Involvement of cytochrome P450 2E1 in the (ω–1)-hydroxylation of oleic acid in human and rat liver microsomes

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Abstract In vitro techniques have been used to investigate the nature of microsomal cytochrome P450 involved in the metabolism of oleic acid, a physiological monounsaturated fatty acid. Like lauric acid, which is currently used as a model substrate of fatty acid metabolism, the alkyl chain of oleic acid is hydroxylated on its ω and (ω–1) carbons. The identity of these hydroxylated metabolites was ascertained by GC/MS and LC/MS. The ω/ω–1 ratio of oleic acid metabolites (1.22 ± 0.01) was found to be similar to that obtained with lauric acid in rat liver microsomes (1.10 ± 0.02), while in human liver microsomes this ratio was 0.75 ± 0.5 for lauric acid and 5.2 ± 2.6 for oleic acid. After treatment of rats with ethanol or clofibrate, inducers of CYP2E1 and CYP4A, respectively, the hydroxylations of oleic acid were shown to be less inducible than those of lauric acid. Five in vitro approaches were used to identify the P450 isoform(s) responsible for the microsomal (ω–1)-hydroxylation of oleic acid: effect of various inducers in rats, correlation studies between specific P450 catalytic activities in a panel of 25 human liver microsomes, chemical inhibitions, immuno-inhibitions and metabolism by cDNA-expressed human P450 enzymes. From the above results, it can be ascertained that P450 2E1 is the main enzyme involved in the (ω–1)-hydroxylation of oleic acid. Furthermore, the ω-hydroxylation of oleic acid was shown to be mainly catalyzed by P450 4A enzymes in human liver microsomes. The turnover number of (ω–1)-hydroxylation of lauric and oleic acids decreased from 7.8 to 1.5 min^{-1}, respectively, suggesting that the dodecane alkyl chain allows optimal binding to the active site of CYP2E1.—Adas, F., F. Berthou, D. Picart, P. Lozac'h, F. Beaugé, and Y. Amet. **Involvement of cytochrome P450 2E1 in the (ω–1)-hydroxylation of oleic acid in human and rat liver microsomes.** ***J. Lipid Res.*** **1998.** 39: **1210–1219.**

Supplementary key words CYP2E1 • lauric acid • ω-oxidation • monooxygenase enzymatic activities • xenobiotics • chlorzoxazone • 4-nitrophenol • mass spectrometry

Cytochrome P450s (1) are the heme-thiolate proteins of the microsomal mixed function monooxygenase system. They are involved in the metabolism of xenobiotics and endogenous compounds, such as steroids and fatty acids. Many P450 enzymes are characterized by specific substrates that are regiospecifically metabolized. Accordingly, lauric acid has been described as a model substrate for hydroxylations catalyzed by P450 in human liver and kidney microsomes (2, 3). More recently, the P450 involved in these hydroxylations has been reported both in the microsomal fractions of rat (4) and in human (5, 6) livers. In both species, lauric acid was regiospecifically metabolized to form ω and (ω–1)-hydroxylated metabolites, and the ratio of these two products varied significantly after starvation, diabetes (7–9), administration of clofibrate and other peroxisome proliferators (10, 11), ethanol or CYP2E1 inducers (4, 12). Fatty acid ω-oxidation is a minor pathway that accounts for less than 10% of total liver fatty acid oxidation under normal physiological conditions (13). However, studies performed with mammalian systems suggest that ω-hydroxylases could be involved in the first step of fatty acid catabolism (14). Moreover, it was described that starvation or intake of certain dietary fat composition could strongly enhance fatty acid hydroxylation activities (15).

Oleic acid is an unsaturated physiological fatty acid present in the free fatty acid fraction and represents approximately 25% of this fraction (16). It is one of the *cis*-unsaturated free fatty acids (with arachidonic acid) which is released from the *sn*-2 position of phospholipids (17). It plays an important physiological role by activating protein

Abbreviations: P450, cytochrome P450 (EC 1.14.14.1) or CYP; 17-OH-oleic acid, 17-hydroxyoleic acid or (ω–1)-hydroxyoleic acid; 18-OH-oleic acid, 18-hydroxyoleic acid or ω-hydroxyoleic acid; PKC, protein kinase C; CHZ, chlorzoxazone; 4-NP, 4-nitrophenol; 17-ODYA, 17-octadecynoic acid; BSTFA, N,O-bis-trimethylsilyl-trifluoroacetamide; TMCS, trimethylchlorosilane; HPLC, high performance liquid chromatography; APCI-LC/MS, atmospheric pressure chemical ionization liquid chromatography/mass spectrometry; GC/EIMS, gas chromatography/electron ionization mass spectrometry.

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kinase C (PKC) and by exerting modulatory effects on ion channels (18) and on ligand-gated receptors (19). Although oleic acid is released at a much lower level than arachidonic acid, it is proposed to be a more important messenger for sustained activation of PKC, as arachidonic acid is a polyunsaturated fatty acid and is therefore more quickly metabolized than oleic acid (17). In addition to the ω and (ω–1)-hydroxylation products, unsaturated fatty acids are generally converted to epoxides by P450 monooxygenases (20–22). However, the epoxide pathway appears to be only a minor microsomal oxidation route for monounsaturated fatty acids such as oleic acid (22). As its metabolism through the P450 pathway has not been clearly established, it was of interest to measure the ω and (ω–1)-hydroxylation of this physiological fatty acid in rat and human liver microsomes. Additionally, the induction effects of ethanol or clofibrate (both known to be inducers of CYP2E1 and CYP4A, respectively) on these metabolic pathways were compared using lauric and oleic acid as substrates.

A further comparison was made of the specific activities of the ω and (ω–1)-hydroxylations of oleic acid in human and rat liver microsomes. In this study, microsomal kinetics, inhibitor and induction techniques, and the use of cells genetically engineered for human P450 were used to investigate the enzymes involved in this metabolism.

MATERIALS AND METHODS

Chemicals

Oleic, lauric, palmitic, elaidic, and stearic acids were purchased from Fluka (Buchs, Switzerland), while [1-^{14}C]oleic acid (50 mCi/mmol) was from Amersham (Amersham, UK). NADPH, 4-nitrophenol (4-NP), chlorzoxazone (CHZ), N,O-bis-trimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were from Sigma (St. Quentin Fallavier, France). Polyclonal antibody against rat CYP2E1 was a gift from Dr. Song (Bethesda, MD), while rat anti-CYP4A polyclonal antibody was from Gentest (Woburn, MA). 17-Octadecynoic acid (17-ODYA) was purchased from Cayman (Cayman Chemical, Ann Arbor, MI). Modified cell microsomes containing human P450s were obtained from Gentest, while cytochrome b5 was from Oxford Biomedical Research, Oxford, MI. All chemicals and solvents were of the highest purity obtainable and were from Merck (Darmstadt, Germany) or Sigma.

Preparation of rat and human liver microsomes

Male Wistar rats (155–170 g) were purchased from Iffa Credo (Les Asbresles, France) and were housed 4 per cage. They were subjected either to ethanol vapor inhalation for 1 month, as previously described (23), or to clofibrate i.p. treatment (4). The rats were killed and the livers were immediately frozen and stored in liquid nitrogen until the preparation of microsomes.

Human liver samples (n = 25) were obtained from subjects who died after traffic accidents. In accordance with French law, ethical committee approval was obtained prior to this study. Upon brain death, the livers were removed, immediately frozen, and stored in liquid nitrogen until the peparation of the microsomal fractions.

Microsomes from rat and human livers were prepared after the homogenization of the tissues, as previously described (24) and stored at −80°C until use. Microsomal protein concentrations were determined using the Bradford method (Bio-Rad, Munich, Germany). Human liver contents, in terms of specific P450 and different monooxygenase activities, have been previously reported (25).

Assay of monooxygenase enzymatic activities

The ω and (ω–1)-hydroxylations of oleic acid were measured by incubating microsomes (0.3 mg of protein) in a reaction mixture containing substrate ([^{14}C]oleic acid 0.075 mm, 0.5 μCi) in 0.12 m potassium phosphate buffer (pH 7.4), and 5 mm $MgCl_2$. The reaction was initiated by the addition of 1 mm NADPH. After 20 min, the reaction was stopped by addition of 0.8 mL of a 10% H_2SO_4 solution. The metabolites and residual substrate were extracted twice with 5 mL of diethylether. The organic phase was dried under a nitrogen stream and the residue was dissolved in acetonitrile before HPLC analysis. For the determination of kinetic parameters in rat and human microsomes, oleic acid was added to the reaction mixture in the range 10–150 μm. Oleic acid was not soluble in the reaction medium beyond 150 μm. All kinetic studies were performed under linear conditions with respect to time and protein concentrations.

Chlorzoxazone 6-hydroxylation was measured according to a previously described method (26), with slight modifications (27). 4-Nitrophenol hydroxylation was determined by HPLC according to the methods described elsewhere (28, 29). P450 2E1 was immunoquantified in human liver microsomes as previously described (6).

Conditions of HPLC analysis

The ω and (ω–1)-hydroxylated metabolites and residual substrate were separated by RP-HPLC using a 5 μm Ultrasphere C18 column 150 × 4.6 mm (Beckman, France). The mobile phase (0.2% acetic acid in water/acetonitrile) program began isocratically with a 46:54 mixture (v/v) for 30 min at a flow rate of 2.0 mL/min, followed by a linear gradient to 5:95 (v/v) water and acetonitrile for 5 min, in order to eluate the residual substrate. The chromatography apparatus was equipped with a Flo-One Beta radiometric detector (Packard, Meriden, CT). Metabolic rates were calculated using the percentage of metabolite area to the total product area, and were expressed as pmol/min per mg of protein.

Gas chromatography/mass spectrometry and HPLC/mass spectrometry

The radioactive fractions were collected after HPLC analysis and freeze-dried. Oxygenated metabolites were then subjected to GC/EIMS (70 eV). Analysis was performed after silylation of the hydroxylated and acidic groups with a mixture of BSTFA/TMCS/pyridine (80:10:10; v/v/v) for 60 min at 60°C. The samples were then dried under a nitrogen stream, taken up by 50 μL of pentane, and run on a CP-Sil 5 CB (Chrompack, Middleburgh, The Netherlands) capillary column (30 m × 0.32 mm i.d., phase ratio = 320) programmed to rise from 140°C to 290°C at 4°C/min. The column was coupled with a Nermag R10-10 mass spectrometer (Ribermag, France).

The formation of the hydroxylated metabolites was also analyzed by LC/APCI-mass spectrometry on a Navigator LC/MS mass spectrometer (Finnigan, Manchester, UK), equipped with an atmospheric pressure ionization source running on negative ion mode. The HPLC chromatographic conditions were the same as described above.

Chemical and immuno-inhibition of oleic acid hydroxylations in human and rat liver microsomes

Compounds such as DMSO and ethanol, known to be competitive inhibitors of CYP2E1, were added prior to incubation at the

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concentrations indicated in Table 4. The incubation was performed using 50 μm oleic acid in both human and rat liver microsomes. Chemical inhibition by 17-ODYA, known to be a mechanism-based inhibitor, was carried out by pre-incubating the inhibitor with microsomal protein and NADPH at 37°C for 20 min. The 0.1 mL incubation medium was then diluted 20-fold in 0.12 m potassium phosphate buffer containing 50 μm oleic acid and 5 mm $MgCl_2$. The reaction was initiated by the addition of 1 mm NADPH and carried out as described above. Control experiments were conducted using the same amounts of organic solvents (never exceeding 0.2%; v/v). Inhibition of oleic acid hydroxylations was also performed using saturated and unsaturated fatty acids such as lauric, palmitic, stearic and elaidic acids at the concentrations indicated in Table 4.

Immuno-inhibition experiments using either anti-CYP2E1 or anti-CYP4A antibodies were assayed on oleic acid hydroxylations in rat and human liver microsomes. A microsomal sample (0.2 mg) from either human liver or ethanol-treated rat liver was incubated at room temperature for 30 min in the absence of NADPH and substrate, but in the presence of either polyclonal anti-rat anti-CYP2E1, anti-CYP4A1 antibodies, or non-immune serum. The reaction was initiated by the addition of 0.05 mm oleic acid and 1 mm NADPH and conducted as described above.

cDNA expression of cells genetically engineered for human P450

Human P450 enzymes (1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 4A11) were supplied by Gentest (Gentest Corp, Woburn). They were obtained from human B-lymphoblastoid cell lines transfected separately with human P450 cDNAs. According to the supplier, they expressed high specific P450 enzymatic activities (Table 5). Some of these preparations, especially P450 2E1, did not contain cytochrome b5. It was added to the incubation mixture in the molar ratio of 1:2 for P450/b5. The incubation method was the same as described above for human and rat liver microsomes, except for the incubation time which was increased to 60 min.

Statistical analysis

Correlation coefficients were calculated using an ANOVA table by the least-square regression analysis from the raw data. They were calculated by including all samples and were considered to be statistically significant when P was <0.05. The results are expressed as mean ± SD.

RESULTS

Identification of oleic acid metabolites

Figure 1 shows the HPLC profiles obtained after incubating oleic acid with microsomes from human (A) and ethanol-treated rat (B) liver microsomes using radiometric detection. The retention times of the two major metabolites and residual substrate were 18, 20, and 46 min, respectively. The generation of these two main metabolites had an absolute requirement for molecular oxygen and NADPH as source of electrons (data not shown).

In order to identify the main metabolites of [^{14}C]oleic acid, the organic extract of the incubation medium was separated by reversed phase-HPLC. The radioactive HPLC peaks 1 and 2 were collected and then freeze-dried. The fraction was derivatized and analyzed by GC-EIMS, as described in Materials and Methods. Another minor chromatographic peak (noted peak 3) could be detected in Fig. 1. Although it has not yet been identified, it is suggested that this peak could be an epoxided derivative of oleic acid. However, as it was below limit of assay detection in human liver microsomes, a more detailed study of the epoxide pathway was not carried out.

As shown in **Fig. 2 A**, the TMS derivatives of oleic acid metabolites were well separated by GC. Their mass spectra showed characteristic fragmentation which could be interpreted structurally (Fig. 2 B). The ion fragment m/z 117 represented the major fragment in the mass spectrum for the TMS derivative of the metabolite 1. This ion resulted from two fragments: [CH_3-CHO-Si-Me_3^+], specific to (ω–1)-hydroxylated fatty acids, as previously described (30), and [COO-Si-Me_3^+] characteristic of the TMS derivative of the carboxylic group. Only this last fragment was detected in the mass spectrum of the TMS derivative of the metabolite 2. Therefore, this fragment represented only 25% of the base peak intensity. On the other hand, an abundant ion fragment m/z 103 was detected on the second spectrum. It was due to the fragment [CH_2-O-Si-Me_3^+], characteristic of ω-hydroxylated fatty acid TMS derivatives. Moreover, TMS derivatives of ω-hydroxylated metabolites produce a strong rearrangement ion at m/z 147 [Me_3-Si-O-Si-Me_2^+] which is common to all ω-hydroxy derivatives. Furthermore, the two compounds have common fragments at m/z 442 [M^+], m/z 427 [M^+–15], m/z 411 [M^+–31] and m/z 337 [M^+–90–15] caused by loss of methyl and methoxy groups. Two other fragments m/z 398 [M^+–44] and m/z 383 [M^+–59], resulting from ion rearrangement, allowed us to confirm the identity of the two main metabolites. They were the (ω–1) and ω-hydroxy derivatives of oleic acid, respectively. The same pattern of fragmentation was observed with the TMS-derivatives of pure 11-OH and 12-OH lauric acid (data not shown). Assuming that the 9–10 double bond remained at its original position, and compared to previously published spectra obtained for metabolites of oleic acid in plants (31, 32), the two major oleate metabolites 1 and 2 were identified as the (ω–1) and ω-hydroxylated metabolites, respectively. Moreover, their order of elution in GC, namely the (ω–1) before the ω-hydroxylated derivative, was the same as described for the TMS derivatives of laurate (33).

The hydroxylated metabolites of oleic acid were also analyzed by LC-APCI/MS (negative ion mode). The mass fragmentogram showed two peaks, noted 1 and 2 (**Fig. 3**). Both were characterized with a pseudo-molecular ion m/z 297 [M–H^-] consistent with a molecular weight of 298, such as $C_{18}H_{34}O_3$, i.e., an hydroxyderivative of oleic acid. The order of HPLC elution of these hydroxylated metabolites was (ω–1) and ω, as previously reported in numerous studies concerning palmitate (34), laurate (4, 5, 35), and arachidonate (36). Moreover, the unknown peaks X and X′ could not be considered as epoxides or oleic acid metabolites, since they were not observed when using radiometric detection.

Kinetic parameters

Table 1 shows the kinetic parameters of 17- and 18-hydroxyoleic acid formation from oleic acid by micro-

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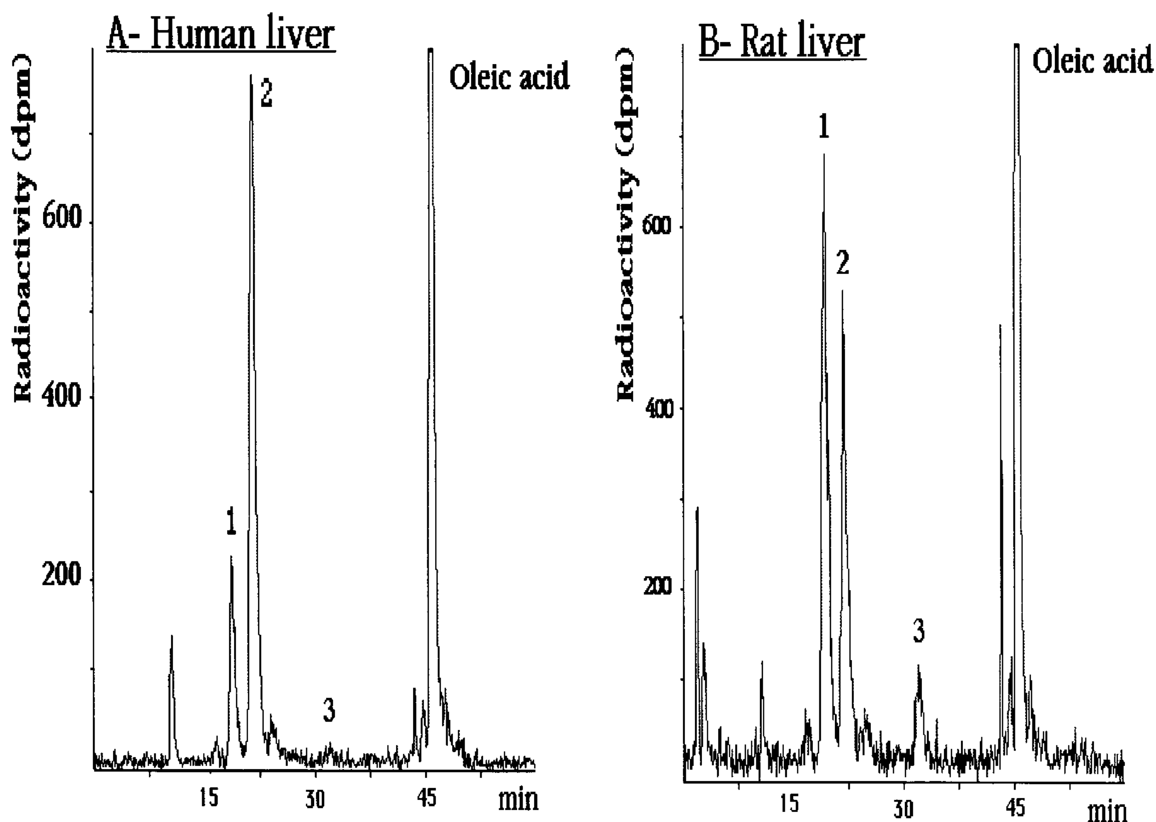

Fig. 1. RP-HPLC profiles of oleic acid (0.075 mm; 0.5 μCi) metabolites produced by human (A) or control rat (B) liver microsomes with radiometric detection. Peaks 1 and 2 were collected for further identification.

somes prepared from either ethanol-treated rat or three human (named FH3, Br039 and Br015) liver microsomes.

In rat liver microsomes, the K_m was 59.8 or 28.7 μm, and the V_m was 1.50 or 0.90 nmol/min per mg for the formation of 17- and 18-OH-oleic acid, respectively. An apparent K_m of 81 ± 39 μm and a V_m of 0.93 ± 0.50 nmol/min per mg of microsomal protein was determined for the formation of 17-OH-oleic acid, while a K_m of 46.6 ± 28 μm with a V_m of 2.60 ± 1.7 nmol/min per mg for the 18-OH-oleic acid was found in human liver microsomes. Oleic acid 17- and 18-hydroxylation displayed simple enzyme kinetic properties with only one enzyme involved in these reactions.

Correlation between oleic acid hydroxylations and different monooxygenase activities in rat and human liver microsomes

In human liver microsomes (n = 25), the oleic acid hydroxylations presented a large interindividual variation (2.6-fold) ranging from 172 to 450 (mean ± SD = 305 ± 162) pmol/min per mg for the 17-hydroxylation, and ranging from 432 to 2205 (mean ± SD = 1357 ± 378) pmol/min per mg (5.1-fold) for the 18-hydroxylation. The ω/ω–1 ratio was 5.2 ± 2.6 in human liver microsomes, while it was 0.75 ± 0.5 with lauric acid as substrate. In contrast, the relative ratios of the two hydroxylated metabolites of oleic acid were very different in rat liver microsomes. The ω/ω–1 ratio was found to be 1.22 ± 0.01 in control rats. Approximately the same value was found when lauric acid was used, namely 1.10 ± 0.02. The above oleic acid hydroxylations were less inducible by both ethanol and clofibrate, than those of lauric acid (**Table 2**).

Table 3 shows the correlation coefficients (r) between 17- and 18-OH-oleic acid formation and three monooxygenase enzymatic activities (CHZ, 4-NP, and 11-OH-lauric acid hydroxylations). Indeed, when two reactions are catalyzed by the same enzyme, the metabolic rates should be correlated to each other in a series of microsomal preparations containing varying levels of the enzyme. Table 3 shows that the 17-OH-oleic acid activity measured in human liver microsomes (n = 25) correlated highly significantly with three monooxygenase enzymatic activities known to be mediated by CYP2E1, namely 4-nitrophenol hydroxylation (r = 0.85; P < 0.001), chlorzoxazone-6-hydroxylation (r = 0.82; P < 0.001) and lauric acid 11-hydroxylation (r = 0.85; P < 0.001). A significant correlation was also found with the CYP2E1 protein content (r = 0.54; P < 0.01). Conversely, the 18-OH-oleic acid formation did not correlate with these CYP2E1 monooxygenase enzymatic activities.

Inhibitions of oleic acid hydroxylations

The inhibitory effect of various P450 compounds was tested on rat and human liver microsomal preparations, with oleic acid at a concentration of 0.05 mm (**Table 4**). Compounds known to be metabolized by CYP2E1, namely dimethylsulfoxide (DMSO) and ethanol, inhibited the

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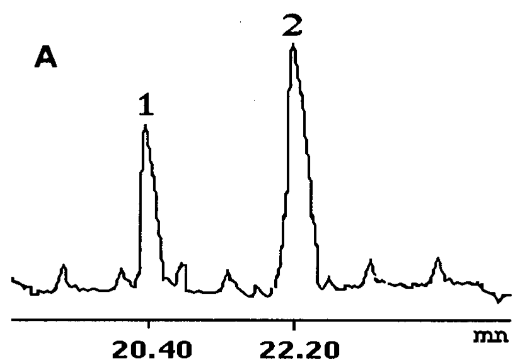

Fig. 2. GC/MS trace recorded as total ion current (A) and mass spectra (B) of the TMS-ester derivatives of oleic acid metabolites. Experimental procedures are described in Materials and Methods. The structure and major cleavage sites of the derivatives are shown. Peak 1 was identified as (ω–1)-OH-oleic acid and peak 2 as ω-OH-oleic acid.

oleic acid (ω–1)-hydroxylation, with approximately 30–40% residual activity in both rat and human liver microsomes. On the other hand, these compounds did not modify the ω-hydroxylation of oleic acid. 17-Octadecynoic acid (17-ODYA), known to be a potent irreversible inhibitor of mammalian fatty acid ω-hydroxylase, inhibited 53 and 75% of oleic acid ω-hydroxylation in rat and human liver microsomes, respectively, while the (ω–1)-hydroxylation was only slightly modified.

Inhibitions using analog fatty acids (Table 4) led to a decrease of the (ω–1)-hydroxylase enzymatic activity with lauric, stearic and elaidic acids, but no effect with palmitic acid was observed. The ω-hydroxylation was not modified with saturated fatty acids, and decreased with elaidic acid.

In order to confirm whether or not the CYP2E1 enzyme is involved in oleic acid metabolism in rat and human liver microsomes, immuno-inhibition experiments using polyclonal anti-rat CYP2E1 or CYP4A1 antibodies were carried out (Table 4). The anti-rat CYP2E1 antibody inhibited the (ω–1)-hydroxylase activity by 80 and 70% in rat and human liver microsomes, respectively, while the ω-hydroxylation was slightly decreased in human liver microsomes

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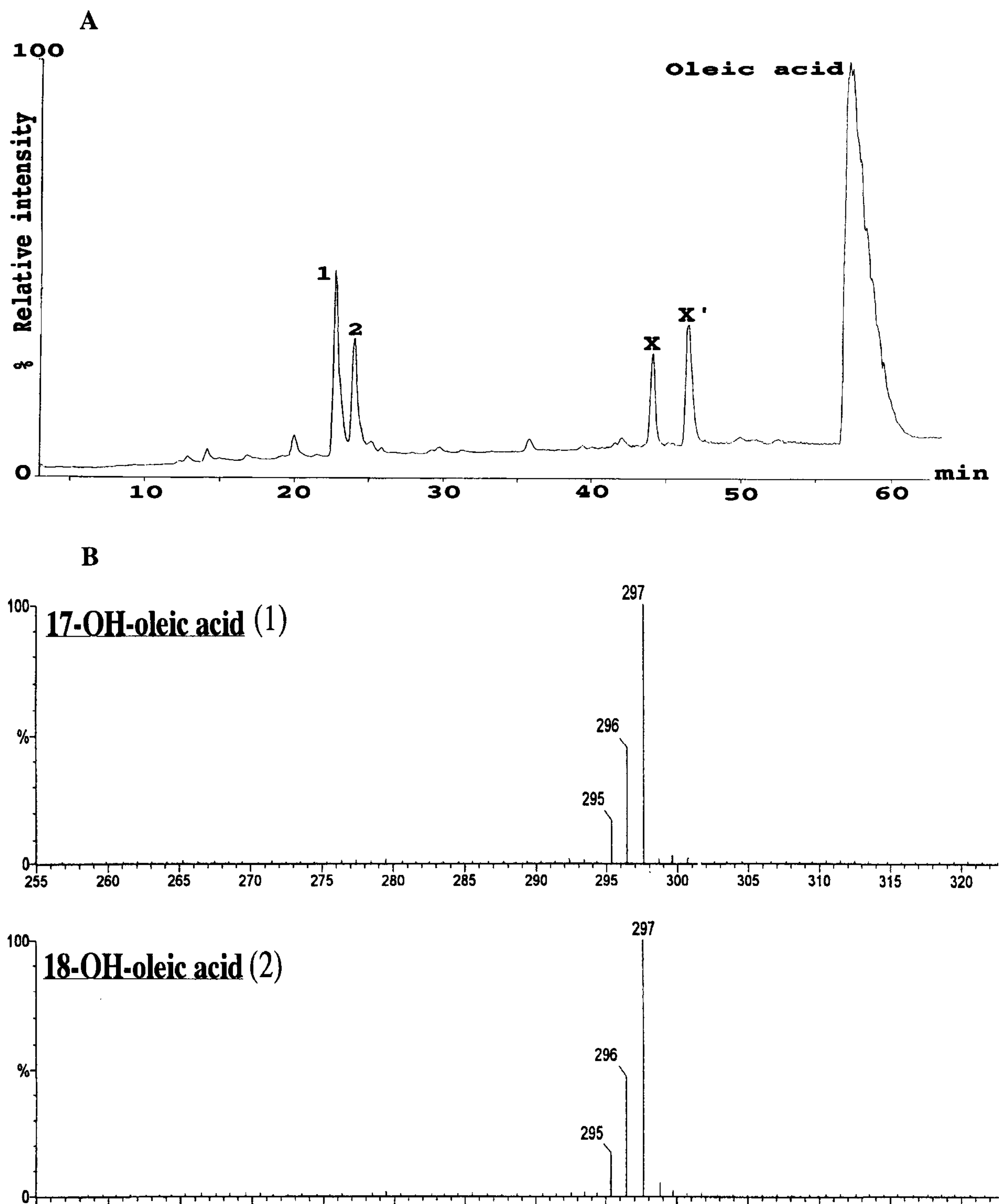

Fig. 3. Liquid chromatographic separation (A) and APCI-mass spectrometry analysis (ion negative mode) (B) of the oleic acid metabolites from human liver microsomes.

only. Using an anti-CYP4A1 antibody, a significant inhibition (65 and 77% inhibition in rat and human liver microsomes, respectively) of the ω-hydroxylation of oleic acid could be observed in microsomes from both rat and human liver. On the contrary, no significant effect on (ω−1)-hydroxylation was detected. Additionally, non-immune serum was found to have no effect on these enzymatic activities.

Metabolism of oleic acid by genetically engineered human P450 isoforms

The microsomal preparation of human cells containing P450 enzymes (1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 4A11) was incubated in the presence of 0.1 mm oleic acid at 37°C for 60 min (**Table 5**). The results showed that the CYP2E1 enzyme was capable of producing the (ω−1)-hydroxylated metabolite of both oleic and

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TABLE 1. Kinetic parameters of ω- and (ω–1)-oleic acid-hydroxylation in rat liver and three human liver microsomal preparations

Liver	(ω–1)-Hydroxylation K_m	(ω–1)-Hydroxylation V_m	ω-Hydroxylation K_m	ω-Hydroxylation V_m
	μM	*nmol/min/mg*	*μM*	*nmol/min/mg*
Rat	59.8	1.50	28.7	0.90
Human				
FH3	122.3	1.50	78.7	4.52
Br039	77.6	0.93	27.2	1.62
Br015	44	0.40	34	1.63
Mean ± SD	81 ± 39	0.93 ± 0.50	46.6 ± 28	2.60 ± 1.7

Samples containing 0.3 mg protein were incubated at 37°C for 20 min with increasing concentrations of oleic acid ranging from 10 to 150 μm.

lauric acids with a turnover of 1.50 and 7.8 min^{-1} (6), respectively. The addition of cytochrome b5 slightly increased the metabolic rate of the (ω–1)-hydroxylated metabolite (1.5-fold). The CYP4A11 enzyme metabolized oleic acid mainly at the ω-position with a minor peak at the (ω–1)-position (with respective turnovers of 1.25 and 0.45 min^{-1}). The other P450 enzymes (1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 3A5) were unable to catalyze the ω and (ω–1)-hydoxylations of oleic acid, whereas they were capable of catalyzing the biotransformation of their specific substrates (Table 5).

DISCUSSION

Early work on fatty acid metabolism focused mainly on the ω-hydroxylation of saturated fatty acids by liver microsomes (37). Following this pioneering work, lauric acid was described to be an in vitro model substrate for rat and human liver CYP2E1 (4–6). As only trace amounts of free and esterified lauric acid were found in the membrane of the hepatic endoplasmic reticulum, it was generally believed that lauric acid must be considered only as a model substrate for the physiologically more relevant endogenous substrates such as oleic and arachidonic acid (5). Oleic acid is an unsaturated physiological fatty acid released from the *sn*-2 position of phospholipids, such as arachidonic acid. However, its metabolism, through the mammalian P450 pathway, has not been subjected to detailed study as has arachidonic acid (21, 22, 38). In order to investigate and identify the nature of the P450 enzyme

TABLE 3. Correlation coefficients between ω- and (ω–1)-hydroxylations of oleic acid and various CYP2E1-mediated monooxygenase enzymatic activities in human liver microsomes (n = 25)

	CHZ	4-NP	(ω–1)-OH-Lauric Acid	(ω–1)-OH-Oleic Acid	CYP2E1 Content
(ω–1)-OH-oleic acid	0.82[b]	0.85[b]	0.85[b]		0.54[a]
ω-OH-oleic acid	0.17[c]	0.13[c]	0.30[c]	0.09[c]	0.28[c]

Correlation coefficients were calculated by the least-squares method.
[a] $P < 0.01$.
[b] $P < 0.001$.
[c] Not significant.

involved in the hydroxylations of oleic acid by human and rat liver microsomes, five in vitro approaches were used, namely induction effect in rats, correlation studies between specific P450 activities in a panel of 25 human livers, chemical and immuno-inhibitions, and metabolism by cDNA-expressed human P450 enzymes.

Two major hydroxylated metabolites, namely ω and (ω–1)-hydroxylated oleic acid, were identified by GC/MS on the basis of the ion fragmentation of the TMS derivatives. Their molecular weight was confirmed using LC/MS analysis. The order of elution by HPLC and GC, i.e., the 17-OH product ahead of the 18-OH product, was in total concordance with data previously published for oleic acid in plants (31, 32), lauric acid (2–5), and other fatty acids (34–36). The kinetic parameters of oleic acid hydroxylations in rat liver, as well as in three human liver microsomes, were studied. The K_m values of the (ω–1)-hydroxylation were not very different from those obtained with lauric acid, while the V_m values were lower. The (ω–1)-hydroxylation rates were 0.93 ± 0.50 and 7.2 ± 4.1 nmol/min per mg protein in human liver microsomes for oleic and lauric acids, respectively (6). Considerable interindividual variations in the V_m values of the human liver microsomes were observed due to the heterogenous origin of the liver donors.

Ethanol, a well-known CYP2E1 inducer (4, 12), was also an effective inducer of (ω–1)-hydroxylation of oleic acid in rat liver microsomes, but this induction was more important when lauric acid was used as substrate (fold increases of 2.78 and 1.35 for lauric and oleic acids, respectively). Additionally, clofibrate, known to induce lauric acid ω-hydroxylase both in mammals (10, 39, 40) and in plants (41, 42), was also found to be an inducer of oleic

TABLE 2. Comparison of the effect of ethanol and clofibrate on the ω- and (ω–1)-hydroxylations of lauric and oleic acids in rats

	(ω–1)-Hydroxylation Lauric Acid	(ω–1)-Hydroxylation Oleic Acid	ω-Hydroxylation Lauric Acid	ω-Hydroxylation Oleic Acid
	pmol/min/mg protein			
Control	1510 ± 310	754 ± 242	1660 ± 270	926 ± 302
	-fold increase		*-fold increase*	
Ethanol-treated	2.78 ± 0.71	1.35 ± 0.13	1.05 ± 0.24	0.71 ± 0.11
Clofibrate-treated	3.32 ± 1.26	0.74 ± 0.09	15.73 ± 8.14	1.98 ± 0.007

Values are expressed as fold increase of enzymatic activities versus control rats.

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TABLE 4. Effect of chemical and immuno-inhibitors on ω- and (ω–1)-hydroxylations of oleic acid in rat and human liver microsomes

		% of Residual Activity			
		Rat Liver		Human Liver	
Inhibitor	Concentration	(ω–1)-OH	ω-OH	(ω–1)-OH	ω-OH
DMSO	10 mm	30	76	47	95
Ethanol	10 mm	29	90	32	97
17-ODYA	50 μm	82	47	68	25
Lauric acid	75 μm			70	109
Palmitic acid	75 μm			105	110
Stearic acid	75 μm			89	90
Elaidic acid	75 μm			47	40
Anti-2E1 antibody	2.5 mg Ig/mg	20	105	30	65
Anti-4A1 antibody	6.5 mg Ig/mg	87	35	91	23
Non-immune serum		102	98	110	93

Oleic acid (50 μm) was incubated with 0.3 mg of microsomal protein and 1 mm NADPH in the presence of various inhibitors at 37°C for 30 min. Values are expressed as % of residual activity versus control assay without inhibitors.

acid ω-hydroxylase (fold increases of 15.7 and 1.98 for lauric and oleic acids, respectively).

Highly significant correlations were found between the (ω–1)-hydroxylation of oleic acid and the various CYP2E1-mediated monooxygenase enzyme activities, namely chlorzoxazone 6-hydroxylation, 4-nitrophenol hydroxylation, and lauric acid 11-hydroxylation, in a panel of 25 human liver microsomes. A correlation was also found with the CYP2E1 protein content. These results suggest that CYP2E1 is mainly involved in the (ω–1)-hydroxylation of oleic acid in both human and rat liver microsomes.

In rat and human liver microsomes, 17-hydroxylation of oleic acid was inhibited by the addition of DMSO and ethanol, compounds known to be competitive substrates for CYP2E1. These results were in accordance with those found when lauric acid was used as substrate (5, 6). Other fatty acids, such as lauric, palmitic, stearic, and elaidic, were tested as competitive molecules of oleic acid in human liver microsomes. A concentration of 75 μm lauric acid only inhibited the (ω–1)-hydroxylation of oleic acid by up to 70% of residual activity, while elaidic acid (an isomer *trans*-monounsaturated fatty acid) was able to inhibit both ω and (ω–1)-hydroxylation. 17-ODYA, a mechanism-based inhibitor of ω-hydroxylase in mammals (43), was found to produce a significant inhibition of oleic acid 18-hydroxylation at a concentration of 50 μm. Mechanism-based inhibitors containing a terminal acetylene function (such as 17-ODYA) were previously described to be potent irreversible inhibitors of both mammalian (44, 45) and plant (46) fatty acid ω-hydroxylases. Moreover, these fatty acid acetylenic analogues have been used successfully to explore the functional role and the reaction mechanisms of mammalian fatty acid ω-hydroxylases (43). Immunoinhibitions using a polyclonal anti-rat CYP2E1 antibody led to a significant inhibition of the (ω–1)-hydroxylation of oleic acid in rat and human liver microsomes, confirming the involvement of CYP2E1 in such a catalytic reaction. The polyclonal anti-CYP4A1 antibody produced a significant inhibition of the ω-hydroxylation, suggesting that this catalytic reaction was carried out by a P450 member of the 4A family, probably the CYP4A11 isoform (47). This result is in full agreement with that reported for lauric acid (3, 5, 10) in rat and human liver microsomes.

Finally, the use of cDNA-expressed human cells allowed us to confirm the involvement of CYP2E1 in the (ω–1)-hydroxylation of oleic acid. Only CYP2E1 was able to produce the (ω–1)-hydroxylated metabolite, while CYP4A11, a human liver (47) and kidney P450 (48), hydroxylated oleic acid mainly at the ω-position. The turnover of the enzyme was lower than with lauric acid (7.8 min^{-1}) (6), suggesting that, among the fatty acids examined, lauric acid was a most appropriate substrate for CYP2E1. Laethem et al. (21) reported also that cytochrome P450 2E1, when reconstituted with cytochrome b5 and NADPH cytochrome P450 oxidoreductase, was able to metabolize lauric, stearic, oleic, linoleic, linolenic, and arachidonic acids to hydroxylated metabolites. A previous study (49) re-

TABLE 5. Incubation of genetically engineered human P450 isoforms with oleic acid (1 mm) at 37°C for 60 min

P450 Isoform	P450 Content	Enzymatic Activity[b]	pmol/min/mg (min^{-1})[b]	(ω–1)-OH-Oleic Acid (min^{-1})	ω-OH-Oleic Acid (min^{-1})
	pmol/mg protein				
1A1	45	Ethoxyresorufin O-deethylase (EROD)	470 (10.4)	–	–
1A2	120	Ethoxyresorufin O-deethylase (EROD)	108 (0.9)	–	–
2B6	86	7-Ethoxy-4-trifluoromethylcoumarin deethylase	210 (2.4)	–	–
2C8	16	5-Chloromethylfluorescein diethylether deethylase	1.2 (0.08)	–	–
2C9	37	Diclofenac 4′-hydroxylase	1830 (49.5)	–	–
2C19	18	S-mephenytoin 4′-hydroxylase	31 (1.7)	–	–
2D6	212	(+) Bufuralol 1′-hydroxylase	4200 (19.8)	–	–
2E1	185	4-Nitrophenol hydroxylase	1810 (9.8)	1.05 ± 0.15	–
2E1 + b5[a]	185	4-Nitrophenol hydroxylase	3600 (19.5)	1.50 ± 0.20	–
3A4	80	Testosterone 6β-hydroxylase	2400 (30)	–	–
3A5	243	Testosterone 6β-hydroxylase	3.6 (0.012)	–	–
4A11	39	Lauric acid 12-hydroxylase	570 (14.6)	0.45 ± 0.10	1.25 ± 0.18

Results are expressed as turnover number (min^{-1} or pmol/min per mg of P450) and resulted from the mean ± SD of two individual experiments. Catalytic activities were not detectable in control microsomal preparations of cells not genetically modified. Values noted (–) are below limits of assay detection (0.02 nmol/min per mg).
[a] Rabbit cytochrome b5 was added with the ratio P450/b5 of 1/2.
[b] Specific catalytic activities according to the supplier are expressed as pmol/min per mg of protein or turnover number (values in parentheses).

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vealed that the turnover number of the CYP2E1 enzyme for the 4-nitrophenol activity could be largely increased by adding cytochrome b5 in the molar ratio of 1:2 (for P450/b5), from 9.3 to 19.5 min^{-1} (or pmol/min per pmol P450) without and with b5, respectively. However, in two previous studies (50, 51), the catalytic properties of a number of purified cytochrome P450s suggested that several of the enzymes were capable of lauric acid (ω–1)-hydroxylation. Among these enzymes, the most important were P450 2E1 and P450 2C8/2C9/2C18. No species differences between human and rat CYP2E1 towards lauric acid hydroxylation were found. Imaoka et al. (51) reported that CYP2C enzymes were able to catalyze the acid (ω–1)-hydroxylation at rates quite similar to that of P450 2E1. Such an involvement of the P450 2C family (47, 51, 52) in lauric acid (ω–1)-hydroxylation was not borne out in rat and human liver microsomes (5, 6, 35), and this noninvolvement was also confirmed for oleic acid. The P450 1A1 and 2C11 isoforms were also shown to be active at producing the C16–C19 monohydroxylated metabolites of arachidonic acid (21) but with overall metabolic rates lower than when using CYP2E1 (a metabolic rate ratio of 1/0.2/10 for P450 1A1, 2C11 and 2E1, respectively). Cytochrome P450s of the 2C family, namely 2C2 and 2CAA (22) or 2C8 and 2C9 (53) have been described to produce regio- and stereoselective epoxidation of arachidonic acid (for 2C8 and 2C9) or C18 unsaturated acids (2C2 and 2CAA), indicating that P450s may be important in the generation of potentially active epoxide metabolites of unsaturated fatty acids other than arachidonic acid. Wang et al. (54) showed that lauric acid had the lowest K_i on NDMA-demethylase activity (a specific CYP2E1 activity) among the various fatty acids studied, ranging from caproic to palmitic acids. They proposed a molecular model in which the size of the dodecane alkyl chain allows the hydrocarbon tail to bind optimally to the CYP2E1 active site, while leaving the negatively charged carboxylic group outside of the substrate access channel, exposing it to the aqueous environment. Similar results were also reported by Fukuda et al. (55). As the alkyl chain length was increased up to 16 or 18 carbons, the enzymatic activities decreased gradually. However, the presence of the *cis* double bond in the middle of the octadecyl chain of oleic acid modifies the geometry of the hydrocarbon chain and increases the interaction between the terminal or subterminal group and the reactive oxygen bound to the iron atom of the heme residue of CYP2E1 (56).

Although it is usually not appropriate to extrapolate animal model findings to humans, it has been shown that rat and human liver CYP2E1 exhibit comparable specificities toward lauric acid and have similar mechanisms of regulation. This observation was confirmed in the present study, in which it can be asserted that at least two different P450 enzymes are involved in the microsomal metabolism of oleic acid. This fatty acid was metabolized almost similarly by both species and also in plants (31, 32).

In conclusion, the involvement of two forms of P450 in the hydroxylation of the terminal and subterminal carbon atoms of oleic acid was clearly demonstrated. CYP2E1 was the main enzyme involved in the (ω–1)-hydroxylation of oleic acid, while a member of the CYP4A family was suggested to be responsible for the ω-hydroxylation. Further studies are needed for a better understanding of the physiological role of these hydroxylated metabolites in mammals.

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