Fatty Acid Hydroperoxides Support Cytochrome P450 2S1-Mediated Bioactivation of Benzo[a]pyrene-7,8-dihydrodiol

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ABSTRACT

In the accompanying report (p. 1031), we showed that a novel dioxin-inducible cytochrome P450, CYP2S1, efficiently metabolizes benzo[a]pyrene-*trans*-7,8-dihydrodiol (BaP-7,8-diol) into the highly mutagenic and carcinogenic benzo[a]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide (BaP-diol-*t*-epoxide), using cumene hydroperoxide in lieu of NADPH/O₂. Lipid hydroperoxide-supported P450 oxidation has been reported in several cases. However, it has not yet been described for the bioactivation of BaP-7,8-diol. In this report, we demonstrate that CYP2S1 can use various fatty acid hydroperoxides to support epoxidation of BaP-7,8-diol at a much higher rate than with cumene hydroperoxide. Kinetic analyses with several fatty acid hydroperoxides revealed that 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13-HpODE) was the most potent oxidant tested (K_m , $3.4 \pm 0.8 \mu\text{M}$; turnover, $4.51 \pm 0.13 \text{ min}^{-1}$), followed by 12S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (K_m , $2.8 \pm 0.7 \mu\text{M}$; turnover, $3.7 \pm 0.1 \text{ min}^{-1}$), 5S-hydroperoxy-6E,8Z,11Z,14Z-

eicosatetraenoic acid (K_m , $2.7 \pm 0.8 \mu\text{M}$; turnover, $3.69 \pm 0.09 \text{ min}^{-1}$), and 15S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (K_m , $11.6 \pm 0.3 \mu\text{M}$; turnover, $0.578 \pm 0.030 \text{ min}^{-1}$). The antioxidant butylated hydroxyanisole inhibited CYP2S1-catalyzed epoxidation by 100%, suggesting that epoxidation proceeds by a free radical mechanism. Other cytochromes P450, including CYP1A1, CYP1B1, CYP1A2, and CYP3A4, were also able to epoxidize BaP-7,8-diol using various fatty acid hydroperoxides, although at slower rates than CYP2S1. The cytotoxicity of BaP-7,8-diol significantly increased in mammalian cells overexpressing CYP2S1, and BaP-diol-*t*-epoxide formation in these cells also increased in the presence of 13-HpODE. Together, these results suggest that fatty acid hydroperoxides can serve as physiological cofactors in supporting in vivo CYP2S1-catalyzed oxidation of BaP-7,8-diol, and that fatty acid hydroperoxides and CYP2S1 may play important roles in benzo[a]pyrene-induced carcinogenesis.

Benzo[a]pyrene (BaP) is a ubiquitous environmental pollutant produced during combustion, including the burning of cigarettes. It has been identified in ambient air, surface water, drinking water, waste water, and char-broiled foods and is classified as a human carcinogen by the International Agency for Research on Cancer (1983). Exposure can occur by ingestion, inhalation, or dermal absorption (Agency for Toxic Substances and Disease Registry, 1990). For BaP to be carcinogenic, it must be converted by oxidative metabolism to

mutagenic and carcinogenic derivatives (Wislocki et al., 1976; Wood et al., 1976; Gelboin, 1980) (see Scheme 1). The first step in this process is the formation of BaP-*trans*-7,8-epoxide, followed by hydrolysis to the BaP-*trans*-7,8-dihydrodiol (BaP-7,8-diol). The latter metabolite is further epoxidized to the mutagenic BaP-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide (BaP-diol-*t*-epoxide), which is extremely reactive with DNA and protein. BaP-diol-*t*-epoxide is very unstable, because it rapidly undergoes hydrolysis to BaP-*r*-7,*t*-8,*t*-9,*t*-10-tetrahydrotetrol (*r*7,*t*8,*t*9,*t*10-tetrol) and BaP-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrotetrol (*r*7,*t*8,*t*9,*c*10-tetrol), whose detection is indicative of BaP-diol-*t*-epoxide formation. The process of BaP bioactivation usually involves cytochromes P450, particularly members of the CYP1 family (Gelboin, 1980; Bauer et al., 1995; Kim et al., 1998). However, the epoxidation of

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ABBREVIATIONS: BaP, benzo[a]pyrene; BaP-7,8-diol, BaP-*trans*-7,8-dihydrodiol; BaP-diol-*t*-epoxide, BaP-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide; *r*7,*t*8,*t*9,*t*10-tetrol, BaP-*r*-7,*t*-8,*t*-9,*t*-10-tetrahydrotetrol; *r*7,*t*8,*t*9,*c*10-tetrol, BaP-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrotetrol; P450, cytochrome P450; HPLC, high-performance liquid chromatography; 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; BHA, butylated hydroxyanisole; COX, cyclooxygenase; MSCV, murine stem cell virus; EGFP, enhanced green fluorescent protein; IRES, internal ribosome entry site.

BaP-7,8-diol also occurs in mammalian tissue extracts during prostaglandin H synthesis (Marnett et al., 1979; Sivara-jah et al., 1979; Marnett and Bienkowski, 1980) and as a result of microsomal lipid peroxidation (Dix and Marnett, 1983).

Cytochromes P450 are heme-thiolate enzymes, functionally classified as monooxygenases (EC 1.14.14.1), which catalyze a wide spectrum of reactions, including heteroatom oxygenation, carbon hydroxylation, epoxidation, and dehalogenation. P450s play a critical role in the bioactivation and detoxication of a wide variety of xenobiotic substances, including environmental toxicants and therapeutic compounds, and also participate in the metabolism of a variety of endogenous compounds, including fatty acids, steroids, eicosanoids, bile acids, and fat-soluble vitamins. One of the most recently discovered human P450s is CYP2S1. It was identified by a homology search in the human genome (Rylander et al., 2001) and by representational difference analysis, a subtractive hybridization procedure used to isolate novel dioxin-inducible genes (Rivera et al., 2002). CYP2S1 has also been shown to be inducible by hypoxia, via hypoxia inducible factor, as well as by all-*trans*-retinoic acid (Smith et al., 2003; Rivera et al., 2007). Moreover, high levels of CYP2S1 expression were detected in smokers' lungs (Thum et al., 2006) and in epithelial cells of tissues that are exposed to the environment, including skin and the respiratory, urinary, and gastrointestinal tracts (Saarikoski et al., 2005b). In addition, CYP2S1 seems to be overexpressed in several tumor types (Downie et al., 2005; Kumarakulasingham et al., 2005; Saarikoski et al., 2005b). Thus, based on its mode of regulation and sites of expression, CYP2S1 has been suggested to have a potential role in the metabolism of carcinogens (Saarikoski et al., 2005a). To date, however, only very limited data on the function of CYP2S1 have been reported.

In the accompanying report (Bui and Hankinson, 2009), we report that CYP2S1 cannot use NADPH for oxidative metabolism because of its inability to accept electrons from NADPH. However, CYP2S1 can use cumene hydroperoxide and hydrogen peroxide to oxidize several carcinogens, including BaP-7,8-diol. Although cumene hydroperoxide supported CYP2S1-catalyzed oxidation, it is not present endogenously and therefore does not serve as a physiological cofactor. Although hydrogen peroxide is found endogenously, and could potentially be a relevant physiological cofactor for CYP2S1, the concentration necessary for effective oxidation *in vivo* is probably considerably greater than endogenous levels under

most circumstances (Bui and Hankinson, 2009). On the other hand, lipid hydroperoxides have been shown in many cases to support cytochrome P450-catalyzed oxidations both *in vivo* and *in vitro* and have been suggested to serve as physiological cofactors for cytochrome P450-mediated oxidations (Dix and Marnett, 1983; Muindi and Young, 1993; Wang and Liehr, 1994; Smith et al., 1995; McCallum et al., 1996; Anari et al., 1997; Tsai et al., 2001; Caro and Cederbaum, 2006). With the ultimate objective of establishing a physiological role for CYP2S1, we investigated in the current report whether this enzyme can use various fatty acid hydroperoxides to oxidize BaP-7,8-diol to the mutagenic BaP-diol-*t*-epoxide. In addition, we sought to determine whether certain other P450s, including dioxin-inducible P450s (i.e., CYP1A1, CYP1A2, and CYP1B1), can also epoxidize BaP-7,8-diol in a lipid hydroperoxide-dependent fashion. We compared the rates of oxidation of BaP-7,8-diol by these P450s with that of CYP2S1 and with the rates of the corresponding NADPH-dependent reactions. Our studies point to the importance of lipid hydroperoxides in benzo[a]pyrene-mediated carcinogenesis.

Materials and Methods

Chemicals and Reagents. Recombinant human CYP1A1, CYP1A2, and CYP3A4 were obtained from Biocatalytics, Inc (Pasadena, CA). All three products also contained the P450 reductase. CYP1B1, including P450 reductase, was purchased from BD Gentest (Woburn, MA). Purified synthetically encoded CYP2S1 was prepared as described in the accompanying article (Bui and Hankinson, 2009). BaP-7,8-diol, *r*,7,*t*,8,*t*,9,*c*,10-tetrol, *r*,7,*t*,8,*t*,9,*t*,10-tetrol, and BaP-diol-*t*-epoxide were purchased from the National Cancer Institute's Chemical Carcinogen Repository (Kansas City, MO). 5*S*-Hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HpETE), 12*S*-hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12-HpETE), 15*S*-hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (15-HpETE), and 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13-HpODE) were purchased from Cayman Chemical (Ann Arbor, MI). HPLC solvents (HPLC grade) were obtained from Sigma Aldrich (St. Louis, MO). All carcinogens were handled with the extreme care appropriate for work with such compounds. c33 cells were derived in our laboratory from the mouse hepatoma cell line, Hepa-1 cells (Lei et al., 2001).

Enzyme Assays. All assays were conducted in 1.5-ml Eppendorf tubes in duplicate, except for those with the c33 cell lysate, which were performed in replicates of four. The purified CYP2S1 used in all enzyme assays was the synthetically encoded protein (Bui and Hankinson, 2009). In experiments designed to determine whether lipid hydroperoxides can support CYP2S1-, CYP1A1-, CYP1A2-, CYP1B1-, or CYP3A4-mediated oxidation of BaP-7,8-diol, 50- μ l re-

action mixtures consisted of 100 mM potassium phosphate buffer, pH 7.5; 0.1 μ M P450; 50 μ M 5-HpETE, 12-HpETE, 15-HpETE, or 13-HpODE; and 100 μ M BaP-7,8-diol. Reactions were carried out at 30°C for 1 min and stopped with 50 μ l of acetonitrile containing 2% acetic acid. Samples were centrifuged at 13,000g for 15 min to remove protein, and supernatants were analyzed using HPLC. To determine whether oxidation of BaP-7,8-diol by CYP2S1 and lipid hydroperoxides was enzymatic, CYP2S1 was heat-treated for 20 min at 85°C before analysis. To determine whether the oxidations occurred via a free radical mechanism, 200 μ M butylated hydroxyanisole (BHA), an antioxidant, was used to trap free radicals.

To determine whether lysates from the c33-derived cell lines (c33-MSCV and c33-H2S1) could epoxidize BaP-7,8-diol using either lipid hydroperoxide or NADPH, 150 μ l of a 1-ml lysate prepared from 5×10^6 cells was incubated with 100 μ M BaP-7,8-diol $\pm$ 20 μ M 13-HpODE, or a NADP/NADPH regeneration buffer (Bui and Hankinson, 2009). Reactions were incubated at 37°C for 5 min for 13-HpODE, or 60 min for NADPH, before being stopped with 150 μ l of acetonitrile (in 2% acetic acid). Samples were analyzed by HPLC to quantify the total amount of 7r,8,t9,c10-tetrol formed.

Analytical Procedures and Product Identification. All samples were analyzed using HPLC. 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). A C₁₈ reversed-phase column (Discovery C₁₈, 2.2×150 mm, 5 μ m; Shimadzu) plus C₁₈ guard column was used to separate BaP-7,8-diol and its metabolites. Solvents were held at 30% B from 0 to 3 min, increased to 85% B from 3 to 20 min, and then held for another 5 min, before returning to the starting condition (solvent A: H₂O; solvent B, 100% acetonitrile; flow rate, 0.4 ml/min). The 7r,8,t9,c10-tetrol product, which is indicative of BaP-diol-t-epoxide formation, was monitored at 340 nm with a diode array detector and with the fluorescence detector setting at 340 nm/402 nm (excitation/emission). The 7r,8,t9,c10-tetrol was confirmed using an authentic product standard, and its concentration was calculated based on that standard curve.

K_m and Turnover Determinations. To determine the K_m of various lipid hydroperoxides for CYP2S1, 50 μ M and six progressive 1:3 dilutions of the lipid hydroperoxides were incubated in 50- μ l reaction mixtures containing 100 μ M BaP-7,8-diol and 100 nM CYP2S1. All the reactions were terminated after 90 s at room temperature by the addition of 50 μ l of ice-cold acetonitrile containing 2% acetic acid. The rates of 7r,8,t9,c10-tetrol formation (nanomoles per minute) were calculated from HPLC data using known concentrations of the authentic standard. The K_m of each substrate was calculated using the Prism software with nonlinear regression and the Michaelis-Menten equation [$V = V_{\max} \cdot S/(K_m + S)$].

To determine the turnover numbers of 7r,8,t9,c10-tetrol with CYP2S1, CYP1A1, CYP1A2, CYP1B1, and CYP3A4 using various lipid hydroperoxides, 0.1 μ M P450 was incubated with 100 μ M BaP-7,8-diol and 50 μ M concentrations of each lipid hydroperoxide. The reactions were incubated for 90 s at room temperature before being stopped with the same volume of ice-cold acetonitrile (2% acetic acid). Turnover numbers were calculated as nanomoles of 7r,8,t9,c10-tetrol formed per nanomole of P450 per minute, or simply expressed per minute.

Construction of the Mammalian Cell Line c33, Overexpressing Human CYP2S1. The human CYP2S1 cDNA was amplified by polymerase chain reaction using the following primers: human CYP2S1: forward, 5'AAGGAAAAAAGCGGCCGCAAAAG-GAAAACCATGGAGGCG ACCGGCACC-3'; reverse, 5'ATAAGAAT-GCGGCCGCTAAACTATCATCTGGTCTG C GTGGTG GAG-3'. All primers incorporate a NotI restriction site. cDNAs were cloned into the pMSCV-IRES/EGFP retroviral plasmid (a kind gift of O. Witte, UCLA, Los Angeles, CA) using a NotI site (Fig. 6A). The empty plasmid and plasmid containing the CYP2S1 gene were cotrans-

ected with an ecotropic packaging plasmid into 293T cells. Recombinant viruses were harvested and used to infect the c33 cell line, which were derived in our laboratory from Hepa-1 cells, and lack Cyp1a1 activity as a result of a mutation in the corresponding gene (Lei et al., 2001). Infection rates ranged from 89 to 94%. The infected cells were subsequently sorted twice for high-level GFP expression with in each case the 5% of cells with the highest level of EGFP fluorescence being isolated. The pool of infected c33 cells were maintained in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Omega, Tarzana, CA), 100 U/ml penicillin, and 100 μ g/ml streptomycin solution (Gemini Bio-Products, West Sacramento, CA).

Preparation of Cell Lysates and Microsomes from Mammalian Cells. Lysates and microsomes were prepared as described by Schenkman and Jansson (1998), with some modifications. For the preparation of lysates, cells were washed twice with ice-cold phosphate-buffered saline and harvested by centrifugation at 800g for 5 min. Pellets were reconstituted in buffer and sonicated on ice using a VibraCell sonicator (Sonics and Materials, Inc., Newtown, CT) four times for 20 s each at 50% power. For immediate use, the reconstitution buffer was either the appropriate assay buffer or 100 mM phosphate buffer, pH 7.4, in both cases supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and 0.1 mg/ml phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). For the storage of cell lysates at -80°C, the buffer was further supplemented with 20% glycerol (Fisher Scientific, Pittsburgh, PA) and 0.1 mM EDTA (Fisher Scientific).

For the preparation of microsomes, cells were harvested as described above. Cell pellets were reconstituted in 100 mM phosphate buffer, pH 7.4, 0.25 M sucrose, and 0.1 mM EDTA, supplemented with 0.1 mg/ml phenylmethylsulfonyl fluoride and the protease inhibitor cocktail. Microsomes were isolated by differential centrifugation at 10,000g for 15 min at 4°C followed by centrifugation at 100,000g for 90 min at 4°C.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL) using bovine serum albumin as standard. For detection of CYP2S1, proteins were separated on a 10% Tris/glycine gel for Western blot analysis. The blots were blocked in 3% bovine serum albumin overnight at 4°C, and the CYP2S1 protein was identified using a rabbit polyclonal antibody against CYP2S1 (kindly provided by Roland Wolff, Biomedical Research Centre, University of Dundee, UK) at a 1:500 dilution. For Western blot analysis of mouse prostaglandin H synthases, also known as cyclooxygenase I and II (COX I and II), proteins were separated on a NuPAGE 4- to 12% Bis-Tris gel (Invitrogen). The COX I and II proteins were identified using rabbit polyclonal antibodies against mouse COX I and II (Cayman Chemical, Ann Arbor, MI) at a 1:500 dilution. The β -actin protein was used as control with a donkey antibody purchased from Sigma (St. Louis, MO) at a 1:2000 dilution. The secondary antibodies were goat anti-rabbit and goat anti-donkey (1:3000 dilution; Promega, Madison, WI), and the blots were visualized using enhanced chemiluminescence detection reagents (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Cytotoxicity Assays. C33 cells overexpressing human CYP2S1 (c33-H2S1) and mock-infected cells (c33-MSCV) were plated in 12-well plates in triplicate at a density of 100 cells/well. To determine whether BaP-7,8-diol could induce cell killing, 24 h after inoculation, the cells were treated with varying concentrations of BaP-7,8-diol (0, 1, 10, and 50 μ M). Colonies were allowed to grow for 10 days, after which time the cells were washed, fixed in 100% methanol, stained with crystal violet, and counted.

In Vivo Metabolism of BaP-7,8-diol in c33-H2S1 Cells. C33 cells overexpressing human CYP2S1 (c33-H2S1) or infected with the parental vector (c33-MSCV) were plated in 6-well plates in triplicate at a density of 100,000 cells/well with 2 ml of media. To determine whether BaP-7,8-diol is epoxidized in these cells, the cells were treated with 40 μ M BaP-7,8-diol 72 h after inoculation. The incuba-

tions were allowed to continue for 16 h, after which time cell metabolism was stopped with 2 ml of acetonitrile containing 2% acetic acid. The dead cells were lifted from the plate, and the mixture containing cells and media was collected, followed by vigorous vortexing for 1 min. The supernatants containing BaP-7,8-diol and metabolites were collected by centrifugation at 13,000g for 30 min. Samples were then analyzed by HPLC to quantify the total amount of 7r,8,t9,c10-tetrol formed.

Statistical analysis. The Student's *t* test was used for all statistical analyses (Prism software; GraphPad Software, San Diego, CA).

Results

Fatty Acid Hydroperoxides Can Support Oxidation of BaP-7,8-diol by CYP2S1. In the accompanying report (Bui and Hankinson, 2009), we show that NADPH cannot support the oxidative activity of CYP2S1. Instead, its epoxidation of BaP-7,8-diol was facilitated by cumene hydroperoxide or hydrogen peroxide (Bui and Hankinson, 2009). Fatty acid hydroperoxides have been reported to be capable of serving as oxygen surrogates for P450-dependent mono-oxygenation (Wang and Liehr, 1994; Anari et al., 1997). We

therefore tested whether CYP2S1 can use certain of these biological peroxides to oxidize BaP-7,8-diol to the mutagenic BaP-diol-*t*-epoxide. 5-HpETE, 12-HpETE, 15-HpETE, and 13-HpODE did in fact support the oxidation of BaP-7,8-diol by purified human CYP2S1. Formation of BaP-diol-*t*-epoxide (detected as 7r,8,t9,c10-tetrol) occurred only in the presence of both fatty acid hydroperoxides and CYP2S1 (Fig. 1, A–D). Although BaP 7r,8,t9,c10-tetrol was a major metabolite, other unidentified fluorescent products were detected at 345 nm. Very little 7r,8,t9,t10-tetrol, an enantiomer of 7r,8,t9,c10-tetrol, was detected (data not shown).

Mechanism of Fatty Acid Hydroperoxide-Dependent CYP2S1-Catalyzed Epoxidation of BaP-7,8-diol. Prostaglandin H synthase has been reported to oxidize BaP-7,8-diol in a hydroperoxide-dependent fashion during prostaglandin synthesis. The reaction involves the iron-porphyrin prosthetic group of the prostaglandin H enzyme (Marnett et al., 1979; Sivarajah et al., 1979; Marnett and Bienkowski, 1980), and occurs via a free radical mechanism. In the presence of the antioxidant BHA, the oxidation of BaP-7,8-diol by

Fig. 1. Fatty acid hydroperoxides support CYP2S1-catalyzed oxidation of BaP-7,8-diol. HPLC chromatograms of 0.1 μ M CYP2S1 incubated with 100 μ M BaP-7,8-diol and 50 μ M 5-HpETE (A), 12-HpETE (B), 15-HpETE (C), or 13-HpODE (D). i, CYP2S1 plus BaP-7,8-diol and fatty acid hydroperoxide; ii, CYP2S1 and BaP-7,8-diol; iii, BaP-7,8-diol and fatty acid hydroperoxide control; iv, 7r,8,t9,c10-tetrol authentic standard metabolite, which is indicated by the arrow. Detection was set at 345 nm.

Fig. 2. CYP2S1 activity is inhibited by the antioxidant, butylated hydroxyanisole (BHA). A, HPLC chromatograms of the incubation of CYP2S1 in the presence of BaP-7,8-diol and 15-HpETE and in the presence (iii) or absence (i) of BHA. CYP2S1 with BaP-7,8-diol but without 15HpETE was used as control (ii). The peak at 5 min in this last mixture is ascribable to the parent compound, BaP-7,8-diol, and its value was subtracted from peaks i and iii. B, inhibition of CYP2S1-mediated BaP-7,8-diol oxidation by BHA is expressed as the percentage of 7r,8,t9,c10-tetrol formed (as determined from A) in the presence of BHA, compared with that in the absence of BHA.

CYP2S1 and 15-HpETE was inhibited 100% (Fig. 2), indicating that BaP-7,8-diol oxidation by CYP2S1 occurs via a free radical mechanism.

We also investigated whether epoxidation of BaP-7,8-diol by 15-HpETE occurred via an enzymatic or nonenzymatic process in the presence of CYP2S1. Heating at 85°C for 20 min CYP2S1 before adding BaP-7,8-diol and 15-HpETE reduced epoxidation by 75%, indicating the activity of an enzymatic process (Fig. 3). This was also observed when CYP2S1 was incubated with cumene hydroperoxide (data not shown). This residual epoxidation activity could be due to incomplete inactivation of CYP2S1 or to a nonenzymatic reaction from the heme released from denatured CYP2S1. High concentrations of hematin, (hydroxo-(porphyrinato)iron III), have been shown to catalyze the epoxidation of 7,8-diol BaP in the presence of 13-HpODE and detergent (Dix et al., 1985).

K_m and V_{max} Determinations for Various Fatty Acid Hydroperoxides with CYP2S1. We compared the effectiveness of all four fatty acid hydroperoxides in the CYP2S1-catalyzed epoxidation of BaP-7,8-diol by measuring their K_m and V_{max} values. 5-HpETE had the lowest K_m ($2.7 \pm 0.8 \mu\text{M}$) followed by 12-HpETE ($2.8 \pm 0.7 \mu\text{M}$), 13-HpODE (3.4 ± 0.8

μM), and 15-HpETE ($11.6 \pm 3 \mu\text{M}$) (Fig. 4). However, 13-HpODE is the most effective oxidant among the four fatty acid hydroperoxides tested, with the highest turnover ($4.51 \pm 0.13 \text{ min}^{-1}$), followed by 12-HpETE ($3.76 \pm 0.10 \text{ min}^{-1}$), 5-HpETE ($3.57 \pm 0.09 \text{ min}^{-1}$), and 15-HpETE ($0.578 \pm 0.030 \text{ min}^{-1}$) (Table 1). All four fatty acid hydroperoxides are more efficient in supporting CYP2S1-catalyzed epoxidation of BaP-7,8-diol than cumene hydroperoxide and H_2O_2 (Bui and Hankinson, 2009).

Fatty Acid Hydroperoxides Support the Epoxidation of BaP-7,8-diol by Other P450s. CYP1A1, CYP1A2, CYP1B1, and CYP2S1 are transcriptionally inducible by dioxin via the aromatic hydrocarbon receptor and its dimerization partner, the aromatic hydrocarbon receptor nuclear translocator (Hankinson, 1995; Rivera et al., 2002). CYP3A4 is the most abundant P450 in the liver, and it is responsible for the metabolism of a wide range of xenobiotics (Thummel et al., 1996). CYP1A1, CYP1A2, CYP1B1, and CYP3A4 have been reported to convert BaP-7,8-diol into both BaP-diol-*t*-epoxide and BaP-diol-*c*-epoxide in the presence of P450 reductase and NADPH (Gautier et al., 1996; Kim et al., 1998). However, we are not aware of any report investigating

Fig. 3. Heat treatment reduces CYP2S1 oxidation activity toward BaP-7,8-diol. A, HPLC chromatograms of BaP-7,8-diol and 15-HpETE incubation with CYP2S1 (i), heat-treated CYP2S1 (ii), or no CYP2S1 (iii). The peak at 5 min in this last incubation is ascribable to BaP-7,8-diol, and its value was subtracted from those for peaks i and ii. B, the loss of CYP2S1 activity due to heat treatment is calculated from the r7,t8,t9,c10-tetrol formation determined from chromatogram (A).

Fig. 4. Determination of the K_m and V_{max} values of 5-HpETE (A), 12-HpETE (B), 15-HpETE (C), and 13-HpODE (D) in supporting epoxidation of BaP-7,8-diol by CYP2S1. The rate of epoxidation of BaP-7,8-diol was determined as the 7r,8t,9t,10c-tetrol formed per minute by $0.1 \mu\text{M}$ CYP2S1. The K_m and V_{max} values were determined with the Prism software using nonlinear regression and the equation $V = V_{max} \cdot S / (K_m + S)$. Data are represented as averages $\pm$ S.E.M.

whether these P450s can use fatty acid hydroperoxides to epoxidize BaP-7,8-diol. In the presence of 5-HpETE, 12-HpETE, 15-HpETE, or 13-HpODE and in the absence of NADPH, we found that CYP1A1, CYP1A2, CYP1B1, and CYP3A4 could all oxidize BaP-7,8-diol to multiple products, including r7,t8,t9,c10-tetrol, which is indicative of the BaP-diol-*t*-epoxide (Fig. 5). To compare the kinetic parameters of CYP1A1, CYP1A2, CYP1B1, and CYP3A4 with those of CYP2S1 in epoxidizing BaP-7,8-diol, we determined their turnover numbers for the fatty hydroperoxides, derived in each case with the same concentrations of P450 proteins and BaP-7,8-diol (Table 1). CYP2S1 was the most efficient enzyme with 5-HpETE, 12-HpETE, and 13-HpODE and had approximately the same efficiency as the other P450s with 15-HpETE. Thus, of the aromatic hydrocarbon receptor/aromatic hydrocarbon receptor nuclear translocator battery P450s, CYP2S1 is the most active in lipid hydroperoxide-dependent metabolism of BaP-7,8-diol to the highly potent ultimate carcinogen, BP-diol-*t*-epoxide.

Kim et al. (1998) reported that CYP1A1 has a higher activity in the epoxidation of BaP-7,8-diol using NADPH (turnover, $1.75 \pm 0.29 \text{ min}^{-1}$) than CYP1B1 ($0.59 \pm 0.04 \text{ min}^{-1}$) or CYP1A2 ($0.21 \pm 0.02 \text{ min}^{-1}$). Our studies show that fatty acid hydroperoxides are more efficient than NADPH in supporting metabolism of BaP-7,8-diol by CYP1A2 (1.31 ± 0.14

min^{-1}) and CYP1B1 ($1.51 \pm 0.15 \text{ min}^{-1}$), but this is not the case for CYP1A1 ($1.20 \pm 0.09 \text{ min}^{-1}$).

Cytotoxicity Assessment of BaP-7,8-diol in Mammalian c33 Cells Overexpressing Human CYP2S1. c33 is a mutant derivative of the mouse hepatoma cell line Hepa-1 that lacks Cyp1a1 activity as a result of frame shift mutations in the coding regions of both *Cyp1a1* alleles (Lei et al., 2001). c33 cells were infected with the MSCV retroviral expression vector carrying human CYP2S1 cDNA (c33-H2S1). In Western blot analysis with a CYP2S1 antibody, the infected cells exhibited an additional 55-kDa band (corresponding to the estimated size of CYP2S1) that was not present in c33 cells or in c33 cells infected with the empty MSCV vector (c33-MSCV) (Fig. 6B). We investigated whether BaP-7,8-diol could induce cytotoxicity in c33-MSCV cells or c33-H2S1 cells. BaP-7,8-diol proved to be toxic to both c33-H2S1 and c33-MSCV cells (Fig. 6C). However, at the highest concentration tested ($50 \mu\text{M}$), BaP-7,8-diol induced more cell death in c33-H2S1 cells than in c33-MSCV cells ($P < 0.0001$; Fig. 7B). Thus, human CYP2S1 exacerbated the cytotoxicity of BaP-7,8-diol in c33 cells.

Epoxidation of BaP-7,8-diol by c33 Cells Overexpressing Human CYP2S1. We next investigated whether the cytotoxicity of BaP-7,8-diol in c33-H2S1 cells was related to the ability of CYP2S1 to epoxidize BaP 7,8-diol. Figure 7A

TABLE 1

Turnover numbers for CYP2S1 and several other cytochromes P450 for the epoxidation of BaP-7,8-diol using various fatty acid hydroperoxides. Data are represented as mean $\pm$ S.D.

P450	Turnover of 7r,8t,9t,10c-Tetrol			
	5-HpETE	12-HpETE	15-HpETE	13-HpODE
	nmol tetrol/nmol P450/min			
2S1	3.59 ± 0.09	3.76 ± 0.10	0.578 ± 0.030	4.51 ± 0.13
1A1	1.20 ± 0.09	0.328 ± 0.092	0.513 ± 0.018	0.734 ± 0.010
1A2	1.31 ± 0.14	1.26 ± 0.24	0.435 ± 0.017	1.19 ± 0.06
1B1	1.51 ± 0.15	0.930 ± 0.22	0.824 ± 0.042	1.22 ± 0.03
3A4	2.31 ± 0.34	0.970 ± 0.169	0.316 ± 0.010	1.31 ± 0.12

Fig. 5. HPLC chromatograms of epoxidation of BaP-7,8-diol supported by CYP1A1 (A), CYP1A2 (B), CYP1B1 (C), and CYP3A4 (D) in the presence of 15-HpETE. i, CYP1A1, CYP1A2, CYP1B1, or CYP3A4 with $20 \mu\text{M}$ 15-HpETE and $100 \mu\text{M}$ BaP-7,8-diol; ii, P450s with BaP-7,8-diol without 15-HpETE controls; iii, the standard, r7,t8,t9,c10-tetrol, which is indicated by an arrow. Other unknown products are indicated by question marks.

shows that lysates of c33-H2S1 cells generated r7,t8,t9,c10-tetrol when incubated with BaP-7,8-diol and 13-HpODE, whereas no increase in tetrol formation occurred in the absence of 13-HpODE or in the presence of NADPH (Fig. 7A). Lysates of c33-MSCV cells also generated r7,t8,t9,c10-tetrol when incubated with BaP-7,8-diol and 13-HpODE (data not shown), but they epoxidized BaP-7,8-diol less effectively than

c33-H2S1 cells ($p < 0.05$; Fig. 7B). This result was further supported by *in vivo* studies of BaP-7,8-diol metabolism, in which it was observed that there was significantly more r7,t8,t9,c10-tetrol produced in c33-H2S1 cells compared with c33-MSCV cells ($p < 0.05$; Fig. 7C). The epoxidation activity observed in c33-MSCV cells could be due to the presence of other P450s or prostaglandin H synthases (COX I and/or II)

NotI site was used for cloning.

Fig. 6. A, structure of the mammalian expression vector for human CYP2S1 (pMSCV-H2S1). The human CYP2S1 cDNA was cloned into the pMSCV-IRES/EGFP vector at the Not I site shown. B, Western blot analysis of c33 cells, c33 infected with the parental retroviral vector, MSCV, and c33 cells infected with pMSCV-H2S1, probed with a polyclonal antibody to CYP2S1. The band indicated by the arrow denotes human CYP2S1, which is approximately 55 kDa. C, toxicity of BaP-7,8-diol to c33-H2S1 and c33-MSCV cells. Cells were incubated with various concentration of BaP-7,8-diol (0, 1, 10, and 50 μ M). Cell viability was determined by counting number of colonies as described under *Materials and Methods*.

Fig. 7. Epoxidation of BaP-7,8-diol by lysates of c33-MSCV and c33-H2S1 cells. A, HPLC chromatograms of c33-H2S1 cells incubated with 13-HpODE and BaP-7,8-diol (i), BaP-7,8-diol alone (ii), or with BaP-7,8-diol and NADPH (iii). B, lysates of c33-H2S1 or c33-MSCV cells (5×10^6 cells/ml) were incubated with 20 μ M 13-HpODE and 100 μ M BaP-7,8-diol for 5 min at 37° C, formation of r7,t8,t9,c10-tetrol was assessed using HPLC. No increase in r7,t8,t9,c10-tetrol above the background occurred in the absence of 13-HpODE. Data are represented as average $\pm$ S.D. *, $P < 0.05$. C, c33-H2S1 or c33-MSCV cells (10^5 cells) were incubated with 40 μ M BaP-7,8-diol for 16 h. *In vivo* epoxidation of BaP-7,8-diol was measured by r7,t8,t9,c10-tetrol formation using HPLC. Data are represented as average $\pm$ S.D. *, $P < 0.05$. D, Western blot analysis of c33 cells, probed with COX I and II antibodies. COX II was not detected in c33 cells with a COX II antibody testing positive with a control extract (data not shown).

in c33 cells, because the latter enzymes are known to be capable of epoxidizing BaP-7,8-diol (Marnett and Bienkowski, 1980). COX I, a constitutively expressed protein, was detected in both c33-H2S1 and c33-MSCV cells, the level of this protein being the same or slightly lower in c33-H2S1 than in c33-M2S1 cells, whereas COX II, an inducible isoform, was not detectable in either cell type (Fig. 7D). The elevated level of BaP-7,8-diol epoxidation in c33-H2S1 are therefore not caused by prostaglandin H synthases (Fig. 7D). Thus, our data demonstrate that CYP2S1 can metabolize BaP-7,8-diol in mammalian cells using a fatty acid hydroperoxide rather than NADPH.

Discussion

Cytochrome P450 2S1 is a new dioxin-inducible P450. A biological role for human CYP2S1 has not yet been convincingly demonstrated. Previous reported studies have tested for substrates in the presence of NADPH and oxygen. The inability to consistently demonstrate an enzymatic activity for the protein in these studies is due to its inability to accept electrons from NADPH via P450 reductase (Bui and Hankinson, 2009), probably due, at least in part, to the fact that CYP2S1 lacks certain amino acids known to be important for the interaction of other P450s with the P450 reductase (Crespi and Miller, 1997; Schulze et al., 2000; Nikfarjam et al., 2006). However, we showed that CYP2S1 can oxidize several substrates using cumene hydroperoxide and hydrogen peroxide.

The activities of certain other P450s, including some microsomal P450s, do not depend on P450 reductase and NADPH. Examples include allene oxide synthase, thromboxane synthase (CYP5A), and prostacyclin synthase (CYP8A) (Brash and Song, 1995; Yeh et al., 2007). Furthermore, biological hydroperoxides such as fatty acid hydroperoxides have been shown to be capable of serving as oxygen surrogates for mono-oxygenation by many other cytochromes P450 (Dix and Marnett, 1983; Muindi and Young, 1993; Wang and Liehr, 1994; Smith et al., 1995; McCallum et al., 1996; Anari et al., 1997; Tsai et al., 2001; Caro and Cederbaum, 2006). For example, all-*trans*-retinoic acid was shown to be catabolized by various human P450s at a faster rate in the presence of lipid hydroperoxides than in the presence of NADPH/O₂ (Muindi and Young, 1993), rat liver microsomes were shown to use lipid hydroperoxide to oxidize BaP-7,8-diol (Dix and Marnett, 1983), and fatty acid hydroperoxides were demonstrated to serve as cofactors in the P450-mediated oxidation of estrogens to quinone metabolites (Wang and Liehr, 1994). In this study, we demonstrated that CYP2S1 uses various physiologically relevant fatty acid hydroperoxides for the oxidation of one of its substrates, BaP-7,8-diol. Fatty acid hydroperoxides are much more effective oxidant cofactors than cumene hydroperoxide or hydrogen peroxide in the epoxidization of BaP-7,8-diol by CYP2S1. In addition, the very low concentrations (K_m values in the low micromolar range) of fatty acid hydroperoxides necessary for the epoxidation of BaP-7,8-diol suggest that these compounds serve as cofactors supporting CYP2S1 oxidative metabolism in vivo, because the levels of certain of these lipid hydroperoxides in human exists in this range (Baer et al., 1990; Wang and Liehr, 1994; Mahle and Dasgupta, 1997; McLemore et al., 1998).

The relevance of CYP2S1 enzymatic activity is further

supported by the observation that incubation of c33 cells overexpressing human CYP2S1 (c33-H2S1) with BaP-7,8-diol resulted in a significant decrease in cell survival compared with control c33 cells (c33-MSCV). In addition, decreased cell survival of c33-H2S1 correlated with a higher level of BaP-7,8-diol epoxidation. Furthermore, we demonstrate that the CYP2S1-dependent conversion of BaP-7,8-diol to the r7,t8,t9,c10-tetrol in the mammalian cells depends on 13-HpODE, but not on NADPH.

Fatty acid hydroperoxides varied with respect to their effectiveness as oxidants in CYP2S1-dependent oxidations of BaP-7,8-diol. The turnovers of each fatty acid hydroperoxide apparently depend to some degree on the structure of the P450s, because 13-HpODE was the most effective with CYP2S1, followed by 5-HpETE, 12-HpETE, and 15-HpETE, whereas for CYP1A1, CYP1A2, CYP1B1, and CYP3A4, 5-HpETE was the most effective. It is noteworthy that 13-HpODE has been reported to be elevated in psoriasis (Baer et al., 1990), whereas the expression of CYP2S1 is also increased in psoriatic skin, and CYP2S1 has been suggested to have a role in all-*trans*-retinoic acid and therapeutic drug metabolism in patients with psoriasis (Smith et al., 2003). It is possible that 13-HpODE serves as a cofactor for CYP2S1-mediated elimination of antipsoriatic drugs in the skin (Smith et al., 2003). Besides fatty acid hydroperoxides, many other biological hydroperoxides exist in vivo, such as cholesterol hydroperoxide, pregnenolone 17 α -hydroperoxide, and progesterone 17 α -hydroperoxide. It will be of interest to investigate whether CYP2S1 can use these biological peroxides to oxidize its substrates in vivo.

The mechanism of fatty acid hydroperoxide-dependent CYP2S1-mediated oxidation of BaP-7,8-diol to the corresponding BaP-diol-*t*-epoxide probably occurs via alkoxy radicals, because the reaction was potently inhibited by an antioxidant, and alkoxy radicals have been shown to be a product of homolytic cleavage of hydroperoxides by P450s (Vaz and Coon, 1987). Epoxidation of BaP-7,8-diol has been considered mainly as a NADPH P450 reductase-dependent, P450-catalyzed reaction, principally involving CYP1A1, CYP1A2, and CYP1B1 (Kim et al., 1998). Although some studies have shown that these P450s are involved in peroxide-dependent oxidation metabolism of various substrates (Muindi and Young, 1993; Wang and Liehr, 1994), we know of no study reporting a role of lipid hydroperoxides in supporting epoxidation of BaP-7,8-diol by these P450s. We show here that CYP1A1, CYP1A2, CYP1B1, and CYP3A4 are able to efficiently epoxidize BaP-7,8-diol using various fatty acid hydroperoxides. The turnover for the conversion of BaP-7,8-diol to r7,t8,t9,c10-tetrol by CYP1A1 is comparable with that for its NADPH dependent activity reported by Kim et al. (1998). However, the rates of tetrol formation for the fatty acid hydroperoxide-dependent activities of CYP1A2 and CYP1B1 are several times higher than their NADPH-dependent reactions (Kim et al., 1998). Thus, lipid hydroperoxides may make an important contribution not only to the carcinogenesis of BaP-7,8-diol and benzo[a]pyrene but also to carcinogenesis mediated by other polycyclic aromatic hydrocarbons, including 7,12-dimethylbenzanthracene, styrene, and naphthalene, which we have shown to be metabolized by CYP2S1 via the peroxide shunt (Bui and Hankinson, 2009). Because CYP2S1 is much more efficient in epoxidation of BaP-7,8-diol than CYP1A1, CYP1A2, CYP1B1, or CYP3A4 in

the presence of lipid hydroperoxides, it may play a physiologically important role in benzo[a]pyrene metabolism and carcinogenesis.

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