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A NOVEL RAT HEPATIC CLOFIBRATE-INDUCIBLE CYTOCHROME P450 THAT IS NOT A LAURIC ACID HYDROXYLASE

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Abstract—2-Methoxy-6-[1-methylethyl]naphthalene (MMEN) was hydroxylated in an NADPH-dependent manner to the (ω-1)-alcohol and the (R)-ω- and (S)-ω-alcohols by rat hepatic microsomes. (S)-ω-Hydroxylation was selectively induced 7-fold by clofibrate treatment. Phenobarbital, 3-methylcholanthrene, dexamethasone, cholestyramine, and MMEN did not induce this activity to the same extent. Incubation of the racemic ω-alcohols with microsomes isolated from rats resulted in a greater rate of degradation of the (S)- than the (R)-ω-alcohol confirming (S)-ω-hydroxylation to be an initial catalytic event. MMEN and lauric acid were not competitive inhibitors of each other in microsomes from clofibrate-treated rats, indicating the (S)-ω-MMEN hydroxylase to be a different enzyme from the characterized clofibrate-inducible lauric acid hydroxylases, CYP4A1 and CYP4A3. This was confirmed by the observations that (1) lauric acid hydroxylation was inhibited by 0.02% Tween 20 or Tween 80 and 25 μM capric or myristic acids, whereas ω-MMEN hydroxylation was not, (2) ω-MMEN hydroxylation was inhibited by ketoconazole, cholesterol and acetone, whereas lauric acid hydroxylation was not, and (3) CYP4A1 and CYP4A3 expressed in Hep G2 cells did not catalyze MMEN hydroxylation. Microsomes from the lungs of rabbits treated with progesterone and kidney of untreated rats did not support selective (S)-ω-MMEN hydroxylation, indicating that this activity is not associated with CYP4A4 or CYP4A2, respectively. Leukotriene B_4 (LTB_4) hepatic microsomal hydroxylation was not inhibited by MMEN and microsomes from human neutrophils did not support the reaction. These data identify a hitherto uncharacterized cytochrome P450 which is selectively induced by clofibrate and does not catalyze the ω-hydroxylation of the fatty acids or prostaglandins investigated. It is proposed that the enzyme catalyzing the selective (S)-ω-hydroxylation of MMEN is a novel rat P450 and that it is either a new member of the CYP4 family or a clofibrate-inducible P450 from another gene family.

Clofibrate is a hypolipidemic agent which upon chronic administration to rats causes liver enlargement, proliferation of the smooth endoplasmic reticulum and peroxisomes, and induction of some of the enzyme systems associated with these organelles [1–3]. A microsomal cytochrome P450, termed P450LAω or CYP4A1§, has been isolated and characterized from the liver of rats treated with clofibrate [5, 6]. Unique to this family of cytochromes P450 is the ω-oxidation of fatty acids [7]. Lauric acid and arachidonic acid ω-hydroxylation have been shown to be induced greater than 10-fold in microsomes isolated from male rats treated with clofibrate; however, the enzyme has little activity towards *d*-benzphetamine, testosterone, or ethoxyresorufin [2, 8]. It has been suggested that the ω-oxidation of fatty acids by clofibrate-inducible P450s may be partly responsible for induction of peroxisomal β-fatty acid oxidases and, in turn, the increased lipid catabolism associated with these compounds [9].

Recently, it has been shown that there are least three clofibrate-inducible CYP4A gene products in the rat [10, 11]; two of these products have been cloned, characterized, and expressed in Hep G2 cells [12]. Both of these products, CYP4A1 and CYP4A3, catalyze the ω-hydroxylation of lauric acid. One was shown to be identical with the previously purified P450LAω. Levels of the CYP4A mRNAs have been analyzed in rat liver and kidney. The CYP4A1, CYP4A2 and CYP4A3 mRNAs were present at very low levels in the liver of untreated rats and are coordinately induced in rats treated with clofibrate. In the kidney, CYP4A1 and CYP4A3 mRNAs were present in low levels and were induced by clofibrate in a manner similar to that in liver. In contrast, the level of CYP4A2 mRNA expression in the kidney of untreated rats was similar to that of the maximally induced CYP4A2 mRNA in liver. A P450 has been purified from untreated rat kidney by two groups [13–15] and based on limited amino acid similarities appears to correspond to CYP4A2. This P450, termed P450K-5, was capable of ω- and (ω-1)-hydroxylation of laric acid as well as other fatty acids.

In this report we present evidence for a rat hepatic clofibrate-inducible P450 that does not catalyze lauric acid hydroxylation, nor does it appear to be one of the characterized members of the CYP4A family. These data suggest this P450 is either a new member of the CYP4A family, or a clofibrate-inducible P450 from another gene family.

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§ The nomenclature used in this report is that described by Nebert *et al.* [4]. P450IVA1, -IVA2 and -IVA3 are herewith referred to as CYP4A1, CYP4A2 and CYP4A3.

EXPERIMENTAL PROCEDURES

Materials

2 - Methoxy - 6 - [1 - methylethyl]naphthalene (MMEN*) and the ω- and (ω-1)-alcohols were synthesized in the Institute of Organic Chemistry, Syntex Research (Palo Alto, CA). [^{14}C]Lauric acid was purchased from Amersham International (United Kingdom) and [^{3}H]leukotriene B_4 (LTB_4) from New England Nuclear, Du Pont (Wilmington, DE). LTB_4, 20-carboxy LTB_4, and 20-hydroxy LTB_4 were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). All other chemicals were of the highest grade available and were purchased from the Sigma Chemical Co. (St. Louis, MO).

Protein preparations

Microsomes and mitochondria were prepared as previously described [16, 17]. Protein concentration was determined by the method of Lowry *et al.* [18] with bovine serum albumin as standard. Adult Sprague–Dawley rats, approximately 200 g (Crl:CD BR Vaf+), were obtained from Charles River Laboratories (Portage, MI). For the induction studies rats were treated with clofibrate (400 mg/kg in corn oil, i.p., once daily for 3 days), phenobarbital (0.1% in drinking water for 6 days), 3-methylcholanthrene (25 mg/kg in corn oil, i.p., once daily for 3 days), dexamethasone (100 mg/kg in corn oil, i.p., once daily for 4 days), cholestyramine (4% (w/w) in diet for 1 week; animals were killed in the middle of the dark cycle), and MMEN (25 mg/kg in corn oil, i.p., daily for 4 days). Adult female New Zealand White rabbits (Hazelton Research Products Inc., Denver, PA) were treated with progesterone (1 mg/kg in corn oil, s.c., for 11 days). Human liver was a gift from Dr. A. Rettie, University of Washington, Seattle, WA. Hepatic microsomes were also obtained from cynomologus monkeys and beagle dogs. Human neutrophils were prepared by density step gradients using a Ficoll Pack in a modification of the method described by Böyum [19].

Cytochromes P450 CYP4A1 and CYP4A3 were cloned and expressed in Hep G2 cells as previously described [12].

Assays

MMEN. Incubations of MMEN contained protein, potassium phosphate buffer, pH 7.4 (50 μmol), magnesium chloride (3 μmol), glycerol (20%), EDTA (0.1 μmol), an NADPH-regenerating system consisting of glucose-6-phosphate (5 μmol), glucose-6-phosphate dehydrogenase (2 units), and NADPH (1 μmol) in a total volume of 1 mL and were agitated at 37° for 10 min. Substrate was added in 20 μL methanol. After terminating the incubations with 6 mL methylene chloride, they were vortexed for 1 min and then centrifuged; the aqueous phase was discarded, and 4.5 mL was evaporated to dryness under a stream of nitrogen. The samples were dissolved in the HPLC buffer system (30% acetonitrile, 70% 50 mM ammonium acetate, pH 5.0) and the products were separated by reverse-phase HPLC at a flow rate of 1.0 mL/min using a Beckman (Ultrasphere 5 μm, 4.6 × 150 mm) column and a Spectraphysics 8800 HPLC. Products were detected by fluorescence (excitation at 233 nm, emission filter cutoff at 340 nm) using a Kratos 970 detector. The amount of product formed was determined by comparing peak areas of metabolites to those generated using an external standard curve. The apparent K_m and V_{max} were determined from nonlinear regression analysis with the computer program Enzfitter (Elsevier-Biosoft).

The identities of the ω-(2-methoxy-6-[1-hydroxy-1-methylethyl]naphthalene) and the (ω-1)-alcohol (2-methoxy - 6 - [2 - hydroxy - 1 - methylethyl]naphthalene) were confirmed by HPLC mass spectrometry against chemically synthesized reference standards.

The LC/MS analyses were carried out using a TSQ 70 triple stage quadruple mass spectrometer (Finnigan-MAT; San Jose, CA) equipped with a thermospray ionization source. Analysis of metabolically generated ω-alcohol and authentic sample gives a spectrum that consists of ions corresponding to $(M + H)^+$ and $(M + NH_4)^+$ at m/z 217 and 234, respectively. The mass spectrum of the (ω-1)-alcohol is dominated by a signal at m/z 199 and suggests the presence of a labile substituent at the 2-position that is lost during the thermospray ionization process. MS/MS spectra of the ion at m/z 199 from either authentic or metabolically generated tertiary alcohol were identical (data not shown).

The *R* and *S* enantiomers of the ω-alcohol were separated by a modification of the procedure of Kern [20]. Briefly, the ω-alcohol peak was collected from the reverse-phase system, dried under nitrogen, and dissolved in chiral HPLC buffer (10% isopropyl alcohol, 90% 4 mM sodium phosphate buffer, pH 7.0). Samples were then chromatographed on a Chiral AGP (ChromTech) column and detected by fluorescence. The ratio of *R*/*S* was determined from peak areas.

Lauric acid. Typical incubations contained microsomal protein (0.05 to 1.0 mg/mL), potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (0.5 mM), [^{14}C]lauric acid (1–100 μM, 6 mCi/mmol), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (2 units) and NADPH (1 mM). Reactions were agitated for 10 min at 37° and then terminated with methylene chloride (6 mL). The samples were vortexed and then centrifuged, the aqueous layer was discarded, and the organic layer (5 mL) was evaporated to dryness under nitrogen at 30°. The residue was reconstituted in methanol (200 μL). The rate of lauric acid hydroxylation was determined by a modification of the method of Romano *et al.* [21]. Lauric acid and its metabolites were separated on an HPLC system consisting of a Series 410 liquid chromatograph, an ISS 100 autoinjector (Perkin–Elmer) and an IC Flo-One Beta radiochemical detector (Radiomatic Instruments). The radioactivity effluent was mixed with scintillation liquid at a ratio of 3:1. Chromatographic separation was accomplished with a Rainin 5 μm, 25 cm Microsorb C_{18} column by

* Abbreviations: MMEN, 2-methoxy-6-[1-methylethyl] naphthalene; and LTB_4, leukotriene B_4.

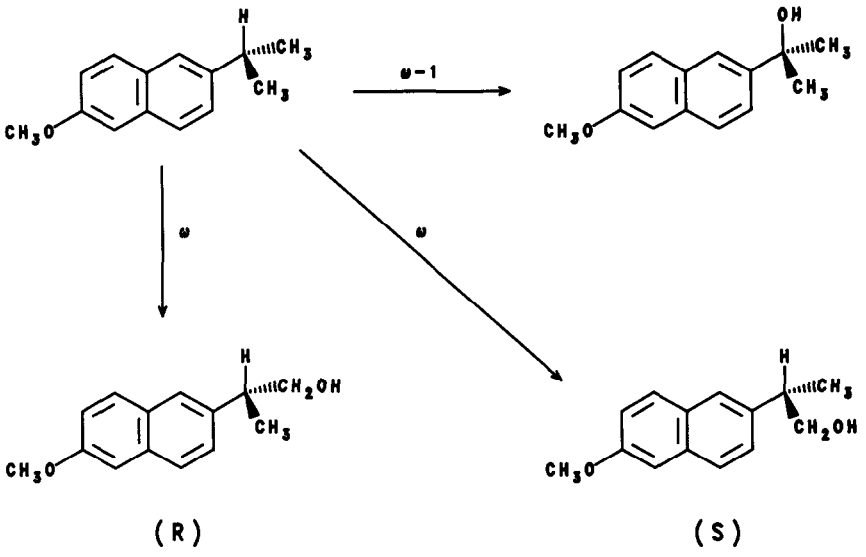

Fig. 1. Hydroxylation pathways of 2-methoxy-6-[1-methylethyl]naphthalene.

gradient elution with acetic acid (1%) and acetonitrile. Following the sample injection (50 μL), acetonitrile was held at 38% for 14 min, then increased linearly to 90% over the next 4 min, and held at 90% for 22 min prior to re-equilibration. The flow rate was 1 mL/min and the counting efficiency was 95%. Gradient conditions did not alter significantly the counting efficiency. The recovery of radioactivity from the column was >98%.

Leukotriene B_4 (LTB_4). Culture tubes contained microsomal protein (2.0 mg/mL), potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (0.5 mM), [^{3}H]LTB_4 (3 μM, 8 mCi/mmol), and a regenerating system consisting of glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (2 units). The reactions were started with NADPH (1 mM). Following agitation for 10 min at 37°, the incubations were terminated with 10% acetic acid (0.4 mL) and methylene chloride (6 mL). The samples were vortexed and then centrifuged, the aqueous layer was discarded, and the organic layer (5 mL) was evaporated to dryness under nitrogen at 30°. The residue was reconstituted in methanol (200 μL). The rate of LTB_4 ω-hydroxylation was determined by a modification of the method of Mukhtar *et al.* [22]. Separation of LTB_4 and its metabolites was achieved with a Rainin 5 μm, 25 cm Microsorb C_{18} column. The column was eluted with 0.01% acetic acid/methanol/acetonitrile under the following conditions: initial 50/50/0; 20 min with a No. 9 concave gradient to 0/0/100; isocratic for 20 min at 0/0/100. The flow rate was maintained at 1 mL/min.

RESULTS AND DISCUSSION

Oxidation of MMEN

Clofibrate-treated rat hepatic microsomes contain an NADPH-dependent activity which catalyzes the ω- and (ω-1)-hydroxylation of MMEN (Fig. 1). These products were identified by co-migration with authentic standards on TLC and HPLC and confirmed with LC/MS. Formation of these products in microsomes isolated from clofibrate-treated rats was linear with time for 20 min and microsomal protein up to 1 mg/mL. These three products accounted for the majority of radiolabeled products formed upon metabolism of [^{14}C]MMEN. Two unidentified more polar peaks (approximately 5% of products) and one unidentified less polar peak were also detectable (approximately 25% of products).

The reactions exhibited saturable kinetics in microsomes isolated from clofibrate-treated rats. The apparent K_m value for formation of both ω- and (ω-1)-alcohol was determined to be 42 μM and the k_{cat} values were 263 and 2760 pmol/min/mg protein, respectively (Fig. 2). The ratio of *S/R* ω-alcohol did not vary with substrate concentration.

Effects of inducers on MMEN oxidation

Adult male rats were treated with chemicals known to induce different families of cytochrome P450 isozymes [23]. The microsomes isolated from these treated animals were incubated with a saturating concentration of MMEN (200 μM), and maximum velocities of product formation were compared (Table 1). The rate of (*S*)-ω-hydroxylation was increased about 7-fold in microsomes obtained from clofibrate-treated male rats as compared to the untreated animals. The rates of (ω-1)- and (*R*)-ω-hydroxylation were unaffected. It is evident that clofibrate induces a P450(s) that specifically catalyzes (*S*)-ω-hydroxylation of MMEN.

Although none of the other chemicals used to induce P450-dependent metabolism selectively induced (*S*)-ω-hydroxylation, they each had a unique

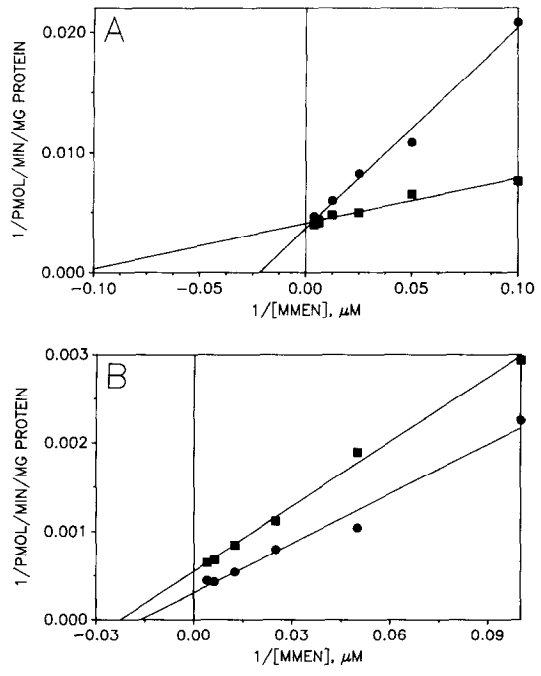

Fig. 2. Lineweaver–Burk plot of the effect of lauric acid upon 2-methoxy-6-[1-methylethyl]naphthalene metabolism. The rates of 2-methoxy-6-[1-methylethyl]naphthalene metabolism to the ω-alcohol (A) and the (ω-1)-alcohol (B) were determined in the presence (■) and absence (●) of 50 μM lauric acid. Microsomes (0.5 mg/mL) from clofibrate-treated rats were incubated for 10 min and products analyzed as described under Experimental Procedures. Metabolism was kept below 20%. The ω-alcohol formed at 10, 40, and 250 μM MMEN with and without lauric acid was subjected to chiral analysis. The percent (*S*)-ω-alcohol was between 76 and 79% in all samples.

effect upon product ratios.* Phenobarbital, an inducer of the CYP2B P450 family, increased the rate of (ω-1)-hydroxylation 2.2-fold, the greatest induction of the compounds tested. The rate of (*S*)-ω-hydroxylation was increased 1.9-fold. 3-Methylcholanthrene, an inducer of the CYP1A family, suppressed hydroxylation at all sites. Dexamethasone, an inducer of the CYP3A family, suppressed (*R*)-ω-hydroxylation. Cholestyramine, an inducer of hepatic P450s involved in cholesterol biosynthesis and degradation, decreased the rates of product formation. Interestingly, in contrast to microsomes from male untreated rats, female rats formed more (*S*)-ω-alcohol than (*R*)-ω-alcohol. In fact the *S*:*R* ratio in female rats was similar to that observed in microsomes from male rats treated with clofibrate. Finally, MMEN when given to rats did not induce its own metabolism.

Metabolism of racemic ω-alcohols

The apparent selective induction of the (*S*)-ω-alcohol could be a result of preferential metabolism of the (*R*)-ω-alcohol to a secondary product or conversion of the (*R*)-ω-alcohol to the (*S*)-ω-alcohol. To address these possibilities 1 nmol of racemic ω-alcohol was incubated with different protein concentrations of microsomes from untreated and clofibrate-treated male rats and the extent of metabolism was determined. Under these conditions, the (*S*)-ω-alcohol disappeared at a greater rate than the (*R*)-ω-alcohol (Fig. 3), indicating that the selective appearance of the (*S*)-ω-alcohol was the result of the initial oxidation and not due to secondary processes.

Effect of detergents

MMEN and lauric acid were incubated with microsomes isolated from clofibrate-treated rats in the presence of 0.02% of four detergents (Table 2). Although Tween 20, sodium cholate, and Triton X-100 had the same effect on ω- and (ω-1)-hydroxylation of MMEN, Tween 80 inhibited (ω-1)-hydroxylation but not ω-hydroxylation. Kinetic analysis of the inhibition showed a change in both K_m and V_{max} indicating a nonspecific inhibition of (ω-1)-hydroxylation (data not shown). This observation confirms that the majority of (ω-1)-hydroxylation in microsomes isolated from clofibrate-treated rats is catalyzed by a different P450 than catalyzes (*S*)-ω-hydroxylation.

Lauric acid ω-hydroxylation was inhibited by both Tween 20 and Tween 80, whereas MMEN-ω-hydroxylation was unaffected by these detergents. This suggests that the clofibrate-inducible P450 catalyzing MMEN-ω-hydroxylation may not be the same one that catalyzes lauric acid ω-hydroxylation.

Effect of lauric acid

As documented in the literature [2, 8], lauric acid ω-hydroxylation is specifically induced by clofibrate (Table 1). Indeed, lauric acid hydroxylation is catalyzed by all the characterized clofibrate-inducible cytochromes P450 [2, 15]. Further characterization of the induction of (*S*)-ω-MMEN activity by clofibrate was pursued by incubating microsomes in the presence of both MMEN and lauric acid. The apparent K_m for lauric acid ω-hydroxylation was determined to be 4.2 μM (data not shown) and, as stated above, the apparent K_m for MMEN ω-hydroxylation was 42 μM. Therefore, incubations with substrate and inhibitor concentrations at or near K_m should result in competitive inhibition if oxidation were occurring in the same enzyme active site. Surprisingly, incubation of 10 μM lauric acid in the presence of 0.5 to 250 μM MMEN resulted in no significant inhibition of lauric acid oxidation (data not shown). In addition, incubation of 50 μM lauric acid with 10–250 μM MMEN resulted in no inhibition of (*S*)-ω-MMEN oxidation (Fig. 2A). Kinetic analysis shows that while the calculated maximum velocities did not change (2629 and 2546 pmol/min/mg protein with and without lauric acid, respectively), addition of lauric acid resulted in a 4-fold lower apparent

* It is possible that there is more than one (*S*)-ω-hydroxylase and that inducers other than clofibrate induce one at the expense of another.

Table 1. Effects of cytochrome P450 inducers upon hepatic metabolism of 2-methoxy-6-[1-methylethyl]naphthalene and lauric acid*

		2-Methoxy-6-[1-methylethyl]naphthalene					Lauric acid		
		Rate of product formation (pmol/min/mg protein)					Rate of product formation (pmol/min/mg protein)		
			ω						
Inducer	Sex	ω-1	S	R	ω-1/ω	%S	ω-1	ω	ω-1/ω
None	M	1238	17	29	27	37	BQL	634	—
Clofibrate	M	1480	118	41	9.3	74	1075	6046	0.18
Phenobarbital	M	739	32	24	49	58	296	849	0.35
3-Methylcholanthrene	M	670	2.2	1.8	176	54	157	499	0.31
Dexamethasone	M	520	22	12	44	65	155	584	0.27
MMEN	M	143	23	31	21	42	200	689	0.29
Cholestyramine	M	816	9	17	32	35	145	250	0.58
None	F	512	13	5	28	73	ND	ND	—
Dexamthasone	F	1615	23.7	6.3	54	79	186	646	0.29

* BQL indicates below quantitation limits (75 pmol for lauric acid hydroxylation); and ND indicates not determined. The data represents the mean of at least two separate experiments of duplicate incubations.

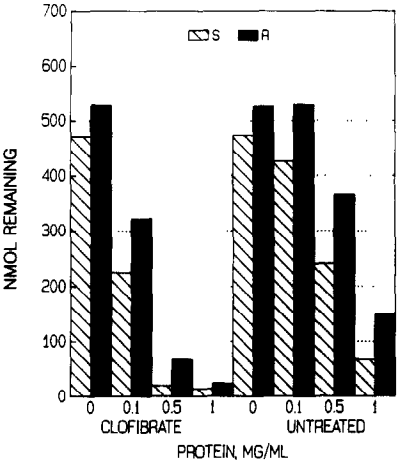

Fig. 3. Metabolism of racemic 2-methoxy-6-[1-methylethyl] naphthalene. Racemic 2-methoxy-6-[1-methylethyl]naphthalene (1 μM) was incubated with different protein concentrations of microsomes isolated from clofibrate-treated rats for 5 min. The amounts of (*R*)- and (*S*)-ω-alcohol remaining were determined as described in Experimental Procedures.

K_m.* In contrast, the apparent K_m and the V_{max} decreased for (ω-1)-hydroxylation (Fig. 2B). The noncompetitive inhibition of MMEN (ω-1)-hydroxylation and the lack of inhibition of ω-hydroxylation support the conclusion that the enzyme(s) catalyzing (ω-1)-hydroxylation is not the same as the enzyme(s) catalyzing (*S*)-ω-hydroxylation. Furthermore, the inability to show competitive inhibition between the two clofibrate-inducible activities suggests that these activities are associated with different enzymes—as was concluded from the detergent experiments.

Interaction with other fatty acids and P450 modulators

Table 3 shows the results obtained when a number of fatty acids and other P450 modulators were incubated with either MMEN or lauric acid in microsomes from clofibrate-induced male rats. Although most of the effects were not pronounced, some differences were observed. Capric and myristic acid inhibited lauric acid ω-hydroxylation by approximately 40%, whereas ω-hydroxylation of MMEN was unaffected. Conversely, ketoconazole, cholesterol, and acetone inhibited ω-hydroxylation of MMEN but not lauric acid. The differences cannot be a result of the relative affinities of the kinetic constants for the inhibitors and the two substrates. The concentration of ketoconazole used (5 μM) was ten times less than the apparent K_m for MMEN while approximately equal to that of lauric acid. Lauric acid should have inhibited MMEN oxidation to a greater extent if the selective inhibition was a result of differences in the relative kinetic constants.

MMEN (S)-ω-hydroxylase activity of two cDNA-expressed clofibrate-inducible P450

Two cytochromes P450, CYP4A1 and CYP4A3 known to be induced by clofibrate, have been characterized and expressed in Hep G2 cells using vaccina virus. A low level of activity was detected in cells containing only the wild type vaccinia vector. The Hep G2 cells infected with recombinant vaccina virus v4A1 and v4A3 had no detectable activity above wild type. These same cells have been shown to efficiently catalyze lauric acid hydroxylation [12]. From these data we conclude that the (*S*)-ω-MMEN hydroxylase is not CYP4A1 or CYP4A3.

Interaction with other ω-hydroxylases

A cytochrome P450 has been purified from untreated rat kidney, P450K-5, and based on limited amino acid sequence data shown to correspond to CYP4A2 [13–15]. This enzyme catalyzes lauric acid ω- and (ω-1)-hydroxylation and has high levels in kidney based on mRNA data. As shown in Table 4, microsomes from the kidney of untreated rat do not selectively catalyze MMEN-(*S*)-ω-hydroxylation. These data indicate that CYP4A2 is not the enzyme catalyzing (*S*)-ω-MMEN hydroxylation.

A number of studies have documented the cytochrome P450-dependent ω-hydroxylation of arachidonic acid and other prostaglandins [19, 24]. These activities have been detected in hepatic microsomes and are well characterized in lung and kidney [25]. One such prostaglandin hydroxylase, CYP4A4 or $P450_{PG\omega}$, has been shown to be induced in lungs of pregnant and progesterone-treated rabbits [26, 27]. We treated a New Zealand white rabbit with 1 mg/kg of progesterone, i.p., for 11 days and prepared microsomes from the lungs, kidneys, and liver. MMEN was metabolized by these microsomes without much enantioselectivity, suggesting that the enzyme(s) induced in this manner is not the (*S*)-ω-hydroxylase (Table 4). Inhibition of MMEN metabolism by arachidonic acid was investigated in microsomes from clofibrate-treated rats and progesterone-treated rabbits. (ω)-Hydroxylation was not inhibited by arachidonic acid with any of these preparations.

LTB_4 is a substrate for an ω-hydroxylase found in neutrophils, and the activity has also been detected in hepatic microsomes [22, 28]. No inhibition of LTB_4 activity was observed in microsomes from clofibrate-treated rats when 10 μM LTB_4 was incubated with 5 and 25 μM MMEN. In fact, a 26% increase in activity was observed (data not shown). Incubation of MMEN with microsomes prepared from human neutrophils resulted in significant

* It is unclear why the apparent K_m decreases from 42 to 11 μM upon addition of lauric acid. Possibly the affinity of the enzyme for MMEN is increased by a direct interaction with lauric acid, or indirectly as a result of lauric acid increasing the apparent concentration of MMEN. The latter could occur by displacement of MMEN from the membrane or by selective inhibition of the (ω-1)-hydroxylase which, due to the 10-fold greater maximum velocity would result in greater substrate availability. However, Tween 80, which inhibits (ω-1)-hydroxylation to a greater extent than lauric acid, had less effect upon the apparent K_m for (*S*)-ω-hydroxylation (a decrease from 62 to 30 μM). Another possibility is that lauric acid is acting like a detergent, partially solubilizing the microsomal membranes and thus allowing easier access of the MMEN substrate, i.e. increasing the affinity.

Table 2. Effects of detergents upon 2-methoxy-6-[1-methylethyl]naphthalene and lauric acid oxidation by microsomes from clofibrate-treated rats*

	2-Methoxy-6-[1-methylethyl]naphthalene		Lauric acid
	Product		Product
Detergent	ω (% Control)	ω-1	ω (% Control)
Tween 20	112	97	20
Tween 80	118	18	58
Triton X-100	55	48	51
Sodium cholate	98	102	116

* Final detergent concentration of 0.02%. Turnover numbers for control incubations were 124 and 1172 pmol/min/mg protein for ω- and (ω-1)-oxidation of MMEN, and 6086 pmol/min/mg protein for lauric acid ω-hydroxylation. Data represent the mean of duplicate incubations.

Table 3. Effects of fatty acids and other cytochrome P450 modulators upon the metabolism of 2-methoxy-6-[1-methylethyl]naphthalene and lauric acid by clofibrate-induced microsomes*

	2-Methoxy-6-[1-methylethyl]naphthalene		Lauric acid	
	Product		Product	
Effector	ω (% Control)	ω-1	ω-1 (% Control)	ω
None	100	100	100	100
Caprylic acid†	82	84	87	91
Capric acid†	80	78	37	61
Lauric acid†	80	87	—	—
Myristic acid†	100	95	94	62
Palmitic acid†	95	81	93	80
Steric acid†	80	90	78	89
Arachidic acid†	86	80	92	94
Arachadonic acid†	113	67	65	72
Linoleic acid†	73	66	86	90
Ketoconazole‡	49	63	105	109
α-Naphthoflavone‡	70	74	94	106
Cholesterol§	55	50	93	108
Acetone‖	63	91	100	95

* The effectors were added to tubes in methanol and the methanol was evaporated under nitrogen before other components were added. Data represent the mean of at least duplicate experiments with duplicate incubations.

† Final concentration 25 μM.

‡ Final concentration 5 μM.

§ Final concentration 50 μM.

‖ Final concentration 2%.

(ω-1)-hydroxylation but no detectable ω-hydroxylation (Table 4). Therefore, we conclude that the enzyme catalyzing (*S*)-hydroxylation of MMEN is not the characterized LTB_4 hydroxylase.

MMEN oxidation was investigated in other tissues and species to determine if (*S*)-ω-hydroxylation was constitutively expressed in any of these tissues or species. As seen in Table 4, the (*S*)-ω-alcohol was not formed to a great extent in the preparations investigated.

In conclusion, the above data show evidence for a clofibrate-inducible P450 which does not catalyze lauric acid hydroxylation. However, the identity of the enzyme is still in question. The data indicate that it is not CYP4A1, CYP4A2, or CYP4A3. Nor does it appear to be one of the other characterized ω-hydroxylases. We can only speculate on its identity as either a new member of the CYP4A family or a member of a different P450 family that is induced by clofibrate. The answer to this will only be resolved after the enzyme catalyzing the activity is purified or cloned and characterized.

The (*S*)-ω-oxidation of MMEN is particularly interesting from a mechanistic viewpoint because (ω-1)-hydroxylation is much more thermodynamically favored than ω-hydroxylation. These data together with the selectivity for (*S*)-ω-hydroxylation rather than (*R*)-ω-hydroxylation, indicate a highly ordered

Table 4. Oxidation of 2-methoxy-6-[1-methylethyl]naphthalene in various tissues and animal species

		2-Methoxy-6-[1-methylethyl]naphthalene				
		Rate of product formation (pmol/min/mg protein)				
			ω			
Tissue source	Prep*	ω-1	S	R	ω-1/ω	%S
M Rat liver	MICS	1962	32	54	23	37
M Human liver	MICS	107	10	5	7.1	68
M Monkey liver	MICS	472	44	34	6.0	57
M Dog liver	MICS	338	18	11	12	63
M Pig liver	MICS	285	5	16	14	22
M Hamster liver	MICS	1425	105	56	8.9	65
M Rabbit liver	SUP	46	6	3	5.1	63
Human placenta	MICS	0.2	0.2†		1.0	—
M Human neutrophil	MICS	153	BQL		—	—
Pig testes	MICS	59	BQL		—	—
M Rat kidney	MICS	6	1.6	0.8	2.5	62
M Cow adrenal	MICS	0.6	0.6	0.4	0.6	63
M Cow adrenal	MITO	0.9	0.25	0.15	2.2	62
M Rat hepatic	MITO	249	6	5	23	53
F Rabbit liver‡	MICS	361	39	21	6.0	65
F Rabbit lung‡	MICS	258	48	34	3.1	59
+Ara‡§	MICS	120	51	40	1.3	56
F Rabbit kidney‡	MICS	23	4.7	2.3	3.4	67
+Ara‡§	MICS	12	2	1	4.1	64

* MICS indicates microsomes; MITO indicates mitochondria; and SUP indicates 9000 *g* supernatant.
† Combined rate for *R* and *S* hydroxylation.
‡ Rabbits were treated with progesterone (1 mg/kg in corn oil, s.c. for 11 days).
§ Arachidonic acid (5 μM) in 2% MeOH.

active site in which the energy differences between catalytically competent binding conformations must be quite high. The thermodynamically unfavorable selectivity indicates that the active site is not as promiscuous as other hepatic drug-metabolizing enzymes.

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