Archives of Biochemistry and Biophysics
Vol. 376, No. 1, April 1, pp. 199–205, 2000
doi:10.1006/abbi.2000.1705, available online at http://www.idealibrary.com on IDEAL®

Identification of CYP2C9 as a Human Liver Microsomal Linoleic Acid Epoxygenase[1]

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Received October 19, 1999, and in revised form December 27, 1999

Leukotoxin (9,10-epoxy-12-octadecanoate) and isoleukotoxin (12,13-epoxy-9-octadecenoate) are monoepoxides of linoleic acid, synthesized by a cytochrome P450 monooxygenase and possibly by an oxidative burst of inflammatory cells. Recent experiments in this laboratory have indicated that the toxicity of leukotoxin and isoleukotoxin is not due to these epoxides, but to the 9,10- and 12,13-diol metabolites. Leukotoxin and isoleukotoxin are metabolized primarily by the soluble epoxide hydrolase to form leukotoxin diol. Investigations with recombinant cytochrome P450 enzymes have demonstrated that leukotoxin and isoleukotoxin can be formed by these enzymes. This study used a combination of experimental approaches to identify the major cytochrome P450 enzyme in human liver involved in linoleic acid epoxidation. The kinetic paramenters were determined; the K_m of linoleic acid epoxidation by pooled human liver microsomes was 170 μM and the V_{max} was 58 pmol/mg/min. Correlation analysis was performed using individual samples of human liver microsomes, and the best correlation of linoleic acid epoxidation activity was with tolbutamide hydroxylase activity, CYP2C9. Recombinant CYP2C9 was the most active in linoleic acid epoxygenation, and antibody and chemical inhibition also indicated the importance of CYP2C9. This enzyme, therefore, may serve as a therapeutic target in the treatment of inflammation in order to reduce the amount of circulating leukotoxin/isoleukotoxin and their related diols.

Key Words: **leukotoxin; cytochrome P450; CYP2C9; 9,10-epoxy-12-octadecenoate; fatty acid oxidation.**

Leukotoxin (9,10-epoxy-12-octadecanoate) is a monoepoxide of linoleic acid, synthesized by a cytochrome P450 monoxygenase and possibly by an oxidative burst of active oxygen produced by cells (1–4). Leukotoxin was identified as a product of neutrophils (5). It has been shown to be cytotoxic in a variety of systems. In experimental animals, the compound is cardiotoxic (6), inhibits mitochondrial respiration (7), and has antitumor activity (8). Recent studies have indicated that the toxicity of leukotoxin and isoleukotoxin is not due to the epoxides, but to the 9,10- and 12,13-diol metabolites. Leukotoxin is metabolized primarily by the soluble epoxide hydrolase (sEH)[3] to form leukotoxin diol. Whereas leukotoxin was not toxic to LacZ-expressing Sf-21 cells, the leukotoxin diol was toxic (LC_{50} ~160 μM). Furthermore, Sf-21 cells that expressed the sEH from either human or murine cDNAs showed greatly increased sensitivity to leukotoxin (LC_{50} ~290 μM) compared to control cells ($LC_{50} \gg 1$ mM). These results indicate that leukotoxin is a protoxin which must be converted to the diol for biological activity (9). This hypothesis that leukotoxin is a protoxin is supported by several lines of evidence. Only the diol and not the parent epoxides are toxic *in vitro* to several mammalian cell types, including renal proximal tubular cells, bronchiolar epithelium, and cardiac myocytes (10, 11). Possibly most convincing is that administration of potent inhibitors of the soluble epoxide hydrolases prevents the *in vivo* toxicity of leukotoxin but not its diol (12). Clinically, leukotoxin has been associated with acute respiratory distress syndrome (ARDS). Leuko-

[1] A. J. Draper was the recipient of an NIEHS postdoctoral fellowship (ES05808) and the Amgen–American Liver Foundation postdoctoral fellowship. This work is also supported by NIEHS Grant R01-ES02710, NIEHS Center for Environmental Health Sciences Grant 1P30-ES05707, and the NIEHS Superfund Basic Research Program P42-ES04699.

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[3] Abbreviations used: sEH, soluble epoxide hydrolase; ARDS, acute respiratory distress syndrome; EET, epoxyeicosatrienoic acid; TNF, tumor necrosis factor.

0003-9861/00 $35.00

toxin is elevated in the plasma of ARDS patients and also in animals breathing pure oxygen (13). Cytotoxicity of leukotoxin diols associated with mitochondrial damage has been reported in renal proximal tubular cells (10). Additionally, it has been hypothesized that leukotoxin plays a role in inflammation (14).

Multiple pathways for linoleic acid oxidation have been elucidated. Recombinant human cytochrome P450 enzymes have been shown to catalyze 9,10- and 12,13-epoxidation, bis-allylic hydroxylation, and ω- and ω-1 hydroxylation (15). Additionally, cytochrome *c* with a hydrogen peroxide-generating system or cytochrome *c* and cardiolipin will catalyze the epoxidation of either double bond of linoleic acid (3, 4). These results suggest that leukotoxin and isoleukotoxin may be formed by enzymatic and nonenzymatic mechanisms *in vivo*. sEH catalyzes the hydrolysis of leukotoxin and isoleukotoxin to their respective diols (16). This study was undertaken in order to determine which human liver cytochrome P450 enzyme is responsible for the epoxidation of linoleic acid. Inhibition of this enzyme may be a therapeutic mechanism for interfering with the inflammatory damage caused by leukotoxin and isoleukotoxin diols.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals used were of the highest purity available. [^{14}C]Linoleic acid was purchased from DuPont–New England Nuclear (Billerica, MA). [^{14}C]Linoleic acid was prepared in methanol by evaporating the commercial stock to dryness and reconstituting the residue with methanol and unlabeled linoleic acid. Solutions were stored in glass vials under nitrogen at −20°C. Linoleic acid, hexanes, ethyl acetate, α-naphthoflavone, nicotine, sulfaphenazole, tolbutamide, quinidine, 4-methylpyrazole, ketoconazole, and lauric acid were purchased from Sigma (St. Louis, MO). *S*-Mephenytoin was purchased from Sanford Ultrafine Chemicals (Manchester, England). Characterized human liver microsomes were purchased from XenoTech, LLC (Kansas City, KS). Anti-cytochrome P450 antibodies and cDNA-expressed cytochrome P450 enzymes were purchased from Gentest (Woburn, MA). Silica gel thin-layer chromatography plates (10-cm plates with nine 1-cm lanes per plate) were purchased from EM Science (Gibbstown, NJ). Mouse recombinant sEH was prepared using the baculovirus expression system as previously described (17) and purified by affinity purification (18).

Enzyme assay procedure. Human liver microsomes (1 mg/ml) were suspended in buffer containing potassium phosphate (100 mM, pH 7.4), magnesium chloride (3 mM), and EDTA (1 mM) at the final concentrations indicated. One microliter of [^{14}C]linoleic acid in ethanol was added with a Hamilton repeating syringe to give the final concentrations indicated. Pure recombinant mouse sEH was added in 2-μl aliquots containing 40 ng sEH every 5 min of incubation. Samples (100 μl total volume) were incubated at 37°C for 10 to 60 min in a shaking water bath. Incubation was terminated by the addition of 200 μl ethyl acetate and 2 μl 5 N HCl. Samples were vortexed well and 140 μl of the ethyl acetate layer was removed for analysis. After samples were dried under a stream of nitrogen, they were reconstituted in 25 μl TLC mobile phase (4:1 hexanes:ethyl acetate + 1% acetic acid) and each entire sample was spotted onto a TLC plate. TLC plates with samples spotted were run twice in developing solvent containing 4:1 hexanes:ethyl acetate + 1% acetic acid. Plates were analyzed with a radiometric TLC plate reader (Bioscan, Washington, DC) to ensure that radioactive spots were in the positions expected. Plates were wet with a light mist of water and then scraped in the regions of interest. Silica scrapings were added to 1 ml scintillation cocktail for scintillation counting.

Confirmation of leukotoxin diol identity. [^{14}C]Linoleic acid and unlabeled linoleic acid were incubated with human liver microsomes in the presence of sEH for 60 min as described above. After analysis as described, the spot doublet at 3.9 and 4.2 cm corresponding to leukotoxin diol (9,10-dihydroxy-12-octadecenoic acid) and isoleukotoxin diol (11,12-dihydroxy-12-octadecenoic acid) was scraped and extracted with ethyl acetate. This extracted sample was divided in two and dried under N_2. One half was treated with butyl buronic acid (200 μg in 50 μl isooctane), while the other half was reconstituted in isooctane. Both samples were then reanalyzed by the TLC procedure described above. A sample of unlabeled linoleic acid was incubated with human liver microsomes in the same experiment, and the unlabeled diol region on the TLC plate was scraped and used for mass spectral analysis.

Nonradiolabeled metabolite samples were derivatized for mass spectral analysis. TLC extract was reduced to dryness under N_2 and immediately redissolved in 50 μl of bis(silyl acetamide) (Supelco) and incubated for 2.5 h at 70°C. Separation was performed with a Hewlett–Packard gas chromatograph (Model 6890, San Jose, CA) using a 30-m J&W DB-5 capillary column (Folsom, CA). Inlet temperature was 250°C and oven temperature ran from 240 to 325°C in 27 min. Mass spectral analysis was performed on a Hewlett–Packard 5973 mass selective detector scanning from *m*/*z* 50 to 650. The presence of characteristic ions for the trimethylsilyl ester/ethers of leukotoxin diol (*m*/*z* 317, 213) and isoleukotoxin diol (*m*/*z* 357, 173) with the appropriate relative intensity and retention time was considered confirmation of structural identity.

Following determination of the leukotoxin diol TLC spots, the region of the TLC lane containing leukotoxin and isoleukotoxin diols was scraped, and the two regioisomers were counted together as leukotoxin diol. Likewise, for determination of leukotoxin and isoleukotoxin, spots were scraped and analyzed together.

Time and protein linearity. To determine whether leukotoxin diol formation was linear with time and protein concentration, pooled human liver microsomes (0.25 to 2 mg/ml) were incubated for 20 min as described above. Additionally, pooled human liver microsomes (1 mg/ml) were incubated for 5–60 min. Metabolite formation was determined as described above.

Comparison of leukotoxin epoxide and diol formation. To determine whether leukotoxin diol formation in the presence of excess sEH was equivalent to epoxide formation in the absence of sEH activity, a chemical inhibitor of sEH was used. It was necessary to use a sEH inhibitor because human liver microsomes contain some sEH activity (19). *N*-Cyclohexyl-*N*′-dodecylurea was added in 1 μl tetrahydrofuran with a Hamilton repeating syringe at a final concentration of 100 μM. Mouse recombinant sEH was added as described above. Because the standard extraction procedure used 5 N HCl, the procedure had to be modified to prevent acid hydrolysis of leukotoxin. For this experiment, NaCl crystals were used instead of HCl to increase the efficiency of ethyl acetate extraction.

Determination of K_m. Human liver microsomes were incubated with [^{14}C]linoleic acid (final concentrations of 3.4 to 500 μM) and the rate of linoleic acid epoxidation was determined as described above. Kinetic constants were determined with the Enzyme Kinetics software package from Trinity Software (Campton, NH). This program weights data toward high substrate concentration (weighting factor = 4). K_m and V_{max} values were determined from nonlinear Michaelis–Menten kinetics. Data are shown in a Lineweaver–Burk plot from which values can be more easily extracted.

Correlation analysis. Sixteen individual samples of human liver microsomes were incubated with 500 μM [^{14}C]linoleic acid and rates of epoxidation were determined as described above. These rates were then compared by correlation with known P450 activities supplied by the manufacturer. The substrate concentration was chosen based on

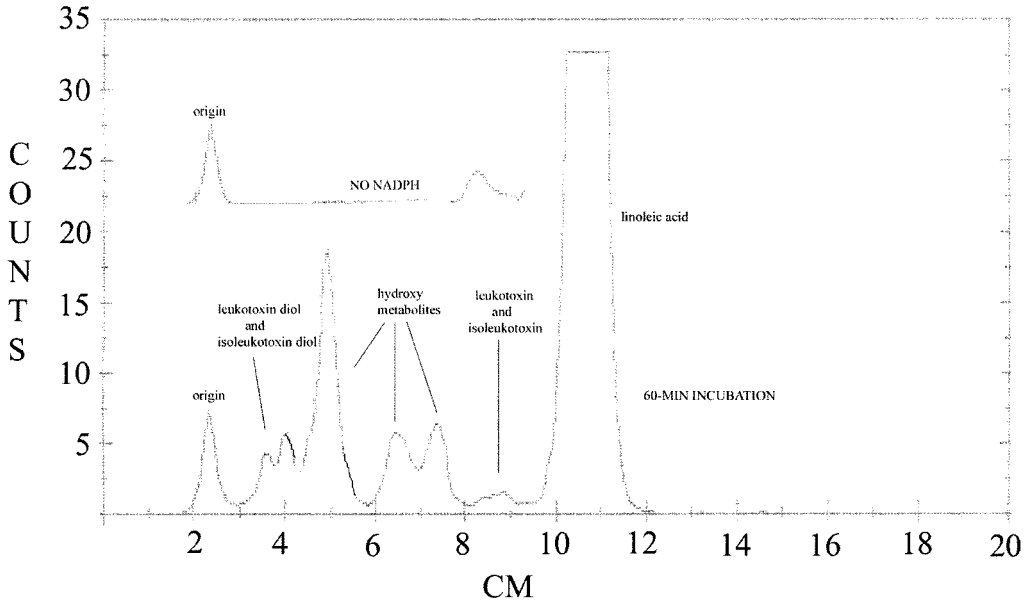

FIG. 1. Human liver microsomal linoleic acid metabolites by thin-layer chromatography. Human liver microsomes were incubated with 500 μM [^{14}C]linoleic acid for 60 min as described under Materials and Methods. Samples were extracted and analyzed by thin-layer chromatography as described under Materials and Methods. Shown are typical Bioscan chromatographs of a 60-min incubation of linoleic acid with human liver microsomes with and without NADPH.

the determination of K_m. Ideally, the substrate concentration would be 10 times the K_m in order that rates fall on the linear part of the kinetic curve, but solubility was problematic, so a concentration roughly 3 times the K_m was chosen.

Antibody and chemical inhibition. Commercially available anti-cytochrome P450 antibodies were suspended in Tris buffer (pH 7.4) with 100 mg human liver microsomes on ice in a total volume of 25 μl. The amount of antibody used was determined from information published by Gentest; an aliquot was selected which would inhibit a known cytochrome P450 activity by greater than 80%. The amounts of antibody used for this experiment were 10 μl α-CYP1A2, 2 μl α-CYP2A6, 2 μl α-CYP2B6, 10 μl α-CYP2C, 2 μl α-CYP2D6, 2 μl α-CYP2E1, and 10 μl α-CYP3A4. The mixtures of antibodies and microsomes were allowed to equilibrate on ice for 20 min, and then the assay procedure described above was followed. Chemical inhibitors were chosen to be selective for one particular cytochrome P450 enzyme under the conditions described. The concentration of inhibitor used was 10-fold its literature K_m or K_i value for its particular enzyme. Chemical inhibitors were added to incubations in 1 μl methanol with a Hamilton repeating syringe. Chemicals used were α-naphthoflavone (0.1 μM), nicotine (240 μM), sulfaphenazole (3 μM), tolbutamide (2 mM), *S*-mephenytoin (1 mM), quinidine (4 μM), 4-methylpyrazole (25 μM), ketoconazole (0.3 μM), and lauric acid (160 μM) at the final concentrations indicated. Solutions of inhibitors were prepared in reagent-grade methanol and stored in glass vials with Teflon-lined caps at −20°C.

Recombinant cytochrome P450 enzymes. Commercially available recombinant cytochrome P450 enzymes expressed in human lymphoblastoid cell microsomes were incubated with 500 μM [^{14}C]linoleic acid as described above for human liver microsomes.

RESULTS

Linoleic acid epoxidation activity in human liver microsomes was determined as described under Materials and Methods. A sample TLC chromatogram (Fig. 1) shows that multiple polar products are formed and that separation of the epoxides and diols was achieved. Product formation was linear with time (up to 2 h) and with protein concentration (up to 1 mM substrate). In addition, a TLC spot corresponding to leukotoxin diol was identified. Following the procedure described above, [^{14}C]leukotoxin diol was extracted from a TLC plate and half was derivatized with butyl buronic acid. Butyl buronic acid is used to derivatize 1,2-diols, but will not affect most other functional groups. Analysis by TLC showed that the leukotoxin diol spots had moved from 3.9/4.2 to 6.8/7.1 cm following derivatization. No radioactivity remained at 3.9/4.2 cm following derivatization, which indicates that primarily, if not exclusively *vic*-diols were formed under these conditions. Additionally, mass spectral anaylsis, performed as described above, confirmed the identification of leukotoxin and isoleukotoxin diols, as shown in Fig. 2. Following identification, the two linoleate diol TLC spots were scraped and counted together as leukotoxin diols.

Comparison of Leukotoxin Diol Formation with Epoxidation

Human liver microsomes were incubated with [^{14}C]linoleic acid in the presence of excess sEH or in the presence of *N*-cyclohexyl-*N'*-dodecylurea, a chemical inhibitor of sEH. Leukotoxin and leukotoxin diol TLC spots were analyzed as described above. As shown in Fig. 3, epoxide formation in the presence of dodecyl cyclohexyl urea was equal to diol formation in the presence of excess sEH. In the absence of either excess

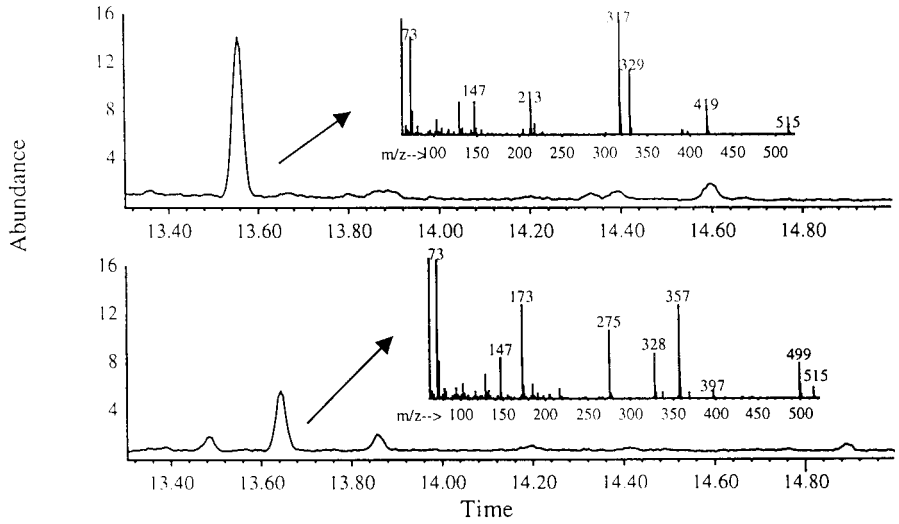

FIG. 2. Idenification of leukotoxin and isoleukotoxin by mass spectral analysis. Human liver microsomes were incubated with 500 μM [^{14}C]linoleic acid for 60 min as described under Materials and Methods. Samples were extracted and analyzed with gas chromatography with a mass spectral detector as described under Materials and Methods. Shown is the chromatography tracing and the mass spectral analysis for leukotoxin diol and isoleukotoxin diol, respectively.

sEH or *N*-cyclohexyl-*N'*-dodecylurea, about 25% of the epoxide formed was hydrated to the diol. All subsequent experiments were done in the presence of excess purified mouse recombinant sEH as described under Materials and Methods.

Determination of Kinetic Constants for Linoleic Acid Epoxidation

Pooled human liver microsomes were incubated with various concentrations of [^{14}C]linoleic acid, and rates of epoxidation were determined by measuring leukotoxin diol formation as described above. A Lineweaver–Burk plot in Fig. 4 shows that product formation was linear with substrate concentration. Kinetic constants were determined with Enzyme Kinetics software, which weights data toward higher substrate concentrations. The V_{max} for linoleic acid epoxidation by human liver microsomes was 58 pmol/mg/min and K_m was 170 μM.

Correlation Analysis

Sixteen individual samples of human liver microsomes were incubated with [^{14}C]linoleic acid and rates of epoxidation were determined by measuring leukotoxin diol formation as described above. The rates of

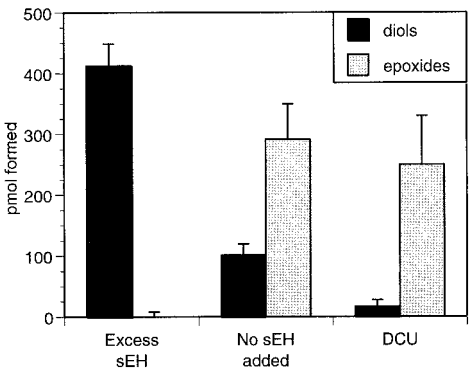

FIG. 3. Comparison of diol formation rate in the presence of excess sEH with epoxide formation rate. Pooled human liver microsomes were incubated with [^{14}C]linoleic acid (500 μM) and the rate of linoleic acid epoxide and diol formation was measured as described under Materials and Methods. Each bar represents the mean (±SD) of three separate determinations. Dodecyl cyclohexyl urea (DCU) (100 μM) was used as an inhibitor of sEH activity in human liver microsomes.

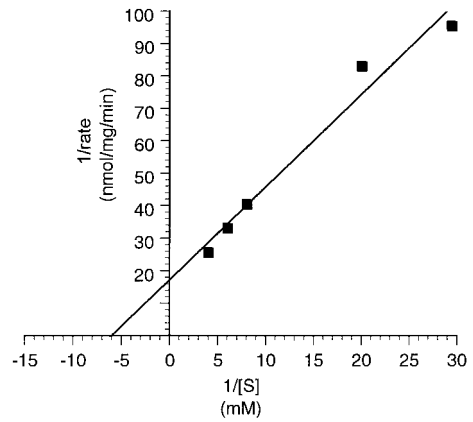

FIG. 4. Determination of kinetic parameters for human liver microsomal epoxidation of linoleic acid. Pooled human liver microsomes were incubated with [^{14}C]linoleic acid (34–500 μM) and the rate of linoleic acid epoxidation was measured as described under Materials and Methods. Each point represents the mean of two separate determinations.

TABLE I

Correlation of Linoleic Acid Epoxidation Activity with Known P450 Activities in Human Liver Microsomes

Enzyme	Correlation[a] coefficient	*y* intercept[b]
CYP1A2	0.171	19
CYP2A6	0.212	12
CYP2B6	0.217	13
CYP2C8	0.257	11
CYP2C9	0.786	0.041
CYP2C19	0.176	13
CYP2D6	0.483	16
CYP2E1	0.206	8.7
CYP3A4	0.278	9.8
CYP4A11	0.457	11

[a] Linoleic acid epoxidation activity in 16 different human liver microsomal samples was measured as described under Materials and Methods. The activity determined was plotted against known P450 activities (supplied by the manufacturer). Correlation coefficients are reported as r^2 values determined as described. Assays used by XenoTech to measure cytochrome P450 activity were as follows: CYP1A2, 7-ethoxyresorufin *O*-dealkylase; CYP2A6, coumarin 7-hydroxylase; CYP2B6, 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylase; CYP2C8, taxol 6α-hydroxylase; CYP2C9, tolbutamide methylhydroxylase; CYP2C19, *S*-mephenytoin 4′-hydroxylase; CYP2D6, dextromethorphan *O*-demethylase; CYP2E1, chlorzoxazone 6-hydroxylase; CYP3A4, testosterone 6β-hydroxylase; and CYP4A11, lauric acid 12-hydroxylase.

[b] *y*-Intercepts of the plots of linoleic acid oxidation activity vs known P450 activities are reported as pmol/mg/min.

epoxidation were compared with known P450 activities, and the correlation coefficients are reported in Table I. The highest correlation was observed with CYP2C9, tolbutamide hydroxylase activity, and as reported in Table I, this was the only correlation to have a *y* intercept near zero.

Antibody Inhibition

Pooled human liver microsomes were incubated with [^{14}C]linoleic acid in the presence of anti-cytochrome P450 antibodies and rates of epoxidation were determined by measuring leukotoxin diol formation as described above. As shown in Fig. 5, the only antibody that caused significant inhibition was the anti-CYP2C antibody. This antibody inhibits both CYP2C9 and CYP2C19. This experiment was limited by the use of commercially available antibodies. There are additional cytochrome P450 enzymes present in human liver for which antibodies were not available.

Chemical Inhibition

Pooled human liver microsomes were incubated with [^{14}C]linoleic acid in the presence of selective chemical inhibitors of cytochrome P450 enzymes and rates of epoxidation were determined by measuring leukotoxin diol formation as described above. As shown in Fig. 6, both sulfaphenazole, a competitive inhibitor of CYP2C9, and tolbutamide, an alternate substrate of CYP2C9, inhibited linoleic acid epoxidation, while the other chemical inhibitors caused less than 20% inhibition.

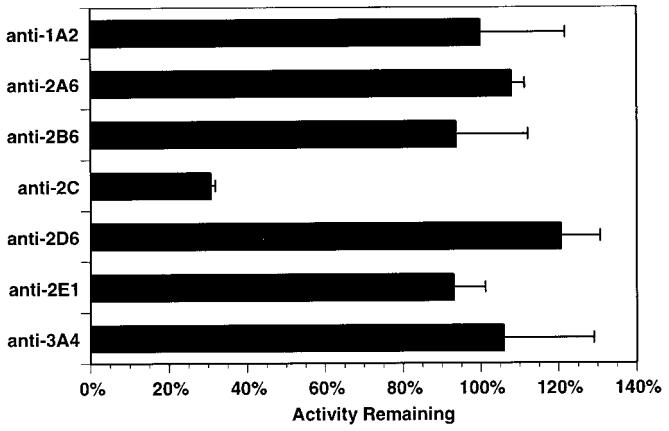

FIG. 5. Effect of anti-cytochrome P450 antibodies on human liver microsomal linoleic acid epoxidation. Pooled human liver microsomes were incubated with [^{14}C]linoleic acid (500 μM) in the presence and absence of catalytically inhibitory anti-cytochrome P450 antibodies and the rate of linoleic acid epoxidation was measured as described under Materials and Methods. Each bar represents the mean (±SD) of three separate determinations.

Recombinant Cytochrome P450 Enzymes

Microsomes from human lymphoblastoid cells containing recombinant cytochrome P450 enzymes were

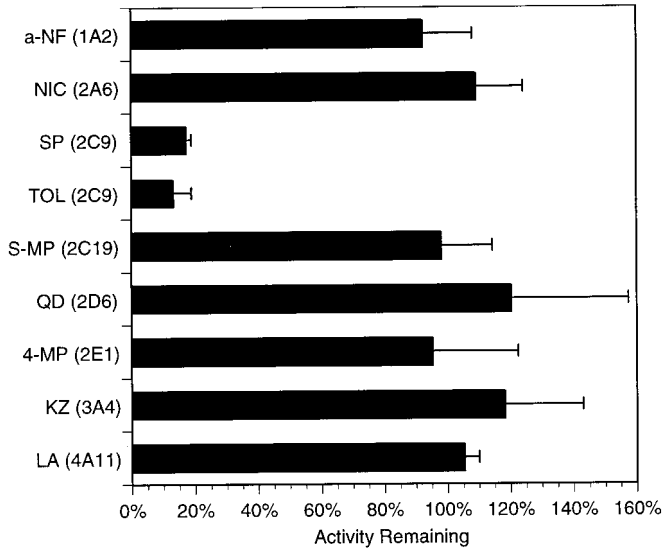

FIG. 6. Effect of cytochrome P450 chemical inhibitors and substrates on human liver microsomal linoleic acid epoxidation. Pooled human liver microsomes were incubated with [^{14}C]linoleic acid (500 μM) in the presence and absence of commonly used cytochrome P450 chemical inhibitors and the rate of linoleic acid epoxidation was measured as described under Materials and Methods. Each bar represents the mean (±SD) of three separate determinations.

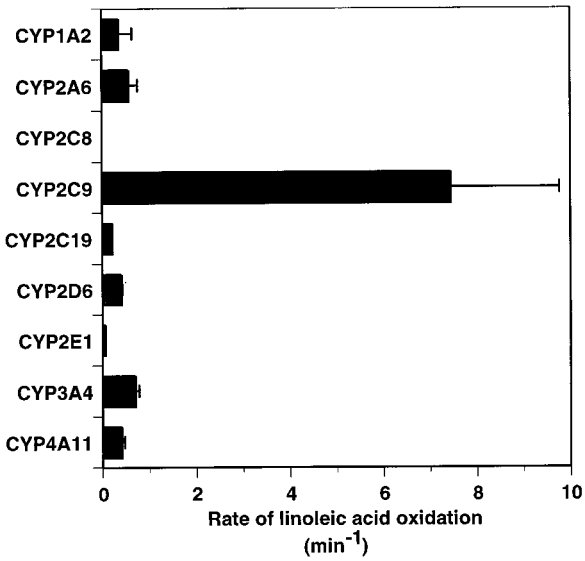

FIG. 7. Linoleic acid epoxidation by recombinant cytochrome P450 enzymes. Microsomes containing recombinant cytochrome P450 enzymes were incubated with [^{14}C]linoleic acid (500 μM) and the rate of linoleic acid epoxidation was measured as described under Materials and Methods. Each bar represents the mean (±SD) of three separate determinations.

incubated with [^{14}C]linoleic acid and rates of epoxidation were determined by measuring leukotoxin diol formation as described above. As shown in Fig. 7, the only recombinant enzyme tested that had significant linoleic acid epoxidation activity was CYP2C9.

DISCUSSION

As suggested by Parkinson (20), four different methods were used to determine which cytochrome P450 enzyme in human liver microsomes is primarily responsible for the epoxidation of linoleic acid: correlation analysis, antibody inhibition, chemical inhibition, and oxidation by recombinant cytochrome P450 enzymes. The results of each of these four experiments provide a solid basis for the identification of CYP2C9 as a key enzyme involved in the formation of leukotoxin by the liver. However, this approach is limited by which enzymes are included. Many human cytochrome P450 enzymes were not included in this study. Not all enzymes or antibodies are commercially available, and there is also the possibility that a yet unidentified CYP may be involved. Recombinant CYP2C9 has previously been shown to have linoleic acid epoxidation activity (21). The results of this study indicate that CYP2C9 is the predominant linoleate epoxygenase in human liver microsomes, but this study does not address the contribution of extrahepatic cytochrome P450 enzymes that could epoxidize linoleic acid. For example, it may turn out that the most important physiological source of leukotoxin is inflammatory cells. It is unknown whether inflammatory cells possess CYP2C9 necessary to synthesize leukotoxin, although one report indicates the presence of a CYP2C enzyme in bronchoalveolar macrophages (22).

In addition to its linoleic acid epoxidation, CYP2C9 has been shown to have arachidonic acid epoxidation activity. The epoxidation of arachidonic acid by recombinant CYP2C9 is regioselective; 14,15-epoxyeicosatrienoic acid (EET) was formed preferentially over 11,12-EET and 8,9-EET. Recombinant CYP2C8 had greater arachidonate epoxidation activity than did CYP2C9. Human liver microsomes also catalyzed the epoxidation of arachidonic acid, and epoxide products constituted 13–28% of total metabolism (23). CYP2C8 and CYP2C9 purified from human liver and reconstituted with NADPH–P450 oxidoreductase, cytochrome b_5, and lipid also showed regioselectivity in arachidonic acid epoxidation. Both CYP2C8 and CYP2C9 formed 14,15-EET preferentially, and while both also formed 11,12-EET, only CYP2C9 formed 8,9-EET. Furthermore, both CYP2C8 and CYP2C9 are stereoselective at the 14,15-position, both preferentially producing 14(*R*),15(*S*)-EET with 86.2 and 62.5% selectivity, respectively. The opposite stereoselectivity was observed with 11,12-EET (24). Recently, a new family of arachidonic acid epoxygenases has been described. CYP2J2 is found in human lung, heart, and alveolar macrophages and produces 14(*R*),15(*S*)-EET with 76% optical purity (25). This enzyme may also play a role in linoleic acid epoxidation, especially given its presence in alveolar macrophages (26). The stereoselectivity of cytochrome P450 epoxidation of linoleic acid is unknown, but the results of our study suggest that this may be an interesting question to pursue with regard to CYP2C9. Furthermore, because our study did not attempt to differentiate between the 9,10- and 11,12-epoxyoctadecenoic acids, the regioselectivity also remains unanswered. Enzymes in the CYP2C family in the rat have also been implicated in the epoxidation of linoleic acid. Both CYP2CAA purified from rabbit renal cortex microsomes and recombinant CYP2C2 catalyzed the formation of both leukotoxin and isoleukotoxin. There was a regioselectivity for both enzymes of 1.6:1 isoleukotoxin:leukotoxin (27).

In our studies, we did observe the formation of other NADPH-dependent metabolites of linoleic acid. As shown by Bylund *et al.* (21), there were many TLC spots showing mobilities between leukotoxin and isoleukotoxin diol and leukotoxin and isoleukotoxin. We assumed these other metabolites to be monohydroxylinoleates based on their relative polarity, but did not determine their structures. Human liver microsomes catalyze the formation of 8-, 9-, 11-, 13-, 15-, 16-, and 17-hydroxylinoleic acids in addition to the 9,10- and 11,12-epoxides and diols (21). In addition, recombinant cytochrome P450 enzymes, including CYP2C8 and

CYP2C9, catalyze the formation of the bisallylic hydroxy metabolite 11-hydroxyoctadecadienoic acid (15).

There are potential pharmacological consequences of CYP2C9-dependent leukotoxin formation. Many cytochrome P450 enzymes are downregulated during inflammation. If leukotoxin diol is involved in inflammation, the downregulation of its epoxygenase during inflammation would be disadvantageous. Recombinant interferons (IFN) from all three families (α, β, and γ) cause a significant depression in hepatic cytochrome P450 levels. Furthermore, endotoxin, which causes the release of IL-6, IL-1, and tumor necrosis factor (TNF), caused cytochrome P450 downregulation (28). Although TNF-α may play an important role in the downregulation of CYP1A2 and 2B, it may not be important to the downregulation of other enzymes. Furthermore, while the endotoxin downregulation of CYP1A2 and 2B was prevented by pentoxifylline, the P450 suppression induced by poly(IC) (polyinosinic acid:polycytylic) was not affected. This indicates that the different agents that cause cytochrome P450 downregulation may act by different mechanisms (29). Recently, interferon-mediated downregulation of rat liver cytochrome P450 was demonstrated to occur through both transcriptional and posttranscriptional mechanisms (30). Although much attention has been given to examining downregulation of cytochrome P450 enzymes in rat, not much is known about human enzymes and how potential downregulation will effect lipid signals and cytotoxic oxylipids. In addition to downregulation, a polymorphism for CYP2C9 has been described which may cause a change in substrate preference for the enzyme (20). The leukotoxins and their diols are correlated with adult respiratory distress syndrome and may be involved in causing the associated inflammation (31). There may also be endogenous regulatory roles for these compounds. In conclusion, CYP2C9 was identified as a linoleic acid epoxygenase in human liver microsomes. This identification may have pharmacological significance in the treatment of inflammation.

ACKNOWLEDGMENTS

The authors thank Mr. John W. Newman for his expert assistance in performing mass spectral analysis and Dr. Christophe Morisseau for providing purified recombinant mouse sEH.

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