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Catalytic roles of rat and human cytochrome P450 2A enzymes in testosterone 7α- and coumarin 7-hydroxylations

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Abstract—Differences in the catalytic roles of rat and human cytochrome P450 2A enzymes in testosterone 7α- and coumarin 7-hydroxylase activities were examined. Liver microsomes from 18 human samples catalyzed coumarin 7-hydroxylation at a mean rate of about 60 pmol/min/nmol P450, but did not show any measurable activity for testosterone 7α-hydroxylation. In rats, both activities were found to be developmentally regulated; 3-week-old rats had the highest activities for these two reactions. Anti-P450 2A1 antibodies and methoxsalen, a potent inhibitor of P450 2A-dependent monooxygenase activities in several animal species, inhibited almost completely both testosterone 7α- and coumarin 7-hydroxylations catalyzed by liver microsomes prepared from 3-week-old male rats. Interestingly, although K_m values for coumarin 7-hydroxylation activities in liver microsomes from 3-week-old rats were not different from those of adult humans, the V_{max} value in rats was only 1/30 of that obtained in 18 human samples. Thus, the present results support the view that marked differences exist in the catalytic roles of rat and human P450 2A enzymes, which, in turn, may sometimes cause species-related differences in susceptibilities toward drug actions and toxicities.

Key words: P450 2A6; P450 2A1; testosterone; coumarin; species differences

Although there are extensive studies suggesting that testosterone 7α-hydroxylation is catalyzed by P450† 2A1 in rat liver microsomes [1, 2], it remains unclear which human P450 enzymes participate in this hydroxylation reaction. Coumarin 7-hydroxylation has also been reported to be catalyzed by P450 2A enzymes in several animal species [3], and lines of evidence have suggested the existence of species-related differences in the catalytic roles of P450 2A enzymes in rats and humans [4, 5]. Several reports have appeared on the catalytic activities of coumarin 7-hydroxylation by rat liver microsomes [3, 4, 6, 7]. Peters *et al.* [6] and Fentem and Fry [7] reported that coumarin 7-hydroxylation activities could not be detected in rat liver microsomes, while other investigators found low, but significant, coumarin 7-hydroxylase activities in rats [3, 4]. Such a discrepancy may be related to the different measurement techniques used for the coumarin 7-hydroxylation assay in these investigations, or to differences in the age of the animals used in these studies because P450 2A1 enzyme has been reported to be regulated developmentally in rats [8].

Therefore, this study was undertaken to examine further the differential roles of P450 2A enzymes in rat and human liver microsomes by measuring testosterone 7α- and coumarin 7-hydroxylation activities. Liver microsomes prepared from male and female rats at different ages were used in this study because of the effect of age on rat P450 2A enzymes, as described in Ref. 8. We also compared liver microsomal activities in 18 human samples with those of purified human P450 enzymes.

Materials and Methods

Chemicals. Testosterone was obtained from the Katayama Chemical Co., Osaka, Japan, and coumarin and methoxsalen were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. 7-Hydroxycoumarin was obtained from the Aldrich Chemical Co., Milwaukee, WI, U.S.A. The 2α-, 2β-, 6β-, 7α-, 16α-, and 16β-hydroxylated metabolites of testosterone were gifts from Dr. Y. Nakamura of the Shionogi Pharmaceutical Co., Osaka, and 15β-hydroxytestosterone was from Dr. D. J. Waxman of the Harvard Medical School, Boston, MA, U.S.A.

Enzyme preparations and antibodies. Human liver samples were obtained from 10 patients (designated as HL-1 to HL-10) undergoing liver resection and from 8 organ donors (designated as HL-11 to HL-18) through the Tennessee Donor Services (Nashville, TN, U.S.A.) as described previously [9]. Male and female Sprague–Dawley rats (Nihon Clea Co., Osaka) of different ages were starved overnight before being killed. In some experiments, male rats treated i.p. with dexamethasone (50 mg/kg, daily for 4 days) were used. Liver microsomes were prepared as described previously [10].

Human P450s 1A2, 2A6, $2C_{MP}$, 2E1, and 3A4 were purified to electrophoretic homogeneity as described [11–16]. Anti-rat P450 2A1 serum was a gift from Dr. Y. Funae. Rabbit liver NADPH-P450 reductase and cytochrome b_5 were purified by the method of Taniguchi *et al.* [17].

Assay methods. Testosterone hydroxylase activities were determined by the method of Brian *et al.* [18] with slight modifications. The incubation mixture consisted of rat and human liver microsomes (0.1 to 0.8 mg protein/mL) and testosterone (0.025 to 0.4 mM) in a final volume of 0.25 mL in the presence of an NADPH-generating system [13]. The reconstituted P450 system was composed of 25 pmol of purified human P450, 50 pmol of cytochrome b_5, 50 pmol of NADPH-P450 reductase, and 10 μg of a phospholipid mixture consisting of L-α-dilauroyl-*syn*-glycero-3-phosphocholine, dioleoylphosphatidylcholine and L-α-phosphatidyl-L-serine (1:1:1) per mL and 100 μg of sodium cholate/mL [19]. Incubations were carried out at 37° for 10–30 min, and terminated by adding 6 vol. of CH_2Cl_2. Product formation, which increased linearly for up to 30 min of incubation, was measured by HPLC and UV absorbance at 254 nm using a 4.6 × 250 mm octyldecylsilyl reverse-phase column (Chromato Tec Co., Tokyo, Japan) eluted with a mixture of CH_3OH/H_2O (64:36) at a flow rate of 1.2 mL/min.

Other assays. Coumarin 7-hydroxylation was assayed by a method described previously; the concentration of 7-hydroxycoumarin was determined fluorometrically (λ_{ex} = 358 nm, λ_{em} = 458 nm) with a Shimadzu RF-5000 spectrofluorometer [15]. P450 and protein contents were estimated by the methods of Omura and Sato [20] and Lowry *et al.* [21], respectively. The contents of individual human P450 proteins were estimated by immunoblotting analysis [12, 22].

† Abbreviation: P450, cytochrome P450.

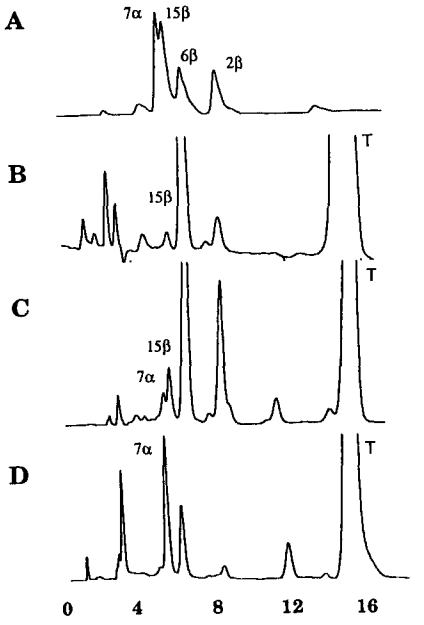

Fig. 1. High-performance liquid chromatographic analysis of testosterone hydroxylation by liver microsomes (0.2 mg protein/mL of incubation mixture) from a human sample, HL-4 (part B), dexamethasone-treated male rats (7 weeks old) (part C), and untreated male rats (3 weeks old) (part D). The elution profile of authentic hydroxylated metabolites of testosterone is also shown (part A). Abbreviations: 7α-hydroxytestosterone (7α), 15β-hydroxytestosterone (15β), 6β-hydroxytestosterone (6β), 2β-hydroxytestosterone (2β), and testosterone (T). In part A, a mixed solution in MeOH containing 0.2 nmol each of the hydroxylated metabolites was applied onto the HPLC column.

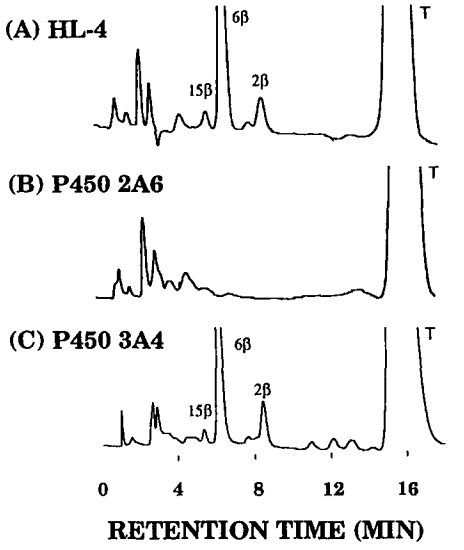

Fig. 2. High-performance liquid chromatographic analysis of testosterone hydroxylation by liver microsomes (0.2 mg protein/mL of incubation mixture) from a human sample, HL-4 (part A), and by a reconstituted monooxygenase system containing 25 pmol of P450 2A6 (part B) or P450 3A4 (part C). Other details are as described in the legend to Fig. 1.

Results and Discussion

Testosterone was incubated with liver microsomes from a human sample (HL-4), from untreated male rats (3 weeks old), and from male rats (7 weeks old) treated with dexamethasone, and the metabolites formed were analyzed on HPLC (Fig. 1). Three major metabolites hydroxylated at 6β-, 2β-, and 15β-positions were identified upon incubation of testosterone with liver microsomes from the human sample (HL-4), but formation of 7α-hydroxytestosterone could not be detected in these microsomes (Fig. 1B). We also determined in 18 human samples that none of the human liver microsomes had detectable levels of testosterone 7α-hydroxylation activities (data not shown). It should be mentioned that the retention times of the 7α- and 15β-hydroxylated metabolites were very close on HPLC analysis, even when different solvent systems were employed for elution of the hydroxylated testosterone metabolites.

Liver microsomes from untreated male rats (3 weeks old), on the other hand, produced 7α-hydroxytestosterone as a major metabolite, but did not form 15β-hydroxylated product at a measurable level (Fig. 1D). When liver microsomes from dexamethasone-treated rats (7 weeks old) were used, the level of 7α-hydroxytestosterone was decreased, and the formation of 15β-, 6β- and 2β-hydroxytestosterone was increased (Fig. 1C).

These results suggested that human liver microsomes did not produce 7α-hydroxytestosterone at a significant level; the limit of detection was defined as <5 pmol/min/nmol P450 in this assay condition. We also determined testosterone hydroxylation activities by a reconstituted monooxygenase system containing P450 2A6 and P450 3A4 (Fig. 2) by the method described in Materials and Methods. P450 3A4 catalyzed 6β-, 2β-, and 15β-hydroxylation, but not 7α-hydroxylation, activities in the reconstituted monooxygenase system (Fig. 2C); the HPLC profile of metabolite formation was very similar to that obtained in liver microsomes from a human sample (HL-4) (Fig. 2A). P450 2A6, on the other hand, did not produce any detectable levels of testosterone metabolites (Fig. 2B). Other purified human P450 enzymes, including P450 1A2, $2C_{MP}$, and 2E1, were also found to have undetectable levels of testosterone hydroxylation activities in this assay condition (data not shown).

It has been reported that the level of P450 2A1 is regulated developmentally by rat liver microsomes of both sexes; the highest activities could be obtained at age 3 weeks [8, 23]. We also found that P450 2A1-dependent testosterone 7α-hydroxylase activities were the highest at age 3 weeks in both male and female rats (Fig. 3B). Recently, Peters *et al.* [6] and Fentem and Fry [7] determined that rat liver microsomes do not catalyze coumarin 7-hydroxylation at measurable levels, while other investigators have found low, but significant, activities in rats [3, 4]. Although it is not known at present why these different results were obtained in several laboratories, it should be mentioned that different assay systems were employed in these studies. With liver microsomes from rats of different ages, we could determine coumarin 7-hydroxylase activities using detection of 7-hydroxycoumarin with a fluorescence detector; the highest activities could be attained at age 3 weeks in both sexes, although the turnover numbers obtained were not very large (Fig. 3C).

These results suggested that coumarin 7-hydroxylation, as well as testosterone 7α-hydroxylation, is catalyzed by P450 2A1 in rat liver microsomes, although the former activities were very low even when 3-week-old rats were used. To obtain more conclusive evidence, we examined the effects of anti-rat P450 2A1 antibodies and methoxsalen,

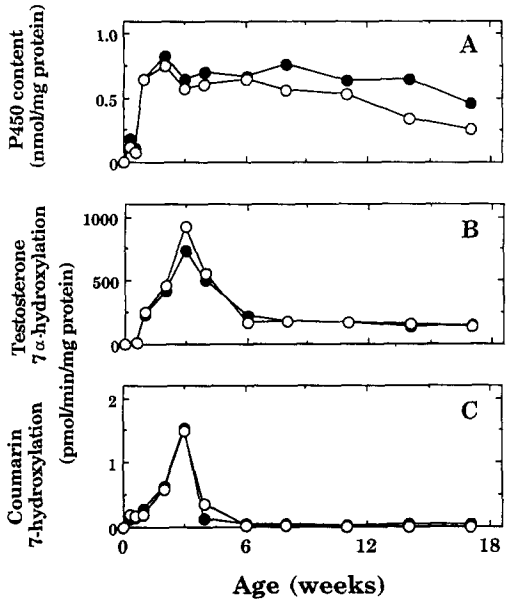

Fig. 3. Effects of aging on the P450 content (part A) and activities of testosterone 7α-hydroxylation (part B) and coumarin 7-hydroxylation (part C) by liver microsomes from male (●) and female (○) rats. Values are means from 2–4 individual rats; the standard deviations in these determinations were less than 15% of the mean values.

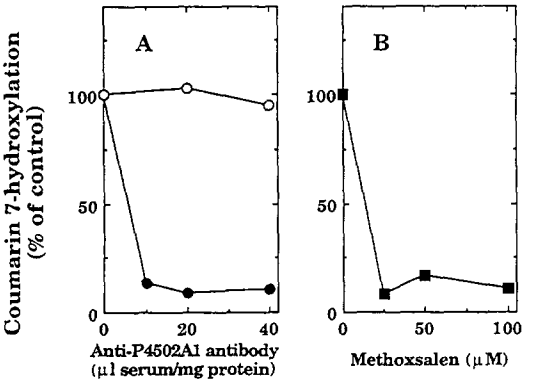

Fig. 4. Effects of anti-P450 2A1 antibodies (part A) and methoxsalen (part B) on the 7-hydroxylation of coumarin by liver microsomes from male rats (3 weeks old). Different amounts of preimmune sera (○), anti-P450 2A1 (●) and methoxsalen (■) were included in the reaction mixtures. The uninhibited activity in the absence of antibodies or inhibitor was 1.54 pmol/min/mg protein. Each point represents the mean of duplicate determinations.

a potent inhibitor of P450 2A-dependent reactions in several animal species [24], on the hydroxylation activities catalyzed by liver microsomes from 3-week-old male rats (Fig. 4). Specificity of anti-P450 2A1 antibodies has already been ascertained previously by enzyme-linked immunosorbent assay (ELISA) in which the previous trivial name P450 IF-3 was used for P450 2A1 [25, 26]. It should also be mentioned that anti-P450 2A1 (IF-3) has been

Table 1. Kinetic analysis of coumarin 7-hydroxylation by rat and human liver microsomes

Microsomes	K_m (μM)	V_{max} (pmol/min/mg protein)
Rat*		
Male	11.3	1.56
Female	11.1	1.80
Human		
HL-18	13.0	174

Kinetic analysis was carried out at substrate concentrations between 2 μM and 1 mM. The kinetic parameters were estimated, using a non-linear regression analysis program ("k · cat," BioMetallics, Princeton, NJ), from mean values obtained in duplicate at each substrate concentration.

* Three-week-old rats were used.

shown to cross-react moderately with P450 2A2 (UT-4), but not with P450 1A1 (MC-5), P450 2B1 (PB-4), P450 2C6 (PB-2), P450 2C11 (UT-2), P450 2C13 (UT-5), P450 2E1 (DM), P450 3A2 (PB-1), and P450 4A2 (K-5), in rat liver microsomes [25]. Coumarin 7-hydroxylation activities were inhibited almost completely by anti-P450 2A1 antibodies and methoxsalen (Fig. 4); similar effects were also seen for testosterone 7α-hydroxylation (data not shown).

To obtain an exact turnover number for coumarin 7-hydroxylation, we carried out kinetic analysis using liver microsomes from male and female rats (both 3 weeks old) and compared the results with those from a human sample (HL-18) that had a relatively high level of P450 2A6 (Table 1). The K_m values obtained in liver microsomes from rats and the human sample (HL-18) were found to be almost the same (~10 μM), but the V_{max} values were very dissimilar (human liver microsomes showed activities about 100-fold higher than those in the rat liver microsomes). We also determined coumarin 7-hydroxylase activities in 18 human samples and found that the mean activity in these samples was about 60 pmol/min/nmol P450.

The results presented in this study suggested that there are species-related differences in the catalytic roles of P450 2A enzymes in rats and humans, and that such differences in P450 2A activities may sometimes cause different susceptibilities toward drug actions and toxicities. In particular, human liver microsomes were found not to be involved in testosterone 7α-hydroxylation, and this may be the result of the inability of P450 2A6 to catalyze the reaction. Further work on the physiological significance of 7α-hydroxytestosterone is needed.

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Osaka Prefectural Institute of Public Health Osaka 537, Japan

HIROSHI YAMAZAKI
MAYUMI MIMURA
CHIKAKO SUGAHARA
TSUTOMU SHIMADA*

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* Corresponding author: Dr. T. Shimada, Osaka Prefectural Institute of Public Health, Nakamichi, Higashinari-ku, Osaka 537, Japan. Tel. 81-6-972-1321; FAX 81-6-972-2393.

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