Effects of Gonadectomy and Sex Hormones on the Induction of Hepatic CYP4A by Clofibrate in Rats

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The effects of sex hormones on the induction of hepatic CYP4A by clofibrate were examined in intact and gonadectomized rats of both sexes by measuring the activity of lauric acid ω-hydroxylation (LAH). Although the specific activity of LAH is the same in both sexes, only in male rats, was the activity significantly increased by administration of clofibrate. In female rats, ovariectomy or testosterone treatment resulted in an enhanced response to clofibrate, similar to that observed in male rats. In ovariectomized female rats, the effect of clofibrate was decreased by estradiol. In orchietomized male rats, testosterone had no effect on the induction of LAH by clofibrate and estradiol suppressed induction. Changes in LAH activities co-related well with those in CYP4A proteins and CYP4A1 mRNA. These results suggest that some factors associated with female sex hormones were involved in the suppressive regulation of CYP4A induced by clofibrate.

Key words CYP4A; lauric acid ω-hydroxylation; sex difference; hormonal regulation

The peroxisome proliferators are a structurally diverse group of chemicals, including the fibrate hypolipidemic drugs (clofibrate, bezafibrate), the phthalate ester plasticizers (DEHP, MEHP), the naturally occurring C19-steroid (DHEA) and perfluoro fatty acids.1–4 Administration of these proliferators to rodents causes hepatomegaly,5–7 induction of peroxisomal β-oxidation enzymes6,7 and the induction of cytochrome P450 (P450) which catalyses the ω-hydroxylation of fatty acids in liver.8 The response to clofibrate and several other peroxisome proliferators is sex-dependent,9–12 and the effects are less pronounced in female animals. The sex-difference in the induction of peroxisomal β-oxidation by perfluoro-octanoic acid is due to the androgen content.11 However, the contribution of sex hormones to the induction of CYP4A1 by clofibrate has not been clarified. Since induction of CYP4A1 in the endoplasmic reticulum is proposed to precede peroxisome proliferation,13 we examined the effect of sex hormones on the clofibrate induction of CYP4A using intact, gonadectomized rats of both sexes.

MATERIALS AND METHODS

Materials Testosterone propionate and 4-bromomethyl-7-methoxy coumarin were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Clofibrin acid, 10-hydroxycouanoic acid and ω-hydroxylauric acid were obtained from Sigma Chemicals (St. Louis, MO). (ω-1)-Hydroxy lauric acid was synthesized as described by Azered et al.14 Sodium laurate was obtained from Nacalai Tesque (Kyoto, Japan). Other reagents used were of the highest grade commercially available.

Animal Treatment Wistar rats were purchased from SLC Japan (Shizuoka, Japan) and were given food and water ad libitum. They were gonadectomized at 6 weeks of age and were treated with sex hormones at 7 weeks of age. To test for age differences rats were also gonadectomized at 3 weeks of age. Testosterone propionate (in corn oil, 5 mg/ml/kg) or estradiol 3-benzoate (in corn oil, 0.5 mg/ml/kg) were given s.c. once every day for 9 d.

CLOFIBRATE treatment was started at 8 weeks. Clofibrate was dissolved in corn oil and given i.p. at a dose of 250 mg/ml/kg for 3 d. Control animals received an equivalent volume of vehicle (1 ml/kg). The animals were sacrificed approximately 24 h after the last injection.

Assay of Lauric Acid Hydroxylation Activity The reaction mixture consisted of 100 μg liver microsomes, 100 nmol sodium laurate in 0.5 ml 0.1 m potassium phosphate buffer (pH 7.4) and incubation was carried out for 20 min at 37°C in the presence of NADPH (final concentration of 1 mm). After stopping the reaction by the addition of 1 n HCl (50 μl), the mixture was extracted three times with 1 ml diethyl ether in the presence of 10 nmol 10-hydroxycouanoic acid as internal standard. After evaporation, the extracts were reacted with 10 mg potassium carbonate and 4-bromomethyl-7-methoxy coumarin (2 μmol) in 0.5 ml aceton for 60 min at 60°C in sealed test-tubes. After evaporation under reduced pressure, the residue was dissolved in methanol (2 ml) and analysed by HPLC (880–PU type intelligent HPLC pump, Nihon Bunko, Tokyo, Japan) on a reverse-phase column (Chromsorb 5-ODS-OH, 4.6 × 150 mm, Chemco Pak) with an 821-FP type intelligent fluorometric detector (Nihon Bunko, Tokyo, Japan) (excitation at 328 nm, emission at 380 nm). ω- and (ω-1)-Hydroxy lauric acids were eluted at retention times of 19 and 21 min, respectively, using a solvent system of methanol-H2O (76:24) at a flow rate of 0.9 ml/min. Peak area ratios of ω- and (ω-1)-hydroxylauric acid to internal standard were calculated using a Chromatopak C-R6A instrument (Shimadzu, Tokyo, Japan).

Primary Culture of Hepatocytes Hepatocytes were isolated from liver of Wistar rats at 8 weeks of age by a collagenase perfusion method described by Williams.15–17 Freshly isolated cells (3 × 10⁶/plate) were seeded on 100 mm-diameter culture dishes (FALCONE) in 10 ml Waymouth's MB752/1 medium containing 10⁻⁷ m insulin, 10⁻⁷ m dexamethasone, 50 ng/ml kanamicin sulphate and 5% newborn bovine serum. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2/95% air, and the medium was replaced every 24 h. After 4 h, the

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culture medium was changed to serum-free medium. Chemicals were dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium so that the final concentration of DMSO was 0.4% (v/v) in all culture groups. Control/control (C/C) groups were exposed to only DMSO at all times. Control/clofibric acid (C/CL) groups were exposed to DMSO from 4 to 24 h and then to clofibric acid (1 mm) up to 48 h. Testosterone/clofibric acid (TP/CL) and estradiol/clofibric acid (EB/CL) groups were exposed to 0.2 μM hormones, respectively, from 4 to 24 h and then to clofibric acid up to 48 h. Cells from eight to ten dishes were harvested with a rubber policeman, washed with PBS and disrupted by sonication. The disrupted cells were centrifuged at 9000 × g for 20 min and the supernatant was centrifuged at 105000 × g for 1 h. The pellet (microsomal fraction) was suspended in 10 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA and stored at −80°C until use.

Analysis of RNA Total RNA was determined by the guanidinium thiocyanate method\(^\text{18}\) from frozen liver. Twenty μg RNA denatured and fractionated in formaldehyde-containing agarose gel (0.9%) was transferred to nylon filters for Northern blotting.\(^\text{19}\) Hybridization was carried out using 5'-end-labelled oligonucleotide of CYP4A1 mRNA (ON-29, 5'-TATGGGAAGGTGCCTGCTGGCTT-3', corresponds to the complement of nucleotides 1701–1720, 3'-untranslated region).\(^\text{20}\)

Western Blot Analysis Western blotting was carried out by the method of Sundseth and Waxman\(^\text{20}\) using polyclonal antibodies to rat liver CYP4A1 (Daichi Pure Chemicals, Tokyo, Japan) that are crossreactive with CYP4A2 and 4A3.

Other Analytical Methods Protein concentration was assayed by the method of Lowry et al.\(^\text{21}\) cytochrome P450 and cytochrome b2 (Cyt.b2) were measured by the method of Omura and Sato\(^\text{22}\) NADPH-cytochrome P450 reductase (p450) was assayed by the method of Omura and Takesue.\(^\text{23}\) The results were statistically analyzed using Student’s t-test.

RESULTS

Effects of Sex Hormones on the Induction of Lauric Acid Hydroxylation by Clofibrate Figure 1 shows the induction of lauric acid ω-hydroxylase (LAH) by clofibrate in intact rats. The LAH activity in intact rats was approximately the same for both sexes. Treatment with clofibrate caused a significant increase in hepatic LAH, but females were less responsive.

To determine if sex hormones play a role in the induction of clofibrate, rats were gonadectomized at 6 weeks of age and treated with sex hormones. In male rats, there were no significant change in the inducibility of LAH activity by clofibrate in intact, orchietomized and testosterone-treated orchietomized animals (21.8, 22.5, 22.4-fold induction, respectively). However, in male rats, as shown in Fig. 2, estradiol treatment of orchietomized animals slightly decreased the induction of LAH by clofibrate. In female rats, as shown in Fig. 3, ovariection caused a

![Fig. 1. The Induction of Lauric Acid Hydroxylase by Clofibrate](image1)

(![](image1.png))

Fig. 1. The Induction of Lauric Acid Hydroxylase by Clofibrate

(ω-1)-OH and ω-OH indicate lauric acid (ω-1-) and ω-hydroxylase activity respectively. Abbreviations: C, untreated; CL, clofibrate treatment. Each bar shows the mean ± S.D. for three rats. **p < 0.01.

![Fig. 2. Effect of Sex Hormones on the Induction of Lauric Acid Hydroxylase by Clofibrate in Male Rats at 6 Weeks of Age](image2)

![Fig. 3. Effect of Sex Hormones on the Induction of Lauric Acid Hydroxylase by Clofibrate in Female Rats at 6 Weeks of Age](image3)
slight increase in the induction of LAH by clofibrate. Testosterone treatment of ovariectomized female rats resulted in the induction of LAH by clofibrate reaching the level of intact male rats. Estradiol treatment of ovariectomized rats promoted inductibility by clofibrate reaching the level observed in intact female rats. Since it has been observed that the age at gonadectomy affected the expression patterns of P450 isozyme, we compared rats at 3 weeks of age, the juvenile period, with animals at 6 weeks of age. The results were similar in both cases (Fig. 4).

Changes in the P450 Content and Components of the Electron Transport System As shown in Table 1, the P450 content was increased by clofibrate treatment in all groups of male rats. These increases in male rats were closely related to induction of LAH by clofibrate (Fig. 2). In female rats, the P450 contents was increased by clofibrate, but to a lesser degree than in males. Fp2 activity was unchanged in males but increased in females by clofibrate. These changes in Fp2 activity were related to altered patterns of LAH activity. The concentrations of Cyt.b2 were unchanged by clofibrate treatment in all groups of rats.

Western Blot Analysis of CYP4A Proteins in Livers To ascertain the relationship between LAH activity and CYP4A protein levels, rat liver microsomes isolated from experimental animals were analyzed by the Western blot technique using a polyclonal antibody against CYP4A1. As shown in lane 2 and lane 4 of Fig. 5, the levels of CYP4A proteins were higher in intact males than in females. In ovariectomized male rats, the level of CYP4A proteins was reduced by estradiol (lane 7), but was unchanged by testosterone (lanes 5 and 6). In contrast, in ovariectomized female rats, the level of CYP4A proteins was slightly increased by clofibrate (lane 8). Treatment with estradiol in ovariectomized rats reduced the induction of protein by clofibrate to the levels seen in intact female rats (lane 10). In ovariectomized female rats, CYP4A protein expression by clofibrate was enhanced by treatment with testosterone compared with that of ovariectomized controls (lane 9). Changes in CYP4A proteins expression agreed well with changes in LAH activity.

Northern Blot Analyses of CYP4A1 mRNA The above data suggest that estradiol treatment suppresses the induction of CYP4A1 by clofibrate in gonadectomized rats. Figure 6 shows the Northern blot analysis of CYP4A1 mRNA in intact, gonadectomized and sex-hormone treated rats. These data are consistent with the above catalytic activity and Western blot data.

<table>
<thead>
<tr>
<th>Rats</th>
<th>Treatment</th>
<th>Clofibrate</th>
<th>Total P450 (nmol/mg)</th>
<th>Fp2 (nmol/min/mg)</th>
<th>Cyt.b2 (nmol/mg)</th>
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</thead>
<tbody>
<tr>
<td>Male</td>
<td>Non-operated</td>
<td>-</td>
<td>0.449 ± 0.020</td>
<td>84.0 ± 5.4</td>
<td>0.299 ± 0.000</td>
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<tr>
<td>Male</td>
<td>Non-operated</td>
<td>+</td>
<td>0.998 ± 0.042a</td>
<td>86.5 ± 4.9</td>
<td>0.299 ± 0.004</td>
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<tr>
<td>Male</td>
<td>Ovariectomized</td>
<td>-</td>
<td>0.385 ± 0.047</td>
<td>62.6 ± 10.1</td>
<td>0.268 ± 0.010</td>
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<tr>
<td>Male</td>
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<td>+</td>
<td>0.928 ± 0.087</td>
<td>74.3 ± 3.9</td>
<td>0.388 ± 0.005</td>
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<tr>
<td>Male</td>
<td>Ovariectomized + TP</td>
<td>+</td>
<td>1.184 ± 0.050a</td>
<td>86.7 ± 5.2</td>
<td>0.303 ± 0.013</td>
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<td>Male</td>
<td>Ovariectomized + EB</td>
<td>+</td>
<td>0.720 ± 0.048a</td>
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<tr>
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<td>0.299 ± 0.020</td>
<td>52.8 ± 4.6</td>
<td>0.241 ± 0.020</td>
</tr>
<tr>
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<td>97.6 ± 5.0a</td>
<td>0.268 ± 0.007</td>
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<tr>
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<td>-</td>
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<td>55.3 ± 2.2</td>
<td>0.284 ± 0.012</td>
</tr>
<tr>
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<td>96.7 ± 11.3</td>
<td>0.285 ± 0.017</td>
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<tr>
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<td>+</td>
<td>0.797 ± 0.094a</td>
<td>114.2 ± 2.8a</td>
<td>0.299 ± 0.013</td>
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<tr>
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<td>+</td>
<td>0.590 ± 0.061a</td>
<td>86.1 ± 8.8</td>
<td>0.304 ± 0.037</td>
</tr>
</tbody>
</table>

Gonadectomy was carried out at 6 weeks of age. Values are expressed as means ± S.D. for three rats. a) Statistically different from non-operated (clofibrate −) rats (p < 0.01). b) Statistically different from non-operated (clofibrate +) rats (p < 0.01). c) Statistically different from non-operated (clofibrate +) rats (p < 0.05).
hormones had no effect on the induction of LAH in cultured hepatocytes isolated from male rats. These results indicate that sex hormones are indirect modulators of hepatic CYP4A induction by clofibrate.

**DISCUSSION**

In gonadectomized rats, estradiol suppressed the induction of CYP4A by clofibrate (Figs. 1—4). This result agrees with the report of Kawashima et al.\(^\text{11}\) which showed an estradiol-dependent suppressive effect on peroxisomal β-oxidation by perfluorooctanoic acid, a peroxisome proliferator. Moreover, they reported that androgen enhanced peroxisomal β-oxidation by the same inducer. However, in our experiments, testosterone had no effect on the induction of CYP4A by clofibrate in male rats. These results lead to the hypothesis that the regulation mechanism of CYP4A by testosterone differs from that of peroxisomal β-oxidation enzymes.

We have examined the relationship between the activity of fp2 and LAH activity in female rats (Table 1). The expression of both CYP4A and fp2 is controlled by both clofibrate and female hormones. As shown in Fig. 5 by Western blot analysis using anti-rat CYP4A1 polyclonal antibody, the changes in LAH activity correlated with the amount of CYP4A proteins. Northern blot analysis also revealed the relationship between changes in LAH activity and the amount of CYP4A1 mRNA (Fig. 6). These results suggest that the suppressive effect of estradiol is due to the pretranslational step of CYP4A. Thus, we examined the hepatic CYP4A induction of clofibrate by using a primary culture system of rat hepatocytes and showed that sex hormones are indirect modulators of the hepatic CYP4A induction by clofibrate (Fig. 7). The sex hormones may act as an indirect modulator, as in the modulation by growth hormone (GH) reported by Sugiyama et al.\(^\text{23}\) in the regulation of peroxisomal β-oxidation by clofibrate. This effect is similar to the low responsiveness to phenobarbital-induced expression of CYP2B1 and 2B2 in female rats.\(^\text{26—28}\) The low inducibility of CYP2B in the female rats is explained by the sustained low levels of GH and thyroxine. Imaoka et al.\(^\text{29}\) have reported that the expression of CYP4A2 in rat kidney is regulated by a thyroid hormone.

The orphan receptor, peroxisome proliferator-activated receptor (PPAR) has been shown to be reactivated by foreign chemicals, such as clofibric acid and to affect the expression of CYP4A and peroxisomal enzyme gene.\(^\text{30—32}\) Although the mechanism of expression in PPAR is unclear, the action of sex hormones may possibly be related to a PPAR-mediated signal transduction pathway.

**REFERENCES**