Catalytic Activity and Isoform Specific Inhibition of Rat CYP4F Enzymes*

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Abbreviations: CYP or P450, cytochromes P450; LTB_4, leukotriene B_4; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; MS-PPOH, N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanoic acid; DDMS, *N*-methylsulfonyl-12,12-dibromododec-11-

enamide; 17-ODYA, 17-octadecynoic acid; 10-SUYS, sodium 10-undecynyl sulfate; ABT, 1-aminobenzotriazole.

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ABSTRACT

Arachidonic acid is ω-hydroxylated to 20-hydroxyeicosatetraenoic acid (20-HETE), which has effects on vasoactivity and renal tubular transport and has been implicated in the regulation of blood pressure. Cytochrome P450 4A (CYP4A) isoforms are generally considered the major arachidonic acid ω-hydroxylases, however, little is known about the role of rat CYP4F isoforms in 20-HETE formation. The rat CYP4F isoforms, CYP4F1, CYP4F4, CYP4F5, and CYP4F6, were heterologously expressed in *E. coli* and their substrate specificity in fatty acid metabolism was characterized. Substrate binding assays indicated that leukotriene B_4 (LTB_4) and arachidonic acid bound CYP4F1 and CYP4F4 in a Type I manner with a K_s of 25-59 μM, and lauric acid bound CYP4F4 poorly. Reconstituted CYP4F1 and CYP4F4 catalyzed the ω-hydroxylation of LTB_4 with a K_m of 24 and 31 μM respectively, and CYP4F5 had minor activity in LTB_4 metabolism. Importantly, CYP4F1 and CYP4F4 catalyzed the ω-hydroxylation of arachidonic acid with an apparent k_{cat} of 9 and 11 min^{-1}, respectively. Lauric acid was a poor substrate for all of the CYP4F isoforms and CYP4F6 had no detectable fatty acid ω-hydroxylase activity. The CYP ω-hydroxylase inhibitors 17-octadecynoic acid, 10-undecynyl sulfate and *N*-methylsulfonyl-12,12-dibromododec-11-enamide showed isoform specific inhibition of CYP4F1 and CYP4F4 catalyzed ω-hydroxylation of arachidonic acid and potency differences between the CYP4A and CYP4F isoforms. These data support a significant role for CYP4F1 and CYP4F4 in the formation of 20-HETE and identify CYP inhibitors that can be used to understand the relative contribution of the CYP4A and CYP4F isoforms to renal 20-HETE formation.

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Members of the cytochrome P450 4F (CYP4F) subfamily have been recently cloned from human, rat, mouse and ram and are of interest because of their role in the metabolism of endogenous fatty acids, prostaglandins and leukotrienes (Chen and Hardwick, 1993; Kikuta et al., 1993; Kawashima and Strobel, 1995; Cui et al., 2000; Bylund and Oliw, 2001; Cui et al., 2001; Hashizume et al., 2001). Four isoforms have been identified in the rat to date (Chen and Hardwick, 1993; Kawashima and Strobel, 1995). The first member of this family to be discovered was CYP4F1, a protein that is constitutively expressed at relatively high levels in rat hepatoma (Chen and Hardwick, 1993). Yeast expressed CYP4F1 catalyzed the ω-hydroxylation of leukotriene B_4 (LTB_4), lipoxin A_4, prostaglandin A_1 (PGA_1), and several hydroxyeicosatetraenoic acids (HETEs) (Kikuta et al., 1993). Rat CYP4F4, CYP4F5, and CYP4F6 were cloned from rat brain (Kawashima and Strobel, 1995). *E. coli* expressed CYP4F5 had low ω-hydroxylation activity toward LTB_4, while the solubilized membrane fraction of CYP4F4 recombinant protein catalyzed the ω-hydroxylation of PGA_1, PGE_1, 6-*trans*-LTB_4 and LTB_4 (Kawashima and Strobel, 1995). More recently, yeast expressed CYP4F5 was found to convert LTB_4 primarily into 18-hydroxy-LTB_4 and a relatively small amount of 19- and 17-hydroxy-LTB_4 and yeast expressed CYP4F6 was found to convert LTB_4 into 19- and 18-hydroxy-LTB_4 (Bylund et al., 2003). Neither CYP4F4 nor CYP4F1 was able to metabolize arachidonic acid or lauric acid, two typical substrates for CYP4A enzymes (Kawashima et al., 1997; Kikuta et al., 1999). Of the five human CYP4F isoforms identified to date, CYP4F2, CYP4F3, and CYP4F12 have all been shown to catalyze the ω-hydroxylation of arachidonic acid (Lasker et al., 2000; Christmas et al., 2001;

Hashizume et al., 2001). Moreover, CYP4F2 is the major enzyme that catalyzes the ω-hydroxylation of arachidonic acid in human kidney microsomes (Lasker et al., 2000).

The ω-hydroxylation of arachidonic acid to form 20-HETE is of increasing interest due to the biological effects of 20-HETE on vascular tone and renal function. 20-HETE inhibits a large conductance, Ca^{2+}-activated K^+ channel (maxi K) in renal microvessels leading to potent vasoconstriction (Zou et al., 1996). In addition, 20-HETE is an endogenous inhibitor of Na^+,K^+-ATPase (Schwartzman et al., 1985), blocks a 70 pS potassium channel in the TALH, and inhibits Na^+-K^+-$2Cl^-$ cotransport (Escalante et al., 1994; Wang and Lu, 1995). 20-HETE has also been implicated in the autoregulation of renal blood flow and tubuloglomerular feedback (Zou et al., 1994a; Zou et al., 1994b). Alterations in renal 20-HETE production may contribute to the development of hypertension in both the spontaneously hypertensive rat (SHR) and the Dahl salt-sensitive rat (Stec et al., 1996; Kroetz et al., 1997). Inhibition of renal 20-HETE formation with specific fatty acid ω-hydroxylase inhibitors or antisense oligonucleotides reduces blood pressure in the SHR and angiotensin II models of hypertension (Su et al., 1998; Wang et al., 2001; Alonso-Galicia et al., 2002; Xu et al., 2002).

The biological functions of 20-HETE are largely characterized in the rat and its renal formation is generally considered to be catalyzed by CYP4A enzymes (Nguyen et al., 1999; Hoch et al., 2000). However, the recent identification of CYP4F isoforms expressed in the rat kidney (Kalsotra et al., 2002; Bylund et al., 2003) and the finding that CYP4F2 is the major arachidonic acid ω-hydroxylase in human kidney (Lasker et al., 2000) led us to hypothesize that the CYP4F enzymes also contribute to renal 20-HETE formation in the rat. In the present study, the four rat CYP4F isoforms were

heterologously expressed in *E. coli* and their metabolism of fatty acids was characterized. The sensitivity of each of the CYP4F isoforms to known CYP inhibitors was also investigated.

METHODS

Materials. [1-^{14}C]Lauric acid (55 mCi/mmol) and [1-^{14}C]arachidonic acid (56 mCi/mmol) were from Amersham Life Science (Arlington Heights, IL). LTB_4 was purchased from Cayman Chemical Co. (Ann Arbor, MI). Human cytochrome b_5 was from Panvera (Madison, WI). 1-Aminobenzotriazole (ABT) and 17-octadecynoic acid (17-ODYA) were purchased from Sigma-Aldrich (St. Louis, MO). 10-Undecynyl sulfate (10-SUYS), N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanoic acid (MS-PPOH), and *N*-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS) were synthesized as described previously (Falck et al., 1997; Xu et al., 2002). Rat cytochrome P450 reductase was expressed and purified as previously reported (Dierks et al., 1998). Ampicillin, δ-aminolevulinic acid, glycerol, lysozyme, dilauroylphosphatidylcholine, catalase, and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). Emulgen 913 was from Karlan Research Products (Santa Rosa, CA). Acetonitrile (HPLC grade) and Scintiverse Cocktail LC were purchased from Fisher Scientific (Fair Lawn, NJ).

***E. coli* expression of CYP4F isoforms.** The CYP4F1 cDNA was kindly provided by Dr. James P. Hardwick (Northeastern Ohio Universities College of Medicine) and the full-length cDNAs of CYP4F4 (1608 bp), CYP4F5 (1660 bp), and CYP4F6 (1707 bp) were isolated from Sprague-Dawley rat liver or kidney as described previously (LeBrun et al., 2002). The CYP4F1, CYP4F4, CYP4F5, and CYP4F6 hexahistidine-tagged proteins were expressed in DH5α cells, purified on a Ni^{2+}-NTA agarose column from Qiagen (Chatsworth, CA), and desalted on a PD-10 column (Amersham Pharmacia Biotech, Solna, Sweden) (LeBrun et al., 2002).

Spectroscopic methods. Absolute spectra of rat CYP4F proteins were recorded on a Hewlett-Packard 8452 diode array spectrophotometer. Reduced CO-difference spectra and substrate perturbation analyses were done on a Varian Cary 1E UV/visible dual-beam spectrophotometer. Both instruments were equipped with a temperature control accessory. The P450 content was determined using the method of Omura and Sato (Omura and Sato, 1964a). To obtain substrate binding difference spectra, the fatty acids were dissolved in dimethylsulfoxide and titrated into the different CYP4F solutions using a 10 µl Hamilton syringe, resulting in a final sample volume change of less than 1%. All spectra were obtained at 25°C. The spectral binding constant K_s was determined from the hyperbolic plot of the differences in the 420- to 390-nm peak to trough absorbance versus the substrate concentration.

Measurement of CYP4F catalytic activity. The activity of CYP4F in the metabolism of fatty acids was measured in a reaction mixture of 20 µg/ml dilauroylphosphatidylcholine, 0.2 mg/ml sodium cholate, 50 pmol/ml CYP4F, 500 pmol/ml cytochrome P450 reductase, 50 pmol/ml cytochrome b_5, and 10 µg/ml catalase. This mixture was incubated for 10 min at room temperature before 100 mM potassium phosphate buffer, pH 7.4, containing 10 mM $MgCl_2$, 8 mM sodium isocitrate, and 0.5 IU isocitrate dehydrogenase was added. LTB_4 (100 µM), [1-^{14}C]arachidonic acid (40 µM), or [1-^{14}C]lauric acid (40 µM) was added to the incubation, and following a 3 min preincubation at 37°C the reaction was initiated by the addition of NADPH to a final concentration of 1 mM. The final volume of the reaction was 0.1 ml. The reaction was stopped after 1-30 min by the addition of 1 N HCl to a final pH of 3-3.5. Metabolites were extracted with ethyl acetate and quantified by HPLC with UV or radiometric

detection as described previously (Sumimoto et al., 1988; Kroetz et al., 1997; Su et al., 1998).

Effect of inhibitors on CYP4F-catalyzed arachidonic acid ω-hydroxylation. Reconstituted CYP4F1 and CYP4F4 were incubated for 30 min at 37°C with 1 mM NADPH, an isocitrate dehydrogenase regenerating buffer as described above, and various concentrations of the known CYP inhibitors MS-PPOH, 17-ODYA, DDMS, 10-SUYS, or ABT. After inactivation, the proteins were diluted 10-fold, and arachidonic acid metabolism was measured as described above. IC_{50} values were estimated by fitting the data to the equation $V = V_o/[1+(I/IC_{50})^n]$ where V is the 20-HETE formation rate in the presence of inhibitor, V_o is 20-HETE formation rate in the absence of inhibitor, I is the inhibitor concentration, and n is the Hill coefficient. The KaleidaGraph fitting program (Abelbeck Software) was used to fit the data by nonlinear regression.

RESULTS

CYP4F Protein Expression

The CYP4F1, CYP4F4, CYP4F5, and CYP4F6 cDNAs were expressed in *E. coli* and the histidine-tagged proteins were purified by Ni^{2+}-NTA agarose affinity column chromatography. Western immunoblotting of the purified proteins with an anti-histidine antibody confirmed that these proteins were histidine-tagged and that the size was in the range of CYP enzymes (data not shown). The proteins, after desalting, exhibited absorption spectra in the oxidized ferric state consistent with their identification as heme-containing CYP enzymes (Figure 1, panels A-D). The four proteins exhibited Soret maxima at 418 or 422 nm as expected for low-spin proteins with a distal water ligand to the heme iron atom (Dawson and Sono, 1987). After reaction with CO and reduction with sodium dithionite all four proteins exhibited a strong absorption with a maximum at 450 nm, as expected for intact CYPs (Figure 1, panels E-H) (Omura and Sato, 1964b). CYP4F1, CYP4F5 and CYP4F6 were present as pure intact, thiolate-ligated P450 proteins. A minor cytochrome P420 shoulder was seen for CYP4F4, indicating this protein was present as a mixture of the active P450 protein, and a denatured inactive protein in which the absorption maximum was at 420 nm. The proportion of the protein in the intact, active state in CYP4F4 was about 83%. The CYP4F4 protein has not previously been obtained in purified, active form (Kawashima et al., 1997).

The spectroscopic binding constants for LTB_4, lauric acid and arachidonic acid are summarized in Table 1. All the difference spectra were Type I (Estabrook et al., 1972), indicating a net shift of the heme from the low to the high spin state. The spectral

perturbation was characterized by a maximum at 390 nm, a trough with an absorbance minimum at 420 nm, and an apparent isosbestic point at 407 nm (Figure 2). No perturbation was observed when lauric acid was titrated into CYP4F1 or when all three substrates were titrated into CYP4F5 and CYP4F6, suggesting that these fatty acids were bound very poorly or, when bound, did not induce a spin state change of the iron atom. In general, arachidonic acid and LTB_4 had similar binding affinity to CYP4F1 and CYP4F4. Lauric acid bound CYP4F4 but not CYP4F1. In contrast, arachidonic acid and lauric acid had higher binding affinity to CYP4A isoforms than CYP4F isoforms (Hoch et al., 2000).

Fatty acid metabolism by CYP4F isoforms

To establish optimal conditions for determination of CYP4F catalytic activity, the effects of varying amounts of NADPH P450 reductase and cytochrome b_5 on arachidonic acid hydroxylation were measured. CYP4F1 and CYP4F4 had catalytic activity toward arachidonic acid in the absence of cytochrome b_5, but an equimolar amount of cytochrome b_5 and a 2-10-fold excess of NADPH P450 reductase were needed for maximal catalytic activity of both enzymes (data not shown). Based on these preliminary studies 50 pmol/ml of purified enzyme and a 1:10:1 ratio of CYP4F:cytochrome P450 reductase:cytochrome b_5 were chosen to characterize the activity of rat CYP4F isoforms in the metabolism of fatty acids.

CYP4F enzymes have been characterized as LTB_4 ω-hydroxylases (Kawashima et al., 1997; Kikuta et al., 1999) so this substrate was used as a positive control to test the catalytic activity of the expressed rat CYP4F isoforms. Recombinant CYP4F1, CYP4F4,

and CYP4F5 proteins all catalyzed the metabolism of LTB_4, while CYP4F6 had no detectable activity (Figure 3). CYP4F4 had the highest LTB_4 ω-hydroxylase activity with a K_m and V_{max} of 31 μM and 40 nmol/min/nmol respectively, followed by CYP4F1 with a K_m and V_{max} of 24 μM and 10 nmol/min/nmol respectively (Figure 4). CYP4F5 had only minor activity towards LTB_4 and the kinetic parameters were not measurable.

Figure 5 shows a representative HPLC chromatogram of arachidonic acid metabolites formed following incubation with the expressed rat CYP4F isoforms. CYP4F1 and CYP4F4 ω-hydroxylated arachidonic acid to form 20-HETE, the major metabolite detected. Both enzymes also formed minor amounts of the ω-1 hydroxylation product, 19-HETE (Figures 5A and 5B). The relative ratios of the ω- and ω-1 hydroxylated metabolites were 21:1 for CYP4F1 and 20:1 for CYP4F4, indicating a strong preference for oxidation at the ω-position of arachidonic acid. In contrast, CYP4F5 and CYP4F6 had no detectable catalytic activity toward arachidonic acid, despite modification of the reconstitution and incubation conditions (Figures 5C and 5D).

The kinetics describing arachidonic acid metabolism by CYP4F1 and CYP4F4 were investigated and the concentration-dependent activity is shown in Figure 6. Increasing ω-hydroxylase activities were measured with increasing arachidonic acid concentrations up to 40 μM. Further increases in the concentration of arachidonic acid resulted in a gradual drop in activity. This decrease in activity may be caused by substrate or product inhibition, by micellar aggregation of the fatty acids, or by physical effects of the fatty acids on the reconstituted enzyme. It has previously been suggested that the detergent activity of long chain fatty acids may disturb the arrangement of the electron transfer partners in the reconstituted system (Hoch et al., 2000).

The catalytic activities of the CYP4F proteins with fatty acids were further characterized by measuring apparent k_{cat} values. CYP4F4 had the highest activity in the metabolism of arachidonate, laurate and LTB_4 (Table 2). LTB_4 was the best substrate for CYP4F4, while arachidonic acid was the best substrate for CYP4F1. Arachidonic acid was a much better substrate for CYP4F1 and CYP4F4 than lauric acid. In contrast, CYP4A1, CYP4A2, CYP4A3, and CYP4A8 all catalyze the ω- and ω-1-hydroxylation of arachidonic acid and lauric acid with the latter being the preferred substrate (Nguyen et al., 1999; Hoch et al., 2000). CYP4F1 and CYP4F4 had similar arachidonic acid ω-hydroxylase activity as CYP4A1, the most active CYP4A isoform with an apparent k_{cat} of 6 min^{-1} (Hoch et al., 2000).

Lauric acid, while not physiologically significant, has been used as a prototype substrate for the CYP4A proteins and was investigated here as a substrate for the rat CYP4F isoforms. Only CYP4F4 had minor lauric acid hydroxylase activity (Figure 7, Table 2). The relative ratio of the ω– and (ω-1)-hydroxylated metabolites was 5:1.

Effect of inhibitors on recombinant CYP4F-catalyzed arachidonic acid ω-hydroxylation

Numerous inhibitors of arachidonic acid ω-hydroxylation and epoxidation have been characterized for their activity against the CYP4A isoforms but their effect on the CYP4F enzymes has not been reported. The terminal acetylenic compound MS-PPOH selectively inhibited microsomal arachidonic acid epoxidation (Wang et al., 1998) and the addition of MS-PPOH (1-500 μM) had no effect on CYP4F1- and CYP4F4-mediated ω-hydroxylation of arachidonic acid (Table 3). Both ω-hydroxylation and epoxidation of

arachidonic acid by Sf9-expressed CYP4A isoforms is inhibited by 17-ODYA, a widely used acetylenic inhibitor of fatty acid metabolism (Zou et al., 1994c; Nguyen et al., 1999). 17-ODYA also potently inhibited the CYP4F1- and CYP4F4-mediated ω-hydroxylation of arachidonic acid with an IC_{50} of 1.81 and 5.59 μM, respectively (Table 3 and Figure 8A). DDMS, a specific inhibitor of microsomal arachidonic acid ω-hydroxylation, potently decreased ω- and ω-1-hydroxylations and epoxidation catalyzed by CYP4A1, CYP4A2, and CYP4A3 with a similar IC_{50} for all isoforms (Wang et al., 1998). In contrast, it had no effect on CYP4F1-mediated ω-hydroxylation of arachidonic acid, and only slightly inhibited CYP4F4-mediated 20-HETE formation with an IC_{50} of 145 μM (Table 3). 10-SUYS, a specific inhibitor of microsomal arachidonic acid ω-hydroxylation (Xu et al., 2002), inhibited the arachidonic acid ω-hydroxylase activity of CYP4F1 and CYP4F4 with an IC_{50} of 231 and 25.0 μM, respectively (Table 3 and Figure 8B). ABT has been characterized as a specific inhibitor of microsomal arachidonic acid ω-hydroxylation (Su et al., 1998) and it inhibited the CYP4F1- and CYP4F4-mediated ω-hydroxylation of arachidonic acid with an IC_{50} of 289 and 371 μM, respectively (Table 3 and Figure 8C). With the exception of ABT, CYP4F1 and CYP4F4 showed different sensitivity to the CYP ω-hydroxylase inhibitors tested (Table 3 and Figure 8).

DISCUSSION

The emerging importance of 20-HETE in the regulation of vascular tone and renal tubular transport has led to a renewed interest in understanding the mechanisms which regulate 20-HETE formation within the kidney. The ω-hydroxylation of arachidonic acid to form 20-HETE has generally been attributed to the CYP4A isoforms (Nguyen et al., 1999; Hoch et al., 2000). More recently, members of the CYP4F family have been cloned and characterized with respect to their catalytic function, tissue expression, and transcriptional regulation (Chen and Hardwick, 1993; Kawashima and Strobel, 1995; Kawashima et al., 1997; Kikuta et al., 1999; Cui et al., 2000; Zhang et al., 2000; Cui et al., 2001). Much of the interest in the CYP4F enzymes has focused on their role in the ω-hydroxylation of LTB_4, an important step in controlling the potent inflammatory properties of this eicosanoid (Sumimoto et al., 1988). In contrast, little is known about the ability of the CYP4F proteins to catalyze arachidonic acid metabolism. Although the rat CYP4F isoforms have been heterologously expressed in both yeast and *E. coli* (Kawashima et al., 1997; Kikuta et al., 1999; Bylund et al., 2003), this represents the first study in which the catalytic function of all four rat isoforms has been directly compared. Despite more than 70% amino acid sequence identity, the rat CYP4F isoforms show differences in substrate specificity and sensitivity to known CYP inhibitors.

Both human and rat CYP4Fs have been characterized as LTB_4 ω-hydroxylases (Kikuta et al., 1993; Kawashima et al., 1997; Kikuta et al., 1999). The current study suggests that the majority of the LTB_4 ω-hydroxylase activity in the rat is catalyzed by CYP4F1 and CYP4F4. The K_m for CYP4F1 in the present study (24 μM) is much lower than that reported for CYP4F1 expressed in yeast (134 μM; (Kikuta et al., 1999)),

possibly due to the different expression systems and incubation conditions. The activity of CYP4F4 was also much higher than that reported previously for an *E. coli* expressed cell lysate (Kawashima et al., 1997). Despite repeated attempts to optimize the reconstitution and incubation conditions for CYP4F5 and CYP4F6, metabolism of LTB_4 was difficult to detect. Minor activity was detected with CYP4F5 while CYP4F6 was devoid of LTB_4 ω-hydroxylase activity. These results are in conflict with the extensive side chain hydroxylation of LTB_4 reported for yeast expressed CYP4F5 and CYP4F6 (Bylund et al., 2003). While CYP4F1 and CYP4F4 are clearly implicated in the metabolism of LTB_4 and regulation of inflammation further studies will be necessary to clarify the role of CYP4F5 and CYP4F6 in this pathway.

A direct comparison of the arachidonic acid ω-hydroxylase activity of all four rat CYP4F isoforms identified CYP4F1 and CYP4F4 as major catalysts of 20-HETE formation. In fact, the k_{cat} values for CYP4F1 and CYP4F4 were very similar to those reported previously for the most active CYP4A isoform, CYP4A1 (Hoch et al., 2000). This is consistent with the identification of CYP4F2 as the major arachidonic acid ω-hydroxylase in human liver (Lasker et al., 2000). However, the inability of yeast expressed CYP4F1 (Kikuta et al., 1999) and *E. coli* expressed CYP4F4 (Kawashima et al., 1997) to metabolize arachidonic or lauric acid highlights the importance of determining optimal conditions for reconstitution of CYP activity with expressed proteins. While the relative importance of the CYP4A and CYP4F isoforms in 20-HETE formation within rat tissues is still unclear, these results support a potential role for both enzyme families in this catalytic function.

A major difference in the activity of the CYP4A and CYP4F isoforms is reflected in the inability of most of the latter enzymes to metabolize lauric acid. CYP4A isoforms readily catalyze the ω-hydroxylation of lauric acid, which is the prototypical substrate for this subfamily (Nguyen et al., 1999; Hoch et al., 2000). Elongation of the fatty acid chain is associated with a loss of CYP4A ω-hydroxylase activity (Hoch et al., 2000). While further studies will be necessary to determine the influence of hydrocarbon chain length on the activity of the CYP4F enzymes, these results establish distinct substrate specificity between the CYP4A and CYP4F subfamilies.

The identification of CYP4F1 and CYP4F4 as arachidonic acid ω-hydroxylases and their detection in rat kidney (Kalsotra et al., 2002) implicates them in the renal formation of 20-HETE. CYP4F1 accounts for 95% of total CYP4F expression in the Sprague-Dawley kidney, while CYP4F4 has the lowest expression (less than 0.01% of total CYP4F expression) (Kalsotra et al., 2002). The abundant expression of CYP4F1 in the rat kidney and our finding that CYP4F1 has similar arachidonic acid ω-hydroxylase activity as the most active CYP4A isoform, CYP4A1, suggests that CYP4F1 is the major CYP4F isoform that contributes to renal 20-HETE biosynthesis in the kidney. Relatively little is known about the regulation of the renal CYP4F enzymes. Clofibrate treatment decreased CYP4F1, CYP4F5 and CYP4F6 and had no effect on CYP4F4 mRNA levels in rat liver (Kalsotra et al., 2002). While tissue specific differences in response to fibric acids make it difficult to predict the effect of this classic inducer on renal CY4F levels, it is interesting to note that in the liver CYP4F1 is repressed while CYP4A1 is induced by clofibrate. This suggests that the increased 20-HETE formation expected from CYP4A1 induction by clofibrate may be somewhat balanced by the decrease in 20-HETE

formation due to CYP4F1 repression. Renal CYP4F expression was also reported to be higher in females relative to males and to be regulated by estrogen. This is in contrast to CYP4A expression which is higher in males than females. This would support a differential role for the CYP4F and CYP4A isoforms in 20-HETE formation in males and females. Interestingly, Kalsotra *et al.* also reported that the expression level of lung CYP4F4 can be induced up to 10-fold 24 hour after traumatic brain injury (Kalsotra et al., 2002). Our results indicate that CYP4F4 has higher arachidonic acid ω-hydroxylase activity than CYP4F1 but its low expression in kidney does not support a major role for this isoform in renal 20-HETE formation. It will be interesting to examine whether CYP4F4 plays its major role in 20-HETE biosynthesis in other tissues during such conditions as traumatic brain injury.

The potent vasoconstrictor effect of 20-HETE on renal arteries is considered prohypertensive while its natriuretic and diuretic effects in renal tubules would contribute to a lowering of blood pressure. The relative importance of the CYP4F and CYP4A isoforms to renal 20-HETE formation will be a function of their expression pattern within the renal tubules and microvessels as well as the catalytic function of each enzyme. CYP4A and CYP4F mRNAs are expressed in a segment and gender specific pattern in the mouse kidney (Stec et al., 2003). Characterization of the corresponding expression pattern in the rat kidney will provide important information about the relative contribution of each of the CYP4F and CYP4A isoforms to renal 20-HETE formation.

CYP inhibitors are an important tool in characterizing the biological significance of 20-HETE. CYP4F1 and CYP4F4 displayed different sensitivity to several inhibitors of CYP ω-hydroxylase activity. Surprisingly, DDMS, a very potent inhibitor of CYP4A-

catalyzed arachidonic acid ω-hydroxylation and epoxidation (Nguyen et al., 1999) had minimal effect on CYP4F1 or CYP4F4-catalyzed 20-HETE formation. 10-SUYS, a specific inhibitor of microsomal arachidonic acid ω-hydroxylation (Xu et al., 2002), was a relatively potent inhibitor of CYP4F4 but had only a minor effect on CYP4F1. This suggests that 10-SUYS can be used at low concentrations to selectively inhibit CYP4F4 fatty acid ω-hydroxylase activity and that DDMS can be used to selectively inhibit CYP4A but not CYP4F arachidonic acid ω-hydroxylase activity. In contrast, 17-ODYA and ABT showed similar effects on CYP4F1 or CYP4F4, consistent with their relatively non-specific inhibition of arachidonic acid hydroxylation and epoxidation (Wang et al., 1998; Nguyen et al., 1999; Alonso-Galicia et al., 2002). The isoform selectivity observed in this study is consistent with the incomplete inhibition of arachidonic acid ω-hydroxylation observed with many CYP inhibitors when used in microsomal systems. The identification of isoform specific inhibitors such as 10-SUYS and DDMS will be invaluable for characterizing the relative contribution of a given CYP4A and/or CYP4F enzyme to 20-HETE formation.

In summary, CYP4F1 and CYP4F4 have been identified as arachidonic acid ω-hydroxylases. In light of the abundant expression of CYP4F1 in the rat kidney (Kalsotra et al., 2002), CYP4F1 may be a major contributor to the biosynthesis of 20-HETE in the rat kidney. Characterization of isoform-selective inhibition of arachidonic acid ω-hydroxylation supports the development of novel CYP inhibitors for widespread use in the *in vivo* and *in vitro* characterization of the biological properties of 20-HETE.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Spectroscopic properties of CYP4F proteins. The absolute absorbance and reduced CO difference spectra of the purified CYP4F proteins were collected at 25°C as described in the Methods section. Absolute absorbance spectra (A-D) and the corresponding reduced CO spectra (E-H) are shown for CYP4F1 (A and E), CYP4F4 (B and F), CYP4F5 (C and G), and CYP4F6 (D and H).

Figure 2. Representative substrate perturbation difference spectra. Aliquots of 0.50 mL of 1.5 μM CYP4F4 were placed in the sample and reference cuvettes of the spectrophotometer at 25°C and the sample absorbance was adjusted to zero (baseline). Arachidonic acid was dissolved in dimethylsulfoxide and titrated into CYP4F4 using a 10-μl Hamilton syringe, resulting in a final sample volume change of less than 1%. The insert shows the determination of a spectral binding constant (K_S) for arachidonic acid derived from plotting the difference in absorbance between peak and trough versus substrate concentration.

Figure 3. Representative chromatogram showing leukotriene B_4 metabolism by *E. coli* expressed CYP4F isoforms. Leukotriene B_4 metabolism by 50 pmol/ml of expressed CYP4F1 (B), CYP4F4 (C), CYP4F5 (D), and CYP4F6 (E) reconstituted with purified NADPH P450 reductase (500 pmol/ml) and cytochrome b_5 (50 pmol/ml) was measured in the presence of NADPH (1 mM). Reactions were carried out for 20 min at 37°C, and metabolites were extracted and separated by HPLC. The identity of leukotriene B_4 and its metabolites was confirmed with authentic standards (A).

Figure 4. Kinetic characterization of LTB_4 metabolism by CYP4F1 and CYP4F4. Purified recombinant CYP4F1 (■) and CYP4F4 (●) were incubated with varying concentrations of LTB_4 and the ω-hydroxylated product was detected by HPLC. The data is shown as mean ± SD (n=3) of the product formation at each substrate concentration. K_m and V_{max} values were estimated by fitting the data to a single substrate Michaelis-Menten equation and were 24 μM and 10 nmol/min/nmol for CYP4F1 and 31 μM and 40 nmol/min/nmol for CYP4F4.

Figure 5. Representative chromatogram showing arachidonic acid metabolism by *E. coli* expressed CYP4F isoforms. Arachidonic acid metabolism by 50 pmol/ml of expressed CYP4F1 (A), CYP4F4 (B), CYP4F5 (C), and CYP4F6 (D) reconstituted with purified NADPH P450 reductase (500 pmol/ml) and cytochrome b_5 (50 pmol/ml) was measured in the presence of NADPH (1 mM). Reactions were carried out for 30 min at 37°C, and metabolites were extracted and separated by HPLC. The identity of the arachidonic acid metabolites has been previously reported (Kroetz et al., 1997).

Figure 6. Concentration-dependent ω-hydroxylation of arachidonic acid by CYP4F1 and CYP4F4. Arachidonic acid metabolism was measured in an incubation of 50 pmol/ml of expressed CYP4F1 (A) and CYP4F4 (B) reconstituted with purified NADPH-P450 reductase (500 pmol/ml) and cytochrome b_5 (50 pmol/ml) and incubated with 5-80 μM of [1-^{14}C]arachidonic acid in the presence of NADPH (1 mM). Reactions were carried out for 10 min at 37°C, and metabolites were extracted and separated by HPLC. The data is shown as mean ± SD (n=3) of the product formation at each substrate concentration.

Figure 7. Representative chromatogram showing lauric acid metabolism by *E. coli* expressed CYP4F isoforms. Lauric acid metabolism by 50 pmol/ml of expressed CYP4F1 (A), CYP4F4 (B), CYP4F5 (C), and CYP4F6 (D) reconstituted with purified NADPH P450 reductase (500 pmol/ml) and cytochrome b_5 (50 pmol/ml) was measured in the presence of NADPH (1 mM). Reactions were carried out for 10 min at 37°C, and metabolites were extracted and separated by HPLC. The identity of the lauric acid metabolites has been previously reported (Okita et al., 1991).

Figure 8. Effect of CYP inhibitors on CYP4F-catalyzed arachidonic acid ω-hydroxylation. Reconstituted CYP4F1 (■) and CYP4F4 (●) were inactivated by incubation with various concentrations of the known CYP inhibitors 17-ODYA (A) 10-SUYS (B) or ABT (C) at 37°C for 30 min in the presence of 1 mM NADPH. The proteins were then diluted 10-fold and arachidonic acid metabolism was measured as described in Methods. The data points represent the means of two separate determinations that do not vary by more than 10%.

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Figure 1

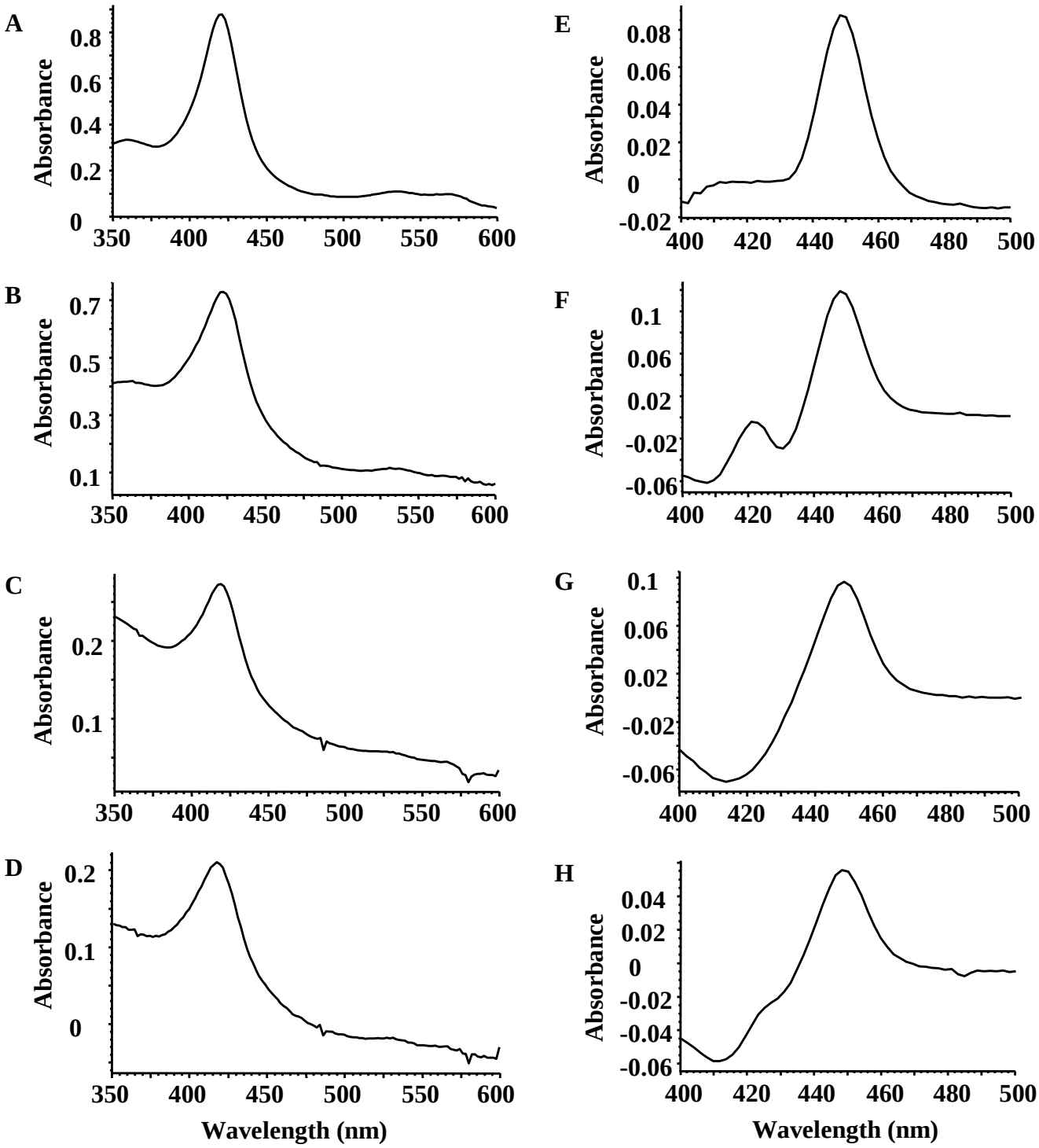

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Figure 2

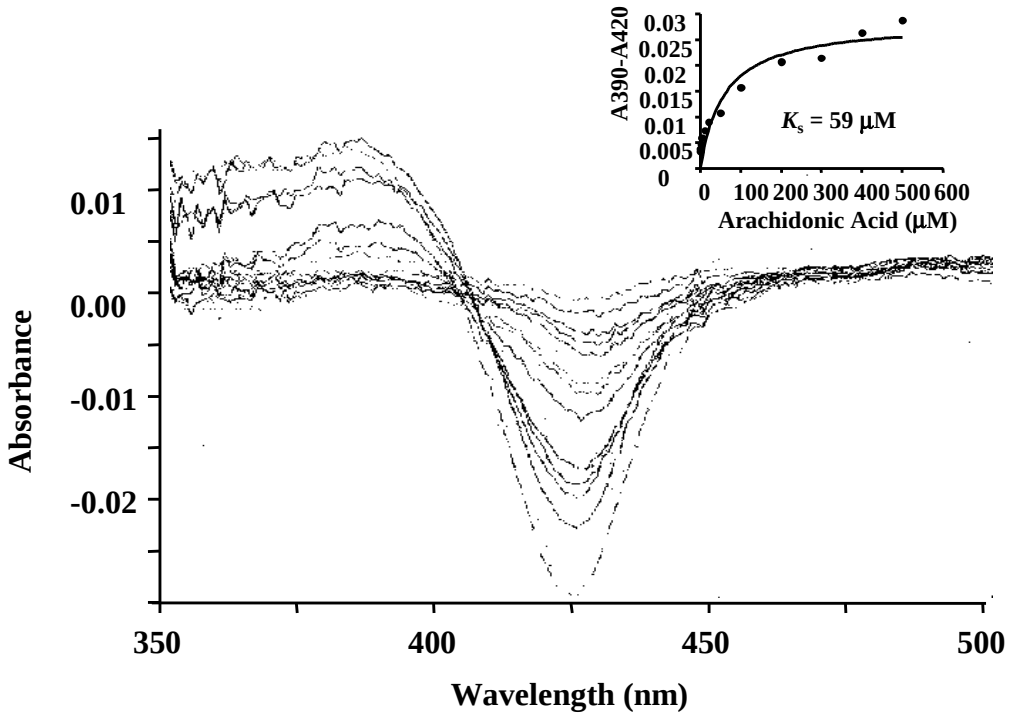

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Figure 3

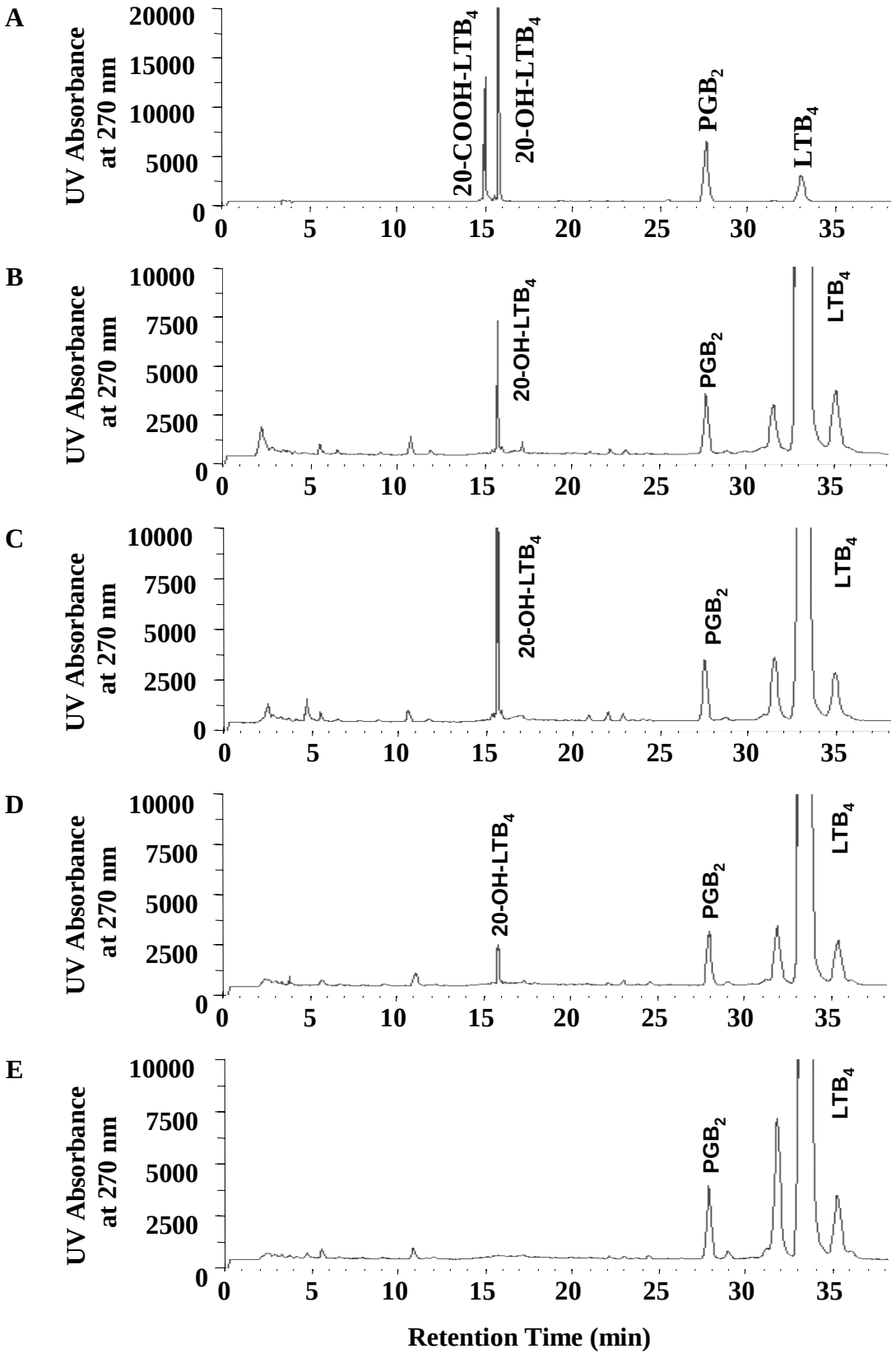

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Figure 4

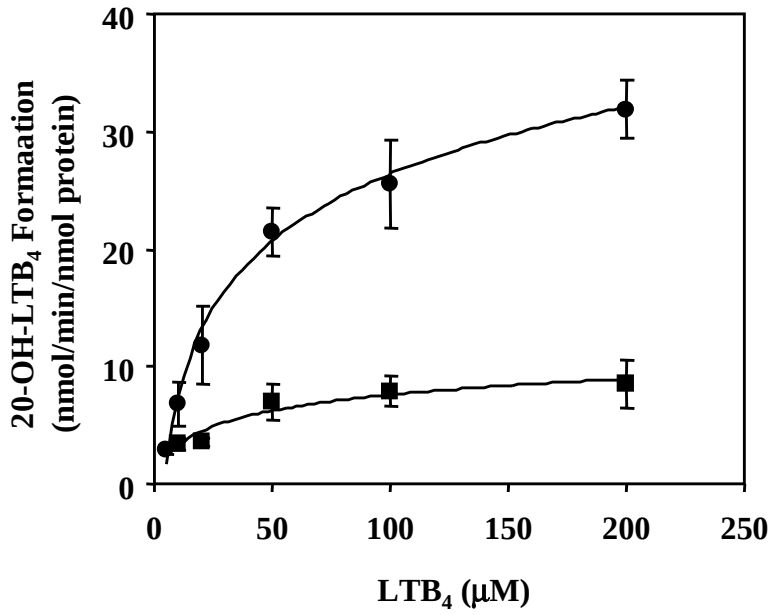

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Figure 5

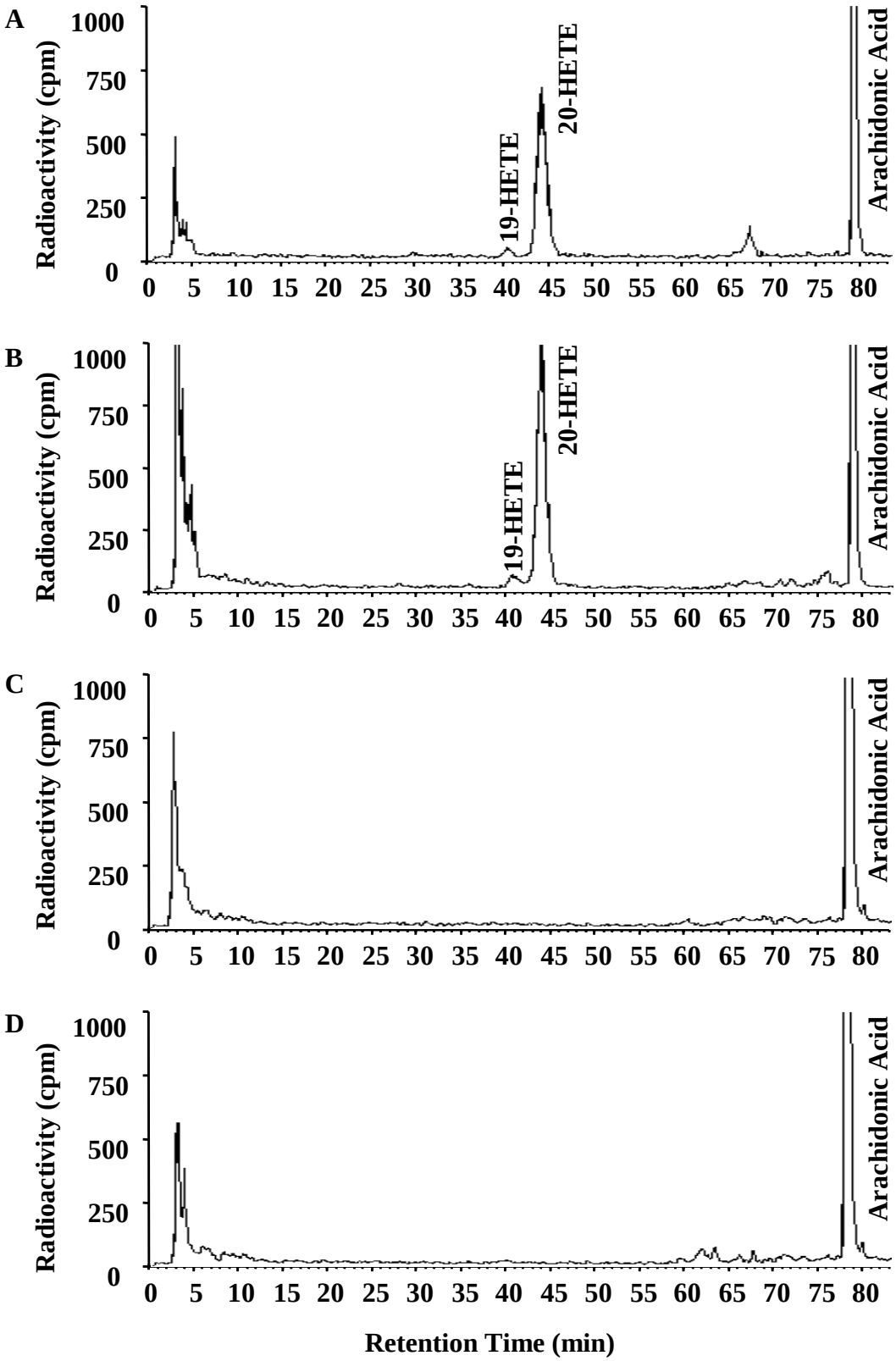

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Figure 6

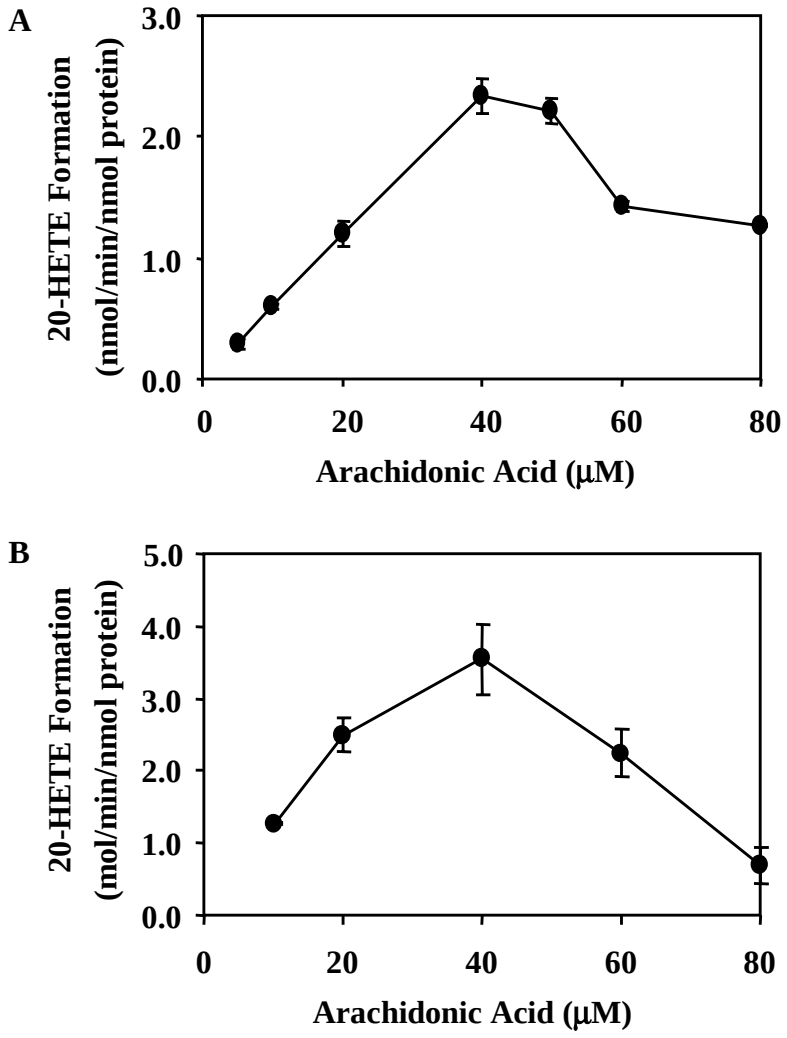

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Figure 7

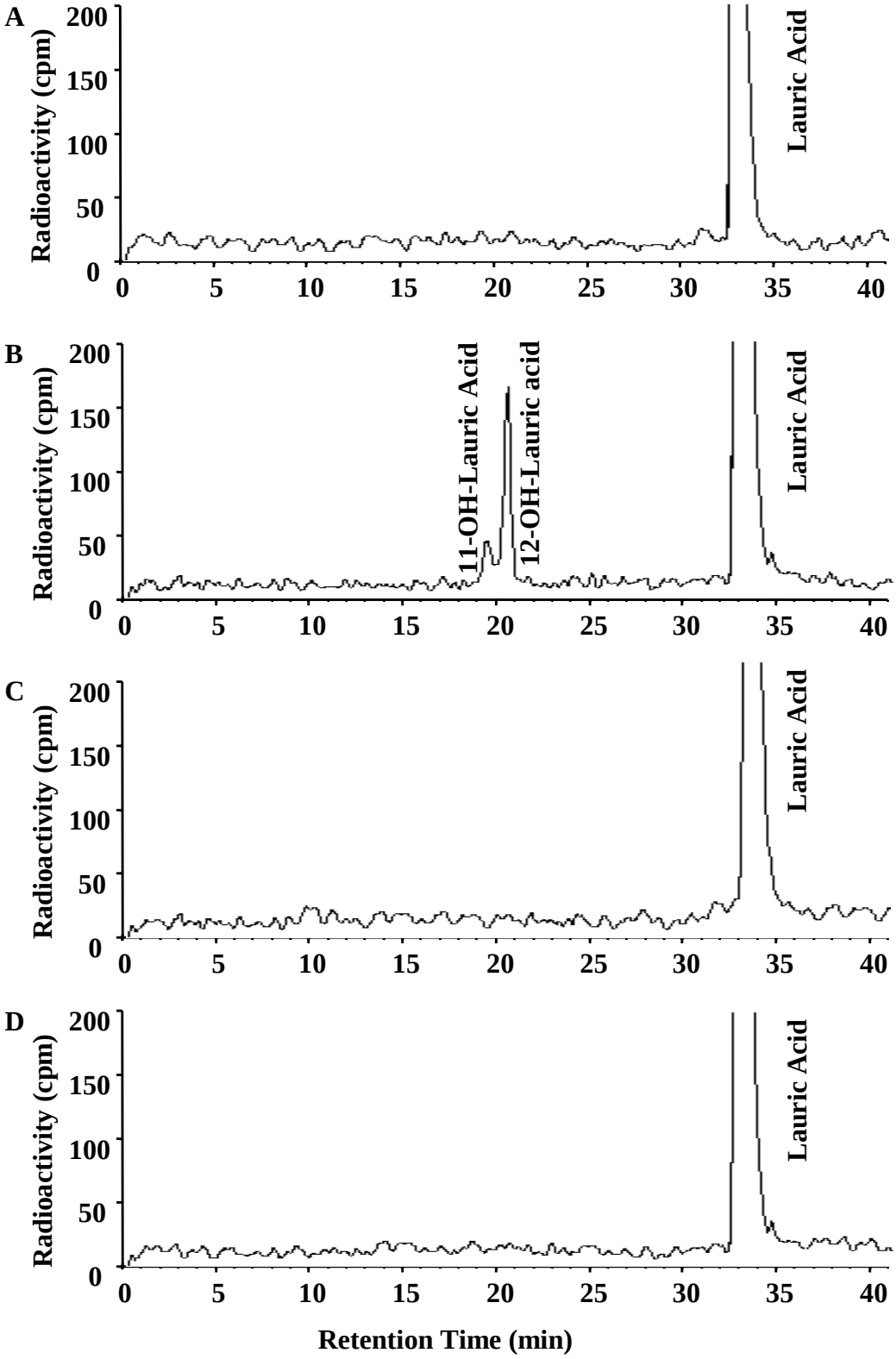

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Figure 8

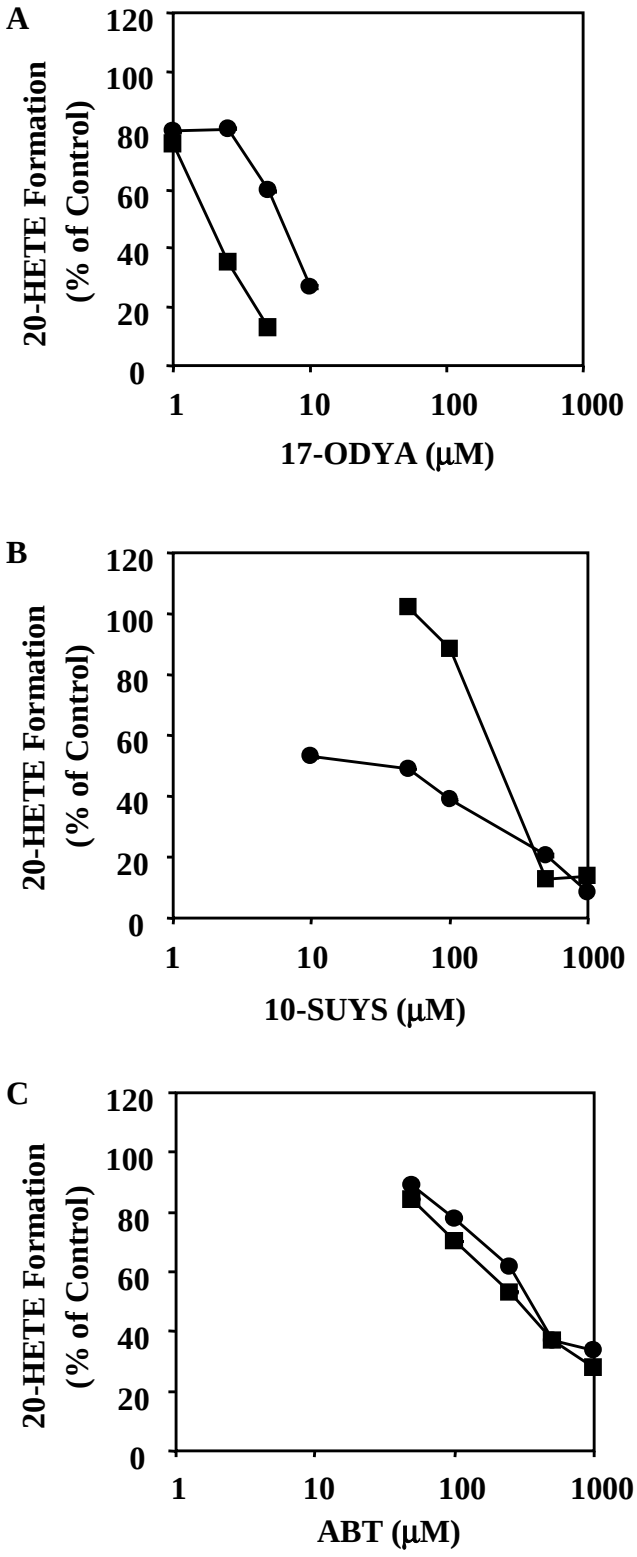

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Table 1. Spectroscopic binding constants (K_s) of rat CYP4F isoforms with the indicated substrates

	K_s (μM)			
Fatty Acid	CYP4F1	CYP4F4	CYP4F5	CYP4F6
Arachidonate	16	59	ND	ND
Laurate	ND[a]	66	ND	ND
LTB_4	25	56	ND	ND

Spectroscopic binding constants were measured as reported under Materials and Methods using a 1.5 μM enzyme solution and 0.5–500 μM fatty acid. The values are the averages of two independent measurements that differed by no more than 10%.

[a]ND, not detectable, no spin state change was observed upon titration with the indicated fatty acid.

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Table 2. Apparent k_{cat} values for fatty acid ω-hydroxylation by rat CYP4F isoforms

	k_{cat} (min^{-1})			
Fatty Acid	CYP4F1	CYP4F4	CYP4F5	CYP4F6
Arachidonate	9	11	ND	ND
LTB_4	2	15	ND	ND
Laurate	ND[a]	1	ND	ND

Apparent k_{cat} values were measured as reported under Materials and Methods using 50 pmol of enzyme. The values are the average of two independent measurements that differed by no more than 10%.

[a]ND, not detectable.

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Table 3. Effect of CYP inhibitors on CYP4F-catalyzed ω-hydroxylation of arachidonic acid

	IC_{50} (μM)	
Inhibitor	CYP4F1	CYP4F4
MS-PPOH	NI[a]	NI
17-ODYA	1.81	5.59
DDMS	NI	145
10-SUYS	231	25.0
ABT	289	371

[a]NI, no inhibition at concentrations studied