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COMPARATIVE STUDIES ON COUMARIN AND TESTOSTERONE METABOLISM IN MOUSE AND HUMAN LIVERS

DIFFERENTIAL INHIBITIONS BY THE ANTI-P450Coh ANTIBODY AND METYRAPONE*

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Abstract—We have studied coumarin 7-hydroxylase (COH) and testosterone 15α-hydroxylase (15αOH) activities in human liver microsomes and compared them with corresponding activities catalysed by members of the P450IIA sub-family in DBA/2N mouse liver microsomes. Human liver contained low levels of 15αOH (about 5–30 pmol/min/mg protein) when compared with control mouse liver microsomes (about 200 pmol/min/mg protein). The anti-P450Coh antibody efficiently inhibited mouse liver 15αOH, also 7αOH (which is a member of the P450IIA sub-family), but it did not inhibit human 15αOH or other testosterone hydroxylases. In mouse liver microsomes, metyrapone preferentially inhibited 15αOH, but in human liver microsomes it inhibited all testosterone hydroxylations measured, including 15αOH (IC_{50} = 2.0–5.0 μM). Metyrapone clearly inhibited COH in mouse liver microsomes, but interestingly it had no effect on COH activity in human liver microsomes, although these two isozymes have earlier been shown to be immunologically similar. On the basis of available evidence human and mouse P450Coh isozymes seem to be orthologous enzymes whereas the present results indicate that the human 15αOH is different from the mouse $P450_{15\alpha}$.

Cytochrome P450 monooxygenases have a central role in the metabolism of endogenous compounds and xenobiotics [1, 2]. Endogenous steroids may serve as natural substrates for liver microsomal P450 and they have been used in studies to define specific P450 isozymes [3–5]. Testosterone is regarded as a useful substrate, because it is metabolized regio- and stereoselectively by a number of isozymes in rat liver microsomes [6].

In the mouse liver testosterone is hydroxylated at the 15α-position by the cytochrome $P450_{15\alpha}$ [7, 8], which belongs to the P450IIA sub-family [9] as well as rat testosterone 7α-hydroxylase (7αOH). Two types of testosterone 15α-hydroxylase (15αOH) have been found in mouse liver [10] and they only differ in 11 amino acid residues out of 494 [11]. The type I enzyme is the $P450_{15\alpha}$ and the type II isozyme is associated with high coumarin 7-hydroxylase (COH‖) activity [12]. The activity of COH is extremely variable in different species [13–15]. It is high in human and mouse liver, while in rats it is almost absent and is not inhibited by anti-P450Coh antibody [16–18].

The extremely high homology of mouse liver $P450_{15\alpha}$ and P450Coh indicates that they diverged considerably later than rodents and man and consequently man could not have the orthologous isozyme. However, it is still possible that human $P450_{15\alpha}$ may be related to P450Coh in man. This is suggested by the intriguing finding that a single amino acid difference in mouse P450Coh drastically alters its substrate specificity to the predominance of 15α-hydroxylation [19]. It is possible that this mutation might have happened in man after the separation of rodents and man or that human P450Coh might possess 15αOH activity.

In the present study we compared COH and 15αOH activities in several human liver samples and in both control and pyrazole treated mice. Previous studies have shown that pyrazole increases the activities related to the IIA sub-family [12, 20]. To further clarify the difference in enzyme activities in

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‖ Abbreviations: COH, coumarin 7-hydroxylase; XαOH and XβOH, testosterone hydroxylase activities with X denoting the carbon atom to be oxidized and α/β the stereospecificity of the reaction P450Coh; P450Coh, mouse P450 isozyme catalysing coumarin 7-hydroxylation; $P450_{15\alpha}$, mouse P450 isozyme catalysing testosterone 15α-hydroxylation.

According to the current P450 nomenclature [9], the genes coding for steroid 15α-hydroxylase and coumarin 7-hydroxylase have been called Cyp2A3 [26, 32]. Because these enzymes are coded for by distinctly different genes (see the text), they will be given new assignments (Nebert *et al.*, in press).

humans and mice the activities *in vitro* were probed by the anti-P450Coh antibody and metyrapone, which is known to inhibit P450-dependent steroid metabolism [21].

MATERIALS AND METHODS

Chemicals. Coumarin was purchased from Serva Feinbiochemica GmbH & Co. (Heidelberg, F.R.G.), testosterone from Merck (Darmstadt, F.R.G.) and metyrapone from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [4-^{14}C]Testosterone (57 mCi/mmol) was from Amersham (Amersham, U.K.). Unlabelled steroid standards were obtained from Prof. D. N. Kirk from Steroid Reference Repository, MRC (London, U.K.). Pyrazole was from Fluka Chemical AG (Buchs, Switzerland). Other chemicals were of the purest grade available.

Liver preparations. Two samples of human liver microsomes were prepared from organ donors who died accidentally (HL 15 and HL 16). The other seven samples were biopsies taken during laparotomy from patients having liver disease. The histology of these samples was investigated and minor damages were noted but no severe damages or autolysis were present. Liver samples were processed within 1 hr and microsomes stored at −70° or they were first frozen on liquid nitrogen and processed later. Tissues from organ donors were removed within 30 min of death. The microsomal fractions were prepared by homogenizing the tissue in a glass homogenizer in 4 volumes of 0.1 M sodium–potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 10,000 *g* for 30 min and the supernatant obtained was centrifuged at 100,000 *g* for 1 hr and the microsomal pellet was washed.

Control and pyrazole treated 8–12-week-old male DBA/2N mice were used in the animal studies. Pyrazole was given as single daily i.p. injections (200 mg/kg dissolved in physiological saline) for 3 consecutive days. Livers from 6 to 8 mice per group were pooled and homogenized and microsomes prepared as for human liver microsomes. Two different pools were used in both control and pyrazole groups. Protein contents were measured according to Bradford [22].

Preparation of the antibody. Pyrazole induced DBA/2N mice were used to purify P450Coh as reported earlier [23] and antiserum against it was raised in rabbits. The IgG fraction was purified by $(NH_4)_2SO_4$ precipitation.

Enzyme assays. Unlabelled steroid standards were used to identify radiolabelled metabolites (15α-, 16α-, 16β-, 7α-, 6β- and 2α-hydroxytestosterone). The purity of the metabolites produced has been measured with HPLC/MS and has been over 90% with 15α-, 16α-, 16β-, 7α-, 6βOH-T and about 80% with 2αOH-T (Honkakoski *et al.*, unpublished).

COH activity was determined according to Aitio [24]. Testosterone hydroxylation activities were determined as described by Waxman *et al.* [6] with slight modifications. Testosterone (50 nmol, dissolved in ethanol) was used in the experiments. The incubation time was 10 min for mouse liver microsomes and 20 min for human liver microsomes. The metabolites were extracted by dichloromethane and were then dried by nitrogen stream. The dried extracts were dissolved in acetone and spotted onto silica gel plates containing a fluorescent indicator (E. Merck, F-254). Plates were run in two different solvent systems: first with dichloromethane/acetone (4:1) and then with chloroform/ethyl acetate/ethanol (4:1:0.7). Metabolites were detected by autoradiography after 1 week exposure using KODAK X-OMAT AR film and the metabolites were scraped out for direct quantitation by liquid scintillation counting. Enzyme activity was calculated for each metabolite as the percentage of total radioactivity (substrate and products) and then converted to picomoles of product based on the testosterone concentration in the incubation mixture. The limit of detection of testosterone metabolites was about 2 pmol/min/mg protein. The coefficient of variation for the assay (the mean deviation of each enzyme activity was divided by the square root of the number of determinations) was dependent on the amount of the metabolite produced. It was about 20% for metabolites produced in low quantities (such as 15αOH-T by human liver) and 4–10% for metabolites produced in larger quantities (more than 100 pmol/mg × min).

Inhibition of enzyme activities by anti-P450Coh antibody and metyrapone. Preliminary studies indicated that the maximal inhibition was achieved with equal protein concentrations of the antibody and microsomes. The antibody was added 2 min before starting reactions with testosterone. Pre-immune serum controls were always assayed parallel with the antibody. Metyrapone was dissolved in water and the concentrations of 0.5, 5, 50 and 500 μM were used. The reaction was initiated by microsomes. The incubation time was 10 min (mouse) or 20 min (human).

RESULTS

Testosterone oxidations in human and mouse liver microsomes

Testosterone hydroxylations were studied in nine human liver samples (Table 1) and a representative autoradiograph of testosterone metabolites formed by liver microsomes from the HL 16 is shown in Fig. 1. Human hepatic 15αOH activity was very low; the activity of the enzyme was higher than 10 pmol/min/mg protein in only three samples. In control mouse liver the 15αOH activity was about 200 pmol/min/mg protein and in the pyrazole induced mouse it was 2.5 times higher (Table 2). 6β-Hydroxytestosterone was the main metabolite in all human liver samples, but the activities varied considerably (between 516 and 2620 pmol/min/mg protein). The human liver also contained some 15βOH- (about 40 pmol/min/mg protein), 16β/2βOH- (about 120 pmol) and 2αOH-activities (about 90 pmol). While we did not have 15β-hydroxytestosterone as a reagent, we did find one metabolite with activity that correlated well with earlier findings [25]. 2β-Hydroxytestosterone has been shown to have the second highest activity, but in the present conditions it co-migrated with 16β-hydroxytestosterone. The correlation coefficients between hydroxylase activities in different human

Table 1. Testosterone oxidations and COH activities in human liver microsomes

Liver number	Activity (pmol/min/mg protein) 15α	15β	6β	16β/2β	2α	A*	COH
1	4.9	37	1160	105	44	160	390
2	10	44	1460	138	95	193	370
3	1.6	22	516	60	18	71	570
6	4.4	32	948	103	67	118	730
7	13	59	1670	147	155	141	520
8	4.4	29	692	66	51	187	450
10	15	56	1430	144	144	98	729
15†	9	40	1030	95	72	237	187
16†	36	60	2620	197	135	67	572

Values are means of duplicate determinations.
* Androstenedione formation.
† Livers from organ donors.

liver samples were relatively high (0.74–0.98). The activity of 6βOH was about 78%, 16β/2βOH 7–8%, 2αOH 4–6% and 15βOH 2–3% of testosterone hydroxylase activity. The androstenedione formation varied from 67 to 237 pmol/min/mg protein and COH activity from 187 to 730 pmol/min/mg protein.

6β-Hydroxylation seemed to be the major route of metabolism of testosterone in control mice, but the formation was clearly lower than in human liver samples. Pyrazole diminished the activities of 6βOH and 16αOH, but it induced 15αOH and 7αOH in the mouse. The activity of 7αOH was 1.5 times higher in the pyrazole group than in the control group.

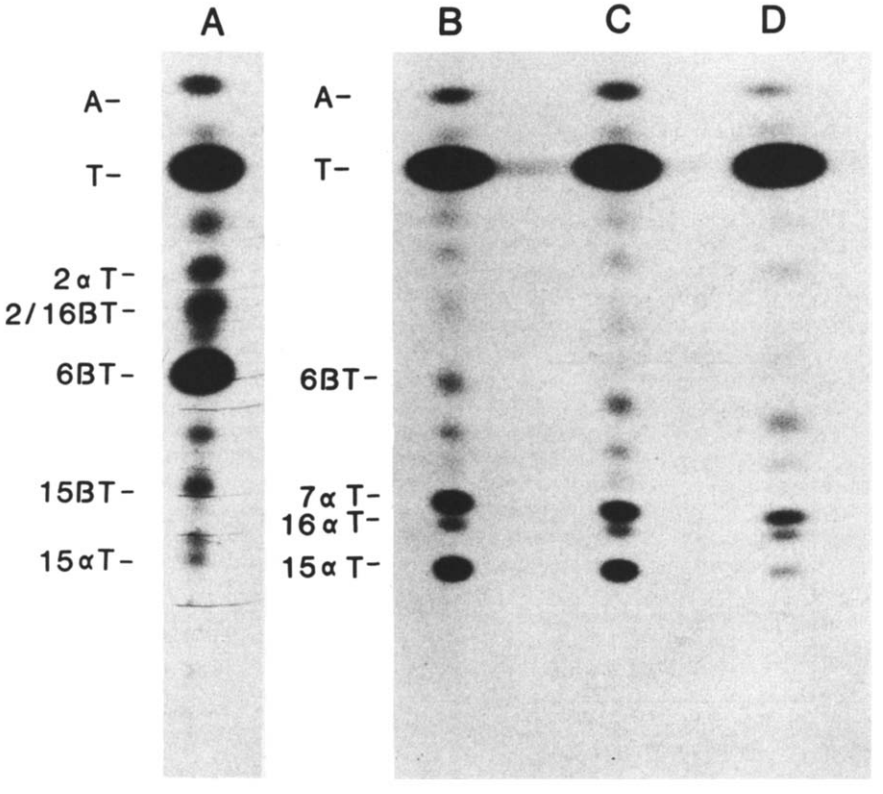

Fig. 1. Autoradiograph of testosterone metabolism (A) in human liver microsomes (HL 16) and the effects of preimmune IgG (C) and the anti-P450Coh antibody (D) on testosterone metabolism in pyrazole-treated mouse liver microsomes. The control activities can be seen on lane B. 6β-Hydroxytestosterone is clearly the main metabolite in human liver (A) while in pyrazole-treated mouse liver 7α- and 15α-hydroxytestosterone seem to be the most abundant metabolites (B). Metabolites identified are marked on the left of lanes A and B. T, testosterone; 15αT, 15α-hydroxytestosterone, etc.; A, androstenedione.

Table 2. Immunoinhibition of mouse testosterone hydroxylases by anti-P450Coh antibody

	Specific activity (pmol/mg protein × min)			
	15α	16α	7α	6β
Control microsomes	201 (100)	285 (100)	295 (100)	709 (100)
Preimmune IgG	200 (100)	274 (96)	253 (86)	785 (111)
Anti-P450Coh	102 (51)	259 (91)	95 (32)	719 (101)
Pyrazole microsomes	492 (100)	81 (100)	431 (100)	75 (100)
Preimmune IgG	405 (82)	73 (90)	331 (77)	64 (85)
Anti-P450Coh	79 (16)	67 (83)	174 (40)	63 (84)

Figures in the parentheses denote percentage of the enzyme activities of the control (control and pyrazole microsomes). Values are means of duplicate determinations on pooled microsomal fractions from 6 to 8 mice in each group.

Immunoinhibition of testosterone hydroxylase activities by anti-P450Coh antibody

In humans. Two human liver samples (HL 15 and 16) were used for immunoinhibition studies. Equal amounts of IgG and microsomal protein were added to the mixture for all incubations. No inhibition was seen in 15αOH or any other testosterone hydroxylase activities (data not shown).

In mice. Contrary to humans testosterone 15α-hydroxylase was inhibited by about 50% in controls and by about 85% after pyrazole treatment (Fig. 1 and Table 2). In addition to 15αOH, 7αOH was also inhibited strongly by the antibody (Table 2). 6βOH and 16αOH activities were inhibited by about 20%, but no real inhibition took place, because preimmune IgG also inhibited them.

Inhibition of COH and testosterone hydroxylases by metyrapone in human and mouse liver microsomes

Metyrapone strongly inhibited COH activity in control and pyrazole treated mouse liver microsomes (IC_{50} = 5.0–10 μM) as seen in Fig. 2A and B. Surprisingly, it had no effect on COH in human liver microsomes measured from seven samples (Fig. 2C). However, 15αOH was clearly inhibited in human, control murine and pyrazole treated mouse micro-

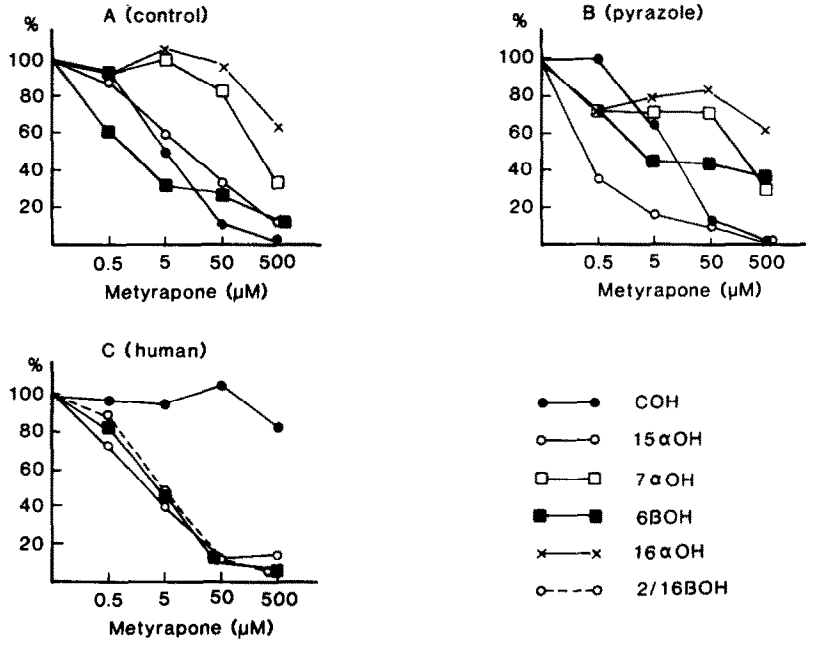

Fig. 2. (A) The effect of metyrapone on 15αOH, 7αOH, 6βOH and 16αOH in control mouse liver microsomes. The maximal enzyme activities can be seen in Table 2, except that of COH which was about 82 pmol/min/mg protein. (B) The effect of metyrapone on COH, 15αOH, 7αOH, 6βOH and 16αOH in pyrazole-treated mouse liver microsomes. The maximal enzyme activities can be seen in Table 2, except that of COH which was about 816 pmol/min/mg protein. (C) The effect of metyrapone on COH, 15αOH, 6βOH, 2β/16βOH in human liver microsomes. The maximal enzyme activities can be seen in Table 1. HL 16 was used in testosterone hydroxylase and COH studies seen in this figure. No inhibition of COH could be detected in seven human liver samples.

somes (IC_{50} = 4, 12 and 0.4 μM, respectively). Metyrapone inhibited testosterone hydroxylases in different ways in human (HL 15 and HL 16) and mouse liver microsomes (Fig. 2). The data for HL 15 are not shown, but the results were similar as for HL 16. The inhibition of 15αOH was selective in the mouse (pyrazole group); 3% activity remaining with the highest concentration. In the control group 6βOH was inhibited more efficiently than 15αOH (IC_{50} = 2 and 12 μM), although 16αOH had 63% of its activity left after incubation with the highest concentration of metyrapone (Fig. 2A). 7αOH-activity was 30–33% of the control activity with the same concentration in both groups. Similar degrees of inhibition of 6βOH, 15αOH and other testosterone hydroxylases in human liver microsomes were seen after metyrapone incubation (IC_{50} values were about 2.0–5.0 μM). Only 5% of 6βOH activity and 14% of 15αOH activity remained. However, the level of 15αOH activity was difficult to measure (only 5 pmol/min/mg protein).

DISCUSSION

It has been shown that there are two closely related genes associated with the mouse liver 15αOH [10], the type I gene product being the $P450_{15\alpha}$ and the type II enzyme (P450Coh) predominantly catalysing 7-hydroxylation of coumarin [11]. Furthermore, it has been shown that the human and mouse P450Coh isozymes are structurally very closely related and immunologically similar [18, 26]. Because a single amino acid mutation (Phe 209 Leu) can alter the P450Coh to the type I enzyme (15αOH) [19], we wanted to study possible similarities between isozymes catalysing 15α-hydroxylation of testosterone in man and mouse and to assess the findings with respect to the known close similarities between murine and human COH isozymes.

Comparison of different testosterone hydroxylase activities between mouse and man demonstrated large and consistent differences. The most important finding was that the activity of 15αOH in human liver microsomes was low; only about 2–15% of that in control DBA/2N mouse. In pyrazole-treated mice the 15αOH activity was even greater, about 20 to 100 times that in human liver microsomes. The activity of 7αOH was about 300 pmol/min/mg protein and in the pyrazole group it was 1.5 times higher. In humans 7αOH was hardly measurable. 6β-Hydroxytestosterone is the major testosterone metabolite in human liver microsomes [25]. In our experiments the activity of 6βOH was the highest, but it varied considerably among different human samples. Its activity was about 74–82% of the total testosterone oxidizing activity. Although the activities of different hydroxylases varied greatly, the proportions of the major metabolites were reasonably constant. Waxman *et al.* [25] identified the metabolite having the second highest activity as 2βOH. In our studies 2β-hydroxytestosterone migrated closely with 16β-hydroxytestosterone. A similar 2αOH activity to that of 15βOH was also seen.

Inhibitory antibodies have been used to define the contributions of specific P450s in microsomal oxidation reactions [27–29]. Our laboratories have previously used polyclonal antibody generated against P450Coh to investigate differences between human and mouse COH [18]. In the present study we showed that the antibody against pyrazole-treated mouse liver P450Coh effectively inhibits the mouse 15αOH. The inhibition of 15αOH was expected because P450Coh and $P450_{15\alpha}$ are structurally very similar. Testosterone 15α-hydroxylase activity of purified P450Coh has been shown previously to be inhibited by the anti-Coh antibody [12]. Interestingly, some effect on 7αOH was also seen in our study. The rat 7αOH belongs to the P450IIA sub-family [30] and it has 75% amino acid similarity with mouse 15αOH [31]. It has been shown that anti-Coh antiserum effectively inhibits COH activity in human liver microsomes [18, 26], but in the present study it had no effect on testosterone hydroxylases in human liver microsomes. A recent study showed, that the antibody to rat P450IIA1 inhibits human COH even more effectively than anti-P450Coh [26]. The type I and type II 15αOHs differ in only 11 amino acid residues. Nine of the corresponding residues in the human P450IIA3 possessing high Coh activity [26, 32] are identical with P450Coh and only one with $P450_{15\alpha}$. In this study the mouse 15αOH was inhibited by anti-P450Coh, but it had no influence on human 15αOH. Consequently human liver $P450_{15\alpha}$ is immunologically different from mouse $P450_{15\alpha}$. It seems warranted to conclude that human liver isozymes responsible for 15αOH and COH are not such closely related enzymes as they are in mouse.

Another technique to compare P450 isozymes is to use diagnostic inhibitors. Metyrapone has been shown to bind to phenobarbital inducible P450 [33] and it inhibits especially steroid metabolizing enzymes [21], but also other enzyme activities [34, 35]. Metyrapone inhibited clearly COH in the mouse microsomes as earlier reported [35], but it had no effect on human liver microsomes. Metyrapone has been shown to moderately inhibit COH activity in humans [15], but only two samples were studied. In the present study no inhibition was seen in seven samples. Metyrapone inhibited testosterone hydroxylases in different ways in human and mouse liver microsomes. In the mouse it inhibited 15αOH quite selectively. In the control group 6βOH was also efficiently inhibited, but other testosterone hydroxylases were much more resistant to inhibition. 15αOH was inhibited in human liver microsomes, but other testosterone hydroxylations were also inhibited to a similar extent. These results are further evidence for the conclusion that human P450Coh and the form catalysing 15αOH activity are not structurally similar isozymes. It is also possible that more isozymes catalyse testosterone oxidation in mouse than in human liver.

In conclusion, we have shown that human and mouse livers contain very different amounts of testosterone 15α-hydroxylase activities, but that 6βOH is clearly the predominant hydroxylase in both species.

Immunoinhibition studies with anti-P450Coh antibody indicated that human 15αOH is quite different from mouse 15αOH. As has been reported

the human COH has considerable similarities with mouse COH but is inhibited in a different way by metyrapone. Although a single amino acid in P450Coh in mouse can alter its catalytic properties to hydroxylate testosterone at the 15α-position, it seems likely that this kind of mutation has not happened in man. It also seems established that human hepatic P450Coh does not catalyse testosterone 15α-hydroxylation.

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