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SHORT COMMUNICATION

Noninvolvement of CYP2E1 in the (ω-1)-hydroxylation of Fatty Acids in Rat Kidney Microsomes

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ABSTRACT. Pyrazole, acetone, and ethanol are known to induce cytochrome P450 2E1 (CYP2E1) and fatty acid (ω-1)-hydroxylation in rat liver microsomes. However, the nature of the P450 enzyme involved in this (ω-1)-hydroxylation has not been clearly established in extrahepatic tissues such as kidney. Four enzymatic activities (hydroxylations of chlorzoxazone, 4-nitrophenol, and two fatty acids) were assayed in kidney microsomal preparations of rats treated with CYP2E1 inducers. *Per os* treatment resulted in large increases (threefold to fivefold) in the chlorzoxazone and 4-nitrophenol hydroxylations, and up to a ninefold increase when ethanol was administered by inhalation. However, neither the ω-hydroxylation nor the (ω-1)-hydroxylation of fatty acids was modified. Immunoinhibition specific to CYP2E1 did not significantly decrease the ω and (ω-1)-lauric acid hydroxylations, while the polyclonal anti-CYP4A1 antibody inhibited in part both the ω- and (ω-1)-hydroxylations. Chemical inhibitions using either CYP2E1 competitive inhibitors (such as chlorzoxazone, DMSO, and ethanol) or P450 mechanism-based inhibitors (such as diethyldithiocarbamate and 17-octadecynoic acid) led to a partial inhibition of the hydroxylations. All these results suggest that fatty acid (ω-1)-hydroxylation, a highly specific probe for CYP2E1 in rat and human liver microsomes, is not mediated by CYP2E1 in rat kidney microsomes. In contrast to liver, where two different P450 enzymes are involved in fatty acid ω- and (ω-1)-hydroxylations, the same P450 enzyme, mainly a member of the CYP4A family, was involved in both hydroxylations in rat renal microsomes. BIOCHEM PHARMACOL **54**;8:947–952, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. CYP2E1; CYP4A; rat kidney; lauric acid; microsomes

Many cytochrome P450† (CYP) isoforms [1] are found in extrahepatic tissues but at lower levels than in liver, making their study difficult. Among the extrahepatic tissues, kidney constitutes the ultimate organ through which xenobiotics pass before their excretion. It has been shown that the P450 content of rat kidney is different from that of rat liver [2, 3]. Furthermore, kidney P450 2E1 (CYP2E1) has been shown to be more inducible by various xenobiotics such as ethanol, pyrazole, and acetone [4, 5] when compared with liver CYP2E1. Even if the formation of the ω-hydroxylated metabolite of lauric acid is widely acknowledged to be a marker of the CYP4A enzyme [6] both in liver and kidney microsomes, the nature of the P450 enzyme involved in the (ω-1)-hydroxylation of fatty acid in kidney microsomes has not been clearly established. Previous studies have reported that (ω-1)-hydroxylation of lauric acid is a useful and highly specific probe for CYP2E1 activity in rat [7] and human [8, 9] liver. However, this assertion was questionable for human kidney microsomes [10], as P450 2E1 is undetectable by immunoblot analysis, and CYP2E1 enzymatic activities are very weak and often close to the detection limit. Therefore, the aim of this study was to measure fatty acid hydroxylation activities in kidney microsomes of control and CYP2E1-induced rats and to compare the levels of induction obtained by using chlorzoxazone (CHZ) and 4-nitrophenol (4-NP) hydroxylations as CYP2E1 probes.

MATERIALS AND METHODS

Chemicals

Lauric and arachidonic acids were purchased from Fluka (Buchs, Switzerland), while their ω- and (ω-1)-hydroxylated metabolites were provided by Dr Salaün (Strasbourg, France). [1-^{14}C]Lauric acid (59 mCi/mmol) and [1-^{14}C]arachidonic acid (55 mCi/mmol) were obtained from Amersham (Amersham, UK). NADPH, diethyldithiocarbamate, and chlorzoxazone were supplied by Sigma (St Louis, MO, USA), 4-nitrophenol was from Fluka, and fluorescent

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† *Abbreviations:* CHZ, chlorzoxazone; CYP or P450, cytochrome P450 (EC 1.14.14.1); DEDTC, diethyldithiocarbamate; 4-NP, 4-nitrophenol; 17-ODYA, 17-octadecynoic acid; 19-OH-AA, 19-hydroxyarachidonic acid or (ω-1)-hydroxyarachidonic acid; 20-OH-AA, 20-hydroxyarachidonic acid or ω-hydroxyarachidonic acid; 11-OH-LA, 11-hydroxylauric acid or (ω-1)-hydroxylauric acid; 12-OH-LA, 12-hydroxylauric acid or ω-hydroxylauric acid.

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reagents were from Aldrich (St. Quentin Fallavier, France). 17-Octadecynoic acid was from Cayman Chemical Co. (Gif-sur-Yvette, France). Electrophoresis reagents were purchased from Bio-Rad (Richmond, CA, USA); nitrocellulose sheets and immunoblot antibodies were from Amersham. Immunoinhibition antibodies were obtained from Dr. B. J. Song (Bethesda, MD, USA) or from Gentest (Woburn, MA, USA) for anti-CYP2E1 and anti-CYP4A1 antibodies, respectively. All other chemicals and solvents were of analytical grade from Merck (Darmstad, Germany) or Sigma (St Louis, MO, USA).

Animal Treatment and Preparation of Rat Kidney Microsomes

Male Sprague Dawley rats were maintained on a water and standard diet *ad lib.* at 22 ± 2° with a light/dark cycle. Each group (five animals per group) was treated with chemical inducers as previously described [5, 7]. Kidney samples were frozen immediately after removal, and the microsomal fraction was prepared according to a previously described method [11]. The subcellular fraction was stored at −80° until use in a 100 mM phosphate buffer, pH 7.4, containing 20% glycerol (v/v). Microsomal protein content was determined using the Bradford method (Bio-Rad, Munich, Germany).

Determination of the Monooxygenase Enzymatic Activities

6-Chlorzoxazone hydroxylation was measured according to the method of Peter *et al.* [12] as previously described [13], with slight modifications. 4-Nitrophenol hydroxylation was determined by HPLC according to methods described elsewhere [14, 15]. Lauric and arachidonic acid hydroxylations were determined as previously described [16]. In some experiments, radioactive substrate (1 μCi) was added, and quantification of the metabolites was performed by simultaneous radiometric and fluorimetric detections after fluorescent derivatization of the carboxylic acids and their metabolites.

Immunoquantification of CYP2E1 and CYP4A Protein

Aliquots of microsomal samples (40 μg) were separated by electrophoresis on 9% sodium dodecyl sulfate-polyacrylamide gels [17] and then transferred electrophoretically to nitrocellulose sheets [18]. These sheets were blocked with PBS containing 3% (w/v) bovine serum albumin, 10% fetal calf serum, and 0.05% Tween 20 (v/v) for 30 min at room temperature and incubated overnight at 4° with either polyclonal anti-rat P450 2E1 or anti-rat P450 4A primary antibodies (Amersham). After washing with PBS, the nitrocellulose sheets were treated with PBS containing anti-rabbit Ig-biotinylated species-specific secondary antibodies conjugated to peroxidase and then washed with PBS. The peroxidase activities were detected by ECL detection with luminol. The quantification of the blots was performed by means of image processing scan analysis (Bioprofil, Vilbert-Lourmat, France). The integrated peak areas of the microsomal preparations were expressed as arbitrary units relative to the amount of protein.

Chemical and Immunoinhibitions of Fatty Acid Metabolism

CYP2E1 competitive inhibitors such as CHZ, DMSO, and ethanol were added prior to incubation at the concentrations indicated in Table 3. Chemical inhibitions by diethyldithiocarbamate (DEDTC) and 17-octadecynoic acid (17-ODYA), mechanism-based inhibitors, were carried out by a preincubation for 30 min at 37° as described elsewhere [9, 19]. All these inhibitor concentrations were chosen according to Newton *et al.* [20]. Incubations were then performed in control, ethanol-treated, and clofibrate-treated rats with substrate concentrations of 0.05 mM.

0.2 mg of a kidney microsomal sample from an ethanol-treated rat placed in 0.12 M phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$ was incubated in the absence of NADPH and lauric acid at room temperature for 30 min and in the presence of increasing amounts of polyclonal anti-rat CYP2E1 antibody (Dr. B. J. Song, Bethesda, MD, USA), anti-rat CYP4A1 antibody (Gentest, Woburn), or nonimmune IgG. The reaction was initiated by the addition of 0.05 mM lauric acid and 1 mM NADPH and was carried out as described above.

Statistical Analysis

The results are expressed as mean ± SD (five animals per group). Correlation coefficients were calculated using an ANOVA table by the least-squares regression analysis from the raw data.

RESULTS AND DISCUSSION

The P450 enzyme family plays a critical role in the metabolism of chemical carcinogens. In particular, CYP2E1 catalyzes the metabolic activation of many chemical compounds, thereby inducing toxicity and/or carcinogenic processes [21]. Although the liver is the organ with the highest drug-metabolizing activity, CYP2E1 is also present in other tissues such as kidney. Basal CYP2E1 levels and enzymatic activities in kidney microsomes have been found to be very low compared with liver and represent about 10–15% of those measured in liver [5, 22, 23].

Animals were treated with various inducers known to either increase the CYP2E1 enzyme (pyrazole, ethanol, and acetone) or the CYP4A enzyme (clofibrate). Both the ω- and (ω-1)-hydroxylations of lauric and arachidonic acids were compared with two monooxygenase enzymatic activities mediated by CYP2E1, i.e. chlorzoxazone 6-hydroxylation and 4-nitrophenol hydroxylation. The results (Table 1) show that CYP2E1 inducers increased CHZ and 4-NP

TABLE 1. Fold increase of CYP2E1 enzymatic activities in kidney microsomes from rats treated with various CYP2E1 inducers vs. control rats

			Lauric acid		Arachidonic acid	
	CHZ	4-NP	ω-1	ω	ω-1	ω
Pyrazole	4.72 ± 0.64	4.85 ± 0.75	1.29 ± 0.26	0.94 ± 0.24	1.80 ± 0.35	1.48 ± 0.16
Ethanol (*per os*)	3.08 ± 0.38	5.14 ± 0.84	1.04 ± 0.33	0.68 ± 0.40	1.27 ± 0.17	1.38 ± 0.27
Ethanol (inhalation)	9.21 ± 2.49	9.37 ± 2.28	1.11 ± 0.15	0.97 ± 0.03	1.55 ± 0.31	0.98 ± 0.08
Acetone	3.38 ± 0.03	4.05 ± 1.07	1.20 ± 0.06	1.0 ± 0.17	1.66 ± 0.22	1.40 ± 0.18
Clofibrate	1.87 ± 0.25	2.12 ± 1.20	1.21 ± 0.16	1.97 ± 0.25	1.20 ± 0.10	1.94 ± 0.35

Values are reported as the mean (±SD) of at least five animals/group.

The substrate concentrations were 0.4 mM, 0.2 mM, 0.1 mM, and 0.075 mM for CHZ, 4-NP, lauric acid, and arachidonic acid, respectively.

Control values were 87 ± 17, 78 ± 8, 1350 ± 240, 2950 ± 440, 100 ± 20, and 330 ± 30 pmol/min/mg for CHZ, 4-NP, 11-OH-LA, 12-OH-LA, 19-OH-AA, and 20-OH-AA, respectively.

monooxygenase activities from threefold to fivefold when compared with controls and up to ninefold when ethanol was administered by inhalation. On the contrary, the rates of laurate and arachidonate hydroxylations or the distribution of the ω- and (ω-1)-hydroxylated metabolites were not modified. Clofibrate, a CYP4A inducer, increased the ω-hydroxylation of fatty acids twofold, whereas the (ω-1)-hydroxylation was not significantly modified when the results are expressed as pmol/min/mg of microsomal protein. As clofibrate treatment led to a slight decrease (by approximately 70–80%) in kidney total P450 [24], this compound was a relatively good inducer of both ω- and (ω-1)-hydroxylase activity with results expressed as pmol/min/nmol P450 (data not shown). However, the induction observed for kidney ω-hydroxylase (twofold) was different from the previously reported liver value (15-fold) [7]. This result is in agreement with the fact that CYP4A is responsible for ω-hydroxylation in both rat liver and kidney microsomes [24–26].

Correlations between the different monooxygenase activities are shown in Table 2. The CHZ and 4-NP activities were found to be strongly correlated in kidney microsomes of control and treated rats ($r = 0.94$; $p < 0.001$), although this was not the case for the ω- and (ω-1)-hydroxylations of fatty acids.

Immunoblot analysis using anti-CYP2E1 antibody (Fig. 1A) revealed an increased CYP2E1 protein content following administration of pyrazole, acetone, ethanol per os or by inhalation (fold increases of approximately 3.2, 2.6, 2.1, and 4.3 relative to control, respectively). Comparison of the two routes of ethanol administration showed that inhalation produced a twofold greater induction than ethanol per os, results which were in agreement with those of Zerilli *et al.* [5]. A difference in the electrophoretic mobilities of rat and human CYP2E1 could be observed. This is in agreement with a previous study [27] in which the authors described that rat CYP2E1 moved faster than human CYP2E1 in polyacrylamide gels. Immunoblot analysis with anti-CYP4A antibody (Fig. 1B) showed an increase in CYP4A protein only after clofibrate treatment, a result which was also in agreement with previous results [25, 26]. The anti-rat CYP4A antibody used in this experiment does not cross-react with other P450 families, as indicated by the supplier; however, it was shown to recognize all the P450 4A enzymes.

Table 3 presents the effects of various compounds known to be metabolized by CYP2E1 (CHZ, DMSO, ethanol, and DEDTC) on lauric acid metabolism in kidney microsomes of control, ethanol-treated, and clofibrate-treated rats. These four inhibitors did not significantly modify the ω-hydroxylation activity, while the (ω-1)-hydroxylation was only slightly inhibited by DEDTC and ethanol. Moreover, this inhibition was more effective in ethanol-treated than in control rats. The effects of 17-ODYA (at 0.1 and 0.2 mM) on the metabolism of lauric acid by renal microsomes are presented in Table 3. Preincubation of microsomes with 17-ODYA in the presence of NADPH significantly inhibited the formation of ω- and (ω-1)-hydroxylated metabolites, and this inhibition was slightly dose-dependent. 17-ODYA has been described to be a potent inhibitor of the cytochrome P450-dependent metabolism of arachidonic acid. It inhibits both the ω-hydroxylase and epoxygenase pathways in microsomes of rat kidney [19], rabbit lung [28], and rat liver [29, 30]. When compared with other chemical compounds that have been used to inhibit cytochrome P450 activity such as

TABLE 2. Correlation coefficients between ω- and (ω-1)-hydroxylations of fatty acids and two CYP2E1 monooxygenase enzymatic activities in kidney microsomes of control and CYP2E1-induced rats

		Lauric acid		Arachidonic acid	
	CHZ	ω-1	ω	ω-1	ω
CHZ	—	0.16 ($n = 27$)	0.25 ($n = 27$)	0.39 ($n = 26$)	0.31 ($n = 26$)
4-NP	0.94 ($n = 25$)	0.15 ($n = 25$)	0.19 ($n = 25$)	0.27 ($n = 25$)	0.24 ($n = 25$)

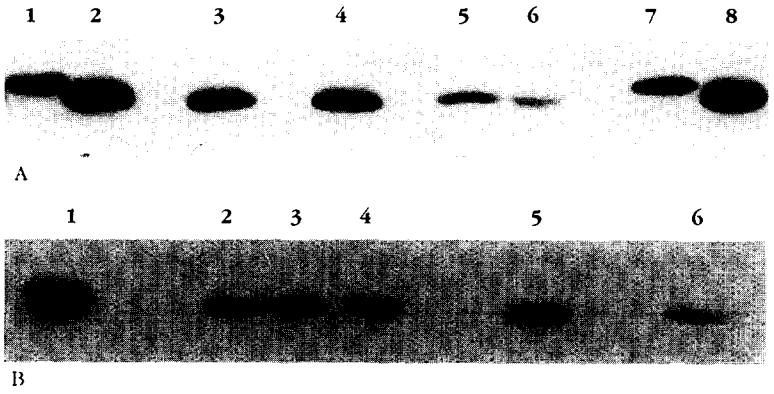

FIG. 1. A, immunoblot analysis of CYP2E1 content in kidney microsomal samples (40 μg of protein per lane) from rats treated with ethanol by inhalation (lane 2), acetone (lane 3), ethanol *per os* (lane 4), clofibrate (lane 6), pyrazole (lane 8), or from untreated rats (lane 5). Lanes 1 and 7 contain control human CYP2E1 (40 μg). B, immunoblot analysis of CYP4A content in kidney microsomal samples (40 μg of protein per lane) from rats treated with clofibrate (lane 1), pyrazole (lane 2), acetone (lane 3), ethanol *per os* (lane 4), ethanol by inhalation (lane 5), or from untreated rats (lane 6).

7-ethoxyresorufin, miconazole, and ketoconazole [19], 17-ODYA was found to be more potent by at least one order of magnitude.

Immunoinhibition of lauric acid metabolism using a polyclonal anti-rat CYP2E1 antibody was also performed in ethanol-treated rats (Fig. 2A). At a concentration of 1.25 and 2.5 mg/mg microsomal protein, this antibody inhibited the CHZ activity (specific to CYP2E1 activity) by up to 50%, while it did not affect the ω- and (ω-1)-hydroxylations of lauric acid.

It was previously shown that an anti-P450 2E1 IgG was able to block 4-NP catalytic activity [4], confirming the role of the CYP2E1 enzyme in the reaction. Interestingly, our results using a polyclonal anti-rat CYP2E1 antibody did not affect laurate (ω-1)-hydroxylation, confirming that CYP2E1 is not responsible for the (ω-1)-hydroxylation of fatty acids in rat kidney microsomes. It is important to note that although CHZ is a specific probe for CYP2E1 in human liver, this is not the case in rat liver, where both CYP2E1 and CYP1A1 are involved in the metabolism of CHZ [13]. Recently, it was found that P450 3A1/3A2 are also involved [31]. The role of CYP1A1 in rat kidney could explain why the anti-rat CYP2E1 antibody did not entirely inhibit the CHZ activity. Using an anti-CYP4A1 antibody (Fig. 2B), a significant inhibition of both ω- and (ω-1)-hydroxylations of lauric acid could be observed in microsomes of ethanol-treated rats. The same inhibition has been observed in control and clofibrate-treated rats, while non-immune serum had no effect (data not shown). These results could be compared with a previous study [32], in which the authors observed a complete inhibition of both ω- and (ω-1)-hydroxylations of lauric acid by the addition of anti-P450 4A2 antibody, indicating that P450 4A2, or a closely related form, mainly contributed to lauric acid hydroxylation in rat kidney. A relative lack of inhibition of kidney lauric acid ω- and (ω-1)-hydroxylases by the antibody to liver CYP4A1 has also been observed [24], with 60 and 20% inhibition in liver and kidney microsomes, respectively.

In conclusion, rat kidney microsomes may hydroxylate fatty acids both at the ω and (ω-1) positions. The use of CYP2E1 inducers led to an increase in CYP2E1 protein as well as enzymatic activities (CHZ and 4-NP), but no significant difference was found when fatty acid hydroxylations were examined. These data therefore demonstrate that fatty acid (ω-1)-hydroxylation cannot constitute a specific marker of CYP2E1 activity in rat kidney microsomes, while CHZ and 4-NP hydroxylations remain specific CYP2E1 probes. The results obtained when using chemical and immunoinhibitors led to the assertion that both ω- and (ω-1)-hydroxylations of lauric acid are mediated by the same P450 enzyme in rat kidney microsomes, whereas two

TABLE 3. Chemical inhibitions of (ω-1)- and ω-hydroxylations of lauric acid (0.05 mM) using different chemical CYP2E1 inhibitors

		% of residual activity					
		Control rat		Ethanol rat		Clofibrate rat	
		ω-1	ω	ω-1	ω	ω-1	ω
CHZ	0.4 mM	92.2	93.6	85.5	101.2	95.4	96.7
DMSO	35 mM	92.2	95.1	74	88.9	90.5	100.8
Ethanol	5 mM	100.5	97.9	77.1	86.3	91.5	90.7
Ethanol	10 mM	75.4	79.8	67.6	79	72.6	82.2
DEDTC	0.1 mM	69.3	86.3	62.5	83.6	60.8	87.8
17-ODYA	0.1 mM	70.4	74.5	85.7	81.3	68	71.1
17-ODYA	0.2 mM	68.4	72	54.9	70.1	62.6	60.5

Control values for (ω-1)- and ω-hydroxylations were 1.30 and 2.85, 1.47 and 3.06, and 1.56 and 5.7 nmol/min/mg for control, ethanol-treated, and clofibrate-treated rats, respectively.

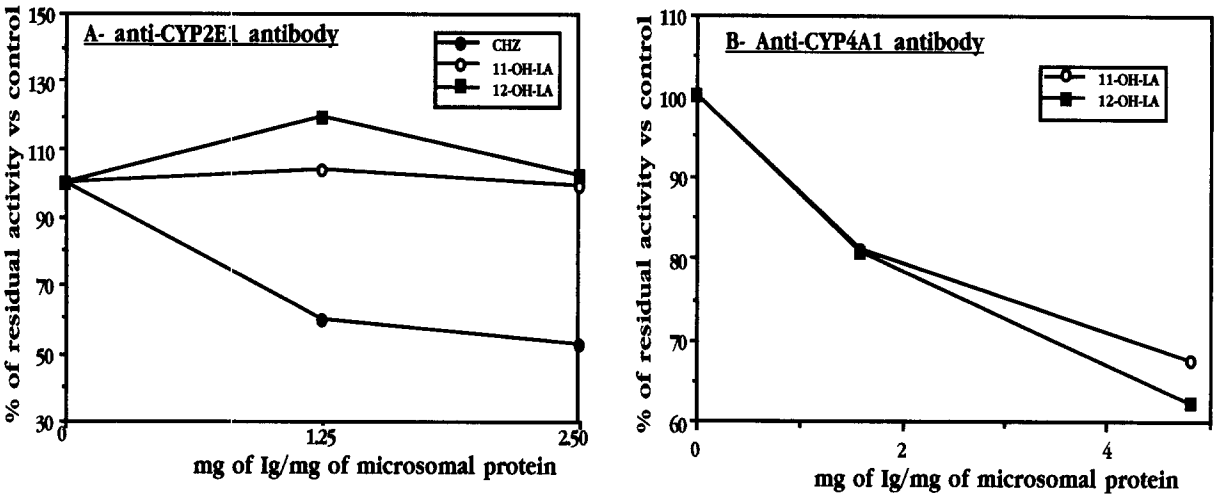

FIG. 2. Effect of polyclonal anti-rat CYP2E1 (A) and CYP4A1 (B) antibodies on lauric acid (0.05 mM) hydroxylations in kidney microsomes from ethanol-treated rats. Results are expressed as percent of residual enzymatic activity vs. control assay without antibody. Control values were 1.47 and 3.06 nmol/min/mg for 11-OH-LA and 12-OH-LA, respectively.

different P450 isoforms are responsible for these hydroxylations in rat and human liver. This rat kidney enzyme can be asserted to be a P450 4A isoenzyme.

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