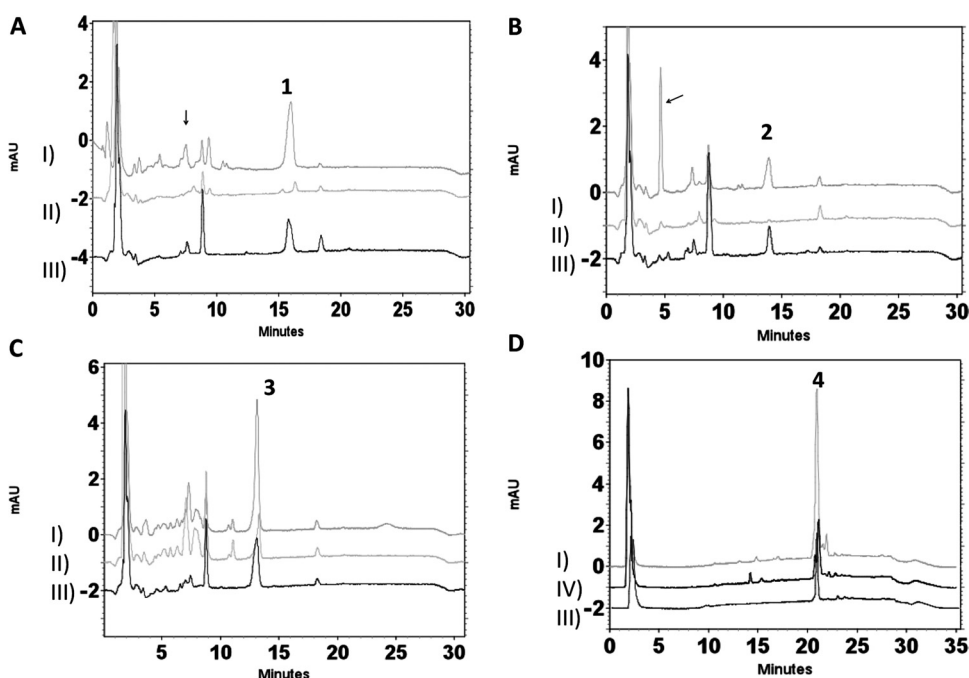


FIG. 6. Metabolism of fatty acid hydroperoxides by recombinant human CYP2S1. 5-HpETE (A), 12-HpETE (B), 15-HpETE (B), or 13-HpODE (D) was incubated with human CYP2S1 (line I), without CYP2S1 (line II), with 5-, 12-, and 15-oxoETEs or 13-oxoETE product standards (line III), or with heat-treated human CYP2S1 (line IV). 1, 5-oxoETE; 2, 12-oxoETE; 3, 15-oxoETE; and 4, 13-oxoODE. Detection was set at 279 nm. A to C were separated using HPLC method 1. D was separated with HPLC method 2. Arrows indicate unknown products. mAU, milli-arbitrary unit.

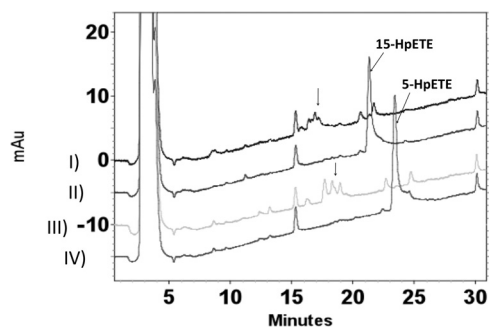


FIG. 7. Other products from the metabolism of 5- and 15-HpETE by recombinant human CYP2S1. Samples were separated with HPLC method 2 and detected at 205 nm. 15-HpETE was incubated with human CYP2S1 (line I) or without human CYP2S1 (line II). 5-HpETE was incubated with human CYP2S1 (line III) or without human CYP2S1 (line IV). The unknown products are indicated by arrows. mAU, milli-arbitrary unit.

PGG<sub>2</sub>, making PGH<sub>2</sub> less likely than PGG<sub>2</sub> to be a preferential CYP2S1 substrate.

Besides the above UV-absorbing product, purified CYP2S1 converted PGH<sub>2</sub> into non-UV absorbing product(s) including TXA<sub>2</sub> (de-

tected as TXB<sub>2</sub> by mass spectrometry) (Fig. 2A). The rate of formation of TXA<sub>2</sub> (turnover = 0.04 min<sup>-1</sup>) is much slower than that of 12-HHT (1.59 ± 0.04 min<sup>-1</sup>) (Table 1). There was much more TXA<sub>2</sub> detected in the incubation of purified CYP2S1 with PGH<sub>2</sub> than with PGG<sub>2</sub> (Fig. 2A). To our knowledge, whether CYP1A1, 1B1, or 3A4 can convert PGH<sub>2</sub> into TXA<sub>2</sub> has not been reported.

Besides TXA<sub>2</sub>, PGE<sub>2</sub> and PGD<sub>2</sub> are some of other products derived from PGH<sub>2</sub>. They are usually formed via enzymatic reaction but also can be generated spontaneously. Their levels were lower in the samples containing either purified recombinant human CYP2S1 or lysates of mammalian cells overexpressing human CYP2S1 (Figs. 2B and 4), suggesting that CYP2S1 does not convert PGH<sub>2</sub> or PGG<sub>2</sub> into either the PGE<sub>2</sub> or PGD<sub>2</sub> metabolites (Fig. 5) but diverts metabolism away from these products.

**Human CYP2S1 Metabolizes Fatty Acid Hydroperoxides.** Previously we showed that fatty acid hydroperoxides, such as certain HpETEs, support human CYP2S1-mediated oxidation of benzo[*a*]pyrene-7,8-diol (Bui et al., 2009). We investigated here whether CYP2S1 can metabolize the same fatty acid hydroperoxides in the absence of benzo[*a*]pyrene-7,8-diol. When purified human CYP2S1 was incubated with 5-, 12-, or 15-HpETE or 13-HpODE, there were

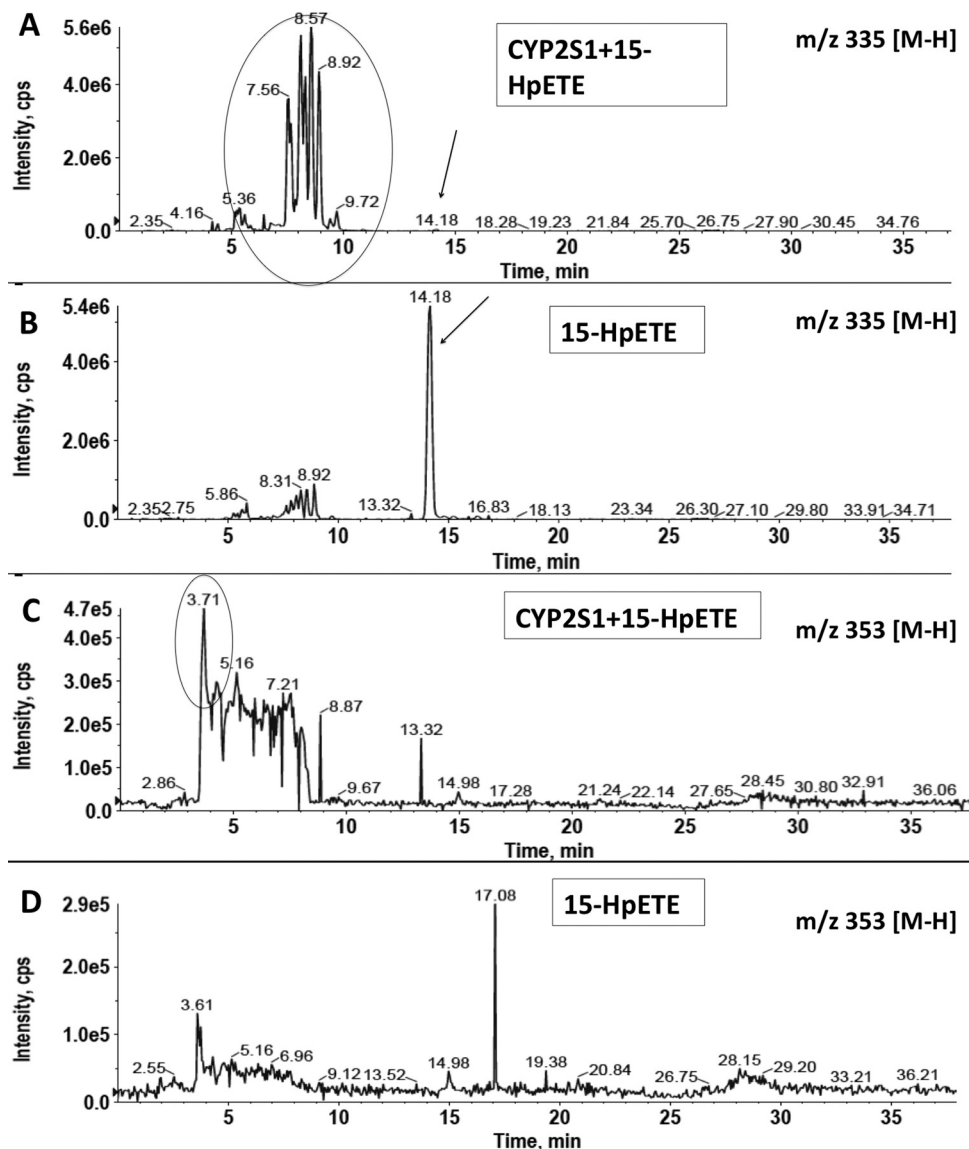


FIG. 8. Product detection of 15-HpETE metabolism by recombinant human CYP2S1 using mass spectrometry. LC-ESI-MS/MS using SIM with a negative ion mode [M - H]<sup>-</sup> on a 15-HpETE sample incubated with purified CYP2S1 (A and C) or without CYP2S1 (B and D). Products were detected by selection of *m/z* 335 [M - H]<sup>-</sup> (A and C) and *m/z* 353 [M - H]<sup>-</sup> (B and D). The *m/z* [M - H]<sup>-</sup> of 15-HpETE is 335, the *m/z* [M - H]<sup>-</sup> of epoxyalcohol or hexoxilin is also 335, and the *m/z* [M - H]<sup>-</sup> of trihydroxy derivatives or trioxilins is 353. The arrows indicate 15-HpETE and the circles indicate epoxyalcohols and trioxilins.

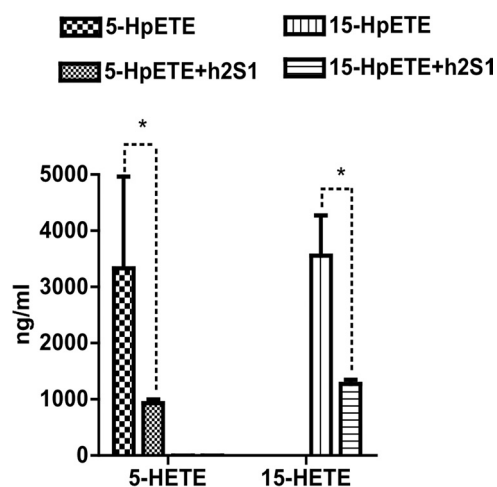


FIG. 9. CYP2S1 reduces the generation of 5-HETE and 15-HETE from 5-HpETE and 15-HpETE. 5-HpETE or 15-HpETE (50  $\mu$ M) was incubated with 0.1  $\mu$ M purified recombinant human CYP2S1 or without enzyme for 10 min. Concentrations were determined by LC-ESI-MS/MS with the MRM negative ion mode. Data are presented as means  $\pm$  S.D. of triplicate determinations. \*,  $p < 0.05$ .

several products detected by HPLC with UV detection or by mass spectrometry (Figs. 6–8). Figure 6 shows that one of the products from each hydroxyperoxy fatty acid has strong UV absorbance at 279 nm, and these products were identified using authentic standards as the keto fatty acids, 5-oxoETE, 12-oxoETE, 15-oxoETE, and 13-oxoODE, respectively. These products are generated via homolytic cleavage of the O–O bond in the hydroperoxy group (Fig. 10). The formation of 13-oxoODE was decreased by more than 80% after human CYP2S1 was heat-treated, indicating that the reaction was enzymatic (Fig. 6D). Similar results were observed with the 5-, 12-, and 15-HpETE incubations with heat-treated CYP2S1 (data not shown). Besides the oxoETEs, there were other more hydrophilic products generated having absorbance at 205 nm that were not generated without CYP2S1 (Fig. 7) and also products having  $m/z$  335 (M – H) and  $m/z$  353 (M – H) as determined by LC-ESI-MS/MS using the SIM mode (Fig. 8). The products with  $m/z$  335 are the putative epoxyalcohols and those with  $m/z$  353 are the putative trihydroxy derivatives (trioxilins, the hydrolytic product of epoxyal-

ols), based on their molecular masses and the rationale of the homolytic O–O cleavage reaction of the hydroperoxy group of 15-HpETE (Fig. 10). This possibility is addressed further below. Figure 8 shows that 50  $\mu$ M 15-HpETE was entirely consumed by 0.2  $\mu$ M human CYP2S1 in 15 min, suggesting that CYP2S1 is very efficient in breaking down 15-HpETE (Fig. 8, A and B). Similar results were observed with 5-HpETE, 12-HpETE, and 13-HpODE incubated with CYP2S1, in which products with the  $m/z$  (M – H) of epoxyalcohols and the  $m/z$  (M – H) of trihydroxy derivatives were detected and for which the rates of substrate disappearance were similar to that of 15-HpETE (data not shown). HETEs are commonly derived from HpETE but by heterolytic O–O cleavage of the hydroperoxy group (Fig. 10) either spontaneously or mediated by peroxidase enzymes. However, our data indicate that CYP2S1 does not participate in the formation of HETEs because in the presence of CYP2S1, the amounts of the HETEs were less than those formed spontaneously (Fig. 9). Thus, human CYP2S1 effectively converts HpETEs and HpODE into oxoETEs and oxoODE, respectively, and into epoxyalcohols, but not into HETEs (Fig. 10).

**Kinetics of Keto Acid Product Formation.** OxoETEs have been demonstrated to be biologically active products of the lipoxygenase pathway. For example, 5-oxoETE has been shown to be a potent eosinophil chemotactic agent, to stimulate calcium mobilization (Powell et al., 1993), and to cause neutrophil degranulation and superoxide formation (Hevko et al., 2001). OxoETEs are generally thought to be generated from HETEs by specific NADP<sup>+</sup>-dependent dehydrogenases (Powell et al., 1992). However, there have been very few reports suggesting the involvement of P450s in oxoETE synthesis directly from HpETEs. 15-OxoETE was reported to be generated from 15-HpETE by rat epidermal microsomes in the absence of NADPH (Van Wauwe et al., 1992). CYP5A (thromboxane synthase) and CYP8A (prostacyclin synthase) (both P450 reductase-independent P450s) were reported to convert 15-HpETE to 15-oxoETE and epoxyalcohols (Yeh et al., 2007). To the best of our knowledge, no other P450s have been reported to participate in oxoETEs synthesis. However, we found that the dioxin-inducible P450s, CYP1A1, 1A2, and 1B1, convert 5-, 12-, and 15-HpETE and 13-HpODE to the respective oxoETEs and oxoODE metabolites at relatively efficient rates, although their turnover rates are slower than that of CYP2S1 for all the fatty acid hydroperoxides tested (Table 2). To further determine the

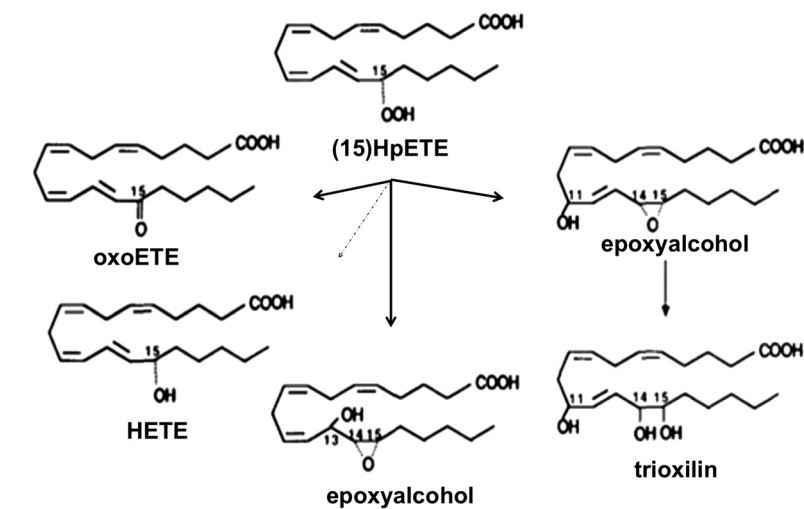


FIG. 10. Reaction scheme for fatty acid hydroperoxide metabolism by CYP2S1. Bold lines indicate reactions catalyzed by CYP2S1; dashed lines represent spontaneous reactions and/or reactions catalyzed by other enzymes.

—→ via homolytic O–O cleavage by CYP2S1  
 - - - - -→ via heterolytic O–O cleavage, non-enzymatic or peroxidase,

TABLE 2

Turnover numbers of 5-, 12-, and 15-oxoETE and 13-oxoODE from the metabolism of 5-, 12-, and 15-HpETE and 13-HpODE, respectively, by CYP1A1, 1A2, 1B1, and 2S1

Turnovers are expressed as nanomole of 12-HHT formed per nanomole of P450 per minute. Data are presented as means  $\pm$  S.D. ( $n = 3$ ).

P450	Turnover			
	5-OxoETE	12-OxoETE	15-OxoETE	13-OxoODE
	$\text{min}^{-1}$			
CYP1A1	11.3 $\pm$ 0.6	6.1 $\pm$ 0.4	10.8 $\pm$ 0.4	7.6 $\pm$ 0.4
CYP1A2	14.4 $\pm$ 0.9	5.2 $\pm$ 0.2	8.6 $\pm$ 0.4	4.8 $\pm$ 0.2
CYP1B1	11.8 $\pm$ 0.3	6.0 $\pm$ 0.3	11.2 $\pm$ 0.6	7.4 $\pm$ 0.3
CYP2S1	16.7 $\pm$ 0.3	11.5 $\pm$ 0.9	16.9 $\pm$ 0.8	20.2 $\pm$ 0.9

kinetic parameters of CYP2S1 toward each substrate, we calculated the corresponding  $K_m$  values (Table 3). All of the  $K_m$  values are in the low micromolar range, suggesting that CYP2S1 may be relevant in metabolizing this endogenous class of chemicals *in vivo*. Of the four substrates tested, CYP2S1 is most efficient (highest turnover/ $K_m$  ratio) with 13-HpODE, followed by 15-HpETE, 5-HpETE, and 12-HpETE.

**Identification of Epoxyalcohols Using Tandem Mass Spectrometry.** The formation of the epoxyalcohols from unsaturated fatty acids in biological systems has been reported; however, their biological functions have not been well defined. In addition, several groups have reported that the formation of the epoxyalcohols is mediated by P450s (Weiss et al., 1987; Chang et al., 1996). We showed that CYP2S1 can isomerize fatty acid hydroperoxides into several epoxyalcohols (Fig. 8), and formation of these products was shown to be enzymatic because heat treatment of CYP2S1 reduced their formation significantly (data not shown). Using tandem mass spectrometry with negative ESI after the collisional activation of the carboxylate ions ( $M - H$ ), we identified several 5-, 12-, and 15-HpETE epoxyalcohol products having  $m/z$  of 335 and 13-HpODE products having  $m/z$  of 311. In addition, based on the tandem mass spectrometry data, we can make predictions about the structures of the epoxyalcohol products. These data are summarized in Table 4 (also see Supplemental Figs. 1–4).

The unique daughter ions formed after collisional activation of the parent  $[M - H]^-$  ion at  $m/z$  335 (Supplemental Fig. 1) indicate that there are at least four different isomers of epoxyalcohols generated from 5-HpETE by CYP2S1. Two of these isomers could be diastereomeric ( $\pm$ )-9-hydroxy-5,6-epoxy-7,11,14,-eicosatrienoic acid and the other two could be ( $\pm$ )-7-hydroxy-5,6-epoxy-8,11,14,-eicosatrienoic acid. For 12-HpETE there were five isomeric products detected (Supplemental Fig. 2). Three are probably isomers of 8-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid (hepoxilin A<sub>3</sub>), one is 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid (hepoxilin B<sub>3</sub>), and one is unknown. For 15-HpETE, there were at least four isomers produced (Supplemental Fig. 3). Three are probably isomers of 11-hydroxy-14,15-epoxy-5,8,12-eicosatrienoic acid, and one is likely to be an isomer of 13-hydroxy-14,15-epoxy-5,8,11,-eicosatrienoic acid.

TABLE 3

$K_m$  and efficiency of CYP2S1 toward the fatty acid hydroperoxides

Michaelis-Menten plots for each compound are presented in Supplemental Fig. 6.

Substrate	$K_m$	Efficiency
	$\mu\text{M}$	$\mu\text{M}^{-1} \cdot \text{min}^{-1}$
5-HpETE	18.0 $\pm$ 3.5	0.93
12-HpETE	38.8 $\pm$ 5.5	0.296
15-HpETE	42.0 $\pm$ 4.2	0.401
13-HpODE	14.1 $\pm$ 1.5	1.43

For 13-HpODE, there were four detectable isomers produced (Supplemental Fig. 4). Three are probably isomers of 9-hydroxy-12,13-epoxy-10-octadecenoic acid and one is an isomer of 11-hydroxy-12,13-epoxy-9-octadecenoic acid. Although we were able to make informed predictions of the structures of the epoxyalcohol products by tandem mass spectrometry, the exact chemical structures with chiral configurations of these products and their quantity could not be determined in this study because of the unavailability of standards for these compounds.

## Discussion

PGG<sub>2</sub> and PGH<sub>2</sub> are generated from arachidonic acid by COX1 and COX2 and represent intermediates in the prostanoid biosynthesis pathway. We demonstrate here that recombinant human CYP2S1 effectively metabolizes PGG<sub>2</sub> and PGH<sub>2</sub> into several products including 12-HHT, TXA<sub>2</sub>, and several unidentified products. Our data suggest that PGG<sub>2</sub> may be a physiologically preferential CYP2S1 substrate because of its nanomolar  $K_m$ . One of the unidentified products of PGG<sub>2</sub> was speculated to be either 15-oxo-PGG<sub>2</sub> or 12-oxo-HT. Based on the mass spectrometry analysis, we suggest 15-oxo-PGG<sub>2</sub> (Supplemental Fig. 6). There is little known about the biological activity of this product. Although we did not directly measure the formation of MDA in our reactions, it is the expected by-product when 12-HHT is generated (Plastaras et al., 2000). MDA has been shown to be mutagenic and reacts with DNA bases to produce adducts of deoxyguanosine (M1G), deoxyadenosine (M1A), and deoxycytidine (M1C) (Niedernhofer et al., 2003; Wang et al., 2004).

TXA<sub>2</sub> is the most well studied among the above-mentioned products, has been shown to have bronchial constriction activity, and has been implicated in the pathogenesis of airway hyperresponsiveness (Barnes et al., 1992; Yeh et al., 2007). Although human CYP2S1 can convert PGH<sub>2</sub> into TXA<sub>2</sub>, its rate of conversion is slower than that of thromboxane synthase (CYP5A) (Yeh et al., 2007) and slower than its own rate of catalysis of 12-HHT. It therefore seems unlikely that CYP2S1 is significantly involved in TXA<sub>2</sub> synthesis *in vivo*. However, our data do suggest that CYP2S1 may be involved in eliminating PGG<sub>2</sub> and PGH<sub>2</sub>, making them unavailable for the downstream synthesis of active prostanoids. This postulate was supported by our observation that there was a considerable diminution in the amounts of PGE<sub>2</sub> and PGD<sub>2</sub> (the two active prostanoids derived from PGH<sub>2</sub>) formed when PGH<sub>2</sub> was incubated with recombinant human CYP2S1 or lysates of mammalian cells overexpressing human CYP2S1. The fact that CYP2S1 and the COX enzymes (1 and 2) are all localized in the membranes of the endoplasmic reticulum (Rouzer and Marnett, 2003; Mbonye et al., 2006) is consistent with the notion that CYP2S1 can interact with COX-derived products.

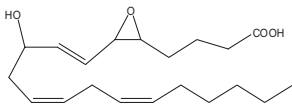
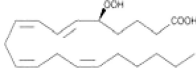
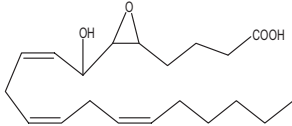
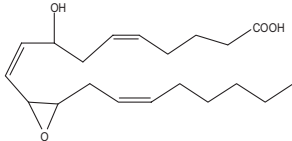
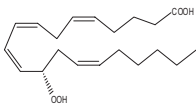
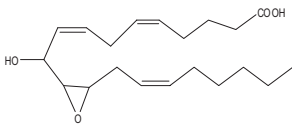
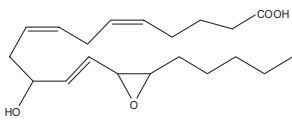
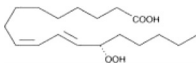
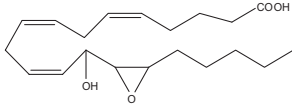
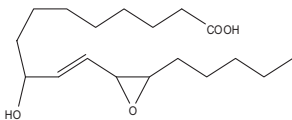
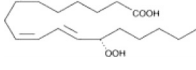
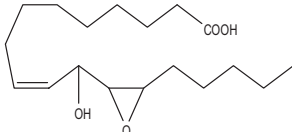
Plastaras et al. (2000) reported that several P450s are capable of converting PGH<sub>2</sub> into 12-HHT and MDA and that this conversion is an enzymatic process and not catalyzed by heme or other heme-containing proteins. Our results are consistent with these findings because we showed that CYP1A1, 1B1, and 3A4 are all capable of converting PGH<sub>2</sub> into 12-HHT and MDA, in addition to CYP2S1. Although CYP2S1 is therefore not the only P450 responsible for the conversion of PGH<sub>2</sub> to 12-HHT and MDA, it is, however, one of the most abundant and widely distributed extrahepatic P450 enzymes (Rylander et al., 2001; Saarikoski et al., 2005). In addition, in certain tissues such as the human lung, the level of CYP2S1 mRNA transcript is higher than that of CYP3A4 and CYP1A2 (Bièche et al., 2007). This finding suggests that the impact of CYP2S1 on 12-HHT and MDA synthesis may be tissue-specific.



TABLE 4

Summary of the epoxy alcohol products generated from HpETEs and HpODE by human CYP2S1

Chemical structures were predicted from LC-ESI-MS/MS (see supplemental data).

Substrate	Product	RT <i>min</i>	Structure without Stereochemistry
5-HpETE	Isomeric 9-hydroxy-5,6-epoxy-7,11,14,- eicosatrienoic acid (9-OH-5,6-O-ET)	9.28 and 12.92	
	Isomeric 7-hydroxy-5,6-epoxy-8,11,14,- eicosatrienoic acid (7-OH-5,6-O-ET)	13.23 and 13.72	
12-HpETE	Isomeric 8-hydroxy-11,12-epoxy-5,8,14- eicosatrienoic acid (hepoxilin A3)	11.5, 11.71, and 12.65	
	Isomeric 10-hydroxy-11,12-epoxy-5,8,14- eicosatrienoic acid (hepoxilin B3)	12.85	
15-HpETE	Isomeric 11-hydroxy-14,15-epoxy-5,8,12,- eicosatrienoic acid (11-OH-14,15-O-ET)	10.55, 11.22, and 11.76	
	Isomeric 13-hydroxy-14,15-epoxy-5,8,11,- eicosatrienoic acid (13-OH-14,15-O-ET)	12.11	
13-HpODE	Isomeric 9-hydroxy-12,13-epoxy-10-octadecenoic acid	9.77, 10.18, and 10.55	
	Isomeric 11-hydroxy-12,13-epoxy-9-octadecenoic acid	10.99, and 11.36	

RT, product retention time.

Besides the prostanoids, we demonstrate that CYP2S1 metabolizes another set of important fatty acid hydroperoxides, including HpETEs and HpODEs, which are derived from lipoxygenase-mediated metabolism of arachidonic acid and linoleic acid, respectively. The fatty acid hydroperoxides are intermediate precursors of many important inflammatory compounds, such as the leukotrienes (proinflammatory) and lipoxins (anti-inflammatory) (Levy et al., 2001; Nebert and Karp, 2008). Our *in vitro* data indicate that CYP2S1 efficiently performs one-electron oxidation of numerous HpETEs and HpODE, converting them into keto fatty acids (e.g., oxoETEs and oxoODE) with higher turnovers than other dioxin-inducible P450s (CYP1A1, 1A2, and 1B1) and higher turnovers than the enzyme's metabolism of PGG<sub>2</sub> and PGH<sub>2</sub>. In addition,

CYP2S1 isomerizes the fatty acid hydroperoxides into epoxyalcohols such as the hepoxilins.

Although there is limited information on the biological functions of these eicosanoid metabolites, there is evidence that several are involved in inflammation (Nigam et al., 2007; Pace-Asciak, 2009). 5-OxoETE was shown to have a role in the asthmatic inflammatory response and has been shown to stimulate neutrophil and eosinophil migration to airways (O'Flaherty et al., 1994, 1996; Jones, 2005). The potential function of 15-oxoETE is unclear. It was shown to be inactive in stimulating neutrophils (O'Flaherty et al., 1996). Likewise, 12-oxoETE, was shown to be inactive in increasing cytosolic calcium levels in human neutrophils, but its reduced form, 12-HETE, was a strong inducer for chemotaxis and cytosolic calcium release (Powell

et al., 1995). In addition, 12-oxoETE and hepoxilin B3 were reported to be the major eicosanoids in epidermis and to be elevated in psoriasis (Antón et al., 2002). This finding is of interest, because the CYP2S1 protein is present at high levels in the epidermis and is increased in psoriasis (Smith et al., 2003). 13-OxoODE was reported to have anti-inflammatory effects by directly interacting with the peroxisome proliferator-activated receptor  $\gamma$  (Altmann et al., 2007). Hepoxilin A3 was shown to be associated with inflammation (Mrsny et al., 2004). Whether CYP2S1 acts in anti- or proinflammatory fashion will depend on the substrates that it encounters.

The mechanism by which the hemoprotein cleaves the peroxide O–O bond plays a decisive role in determining the catalytic outcome of the corresponding reactions and is highly dependent on the nature of the P450 isoform, the chemical properties of the organic peroxide, and the nature of the oxygen acceptor (Capdevila et al., 2005). Prostacyclin synthase (CYP8A) and thromboxane synthase (CYP5A) have been shown to convert 15-HpETE into 15-HETE, 15-oxoETE, and 13-hydroxy-14,15-epoxy-5,8,11-eicosatrienoic acid (Yeh et al., 2007). The first product occurs via heterolytic cleavage of the O–O bond, whereas the last two result from homolytic cleavage. In our study, CYP2S1 did not metabolize 15-HpETE or other HpETEs to the corresponding HETEs, indicating that CYP2S1 does not heterolytically cleave the O–O bond but exclusively catalyzes homolytic cleavage of the O–O bond. Evidence in support of this notion was also obtained in our previous studies (Bui et al., 2009), in which CYP2S1 HpETE-dependent oxidation of benzo[a]pyrene-7,8-diol was 100% inhibited by the antioxidant, butylated hydroxyanisole. Thus, CYP2S1 may divert the metabolism of arachidonic acid away from HETEs by converting HpETEs into oxoETEs and epoxy alcohols. This result is of interest because several studies suggest that 5-, 12-, and 15-HETEs are inflammatory mediators and that their levels are elevated in several inflammatory diseases such as asthma and atherosclerosis (Burhop et al., 1988; Damon et al., 1989; Rubin and Mollison, 2007).

In conclusion, this report presents the first identification of endogenous substrates for CYP2S1. Our data suggest that human CYP2S1 is an important enzyme in the metabolism of some of the key intermediate eicosanoids of the COX and lipoxygenase pathways and thus suggest that CYP2S1 may play an important role in modulating the inflammatory process. However, in vivo studies will be needed to confirm this finding and to establish whether CYP2S1 acts in a pro- or anti-inflammatory fashion.

While our article was under review, Nishida et al. (2010) reported that CYP2S1 is reduced by NADPH in the presence of NADPH cytochrome P450 reductase and under these conditions it can reduce the anticancer agent 1,4-bis[2-(dimethylamino-*N*-oxide)ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione (AQ4N). However, they used anaerobic conditions for these experiments, whereas we used aerobic conditions. In addition, they used rat cytochrome P450 reductase, whereas we used the human reductase. These differences may explain the discrepancies between our previous observations and theirs. Possible reasons for these discrepancies were also discussed by Gomez et al. (2010).

#### Authorship Contributions

Participated in research design: Bui.

Conducted experiments: Bui, Imaizumi, and Beedanagari.

Performed data analysis: Bui and Imaizumi.

Wrote or contributed to the writing of the manuscript: Bui, Imaizumi, Reddy, and Hankinson.

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