Omega oxidation of 3-hydroxy fatty acids by the human CYP4F gene subfamily enzyme CYP4F11

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Abstract **Long-chain 3-hydroxydicarboxylic acids (3-OHDCAs) are thought to arise via β-oxidation of the corresponding dicarboxylic acids (DCAs), although long-chain DCAs are neither readily transported into nor β-oxidized in mitochondria. We thus examined whether ω-hydroxylation of 3-hydroxy fatty acids (3-OHFAs), formed via incomplete mitochondrial oxidation, is a more likely pathway for 3-OHDCA production. NADPH-fortified human liver microsomes converted 3-hydroxystearate and 3-hydroxypalmitate to their ω-hydroxylated metabolites, 3,18-dihydroxystearate and 3,16-dihydroxypalmitate, respectively, as identified by GC-MS. Rates of 3,18-dihydroxystearate and 3,16-dihydroxypalmitate formation were 1.23 ± 0.5 and 1.46 ± 0.30 nmol product formed/min/mg protein, respectively (mean ± SD; n = 13). Polyspecific CYP4F antibodies markedly inhibited microsomal ω-hydroxylation of 3-hydroxystearate (68%) and 3-hydroxypalmitate (99%), whereas CYP4A11 and CYP2E1 antibodies had little effect. Upon reconstitution, CYP4F11 and, to a lesser extent, CYP4F2 catalyzed ω-hydroxylation of 3-hydroxystearate, whereas CYP4F3b, CYP4F12, and CYP4A11 exhibited negligible activity. CYP4F11 was the lone CYP4F/A enzyme that effectively oxidized 3-hydroxypalmitate. Kinetic parameters of microsomal 3-hydroxystearate metabolism were K_m = 55 μM and V_{max} = 8.33 min^{-1}, whereas those for 3-hydroxypalmitate were K_m = 56.4 μM and V_{max} = 14.2 min^{-1}. CYP4F11 kinetic values resembled those of native microsomes, with K_m = 53.5 μM and V_{max} = 13.9 min^{-1} for 3-hydroxystearate and K_m = 105.8 μM and V_{max} = 70.6 min^{-1} for 3-hydroxypalmitate. Our data show that 3-hydroxystearate and 3-hydroxypalmitate are converted to ω-hydroxylated 3-OHDCA precursors in human liver and that CYP4F11 is the predominant catalyst of this reaction. CYP4F11-promoted ω-hydroxylation of 3-OHFAs may modulate the disposition of these compounds in pathological states in which enhanced fatty acid mobilization or impairment of mitochondrial fatty acid β-oxidation increases circulating 3-OHFA levels.—**Dhar, M., D. W. Sepkovic, V. Hirani, R. P. Magnusson, and J. M. Lasker. **Omega oxidation of 3-hydroxy fatty acids by the human CYP4F gene subfamily enzyme CYP4F11. *J. Lipid Res.* 2008.** 49: **612–624.**

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Fatty acid oxidation becomes a pivotal source of energy during the fasting state in mammalian cells, particularly in heart and skeletal muscle. In fact, fatty acid oxidation accounts for nearly 80% of the total energy used in infants and children during the initial hour of fasting (1). After a fast of 36–48 h, fatty acids are mobilized from adipose tissue stores to maintain the energy supply via their catabolism, mainly via mitochondrial β-oxidation and, to a lesser extent, by microsomal ω-oxidation. In the latter pathway, the terminal, or ω, carbon atom of the fatty acid chain undergoes hydroxylation by P450 enzymes. Although numerous P450s are capable of oxidizing C10–C26 fatty acids at diverse positions along the alkyl chain, only those enzymes belonging to the CYP4F/A subfamilies preferentially hydroxylate the terminal methyl group (2–5). This catalytic function is highly conserved among the CYP4F/A enzymes found in numerous animal species, including humans, and may provide another route to eliminate the potentially toxic levels of free fatty acids observed under certain conditions, such as uncontrolled diabetes, starvation, and alcohol abuse. Indeed, as described below, the ω-hydroxylation of medium- and long-chain free fatty acids appears to constitute the initial step in the formation of the cognate dicarboxylic acids (DCAs), which are more easily eliminated than the parent compounds or can undergo additional metabolism via peroxisomal β-oxidation (6). The human CYP4A gene subfamily consists of only a single major member, CYP4A11 (7, 8), whereas the CYP4F subfamily is composed of at least six different enzymes

Abbreviations: b_5, cytochrome b_5; BSTFA, *N,O*-bis-(trimethylsilyl) trifluoroacetamide; DCA, dicarboxylic acid; LCHAD, long-chain 3-hydroxyacyl-coenzyme A dehydrogenase; MTP, mitochondrial trifunctional protein; 3-OHDCA, 3-hydroxy dicarboxylic acid; 3-OHFA, 3-hydroxy fatty acid; P450 reductase, PDAM, 1-pyrenyldiazomethane; NADPH:P450 oxidoreductase; R_T, retention time on HPLC or GC.

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with 74% overall amino acid sequence homology: CYP4F2, CYP4F3a, CYP4F3b, CYP4F8, CYP4F11, and CYP4F12 (9) (P450 enzymes belonging to the CYP4F and/or CYP4A subfamilies are designated here based on the nomenclature given on Dr. David Nelson's website for cytochrome P450s: http://drnelson.utmem.edu/human.P450.table.html). CYP4A11, CYP4F2, CYP4F3a, and CYP4F3b are the best studied of the CYP4F/A P450s, are expressed predominantly in liver and kidney (although CYP4F3a is found in myeloid tissue), and are best known at present for their capacity to ω-hydroxylate the essential fatty acid arachidonate to 20-hydroxyeicosatetraenoic acid, a potent vasoactive eicosanoid, and/or to convert the proinflammatory agent leukotriene B_4 to inactive products via ω-hydroxylation (4, 10–13).

In normal subjects, fatty acids hydroxylated at carbon position 3 (3-OHFAs) are found in plasma at very low concentrations. However, subjects exhibiting increases in fatty acid mobilization or deficiencies in mitochondrial fatty acid β-oxidation show marked increases of plasma 3-OHFA content, especially those with chain lengths of >10 carbons (1, 14). These 3-OHFAs are converted via a poorly characterized pathway to the respective 3-hydroxy dicarboxylic acids (3-OHDCAs), which results in the accumulation of medium- and long-chain 3-OHDCAs, a phenomenon that occurs in pathologies such as diabetic ketoacidosis and glycogen storage disease (15). Although it was originally suggested that 3-OHDCAs originate from β-oxidation of the corresponding dicarboxylic fatty acids (16), the fact that medium- and long-chain DCAs are neither readily transported into mitochondria nor β-oxidized in that organelle (6) indicates that another pathway must be involved in 3-OHDCA formation. One such route could involve P450-dependent ω-hydroxylation of the cognate 3-OHFA (**Fig. 1**). In this pathway, endogenous medium- and long-chain fatty acids first undergo incomplete β-oxidation in mitochondria, accumulate in that organelle as 3-hydroxyacyl-CoA ester intermediates, and, upon carnitine transesterification or hydrolytic cleavage, are released as free 3-OHFAs. Conditions favoring the buildup of 3-hydroxyacyl-CoA esters and, thus, 3-OHFA, are a decreased NAD^+/NADH ratio, which inhibits long-chain 3-hydroxyacyl-coenzyme A dehydrogenase (LCHAD) activity, as well as a deficiency in this mitochondrial enzyme and that of mitochondrial trifunctional protein (MTP) (17). The 3-OHFAs are then ω-oxidized by CYP4F/A P450s to their respective dihydroxylated fatty acids. Subsequently, either alcohol dehydrogenase or a CYP4F/A enzyme converts the ω-hydroxy group into an aldehyde derivative, which is then oxidized by aldehyde dehydrogenase to form a ω-carboxylic acid, thus giving the DCA product. In fact, the participation of human CYP4F/A enzymes in DCA formation was recently reported by Sanders et al. (18), who demonstrated that both CYP4F2 and CYP4F3b catalyze the ω-hydroxylation of hexacosanedioic acid, a very-long-chain (C26:0) fatty acid. The subsequent, sequential oxidation of 26-hydroxyhexacosanedioate by either alcohol dehydrogenase or CYP4F2 to the 26-CHO derivative, and then by aldehyde dehydrogenase to the 26-COOH product, gave rise to a very-long-chain DCA (18).

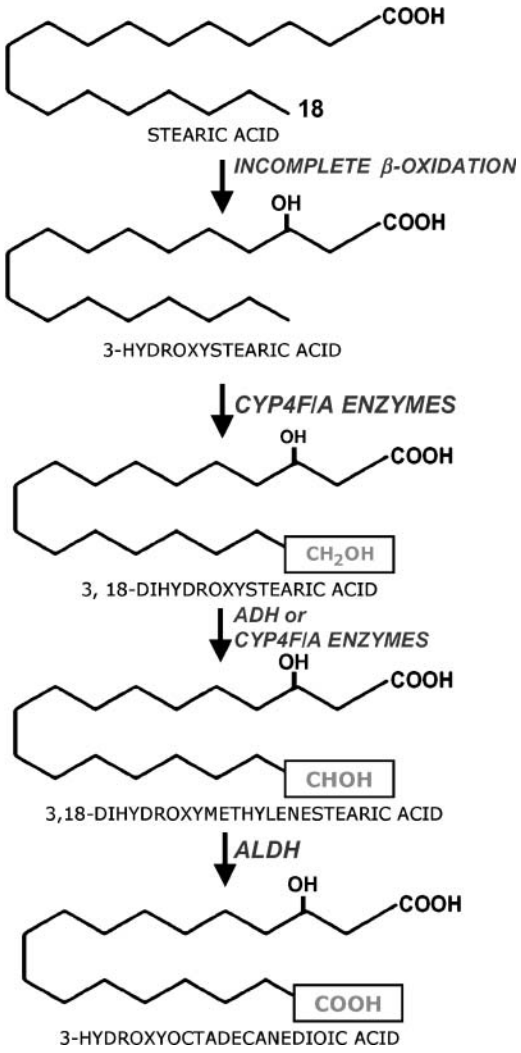

Fig. 1. Proposed scheme for the formation of long-chain 3-hydroxydicarboxylic acids: involvement of CYP4F/A enzymes. In this scheme, 3-hydroxystearate is formed during the incomplete mitochondrial β-oxidation of stearate, an exemplary long-chain fatty acid. A 3-hydroxysteroyl-CoA ester intermediate accumulates in mitochondria and, upon carnitine transesterification or hydrolytic cleavage, is released as free 3-hydroxystearate. The 3-hydroxylated fatty acid is then ω-oxidized at position 18 by CYP4F and/or CYP4A subfamily enzymes to form the metabolite 3,18-dihydroxystearate. The latter compound is again oxidized by either a CYP4F/A P450 or alcohol dehydrogenase (ADH) to form 3,18-dihydroxymethylenestearic acid, which is finally converted by aldehyde dehydrogenase (ALDH) to 3-hydroxyoctadecanedioic acid, a dicarboxylic fatty acid.

The aim of the present study was to characterize the human CYP450 enzyme(s) underlying hepatic ω-hydroxylation of two exemplary, long-chain 3-OHFAs, 3-hydroxystearate and 3-hydroxypalmitate. Using different approaches, namely purified P450 reconstitution, immunochemistry, and enzyme kinetics, we found that a member of the CYP4F gene subfamily, CYP4F11, was the predominant catalyst of this fatty acid oxidation reaction in human liver.

MATERIALS AND METHODS

Human liver specimens

Normal human liver tissue was obtained from organ donors through the Liver Tissue Procurement and Distribution System (Minneapolis, MN), which was funded by National Institutes of Health Contract N01 DK-9-2310. None of the subjects had a history of overt drug or alcohol abuse. Livers were removed within 30 min of death, cut into small pieces, frozen in liquid nitrogen, and stored at −80°C until microsomes were prepared. P450 content was determined from CO-reduced difference spectra, and protein concentration was measured using the bicinchoninic acid procedure.

Microsomal enzymes

Human CYP4F2, CYP4F3b, CYP4F11, and CYP4F12 were expressed in *Spodoptera frugiperda* insect cells that had been infected with the cognate CYP4F cDNA-containing baculovirus constructs. The enzymes were purified to near homogeneity from *S. frugiperda* cellular lysates using hydrophobic, adsorption, and/or Ni^{2+}-nitrilotriacetic acid agarose chromatography. Additional details regarding CYP4F/A P450 cDNA cloning, baculovirus construct preparation, and expressed enzyme purification will be published elsewhere. CYP4A11, cytochrome b_5 (b_5), and NADPH:P450 oxidoreductase (P450 reductase) were purified to near electrophoretic homogeneity from human liver microsomes as reported previously (4, 5). The specific contents of CYP4A11, b_5, and P450 reductase were 12.6, 25.7, and 33.5 nmol/mg protein; in the latter case, this was equivalent to 113,000 U/mg protein.

Analysis of 3-OHFA ω-hydroxylation

Conversion of 3-OHFAs to their corresponding ω-hydroxylated metabolites was performed as described for oleate ω-hydroxylation.[2] In brief, reaction mixtures (0.25 ml) contained human liver microsomes (protein equivalent to 50–100 pmol of P450) or P450 reconstituted systems, 100 μM 3-OHFA substrate, 100 mM potassium phosphate buffer (pH 7.4), and 0.5 mM NADPH. Reconstituted systems consisted of 25–50 pmol of purified P450, 75–150 pmol of P450 reductase, 7.5–15 μg of synthetic L-α-dilauroylphosphatidylcholine, and 100–200 pmol of b_5. The following 3-OHFA substrates were used: 3-hydroxypalmitate (3-hydroxyhexadecanoic acid), 3-hydroxystearate (3-hydroxyoctadecanoic acid), and 3-hydroxylaurate (3-hydroxydodecanoic acid). Reactions were initiated with NADPH and were terminated after 10 min at 37°C by the addition of ethyl acetate. For kinetic experiments, substrate concentrations were varied from 6 to 200 μM. For antibody inhibition studies, liver microsomes or reconstituted recombinant P450s were first incubated with either immune-specific or preimmune IgG for 3 min at 37°C and then for 10 min at room temperature, followed by addition of the remaining reaction components. Incubation mixtures were extracted with 4 volumes of ethyl acetate, after which the organic extracts were evaporated to dryness with nitrogen gas at room temperature. The residues were resolubilized in 80 μl of methanol to which 20 μl of a freshly prepared solution of 1-pyrenyldiazomethane (PDAM; 1 mg/ml in ethyl acetate) was added (19). Fatty acid-PDAM derivatives were allowed to form overnight at room temperature in the dark and were then processed using a Waters Alliance HPLC apparatus equipped with a model 2690 Separation Module and a model 2487 UV/visible light detector (Waters Associates, Milford, MA). Samples were resolved on a Novapak C18 column (3.9 × 150 mm; Waters Associates) using a convex gradient (0.3%/min) from 90% to 99% acetonitrile in water at a flow rate of 1.0 ml/min. Column eluents were continuously monitored for ultraviolet absorbance at 275 nm. Under these conditions, baseline separation of PDAM-derivatized, ω-hydroxylated 3-OHFA metabolites was achieved. Rates of 3-OHFA ω-hydroxylation were determined from standard curves prepared by adding varying amounts of 16-hydroxypalmitate to incubations conducted without NADPH.[3] Laurate ω-hydroxylation was assessed as described by Powell, Wolf, and Lasker (5), except that 11-hydroxylaurate and 12-hydroxylaurate were resolved on the Novapak column described above using a convex gradient (1%/min) from 70% to 90% acetonitrile in water at a flow rate of 1.0 ml/min. Enzyme kinetic data were analyzed using Prism Version 5 (GraphPad Software, San Diego, CA).

Mass spectral characterization of 3-OHFA metabolites

Because authentic dihydroxylated fatty acid metabolites were not commercially available, we used GC-MS to confirm the identities of the major 3-OHFA oxidative products formed by microsomal CYP4F/A enzymes. Dried, organic extracts derived from pooled incubations of 3-hydroxystearate or 3-hydroxypalmitate with human liver microsomes in the presence and absence of NADPH were resolubilized at 80°C for 1 h in 40 μl of *N,O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane and 10 μl of pyridine. The derivatized samples were then subjected to GC-MS using an Agilent Technologies 6890N gas chromatograph equipped with a model 5973 mass selective detector and a model G2589A MSD Chemstation. The sample injection port was equipped with a split/splitless capillary inlet system and a silanized glass insert. Samples were resolved on an Ultra 2 capillary column packed with cross-linked 5% phenyl-methyl silicone (25 m × 0.2 mm × 0.33 μm film thickness; Agilent Technologies, Palo Alto, CA) using helium as the carrier gas at flow rate of 1 ml/min. The injection port, ion source, and GC-MS interface temperatures were maintained at 300, 280, and 270°C, respectively. The oven temperature was programmed as follows: the initial temperature of 100°C was held for 1 min, followed by an increase of 20°C/min to 260°C, which was again held for 1 min, and finally increased by 1°C/min to 280°C. The MS source temperature was 230°C, and the quadrupole temperature was 150°C. Metabolite peaks were first identified by comparing gas chromatograms of incubations conducted with and without NADPH. Metabolite mass spectra were then taken in the scanning electron-impact mode, and the spectra were compared with the National Institute of Standards and Technology mass spectral database.

Immunochemical methods

Polyclonal antibodies to human CYP4F,[4] CYP4A11, and CYP2E1 were raised in male New Zealand White rabbits as de-

[2] Hirani V., A. Kozeska, and J. M. Lasker, unpublished observations.

[3] We used 16-hydroxypalmitate for standard curves rather than 3,16-dihydroxypalmitate or 3,18-dihydroxystearate because the latter compounds were not commercially available and because the PDAM derivatives of medium- and long-chain fatty acids, such as laurate, oleate, and palmitate, exhibit nearly identical extinction coefficients at 275 nm (19).

[4] When this antibody was first produced, only two human CYP4F P450s had been identified, CYP4F2 and CYP4F3a, the latter of which is found exclusively in myeloid cells. Later studies revealed the expression in human liver and kidney of CYP4F3b (the alternatively spliced form of CYP4F3a) (10), CYP4F11, and CYP4F12 (11, 40–42). Because of the extensive (75%) sequence homology among the CYP4F P450s, it is now known that our original polyclonal CYP4F2 antibody cross-reacts with all of these enzymes.

scribed elsewhere (5, 13, 20). Characteristics of the peptide antibody targeted specifically against CYP4F2 are forthcoming.[5] Preimmune (control) IgG was prepared from rabbit sera obtained before immunization. Protein blotting of microsomal proteins and purified P450 enzymes to nitrocellulose and subsequent immunochemical staining with polyspecific anti-CYP4F or anti-CYP4F2 peptide IgG were performed as described elsewhere (13, 21).

Materials

3-Hydroxystearate, 3-hydroxypalmitate, and 3-hydroxylaurate were obtained from Matreya LLC (Pleasant Gap, PA). PDAM was obtained from Molecular Probes/Invitrogen (Carlsbad, CA). All other chemicals used were of the highest grade commercially available.

RESULTS

Characteristics of microsomal 3-OHFA ω-hydroxylation

Human liver microsomes fortified with NADPH converted 3-hydroxystearate and 3-hydroxypalmitate to their corresponding ω-hydroxylated metabolites (3,18-dihydroxystearate and 3,16-dihydroxypalmitate, respectively) in a time- and P450-dependent manner. Metabolite formation was linear for at least 10 min with up to 150 pmol of microsomal P450 per reaction (data not shown) and did not occur upon omission of NADPH from the reaction mixtures (**Fig. 2A, C**). As shown in Fig. 2B, D, only a single major PDAM-derivatized peak was formed from 3-hydroxystearate as well as from 3-hydroxypalmitate, with retention times on HPLC or GC (R_T) of 7.1 and 4.1 min, respectively. The absence of earlier eluting peaks whose formation was NADPH-dependent suggests that neither of these 3-OHFAs was converted to ω-1 hydroxylated products to any significant extent. Indeed, the HPLC conditions used here can give baseline resolution of the ω- and ω-1 metabolites of numerous fatty acids, including oleate (22) and laurate (see below). Rates of 3-hydroxystearate oxidation among the 13 different human subjects examined ranged from 1.5 to 7.3 nmol 3,18-dihydroxystearate formed/min/nmol P450 (0.2–2.1 nmol/min/mg protein), with a mean rate of 3.91 ± 1.8 nmol product formed/min/nmol P450 (0.87 ± 0.6 nmol/min/mg protein). With 3-hydroxypalmitate, ω-hydroxylation activities among the subjects also varied extensively (1.9–13.7 nmol product formed/min/nmol P450; 0.3–4.3 nmol/min/mg protein), with a mean rate of 6.59 ± 3.6 nmol 3,16-dihydroxypalmitate formed min/nmol P450 (1.46 ± 1.1 nmol/min/mg protein). Another 3-OHFA, 3-hydroxylaurate, was not a substrate for ω-hydroxylation by human liver microsomes, as indicated by the lack of 3,12-dihydroxylaurate formation with this fatty acid (data not shown).

We used GC-MS to confirm that the major HPLC peaks with R_T of 7.1 and 4.1 min shown in Fig. 2B, D were indeed the PDAM derivatives of 3,18-dihydroxystearate and 3,16-dihydroxypalmitate, respectively. For these experiments, organic extracts prepared from incubations of human liver microsomes, 3-hydroxystearate or 3-hydroxypalmitate, and NADPH were derivatized with BSTFA and then resolved by gas chromatography. Metabolites formed by CYP4F/A enzymes were initially identified by comparing the GC profiles of incubations performed in the presence and absence of cofactor (**Fig. 3A, C**). Electron-impact MS of the peak with R_T = 15.2 min shown in Fig. 3A gave a structure identified as octadecanoic acid 3,18-(trimethylsiloxy)-methyl ester based upon the following ion fragments: a mass ion fragment ($M^{+\bullet}$-1) at *m/z* 459; ion rearrangement fragments at *m/z* 369 and 175, which arise from the loss of methyl and/or methoxy groups; and a prominent ion at *m/z* 103, which stems from the terminal fragment CH_2-O-Si-Me_3^+ characteristic of ω-hydroxylated fatty acid-trimethylsilylated derivatives (23, 24). That this compound was not the ω-1 hydroxylated metabolite 3,17-dihydroxystearate was indicated by the low abundance of the ion fragment at *m/z* 117, which is representative of the CH_3-CHO-Si-Me_3^+ fragment specific to ω-1-hydroxylated fatty acid-trimethylsilylated derivatives (as well as of COO-Si-Me_3^+, the fragment characteristic of carboxylate trimethylsilylated derivatives) (23, 25). Similarly, the mass spectra of the peak with R_T = 12.7 min depicted in Fig. 3C was identified as hexadecanoic acid 3,16-(trimethylsiloxy)-methyl ester based upon the ion fragments at *m/z* 431 ($M^{+\bullet}$-1), *m/z* 341 and 175 (loss of methyl or methoxy groups), and *m/z* 103, the terminal CH_2-O-Si-Me_3^+ ion. Again, the lack of an ion fragment at *m/z* 117 demonstrated that this compound was not the ω-1 hydroxylated metabolite of 3-hydroxypalmitate and that the conditions we used here to derivatize these dihydroxylated fatty acids gave little sialylation of the carboxyl moiety. It should also be noted here that the GC peak observed at R_T = 12.8 min in Fig. 3C is most likely not 3,15-dihydroxypalmitate, because this ω-1 hydroxylated product would elute from this particular GC column with a shorter R_T than the corresponding ω-hydroxylated metabolite.

Immunoinhibition of microsomal 3-OHFA ω-hydroxylation

The effects of P450 antibodies on 3-OHFA ω-hydroxylation by human liver microsomes are shown in **Fig. 4**. Incubation of microsomes from subject A with an optimized ratio (5 mg IgG/nmol P450) of anti-CYP4F IgG, an antibody prepared by immunization of rabbits with CYP4F2 purified from human liver (4, 13), gave marked inhibition of 3,18-dihydroxystearate formation. In contrast, antibodies to CYP4A11, which is also a fatty acid ω-hydroxylase (4, 5, 26, 27), had little effect on this same ω-hydroxylation reaction. CYP4F antibodies were even more potent inhibitors of 3-hydroxypalmitate metabolism, as they elicited nearly complete inhibition of microsomal 3,16-dihydroxypalmitate formation. In contrast, anti-CYP4A11 and anti-CYP2E1 (whose immunogen is a ω-1 hydroxylase) (28–30),

[5]Hirani V., A. Yarovoy, A. Kozeska, and J. M. Lasker. Expression of CYP4F2 in human liver and kidney: assessment using specific peptide antibodies, submitted, 2007.

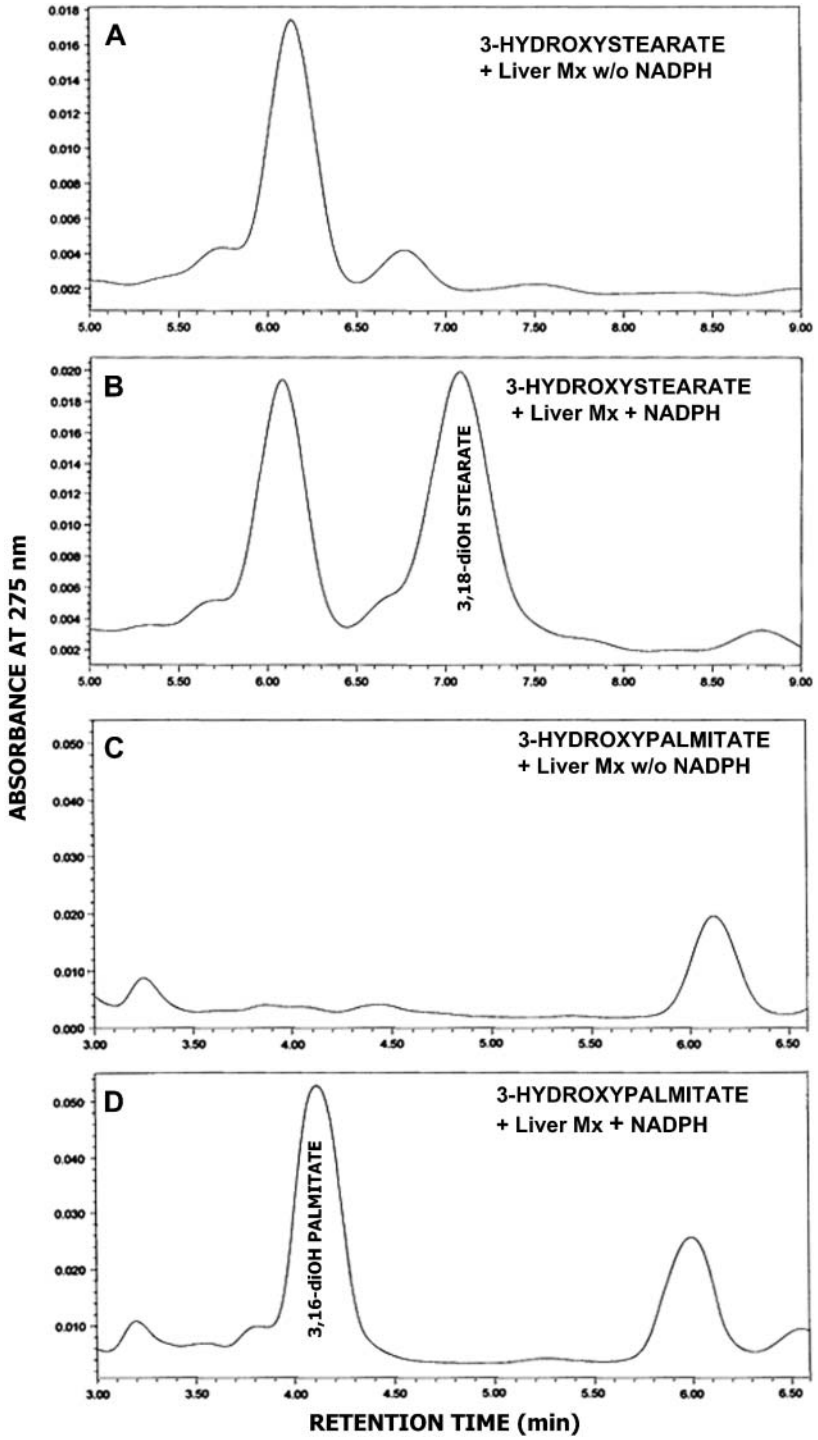

Fig. 2. HPLC analysis of 3-hydroxystearate and 3-hydroxypalmitate ω-hydroxylation by human liver microsomes. 3-Hydroxystearate, 3-hydroxypalmitate, and their respective ω-hydroxylated metabolites (3,18-dihydroxystearate and 3,16-dihydroxypalmitate) were separated on a Novapak C18 column using the HPLC conditions described in Materials and Methods. A: Chromatogram showing the absence of 3,18-dihydroxystearate production upon incubation of 100 μM 3-hydroxystearate with liver microsomes (mx) from subject A but without NADPH fortification. B: Chromatogram showing 3,18-dihydroxystearate formation [retention time on HPLC or GC (R_T) = 7.1 min] upon inclusion of NADPH in the microsomal reaction mixture. C: Chromatogram showing the lack of metabolite formation from 3-hydroxypalmitate (100 μM) upon its reaction with subject A liver microsomes in the absence of NADPH. D: Chromatogram showing 3,16-dihydroxypalmitate production (R_T = 4.2 min) upon inclusion of NADPH in the microsomal reaction mixture. Although not depicted here, 3-hydroxystearate-1-pyrenyldiazomethane (PDAM) and 3-hydroxypalmitate-PDAM elute with R_T values of 24 and 18.7 min, respectively. The PDAM derivatives observed at 5.8–6.4 min in all four chromatograms are unrelated to 3-hydroxy fatty acid (3-OHFA) metabolism, because they are also observed in the absence of cofactor.

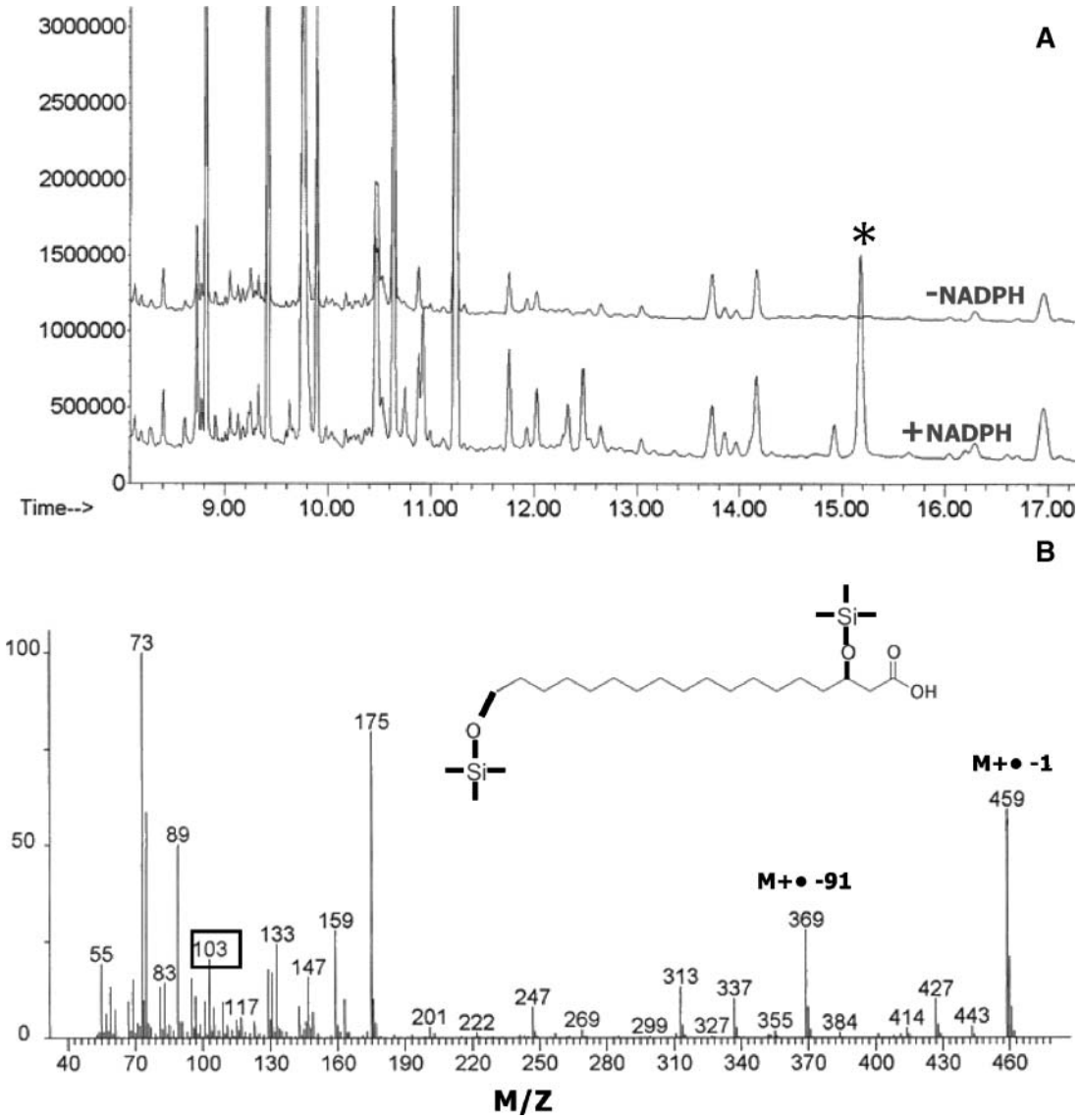

Fig. 3. GC-MS analysis of 3,18-dihydroxystearate and 3,16-dihydroxypalmitate. Organic extracts prepared from incubations of human liver microsomes with 3-hydroxystearate or 3-hydroxypalmitate in the presence and absence of NADPH were derivatized with *N,O*-bis-(trimethylsilyl) trifluoroacetamide and then subjected to GC-MS analysis as described in Materials and Methods. A: Gas chromatogram of the ω-hydroxylated metabolite formed (see asterisk-demarcated peak) from 3-hydroxystearate. B: Mass spectrum and presumed structure of octadecanoic acid 3,18-(trimethylsiloxy)-methyl ester, which is the trimethylsilylated (TMS) ester derivative of 3,18-dihydroxystearat eluting at 15.2 min. C: Gas chromatogram of the ω-hydroxylated metabolite formed (see asterisk-demarcated peak) from 3-hydroxypalmitate. D: Mass spectrum and structure of hexadecanoic acid 3,16-(trimethylsiloxy)-methyl ester, the TMS ester derivative of 3,16-dihydroxypalmitate, which eluted from the GC column at 12.6 min.

failed to evoke any inhibition of 3-hydroxypalmitate oxidation by human liver microsomes (Fig. 4).

Kinetic analysis of 3-OHFA ω-hydroxylation

Liver microsomes from subject A were initially used to determine the kinetic parameters of the 3-hydroxystearate ω-hydroxylation and 3-hydroxypalmitate ω-hydroxylation reactions. Over the range of substrate concentrations used (6–200 μM), oxidation of 3-hydroxystearate and 3-hydroxypalmitate exhibited simple Michaelis-Menten kinetics (**Fig. 5A, B**). Eadie-Hofstee plots for the reactions were linear in nature (data not shown). Analysis of rate data using nonlinear regression gave apparent K_m values of 54 and 56.4 μM, with corresponding V_{max} values of 8.3 and 14.2 min^{-1}, for 3,18-dihydroxystearate and 3,16-dihydroxypalmitate formation, respectively. Monophasic microsomal ω-hydroxylation kinetics were also noted with 3-hydroxystearate in the case of subject B. With liver microsomes from this subject, kinetic parameters derived for 3,18-dihydroxystearate formation were K_m = 52.5 μM and V_{max} = 7.8 min^{-1}. The monophasic kinetic properties of 3-OHFA ω-hydroxylation by human liver microsomes, which were similar to those observed for ω-hydroxylation of laurate (5) and oleate (unpublished data), suggested the involvement

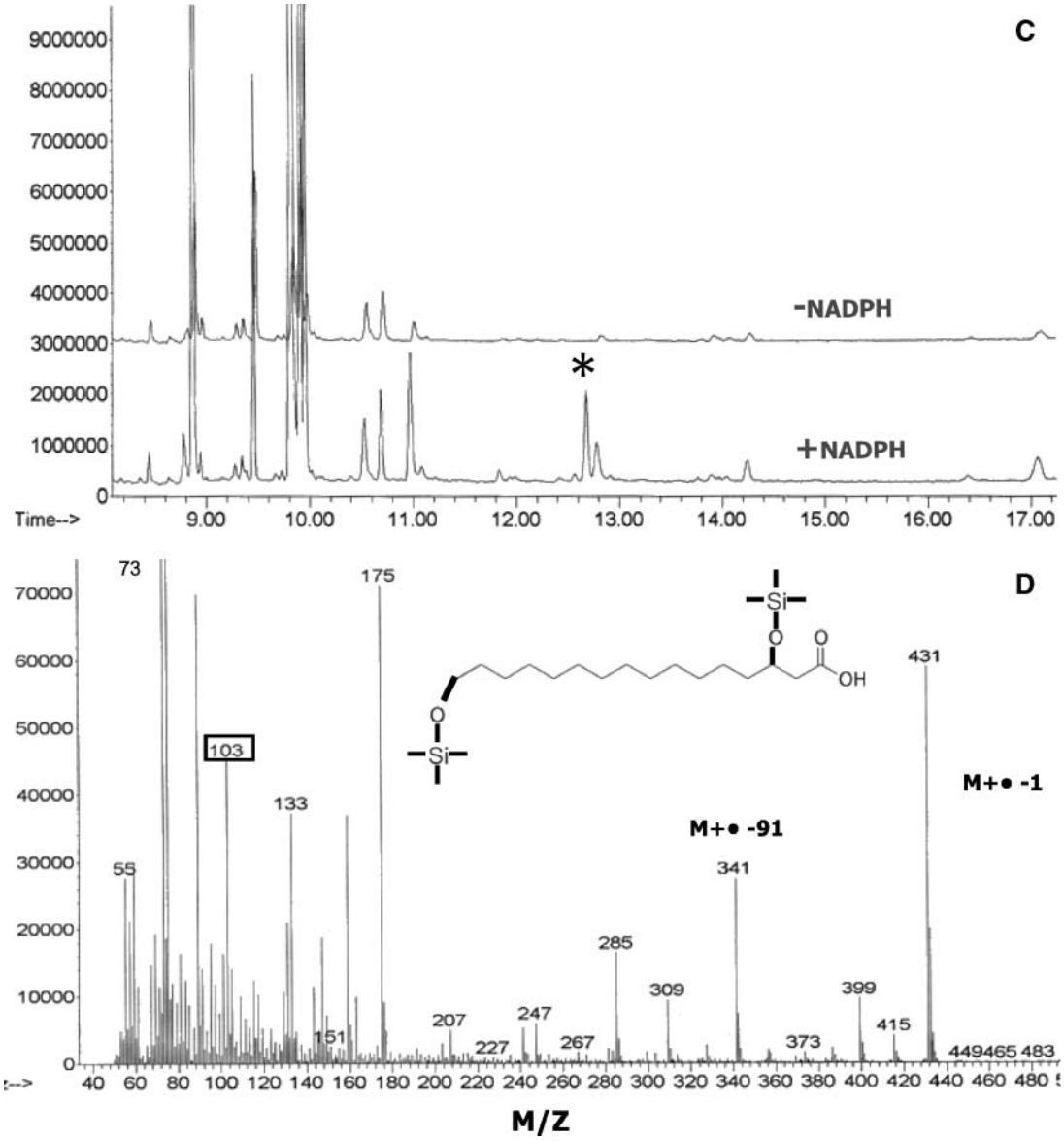

Fig. 3.—*Continued.*

of only a single P450 enzyme in the reaction (or two enzymes with similar kinetic features). That the enzyme was a CYP4F P450 was indicated by the immunoinhibition experiments outlined above (Fig. 4). Indeed, upon reconstitution with P450 reductase, phospholipid, and b_5, CYP4F11 converted 3-hydroxystearate to its 18-hydroxylated metabolite at rates nearly 2-fold higher than liver microsomes and nearly 3-fold higher than CYP4F2, the other CYP4F enzyme that efficiently catalyzed 3-hydroxystearate ω-hydroxylation (**Table 1**). CYP4F3b exhibited some activity with this substrate, whereas CYP4F12 and CYP4A11 were essentially inactive. In the case of 3-hydroxypalmitate, the substrate specificity of the CYP4F enzymes was more obvious, because this compound was ω-hydroxylated by CYP4F11 at rates nearly 10-fold higher than those noted with CYP4F2, CYP4F3b, and CYP4A11 (Table 1). As observed with intact liver microsomes, the purified recombinant CYP4F/A enzymes metabolized these two 3-OHFAs only at the ω-carbon, and not at the ω-1 position (data not shown). That CYP4A11 was indeed catalytically active is illustrated by its high ω-hydroxylase activity with laurate, an exemplary CYP4A11 substrate (Table 1). Neither CYP4F2 nor CYP4F3b was an efficient catalyst of laurate ω-hydroxylation, although CYP4F11 converted laurate to 12-hydroxylaurate at rates that were 50% of those observed with CYP4A11.

In light of the extensive 3-OHFA ω-hydroxylating properties of CYP4F11 and CYP4F2, we examined the kinetics parameters associated with 3-hydroxystearate and 3-hydroxypalmitate metabolism by these two CYP4F enzymes (**Fig. 6**). Monophasic kinetics were observed in the case of CYP4F11-catalyzed 3-hydroxystearate oxidation, with an apparent K_m of 53.5 μM and a V_{max} of 13.9 min^{-1} (Fig. 6A). This K_m value for CYP4F11 is quite similar to that determined with liver microsomes from subjects A and B (Fig. 5A). In con-

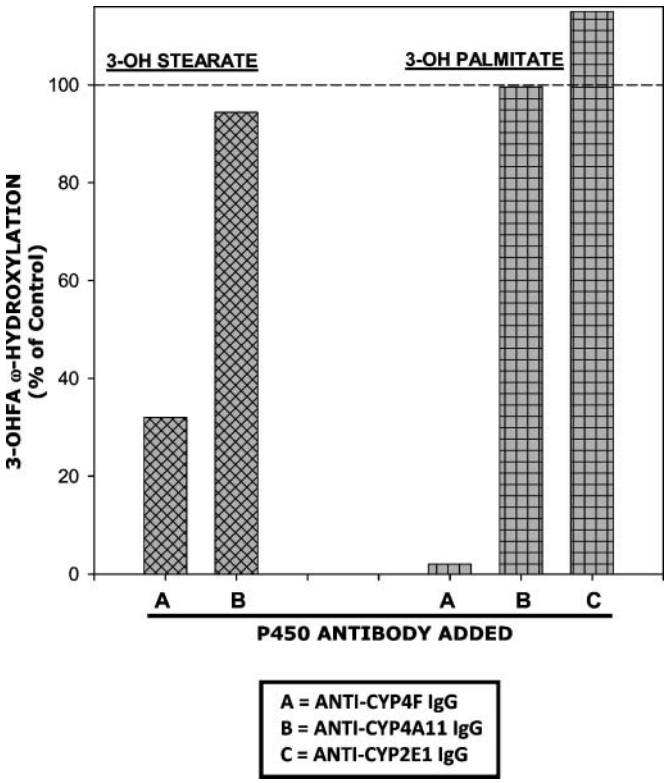

Fig. 4. Inhibition of 3-OHFA ω-hydroxylation by P450 antibodies. Metabolism of 3-hydroxystearate and 3-hydroxypalmitate to their respective ω-hydroxylated products was assessed in incubation mixtures containing liver microsomes from subject A (protein equivalent to 50 pmol of P450), 100 μM 3-OHFA substrate, 100 mM potassium phosphate buffer (pH 7.4), and 1 mM NADPH. Antibodies were added at an IgG/P450 ratio of 5 mg IgG/nmol P450 (1.25 mg of either preimmune or immune-specific IgG). Reactions were performed as described in Materials and Methods, except that microsomes were preincubated with antibodies for 3 min at 37°C, followed by 10 min at ambient temperature before initiating the reactions. Control rates of metabolism by subject A liver microsomes were 1.69 nmol 3,18-dihydroxystearate formed/min/nmol P450 and 2.45 nmol 3,16-dihydroxypalmitate formed/min/nmol P450.

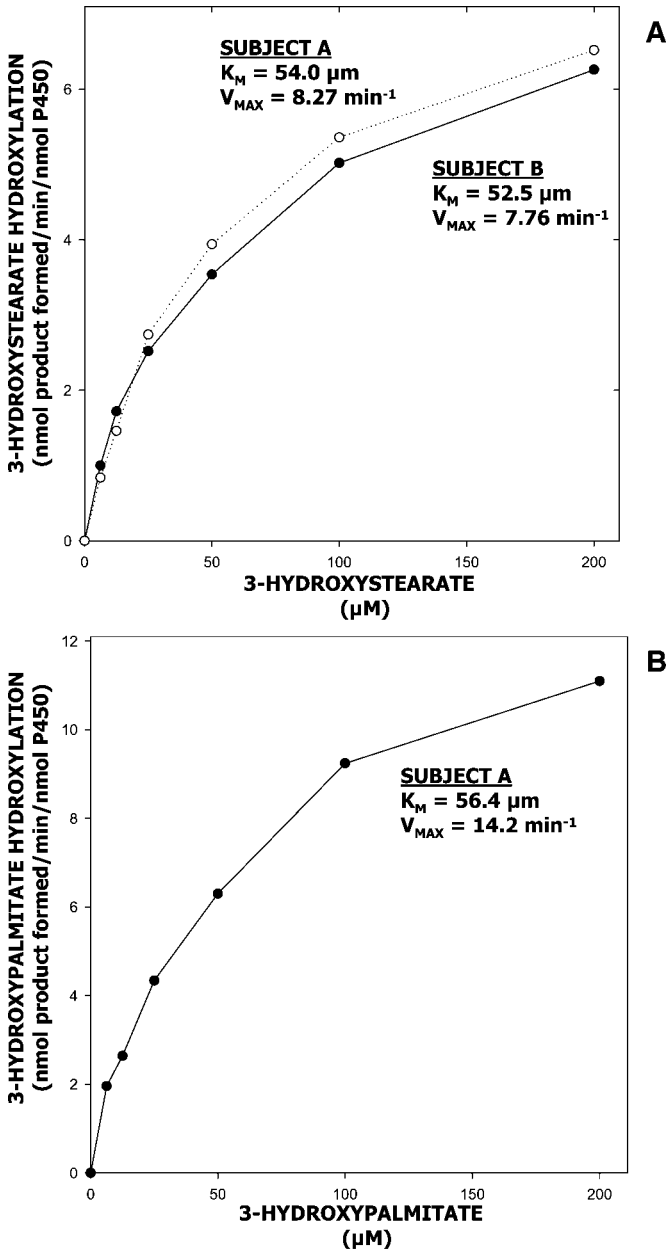

Fig. 5. Kinetic analysis of 3-OHFA ω-hydroxylation by human liver microsomes. 3-Hydroxystearate and 3-hydroxypalmitate ω-hydroxylation by liver microsomes from subjects A and B were assessed as described in Materials and Methods. Simple Michaelis-Menten kinetics were observed throughout the range of 3-OHFA concentrations used (6–200 μM). Nonlinear regression analysis was used to derive the apparent K_m and V_{max} values shown for 3-hydroxystearate (A) and 3-hydroxypalmitate (B).

trast, CYP4F2 exhibited a K_m of 29.1 μM and a V_{max} of 5.8 min^{-1} with 3-hydroxystearate. The intrinsic clearance values (V_{max}/K_m) determined with 3-hydroxystearate for CYP4F11 and CYP4F2 were 0.259 and 0.122 μl/min/nmol P450, respectively, which reflect the greater capacity of CYP4F11 to metabolize this 3-hydroxylated fatty acid. Monophasic ω-hydroxylation kinetics were also noted with CYP4F11 and 3-hydroxypalmitate (Fig. 6B), whose rate data analysis using nonlinear regression gave an apparent K_m value of 105.8 μM with a corresponding V_{max} value of 70.6 min^{-1}. The intrinsic clearance value determined for 3-hydroxypalmitate metabolism by CYP4F11 was 0.667, which is nearly 3-fold greater than that exhibited by subject A liver microsomes (0.252). With both CYP4F11 and CYP4F2, Eadie-Hofstee plots of the ω-hydroxylation data reflected simple Michaelis-Menten kinetics (data not shown).

Correlation between liver CYP4F content and 3-OHFA ω-hydroxylation

Figure 7A shows the significant correlation observed ($r = 0.658$, $P < 0.01$, n = 14) between the total content of CYP4F enzymes in liver microsomes from 14 different individuals and rates of microsomal 3,18-dihydroxystearate formation. For these studies, aggregate hepatic CYP4F content was assessed on Western blots immunostained with

TABLE 1. 3-OHFA ω-hydroxylation by human P450 enzymes

Enzyme	3-OH Fatty Acid ω-Hydroxylation		
	3,18-Dihydroxystearate	3,16-Dihydroxypalmitate	Laurate ω-Hydroxylation
CYP4F2	3.6	3.7	bd[a]
CYP4F3b	1.4	2.9	bd
CYP4F11	9.7	35.0	10.7
CYP4F12	bd	—	—
CYP4A11	bd	1.7	19.8
UC8911 liver microsomes	5.8	9.5	11.7

3-OHFA, 3-hydroxy fatty acid. 3-Hydroxystearate and 3-hydroxypalmitate ω-hydroxylation was assessed in reaction mixtures containing a P450 reconstituted system, 100 μM 3-OHFA substrate, 100 mM potassium phosphate buffer (pH 7.4), and 0.5 mM NADPH. Reconstituted systems consisted of 25 pmol of purified CYP4F/A enzyme, 75 pmol of NADPH:P450 oxidoreductase, 7.5 μg of synthetic L-α-dilauroylphosphatidylcholine, and 100 pmol of cytochrome b_5. With human liver microsomes, an amount of protein equivalent to 50 pmol of P450 was used. Reactions were initiated with NADPH and were terminated after 10 min at 37°C. Formation of 3,18-dihydroxystearate and 3,16-dihydroxypalmitate was quantitated by HPLC as described in Materials and Methods. Data are expressed as nmol product formed/min/nmol P450 and denote averages of three to six individual determinations.

[a] bd, below detection limits.

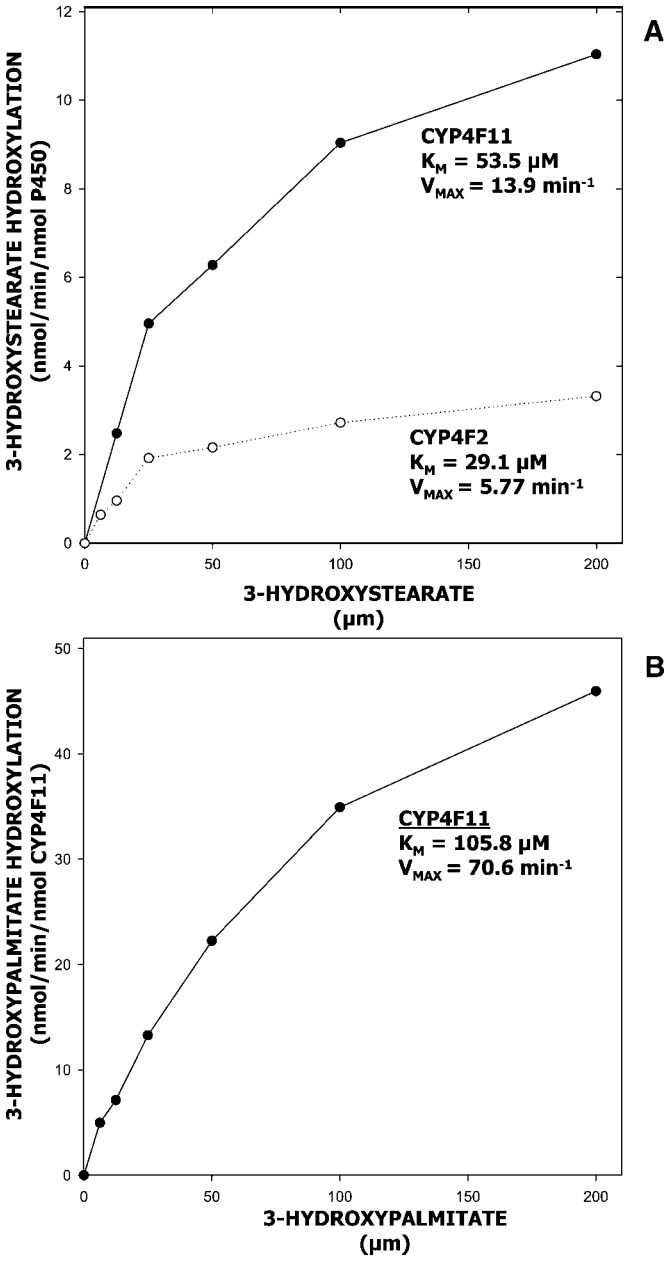

Fig. 6. Kinetic analysis of 3-OHFA ω-hydroxylation by purified CYP4F enzymes. ω-Hydroxylation of 3-hydroxystearate and 3-hydroxypalmitate by purified CYP4F11 and CYP4F2 upon reconstitution with NADPH:P450 oxidoreductase and cytochrome b_5 was measured as described in Materials and Methods. Simple Michaelis-Menten kinetics were exhibited by both CYP4F enzymes across the range of 3-OHFA concentrations examined (3–200 μM). The apparent K_m and V_{max} values shown for CYP4F11- and CYP4F2-catalyzed 3-hydroxystearate oxidation (A) and for CYP4F11-catalyzed 3-hydroxypalmitate oxidation (B) were derived using nonlinear regression analysis. Additional details are provided in Table 1.

our polyspecific anti-CYP4F antibody, which recognizes all CYP4F enzymes expressed in human liver. In contrast, no correlation was observed between total microsomal CYP4F content and rates of 3-hydroxypalmitate ω-hydroxylation ($r = 0.366$, $P = 0.198$) (data not shown). Furthermore, no relationship was found between microsomal CYP4F2 content (measured with a CYP4F2-specific peptide antibody) (21) and either 3-hydroxystearate ω-hydroxylase ($r = 0.167$, $P = 0.604$) (Fig. 7B) or 3-hydroxypalmitate ω-hydroxylase activity ($r = 0.050$, $P = 0.824$) (data not shown). Finally, among the 14 individuals analyzed here, a strong correlation ($r = 0.763$, $P < 0.002$) was noted between rates of 3-hydroxystearate and 3-hydroxypalmitate ω-hydroxylation (Fig. 7C), suggesting that both activities are catalyzed by the same microsomal CYP4F enzyme(s).

DISCUSSION

The results presented here demonstrate that NADPH-fortified human liver microsomes convert two exemplary 3-OHFAs, 3-hydroxystearate and 3-hydroxypalmitate, to their corresponding ω-hydroxylated metabolites, as substantiated by GC-MS analysis. Polyclonal antibodies to CYP4F2, which also recognize CYP4F3a, CYP4F3b, CYP4F11, and CYP4F12, markedly inhibited the formation of 3,18-dihydroxystearate and 3,16-dihydroxypalmitate by liver microsomes, whereas antibodies to other fatty acid hydroxylases, including CYP4A11 and CYP2E1, had little effect on microsomal ω-oxidation of these long-chain 3-OHFAs (Fig. 4). Upon reconstitution with P450 reductase, b_5, and phospholipid, the CYP4F gene subfamily members CYP4F11 and, to a lesser extent, CYP4F2 converted 3-hydroxystearate to 3,18-dihydroxystearate at substantial rates, whereas the other CYP4F/A P450s examined were not effective catalysts of this ω-hydroxylation reaction (Table 1). In the case of 3-hydroxypalmitate, the principal P450 enzyme promoting its ω-terminal oxidation was CYP4F11 as well. Among the 14 different human subjects examined here, a significant correlation was found

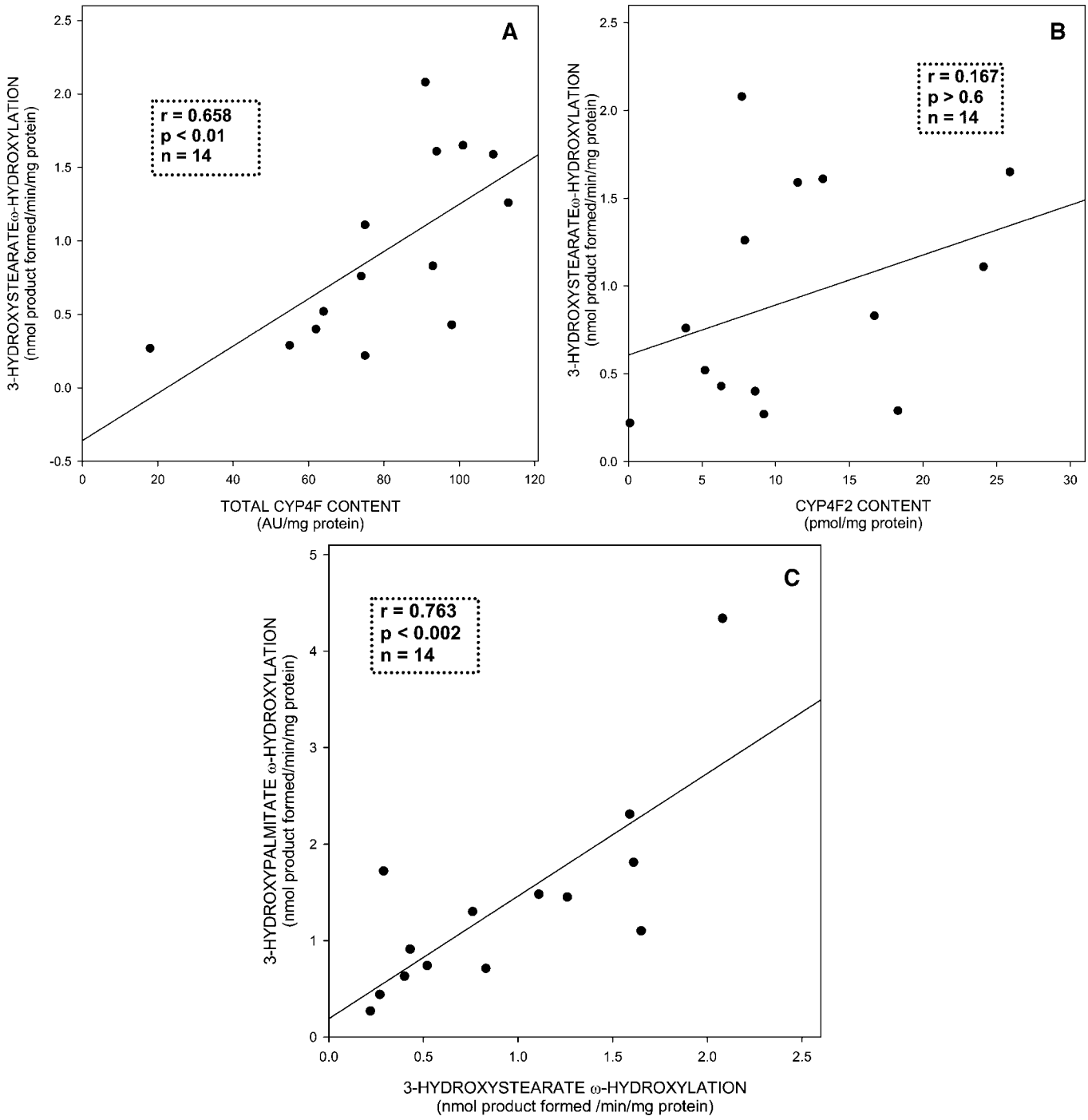

Fig. 7. Correlation between total CYP4F content (A), CYP4F2 content (B), and 3-OHFA ω-hydroxylation in human liver microsomes. Total CYP4F and CYP4F2 contents in liver microsomes from 13 different subjects were determined by Western blotting, whereas rates of 3,18-dihydroxystearate and 3,16-dihydroxypalmitate formation (C) by the same samples were assessed as described in Materials and Methods. The correlation coefficient (r) was derived from the line of best fit by regression analysis, and a test of slope was used to determine the level of significance (P).

between aggregate CYP4F content in liver microsomes (measured immunochemically) and rates of 3,18-dihydroxystearate formation (Fig. 7), although the same relationship was not observed with 3,16-dihydroxypalmitate formation. Finally, microsomal 3-hydroxystearate ω-hydroxylase activity was highly correlated ($r = 0.763$, $P < 0.002$, n = 14) with 3-hydroxypalmitate ω-hydroxylase activity (Fig. 7C), providing additional evidence that both 3-OHFA oxidation reactions are catalyzed by the same P450 enzyme, most likely CYP4F11.

The capacity of CYP450 enzymes to oxidize fatty acids has been known for quite some time; in fact, fatty acids

were among the first substrates tested with these enzymes upon their purification (31). More recent studies have revealed that P450 enzymes belonging to the CYP4F/A subfamilies have the unique ability to ω-hydroxylate the thermodynamically unfavorable terminal methyl groups of medium-, long-, and very-long-chain fatty acids (2–5, 18) as well as those of bioactive eicosanoids (e.g., leukotriene B_4) derived from the essential C20:4 fatty acid, arachidonate (4, 10–13). Nearly all investigations of fatty acid ω-hydroxylation have focused on the CYP4A gene subfamily, which in humans is composed of essentially a single P450, CYP4A11. Although a second CYP4A gene subfamily product designated CYP4A22 has been described (7, 8), it is expressed at very low levels. The substrate specificity of CYP4A11 encompasses both medium- and long-chain fatty acids and is similar to that observed with the rat CYP4A P450s (5, 26, 32). In contrast, much less is known about the capacity of CYP4F enzymes to ω-hydroxylate free fatty acids. Here, we found that two human CYP4F gene subfamily members, CYP4F11 and, to a lesser extent, CYP4F2, were efficient catalysts of 3-OHFA ω-hydroxylation. The involvement of other P450 enzymes in the ω-oxidation of these 3-hydroxylated fatty acids was ruled out in immunoinhibition experiments, in which it was demonstrated that anti-CYP4F IgG was the lone P450 antibody capable of markedly inhibiting ω-hydroxylated product formation (Fig. 4). That CYP4F11 is the more dominant of the two enzymes with regard to 3-OHFA oxidation was indicated by the following observations: *a*) CYP4F11 converted 3-hydroxystearate to its 18-hydroxylated metabolite at rates that were nearly 3-fold higher than CYP4F2 and 2-fold higher than native liver microsomes; CYP4F11 was also the only CYP4F/A enzyme that extensively oxidized 3-hydroxypalmitate to its corresponding dihydroxylated product (Table 1); and *b*) kinetic analyses revealed that the K_m values for 3-hydroxystearate and 3-hydroxypalmitate hydroxylation with liver microsomes were very similar to those determined with purified, reconstituted CYP4F11 (Figs. 5, 6). The significant correlation ($r = 0.658$, $P < 0.01$) observed between the immunochemically determined, aggregate CYP4F content and 3-hydroxystearate ω-hydroxylase activity but not 3-hydroxypalmitate ω-hydroxylase activity ($r = 0.366$, $P = 0.198$) in liver microsomes from 14 different subjects (Fig. 7) may stem from the weak reactivity of CYP4F11 with anti-CYP4F on Western blots compared with the strong immunoreactions observed with CYP4F2, CYP4F3b, and CYP4F12 (21).

It should be emphasized here that this is the first instance in which a prospective endogenous function has been described for CYP4F11. The drug-metabolizing activities attributed to CYP4F11 by Kalsotra et al. (33), including demethylation of erythromycin, benzphetamine, ethylmorphine, chlorpromazine, and imipramine, are not likely intrinsic functions of the enzyme but rather confounding characteristics of heterologous expression of the enzyme in yeast. In one of the few reports on CYP4F-catalyzed fatty acid metabolism, Sanders et al. (18) showed that the very-long-chain fatty acids docosanoic acid (C22:0), tetracosanoid acid (C24:0), and hexacosanedioic acid (C26:0) are all converted to their respective ω-hydroxylated metabolites by human liver microsomes and that heterologously expressed CYP4F3b and CYP4F2 were the only P450s that catalyzed ω-hydroxylation of the C26:0 fatty acid substrate; CYP4F11 was not tested in that study. Furthermore, the catalytic specificity of human CYP4F11 toward fatty acids appears to be somewhat broader than that of the other CYP4F/A P450s, because the enzyme displayed extensive ω-hydroxylase activity not only with 3-hydroxystearate, 3-hydroxypalmitate, and laurate but also with leukotriene B_4 and arachidonate.[4] Although laurate, an exemplary fatty acid P450 substrate, is extensively metabolized by CYP4F11 (Table 1), its 3-hydroxylated analog is not, indicating that chain length may be one determinant governing 3-OHFA ω-hydroxylation by CYP4F11. Analogous to other CYP4F enzymes (3, 4, 34), CYP4F11 exhibited complete regioselectivity for the 3-OHFA ω-terminal carbon and did not oxidize these substrates at the adjacent ω-1 position. In fact, the lack of conversion of 3-hydroxystearate and 3-hydroxypalmitate to their ω-1 hydroxylated derivatives by native hepatic microsomes suggests that these 3-OHFAs are not substrates for CYP2E1, the principal fatty acid ω-1 hydroxylating enzyme expressed in human liver (29, 30, 35, 36).

Omega hydroxylation of 3-OHFAs by CYP4F enzymes may play an important role in eliminating these potentially toxic fatty acid intermediates from the circulation. Plasma concentrations of 3-OHFAs, especially those with chain lengths of >10 carbons, are markedly increased in patients exhibiting enhanced β-oxidation flux (e.g., ketotic states such as prolonged fasting and diabetic ketoacidosis) or those with specific defects in mitochondrial LCHAD (1, 14). In fact, circulating 3-OHFA levels have been proposed as a diagnostic marker for both LCHAD and MTP deficiencies (14, 37, 38). In these patients, endogenous medium- and long-chain fatty acids that typically undergo complete β-oxidation to give acetyl-CoA are instead only partially oxidized, resulting in mitochondrial accumulation of the corresponding 3-hydroxyacyl-CoA ester intermediates. Upon hydrolytic cleavage of these intermediates or their conversion into carnitine esters, the resultant 3-OHFAs are released from mitochondria and are ultimately converted to 3-OHDCAs. As shown here, CYP4F11 and CYP4F2 are likely involved in the initial step of this process, namely the conversion of 3-OHFAs to dihydroxylated fatty acids (Fig. 1). In patients with LCHAD or MTP deficiency, plasma 3-hydroxystearate levels increase up to 230-fold (from 0.02 to nearly 5 μM), whereas 3-hydroxypalmitate levels can increase as much as 80-fold (from 0.18 to 15 μM) (1). Although such plasma concentrations are somewhat less than the apparent K_m values determined here for 3-hydroxypalmitate and 3-hydroxystearate ω-hydroxylation by CYP4F11 and CYP4F2 (28.9–105.8 μM; Figs. 5, 6), the overall K_m values are still within the same order of magnitude, implying that CYP4F-catalyzed oxidation of 3-OHFA is likely to be operative under conditions of impaired β-oxidation in vivo. The capacity of CYP4F2 and CYP4F3b, in combination with alcohol and aldehyde dehydrogenases, to convert very-long-chain dihydroxyl-

ated fatty acids to DCA was described recently (18). As such, it is likely that long-chain dihydroxylated fatty acids formed from 3-OHFAs, such as 3,16-dihydroxypalmitate and 3,18-dihydroxystearate, undergo the same process (Fig. 1). Despite the original proposal that 3-OHDCAs were derived during mitochondrial β-oxidation of the cognate DCA (16, 39), the fact that neither medium- nor long-chain DCAs are readily transported into or β-oxidized in mitochondria (6) appears to eliminate such a pathway as a source of 3-OHFAs and, thus, 3-OHDCAs as well.

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