Fenofibrate, a ligand for PPARα, inhibits aromatase cytochrome P450 expression in the ovary of mouse

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Abstract Peroxisome proliferator-activated receptors (PPARs) play important roles in the metabolic regulation of lipids including steroids. In this study, we investigated whether fenofibrate, a ligand for PPARα, could influence estrogen synthesis in vivo in the ovary of mice. As reported, chronic treatment of C57BL6/J female mice with various amounts of fenofibrate as a diet reduced the serum triglycerides level and induced hepatomegaly in a dose-dependent manner. Northern blot analyses using hepatic RNA confirmed the induction of classical PPARα-target genes including acyl-CoA oxidase and lipoprotein lipase. The analyses using ovarian RNA revealed the suppression of gene expression for enzymes involved in steroidogenesis including CYP11A, CYP19, steroidogenic acute regulatory protein, and HDL receptor, but the CYP17 expression was evidently induced. Consistent with the suppression of CYP19 mRNA expression, the aromatase activity in the ovary was dose-dependently inhibited, resulting in significant decreases in the uterine size and bone mineral density. When PPARα null mice were treated with dietary fenofibrate, neither hepatomegaly nor inhibition of ovarian aromatase activity was observed, rather the activity was enhanced. These results demonstrate that fenofibrate inhibits ovarian estrogen synthesis by suppressing the mRNA expressions and that functional PPARα is indispensable for the inhibitory action of the agent in vivo.—Toda, K., T. Okada, C. Miyaura, and T. Saibara. **Fenofibrate, a ligand for PPARα, inhibits aromatase cytochrome P450 expression in the ovary of mouse.** ***J. Lipid Res.*** **2003.** 44: **265–270.**

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Fibrates and their derivatives (bezafibrate, fenofibrate, clofibrate, and clinofibrate) constitute a group of hypolipidemic agents that are widely used in the treatment of hypertriglyceridemia and combined hyperlipidemia, being particularly effective in lowering the plasma triglyceride and cholesterol levels (1, 2).

Administration of fibrates to rodents results in numerous hepatic alterations, including hepatomegaly, an increase in the number and size of peroxisomes, and an increase in the expression of genes encoding peroxisomal, mitochondrial, and microsomal fatty acid-metabolizing enzymes. These alterations in the liver cause reduction of lipid levels in the serum as well as in adipose tissues (3). Previously, we reported that bezafibrate or fenofibrate was effective in restoring hepatic steatosis due to estrogen-insufficiency in mice (4–6).

The pharmacological actions of fibrates have been found to be mediated through activation of the peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear hormone receptor superfamily (3). Activated PPARα binds as a heterodimer with the retinoid X receptor to a peroxisome proliferator-response element located in the promoter region of target genes, which results in transcriptional activation of the target (7) genes (8).

Apart from the genes coding for enzymes involved in fatty-acid metabolism, recent studies have demonstrated that activated PPARα regulates the expression of genes for enzymes involved in steroid metabolism. Those include 17β-hydroxysteroid dehydrogenase IV, 11β-hydroxysteroid dehydrogenase I, and 3β-hydroxysteroid dehydrogenase V (9–11). Furthermore, progesterone production in human choriocarcinoma JEG-3 cells (12) and in Leydig cells (13) is reported to be inhibited by a PPARα-mediated pathway.

To better understand the molecular mechanisms of the pleiotropic responses induced by activated PPARα, we investigated its effects on the ovarian estrogen synthesis in vivo using mice. The data demonstrate that fenofibrate inhibits aromatase activity by suppressing the mRNA expression in a dose-dependent manner, and further that functional PPARα is required for the agent to exhibit inhibitory action in vivo.

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MATERIALS AND METHODS

Materials

A standard rodent chow (NMF) was obtained from Oriental Yeast (Tokyo, Japan). A kit for the measurement of triglyceride was purchased from Nissui Ltd. (Tokyo Japan). Fenofibrate was from Sigma-Aldrich Japan (Tokyo, Japan). ISOGEN was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A Zeta-Probe blotting membrane was obtained from Bio-Rad (Hercules, CA). [α-^{32}P]dCTP was purchased from ICN Biomedicals, Inc (Costa Mesa, CA). pBluescript SKII(−) was obtained from Stratagene (La Jolla, CA). All other chemicals were of analytical grade and obtained commercially.

Animals

Animal care and experiments were carried out in accordance with institutional animal regulations. PPARα null mice (14) were obtained from the Jackson Laboratory. C57BL6/J and PPARα null female mice were maintained on a 12 h light/dark cycle at 22–25°C and given water and rodent chow diet with or without fenofibrate ad libitum.

The chow diets supplemented with fenofibrate were prepared by impregnation with fenofibrate, which was dissolved in acetone as described previously (15). C57BL6/J female mice at 7 weeks of age were divided into five diet groups and fed a diet containing various amounts of fenofibrate for 4 weeks: 0% (n = 6), 0.005% (n = 11), 0.02% (n = 14), 0.1% (n = 19), and 0.4% (n = 13). PPARα null female mice at the same age were fed a normal diet with 0% (n = 5) or 0.1% (n = 5) fenofibrate for 4 weeks and used for analysis.

Aromatase activity

Four to six ovaries were used to prepare microsomal fractions (16). Aromatase activity in the fractions was measured by a tritiated water-release assay using 1β-[^{3}H]androst-4-ene-3,17-dione as a substrate as described previously (17). An inhibitor of aromatase, 4-androsten-4-ol-3,17-dione, was included at 10 μM. Aromatase activity was expressed as picomoles of [^{3}H]water released per mg protein per h.

Histological examination

Ovaries from the mice treated with fenofibrate were fixed in a solution of 10% buffered formalin for 24 h, dehydrated in graded ethanol, and embedded in paraffin. Sections were cut 4 μm thick and stained with hematoxylin-eosin.

Measurement of plasma triglyceride concentration

Plasma was prepared from the blood samples collected from the tail vein of mice after they were fasted for 5 h. Concentrations of triglyceride were measured by colorimetric methods using 10 μl of the plasma with a kit.

Northern blots

Total RNA was prepared from the livers and ovaries using ISOGEN. Equal aliquots of total RNA (15 μg) were denatured with formaldehyde and formamide, subjected to electrophoresis in a 1.0% agarose gel, and transferred to a blotting membrane for hybridization (18). cDNA probes were prepared by PCR amplification using oligo d(T)-primed cDNA derived from ovarian RNA or hepatic RNA as a template with the following sets of primers: P450scc (CYP11A) (a 1,273-bp fragment with sense primer: 5′-TACGTGGCAGGGTCCAGTGCTGAGT-3′, and antisense primer: 5′-GAAGTGGGTGGTATTTTGGCTTTTT-3′), $P450_{17\alpha}$ (CYP17) (a 407-bp fragment with sense primer: 5′-TGGTGCACAATCCTGAGGTG-3′, and antisense primer: 5′-AAAGGGTCGATCAGAAAGACCACCTTGGGG-3′), HDL receptor (a 604-bp fragment with sense primer: 5′-CCATGAAGCTGACCTACAACGAATCAA-3′, and antisense primer: 5′-TCCTGGGAGCCCTTTTTACTACCACTC-3′), steroidogenic acute regulatory protein (StAR) (a 484-bp fragment with sense primer: 5′-AAGCCAGCAGGAGAACGGGGACGAA-3′, and antisense primer: 5′-TAGGACCTGGTTGATGATTGTCTTC-3′), PPARα (a 720-bp fragment with sense primer: 5′-CCTGTCTGTCGGGATGTCACACAATGC-3′, and antisense primer: 5′-TGCAACTTCTCAATGTAGCCTATGTTT-3′), lipoprotein lipase (LPL) (a 1,101-bp fragment with sense primer: 5′-GGCCGCAGCAGACGCAGGAAGAGATTT-3′, and antisense primer: 5′-AAGAAGGAGTAGGTTTTATTTGTGGAA-3′), and 17β-hydroxysteroid dehydrogenase (17β-HSD) (a 765-bp fragment with sense primer: 5′-AAATAGCCATAGATGCTGGTTTCC-3′, and antisense primer: 5′-TCATGTCCTCTGAAGTCAACTGAA-3′). The amplified products were cloned into the *Eco*RV site of pBluescript SKII(−). The cDNA fragments for acyl-CoA oxidase, $P450_{arom}$(CYP19), FSH receptor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared as described previously (4, 19). The cDNA probes were labeled with [α-^{32}P]dCTP using a Klenow fragment. The filters were hybridized with the indicated ^{32}P-labeled probes (∼1×10^6 cpm/ml) for 12 h at 42°C, washed three times with 0.1% (w/v) SDS/0.1× SSC at 50°C for 30 min, and exposed at −80°C to Fuji X-ray film (Fuji Photofilm Inc.) with intensifying screens for 12–36 h. The quantification of the signals was done with BAS2000 (Fujifilm Inc. Tokyo, Japan). The results were normalized to the signal generated from GAPDH mRNA.

Radiographic analysis of the femur

Radiographs of the femurs were taken with a soft X-ray generator (model CMB-2; SOFTEX, Tokyo, Japan) (20). The bone mineral density (BMD) of the femurs was measured using a dual X-ray absorptiometer (model DCS-600R; Aloka, Tokyo, Japan), as reported previously (20).

Statistic analysis

Data are expressed as the means ± SEM. The significance of the differences was analyzed using the Student's *t*-test employing InStat software (GraphPad Software, Inc., San Diego, CA). A P value less than 0.05 was considered significant.

RESULTS

Effects of fenofibrate on gene expression involved in hepatic fatty acid metabolism

When wild-type female mice were fed a chow diet containing 0%, 0.005%, 0.02%, 0.1%, and 0.4% of fenofibrate for 4 weeks, the liver-body weight ratio increased from 0.052, 0.051, 0.064 ($P < 0.02$, vs. 0%), 0.103 ($P < 0.001$), and 0.138 ($P < 0.001$), respectively, showing induction of dose-dependent hepatomegaly. Furthermore, fenofibrate treatment resulted in a significant decrease in the fasted serum levels of triglycerides from 29.8 ± 1.7 mg/dl (0%) to 15.7 ± 3.3 mg/dl (0.005%, $P < 0.02$ vs. 0%), 9.8 ± 0.8 mg/dl (0.02%, $P < 0.001$ vs. 0%), 13.9 ± 1.5 mg/dl (0.1%, $P < 0.001$ vs. 0%), and 11.9 ± 1.4 mg/dl (0.4%, $P < 0.001$ vs. 0%). The effects of fenofibrate on the liver were also examined by measuring the mRNA expression of genes for acyl-CoA oxidase, LPL, 17β-HSD, and HDL receptor (**Fig. 1**). The expression level of acyl-CoA oxidase and LPL was dose-dependently elevated in

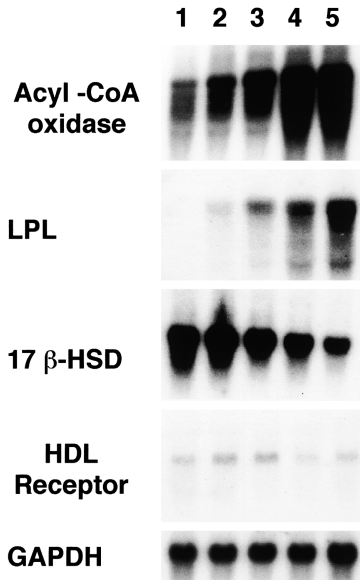

Fig. 1. Alterations in the gene expression in the liver of mice treated with fenofibrate. The expression of acyl-CoA oxidase, LPL, HDL receptor, 17β-HSD, and GAPDH mRNAs was analyzed by Northern blot hybridization using 15 μg of total RNA from the livers of C57BL6/J female mice. The mice were fed a chow diet supplemented with 0% (lane 1), 0.005% (lane 2), 0.02% (lane 3), 0.1% (lane 4), 0.4% (lane 5) fenofibrate from 7 weeks of age for 4 weeks.

the mice treated with fenofibrate. In contrast, the expression of 17β-HSD, which participates in the metabolism of steroids (21), was suppressed in a dose-dependent manner. Fenofibrate did not influence the expression of HDL receptor in the liver. These hepatic effects of fenofibrate are in good agreement with earlier studies (8, 22).

Effects of fenofibrate on the ovary, uterus, and bone

Northern blot hybridization using ovarian RNA from mice treated with fenofibrate revealed the suppression of mRNA expression involved in steroidogenesis, including CYP11A, CYP19, HDL receptor, and StAR (**Fig. 2**). In contrast, the expression of CYP17 was increased ~2-fold over that of the untreated mice by the treatment with more than 0.02% fenofibrate. Fenofibrate did not alter the expression level of PPARα mRNA in the ovary. The expression of FSH receptor mRNA was not clearly influenced by fenofibrate as observed in the genes involved in steroidogenesis. These results demonstrate that fenofibrate does not affect the general gene expression levels, but it suppresses those involved in steroidogenesis.

Because the expression level of the CYP19 transcripts is reduced by the treatment with fenofibrate, the aromatase activity was measured using ovarian microsomal fractions. As shown in **Fig. 3A**, the aromatase activity of control ovaries was 6.6 ± 1.3 pmol/mg protein/h under our experimental conditions. When 4-androsten-4-ol-3,17-dione, an inhibitor of aromatase, was included in the reaction mixtures, more than 95% of the control activity was inhibited. Treatment with fenofibrate caused a dose-dependent inhibition of the aromatase activity, with 0.4% fenofibrate resulting in a reduction of the activity to a nearly undetectable level. A dose-dependent reduction in the uterine weight was observed in the mice treated with fenofibrate (Fig. 3B). Furthermore, bone mineral density at femur was also reduced in a dose-dependent manner (Fig. 3C). The uterine and skeletal phenotypes strongly support the idea that fenofibrate inhibits estrogen synthesis in vivo.

Histological analysis of the ovaries demonstrated the absence of typical corpus lutea and presence of many atretic follicles in the mice treated with 0.4% fenofibrate (**Fig. 4**).

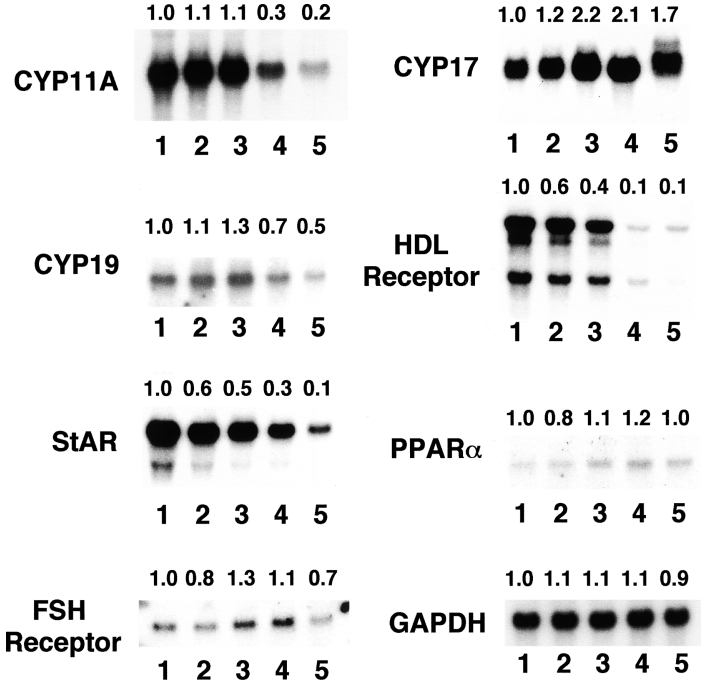

Fig. 2. Alterations in the gene expression in the ovaries of mice treated with fenofibrate. The expression of CYP11A, CYP17, CYP19, HDL receptor, StAR, PPARα, FSH receptor, and GAPDH mRNAs was analyzed by Northern blot hybridization using 15 μg of total RNA from the ovaries of C57BL6/J female mice. The mice were fed a chow diet supplemented with 0% (lane 1), 0.005% (lane 2), 0.02% (lane 3), 0.1% (lane 4), 0.4% (lane 5) fenofibrate from 7 weeks of age for 4 weeks. The number at the top of each lane represents the mean of expression level of the gene relative to that of the untreated mice. The quantification of the expression levels was made in two independent experiments.

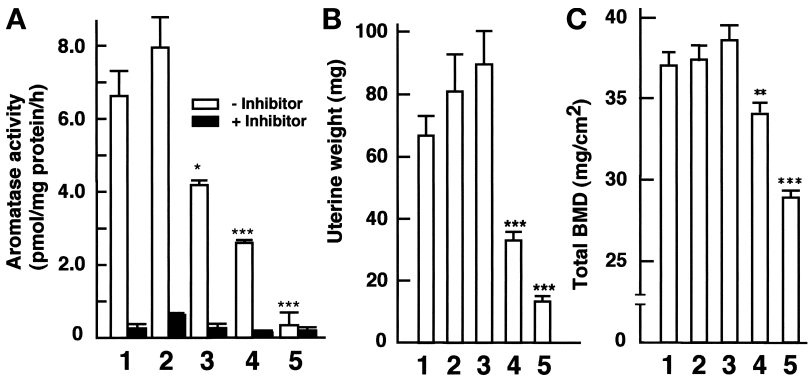

Fig. 3. Alterations in the ovarian aromatase activity, uterine size, and bone mineral density. A: The female mice were fed a diet containing 0% (lane 1), 0.005% (lane 2), 0.02% (lane 3), 0.1% (lane 4), 0.4% (lane 5) fenofibrate from 7 weeks of age for 4 weeks. The aromatase activity was measured using the tritiated water-releasing method. Microsomal fractions prepared from the ovaries were incubated with 1β-[^{3}H]androst-4-ene-3,17-dione,[1β-^{3}H(N)] at 37°C for 1 h in the absence (open bar) or presence (closed bar) of 10 μM 4-androsten-4-ol-3,17-dione, an aromatase inhibitor. The radioactivity released from the substrate in the form of H_2O was determined. The activity is expressed as picomoles of tritiated water released/mg protein/h. Each bar represents the mean ± SEM of three experiments. B: Effects of dietary fenofibrate on uterine weight were measured. C: Bone mineral density (BMD) in the total area of the femur was measured. The data are expressed as the mean ± SEM. Significantly different from untreated mice. $^{**}P < 0.02$, $^{***}P < 0.001$.

Presence of many atretic follicles is a symptom observed in the ovaries of mice with estrogen-insufficiency (19, 23).

In order to assess functional consequences of the alterations of folliculogenesis, the estrous cycle was examined by histological analysis of cell populations collected from vaginas. The mice that received fenofibrate at the concentration less than 0.02% showed a regular estrous cycle, whereas the mice treated with 0.4% fenofibrate displayed a complete lack of the cycle, showing the presence of leukocytes in mucous secretions, which is an image similar to that observed in the mice lacking the functional CYP19 gene (19).

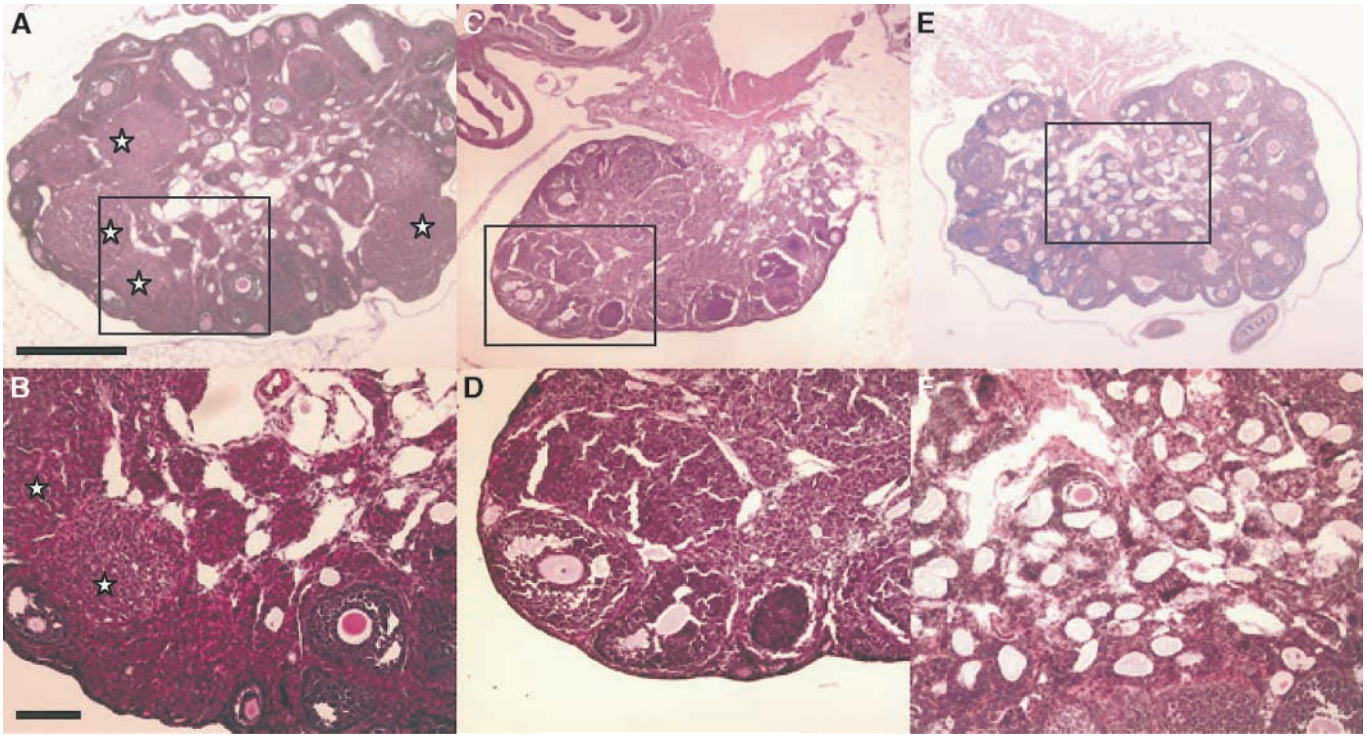

Fig. 4. Micrograph of ovaries of mice treated with fenofibrate. The mice were treated with 0% (A, B), 0.02% (C, D), and 0.4% (E, F) fenofibrate from 7 weeks of age for 4 weeks. The ovaries were collected and processed for histological analysis. The sections were stained with hematoxylin-eosin. Typical corpus lutea (stars) were observed in the control ovary (A and B) but not in the ovary of mice treated with 0.4% fenofibrate (E and F). Furthermore, many atretic follicles were observed in the ovaries treated with 0.4% fenofibrate. The ovaries of mice treated with 0.02% fenofibrate showed intermediate values between those shown in A and E. Bars, 500 μm in A, and 10 μm in B.

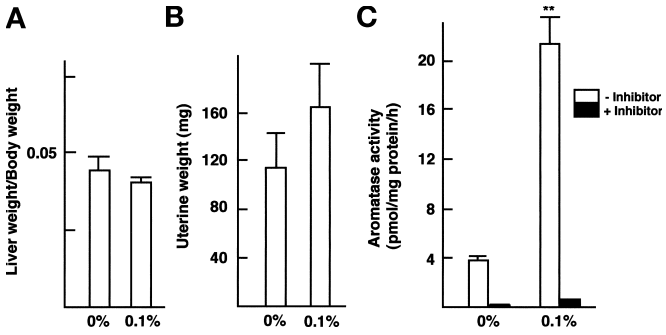

Fig. 5. Effects of dietary fenofibrate on PPARα null mice. The PPARα null female mice at 7 weeks of age were fed a diet containing 0% or 0.1% fenofibrate for 4 weeks. A: The ratio of liver-body weight, an indicator of hepatomegaly, was determined. B: Uterine weights of mice treated with fenofibrate were measured. C: Aromatase activity in the ovarian microsomal fractions was measured in the absence (open bar) or presence (closed bar) of 10 μM 4-androsten-4-ol-3,17-dione, an aromatase inhibitor. The radioactivity released from the substrate in the form of H_2O was determined. The activity is expressed as picomoles of tritiated water released/mg protein/h. Each bar represents the mean ± SEM of three experiments. Significantly different from untreated mice $^{**}P < 0.02$.

Effects of fenofibrate on the liver, uterus, and ovary of PPARα null mice

PPARα null mice were similarly treated with 0.1% fenofibrate to examine whether the effects observed in the wild-type mice were exerted through the activation of the PPARα pathway or not. The liver-body weight ratios in the control and fenofibrate-treated mice were 0.044 ± 0.004 and 0.04 ± 0.001 (n = 5), respectively, indicating no induction of hepatomegaly (**Fig. 5A**). The uterine weights were 111.3 ± 27.9 mg and 162.2 ± 34.8 mg for the control and treated mice, respectively, although the difference was not significant (Fig. 5B). Unexpectedly, the aromatase activity in the ovaries of the PPARα null mice was stimulated ~5.5-fold over the control level by dietary fenofibrate (Fig. 5C). The induction of steroidogenesis in PPARα null mice by agonists for PPARα has been reported recently (13). Nevertheless, our present findings strongly indicate that functional PPARα is required for fenofibrate to inhibit the aromatase activity in the ovary of mice.

DISCUSSION

In the present study, we demonstrated that fenofibrate, a common hypolipidemic drug, inhibits estrogen-synthesis in the ovary of mice in vivo. The agent suppresses the expression of the CYP19 gene, and also genes for HDL receptor and StAR, which respectively participate in cholesterol transportation from plasma HDL to the ovarian cells (24) and from the cytosol to mitochondria (25).

Agonists of PPARγ such as prostaglandin J2 or ciglitazone were shown to stimulate secretion of 17β-estradiol in cultured rat granulosa cells (26). Nonetheless, the stimulatory effect of the agonists on the estrogen biosynthesis is still controversial, as selective ligands for PPARγ such as thiazolidinedione inhibited transcription of the CYP19 gene in cultured human adipose stromal cells (27) and in human ovarian granulosa cells (28). Furthermore, a study employing in situ hybridization demonstrated that PPARγ is a major subtype of PPARs expressed in the rat ovary (26). These studies, therefore, imply that fenofibrate might inhibit the mRNA expression and activity of aromatase not through the activation of PPARα, but PPARγ. However, the results obtained with the PPARα null mice indicate that functional PPARα is obligatory for the inhibitory actions of fenofibrate in vivo. It remains to be clarified whether the drug acts directly on the ovary. A recent study has provided evidence to support the direct action of activated PPARα on steroidogenic cells such as Leydig cells, where PPARα inhibits steroidogenesis through suppression of the transport of cholesterol into the mitochondria (13). Alternatively, fenofibrate possibly modifies the hypothalamic-pituitary functions to inhibit the production of gonadotropin, which in turn causes the inhibition of estrogen synthesis in the ovary. The importance of PPARα in the transcriptional regulation in the pituitary cells has been reported in an in vitro study (29). Other indirect effects of fenofibrate and/or its metabolized products on certain signaling molecules that might interfere with the transcription of *Cyp19* in the ovary could also account for the phenomenon. Likewise, this possible indirect action of fenofibrate might explain the unexpected response of PPARα null mice, in which the aromatase activity is significantly enhanced by the drug. As diminution in uterine size and loss of BMD are characteristic manifestations of estrogen insufficiency, as occurred in aromatase gene-knockout (ArKO) mice (19, 20), we assume that the symptoms observed in the mice treated with fenofibrate are ascribed to a suppression in estrogen synthesis. Nevertheless, it is possible that the drug acts directly on the uterus and bone, because the diminution in uterine size and loss of BMD are also observed in ArKO mice treated with fenofibrate (K. Toda, unpublished observations).

Administration of fibrates such as bezafibrate is proposed to be beneficial in continuing tamoxifen treatment for obese early breast cancer patients (30), because adjuvant tamoxifen often induces severe liver steatosis and nonalcoholic steatohepatitis (31). In addition, tamoxifen is suggested to be an intrinsic estrogenic agonist, and a minimal but evident risk of endometrial cancer has been reported with adjuvant tamoxifen (32). Our present study supports the beneficial effects of a coadministration of PPARα activators with antiestrogenic drugs or aromatase inhibitors for the treatment of estrogen-dependent diseases, because they not only activate fatty acid β-oxidation in the liver, but also inhibit estrogen synthesis.

Apart from the clinical aspects, we should point out the possible disruption of the endocrine system by unintended activation of PPARα, as ligands for PPARα are found in diverse classes of chemical compounds, including commercially used plasticizers (e.g., phthalate esters), industrial solvents used as surfactants or wetting agents (e.g., trichloroethylene), herbicides (e.g., perfluorodecanoic acid),

and naturally occurring chemicals (e.g., phytanic acid) (33, 34). Thus, unlimited exposure to these compounds might induce the suppression of estrogen synthesis, which results in unexpected side effects in vivo, including detrimental effects on the reproductive system.

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