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Lauric acid hydroxylase activity and cytochrome P450 IV family proteins in human liver microsomes

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Cytochrome P450 enzymes are important in the metabolism of many endogenous substrates, as well as a large variety of drugs, chemical carcinogens and environmental pollutants. Ten families of cytochrome P450 enzymes have been identified so far in mammals [1]. Six of these are small families of one or two members and are involved in pathways of steroidogenesis or bile acid synthesis in highly specialized tissues [2]. One family, cytochrome P450 IV, consists of enzymes that are involved in hydroxylating fatty acids [2]. The remaining three families encode xenobiotic metabolizing enzymes. In these latter four families, it appears that a tremendous variability exists between species in the number of P450 genes and the substrate specificities of individual P450 forms [2]. It is therefore difficult to extrapolate toxicology and carcinogenicity studies from rodents to man. These species differences emphasize the necessity to study human cytochrome P450 enzymes.

One of the most active cytochrome P450 IV fatty acid-metabolizing enzymes is P450 IVA1, formerly termed cytochrome P452 [3–5]. The ω-hydroxylase activity towards lauric acid is suggested to be an indicator of cytochrome P450 IV activity [2, 3, 5]. After treatment of rats with hypolipidaemic, peroxisome proliferating compounds like clofibrate, nafenopin or di(2-ethylhexyl)phthalate (DEHP) both the ω-hydroxylase activity towards lauric acid, and cytochrome P450 IVA1 protein content are substantially induced in liver and kidney, whereas the (ω-1)-hydroxylase activity is much less induced [5–8].

Since the hypolipidaemic, peroxisome proliferating compounds are classified as epigenetic hepatocarcinogens [9, 10], attention has been focussed to elucidate the possible mechanism(s) of carcinogenesis. It has been suggested that the carcinogenicity might result from an increased hydrogen peroxide production generated via the peroxisomal fatty acid β-oxidation system in the liver [10]. Sharma *et al.* [6] proposed that the enhanced peroxisomal β-oxidation is a result of the perturbation of lipid metabolism, which in turn might be the result of an enhanced ω-hydroxylation of fatty acids by cytochrome P450 IVA1.

Species differences in the induction of peroxisome proliferation have been reported. Non-rodent species are reported to be less sensitive to peroxisome proliferators than rodents [11–13]. Since peroxisome proliferation is causally linked to hepatocarcinogenicity there is considerable debate about the significance of peroxisome proliferators as hepatocarcinogens in humans. More information regarding the cause of the interspecies differences of peroxisome proliferation is needed.

It seems possible that species differences in peroxisome proliferation are the result of differences in the presence or inducibility of cytochrome P450 IVA1 [11–13]. In this paper we have studied the presence of cytochrome P450 IV family enzymes in hepatic microsomes derived from human samples, as a preliminary step in determining the potential human risk posed by peroxisome proliferators.

Materials and Methods

Chemicals and reagents. Nafenopin was a gift of Ciba Geigy (Basle, Switzerland). Lauric acid, ω-hydroxylauric acid and NADPH were obtained from the Sigma Chemical

Co. (St Louis, MO, U.S.A.). 18-Crown-6 ether and 4-(bromomethyl)-6,7-dimethoxycoumarin were obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). DEHP was purchased from Janssen Chimica (Beerse, Belgium).

Antibodies against a highly purified, electrophoretically homogenous preparation of rat hepatic cytochrome P450 IVA1 were raised in sheep and the specificity of the antibodies is described elsewhere [6, 7]. All other chemicals were of the highest purity obtainable.

Liver samples. Human liver samples were obtained from kidney transplant donors (N = 13). The investigations were approved by the local ethical committee on human experimentation.

Rat liver samples were from male random-bred Wistar rats (Cpb:WU (SPF), body wt 150–200 g). Rats were pretreated by gastric intubation once daily for 3 days with either di(2-ethylhexyl)phthalate (500 mg/kg body wt), nafenopin (100 mg/kg body wt), olive oil (5 mL/kg body wt) or saline. Twenty-four hours after the last dose rats were killed by decapitation. Livers were perfused with 0.9% (w/w) NaCl for 10 min.

Whole liver homogenates were prepared in 0.25 M sucrose, 2 mM EDTA and 10 mM Tris–HCl (pH 7.4). Homogenate was centrifuged at 10,000 *g* for 20 min. Microsomal fractions were prepared from the 10,000 *g* supernatant by centrifugation at 105,000 *g* for 60 min.

Assays. The ω- and (ω-1)-hydroxy metabolites of lauric acid were separated by reverse phase HPLC after derivatization with the fluorescent compound 4-(bromomethyl)-6,7-dimethoxy-coumarin, as described by Dirven *et al.* [8].

Total cytochrome P450 content was determined by the method of Omura and Sato [14].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting with a polyclonal antibody against rat hepatic cytochrome P450 IVA1 was performed according to Peters and Jansen [15]. A semi-quantitative estimate of the intensity of the cytochrome P450 IV bands on the immunoblot was made using a laser densitometer (Ultrascan XL, LKB, Bromma, Sweden).

Protein concentrations were determined by the method of Bradford [16].

Results and Discussion

Table 1 gives the lauric ω- and (ω-1)-hydroxylase activities in microsomal preparations derived from either human liver or saline-treated rat liver. The activities determined in human liver are 2–4 times higher than those measured in rat liver. Also the ratios between ω- and (ω-1)-hydroxylase activities are different in rat as compared to man: in man, the ω-hydroxylase activity is twice as large as the (ω-1)-hydroxylase activity, whereas in rat both activities are comparable.

Immunoblot analysis of human and rat microsomal fractions was performed using a sheep antibody raised against purified rat hepatic cytochrome P450 IVA1. Figure 1 shows that this antibody reacts with two protein bands (molecular weights 51.5 and 52 kDa) for olive oil-, DEHP- or nafenopin-treated rat liver microsomes. This pattern is identical as described by Makowska *et al.* [7] and Hardwick *et al.* [4]. Both bands are induced by the treatment with either DEHP or nafenopin. The lower band (51.5 kDa) co-migrates with rat hepatic cytochrome P450 IVA1. The additional band of approximately 52 kDa might be another member of the cytochrome P450 IV subfamily [7] like cytochrome P450 IVA2 or P450 IVA3 [17], but this has to be verified.

In all human liver samples a strong reacting and a weak reacting band was detected. The strong reacting band has a slightly lower molecular weight (approximately 51 kDa) than rat hepatic cytochrome P450 IVA1. The weak reacting band has a molecular weight of approximately 56.5 kDa. These findings suggest the existence of proteins in human liver that are immunochemically related to rat hepatic cytochrome P450 IVA1.

A correlation was found beween the staining intensities of the 51.5 kDa band and the rate of lauric acid ω-

Table 1. ω- and (ω-1)-hydroxylase activities towards lauric acid in human and rat liver microsomes

Patients No.	Sex	Age	Total cytochrome P450 (nmol/mg protein)	Hydroxylase activity (nmol product/min. nmol total cytochrome P450)		Intensity of 51 kDa band (absorbance units/pmol total cyt P450)
				(ω-1)-Hydroxylauric acid	ω-Hydroxylauric acid	
1	F	30	0.24	1.83	2.53	ND
2	F	18	0.78	1.15	1.85	0.15
3	—	—	0.51	0.70	2.14	0.15
4	M	27	0.26	1.24	2.87	0.13
5	M	15	0.33	1.55	1.82	0.10
6	M	20	0.34	0.33	2.39	0.16
7	M	46	0.84	1.38	2.16	0.07
8	—	—	0.27	1.89	4.08	0.08
9	M	18	0.39	2.25	4.99	0.06
10	—	—	0.50	1.15	2.8	0.06
11	—	—	0.42	2.32	3.79	0.12
12	M	47	0.20	3.29	4.5	ND
13	F	18	0.26	2.25	4.57	0.08
Mean ± SD			0.41 ± 0.20	1.64 ± 0.78	3.11 ± 1.12	
Rats						
Mean ± SD*			0.74 ± 0.16	0.66 ± 0.11	0.74 ± 0.49	

ND, not determined.
—, Sex and age of these donors are not known.
* Mean ± SD of five rats treated with saline.

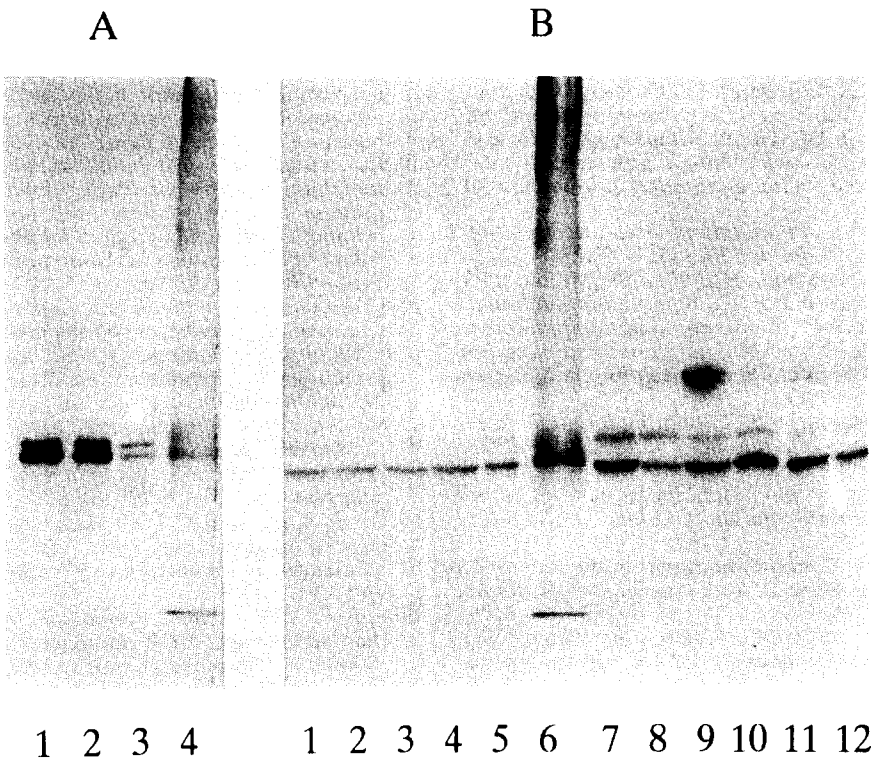

Fig. 1. Immunodectection of hepatic cytochrome P450 IV proteins in rat and human liver microsomes. Microsomal fractions were subjected to SDS–polyacrylamide gel electrophoresis (7% acrylamide) and immunoblotted with an antibody to rat hepatic cytochrome P450 IVA1. (A) Track 1, nafenopin-treated rat liver microsomes [2 pmol]*; track 2, DEHP-treated rat liver microsomes [2 pmol]†; track 3, olive oil-treated rat liver microsomes [5 pmol]‡; track 4, authentic rat hepatic cytochrome P450 IVA1 standard [6 pmol]. (B) Tracks 1–5, human liver microsomes corresponding with patient Nos. 13, 10, 9, 8 and 7, respectively [5 pmol]; track 6, authentic rat hepatic cytochrome P450 IVA1 [6 pmol]; track 7–12, human liver microsomes corresponding with patient Nos. 6, 5, 4, 3, 2 and 11, respectively [5 pmol]. Amounts of microsomes loaded onto the gel are expressed as pmol total cytochrome P450, and is given in brackets. *Lauric acid ω-hydroxylase activity is 8.35 nmol/min. nmol total cytochrome P450, and intensity of 51.5 KDa band is 0.55 absorbance units/pmol. †Lauric acid ω-hydroxylase activity is 4.07 nmol/min. nmol total cytochrome P450, and intensity of 51.5 KDa band is 0.32 absorbance units/pmol. ‡Lauric acid ω-hydroxylase activity is 0.70 nmol/min. nmol total cytochrome P450, and intensity of 51.5 KDa band is 0.05 absorbance units/pmol.

hydroxylation for the treated and untreated rat samples (see text of Fig. 1). However, an observable relation was not found between the staining intensities of the 51 kDa band and the rate of lauric acid ω-hydroxylation for the human samples, suggesting that the 51 kDa protein has hardly any lauric acid ω-hydroxylase activity.

Apart from cytochrome P450 IVA1 other cytochrome P450 IV family enzymes may have lauric acid ω-hydroxylase activity [2, 5], and it is possible that these enzymes catalyse the pronounced lauric acid hydroxylation activities, as determined in the human liver samples. In addition it should be notified, when analysing human cytochrome P450 IV enzymes by using an antibody raised to rat hepatic cytochrome P450 IVA1, the human cytochrome P450 IV levels may be underestimated due to lack of specificity of the antibody for the human cytochrome P450 IV proteins.

We conclude that in human liver cytochrome P450 IV proteins are present, which are related to rat hepatic cytochrome P450 IVA1, but have different physicochemical properties.

As a consequence, the reported species differences in peroxisome proliferation between rodents and primates [11–13] might be due to differences in the inducibility of these proteins.

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The antihypertensive compounds hydralazine, dihydralazine and cadralazine and their metabolites inhibit myeloperoxidase activity as measured by chemiluminescence

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Antineutrophil cytoplasma antibodies (ANCA) now called C-ANCA were described in 1985 [1]. They have become a marker of systemic vasculitis, especially Wegener's granulomatosis and are directed against the elastinolytic enzyme, Proteinase 3.

Antibodies against the lysosomal enzyme myeloperoxidase (MPO) were recently reported in patients with idiopathic necrotizing and cresentic glomerulonephritis [2]. We have found antimyeloperoxidase antibodies (anti-MPO) in patients with genuine systemic lupus erythematosus (SLE) [3]. We have also recorded circulating anti-MPO in the hydralazine-induced SLE-like syndrome [3], and in isolated hydralazine-induced kidney damage [4].

Hydralazine (1-hydrazinophthalazine) is an antihypertensive agent, which has been in clinical use since 1950. Cadralazine, (2,3-[6-(2-hydroxypropyl)ethylamino]-pyridazinyl)ethylcarbazate (ISF 2469) is structurally related to hydralazine and with the same pharmacological principles [5, 6]. Myeloperoxidase is a heme enzyme localized in azurophil granula in neutrophil granulocytes and monocytes [7]. Its main function is the killing of microorganisms taken up via phagocytosis. Since a connection between hydralazine-induced autoimmunity and circulating anti-MPO has been found [3], it was of interest to investigate the interaction between MPO and hydralazine and its relevant metabolites, and their influence upon enzymatic