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Sex and Strain Differences in Constitutive Expression of Fatty Acid ω-Hydroxylase (CYP4A-Related Proteins) in Mice

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The constitutive expression of hepatic fatty acid hydroxylase was examined in both sexes of ddY mice by measuring the activities of lauric acid ω-hydroxylase (LAH). The activity of male mice was significantly higher than that of female mice. Such a sex difference of hepatic LAH activity was not observed in other strains of mice, including BALB/c and C57BL/6. To examine whether decreased total P450 activities caused low LAH activity levels in female ddY mice, ethoxycoumarin *O*-deethylase activity, which is exhibited by many P450s, was measured in both sexes of mice. This activity had no sex difference. The developmental regulation of hepatic fatty acid hydroxylase was then examined by making consecutive measurements of LAH activity in ddY mice. The activity is the same in immature male and female mice, but is differentiated in the sexually mature state. Furthermore, in male mice, orchiectomy caused a dramatic decrease in hepatic LAH activity and the activity was restored by testosterone treatment to the level of the intact animal. In female mice, ovariectomy and estradiol treatment had no effect on the activity, but testosterone treatment caused an increase in the activity. The above data are consistent with the constitutive expression of CYP4A-related proteins measured by using anti-rat CYP4A1 polyclonal antibody. Anti-CYP4A1 antibody inhibited LAH activity, but not lauric acid (ω-1)-hydroxylase activity. These results suggest that some factors associated with male sex hormone are involved in the regulation of hepatic fatty acid ω-hydroxylase in ddY mice.

Key words: hormonal regulation, lauric acid ω-hydroxylase, mouse CYP4A, sex difference, strain difference.

Cytochrome P450s (CYPs) represent a large group of monooxygenases which are active in metabolizing hormones, fatty acids, drugs, and other xenobiotics. CYPs consist of a gene superfamily divided into families and subfamilies on the basis of sequence similarity (*1*). The CYP4A gene family is highly active in the metabolism of ω and (ω-1) hydroxylation of fatty acids, including arachidonic acid and prostaglandins, but does not metabolize foreign compounds (*2, 3*). In rodents, hepatic CYP4A proteins are induced by various peroxisome proliferators (PP) including fibrate hypolipidemic drugs (clofibrate and bezafibrate), phthalate ester plasticizers [di-(2-ethylhexyl)-phthalate, mono-(2-ethylhexyl)phthalate], a naturally occurring C19-steroid (dehydroepiandrosterone) and perfluoro fatty acids (*4, 5*). The CYP4A subfamilies in rats have been studied in detail and classified as CYP4A1, 4A2, and 4A3 (*6-8*). However, the expression of Cyp4a in mice has not been studied as much as that of rat CYP4A. Two clones of Cyp4a in mice were recently classified into Cyp4a10 and 4a12 (*9, 10*). In addition, it was reported that the regulation of this CYP subfamily is sexually controlled and is affected by testosterone in the kidney of C57BL/6 mouse (*11, 12*). In the present study, we examined developmental and hormonal regulation in the constitutive expression of hepatic fatty acid hydroxylase.

Abbreviations: CYP, cytochrome P450; ECOD, ethoxycoumarin *O*-deethylase; GH, growth hormone; LAH, lauric acid ω-hydroxylase; PP, peroxisome proliferator; PPAR, peroxisome proliferator-activated receptor.

MATERIALS AND METHODS

Materials—Testosterone propionate and 4-bromomethyl-7-methoxycoumarin were obtained from Tokyo Kasei Kogyo (Tokyo). 10-Hydroxydecanoic acid and ω-hydroxylauric acid were obtained from Sigma Chemicals (St. Louis, MO). (ω-1)-Hydroxylauric acid was synthesized as described by Azerad *et al.* (*13*). Sodium laurate was obtained from Nacalai Tesque (Kyoto). Other reagents were obtained from Wako Pure Chemicals (Tokyo).

Animal Treatment—Wistar rats and ddY mice were purchased from SLC Japan (Shizuoka). BALB/c and C57BL/6 mice were purchased from the Laboratory Animal Research Center of the Medical School, Tohoku University (Sendai). Food and water were given *ad libitum*. ddY mice were gonadectomized at 5 weeks of age and sex hormone treatments were started at 6 weeks of age. Testosterone propionate (in corn oil, 5 mg/2 ml/kg) or estradiol 3-benzoate (in corn oil, 0.5 mg/2 ml/kg) was given s.c. once every day for a week. Control animals were given just the vehicle (2 ml/kg). The animals were killed approximately 24 h after the last injection.

Assay of Lauric Acid Hydroxylase Activity—The reaction mixture (0.5 ml), which consisted of 50 μg of liver

microsomes and 100 nmol of sodium laurate in 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.4), was incubated for 20 min at 37°C in the presence of NADPH (final concentration of 1 mM). In the experiments on the inhibition of lauric acid hydroxylase by anti-CYP4A1 antibody (Daiichi Pure Chemicals, Tokyo), mouse liver microsomes were preincubated with anti-CYP4A1 antibody at 37°C for 30 min. The reaction was stopped by the addition of 1 N HCl (50 μl) and the reaction mixture was extracted three times with 1 ml of diethyl ether in the presence of 10 nmol of 10-hydroxydecanoic acid as an internal standard. After evaporation, the extracts were reacted with 10 mg of potassium carbonate and 4-bromomethyl-7-methoxycoumarin (2 μmol) in 0.5 ml of acetone for 60 min at 60°C in a sealed test-tube. After evaporation under reduced pressure, the residue was taken up in methanol (2 ml) and analyzed by HPLC (880-PU type intelligent HPLC pump, Nihon Bunko, Tokyo) on a reverse-phase column (CHEMCOSORB 5-ODS-OH, 4.6×150 mm, Chemco Pak) with an 821-FP type intelligent fluorometric detector (Nihon Bunko) (excitation at 328 nm, emission at 380 nm). The retention times of ω- and (ω-1)-hydroxylauric acids were 19 and 21 min, respectively, using a solvent system of methanol : H_2O (76 : 24) at a flow rate of 0.9 ml/min. Peak areas of ω- and (ω-1)-hydroxylauric acid relative to that of the internal standard were calculated using a Chromatopak C-R6A (Shimadzu, Tokyo).

Western Blot Analysis—SDS/PAGE was carried out according to the method of Laemmli (*14*), using 7.5% separating gels. Western blotting was performed as described by Towbin *et al.* (*15*). After separation, proteins were transferred electrophoretically to a nitrocellulose membrane and incubated with polyclonal antibodies against rat liver CYP4A1 (Daiichi Pure Chemicals). The protein that had reacted with the antibody was stained with rabbit anti-goat IgG antibody and horseradish peroxidase-labeled protein A plus 3,3′-diaminobenzidine.

Other Analytical Methods—Ethoxycoumarin *O*-deethylase (ECOD) was assayed by the method of Ullrich and Weber (*16*) as modified by Aitio (*17*). The protein concentration was assayed by the method of Lowry *et al.* (*18*). Cytochrome P450 contents were measured by the method of Omura and Sato (*19*). The results were statistically analyzed by the use of Student's *t* test.

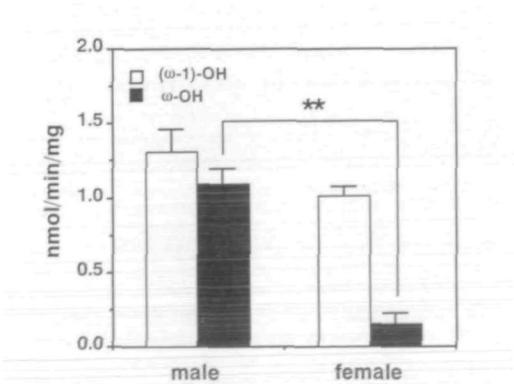

Fig. 1. **Lauric acid hydroxylase activity of hepatic microsomes in ddY mice.** (ω-1)-OH and ω-OH indicate lauric acid (ω-1)- and ω-hydroxylase activity, respectively. Each bar shows the mean ± SD for three mice. $^{**}p<0.01$. Details are given under "MATERIALS AND METHODS."

RESULTS

Sex Difference of Lauric Acid Hydroxylase Activity and CYP4A-Related Protein Levels in ddY Mice—Lauric acid ω-hydroxylase (LAH) activities in both sexes of ddY mice are shown in Fig. 1. LAH activities of male mice were significantly higher than those of female mice (approximately 7.2-fold), while lauric acid (ω-1)-hydroxylase activities were similar in both sexes. To ascertain the interrelation between LAH activity and CYP4A-related protein levels, liver microsomes isolated from ddY mice, and Wistar rats were analyzed by Western blotting using polyclonal antibodies raised against rat CYP4A1. As shown in Fig. 2, the level of CYP4A-related protein in ddY mice was higher in male mice (lane 1) than in female mice (lane 2). On the other hand, the constitutive levels of CYP4A proteins were similar (lanes 3 and 4) in both sexes of Wistar rats.

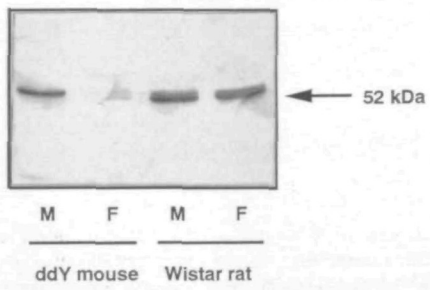

Fig 2 **Western blot analysis of CYP4A-related proteins of hepatic microsomes in ddY mice and Wistar rats.** Microsomal proteins (100 μg/lane) were separated on 7.5% gels by SDS/PAGE, transferred to nitrocellulose and incubated with a polyclonal antibody to CYP4A1 as detailed in "MATERIALS AND METHODS." Abbreviations M, male; F, female.

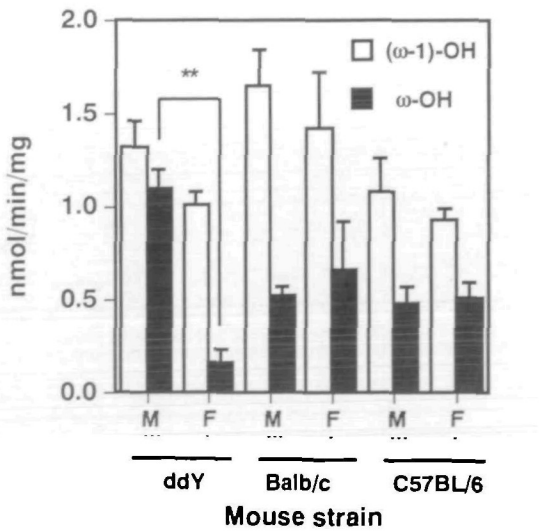

Fig 3. **Lauric acid hydroxylase activity of hepatic microsomes in mice of different strains.** (ω-1)-OH and ω-OH indicate lauric acid (ω-1)- and ω-hydroxylase activity, respectively. Abbreviations: M, male; F, female Each bar shows the mean ± SD for three mice $^{**}p<0.01$. Details are given under "MATERIALS AND METHODS."

Differences of Expressions of Lauric Acid Hydroxylase Activity and CYP4A-Related Protein Levels in Various Strains of Mice—We examined other strains of mice, including BALB/c and C57BL/6, to see whether the sex difference of LAH activities and CYP4A-related protein levels observed in ddY mice was also present in them. As shown in Fig. 3, LAH and lauric acid (ω-1)-hydroxylase activities were similar in both sexes of BALB/c and C57BL/6 mice. The polyclonal antibody to CYP4A1 identified two distinct proteins expressed in BALB/c and C57BL/6 liver (Fig. 4). The CYP4A-related protein of high molecular weight (band X) in male and that of low molecular weight (band Y) in female were present at approximately similar levels in both sexes of those strains of mice. However, a single band X was strongly detectable in male ddY mice. On the other hand, both bands X and Y were weakly detectable in female ddY mice. Levels of CYP4A-related proteins correlated well with the LAH activities in the three strains of mice. To examine whether the LAH activity observed in female ddY mice reflects a decrease of total P450 content, ECOD activities were measured in three strains of mice. As shown in Fig. 5, total P450 content and ECOD activity were similar in both sexes of the three strains. The above data showed that the low LAH activity observed in female ddY mice is not due to a decrease in P450 activity and the strain differences resulted from differences of constitutive expression of CYP4A-related proteins.

Development of LAH Activity and Expression of CYP4A-Related Proteins in ddY Mice Liver—The developmental expression of LAH activity was measured in ddY mice ranging from 3 to 9 weeks of age. LAH activities in both sexes were different at different ages (Fig. 6). LAH activities in both sexes were similar (approximately 0.5 nmol/min/mg) at 3 weeks of age. LAH activities in male mice were slightly increased at 5 weeks of age and reached higher levels (approximately 1.1 nmol/min/mg) at 7 and 9 weeks of age. On the other hand, LAH activities in female

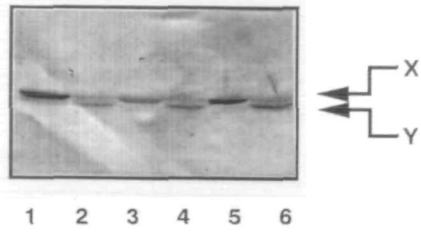

Fig 4 **Western blot analysis of CYP4A-related proteins in mice of different strains.** Liver microsomes were prepared from ddY, BALB/c, and C57BL/6 mice. Microsomal proteins (100 μg/lane) were separated on 7 5% gels by SDS/PAGE, transferred to nitrocellulose and incubated with a polyclonal antibody to CYP4A1 as detailed in "MATERIALS AND METHODS." Lane 1, male ddY; lane 2, female ddY; lane 3, male BALB/c, lane 4, female BALB/c, lane 5, male C57BL/6; lane 6, female C57BL/6.

P450 Content (nmol/mg)	0.509 ±0.012	0 485 ±0.057	0.585 ±0 039	0 474 ±0 06	0 475 ±0.037	0 489 ±0 038

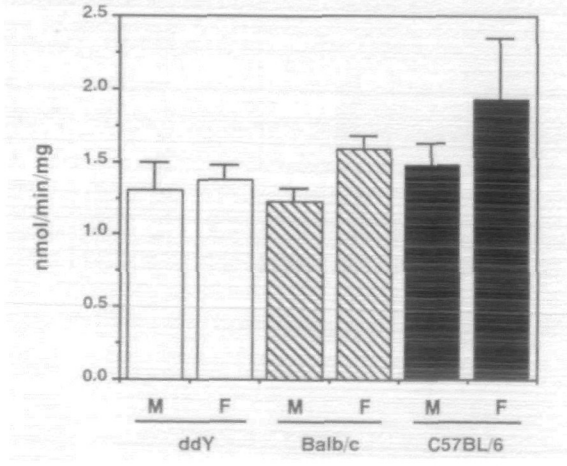

Fig. 5. **Ethoxycoumarin *O*-deethylase (ECOD) activity of hepatic microsomes in mice of different strains.** Abbreviations M, male, F, female. Each bar shows the mean±SD for three mice. Details are given under "MATERIALS AND METHODS."

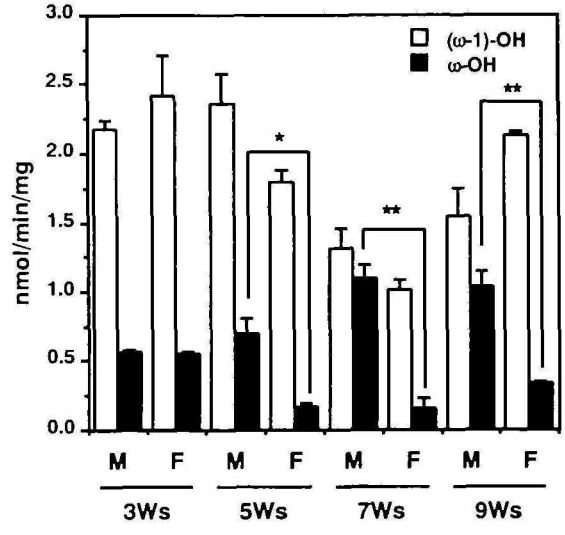

Fig. 6 **Developmental expression of hepatic lauric acid hydroxylase activity in ddY mice.** (ω-1)-OH and ω-OH indicate lauric acid (ω-1)- and ω-hydroxylase activity, respectively. Abbreviations: M, male; F, female Each bar shows the mean±SD for three mice. $*p<0$ 05, $**p<0.01$ Details are given under "MATERIALS AND METHODS."

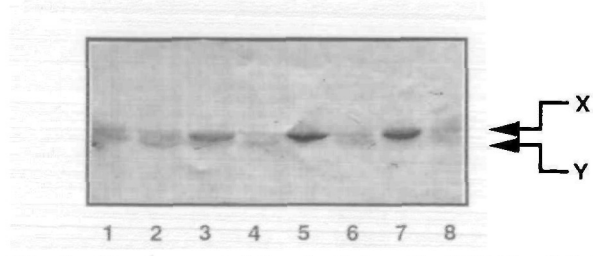

Fig. 7. **Developmental level of hepatic CYP4A-related proteins in ddY mice.** Liver microsomes were isolated from mice at the ages of 3, 5, 7, and 9 weeks. Microsomal proteins (100 μg/lane) were separated on 7.5% gels by SDS/PAGE, transferred to nitrocellulose and incubated with a polyclonal antibody to CYP4A1 as detailed in "MATERIALS AND METHODS." Lane 1, 3 weeks male; lane 2, 3 weeks female; lane 3, 5 weeks male; lane 4, 5 weeks female; lane 5, 7 weeks male; lane 6, 7 weeks female; lane 7, 9 weeks male; lane 8, 9 weeks female.

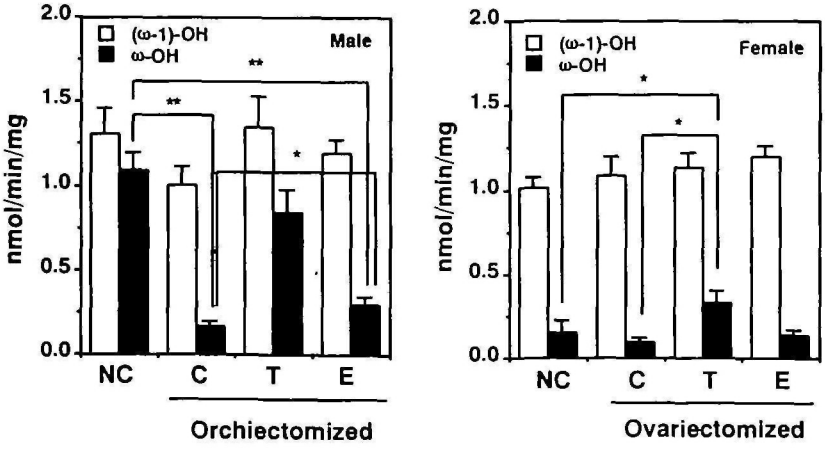

Fig. 8. **Effects of gonadectomy and sex hormone treatment on lauric acid hydroxylase activity in ddY mice.** (ω-1)-OH and ω-OH indicate lauric acid (ω-1)- and ω-hydroxylase activity, respectively. Abbreviations: NC, normal control; C, untreated control, T, testosterone propionate treatment, E, estradiol 3-benzoate treatment Each bar shows the mean±SD for three mice $*p<0.05$, $**p<0$ 01. Details are given under "MATERIALS AND METHODS "

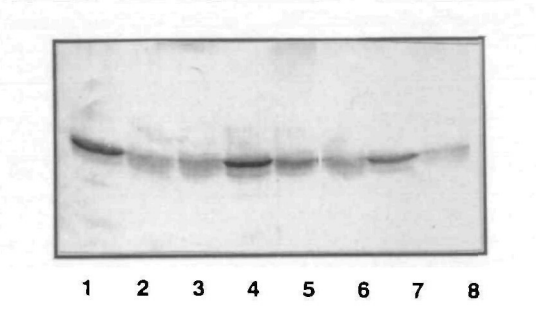

Fig 9. **Effects of gonadectomy and sex hormone treatment on the level of CYP4A-related proteins in ddY mice.** Liver microsomes were prepared from ddY mice. Microsomal proteins (100 μg/lane) were separated on 7.5% gels by SDS/PAGE, transferred to nitrocellulose, and incubated with a polyclonal antibody to CYP4A1 as detailed in "MATERIALS AND METHODS " Lane 1, intact male, lane 2, intact female; lane 3, orchiectomized male, lane 4, orchiectomized male treated with testosterone propionate; lane 5, orchiectomized male treated with estradiol 3-benzoate, lane 6, ovariectomized female; lane 7, ovariectomized female treated with testosterone propionate; lane 8, ovariectomized female treated with estradiol 3-benzoate

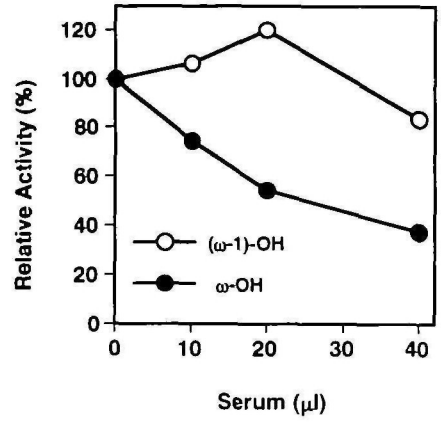

Fig. 10 **Inhibitory effect of anti-CYP4A1 antibody on lauric acid hydroxylase activities in male ddY mouse liver microsomes.** (ω-1)-OH and ω-OH indicate lauric acid (ω-1)- and ω-hydroxylase activity, respectively Mouse liver microsomes were preincubated with the indicated amounts of anti-CYP4A1 antibody at 37°C for 30 min Details are given under "MATERIALS AND METHODS "

mice decreased to approximately 0.15 nmol/min/mg at 5 weeks of age and maintained that level at 7 and 9 weeks of age. At 7 and 9 weeks of age, the ratios of LAH activity between male and female were approximately 7.2. As shown in Fig. 7, the CYP4A-related proteins, proteins in bands X and Y, were weakly detectable in both sexes at 3 weeks of age. However in male mice, the band X protein was increased and the band Y protein had disappeared at 5 weeks of age. The band X protein was markedly increased at 7 and 9 weeks of age. In female mice, these proteins (bands X and Y) disappeared gradually with sexual maturity.

Effects of Gonadectomy and Sex Hormone Treatment on Lauric Acid Hydroxylase Activity and CYP4A-Related Protein Levels in ddY Mice—To determine whether sex hormones regulate the level of male-specific CYP4A-related proteins, mice were gonadectomized, and treated with sex hormones. In male mice, as shown in Fig. 8, orchiectomy resulted in a significant decrease of LAH activity. The LAH activity of orchiectomized mice was restored by testosterone treatment to the level in intact males. On the other hand, estradiol treatment in orchiectomized mice resulted in a slight increase of the activity. In female mice, there was no significant change in LAH activity upon ovariectomy and estradiol treatment. However, in ovariectomized animals, testosterone treatment increased the activity (approximately 3.4-fold). The above data suggested that testosterone treatment enhanced the expression of LAH activity in gonadectomized mice. Figure 9 shows a Western blot analysis of CYP4A-related proteins in intact, gonadectomized and sex hormone-treated mice. These data are consistent with those on LAH activities.

Inhibition of Lauric Acid Hydroxylase Activity by Anti-CYP4A1 Antibody in Male ddY Mouse Liver Microsomes—As shown in Fig. 10, anti-CYP4A1 antibody dose-dependently inhibited LAH activity in male ddY mouse liver microsomes; 40 μl of anti-CYP4A1 antiserum inhibited more than 60% of the microsomal LAH activity. In contrast, lauric acid (ω-1)-hydroxylase activity was not inhibited by anti-CYP4A1 antibody.

DISCUSSION

This study has demonstrated that the constitutive expres-

sion of lauric acid ω-hydroxylase (CYP4A-related protein) is controlled by male sex hormone in ddY mice. However, it has been reported that C57BL/6 mice show no sex-related difference in the level of expression of LAH activity (*20*). The constitutive levels of CYP4A-related proteins were similar in C57BL/6 and BALB/c mice (Fig. 3). Furthermore, the relationship between CYP4A-related proteins (bands X and Y) and levels of LAH activity in the three strains of mice suggested that LAH activities are due mainly to the band X protein and the band Y protein in female mice. The lower LAH activity in female ddY mice may be due to a lower level of CYP4A-related proteins, especially the band Y protein. These results were supported by the developmental changes of the band Y protein from 3 to 9 weeks of age in ddY mice. In ddY mice, hepatic microsomal P450 contents, levels of components of the electron transport system (data not shown), and ECOD activity were similar in both sexes. These results suggest that the sex difference of LAH activity was a result of differences in the expression level of a P450 isozyme.

In the study of developmental changes of lauric acid hydroxylase activity, it was observed that levels of lauric acid (ω-1)-hydroxylase activity are vary with age. Changes in these levels may be related to the levels of various CYP isozymes that catalyze lauric acid (ω-1)-hydroxylation.

The changes in LAH activity were very similar to those in CYP4A-related proteins. The good correlation and the specific inhibition of LAH activity by anti-CYP4A1 antibody (Fig. 10) indicate that LAH activity is almost completely dependent on CYP4A-related proteins in liver microsomes.

Based on gonadectomy experiments, testosterone was considered to be a major regulatory factor in the constitutive level of hepatic lauric acid ω-hydroxylase. However, estradiol treatment of orchiectomized mice resulted in a slight increase of the LAH activity. This result suggested that the constitutive level of LAH proteins is regulated by the balance of hormones and is not determined by testosterone alone. Moreover, in ovariectomized mice testosterone treatment did not increase the level of the LAH activity to that in male mice. Various explanations are possible for these results. The effects of testosterone may be indirectly mediated by many regulatory factors, including growth hormone (GH). The inter-pulse interval of the circulating GH secretion pattern has been reported to be critical in regulating sexually dimorphic hepatic P450 expression in rats and mice (*21-23*). In relation to hormonal regulation of CYP4A families, it is known that the expression of hepatic CYP4A2 in the rat is male-specific and is regulated by growth hormone (*24*). The level of CYP4A-related protein in the mouse kidney is controlled by testosterone (*11, 12*). We also observed that the constitutive level of hepatic CYP4A-related protein in ddY mice is controlled by testosterone. As to the mechanism of this sex hormonal regulation, an element located at the 5′-flanking region of the Cyp4a gene may interact with a factor indirectly derived from sex hormones. Henderson *et al.* reported that the amino acid sequence derived from cloned cDNA of Cyp4a10 from C57BL/6 mouse was 92% identical with that of rat CYP4A1, and that the expression of Cyp4a proteins in liver and kidney was sexually differentiated in mature animals (*10*). They also found that the constitutive expression of Cyp4a10 mRNA was predominant in females. The CYP4A-related protein examined in this study seems to be different from Cyp4a10 and the regulatory interrelation between constitutive and inducible expression of Cyp4a is unclear.

In addition to the hormonal regulation, exogenous agents also play a role in the expression of these proteins in both liver and kidney. Of particular interest is the regulation of fatty acid metabolism by PP, such as clofibrate. Further, the orphan receptor, a peroxisome proliferator-activated receptor (PPAR) has been shown to react with PP and to affect the expression of CYP4A and peroxisomal enzyme genes (*25-27*). Although the mechanism of the expression in PPAR is unclear, the action of sex hormones may possibly be related to the PPAR-mediated signal transduction pathway.

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