Interspecies Homology of Cytochrome P-450: Inhibition by Anti-P-450-Male Antibodies of Testosterone Hydroxylases in Liver Microsomes from Various Animal Species Including Man

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Abstract—P-450-male is one of the male-specific forms of cytochrome P-450 in liver microsomes of adult rats and functions as testosterone 2α- and 16α-hydroxylases. The purpose of this study was to examine whether forms of cytochrome P-450 cross-reactive with anti-P-450-male antibodies are present in liver microsomes of other animal species, such as male and female mice, rabbits, guinea pigs, hamsters, dogs and humans. The antibodies cross-reacted with a protein(s) in the liver microsomes of all animal species to show one or more bands on nitrocellulose paper by Western blot peroxidase-anti-peroxidase staining analysis. Qualitative as well as quantitative species differences were noted in the testosterone hydroxylases. The antibodies inhibited 2α-hydroxylase in male rats, 16α-hydroxylase in male rats and male and female dogs, 7α-hydroxylase in female rats and male and female mice and hamsters, and 15α-hydroxylase in female mice and hamsters. No clear inhibition of 6β-hydroxylase, which was present in all animal species, was observed. These results indicate that forms of cytochrome P-450 that are immunochemically related with P-450-male catalyze the hydroxylation of testosterone at varying positions depending on the animal species, with the exception of 6β-hydroxylation, which may be catalyzed by a distinct form of cytochrome P-450.

Marked species differences are generally observed in the activities of enzymes involved in the metabolism of a wide variety of endogenous and exogenous substrates (1). Cytochrome P-450 is known to play central roles in the metabolism of certain steroids and xenobiotics. Recent investigations have indicated that there are multiple forms of cytochrome P-450 in liver microsomes (2-4). It has also been shown that forms of cytochrome P-450, corresponding to phenobarbital- or 3-methylcholanthrene-inducible cytochrome, are present in most animal species (5-10), although the properties of this enzyme seem to differ slightly from one species to the other.

P-450-male** is one of the constitutive forms of cytochrome P-450, and it has been shown to exist specifically in adult male rats (19-21). A characteristic of this cytochrome is its ability to hydroxylate testosterone at the 2α- and 16α-positions (14, 22, 23). Interestingly, P-450-female, which is present specifically in adult female rats (21) and is able to catalyze the 15β-hydroxylation of 5α-androstane-3α,17β-diol disulfate (24, 25) but not the hydroxylation of testosterone (23), shows immunochemical relatedness to P-

** P-450-male corresponds to RLM5 (11, 12), UT-A (3), P-450h (13), P-450 2c (14), M-1 (15), UT-2 (16), P-45026 (17) and P-450c26 (18); and P-450-female corresponds to P-450 2d (14), P-450i (13) and F-1 (15), respectively.

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450-male (21). In addition, it is of interest to note that there is a remarkable similarity in the N-terminal amino acid sequences between P-450-male and P-450-female (26, 27), probably suggesting that these cytochromes are derived from the same ancestral gene.

Thus, the purpose of this study was to examine the possibility of whether a form(s) of cytochrome P-450 corresponding to P-450-male is present in the liver microsomes of various animal species.

We report herein that anti-P-450-male antibodies react with a protein(s) in the liver microsomes from all species of animals examined and inhibit testosterone hydroxylase activities. In addition, we found significant sex-related differences in a protein cross-reactive with anti-P-450-male antibodies and in testosterone 6β-hydroxylase activities in hamster liver microsomes.

Materials and Methods

Materials: Testosterone, cortisone and cortisol were purchased from commercial sources and were used after purification by recrystallization. The 6β-, 7α-, 11α-, 11β-, 16β-, and 19-hydroxytestosterones were obtained from Steraloids, Inc., Wilton, U.S.A.; and 16α-hydroxytestosterone was from Sigma. The 2α-, 2β-, 6α- and 15α-hydroxytestosterones were kindly donated by Dr. M. Kitada, Chiba University. NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were the products of Oriental Yeast, Tokyo, Japan. Goat antirabbit immunoglobulin G (IgG) and peroxidase-rabbit anti-peroxidase complex were purchased from Cappel Laboratories, West Chester and Daiichi Chemicals, Tokyo, Japan, respectively. Nitrocellulose paper was obtained from Bio-Rad. P-450-male was purified from adult male Sprague-Dawley rats according to the method of Funae et al. (16). Antibodies to P-450-male were prepared by immunization of female Japanese White rabbits according to the method of Kamataki et al. (21). The IgG fraction from immune or preimmune sera was partially purified by ammonium sulfate precipitation.

Preparation of microsomes: Adult (8–12 week-old except rabbits and dogs) male and female Sprague-Dawley rats, ddY mice, Golden harriers, Hartley guinea pigs, Japanese White rabbits (about 3 kg) and Beagle dogs (10 kg for male and 8 kg for female) were used. The animals were stunned by a blow on the head and decapitated, except dogs which were anesthetized with hexobarbital before being killed. The livers were immediately removed and homogenized in 1.15% potassium chloride to prepare 25% homogenates. Liver microsomes obtained by sequential centrifugation at 9,000 × g for 20 min and at 105,000 × g for 60 min at 4°C were washed once by resuspension with 1.15% potassium chloride and recentrifugation at 105,000 × g for 30 min at 4°C. Washed microsomes were suspended in ice-cold distilled water and stored at −80°C until use. No marked decline in the microsomal proteins were observed during the storage. Human livers from four male (M-1, M-2, M-3, M-4) patients and one female (F-1) patient were isolated during pathological examinations. The ages of the patients were 69, 76, 76, 65 and 87 years, and the causes of death were esophagus tumor, cerebral tumor, pulmonary tuberculosis, rupture of the aorta and pulmonary tuberculosis, respectively. Drugs given prior to death were cefmetazole (M-1), cefaclor, tobramycin and ampicillin (M-2), erythromycin and rifampicin (M-3), lata-moxef and tobramycin (M-4), and cefaclor (F-1), respectively. The delay between death and sampling was less than 4 hr. The livers were frozen immediately after removal and stored at −80°C until use. Human liver microsomes were prepared in the same manner as described above. Concentrations of microsomal protein and contents of cytochrome P-450 were measured by the methods of Lowry et al. (28) and Omura and Sato (29), respectively.

Measurements of testosterone hydroxylase activities: Testosterone hydroxylase activities were measured according to the method of Hayashi et al. (18) with slight modifications. A typical incubation mixture (0.5 ml) for the assay of microsomal testosterone hydroxylase activity consisted of 0.5 mg of microsomal protein, 0.05 mM EDTA, 100 mM phosphate buffer (pH 7.4), 300 nmol of testosterone dissolved in 10 μl of methanol, and an NADPH generating system (6.0 mM...
magnesium chloride, 0.8 mM NADP, 8.0 mM glucose 6-phosphate and 0.5 unit of glucose 6-phosphate dehydrogenase). The mixture was incubated at 37°C for 15 min and then extracted with 3 ml of benzene. The benzene layer was evaporated to dryness under reduced pressure. The residue was dissolved in 100 μl of methanol containing cortisol (for all animals except hamsters) or cortisone (for hamsters) as an internal standard. An aliquot was analyzed in a high-performance liquid chromatograph (Shimadzu Model LC-3A) equipped with a Model SPD-2A UV absorbance detector (Shimadzu) and a 0.6×25-cm ERC-silica 1181 column (particle size of 3 μm, Tokyo Rika, Tokyo, Japan). A solvent system of n-hexane : dichloromethane : ethanol (62:35:3) was used at a flow rate of 1.5 ml/min, and the eluate was monitored at 240 nm. The retention times (min) of the reference substances used were as follows: androst-4-ene-3,17-dione (3.5), testosterone (4.7), 2α-hydroxytestosterone (7.5), 2β-hydroxytestosterone (8.2), 6β-hydroxytestosterone (9.2), 6α-hydroxytestosterone (10.2), cortisol (12.5), 19-hydroxytestosterone (14.4), 16α-hydroxytestosterone (15.4), 7α-hydroxytestosterone (16.2), 15α-hydroxytestosterone (27.4). In the experiments for the inhibition of testosterone hydroxylase by anti-P-450-male antibodies, liver microsomes were pre-incubated with anti-P-450-male IgG at 25°C for 30 min and then assayed in the manner described above.

Western blots of microsomal proteins: Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (30), using a 7.5% polyacrylamide gel for analysis of microsomal proteins. Microsomal proteins separated in the gel were electrophoretically transferred to a sheet of nitrocellulose paper, incubated with 1/200 dilution of anti-P-450-male IgG for 1 hr at 25°C, and then stained by the use of the peroxidase-anti-peroxidase technique according to the method of Towbin et al. (31) as modified by Guengerich et al. (32).

Results

Western blot analysis of liver microsomal proteins reactive with anti-P-450-male antibodies: Figure 1 shows the results of Western blot analyses of liver microsomal proteins from seven mammalian species in-
cluding humans, probed with anti-P-450-male antibodies. The same amount of microsomal protein was applied to each well, except for the microsomes from one patient (M-1). Since the human liver microsomes showed low specific content of cytochrome P-450, a larger amount (five times) of microsomes was applied. All the microsomal samples tested showed a peroxidase-positive band(s) in the molecular weight region of cytochrome P-450, indicating that the liver microsomes of all the animal species contain a form(s) of cytochrome P-450 that is immunochemically related to P-450-male. Liver microsomes from a male patient (M-1) showed two bands though their intensities were still low. Both male and female dogs showed a single band. A difference in the pattern of immuno-stained bands between male and female animals was observed in rats, mice and hamsters but not in guinea pigs, rabbits and dogs.

Testosterone hydroxylase activities in liver microsomes from male and female animals of six species: Figure 2 shows the typical high-performance liquid chromatograms of testosterone metabolites formed by incubations with liver microsomes from male and female rats. Marked sex-difference in the metabolic pattern was observed in rats as shown previously (22, 33). Namely, male rats showed high activities to yield androstenedione, 2α-, 6β- and 16α-hydroxytestosterones as major metabolites (34), while female rats showed low activities and yielded 6β- and 7α-hydroxytestosterones with trace amounts of androstenedione (35). Activities of major testosterone hydroxylases in liver microsomes from various animal species are shown in Fig. 3. A significant sex-related difference was found in the testosterone 6β- and 7α-hydroxylase activities in hamsters. In 6β-hydroxylation, the activity was higher in females than in males, whereas in 7α-hydroxylation, males showed higher activity than females. Compared to rats and hamsters, other animals did not show such a significant sex difference. Androstenedione was formed as a common metabolite in all animal species, while qualitative as well as quantitative differences were observed among the species in the hydroxylases (not shown). The major metabolites in both male and female mice and guinea pigs were 6β-, 16α- and 7α-hydroxytestosterones. In addition to these metabolites, mice also produced a small amount of 15α-hydroxytestosterone. Rabbits afforded

![High-performance liquid chromatograms of testosterone metabolites formed by liver microsomes from male (A) and female (B) rats.](image)

Fig. 2. High-performance liquid chromatograms of testosterone metabolites formed by liver microsomes from male (A) and female (B) rats. Incubations were carried out as described under Materials and Methods. s, solvent; a, androstenedione; b, testosterone; c, 2α-hydroxytestosterone; d, 6β-hydroxytestosterone; e, cortisone (internal standard); f, 16α-hydroxytestosterone; g, 7α-hydroxytestosterone.
only two hydroxylated metabolites, 6β- and 16α-hydroxytestosterones. Though hamsters did not hydroxylate at the 16α-position, the animal gave a variety of hydroxylated products, 2β-, 6β-, 7α-, 15α- and 19-hydroxytestosterones. Liver microsomes from dogs showed 2β-, 6β- and 16α-hydroxylase activities with no sex difference. In general, 6β-hydroxylase activity was higher in all animal species employed.

**Testosterone hydroxylase activities in human liver microsomes:** Human liver microsomes showed the activities of 2β- and 6β-hydroxylases (Fig. 4), although the activities varied among individuals as shown in Fig. 5. Liver microsomes from two patients (M-3 and F-1) showed relatively high activities of the 6β-hydroxylase. However, 16α-hydroxylase was not detectable in all samples tested as in the case with hamster liver microsomes. Both the 6β- and 2β-hydroxylase activities did not correlate with the contents of cytochrome P-450, which were determined to be 0.034, 0.48, 0.38, 0.045 and 0.54 nmol per mg of microsomal protein for M-1, M-2, M-3, M-4 and F-1, respectively.

**Inhibition of liver microsomal testosterone**

| Species       | Sex | n | 6β-Hydroxylase | 7α-Hydroxylase | 16α-Hydroxylase |
|---------------|-----|---|----------------|----------------|-----------------|
| Rat           | M   | 5 |                |                |                 |
|               | F   | 5 |                |                |                 |
| Mouse         | M   | 5 |                |                |                 |
|               | F   | 5 |                |                |                 |
| Rabbit        | M   | 2 |                |                |                 |
|               | F   | 4 |                |                |                 |
| Guinea pig    | M   | 5 |                |                |                 |
|               | F   | 5 |                |                |                 |
| Hamster       | M   | 10|                |                |                 |
|               | F   | 10|                |                |                 |
| Dog           | M   | 4 |                |                |                 |
|               | F   | 4 |                |                |                 |

Fig. 3. Testosterone hydroxylase activities in liver microsomes from male (M) and female (F) rats, mice, rabbits, guinea pigs, hamsters and dogs. Statistical significance (P<0.01) was examined by Student’s t-test between male and female animals as indicated by (*). N.D.: not detectable.

**Fig. 4.** High-performance liquid chromatograms of testosterone metabolites formed by liver microsomes of patients M-3 (A) and F-1 (B). The incubations were carried out as described under Materials and Methods. s, solvent; a, androstenedione; b, testosterone; d, 6β-hydroxytestosterone; e, cortisone (internal standard); h, 2β-hydroxytestosterone. 16α-Hydroxytestosterone (f) was not observed in either of the chromatograms.

hydroxylases by anti-P-450-male IgG: Figure 6 shows the inhibitory effects of anti-P-450-male IgG on testosterone hydroxylases in liver microsomes from various animals, where
### Fig. 5. Testosterone hydroxylase activities in liver microsomes of male (M-1, 2, 3, 4) and female (F-1) patients. N.D.: not detectable.

|        | 2α-Hydroxylase (nmol/min/mg protein) | 6α-Hydroxylase (nmol/min/mg protein) |
|--------|-------------------------------------|-------------------------------------|
| M-1    | N.D.                                |                                     |
| M-2    | N.D.                                |                                     |
| M-3    |                                     |                                     |
| M-4    | N.D.                                |                                     |
| F-1    |                                     |                                     |

### Fig. 6. Inhibition by anti-P-450-male antibodies of testosterone hydroxylase activities in liver microsomes from male and female animals. Microsomes, pooled from at least 5 animals except for dogs, in which one male and one female dog were used, were preincubated with the indicated amount of anti-P-450-male IgG (closed circle) or rabbit preimmune IgG (open circle) at 25°C for 30 min prior to the enzymatic reaction. The activities obtained in the absence of the antibodies are shown as 100% (control) in the Figure. OHase: hydroxylase.
the hydroxylases that were clearly inhibited by the antibodies are presented. In the controls, the 16α-hydroxylase activities in male rats, male and female rabbits, and male and female dogs were 1.85, 0.711, 0.586, 0.628 and 0.337 nmol/min/mg protein, respectively; the 7α-hydroxylase activities in female rats, male and female mice, and male and female hamsters were 0.137, 0.050, 0.116, 1.20 and 0.443 nmol/min/mg protein, respectively; the 15α-hydroxylase activities in female mice and male and female hamsters were 0.058, 0.162 and 0.081 nmol/min/mg protein, respectively; the 2α-hydroxylase activity in male rats was 0.847 nmol/min/mg protein; and 2β-hydroxylase activity in humans (M-3) was 0.530 nmol/min/mg protein. Anti-P-450-male IgG inhibited not only testosterone 2α- and 16α-hydroxylases in liver microsomes from male rats but also various testosterone hydroxylases in microsomes from other animals. The result that the 2α- and 16α-hydroxylases in male rats were markedly inhibited by the antibodies indicates P-450-male is mainly responsible for these hydroxylations in the liver microsomes of male rats. A potent inhibition by the antibodies of the 7α-hydroxylase in the liver microsomes from female rats, male and female mice, and female hamsters was noted. The antibodies also inhibited partially the 7α-hydroxylase in male hamsters and completely inhibited the 15α-hydroxylase in female mice. The 15α-hydroxylase in microsomes from male and female hamsters was partially inhibited by the antibodies. The antibodies inhibited the activities of the 16α-hydroxylases in male and female dogs and rabbits though the extents of inhibition were weak. In contrast, the antibodies were without any effects on the 6β-hydroxylase in liver microsomes from all animal species tested including humans (not shown).

Discussion

Significant sex-related differences in the activities of drug metabolizing enzymes have been reported in rats and limited strains of mice (33, 36, 37). Some differences have been reported in renal as well as hepatic enzymes of mice (38–41). In the present study, however, we did not find any significant sex-related differences of testosterone hydroxylase activities in the ddY strain of mice. The discrepancy between the present and the reported results seems to be caused by the difference of the strain of mouse employed.

One or more bands were detectable on the nitrocellulose paper when liver microsomes from various animal species were analyzed immunochemically with antibodies to P-450-male. Though closely related cytochrome P-450 can be distinguished not by using polyclonal antibodies but by a monoclonal antibody (42), the polyclonal antibodies can be used to detect cytochromes P-450 corresponding to P-450-male. Unlike the monoclonal antibodies to P-450-male (21), the antibodies cross-reacted with proteins in the liver microsomes from female rats to show two bands on the nitrocellulose paper (Fig. 1). Although not shown in Fig. 3, the activity of testosterone 15α-hydroxylase in male and female mice were 0.050±0.022 and 0.017±0.010 nmol per min per mg of microsomal protein (n=5), respectively. As shown in Fig. 6, the activities of the 15α-hydroxylase was almost completely inhibited by the antibodies in female mice, while no apparent inhibition was seen in male mice. These results probably indicate that the 15α-hydroxylase in female mice is immuno-stained as a band located at the top among the visible bands.

Furthermore, we found a difference in the immuno-stained bands between male and female hamsters (Fig. 1). As can be seen, an immuno-stained band existed in the microsomes from female hamsters, which was nearly absent in males. In accordance with this result, we found a significant sex-related difference in the activities of testosterone 6β-hydroxylase in hamsters. From these results, we expected the inhibition of the 6β-hydroxylase by the antibodies. The results shown in Fig. 6, however, indicated that the antibodies inhibited the 7α-hydroxylase but not the 6β-hydroxylase. Thus, it can be assumed that a form of cytochrome P-450 catalyzing the 6β-hydroxylation is not visible on the nitrocellulose paper, and an immuno-stained band, probably a form of cytochrome P-450 that preferentially occurs in female hamsters, possesses other function(s). These results suggest that there are at least two
forms of cytochrome P-450 that are present in female hamsters in larger amounts than in males. In support of this view for the existence of a sex-difference in the cytochrome P-450 in hamsters, Colby et al. (43) demonstrated that corticosterone side chain metabolism was affected by gonadectomy and by treating hamsters with testosterone.

Recently, Yamazoe et al. (44) reported that testosterone 6β-hydroxylase in rats was regulated by pituitary hormones, but in a manner distinct from P-450-male. The present results, together with the results by Yamazoe et al. (44) and Colby et al. (43), support the view that a form(s) of cytochrome P-450 responsible for the 6β-hydroxylation is regulated by gonadal hormones via the hypophysis and leads to the occurrence of sex-related differences of steroid metabolism in some species of animals, including rats and hamsters.

We found detectable but only slight inhibitions by the antibodies of testosterone 16α-hydroxylase in the liver microsomes of male beagle dogs. Our recent experiments have realized the purification of a form of cytochrome P-450 (P-450-D1) from male beagle dogs, which was cross-reactive with the anti-P-450-male antibodies and showed the activity of testosterone 16α-hydroxylase. Details of the results will be published elsewhere.

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