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VALIDATION OF THE (ω-1)-HYDROXYLATION OF LAURIC ACID AS AN *IN VITRO* SUBSTRATE PROBE FOR HUMAN LIVER CYP2E1

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Abstract—The (ω-1)-hydroxylation of lauric acid (11-OH-LA), a model substrate of fatty acids, was previously shown to be due to CYP2E1 in rat liver microsomes. The present study examined changes in hepatic CYP2E1 content and 11-OH-LA in a panel of 29 human liver microsomes. The 11-OH-LA activity was strongly correlated with the CYP2E1 content, quantitated by immunoblot ($r = 0.75$) and with four monooxygenase activities known to be mediated by CYP2E1: chlorzoxazone-6-hydroxylation ($r = 0.73$), 4-nitrophenol hydroxylation ($r = 0.84$), *N*-nitrosodimethylamine demethylation ($r = 0.79$) and n-butanol oxidation ($r = 0.73$). The (ω-1)-hydroxylation of lauric acid was inhibited by ethanol (K_i = 3.5 mM), acetone (IC_{50} = 10 mM), dimethylsulfoxide, chlorzoxazone (competitive inhibitors of CYP2E1), diethyldithiocarbamate, and diallylsulfide (both selective mechanism-based inactivators of CYP2E1). The weak value of ethanol K_i on the (ω-1)-hydroxylation of lauric acid suggested that low levels of alcohol could modify fatty acid metabolism in the liver. Furafylline and gestodene, suicide substrates of CYP1A and CYP3A4, respectively, did not modify the 11-hydroxylation of lauric acid. Polyclonal antibody directed against rat CYP2E1 inhibited the formation of 11-OH-LA without affecting 12-OH-LA activity. Taken together, these results suggest that CYP2E1 is involved in the (ω-1)-hydroxylation of lauric acid in human liver microsomes, and ω-hydroxylation is mediated by another enzyme. Finally, the use of yeasts and mammalian cells genetically engineered for expression of 9 human P450s demonstrated that CYP2E1 was the one enzyme involved in the (ω-1)-hydroxylation of lauric acid.

Key words: liver; human CYP2E1; (ω-1)-hydroxylation; lauric acid; cDNA expression

Microsomal P450s[‖] constitute a super-family of heme-thiolate enzymes that catalyze the monooxidation of a wide variety of lipophilic compounds such as steroids and fatty acids. This capability for metabolism of diverse substrates is due to the existence of multiple forms of P450s with different, but broad and overlapping, substrate specificities. In addition to their specific roles in biosynthetic pathways, constitutive forms of these proteins are known to be involved in the process of ω-hydroxylation of fatty acids where LA is commonly used as a model substrate. Under physiological or pathological conditions where the β-oxidation of fatty acids is impaired (starvation, diabetes, or treatment with peroxisomal proliferators), the ω-oxidation of fatty acids is increased [2, 3]. Many studies have suggested that the ω- and (ω-1)-hydroxylations of LA are mediated by different forms of P450 [4, 5]. Recently, the involvement of CYP2E1 in the (ω-1)-hydroxylation of LA was described in rat liver microsomes [6]. Moreover, recent studies have shown similar results in rabbits [7] and human liver microsomes [8]. A better understanding of the mechanisms of drug metabolism requires a precise knowledge of the catalytic activities of each isoform. A promising approach to this problem is the expression of cloning cDNA coding for P450 in an adequate heterologous system, as yeasts or mammalian cells genetically engineered for expression of human P450 2E1.

The ethanol-inducible P450 enzyme, CYP2E1, is of considerable relevance to human health, first because it is inducible by acute and chronic alcohol ingestion, diabetes, fasting, acetone, and isoniazid [9] and, second, because it is involved in the oxidation of many toxic and carcinogenic chemicals [10]. It may also be involved in alcoholic liver disease pathogenesis [11]. It is, therefore, of great interest to correlate CYP2E1 activity with the metabolism of fatty acid, such as LA in human liver microsomal preparations. Given the clinical relevance of CYP2E1 in many diseases, the aim of this study was to establish its involvement in the (ω-1)-hydroxylation of LA through a number of approaches including correlation studies in a wide panel of human liver microsomes, chemical and immunological inhibitions, kinetic experiments and cDNA expression of 9 P450 isoforms.

MATERIAL AND METHODS

Chemicals

LA was obtained from Fluka (Buchs, Switzerland) and its metabolites (11- and 12-hydroxylauric acid) were

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‖ Abbreviations: Cytochrome P450 (EC 1.14.14.1), P450 or CYP. The name cytochrome must be abandoned according to the Nomenclature Committee of the International Union of Biochemistry, the appropriate name being "heme-thiolate protein." Individual forms of P450 are designated according to the nomenclature recommended by Nelson *et al.* [1]. 11-OH-LA, 11-hydroxy-lauric acid or (ω-1)-hydroxylauric acid or 11-hydroxydodecanoic acid; 12-OH-LA, 12-hydroxylauric acid or ω-hydroxylauric acid or 12-hydroxydodecanoic acid; LA, lauric acid or dodecanoic acid; DEDTC, diethyldithiocarbamate; DMSO, dimethylsulfoxide; NADPH, nicotinamide adenine diphosphate hydrogen; DAS, diallylsulfide; NDMA, N-nitrosodimethylamine; HPLC, high performance liquid chromatography; EDTA, ethylene diamine tetracetic acid.

furnished by Dr Salaün (Strasbourg, France). [^{14}C]-lauric acid (50 mCi/mmol) was purchased from Amersham (Amersham, U.K.). DAS and DEDTC were obtained from Aldrich (St Quentin Fallavier, France). NADPH and chlorzoxazone were supplied by Sigma (St Louis, MO, U.S.A.), gestodene by Schering SA (Lys-Lez-Lannoy, France) and furafylline by Hoffmann-La Roche (Basel, Switzerland). Antibody against rat CYP2E1 was a generous gift from Dr Song (Bethesda, MD, U.S.A.). Modified cell microsomes containing human CYP2E1 and NADPH P450 reductase were obtained from Gentest Corp (Woburn, MA, U.S.A.). All chemicals and solvents were of the highest purity obtainable (Merck, Darmstadt, Germany).

Human liver samples and microsome preparation

Human liver samples (n = 29) were obtained from subjects who died following traffic accidents, in accordance with French law. Ethical committee approval was obtained prior to this study. At brain death, the liver was removed, frozen immediately, and stored in liquid nitrogen until use for preparation of microsomes. Microsomal fractions were prepared as previously described [12] and stored at −80°C until use. Their contents in terms of specific P450 and different monooxygenase activities have been previously reported [13]. Microsomal protein contents were determined using the Bradford method according to the procedure recommended by the supplier (Biorad, Munich, Germany). Total P450 concentrations were determined by Fe^{2+} difference spectrophotometry with a molecular extinction coefficient of 91 $mM^{-1}.cm^{-1}$.

Determination of monooxygenase P450 activities

Four monooxygenase activities for CYP2E1 were determined in human liver microsomes. All P450 activities were carried out at 37°C and initiated by the addition of NADPH (1 mM). Lauric acid hydroxylase was determined by HPLC as previously described [6] with a substrate concentration of 0.1 mM. Slight modification were brought for yeasts and modified cell microsomes which were incubated in the presence of [1-^{14}C]-lauric acid (specific activity: 5 mCi/mmol) for 60 min. For the determination of kinetic parameters on five samples, LA was added to the reaction mixture in the range 5–200 μM. The quantification of metabolites was performed by HPLC with simultaneous radiometric (Flo-one Beta, Packard, Meriden, CT, U.S.A.) and fluorimetric detections. Briefly, carboxylic compounds were converted into fluorescent coumarinic derivatives, separated by HPLC and detected by fluorimetry as previously described [6]. The 6-hydroxylation of chlorzoxazone was measured according to Peter *et al.* [14], with slight modifications as previously described [15]. The 4-nitrophenol hydroxylation was determined by HPLC according to methods described elsewhere [16, 17], and the NDMA demethylation and n-butanol oxidation measurements were obtained according to Lucas *et al.* [18].

Immunoblot quantification of P450 2E1, 3A, and 4A

Microsomal proteins (20 and 40 μg) were separated by sodium dodecyl sulfate-polyacrylamide (9% acrylamide) gel electrophoresis according to Laemmli [19], and then transferred electrophoretically to nitrocellulose sheets (Hybond C, Amersham International, Amersham, U.K.) according to Towbin *et al.* [20]. Nitrocellulose sheets were then blocked with PBS containing 3% (w/v) bovine serum albumin, 10% fetal calf serum (Sigma) and 0.05% Tween 20 (v/v) for 30 min at room temperature and incubated overnight at 4°C with either polyclonal anti-rat P450 2E1 antibody (Oxygene, Dallas, U.S.A.), monoclonal anti-P450 NIF [21], or polyclonal anti-rat P450 4A antibody (Amersham, U.K.). After washing with PBS, the nitrocellulose sheets were treated with PBS containing either anti-rabbit IgG conjugated to peroxidase (Dako, Versailles, France), rabbit anti-mouse immunoglobulins conjugated to peroxidase (Dako, Versailles, France), or anti-sheep IgG conjugated to peroxidase (Amersham, U.K.) for P450 2E1, 3A4, and 4A, respectively. Finally, the sheets were washed with PBS and peroxidase activities detected with 4-chloro-naphtol and H_2O_2 (for P450 2E1 and 3A4) and by ECL detection with luminol (for P450 4A). The quantification of blots was performed by densitometry, and the integrated peak area of the various microsomal preparations was expressed as arbitrary units relative to the amount of protein.

Inhibition of lauric acid hydroxylations by different chemical compounds

Incubations were performed with 0.05 mM LA. Compounds such as DMSO, chlorzoxazone, or ethanol—competitive inhibitors—were added prior to incubation at concentrations indicated in Table 3. These concentrations were chosen according to Newton *et al.* [22]. Chemical inhibitions by gestodene, DAS, furafylline, or DEDTC—mechanism-based inhibitors—were carried out with preincubation of the substrate, proteins, and NADPH. Briefly, 0.3 mg of microsomal proteins was incubated with 100 μM gestodene, 250 μM DAS, 200 μM furafylline, or 100 μM DEDTC, and 1 mM NADPH for 30 min at 37°C. The 0.1 mL incubation medium was then diluted twentyfold in 0.12 M phosphate buffer pH 7.4 containing 0.05 mM LA and 5 mM $MgCl_2$. The reaction was initiated by the addition of 1 mM NADPH. After a 10-min incubation at 37°C, the metabolites were extracted and measured by HPLC as described above. Control experiments were conducted as above with the same amounts of organic solvent (never exceeding 0.2%, v/v).

For inhibition of LA hydroxylations by acetone or ethanol, 0–100 mM acetone or 0–2 mM ethanol were added to the incubation medium containing 0.1 mM LA. Liver samples used in these experiments (Br035, Br048, FH3) were chosen on the basis of their high LA activities.

Immuno-inhibition of LA hydroxylations

103 pmol of total P450 from the human liver sample Br035 in 0.12 M phosphate buffer pH 7.4 containing 5 mM $MgCl_2$, were incubated in the absence of LA and NADPH at room temperature for 30 min and in the presence of 3.65 and 7.3 mg IgG/nmol P450 of anti-rat CYP2E1 polyclonal antibody or non-immune IgG. The reaction was started by the addition of 0.1 mM LA and 1 mM NADPH and carried out as described above.

cDNA expression of human CYP2E1

Nine P450 enzymes (1A1, 1A2, 2C8, 2C9, 2C18, 2D6, 2E1, 3A4, and 3A5) were expressed separately in yeasts using an adaptation of the procedures described

elsewhere [23, 24]. P450 coding sequences were cloned by the polymerase chain reaction either from human cDNA templates obtained by reverse transcription of mRNA or from plasmids. All these sequences, checked by DNA sequencing, were inserted in the yeast expression vector YeDP60. The expression vector was introduced into the engineered *Saccharomyces cerevisiae* strain W(R) which overexpresses yeast NADPH-P450 reductase when grown with galactose as a carbon source [23]. Culture medium S5 (containing galactose) without adenine, spheroplast preparation by enzymatic digestion of yeast cell walls, and subcellular fractionation were obtained as previously described [24].

Microsomes supplied by Gentest were obtained from a DMSO pretreated human B-lymphoblastoid cell line transfected with human CYP2E1 cDNA, which was introduced into the vector as a 1.6 kb EcoR1/Ndel fragment (Gentest Corporation, Woburn, MA, USA). This cell line expressed high CYP2E1 activity (0.109 nmol P450.mg^{-1}) because two CYP2E1-containing transcriptional cassettes were present per vector molecule.

Statistical analysis

Correlation coefficients were calculated using an ANOVA table by the least-squares regression analysis from the raw data. A normal gaussian distribution in the population was observed (skewness = 1.12 for 11-OH-LA activity). Correlation coefficients were, therefore, calculated by including all the samples and were considered to be statistically significant when P was <0.005. The results are expressed as mean ± SD.

RESULTS

Kinetic parameters

Table 1 shows the kinetic parameters of 11-OH- and 12-OH-lauric acid formation from LA by microsomes prepared from 5 human livers (Br035, Br048, Br050, Br051, and FH3). A K_m of 84 ± 45 μM and a V_m of 7.2 ± 4.1 nmol.min^{-1}.mg^{-1} of microsomal protein were determined for the 11-OH-LA formation. K_m was 13 ± 6.6 μM with a V_m of 2.1 ± 1.2 nmol.min^{-1}.mg^{-1} for the 12-hydroxylauric acid formation.

Correlations between LA hydroxylations and different monooxygenase activities in human liver microsomes

Table 2 shows the correlation coefficients between 11- and 12-OH-LA activities and different monooxygenase activities. The LA hydroxylations presented a large interindividual variation of 8.6-fold ranging from 476 to 4076 (mean ± SD = 1530 ± 840; n = 29) pmol.min^{-1}.mg^{-1} for the 11-hydroxylation reaction and of 10.9-fold ranging from 232 to 2520 (mean ± SD = 1070 ± 640; n = 29) pmol.min^{-1}.mg^{-1} for the 12-hydroxylation reaction, respectively. If two reactions are catalyzed by the same enzyme, the metabolite rates should be correlated to each other when a series of microsomal preparations containing varying levels of the enzyme are compared. Table 2 also shows that the 11-OH-LA activity measured in human liver microsomes correlated significantly with 4 catalytic activities known to be mediated by CYP2E1: 4-nitrophenol hydroxylation (r = 0.84; P < 0.001), chlorzoxazone-6-hydroxylation (r = 0.73; P < 0.001), NDMA demethylation (r = 0.79; P < 0.001) and n-butanol oxidation (r = 0.73; P < 0.001). Furthermore, lauric (ω-1)-hydroxylation activity was highly correlated with CYP2E1 immunoquantified by the Western Blot technique in 24 human hepatic microsomal preparations (r = 0.75; P < 0.001). The regression line equation, determined by the least-squares method, is y = –0.004 + 1.14x. As the y intercept of this equation is approximately zero, it is suggested that other P450s were not involved in the (ω-1)-hydroxylation of LA.

Conversely, all these monooxygenase activities did not correlate with the 12-OH-LA formation, with r values between 0.53 and 0.07.

Inhibition of LA hydroxylations by different chemical compounds

The inhibitory effect of different P450 substrates was tested on the Br048 and Br035 microsomal samples. The concentration of the LA substrate was 0.05 mM (*i.e.* approximately K_m/2). Compounds known to be metabolized by CYP2E1, namely DMSO, DEDTC, chlorzoxazone, and ethanol, inhibited the 11-hydroxylation of lauric acid by approximately 65–75% *vs* control (Table 3). On the contrary, these compounds did not modify the 12-hydroxylation of LA. DAS, a suicide substrate of CYP2E1, produced a weak inhibition (63% of residual activity) of the 11-hydroxylation of LA. Furafylline, an inhibitor of CYP1A, had no effect on the 11- and 12-hydroxylations of lauric acid. Gestodene, a 17α-acetylenic steroid known to be a mechanism-based inactivator of CYP3A4, did not affect 11-OH-LA (87% of residual activity *vs* control) or 12-OH-LA activities.

Ethanol, a prototype substrate of CYP2E1, was shown to competitively inhibit the (ω-1)-hydroxylation of LA with a K_i of 3.5 mM (i.e. 0.15 g.L^{-1}) in Br048 microso-

Table 1. Kinetic parameters of 11- and 12-lauric acid hydroxylations on 5 human liver microsomal preparations. 0.3 mg of microsomal proteins was incubated for 10 min at 37°C with concentrations of LA ranging from 5 to 200 μM.

	11-OH-LA		12-OH-LA	
	K_m (μM)	V_m (nmol/min/mg)	K_m (μM)	V_m (nmol/min/mg)
FH3	48	4.8	23.3	0.8
Br035	152	12.8	7.3	2.0
Br048	92	10.2	14	2.0
Br050	91	4.9	7.0	4.1
Br051	37	3.2	13.4	1.8
Mean ± SD	84 ± 45	7.2 ± 4.1	13 ± 6.6	2.1 ± 1.2

Table 2. Correlation coefficients (r) between ω- and (ω-1)-hydroxylations of LA and different monooxygenase activities mediated by CYP2E1 in human liver microsomal samples. Correlation coefficients were calculated by the least squares method. For $r > 0.65$, $P < 0.005$.

	Mean value* ± SD (a)	11-OH-LA	12-OH-LA
P450 2E1 content	1.65 ± 0.99 (18.8)	0.75 ($n = 24$)	0.17 ($n = 24$)
P450 4A content	2.23 ± 0.58 (3.3)	0.07 ($n = 29$)	0.03 ($n = 29$)
P450 3A4 content	2.17 ± 1.17 (14)	0.22 ($n = 27$)	0.28 ($n = 27$)
Chorzoxazone-6-hydroxylation	456 ± 352 (19.5)	0.73 ($n = 27$)	0.13 ($n = 28$)
4-nitrophenol hydroxylation	129 ± 96 (20.7)	0.84 ($n = 31$)	0.53 ($n = 29$)
N-Nitrosodimethylamine demethylation	446 ± 290 (31.8)	0.79 ($n = 21$)	0.17 ($n = 21$)
n-butanol oxidation	1110 ± 506 (6.6)	0.73 ($n = 21$)	0.07 ($n = 21$)

* Mean values are expressed as arbitrary units relative to the amount of protein for P450 2E1, 4A, and 3A4 contents, and as pmol · min^{-1} · mg^{-1} for the other monooxygenase activities. (a) values in brackets represent the ratio between the minimum and maximum of activities.

mal preparation, but did not modify the ω-hydroxylation activity of LA (Fig. 1).

Acetone, another chemical substrate of CYP2E1 known to play a key role in diabetes, was tested on LA activity at concentrations varying from 1 to 100 mM. The 12-hydroxylation of lauric acid was not modified, whereas 11-OH-LA activity was inhibited with an IC_{50} of 10 mM (Fig. 1).

Immuno-inhibition of LA hydroxylations

To confirm whether or not the CYP2E1 enzyme is involved in LA metabolism in human liver microsomes, immuno-inhibition studies with an antirat CYP2E1 polyclonal antibody were carried out. Fig. 2 shows that the antibody inhibited 11-OH-LA activity in the Br035 liver sample up to 20% and 7% of the residual activity with 3.65 and 7.3 mg IgG/nmol P450 respectively, and 12-OH-LA activity was not affected. This result further demonstrates that the (ω-1)-hydroxylation of LA was mainly due to the CYP2E1 enzyme. Other P450 antibodies, CYP1A and CYP3A, were not inhibitory (data not shown).

Metabolism of LA by yeasts genetically engineered for human CYP2E1

Figure 3 shows the HPLC profiles of LA metabolism in microsomes of yeasts genetically engineered to express human CYP2E1, compared to control yeasts and human liver microsomes. Detection of the peaks was performed using simultaneous radiometric and fluorimetric methods. The 11- and 12-hydroxylauric acid metabolites were identified on the basis of their retention times and their radiometric and fluorimetric labellings. The genetically engineered yeasts produced only the 11-hydroxylated metabolite from LA, whereas Br048 human liver microsomes produced the two hydroxylated compounds (Fig. 3).

Table 4 shows the different monooxygenase activities measured in microsomes from yeasts and human cells, both engineered to express CYP2E1. These results reveal that CYP2E1 was quantitatively expressed in transformed yeasts at a relatively high level, and control yeasts had no effect on the different substrates, suggesting that the production of the 11-OH-LA is solely due to CYP2E1 enzyme. Other human P450 enzymes, 1A1, 1A2, 2C8, 2C9, 2C18, 2D6, 3A4, and 3A5, were unable to catalyze the 11-hydroxylation of LA. These results emphasize that this catalytic activity is highly specific to CYP2E1.

DISCUSSION

Distinct isoforms of mammalian P450s are believed to catalyze the ω and (ω-1) hydroxylations of LA [4]. However, a single P450, capable of catalyzing LA hydroxylation at both the ω and (ω-1) positions, was described in rat kidney cortex microsomes [25, 26]. Porcine kidney cortex microsomes possess two P450s; one is specific for (ω-1) hydroxylation and the other hydroxylates both the ω and (ω-1) carbon atoms of fatty acids [27]. Rat liver P450 may hydroxylate fatty acids, such as LA, at the ω and (ω-1)-positions, and the ratio ω-1/ω has been observed to depend on the treatment of the rats [6]. Recently, Clarke *et al.* [8] showed that LA could be a model substrate for the simultaneous determination of both CYP2E1 and 4A in rat and human liver microsomes. These authors suggest that there are at least two enzymes capable of ω-hydroxylating lauric acid, a low K_m enzyme and a high K_m enzyme. These authors further suggest that the first could be P4504A, and the high K_m enzyme, highly correlated with cyclosporin oxidation, could be P4503A. In the present paper, the kinetic parameters of LA hydroxylation in 5 liver microsomal preparations were studied. The K_m values obtained were in agreement with previous data [8]. Considerable interindividual variation in the V_m values was observed,

Table 3. Effect of chemical inhibitors of CYP2E1 on ω- and (ω-1)-hydroxylations of LA in human liver microsomes. *competitive inhibitors; **mechanism-based inhibitors. LA 0.05 mM was incubated with 0.3 mg of microsomal proteins and 1 mM NADPH in the presence of various inhibitors at 37°C for 10 min. The metabolites were extracted, then separated by HPLC, and detected by fluorimetry.

		% of residual activity (*vs* control)	
Inhibitors	mM	11-OH-LA	12-OH-LA
DMSO*	40	45	97
	235	29	98
Chlorzoxazone*	0.2	77	102
	0.4	25	93
Ethanol*	5	44	88
	10	33	96
DAS**	0.25	63	82
DEDTC**	0.1	28	92
Furafylline**	0.2	99	100
Gestodene**	0.1	87	98

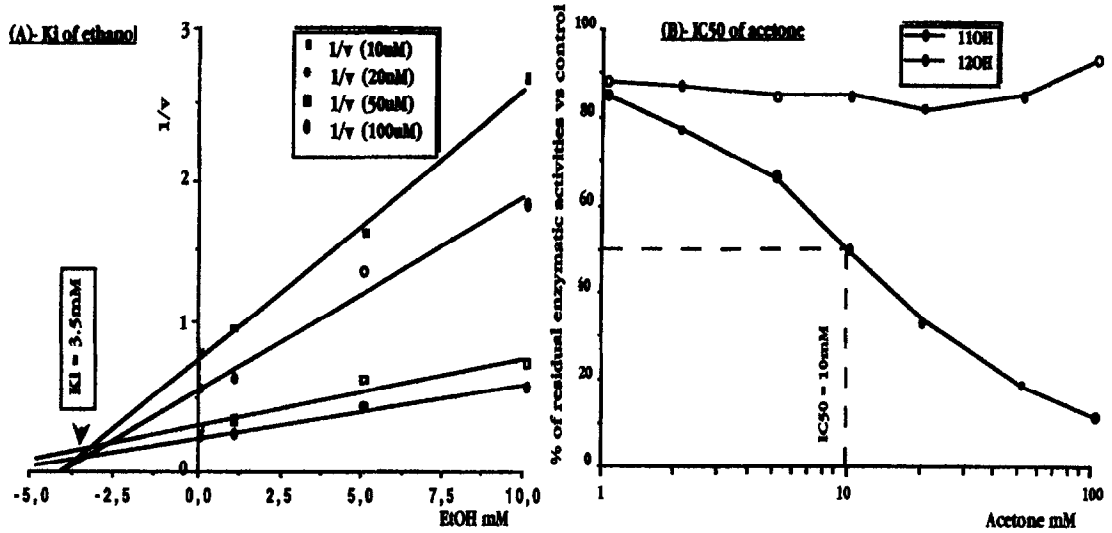

Fig. 1. Inhibition of 11-hydroxylauric acid activity by ethanol (A, Dixon plot) or by acetone (B, IC_{50}) in microsomal human liver Br048. Lauric acid was used at various concentrations (■ 10 μM; ○ 20 μM; □ 50 μM; ● 100 μM).

however, which is probably due to the heterogenous origin of the liver donors, an observation that is in agreement with those of a previous study [28].

Highly significant correlations were found between the 11-hydroxylation of lauric acid and the different CYP2E1-mediated monooxygenase activities, namely chlorzoxazone-6-hydroxylation, 4-nitrophenol hydroxylation, NDMA demethylation and n-butanol oxidation. A significant correlation was also observed between the 11-hydroxylation of LA and the CYP2E1 content. It has been suggested that NDMA demethylation is the most specific probe of CYP2E1 in rat liver microsomes for induction studies [18]. P-nitrophenol hydroxylation has also been reported to be very specific to CYP2E1 [16, 17]. In the present study, LA 11-hydroxylation is shown to be another useful and highly specific probe of CYP2E1 activity.

In human liver microsomes, LA 11-hydroxylation was inhibited by DEDTC, which is known to be a mechanism-based inhibitor of CYP2E1 [29]. The residual activity was 28% at the 0.1 mM concentration of DEDTC, which represents a more elevated inhibition than that reported elsewhere [8]. This is probably due to the choice of liver samples, which had high interindividual variations in enzymatic activities. Other specific inhibitors were used in an attempt to identify the isoforms of P450 involved in LA metabolism. 11-OH-LA was sensitive to the addition of DMSO, chlorzoxazone, ethanol,

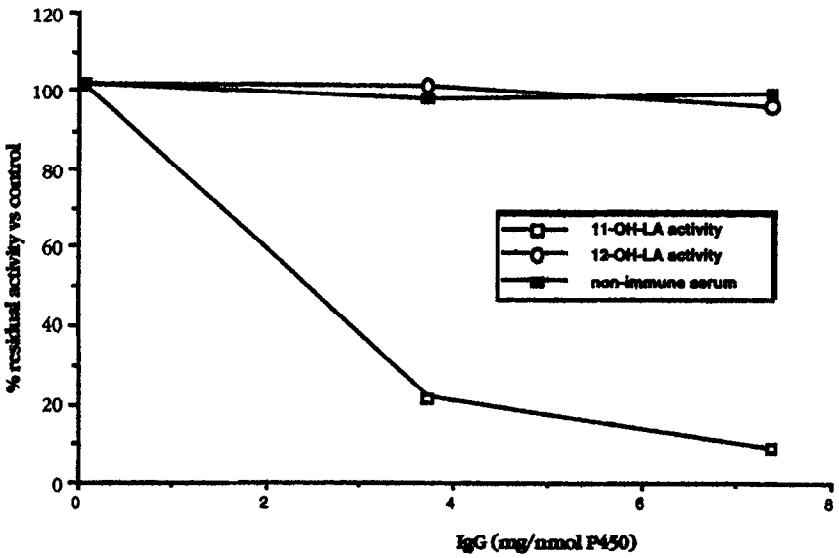

Fig. 2. Immuno-inhibition of lauric acid metabolism in microsomal preparations from Br048 human liver by polyclonal anti-P450 2E1 antibody. Liver microsomes were incubated for 30 min at room temperature in the presence of non-immune IgG (■) or increasing amounts of immune IgG antibody (3.65 and 7.3 mg), and the residual activity was calculated for the 11-OH-LA (□) and the 12-OH-LA (○).

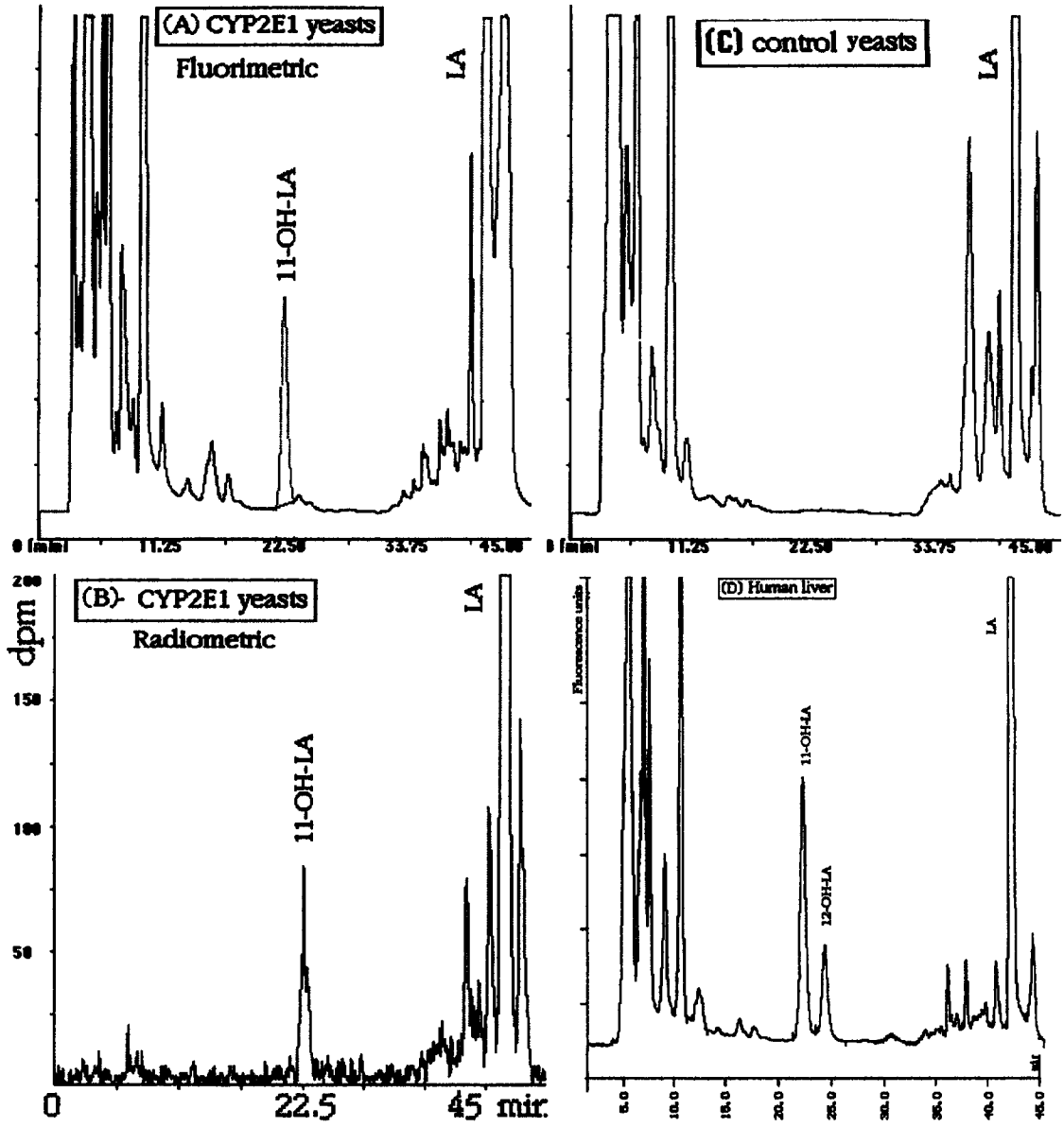

Fig. 3. HPLC profiles of metabolites of lauric acid 0.1 mM (1 μCi [^{14}C]-lauric acid) produced by microsomes (1 mg proteins incubated for 60 min) from yeasts genetically engineered for expressing human CYP2E1 with (A) fluorimetric detection and (B) radiometric detection, and control yeasts with (C) fluorimetric detection. For comparison, HPLC profile of metabolites of lauric acid 0.1 mM produced by microsomes from Br048 human liver (0.3 mg proteins). (D) Compounds were detected by fluorimetry after their conversion into fluorescent coumarinic derivatives.

and acetone, all of which are competitive substrates for CYP2E1. These results are in accordance with those found in rat liver [6] and in a transduced HepG2 cell line [30] that expressed the coding sequences of the human CYP2E1. Acetone is commonly used as an inducer of CYP2E1 and has also been observed to induce the CYP2B form [31]. The weak K_i value of ethanol (3.5 mM) could be an important factor in the role of CYP2E1 (or P450-alcohol) in some physiological disorders resulting from alcohol abuse. This concentration, equivalent to 0.15 g.L^{-1}, can easily be attained by low levels of alcohol intake. Other fatty acid substrates, however, such as palmitic, stearic, and arachidonic acid should be tested in the future with human liver microsomes to increase our knowledge of this (ω-1)-hydroxylation under physiological conditions. DAS, a major flavor ingredient of garlic, has previously been shown to inhibit chemically-induced carcinogenesis and cytotoxicity in animal model systems [32, 33]. It inhibits the activity of CYP2E1 by competitive inhibition in CYP2E1-induced rat microsomes and by decreasing the microsomal level of CYP2E1 *in vivo;* it may also induce the hepatic level of CYP2B1. In the present study, DAS, at a concentration of 250 μM, weakly decreased (ω-1)-hydroxylase. Conversely, gestodene, a 17α-acetylenic steroid known to inactivate CYP3A4 [34], did not modify the LA hydroxylations, suggesting that the P450 3A4 does not contribute to LA hydroxylations in the human liver. This result is in

Table 4. Monooxygenase P450 activities of microsomes from yeasts expressing human CYP2E1 and from modified cell microsomes containing CYP2E1, compared to human liver Br048. Values are expressed as pmol · min^{-1} · mg^{-1} and in brackets as turnover number (min^{-1}). These values are the result of a single determination. nd = not detectable.

Monooxygenase activities	Microsomes yeasts CYP2E1	Human B-lymphoblastoid cell microsomes	Human liver Br048
Chlorzoxazone-6-hydroxylation	39.6	425 (3.9)	1809
4-nitrophenol hydroxylation	362	1150 (10.5)	466
11-lauric acid hydroxylation	347	850 (7.8)	4070
12-lauric acid hydroxylation	nd	nd	2520

agreement with a previous study [8] which suggested that when LA substrate concentrations were greater than 20 μM, the 12-hydroxylation of LA was due solely to CYP4A and not to CYP3A. Furafylline, a mechanism-based inhibitor of CYP1A1 and of 1A2 in particular [35], had no effect on LA hydroxylation, a result that is in agreement with previous studies demonstrating that concentrations of furafylline less than approximately 20 μM have no effect on CYP2E1 [36, 37] or ω-hydroxylase reactions in human liver microsomes [38].

Although it is usually not appropriate to extrapolate animal model findings to humans, it has been shown that the rat and human liver CYP2E1s exhibit comparable specificities and have similar mechanisms of regulation. This is confirmed by our studies on rat and human liver microsomes where LA is observed to be metabolized similarly by both species. In the rabbit, Fukuda *et al.* [7] described the involvement of P4502C enzymes, as well as 2E1, as laurate (ω-1) hydroxylating P450s. By using sulfaphenazole, a selective inhibitor of P450 2C9, however, Clarke *et al.* [8] found no significant inhibition of (ω-1)-OH-LA in human liver microsomes. Data reported in this study, especially by using P450 isoforms produced by genetic engineering, demonstrate that any P450 of the 1A, 2C, 2D, and 3A families is able to hydroxylate the penultimate carbon atom of LA. On the whole, these results demonstrate that CYP2E1 is the predominant, if not singular, human P450 enzyme responsible for the 11-hydroxylation of LA. Furthermore, this hydroxylation reaction may be affected by low levels of ethanol, a finding that merits further research.

A better and more powerful tool in the elucidation of the P450 isoform enzymatic characteristics and their substrate specificities is the use of cDNA expression vectors. In the present study, the use of yeasts and modified cell microsomes genetically engineered to express human CYP2E1 clearly demonstrates the capacity of such a system to hydroxylate LA solely at the (ω-1) position. In conclusion, our results suggest that LA could be considered to be a highly specific probe of CYP2E1 in human liver.

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