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Human liver microsomal cytochrome P-450IIE1. Immunological evaluation of its contribution to microsomal ethanol oxidation, carbon tetrachloride reduction and NADPH oxidase activity

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Cytochrome P-450 constitutes a family of hemoproteins acting as monooxygenases with different but partially overlapping substrate specificities. Several types of rat and rabbit liver forms of cytochrome P-450 have been thoroughly characterized, whereas only a limited number of human liver cytochromes P-450 have been described. These include cytochromes P-450 participating in microsomal bufuralol hydroxylation [1], testosterone 6β-hydroxylation [2] and nifedipine oxidation [3]. Immunological evidence has been given for the existence of 3-methylcholanthrene-inducible P-450 in human liver [4], and a human form of cytochrome P-450 inducible by dexamethasone and macrolide antibiotics, that resembles pregnenolone-16α-carbonitrile-inducible P-450p in rat liver, has been described [5].

Ethanol-inducible cytochrome P-450 (P-450IIE1) was first purified from rabbit liver microsomes [6, 7] and later from rat liver [8]. In the present paper, we have used anti-IgG raised against the ethanol-inducible form of P-450 from either rabbit liver (P-450 LMeb)* or rat liver (P-450j), in order to be able to identify the human form of the enzyme. The inhibitory properties of the antibodies allowed examination of the role of the human ethanol-inducible cytochrome P-450 in human microsomal metabolism of, for example, ethanol and carbon tetrachloride.

Materials and methods

NADPH was obtained from Sigma (St. Louis, MO), EDTA from Fluka (Buchs, Switzerland) and protein A peroxidase from Bio-Rad (Richmond, CA). Human adult liver tissue was obtained shortly after circulatory arrest from cadaveric renal transplant donors [9] (see Table 1). Liver microsomes were prepared in the absence of EDTA as described in [10].

SDS-polyacrylamide gel electrophoresis was performed on slab gels containing 8.5% acrylamide using the discontinuous system according to Laemmli [11]. After electrophoresis, the proteins were transferred to a nitrocellulose filter (Bio-Rad) in a buffer containing SDS and methanol [12]. The ethanol-inducible P-450 was detected by using anti-P-450j IgG, protein A peroxidase and 4-chloro 1-naphtol [12].

Antibodies against P-450j and P-450 LMeb were raised in rabbit [13] and sheep [14], respectively. The IgG fraction was prepared from the antisera by ammonium sulfate precipitation [15]. Preimmune IgG was prepared in an identical manner.

Protein was determined by the method of Lowry *et al.* [16] and cytochrome P-450-content was analyzed according to Omura and Sato [17]. NADPH-oxidase activity was determined spectrophotometrically at 340 nm at 37°. Microsomal NADPH-dependent oxidation of ethanol was analyzed in the presence of 0.5 mM sodium azide as previously described [18]. CCl_4-dependent lipid peroxidation was determined as the formation of TBA-reactive substances using incubations with microsomes corresponding to 0.5 mg of protein, 2.15 mM CCl_4 and 10 μM EDTA [19]. Immunoquantification of ethanol-inducible cytochrome P-450 from human liver was performed by radial immunodiffusion [14] using anti P-450 LMeb-IgG (0.3 mg/ml gel). Rabbit liver P-450 LMeb was used as standard. The data presented in this paper constitute mean values of 2–3 different experiments performed in duplicate. Linear regression analyses were performed with the least-square method, and P values less than 0.05 were considered significant.

Results and discussion

Microsomes from rat, rabbit and human liver were analyzed by Western blot using anti-P-450j IgG (Fig. 1). Only one single protein band was recognized in each species. The immunoreactive band from human liver microsomes migrated according to an apparent molecular weight of $M_r = 54{,}000$. This is identical with that of the purified human homologue of P-450j (P-450Hj) as determined electrophoretically and recently presented by Wrighton *et al.* [20] and Lasker *et al.* [21].

Immunoquantification of cytochrome P-450Hj in liver microsomal samples from 23 different individuals revealed a striking interindividual difference in the microsomal content of this enzyme (Table 1 and Fig. 2). The relative amount, when expressed per nmol of total P-450 in the microsomes, differed by more than 50-fold between the specimens. In seven of the samples, apparently no detectable P-450Hj was present, whereas in liver microsomes prepared from a known alcoholic, the relative content of P-450Hj was 3-fold higher than in any of the other individuals (cf. Table 1).

Cytochrome P-450 LMeb and P-450j are characterized by a high rate of NADPH consumption in the absence of added substrate [22, †]. When the rate of microsomal NADPH oxidation in the different microsomal samples was plotted against the level of P-450Hj in the membranes, a correlation coefficient of 0.79 ($P < 0.001$) was reached (Fig. 2A). A similar extent of correlation ($r = 0.77$, $P < 0.001$) was obtained when the rate of microsomal NADPH-dependent ethanol-oxidation was plotted against the content of P-450Hj in the microsomes (Fig. 2B). The highest activities were observed in microsomal samples from the known alcoholic, where 14 nmol of ethanol was oxidized and 57 nmol NADPH consumed, per nmol P-450 and minute. Indeed, a correlation ($r = 0.85$, $P < 0.001$) was observed between all three variables, indicating that P-450Hj is responsible for a major part of these activities (Fig. 2C). By contrast, when the total amount of cytochrome P-450 in the microsomes was plotted against the rate of microsomal ethanol oxidation or NADPH-oxidase activity, no significant correlations were obtained ($r = 0.43$) and ($r = 0.31$), respectively (not shown). However, it cannot be excluded that additional forms of cytochrome P-450 might contribute to microsomal ethanol oxidation, as is also the case with rat liver microsomes [13], preferentially in liver species where this form of P-450 appears to be absent (cf. Fig. 2).

* Abbreviations used: P-450j, ethanol-inducible cytochrome P-450 from rat liver; P-450 LMeb, ethanol and benzene-inducible cytochrome P-450 from rabbit liver; P-450Hj, ethanol-inducible cytochrome P-450 from human liver; CCl_4, carbon tetrachloride, TBA, thiobarbituric acid.

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Table 1. Characteristics of specimens used in the present study

Sample no.	Sex	Age	Known alcohol, cigarette or drug intake	Liver microsomes nmol P-450 per mg	Liver microsomes pmol P-450Hj per mg
HL 16	M	51	alcohol, oxazepam propranolol, promethazine propiomazin, melperon, orphenadine	0.22	77
HL 18	F	59	smoker	0.54	<5
HL 19	M	13	—	0.36	23
HL 20	F	56	barbiturates	0.93	39
HL 21	M	65	smoker, glucocorticoids phenytoin	0.36	28
HL 24	F	44	—	0.44	<5
HL 25	F	52	diazepam, promethazine	0.35	<5
HL 28	F	44	—	0.64	28
HL 29	F	62	smoker	0.68	<5
HL 31	F	61	smoker	0.62	12
HL 32	F	65	—	0.28	34
HL 33	F	25	—	0.31	28
HL 34	F	62	—	0.85	42
HL 35	M	56	hydrochlorthiazide, hydralizine, glucocorticoids	0.31	<5
HL 36	M	21	—	0.59	12
HL 37	F	59	—	0.40	9
HL 38	F	38	—	0.28	31
HL 39	F	62	—	0.45	34
HL 41	M	34	pentobarbital	0.16	9
HL 42	M	31	phenytoin	0.40	<5
HL 43	M	41	diazepam	0.18	<5
HL 44	F	49	—	0.32	35
HL 45	M	46	pentobarbital	0.61	34

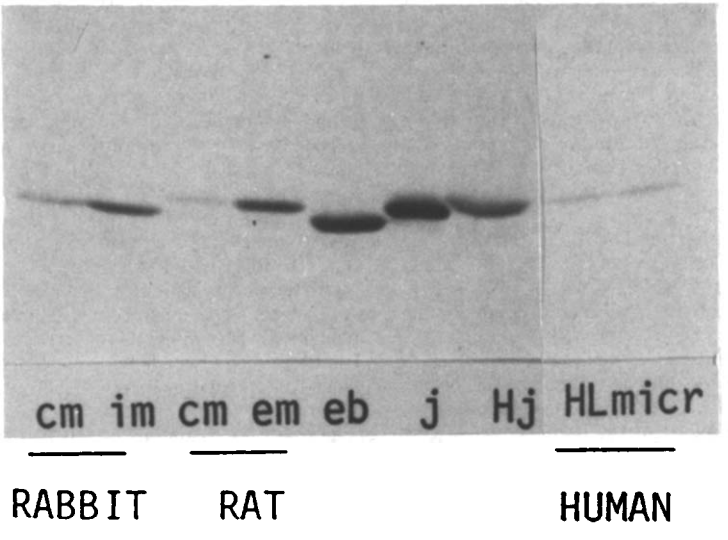

Fig. 1. Western blot analysis of liver microsomes from rabbit, rat and human and the related purified enzymes. The lanes contained microsomes corresponding to 15 μg protein (control (cm) and imidazole-treated (im) rabbit), 7 μg (control (cm) and ethanol-treated (em) rat) and 30 μg (human, (HLmicr)), and purified P-450 LMeb (10 pmol), P-450j (1 pmol) and partially purified P-450Hj (24 pmol). The proteins were immunoblotted as described under Materials and Methods with anti-P-450j IgG.

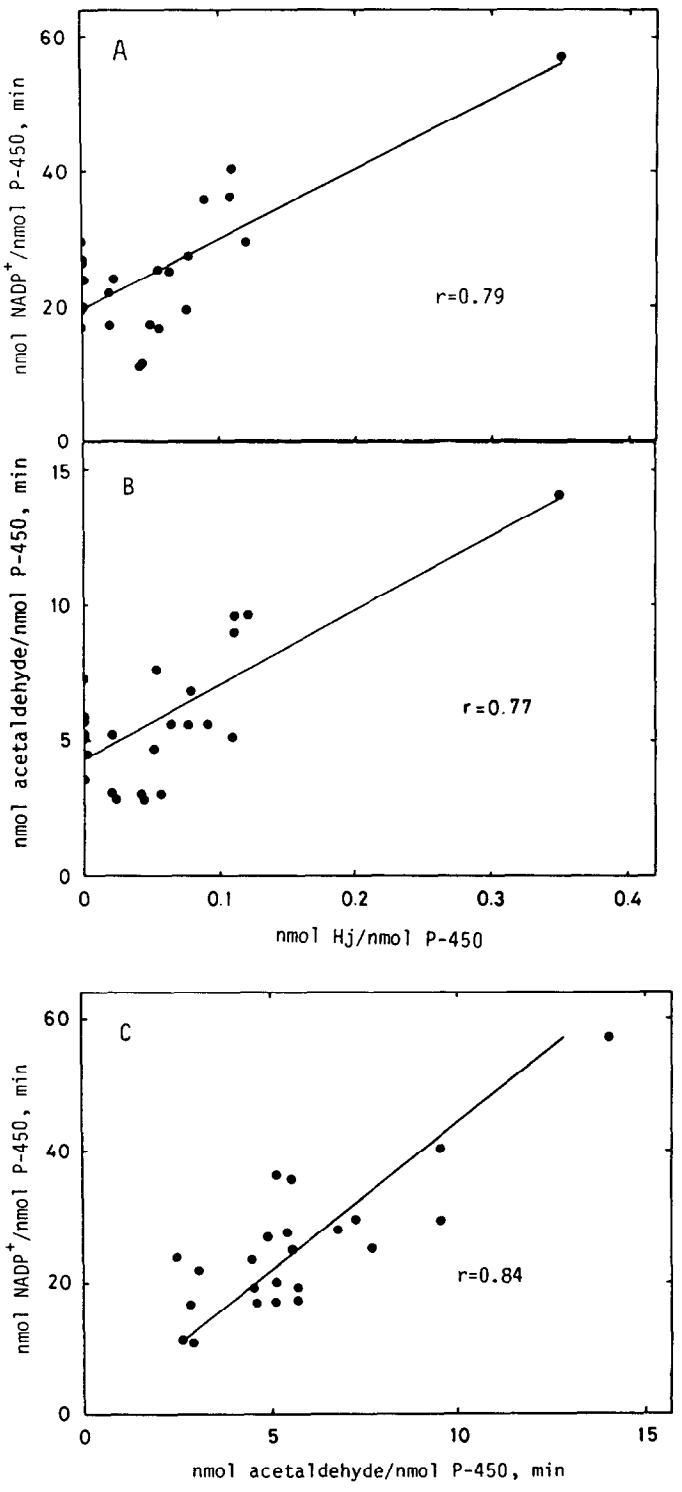

Fig. 2. Rate of NADPH-oxidase activity in human liver microsomes as a function of the content of immunodetectable P-450Hj (A), and rate of NADPH-dependent oxidation of ethanol in human liver microsomes (B). The amount of immunoreactive P-450 in the microsomal fractions was analyzed using radial immunodiffusion and anti-P-450 LMeb-IgG and is expressed per nmol of spectrophotometrically determined cytochrome P-450. NADPH-oxidase activity as a function of the rate of microsomal ethanol oxidation (C). Each data point represent one individual.

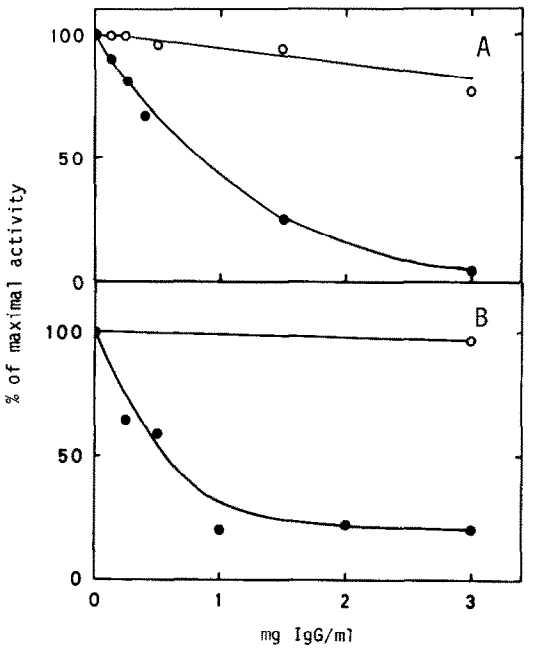

Fig. 3. Effect of anti-P-450 LMeb-IgG (●---●) or preimmune IgG (○---○) on the NADPH-dependent oxidation of ethanol (A) and carbon tetrachloride-dependent lipid peroxidation (B) in human liver microsomes. The rate of ethanol oxidation in the absence of IgG was 2.8 nmol acetaldehyde formed per mg protein and minute and the rate of formation of thiobarbituric acid reactive products in the absence of IgG was 65 pmol per mg protein and minute. Experiments with anti P-450j was 65 pmol per mg protein and minute. Experiments with anti-P-450j IgG yielded essentially identical results (not shown in the figure). Inhibition studies, with similar results, were carried out with liver microsomes from specimens HL 16, 31–34, 38 and 39 (cf. Table 1).

Anti-P-450j IgG and anti-P-450 LMeb IgG were used to evaluate the role of P-450Hj in the microsomal oxidation of ethanol and reduction of carbon tetrachloride as determined by the formation of TBA reactive substances (Fig. 3). Inhibition of both activities was observed when increasing amounts of anti-IgG were added, whereas preimmune IgG had no significant effect. At maximum, NADPH-dependent ethanol oxidation was almost completely inhibited by anti-P-450 LMeb IgG and the rate of microsomal carbon tetrachloride-dependent lipid peroxidation was inhibited maximally by about 80%. This indicates the participation of cytochrome P-450Hj in CCl_4-mediated hepatotoxicity in human liver and in the human microsomal oxidation of ethanol. Previously, immunological evidence has also been given for the participation of P-450Hj in human liver microsomal *N*-nitrosodimethylamine demethylation [23, 24] and aniline hydroxylation [24] and, in addition, for P-450j in the metabolic activation of CCl_4 in microsomes from acetone-treated rats [13].

In conclusion, we have presented evidence for the presence of a human homologue of the ethanol-inducible form of cytochrome P-450 that participates in the microsomal metabolism of ethanol and carbon tetrachloride and is the subject of an extensive interindividual variation. The ethanol-dependent induction of this isozyme appears to be of only limited importance for any enhanced elimination of ethanol from the liver,* but of great importance for synergistic action of ethanol in the toxicity of, for example, acetaminophen [25], nitrosoamines, CCl_4 and other organic solvents.

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Adenosine-A_1 receptors are not coupled to Ca^{2+} uptake in rat brain synaptosomes

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Adenosine (ADO) and analogues inhibit the release of neurotransmitters in the central nervous system and depress neuronal activity in a number of experimental models [1–4]. Adenosine exerts its effects via membrane bound receptors, which are generally divided into two subtypes, that are coupled to adenylate cyclase in an inhibitory (A_1 receptor) or a stimulatory (A_2 receptor) way. The relationship, however, between A_1 or A_2 adenosine receptor mediated modulation of adenylate cyclase and these calcium sensitive processes, is not clear. It has been proposed that the inhibition of presynaptic inward calcium currents is involved [3, 4].

From the structure activity relationships for the neurodepressing effects of ADO analogues, it can be deduced that an A_1-receptor-like subtype is involved [1]. As reports on this subject are not very consistent, the existence of a third receptor subtype was proposed, which is coupled to a calcium channel [5].

$^{45}Ca^{2+}$ uptake by synaptosomes can be measured for the investigation of calcium dependent effects of ADO. Wu *et al.* [6] and Ribeiro *et al.* [7] reported, that $^{45}Ca^{2+}$ uptake by synaptosomes is inhibited by ADO and analogues. Barr *et al.* [8], in contrast, were unable to demonstrate any influence of ADO on $^{45}Ca^{2+}$ uptake. However, it was not confirmed, whether the ADO analogues that were tested, actually bound to synaptosomes under the experimental conditions. In neither study the presence of endogenous ADO was taken into account. As synaptosomes are metabolically active [9], the ATP content of these preparations is high. Williams *et al.* [10] demonstrated that ATP interferes with binding of A_1 receptor ligands, due to metabolic breakdown of ATP to ADO. Consequently, endogenous ADO and ATP may compete with the effects of externally added ADO or analogues. Moreover, the incubation media contain ions, like Mg^{2+}, Ca^{2+}, Na^+ and K^+, that may interfere with ADO receptor binding [11, 12]. The availability of the tritiated, highly A_1-selective antagonist [^{3}H]DPCPX (1,3-dipropyl-8-cyclopentyl-xanthine) [13] enabled us to compare the effects of ADO analogues on $^{45}Ca^{2+}$ uptake with their binding to the A_1-receptor, represented by displacement of [^{3}H]DPCPX binding. When virtually identical preparations are used for both tests, differences in tissue penetration can be avoided and effects on $^{45}Ca^{2+}$ uptake and radioligand binding can be directly compared.

Methods

All solutions were made in distilled water and buffered with 10 mM Hepes, adjusted to pH 7.4 at the required temperature with Tris. The incubation medium contained (in mM) NaCl 140, KCl 5, $MgCl_2$ 1.2, Na_2HPO_4 1.2, $CaCl_2$ 0.1 or 1.0 and glucose 10. The wash buffer contained (in mM) NaCl 140 and KCl 5, the stop buffer NaCl 140, KCl 5 and EGTA 5. $^{45}Ca^{2+}$ uptake solutions had the same composition as the incubation medium, but contained *ca.* 10^6 cpm $^{45}Ca^{2+}$ (NEN, F.R.G.). For basal uptake the final KCl concentration was 5 mM, for KCl-stimulated uptake 30 mM.

Synaptosomes were prepared by a combination of differential and density gradient centrifugation [14]. Rat