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Role of Human CYP4F2 in Hepatic Catabolism of the Proinflammatory Agent Leukotriene B4[1]

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Leukotriene B4 (LTB4), an arachidonic acid derivative, is a potent proinflammatory agent whose actions are terminated by catabolism via a microsomal ω-hydroxylation pathway. Although the liver serves as the principal site for LTB4 clearance from the systemic circulation, the attributes of hepatic LTB4 metabolism are ill defined in humans. Thus, we examined metabolism of LTB4 to its ω-hydroxylated metabolite 20-hydroxyleukotriene B4 (20-OH LTB4) by human liver microsomes and also purified the hepatic P450 enzyme underlying this reaction. Liver microsomes from 10 different subjects converted LTB4 to 20-OH LTB4 at similar rates (1.06 ± 0.3 nmol/min/nmol P450; 0.25 ± 0.1 nmol/min/mg protein). Analysis of the microsomal LTB4 20-hydroxylation reaction revealed kinetic parameters (apparent K_m of 74.8 μM with a V_{MAX} of 2.42 nmol/min/nmol P450) consistent with catalysis by a single P450 enzyme. Conventional chromatography combined with immunochemical screening with rat CYP4A1 antibodies was then used to isolate a P450 enzyme from human liver microsomes with a molecular weight of 57,000 and an NH_2-terminal amino acid sequence 94% homologous (^{12}Trp → ^{12}Gly) over the first 17 residues with the human *CYP4F2* cDNA-derived sequence. Upon reconstitution with P450 reductase and phospholipid, CYP4F2 converted LTB4 to 20-OH LTB4 at a turnover rate of 392 pmol/min/nmol P450, whereas the other human liver P450s tested, including CYP4A11, exhibited neglible LTB4 ω-hydroxylase activity. Polyclonal antibodies to CYP4F2 were found to markedly inhibit (91.9 ± 5%; $n = 5$) LTB4 20-hydroxylation by human liver microsomes. Microsomal 20-OH LTB4 formation was also inhibited 30% by arachidonic acid, a known CYP4F2 substrate, and 50% by prostaglandin A_1 but was unaffected by lauric acid, palmitic acid, and $PGF_{2\alpha}$. Finally, a strong correlation ($r = 0.86$; $P < 0.002$; $n = 10$) was observed between CYP4F2 content and LTB4 20-hydroxylase activity in the human liver samples. Our results indicate that CYP4F2 is the principle LTB4 ω-hydroxylating enzyme expressed in human liver and, as such, may play an important role in regulating circulating as well as hepatic levels of this powerful proinflammatory eicosanoid.

Lipid-derived mediators, or eicosanoids, are intimately involved in the regulation of cell and/or organ physiology. Among these compounds are the proinflammatory agents LTB4[3] and the cysteinyl leukotrienes, which are derived from arachidonic acid through the action of 5-lipoxygenase (1). LTB4 is a potent activator of polymorphonuclear leukocytes, induces chemotaxis and chemokinesis, and also stimulates superoxide generation, cellular aggregation, and calcium mobilization in neutrophils (2). Thus, it is likely that LTB4 plays an important role in those inflammatory disease states where neutrophil infiltration into tissues is prominent.

The turnover of LTB4 appears to determine the extent and duration of the inflammatory response (3). LTB4 is initially degraded to 20-OH LTB4, an ω-hydroxylated metabolite with substantially reduced biological activity (3, 4). 20-LTB4 then undergoes dehy-

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[3] Abbreviations used: LTB4, leukotriene B4; 20-OH LTB4, 20-hydroxyleukotriene B4; P450, cytochrome P450; b_5, cytochrome b_5; P450 reductase, NADPH:P450 oxidoreductase; IgG, immunoglobulin G; DLPC, L-α-dilauroylphosphatidylcholine; PGA_1, PGE_1, and $PGF_{2\alpha}$, prostaglandins A_1, E_1, and $F_{2\alpha}$, respectively; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonia]propanesulfonate; DOPC, L-α-dioleoylphosphatidycholine; KPO_4, potassium phosphate.

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drogenation to 20-carboxyleukotriene B4, which is further catabolized by β-oxidation in peroxisomes (5). Studies with human neutrophils first suggested that conversion of LTB4 to 20-OH LTB4 was catalyzed by a P450 enzyme (6, 7). It was later reported that the P450 enzyme underlying this reaction was a *CYP4A*[4] gene family product, since LTB4 hydroxylation by human neutrophil microsomes was specifically inhibited by antibodies to rabbit lung (CYP4A4) and kidney (CYP4A5) fatty acid ω-hydroxylases (8). Kikuta *et al.* (9) then isolated a cDNA termed LTB4 ω-hydroxylase from human neutrophils that, upon transfection into yeast, gave rise to a protein that catalyzed 20-hydroxylation[5] of LTB4. Sequence analysis of the human neutrophil LTB ω-hydroxylase cDNA revealed that it encoded not a *CYP4A* gene subfamily product but rather the first member of a new *CYP4* gene subfamily, namely *CYP4F*.[6]

While LTB4 degradation occurs primarily in neutrophils at the site of inflammation, the liver can also catabolize this eicosanoid upon its release into the systemic circulation (10–14). In fact, rat liver microsomes exhibit high levels of LTB4 ω-hydroxylase activity (15, 16). The P450 underlying LTB4 20-hydroxylation in rat liver was studied in detail by Sumimoto *et al.* (16) who, short of actually identifying the enzyme(s), found that it displayed a b_5 requirement, was also a catalyst of PGA_1 ω-hydroxylation, and, in contrast to the human neutrophil enzyme, was not inhibited by antibodies to rabbit lung CYP4A4. More recently, a cDNA (subsequently designated *CYP4F2*) was isolated from human liver that displayed extensive sequence homology (87%) with the human neutrophil *CYP4F3* cDNA (17). Microsomes from yeast transfected with this *CYP4F2* cDNA possessed substantial LTB4 20-hydroxylase activity but failed to metabolize other lipids such as PGA_1, lauric acid, or arachidonic acid. In contrast, the human liver *CYP4F2* cDNA isolated by Chen and Hardwick (18), which may be identical to the one cloned by Kikuta *et al.* (17), was found to catalyze ω-hydroxylation of not only LTB4 but also lauric, palmitic, and arachidonic acids upon heterologous expression in insect cells.

While it is evident that recombinant CYP4F2, when expressed in heterologous systems, can convert LTB4 to its 20-hydroxylated metabolite, little else is known about the characteristics of this human P450 enzyme and/or its overall role in hepatic LTB4 catabolism. To that end, we utilized classical purification methodology to isolate CYP4F2 from human liver microsomes. The purified hemoprotein was identified as CYP4F2 based upon its sequence homology over the first 17 amino acid residues with the corresponding cDNA (17, 18). Upon reconstitution with P450 reductase and phospholipid, native CYP4F2 proved to be an efficient catalyst of LTB4 ω-hydroxylation. Antibodies produced against CYP4F2 were then utilized to reveal that this P450 enzyme indeed underlies most LTB4 ω-hydroxylation occurring in human liver.

EXPERIMENTAL PROCEDURES

Human liver specimens. Human liver specimens were obtained from the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis). The livers were removed within 30 min of death, frozen in liquid nitrogen, and stored at −80°C until microsomes were prepared as described elsewhere (19). Protein concentration was measured using the bicinchoninic acid procedure (20) while P450 concentration was determined from the CO-reduced difference spectrum (21).

Enzyme purification. CYP2C9, b_5, and P450 reductase were purified to electrophoretic homogeneity from human liver microsomes as previously reported (19, 22). CYP4A11 was purified according to Powell *et al.* (23), and CYP2A6 was isolated essentially as described by Yun *et al.* (24). The specific contents of these purified human liver enzymes were 11.4 (CYP2C9), 6.1 (CYP2A6), 12.6 (CYP4A11), and 37.1 (b_5) nmol/mg protein while the specific activity of P450 reductase was 32,000 units (9.5 nmol)/ml; 1 unit of P450 reductase activity was defined as that amount catalyzing reduction of 1 nmol ferricytochrome c/min at 22°C in 300 mM potassium phosphate buffer (pH 7.7).

CYP4F2 was purified from human liver sample UC9411 according to the following method. KPO_4 buffers used for the purification contained 1 mM DTT, 1 mM EDTA, and 20% glycerol unless denoted otherwise. As reported previously (19, 23), a P450-enriched fraction derived from tryptamine CH-Sepharose 4B chromatography of CHAPS-solubilized human liver microsomes was subjected to hydroxylapatite chromatography. The material recovered during stepwise elution of the hydroxylapatite column (Hypatite C; Clarkson Chemical Co., Williamsport, PA) with 25 mM KPO_4 and 50 mM KPO_4 buffers, pH 7.4, containing 0.5% Lubrol PX was found to be enriched in a 57-kDa protein cross-reactive with anti-rat CYP4A1 IgG on immunoblots (see below). This P450-containing sample was concentrated 15-fold by ultrafiltration through an Amicon YM-30 membrane, dialyzed against 5 mM KPO_4 buffer, pH 7.7, and then subjected to anion-exchange chromatography on a DE-53 cellulose column (1.0 × 19 cm) as described for CYP4A11 (23), except that the column was developed with a linear gradient of 0–100 mM KCl prepared in 6 column volumes of equilibration buffer. SDS–PAGE and immunoscreening with anti-rat CYP4A1 revealed that A_{417}-containing fractions eluting from column at a KCl concentration of 70–75 mM contained mainly CYP4F2 plus several minor contaminants. These fractions were pooled, dialyzed overnight at 4°C versus 100 volumes of 10 mM KPO_4 buffer, pH 7.4, and subsequently applied to a 1.0 × 4-cm Hypatite C column equilibrated with 10 mM KPO_4 buffer, pH 7.4, containing 0.5% Lubrol PX. The charged column was washed consecutively with 8 volumes of equilibration buffer, 8 volumes of equilibration buffer containing 25 mM KPO_4,

[4] The P450 enzymes described in this report are designated according to the nomenclature of Nelson *et al.* (37). Furthermore, for sake of simplicity, we refer to CYP4F2 and other CYP4 proteins purified from human liver as native enzymes and to the corresponding CYP4 proteins derived by molecular cloning techniques as recombinant enzymes.

[5] In this paper, the terms LTB4 ω-hydroxylation and 20-hydroxylation have been used interchangeably and denote hydroxylation of LTB4 at the primary carbon–hydrogen bond.

[6] The LTB4 ω-hydroxylases cloned from human neutrophils (i.e., polymorphonuclear leukocytes) and human liver by Kikuta and coworkers (9, 17) represent distinct enzymes that have been designated CYP4F3 and CYP4F2, respectively.

and 40 volumes of equilibration buffer with Lubrol PX omitted. Purified CYP4F2 was then eluted from the hydroxylapatite resin with 350 mM KPO_4 buffer, pH 7.4, containing 0.5% cholate. The final enzyme preparation was extensively dialyzed against 100 mM KPO_4 buffer, pH 7.4, to remove detergent and concentrated by ultrafiltration through an Amicon PM-30 membrane. The yield of purified CYP4F2 was 0.5%, or 7.3 nmol from the 1350 nmol of microsomal P450 originally processed. Essentially the same method was used to isolate a second CYP4F2 preparation from liver sample UC8907; in this case, the yield of purified enzyme was 0.5% (9.2 of 1855 nmol processed). NH_2-terminal sequencing was performed after binding CYP4F2 to Immobilon P^{SQ} (Millipore, Marlboro, MA) using a Hewlett–Packard Model 1000A sequenator/on-line PTH amino acid analyzer.

LTB4 20-hydroxylase assay. The conversion of LTB4 to 20-OH LTB4 by human liver microsomes was performed as outlined by Sumimoto *et al.* (16) but with certain modifications. Reaction mixtures (0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4), 30 μM LTB4, 2.5 mM NADPH, and either human liver microsomes (protein equivalent to 100 pmol P450) or reconstituted P450 enzymes. Reconstituted systems were composed of 50 pmol purified P450, 150 pmol P450 reductase, and 15 μg DLPC. In some assays, 200 pmol b_5 was included and/or 15 μg DOPC was substituted for DLPC. Reactions were initiated with NADPH and were terminated after 10–20 min at 37°C with 10 μl of 4.0 N HCl. In kinetic experiments, the concentration of LTB4 was varied from 3.75 to 120 μM. For antibody inhibition studies, microsomes were first incubated with either anti-human CYP4F2 or preimmune IgG (described below) for 10 min at room temperature, followed by the addition of the remaining reaction components. LTB4 and its metabolites were isolated from incubation mixtures by extraction with 5 vol of ethyl acetate using a multiple vortexer device, after which the organic extracts were separated and evaporated to dryness with nitrogen gas at room temperature. Residues were resolubilized in 150 μl of methanol, and 50-μl aliquots subjected to HPLC analysis using a Beckman System Gold instrument equipped with a Model 122 programmable UV detector. Samples were resolved on μBondapak C_{18} column (3.9 × 300 mm; Waters Corp., Milford, MA) by isocratic elution with methanol:0.5% phosphoric acid (60:40, v/v) at a flow rate of 1.2 ml/min with monitoring at 270 nm. Under these conditions, 20-OH LTB4 and LTB4 exhibited retention times of 8.1 and 14.6 min, respectively. Rates of product formation were determined from standard curves prepared by adding varying amounts of authentic 20-OH-LTB4 to incubations conducted without NADPH. Enzyme kinetic results were analyzed by nonlinear regression using weighted ($1/y$) untransformed data (GraFit 3.0; Erithacus Software Ltd., Cambridge, UK); Michaelis–Menten parameters were determined using a simple one-enzyme model. Arachidonic acid ω-hydroxylation by human liver microsomes was determined as described by Powell *et al.* (25).

Immunochemical methods. Polyclonal antibodies to human CYP4F2 were raised in male New Zealand White rabbits using an immunization procedure described elsewhere (26). Preimmune IgG was prepared from rabbit sera obtained prior to immunization. All IgG fractions were purified from sera using caprylic acid/ammonium sulfate fractionation (27). Anti-CYP4F2 was nearly monospecific as isolated but required back-adsorption against human epidermal keratin covalently linked to Sepharose 4B to remove the keratin cross-reactivity that interfered with immunoquantitation. Protein blotting of microsomal proteins and purified P450 enzymes to nitrocellulose and subsequent immunochemical staining with anti-CYP4F2 were performed as reported previously (26, 28). For immunoquantitation, CYP4F2 enzyme levels were first quantitated in our reference liver sample, UC9410, by applying various amounts of purified CYP4F2 (0.25–0.65 pmol) and UC9410 liver microsomes (2.5–7.5 μg) to the same polyacrylamide gel, followed by staining of the ensuing Western blots with anti-CYP4F2 IgG. The blots were scanned with an Agfa Arcus II flat-bed scanner interfaced to a computer, and immunoreactive areas on the image were measured using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). CYP4F2 enzyme content was then assessed in the other liver specimens (applied at 5 and/or 10 μg/gel lane) by comparing immunostaining intensities to that of the UC9410 reference sample. All immunochemical staining was performed using conditions under which the peroxidase reaction density was directly proportional to the amount of protein applied to the original polyacrylamide gels. The contents of CYP4A11, CYP2C8, CYP2C9, CYP2C19, CYP3A4, and CYP2A6 in human liver microsomes were determined as described elsewhere (22, 25, 29).

Materials. LTB4 was purchased from Spacecoast Biochemicals (Vero Beach, FL). Authentic 20-OH LTB4, arachidonic acid, PGA_1, PGE_1, and $PGF_{2\alpha}$ were obtained from Cayman Chemical Co. (Ann Arbor, MI), while sodium laurate was from Fluka Chemical Corp. (Ronkonkoma, NY). NADPH and palmitic acid were obtained from Sigma (St. Louis, MO), and synthetic DLPC and DOPC were from Avanti Polar Lipids, Inc. (Alabaster, AL). Ketoconazole was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). All other chemicals were of the highest purity commercially available.

RESULTS

LTB4 hydroxylation by human liver microsomes. To characterize the role of human P450 enzymes in hepatic LTB4 ω-hydroxylation, we initially assessed the properties of this eicosanoid-metabolizing reaction in intact liver microsomes. Hepatic microsomes from subject UC9209, when fortified with NADPH, converted LTB4 to a single product with a retention time on HPLC identical to that of authentic 20-OH LTB4 (data not shown). Product formation was not observed upon omission of NADPH from the reaction, nor was there evidence for formation of either 19-hydroxy LTB4 or 20-carboxy LTB4 (15, 30). Rates of microsomal 20-OH LTB4 formation were linear for at least 10 min with up to 100 pmol P450 (0.33 mg microsomal protein) per assay. The other nine human liver samples displayed a similar LTB4 metabolic profile, with rates ranging from 0.69 to 1.59 nmol 20-OH LTB4 formed/min/nmol P450 (0.14–0.42 nmol 20-OH LTB4 formed/min/mg protein). Overall, microsomal LTB4 3-hydroxylation rates were 1.06 ± 0.3 nmol product/min/nmol P450 or 0.25 ± 0.1 nmol product/min/mg protein (mean ± SD; $n = 10$).

Kinetic parameters of the LTB4 ω-hydroxylation reaction were subsequently examined using liver microsomes from subject UMi592. The conversion of LTB4 to 20-OH LTB4 was found to exhibit simple Michaelis–Menten kinetics over the range of LTB4 concentrations employed (3.75–120 μM) (Fig. 1A). The Eadie–Hofstee plot shown in Fig. 1B was used to derive an apparent K_m of 74.8 μM and a V_{MAX} of 2.42 nmol 20-OH LTB4 formed/min/nmol P450 (0.57 nmol/min/mg protein). Such monophasic kinetics indicated the involvement of only a single P450 enzyme in human hepatic LTB4 ω-hydroxylation, possibly CYP4F2.

The effects of various fatty acids and prostaglandins on LTB4 ω-hydroxylation by human liver microsomes are presented in Table I. These compounds were added to our incubation mixtures at concentrations 33 and

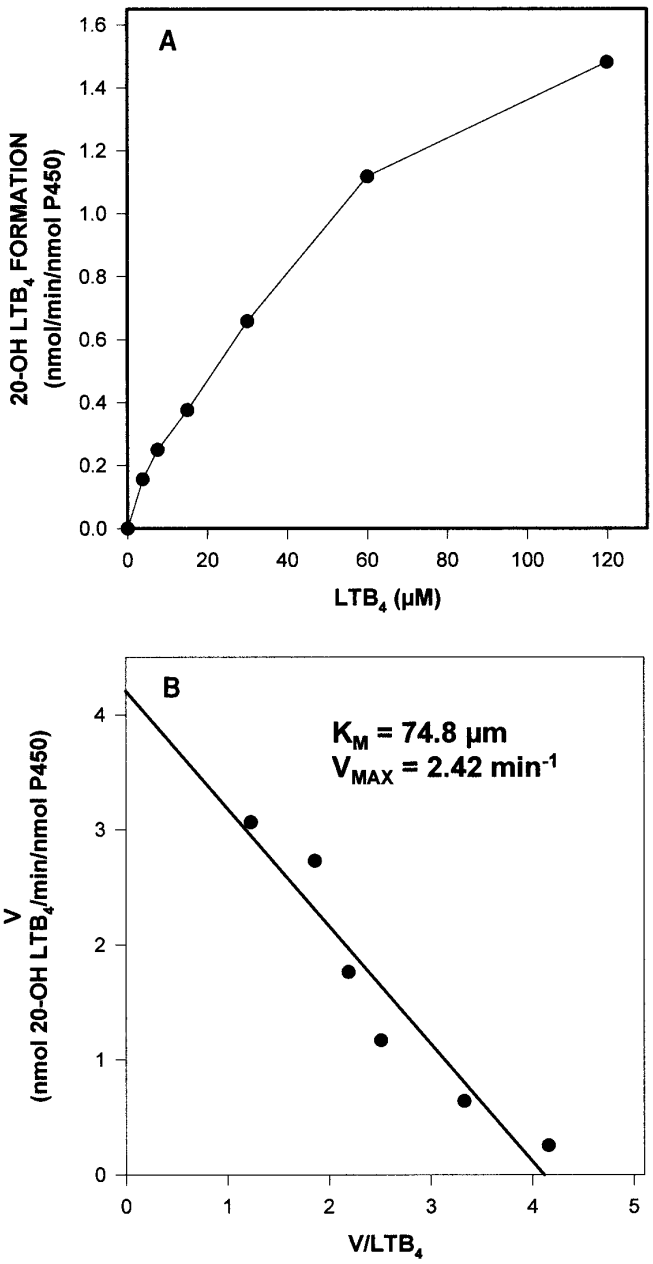

FIG. 1. Kinetic analysis of LTB4 20-hydroxylation by human liver microsomes. LTB4 20-hydroxylation by liver microsomes from subject UMi592 was assessed as described under Experimental Procedures. The LTB4 concentration was varied from 3.75 to 120 μM. (A) Plot of reaction velocity versus substrate concentration; (B) Lineweaver–Burke transformation of the data shown in A. The apparent K_m and V_{MAX} shown in B were derived by fitting the results to a single-component Michaelis–Menten equation.

333% that of LTB4. At high concentrations (100 μM), arachidonic acid elicited moderate (30%) yet significant inhibition of 20-OH LTB4 formation, whereas lauric and palmitic acids, which are substrates for CYP4A11 (23, 31), had essentially no effect on the reaction. Modest (20%) inhibition of microsomal LTB4 ω-hydroxylase activity was also noted with PGE_1 at each of the concentrations employed, while the inhibition (50%) observed with 100 μM PGA_1 was more substantial. The other prostaglandin examined, $PGF_{2\alpha}$, evoked little, if any, decrease in ω-hydroxylation of LTB4 (Table I). Inhibition of microsomal 20-OH LTB4 formation ranging from 53 to 72% was also elicited by both concentrations of ketoconazole, a fungicide which is a specific inhibitor of human CYP3A4-catalyzed reactions at low (1–10 μM) concentrations but a generalized human P450 inhibitor at higher (100 μM) concentrations (32).

Characteristics of purified human liver CYP4F2. Conventional purification methods, combined with screening of partially purified P450 fractions with rat CYP4A1 antibodies on Western blots, were employed to isolate CYP4F2 from human hepatic microsomes. It should be emphasized here that upon reaction with human liver microsomes, anti-rat CYP4A1 IgG recognized only a single protein, namely CYP4A11 (23). However, when partially purified P450 fractions were screened with rat CYP4A1 antibodies, another immunoreactive protein with a greater M_r (i.e., CYP4F2) was detected. Purified CYP4F2 had a specific content of 7.2 nmol P450/mg protein, exhibited a ferrous carbonyl Soret maximum at 450.5, and displayed an oxidized spectrum typical of a low-spin ferric hemoprotein with a Soret maximum at 417 nm. The hemoprotein was nearly homogeneous on SDS–PAGE, migrating with a molecular weight of 57,000 (see lane 4 in Fig. 2A), which was greater than that of CYP4A11 (M_r 52 kDa)

TABLE I

Effect of Fatty Acids and Prostaglandins on LTB4 20-Hydroxylation by Human Liver Microsomes

	LTB4 20-hydroxylation (% of control activity)	
Addition	10 μM	100 μM
Lauric acid	87.2 ± 3[a]	86.1 ± 7
Arachidonic acid	91.3 ± 4	69.2 ± 8[b]
Palmitic acid	101.1 ± 9	93.9 ± 21
PGA_1	91.3 ± 8	52.5 ± 7[b]
PGE_1	82.7 ± 3[b]	79.2 ± 5[b]
$PGF_{2\alpha}$	93.8 ± 8	86.5 ± 7
Ketoconazole	47.5 ± 21[b]	27.6 ± 5[b]

Note. LTB4 20-hydroxylase activity was assessed in incubation mixtures (0.2 ml) containing liver microsomes from subject UMi592 (protein equivalent to 0.1 nmol P450), 30 μM LTB4, 2.5 mM NADPH, 100 mM potassium phosphate buffer, and the compounds listed (added in ethanol) at the indicated concentrations. Reactions were initiated with NADPH and terminated after 10 min at 37°C, and 20-OH LTB4 formation was then assessed by HPLC as described under Experimental Procedures.

[a] Values denote the mean ± SD of three separate determinations. Control activity (vehicle only) was 0.53 ± 0.1 nmol 20-OH LTB4 formed/min/nmol P450.

[b] Significantly different ($P < 0.01$) from control activity by two-tailed t test.

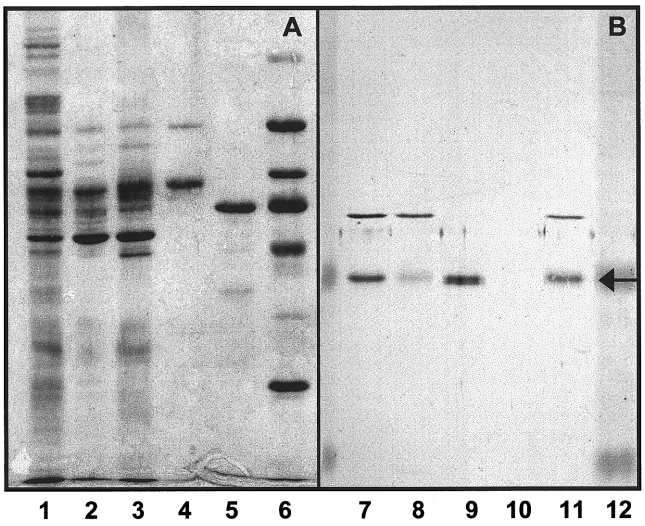

FIG. 2. SDS–PAGE and Western blot analysis of CYP4F2 purified from human liver microsomes. (A) Samples were subjected to electrophoresis on a slab gel (0.75 mm thick) containing 7.5% acrylamide using a discontinuous buffer system. Lane 1, human liver microsomes from subject UC9411 (10 μg); lane 2, tryptamine Sepharose buffer D eluate (3 μg); lane 3, Hypatite C 25 + 50 mM KPO_4 buffer eluate (4 μg); lane 4, purified CYP4F2 (0.5 μg); lane 5, purified CYP4A11 (0.5 μg); lane 6, protein standards (0.5 μg each) with molecular masses of 98, 68, 58, 53, 43, and 29 kDa (top to bottom). (B) Samples were subjected to SDS–PAGE as described above, followed by electrophoretic transfer to a nitrocellulose filter. The filter was then immunochemically stained with anti-CYP4F2 IgG as described under Experimental Procedures. Lane 7, human liver microsomes from subject UC9209 (10 μg); lane 8, human liver microsomes from subject UC9402 (10 μg); lane 9, purified CYP4F2 (0.1 μg); lane 10, purified CYP4A11 (0.1 μg); lane 11, human liver microsomes from subject UC9411 (10 μg); lane 12, prestained protein standards. The arrow denotes the position of CYP4F2. Note that the molecular masses of the Coomassie blue- and immunostained proteins have not been aligned.

(23). The minor contaminant of larger molecular weight (70 kDa) observed in the CYP4F2 preparation represented less than 5% of the total protein applied. A second CYP4F2 preparation isolated from a different human subject (UC8907) had a higher specific content (9.5 nmol P450/mg protein) but exhibited identical spectral properties and electrophoretic behavior (data not presented).

The results of CYP4F2 sequence analysis are given in Table II. As shown, 16 of the first 17 NH_2-terminal amino acids were identical to amino acids 5 through 21 deduced from the human liver *CYP4F2* cDNA sequence (17). The one exception was at position 12, where a glycine was found on the purified protein versus the tryptophan predicted from the cDNA. This glycine at position 12 was found on both CYP4F2 preparations subjected to sequencing, although the P450s were derived from different individuals. Interestingly, the *CYP4F2* cDNA sequence reported by Chen and Hardwick (18) has neither glycine nor tryptophan at position 12 but rather cysteine as well as aspartate at position 13 (Table II). The CYP4F2 NH_2-terminal sequence bore little resemblance to that of CYP4A11 although, like the latter P450, the first 4 residues (Met-Ser-Glu-Leu) deduced from the corresponding cDNA were not found on the hepatic protein. This phenomenon has also been described in the case of other purified CYP4 enzymes (33–36).

Upon reconstitution with P450 reductase and DLPC, CYP4F2 converted LTB4 to a single metabolite, namely 20-OH LTB4, at a rate of 392 pmol product formed/min/nmol P450 (Table III). Inclusion of b_5 in the CYP4F2 reconstituted system resulted in a marked (80%) decrease in rates of 20-OH LTB4 formation. In contrast to DLPC, the phospholipid L-α-dioleoylphosphatidylcholine failed to support LTB4 ω-hydroxyla-

TABLE II

Sequence Homology between Human Liver CYP4F2 and Other CYP4 Proteins

	Amino acid residue																					
	1	2	3	4	5	6	7	8	9	0	11	12	13	14	15	16	17	18	19	20	21	
CYP4F3 cDNA	**M**	P	**Q**	**L**	**S**	**L**	**S**	S	**L**	**G**	**L**	**W** (C)	**P** (D)[b]	M	**A**	**A**	**S**	**P**	**W**	**L**	**L**	18 / 21[a]
CYP4F2 cDNA	M	S	Q	L	S	L	S	W	L	G	L	W	P	V	A	A	S	P	W	L	L	—
CYP4F2	—	—	—	—	**S**	**L**	**S**	**W**	**L**	**G**	**L**	G	**P**	**V**	**A**	**A**	**S**	**P**	**W**	**L**	**L**	16 / 17
CYP4A11	—	—	—	—	V	**L**	**S**	P	S	R	**L**	L	G	D	V	S	G	I	L	Q	A	3 / 17

Note. The human liver *CYP4F2* (LTB_4) (17) and human neutrophil *CYP4F3* (9) sequences were deduced from the corresponding cDNAs, while the human liver CYP4F2 and CYP4A11 (23) sequences were derived from the purified hepatic proteins. Gaps have been introduced into the CYP4F2 and CYP4A11 sequences to optimize homology. Amino acid residues in common among *CYP4F2* and the other sequences shown are in boldface. CYP4F2 sequence analysis was performed with 26 pmol of PVDF-bound protein, and yields per residue ranged from 1.5 to 10.5 pmol.

[a] Number of matches with the amino acid sequence deduced from the *CYP4F2* cDNA.

[b] The CYP4F2 amino acid sequence deduced by Kikuta *et al.* (17) has tryptophan and proline at residues 12 and 13, respectively, while that found in the GenBank data base (Accession No. U02388) and attributed to Chen and Hardwick (18) has cysteine and aspartate at these residues.

TABLE III

LTB4 20-Hydroxylation by Purified Human Liver P450 Enzymes

Enzyme	LTB4 20-hydroxylation
CYP4F2	392[a]
+ b_5	85
CYP4A11	<10[b]
CYP2C9	<10
CYP2A6	<10
UC9209 liver microsomes	806

Note. LTB4 20-hydroxylase activity was assessed in incubation mixtures (0.2 ml) containing 100 mM potassium phosphate buffer (pH 7.4), 30 μM LTB4, 2.5 mM NADPH, and purified P450 enzymes or liver microsomes (protein equivalent to 100 pmol). Reconstituted systems were composed of 50 pmol purified P450, 150 pmol P450 reductase, and 15 μg DLPC. Where indicated, 200 pmol b_5 was included in the CYP4F2 reconstituted system. Reactions were initiated with NADPH and terminated after 20 min at 37°C. Formation of 20-OH LTB4 was quantitated by HPLC as described under Experimental Procedures.

[a] Values are expressed as pmol 20-OH LTB4 formed/min/nmol P450 and denote the average of at least two individual determinations.

[b] Below limits of detection.

tion by CYP4F2. None of the other human P450 enzymes examined here, including CYP4A11, CYP2C9, and CYP2A6, catalyzed ω-hydroxylation of LTB4 (Table III).

Immunochemical analysis of CYP4F2 participation in microsomal LTB4 ω-hydroxylation. The metabolic properties of purified, reconstituted CYP4F2, when viewed in light of the kinetic parameters of LTB4 ω-hydroxylation derived with hepatic microsomes, suggested that this P450 enzyme played a major role in LTB4 catabolism in human liver. To further assess this role, we employed antibodies raised against CYP4F2. The Western blot shown in Fig. 2B reveals that anti-CYP4F2 IgG recognized a single protein, expressed at variable levels in human liver microsomes (lanes 7, 8, and 11), with the same molecular weight as the immunogen (lane 9). No cross-reaction of anti-CYP4F2 with CYP4A11 (or with any other hepatic P450 enzyme) was noted, although the antibody did react with a 70-kDa non-P450 protein. Incubation of anti-CYP4F2 with liver microsomes from subject UMi592 resulted in a dramatic inhibition of LTB4 ω-hydroxylase activity (Fig. 3A). Inhibition approached maximum levels (95.1%) at an anti-CYP4F2:P450 ratio of only 2.5 mg IgG/nmol. In contrast, antibodies to another P450 enzyme, namely CYP2A6, were without effect on the microsomal LTB4 20-hydroxylation reaction (data not shown). Marked inhibition of LTB4 ω-hydroxylation by anti-CYP4F2 IgG was also observed in four other subjects (Fig. 3B), with overall inhibition averaging 91.9 ± 5% (n = 5).

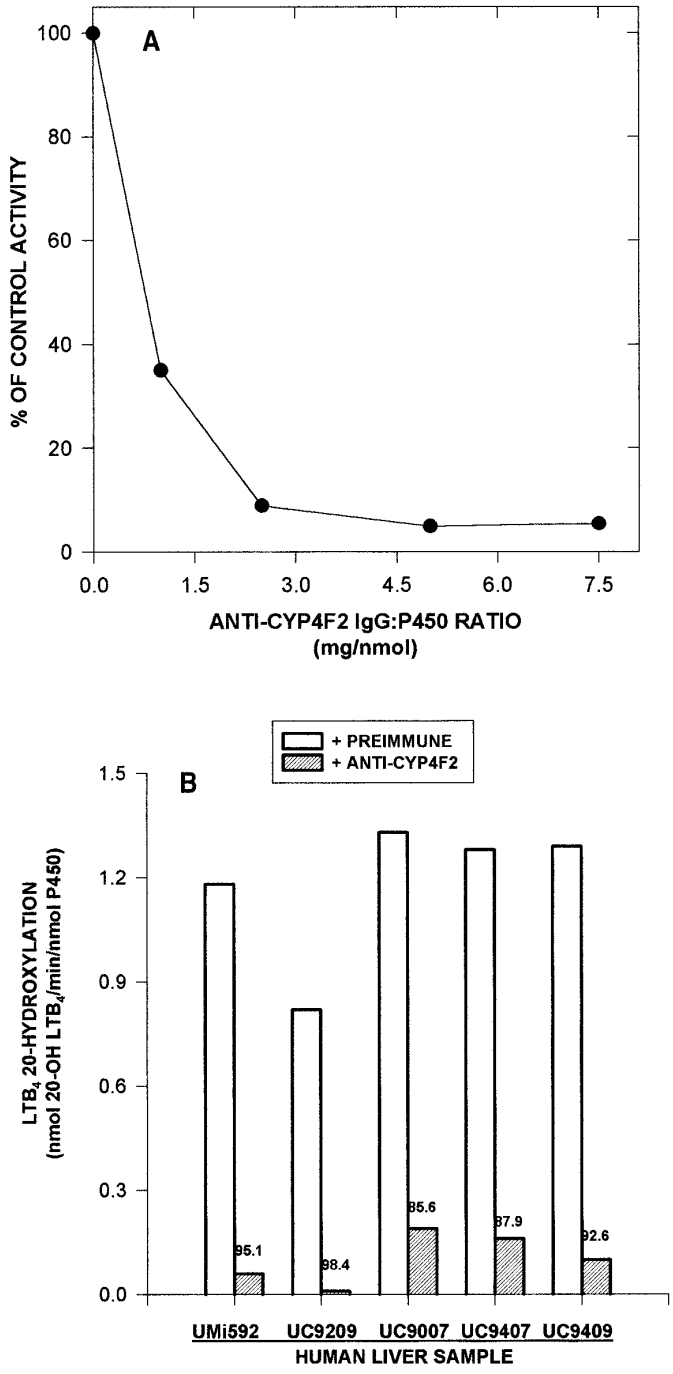

FIG. 3. Inhibition of LTB4 20-hydroxylation in human liver microsomes by anti-CYP4F2 IgG. (A) LTB4 20-hydroxylation was assessed in incubation mixtures containing liver microsomes from subject UC9209 (100 pmol P450), 30 μM LTB4, 2.5 mM NADPH, 100 mM KPO_4 buffer (pH 7.4), and anti-CYP4F2 IgG and/or preimmune (control) IgG. The total amount of immune-specific IgG plus preimmune IgG added was maintained at 0.75 mg. Reactions were performed as described under Experimental Procedures except that microsomes were preincubated with antibodies for 3 min at 37°C and for 10 min at ambient temperature before initiation. 100% of control activity was 1.18 nmol 20-OH LTB4 formed/min/nmol P450. (B) LTB4 20-hydroxylation was assessed using incubation conditions identical to those described for A except that 0.5 mg of preimmune IgG or anti-CYP4F2 IgG was included in the reactions. The human liver samples used are indicated. Values denote the average of three individual determinations.

We next employed CYP4F2 antibodies to quantitate levels of the corresponding antigen in hepatic microsomes from 10 different subjects. Western blots similar to that shown in Fig. 2B, coupled with scanning densitometry, were used for immunoquantitation. Expression of CYP4F2 was noted in all of the human liver samples and ranged from 35.7 to 194.5 pmol/mg microsomal protein (mean = 141.8 ± 52 pmol/mg). A comparison between CYP4F2 content and LTB4 ω-hydroxylase activity in these subjects revealed a strong correlation ($r = 0.860$, $P < 0.002$) (Fig. 4A). Aside from CYP4A11, correlations were not found between rates of 20-OH LTB4 formation in the liver samples and their content of other P450 enzymes, including CYP2A6 ($r = 0.050$), CYP2C8 ($r = 0.346$), CYP2C9 ($r = -0.539$), CYP2C19 ($r = 0.165$), CYP2D6 ($r = 0.545$), and CYP3A4 ($r = -0.024$) (data not shown). In the case of CYP4A11, the significant relationship ($r = 0.651$, $P < 0.05$) found between microsomal LTB4 20-hydroxylation and levels of this P450 stemmed from the correlation ($r = 0.682$, $P < 0.05$) between hepatic CYP4A11 and CYP4F2 contents in our sample population, as described elsewhere (25). Finally, a close relationship ($r = 0.927$, $P < 0.001$) was also noted between ω-hydroxylation of LTB4 and arachidonic acid, another CYP4F2 substrate (25), in the eight liver samples subjected to this correlation analysis (Fig. 4B).

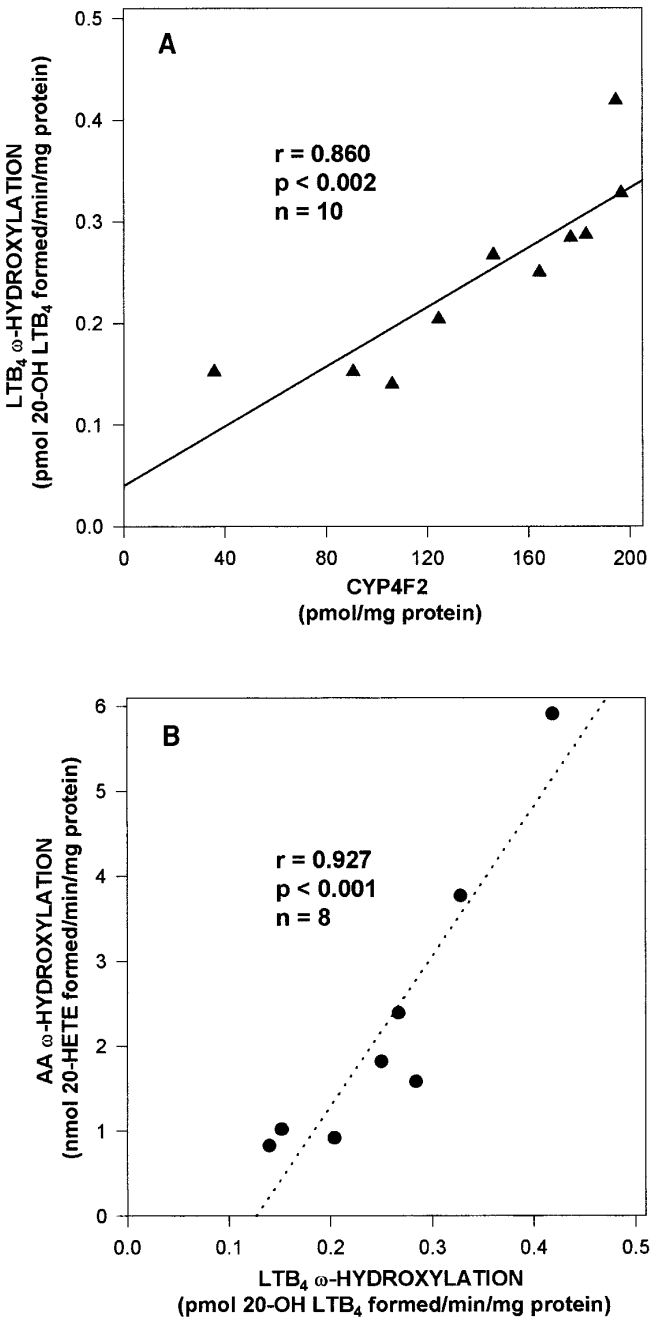

FIG. 4. Correlation between CYP4F2 content, LTB4 20-hydroxylation, and arachidonic acid 20-hydroxylation in human liver microsomes. (A) Microsomal CYP4F2 content was assessed in 10 human liver samples on immunoblots similar to that shown in Fig. 2B, while the conversion of LTB4 to 20-OH LTB4 by the samples was measured as described under Experimental Procedures. (B) Conversion of arachidonic acid to 20-HETE was assessed as detailed elsewhere (25).

DISCUSSION

It has become increasingly evident that microsomal P450 enzymes can play a pivotal role in the disposition of endobiotics with important physiological functions. The results presented herein augment that role by revealing that the *CYP4* gene subfamily member CYP4F2 is the principal enzyme in human liver underlying catabolism of the potent proinflammatory agent LTB4. Our approach for isolating CYP4F2 from human liver took advantage of the structural relatedness among the mammalian CYP4 proteins (37) and involved the use of antibodies to the rat fatty acid ω-hydroxylase CYP4A1. These antibodies, which cross-reacted with both human CYP4A11 (23) and a second P450 of larger molecular weight (57,000), were employed to track the latter enzyme during its multistep purification from hepatic microsomes. Sequence analysis of this second immunoreactive P450 confirmed its identity as CYP4F2, since the hemoprotein exhibited nearly complete homology over the first 17 amino acids with the human liver *CYP4F2* cDNA-derived sequence (17, 18). Purified CYP4F2 proved to be an efficient catalyst of LTB4 oxidation, hydroxylating this eicosanoid solely at the ω- or 20-position of the molecule. That CYP4F2 was the predominant LTB4 ω-hydroxylase in human liver was then revealed in immunoinhibition studies, in which it was shown that antibodies to this enzyme elicited marked (92%) inhibition of microsomal 20-OH LTB4 formation. These antibodies were also utilized to establish, for the first time, a relationship between expression of a specific P450 enzyme, namely CYP4F2, and LTB4 ω-hydroxylase activity in human liver.

Our studies on hepatic LTB4 ω-hydroxylation, which is the first step of the LTB4 catabolic pathway, initially focused on the properties of this reaction in intact human liver microsomes. It was found that hepatic microsomes from each of 10 different subjects converted LTB4 to a single major product, namely 20-OH LTB4, and that rates of product formation varied only threefold among individuals (see Results). In agreement with Kikuta *et al.* (17), we found no evidence of LTB4 oxidation by human liver microsomes to 19-hydroxy LTB4, a metabolite generated by rat liver microsomes via a P450 enzyme reportedly distinct from that catalyzing 20-OH LTB4 formation (15). Kinetic analysis of LTB4 ω-hydroxylation by human liver microsomes gave results (an apparent K_m of 74.8 μM and a V_{MAX} of 2.42 nmol 20-OH LTB4 formed/min/nmol P450) consistent with reaction catalysis by only a single hepatic P450 enzyme (Fig. 1). In fact, this Michaelis constant is similar not only to the apparent K_m values (40–42 μM) reported for rat liver LTB4 ω-hydroxylation (15, 16) but also to the K_m (45 μM) described for LTB4 20-hydroxylation by microsomes from yeast transfected with a human liver *CYP4F2* cDNA (17). Although the apparent K_m for LTB4 metabolism by human liver microsomes greatly exceeds that (0.7 μM) determined with human neutrophil microsomes (7), the comparable V_{MAX} values of 0.57 vs 0.42 nmol 20-OH LTB4 formed/min/mg protein for hepatic and neutrophil microsomes, respectively, indicate that the liver possesses an enormous capacity for LTB4 catabolism. With regard to the substrate specificity of human liver LTB4 ω-hydroxylase, both PGA_1 and arachidonic acid were found to elicit at least 30% inhibition of 20-OH LTB4 formation (Table I). Such results are in accord with the capacity of PGA_1 to serve as both a substrate and a competitive inhibitor of LTB4 ω-hydroxylase activity in rat hepatic microsomes (16) as well as with our recent finding that arachidonic acid is extensively ω-hydroxylated by human liver CYP4F2 (25). In contrast to CYP4A11 (23, 31, 38), medium-chain saturated fatty acids (e.g., lauric and palmitic acids) do not appear to be substrates for CYP4F2.

The kinetic properties of hepatic LTB4 ω-hydroxylation, when considered together with the underlying enzyme's substrate specificity, led us to surmise that CYP4F2 (or a closely related P450) was the principal catalyst of this reaction in human liver. We subsequently found that purified CYP4F2, upon reconstitution with P450 reductase and DLPC, indeed converted LTB4 to 20-OH LTB4 (Table III). Although rates of LTB4 ω-hydroxylation by CYP4F2 were only 50% of those observed with intact liver microsomes, this was most likely due to our inability to derive enzyme reconstitution conditions optimal for LTB4 catalysis rather than to the contamination of CYP4F2 with other P450s devoid of LTB4-metabolizing activity. That LTB4 ω-hydroxylation was catalyzed specifically by CYP4F2 was indicated by the failure of the other human liver P450s tested, including CYP4A11, to support this reaction (Table III). Surprisingly, addition of b_5 to the CYP4F2-reconstituted system decreased the enzyme's activity toward LTB4, an observation in direct contrast to the marked (nearly threefold) stimulation of CYP4F2-mediated arachidonic acid ω-hydroxylation by b_5 (25). Whether recombinant hepatic CYP4F2 and recombinant neutrophil CYP4F3 also hydroxylate LTB4 independent of b_5 participation is not known, as these enzymes have been examined only in unmodified microsomal preparations derived from yeast transfected with the corresponding cDNAs (9, 17). In fact, other studies on the involvement of b_5 in the LTB4 ω-hydroxylation reaction have yielded equivocal results, since antibodies to rat liver b_5 failed to affect LTB4 20-hydroxylation by human neutrophil microsomes (7) while the same antibodies markedly inhibited formation of 20-OH LTB4 by rat liver microsomes (16).

The CYP4F2 protein purified here from human liver exhibited properties similar to those of the analogous recombinant enzyme (17, 18), yet differed in several important aspects. LTB4 ω-hydroxylation by native CYP4F2, like recombinant CYP4F2, was completely regiospecific in nature, as only the LTB4 terminal methyl group and not the adjacent ω-1 methylene group was hydroxylated. In the case of native CYP4F2, this absolute regiospecificity extends to other substrates, including arachidonic and oleic acids, which are hydroxylated exclusively at the ultimate carbon (25, 39). The capacity of the corresponding recombinant enzyme to metabolize substrates other than LTB4 remains uncertain, however. While Kikuta *et al.* (17) reported that microsomes from yeast transfected with the human *CYP4F2* cDNA were inactive toward arachidonic acid, lauric acid, and PGA_1, Chen and Hardwick (18) found that microsomes from insect cells transfected with the same cDNA metabolized not only arachidonic and lauric acids but also steric and palmitic acids. Whether the hepatic *CYP4F2* cDNAs cloned by these investigators are indeed identical is also not clear, as the amino acid sequences derived from the respective cDNAs differ at residues 12 and 13 (Table II). The amino acid sequence of native CYP4F2 more closely resembles the cDNA-derived sequence of Kikuta *et al.* (17) but still differs from the latter at residue 12 (glycine instead of the predicted trytophan). Since glycine is rarely misassigned as trytophan and both hepatic CYP4F2 preparations isolated from different subjects in this study showed glycine at residue 12, our results may be indicative of CYP4F2 microheterogeneity in human liver.

Several lines of evidence presented in this study indicate that CYP4F2 is the principal catalyst of LTB4 ω-hydroxylation in human liver. First, antibodies to CYP4F2 were shown to invariably inhibit the bulk of LTB4 ω-hydroxylase activity in hepatic microsomes

(Fig. 3). In fact, inhibition of 20-OH LTB4 formation by anti-CYP4F2 averaged 92 ± 5% among the 5 human subjects examined. The anti-CYP4F2 IgG used in these experiments was highly specific for the corresponding P450 antigen, as shown on Western blots herein (Fig. 2) and elsewhere (25). Second, we demonstrated that CYP4F2 content in liver microsomes from 10 different subjects was strongly correlated ($r = 0.86$, $P < 0.002$) with rates of LTB4 ω-hydroxylation (Fig. 4A). Moreover, a close relationship ($r = 0.93$, $P < 0.001$) was observed between microsomal LTB4 ω-hydroxylation and arachidonic acid ω-hydroxylation (Fig. 4B). As CYP4F2 is the predominant arachidonate 20-hydroxylase in human liver (25), these data provide rather convincing evidence of an analogous role for CYP4F2 with regard to LTB4 20-hydroxylation. Interestingly, we found that rates of microsomal 20-OH LTB4 formation were correlated not only with hepatic levels of CYP4F2 but also with those of CYP4A11 ($r = 0.65$, $P < 0.05$), a P450 that exhibits negligible LTB4 ω-hydroxylase activity. Although the latter correlation can be accounted for by the relationship noted between hepatic CYP4F2 and CYP4A11 content ($r = 0.68$, $P < 0.05$) among the subjects examined here, these data nevertheless raise the intriguing possibility that the expression of CYP4F2 and CYP4A11 in human liver are coordinately regulated.

In light of their finding that LTB4 is a potent ligand of the peroxisome proliferator-activated receptor α (PPARα) in rats and mice, Devchand *et al.* (40) have suggested that activation of PPARα results in enhanced degradation of this inflammatory agent via the associated induction of the ω-hydroxylation (i.e., CYP4F) pathway. In fact, these investigators proposed a feedback mechanism whereby PPARα activation by LTB4 regulates not only the duration of the inflammatory response but also the clearance of LTB4 in the liver (40). However, certain other observations cast doubt on this mechanism, including the failure of clofibrate, a classical PPARα agonist as well as an inducer of medium- and long-chain fatty acid oxidation (41), to induce hepatic LTB4 20-hydroxylation in rats (5, 42). Furthermore, the pleiotropic response (including enhanced microsomal ω-hydroxylation and peroxisomal β-oxidation of fatty acids) noted in rodent liver upon administration of peroxisomal proliferators such as clofibrate and ciprofibrate has not been observed in human hepatocytes (43–45), possibly because of the low levels of PPARα expression in human liver (46). Nevertheless, when one considers the biological manifestations of LTB4 catabolism (and arachidonic acid oxidation) by CYP4F2, the potential induction of this P450 enzyme in human hepatic and extrahepatic tissues by hypolipidemic agents and/or pathophysiological states (e.g., hypertension and diabetes) (47–49) demands further investigation.

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