

**Cholesterol-lowering effect of bezafibrate is independent of peroxisome proliferator-activated receptor activation in mice**

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**Abbreviations:**

ABC, ATP-binding cassette; ABCA1, ABC transporter A1; ACAT2, acyl-CoA:cholesterol acyltransferase 2; ACC2, acetyl-CoA carboxylase 2; AOX, acyl-CoA oxidase; AOX2, branched-chain AOX; aP2, adipocyte fatty acid-binding protein; apo, apolipoprotein; AUC, area under the concentration-time curve; BAAT, bile acid-CoA:amino acid *N*-acyltransferase; BSEP, bile salt export pump; CD36, fatty acid translocase; Ch25h, cholesterol 25-hydroxylase; C<sub>max</sub>, maximum plasma concentrations; CTE, cytosolic thioesterase; CYP7A1, cholesterol 7 $\alpha$ -hydroxylase; CYP27A1, sterol 27-hydroxylase; CYP7B1, oxysterol 7 $\alpha$ -hydroxylase; CYP8B1, sterol 12 $\alpha$ -hydroxylase; DAB, 3, 3'-diaminobenzidine; FPPS, farnesyl pyrophosphate synthase; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; 3 $\beta$ -HSD, 3 $\beta$ -hydroxysteroid dehydrogenase; LDLR, low-density lipoprotein receptor; L-FABP, liver fatty acid-binding protein; LXR, liver X receptor; Mdr, multidrug resistance; Mrp, multidrug resistance-associated protein; NPC1, Niemann-Pick type C1 protein; NTCP, sodium-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; PCR, polymerase chain reaction; PDK4, pyruvate dehydrogenase kinase 4; PPAR, peroxisome proliferator-activated receptor; SCP2, sterol carrier protein 2; SQS, squalene synthase; SR-B1, scavenger receptor B1; SREBP, sterol regulatory element-binding protein; TC, total cholesterol

## Abstract

The hypocholesterolemic potential of peroxisome proliferator-activated receptor (PPAR) pan-activator bezafibrate has been documented. However, in addition to uncertainty about the contribution of PPAR $\alpha$  to its effect, there is a marked discrepancy in bezafibrate dosages used in previous rodent experiments ( $\geq 50$  mg/kg/day) and those in clinical use ( $\leq 10$  mg/kg/day). To investigate the association between bezafibrate-induced cholesterol reduction and PPAR $\alpha$  activation, wild-type and *Ppara*-null mice were treated with bezafibrate at high (100 mg/kg/day) or low (10 mg/kg/day) doses and analyzed. High-dose treatment decreased hepatic cholesterol content in wild-type mice, but increased serum cholesterol concentration. In liver samples, simultaneous increases in the expression of numerous proteins involved in cholesterol biosynthesis and catabolism, as well as cholesterol influx and efflux, were observed, which made interpretation of phenotype changes subtle. These complicated responses were believed to be associated with intensive PPAR activation and accompanying up-regulation of liver X receptor  $\alpha$ , farnesoid X receptor, and sterol regulatory element-binding protein 2 (SREBP2). In contrast, low-dose bezafibrate treatment decreased serum and hepatic cholesterol concentrations in a PPAR $\alpha$ -independent manner, probably from suppression of SREBP2-regulated cholesterologenesis and enhancement of cholesterol catabolism due to elevated 7 $\alpha$ -hydroxylase levels. Interestingly, the low-dose treatment did not affect the expression of PPAR target genes or number of peroxisomes, suggesting the absence of PPAR activation. These results demonstrate that the action of bezafibrate on cholesterol metabolism may vary with dosage, and that the cholesterol-reducing effect found in mice at dosages similar to those administered to humans is independent of significant PPAR activation.

***Keywords:***

bezafibrate; peroxisome proliferator-activated receptor (PPAR); *Ppara*-null mice;  
cholesterol metabolism; sterol regulatory element-binding protein (SREBP) 2

## 1. Introduction

Bezafibrate, a second-generation fibrate drug, is used clinically as a hypolipidemic agent. Bezafibrate treatment lowers serum triglyceride and cholesterol levels by more than 25% and 10%, respectively, in patients with hyperlipidemia [1-4], and the Bezafibrate Infarction Prevention study has demonstrated a relationship between the lipid-lowering effects of bezafibrate and a reduction of the risk of cardiovascular events in dyslipidemic patients with coronary artery disease [5]. The molecular mechanism underlying the triglyceride-reducing effect of bezafibrate is due in part to the induction of lipoprotein lipase activity mediated by the activation of peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), a member of the nuclear receptor superfamily [6, 7]. However, the molecular mechanism underlying its effect on cholesterol metabolism has not yet been fully elucidated.

PPAR $\alpha$  is highly expressed in the liver, kidney, and heart, and functions as a critical regulator of fatty acid metabolism [8-11]. Recently, several studies using a *Ppara*-null mouse model established a possible direct involvement of PPAR $\alpha$  in the regulation of cholesterol metabolism. For example, PPAR $\alpha$  activation enhances hepatic expression of mRNA encoding sterol 12 $\alpha$ -hydroxylase (CYP8B1), an enzyme involved in bile acid synthesis from cholesterol [12], and multidrug resistance 2 (Mdr2), a phospholipid transporter for bile formation [13]. On the other hand, it is known that bezafibrate can also activate other PPAR subtypes [ $\beta$  ( $\delta$ ) and  $\gamma$ ] [14-16]. Thus, it remains unclear whether the effect of bezafibrate on cholesterol reduction is singularly due to its effect on PPAR $\alpha$ .

In the present study, the contribution of PPAR $\alpha$  to the cholesterol-reducing effect

of bezafibrate was examined using *Ppara*-null mice. With respect to this issue, there is a marked discrepancy between bezafibrate dosages used in previous rodent studies and those typically administered in the clinic; although the appropriate dosage for humans is estimated to be less than or equal to 10 mg/kg/day, studies in rodent models have often opted for high-dose regimens (50 mg/kg/day or more) [16-19]. In the present study, therefore, wild-type and *Ppara*-null mice were treated with bezafibrate at high (100 mg/kg/day) or low (10 mg/kg/day) doses. Unexpectedly, low-dose bezafibrate treatment reduced serum and hepatic cholesterol concentrations in the absence of prominent PPAR activation.

## **2. Materials and methods**

### *2.1. Materials*

Bezafibrate (2-{4-[2-(4-chlorobenzamido)ethyl]phenoxy}-2-methylpropionic acid) was kindly provided by Kissei Pharmaceutical Co., Ltd. (Matsumoto, Japan). Carboxymethylcellulose, Cholesterol E-Test kit, Free Cholesterol E-Test kit, Triglyceride E-Test kit, and Triton X-100 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and 3, 3'-diaminobenzidine (DAB) tetrahydrochloride was from Dojindo Laboratories (Kumamoto, Japan). RNeasy Mini Kit was obtained from QIAGEN (Hilden, Germany). Reverse transcript reagents were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Power SYBR Green PCR Master Mix was obtained from Applied Biosystems (Foster City, CA, USA). BCA Protein Assay Kit and 1-Step NBT/BCIP reagent were purchased from Pierce Biotechnology (Rockford, IL, USA). Nitrocellulose membrane was obtained from GE Healthcare (Chalfont St. Giles, UK). Rabbit polyclonal antibodies against 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), cholesterol 7 $\alpha$ -hydroxylase (CYP7A1), sterol regulatory element-binding protein 2 (SREBP2), fatty acid translocase (CD36), actin, and histone H1, as well as goat polyclonal antibodies against squalene synthase (SQS), oxysterol 7 $\alpha$ -hydroxylase (CYP7B1), acetyl-CoA carboxylase 2 (ACC2), pyruvate dehydrogenase kinase 4 (PDK4), and adipocyte fatty acid-binding protein (aP2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against acyl-CoA oxidase (AOX) and cytosolic thioesterase (CTE) were described elsewhere [20, 21]. Alkaline phosphatase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

## 2.2. *Animals and bezafibrate treatment*

*Ppara*-null mice on a Sv/129 genetic background were described elsewhere [22]. Twelve-week-old male Sv/129 wild-type and *Ppara*-null mice (25-30 g body weight) were assigned to one of three groups (control group, low-dose bezafibrate group, or high-dose bezafibrate group;  $n = 6$  in each). The mice were housed in a temperature- and light-controlled environment (25 °C; 12 h light/dark cycle) and maintained with tap water *ad libitum* and a 7% fat-containing standard rodent diet. Bezafibrate was suspended in 1% (w/v) carboxymethylcellulose at final concentrations of 1.5 and 15 mg/mL for the 10 and 100 mg/kg/day treatments, respectively, and 0.2 mL of each suspension was administered by gavage once a day (10:00 a.m.) for 10 days. The same amount of 1% (w/v) carboxymethylcellulose without bezafibrate was administered in a similar manner to control mice. At the end of treatment, food was withdrawn from all mice overnight, mice were killed under anesthesia, and blood and livers were collected for analysis. All animal experiments were conducted in accordance with the animal care guidelines approved by the Shinshu University School of Medicine.

## 2.3. *Measurement of lipids*

Serum total cholesterol (TC) and triglyceride concentrations were measured using the Cholesterol E-Test Wako and Triglyceride E-Test Wako kits, respectively. Serum lipoprotein profiles were analyzed by high-performance liquid chromatography with an on-line dual detection system (Skylight Biotech., Akita, Japan) [23]. To measure hepatic cholesterol content, total lipid in liver tissue (50 mg) was extracted according to the hexane/isopropanol method [24]. The lipid extract was solubilized in distilled water by

the addition of Triton X-100 as described previously with minor modifications [25], and cholesterol was then measured. Measurement of liver free cholesterol content was carried out using the Free Cholesterol E-Test Wako kit. Liver cholesteryl ester levels were calculated as the difference between liver TC and free cholesterol levels.

#### *2.4. Analysis of mRNA expression*

Total liver RNA was extracted using the RNeasy Mini Kit, and mRNA was reverse-transcribed using oligo-dT priming. Levels of mRNA were determined by quantitative real-time polymerase chain reaction (PCR) using SYBR Green chemistry on an ABI PRISM 7000 sequence detection system (Applied Biosystems). Specific primers were designed by Primer Express software (Applied Biosystems; Table 1). Each mRNA level was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and then normalized to that of control wild-type mice.

#### *2.5. Immunoblot analysis*

Preparations of whole liver lysate and hepatic nuclear fraction were performed as described previously [26-28]. Protein concentrations were determined using the BCA Protein Assay Kit. Whole liver lysates or nuclear fractions (50  $\mu$ g protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, these membranes were incubated with primary antibodies followed by alkaline phosphatase-conjugated secondary antibodies, and then treated with 1-Step NBT/BCIP substrate. The position of protein bands was determined by molecular weight. Band intensity was quantified densitometrically, normalized to that of actin or histone H1, and then normalized to that of control

wild-type mice.

## 2.6. Cytochemical staining and morphometric analysis of hepatic peroxisomes

Cytochemical staining of hepatic peroxisomes was carried out using the DAB technique as described elsewhere [28]. DAB-stained liver sections were visualized with both light and electron microscopy. Morphometric analysis of peroxisomes was also performed using electron micrographs at an original magnification of 4000 $\times$ . For each mouse, 10 independent fields in the pericentral area of liver lobuli were photomicrographed, and the number of peroxisomes and area of each individual peroxisomal profile were measured. The numerical density and volume density of peroxisomes were calculated as follows: numerical density (number/ $\mu\text{m}^2$ ) =  $N_P/(A_T - A_{\text{empty}})$ , and volume density (%) =  $A_{TP}/(A_T - A_{\text{empty}}) \times 100$ , where  $N_P$  was the peroxisome number in the test area,  $A_T$  was the test area,  $A_{\text{empty}}$  was the area composed of vascular and biliary lumens and hepatocyte nuclei, and  $A_{TP}$  was the area of total peroxisomal profiles in the test area. All areas were measured by a Luzex AP image analyzer (Nireco, Tokyo, Japan).

## 2.7. Statistical analysis

Results are expressed as mean  $\pm$  S.D. Statistical analysis was performed using SPSS software 11.5J for Windows (SPSS inc., Chicago, IL, USA). Comparison between the groups was made by means of Student's  $t$  test. A  $P < 0.05$  was considered to be statistically significant.

### 3. Results

#### 3.1. Phenotype changes

All mice appeared to be healthy during the experimental period, and no significant differences in food consumption or body weight were observed. Liver/body weight ratio was increased only in wild-type mice that received high-dose bezafibrate (Fig. 1A). Serum TC concentrations were slightly increased by high-dose bezafibrate treatment in wild-type, but not in *Ppara*-null mice, and were decreased by low-dose bezafibrate in both genotypes (Fig. 1B). Serum triglyceride concentrations were decreased in mice treated with low-dose bezafibrate compared with controls ( $94.1 \pm 10.3$  vs.  $123.5 \pm 16.8$  mg/dL in wild-type mice,  $P = 0.037$ ;  $71.6 \pm 11.2$  vs.  $94.6 \pm 10.3$  mg/dL in *Ppara*-null mice,  $P = 0.033$ ). There was no pronounced alteration in lipoprotein composition from the treatment, since amounts of very-low-density lipoprotein and low-density lipoprotein were far less than those of high-density lipoprotein (data not shown). Liver TC content was significantly reduced by high-dose treatment in wild-type mice, and by low-dose treatment in both genotypes (Fig. 1C). Hepatic free cholesterol content was also decreased in mice treated with low-dose bezafibrate compared with controls ( $1.19 \pm 0.06$  vs.  $1.47 \pm 0.08$  mg/g tissue in wild-type mice,  $P = 0.004$ ;  $1.79 \pm 0.36$  vs.  $3.03 \pm 0.45$  mg/g tissue in *Ppara*-null mice,  $P = 0.005$ ). The ratio of cholesteryl ester to TC was decreased in wild-type mice only ( $14.7 \pm 3.2$  vs.  $25.9 \pm 4.8\%$ ,  $P = 0.008$ ). These two parameters were also reduced in wild-type mice that received high-dose bezafibrate (data not shown). These findings indicate that the cholesterol-lowering effect of bezafibrate occurs in a PPAR $\alpha$ -independent manner in cases of low-dose administration.

### 3.2. Effects on cholesterol biosynthesis

To elucidate the molecular mechanism of the effects of bezafibrate, hepatic expression of mRNA encoding cholesterol biosynthetic enzymes was first measured. High-dose bezafibrate treatment markedly increased the expression of 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), HMGR, farnesyl pyrophosphate synthase (FPPS), and SQS mRNA in wild-type mice only (Fig. 2A), suggesting that these changes may be mediated by PPAR $\alpha$ . In contrast, low-dose bezafibrate treatment significantly decreased the expression of HMGS, HMGR, FPPS, and SQS mRNA in both genotypes (Fig. 2A). Immunoblot analysis revealed that changes in HMGR and SQS protein expression were consistent with those in mRNA levels (Fig. 2B and C). These results demonstrate that de novo cholesterologenesis pathway is markedly suppressed specifically by low-dose bezafibrate treatment in both genotypes.

### 3.3. Effects on cholesterol catabolism

To determine whether bezafibrate treatment affected cholesterol catabolism, the expression of mRNA encoding bile acid synthetic enzymes was examined. High-dose bezafibrate treatment markedly increased the expression of CYP7A1, CYP8B1, and branched-chain AOX (AOX2) in wild-type mice, while their *Ppara*-null counterparts exhibited an increase in CYP7A1 expression, but a decrease in CYP8B1 (Fig. 3A). On the other hand, low-dose treatment significantly enhanced the expression of CYP7A1 and CYP7B1 mRNA in both genotypes (Fig. 3A). These increases were confirmed by immunoblot analysis (Fig. 3B and C). Bezafibrate treatment did not affect the expression of mRNA encoding sterol 27-hydroxylase (CYP27A1), 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD), or cholesterol 25-hydroxylase (Ch25h). These results suggest

that increased expression of two cholesterol-catabolizing enzymes (i.e., CYP7A1 and CYP7B1) may at least partially contribute to the cholesterol-reducing effect observed in wild-type and *Ppara*-null mice administered with low-dose bezafibrate.

#### *3.4. Effects on cholesterol influx, efflux, and intracellular sterol transport*

Next, the expression of mRNA encoding proteins involved in cholesterol influx and efflux was investigated. In wild-type mice, high-dose bezafibrate treatment markedly enhanced the expression of apolipoprotein A2 (apoA2), apoE, ATP-binding cassette (ABC) transporter A1 (ABCA1), scavenger receptor B1 (SR-B1), and low-density lipoprotein receptor (LDLR) mRNA, while low-dose treatment increased that of ABCA1 (Fig. 4A and B). These changes were not observed in similarly treated *Ppara*-null mice.

The expression of mRNA encoding sterol transporters and acyltransferases was also evaluated to determine the effect of bezafibrate on the intracellular mobility of cholesterol and its metabolites. High-dose bezafibrate treatment significantly elevated mRNA levels of Niemann-Pick type C1 protein (NPC1), sterol carrier protein 2 (SCP2), liver fatty acid-binding protein (L-FABP), and acyl-CoA:cholesterol acyltransferase 2 (ACAT2) in wild-type mice (Fig. 4C and D). *Ppara*-null mice that received high-dose bezafibrate showed an increase in L-FABP mRNA expression. Low-dose bezafibrate treatment also elevated mRNA levels of NPC1 and L-FABP in wild-type mice, but not in *Ppara*-null mice (Fig. 4C and D). Bile acid-CoA:amino acid *N*-acyltransferase (BAAT) mRNA expression was unchanged.

#### *3.5. Effects on bile secretion and bile acid reabsorption*

High-dose bezafibrate treatment markedly increased mRNA expression of transporters related to bile secretion, including Mdr1, Mdr2, multidrug resistance-associated protein 2 (Mrp2), Mrp3, ABCG5, and ABCG8 in wild-type mice (Fig. 5A). No such effects from low-dose treatment were noted. Expression of bile salt export pump (BSEP) mRNA was constant.

Next, the expression of mRNA encoding bile salt transporters was examined since approximately 95% of bile salts are reabsorbed in the liver through enterohepatic circulation [29]. High-dose bezafibrate treatment significantly enhanced sodium-taurocholate cotransporting polypeptide (NTCP) mRNA expression in both genotypes, while low-dose treatment did not (Fig. 5B). Levels of organic anion transporting polypeptide 1 (OATP1) and OATP2 mRNA were unchanged by the treatment.

### *3.6. Alterations in transcription factor expression*

In order to investigate changes in transcription factors, the expression of mRNA encoding PPARs and other factors related to cholesterol metabolism was evaluated. High-dose bezafibrate treatment markedly elevated mRNA levels of PPAR $\alpha$ , liver X receptor  $\alpha$  (LXR $\alpha$ ), farnesoid X receptor (FXR), and SREBP2 in wild-type mice only (Fig. 6A). Interestingly, the low-dose treatment significantly down-regulated SREBP2 mRNA levels in both genotypes (Fig. 6A). PPAR $\beta$  mRNA was considerably decreased by bezafibrate in both genotypes at both dosages, while PPAR $\gamma$  mRNA remained unchanged. Immunoblot analysis showed that the changes in nuclear SREBP2 levels were in accordance with those at the transcriptional levels (Fig. 6B and C). Additionally, nuclear SREBP2 levels were correlated with the expression of its target genes, such as

HMGS, HMGR, FPPS, and SQS (Fig. 2).

### *3.7. Effects on PPAR target gene expression and peroxisome proliferation*

As described above, several PPAR $\alpha$ -independent effects were found in the low-dose bezafibrate treatment group, leading to the hypothesis that low doses of bezafibrate function via mechanisms other than PPAR $\alpha$  activation. To address this issue, the expression of representative PPAR $\alpha$  target genes, including AOX and CTE [30], was investigated in wild-type mice. As expected, high-dose bezafibrate treatment markedly elevated expression of these genes, but low-dose treatment did not (Fig. 7A-C). These findings were in agreement with the result that PPAR $\alpha$  mRNA was not elevated by low-dose administration (Fig. 6A) since PPAR $\alpha$  activation is known to activate its own transcription [11, 31]. Additionally, peroxisome proliferation, a well-known indicator of PPAR $\alpha$  activation [22], was not observed in the low-dose group (Fig. 7D and E). Furthermore, to determine the contribution of low-dose bezafibrate on PPAR $\beta$  and/or PPAR $\gamma$  activation, the expression of target genes of PPAR $\beta$  (ACC2 [32] and PDK4 [33]), PPAR $\alpha/\gamma$  (CD36 [34]), and PPAR $\gamma$  (aP2 [35]) were analyzed in both genotypes, but showed no increase in mice treated with low-dose bezafibrate (Fig. 8). Overall, these results strongly suggest that low doses of bezafibrate do not induce significant activation of murine PPARs.

#### 4. Discussion

The present study provides novel and unexpected evidence that the pharmacological action of bezafibrate on cholesterol metabolism varies considerably according to its dosage. Treatment with high-dose bezafibrate (100 mg/kg/day), a dosage similar to that used in previous rodent experiments, resulted in both a slight increase in serum TC concentration and a marked decrease in hepatic TC in a PPAR $\alpha$ -dependent manner, accompanied with strong PPAR activation. In contrast, treatment with low-dose bezafibrate (10 mg/kg/day), corresponding approximately to the dosage used in human clinical practice, decreased serum and hepatic cholesterol concentrations in the absence of significant PPAR activation. As far as we know, this is the first study that explores the dose-dependent effects of bezafibrate in mice.

High-dose bezafibrate treatment caused several conflicting changes in the mRNA levels of various enzymes, transporters, and receptors related to hepatic cholesterol metabolism in a PPAR $\alpha$ -dependent fashion. Specifically, mRNA levels of proteins involved in cholesterol biosynthesis (HMGS, HMGR, FPPS, and SQS), catabolism (CYP7A1, CYP8B1, and AOX2), uptake (SR-B1 and LDLR), and excretion (apoA2, apoE, ABCA1, and ABCG5/8) were all elevated. Although this simultaneous induction was too complicated to interpret all of the molecular mechanisms involved, these responses were likely associated with the both direct and indirect effects of intensive PPAR activation. It is conceivable that the elevated levels of LXR $\alpha$ , FXR, and SREBP2 found following high-dose bezafibrate treatment affected the expression of numerous proteins involved in cholesterol/bile acid metabolism; all three have been shown to regulate expression of CYP7A1 and ABC transporters [36, 37], apoE, Mdr2, and Mrp2 [38-40], and cholesterologenic enzymes and LDLR [41, 42]. Moreover, hepatic

expression of the xenobiotic transporter Mdr1 was up-regulated by high-dose treatment in a PPAR $\alpha$ -dependent manner, suggesting the possible influence of PPAR $\alpha$  activation-induced stress, such as rodent-specific hepatomegaly [13]. Therefore, previous results in rodents treated with high-dose bezafibrate may not accurately reflect the pharmacological effect of this drug in humans.

The molecular mechanism of the cholesterol-reducing effect of low-dose bezafibrate seemed to be due to suppression of SREBP2-regulated de novo cholesterologenesis and stimulation of cholesterol catabolism through increased expression of two  $7\alpha$ -hydroxylases, CYP7A1 and CYP7B1. A previous study suggested that the expression of human CYP7B1 is suppressed by SREBPs [43]. In the present study, CYP7B1 expression in both genotypes under low-dose bezafibrate treatment reacted inversely to SREBP2; therefore, increases in CYP7B1 expression might be accompanied by attenuation of SREBP2. In contrast to CYP7B1, CYP7A1 expression was up-regulated by bezafibrate treatment in a PPAR $\alpha$ -independent, dose-dependent manner. Thus, these changes might be attributable to some as yet unknown bezafibrate-specific action. The down-regulation of SREBP2 by low-dose treatment occurred at the transcriptional level. Transcription of SREBP2 is known to be regulated by mature SREBP2 translocated to the nucleus through a proteolytic processing step controlled by several proteins, including SREBP cleavage-activating protein and site-1 protease [41, 42, 44, 45]. Bezafibrate might influence the proteolysis of SREBP2 through modulation of such factors, though further experiments are needed in this regard.

The present study revealed that low-dose bezafibrate treatment increased the expression of ABCA1, NPC1, and L-FABP in the presence of PPAR $\alpha$ . Previous reports

have established a prominent role for hepatic ABCA1 in the regulation of plasma high-density lipoprotein cholesterol levels [46, 47]. In addition, NPC1 has been shown to transport free cholesterol from lysosomes to the plasma membrane, contributing to cholesterol efflux through the ABCA1 pathway [48]. Therefore, these changes may be partly related to bezafibrate enhancement of high-density lipoprotein cholesterol in humans. L-FABP is known as a major bile acid transporter [49, 50], and the enhancement of its expression may be implicated in facilitation of intracellular bile acid mobility. Recently, the efficacy of bezafibrate has been demonstrated in patients with chronic cholestatic liver diseases, such as primary biliary cirrhosis [51-53]. The present results concerning L-FABP induction might partially explain the beneficial effects of bezafibrate against such diseases. Taken together, our results suggest the importance of PPAR $\alpha$  on understanding the pharmacological effects of bezafibrate.

The above-mentioned dose discrepancy between rodents and humans could be due to pharmacokinetic differences; dose justification alone might be inadequate to compare the conditions. However, our preliminary analysis and the previous reports have demonstrated that maximum plasma concentrations ( $C_{max}$ ) and area under the concentration-time curve (AUC) values in rats after the single high-dose administration of bezafibrate were quite different from those in healthy volunteers after the single intake at the clinical dose (mean  $C_{max}$ , 140 vs. 11  $\mu\text{g}/\text{mL}$ ; mean AUC values, 1520 vs. 39  $\mu\text{g}\cdot\text{h}/\text{mL}$ ) [54, 55]. On the other hand, these parameters in rats after the single low-dose administration were relatively similar to those in healthy volunteers (21  $\mu\text{g}/\text{mL}$  and 107  $\mu\text{g}\cdot\text{h}/\text{mL}$ , respectively). Therefore, the phenomena observed in mice under low-dose administration might reflect the conditions in bezafibrate-treated humans more accurately than those in mice with high-dose administration.

This study showed that low-dose bezafibrate treatment reduced circulating cholesterol and altered hepatic cholesterol metabolism through a PPAR $\alpha$ -independent mechanism in mice. Although no significant activation of any PPAR subtype was determined under low-dose treatment as estimated by the expression of various PPAR target genes and morphometry of hepatic peroxisomes, we cannot rule out the possibility that the PPAR $\alpha$ -independent effect was nonetheless somehow mediated by PPAR $\beta$ , PPAR $\gamma$ , or both. Further studies using mice lacking all PPAR subtypes are needed to address this issue. Additionally, cholesterol-metabolizing pathways in mice partly differ from those in humans. For example, the degree of contribution of the alternative pathway to overall bile acid synthesis is thought to be low in mice compared to humans [36]. The same experiment using other mammals such as guinea pigs, which have more similarities in cholesterol metabolism to humans than mice [56, 57], would confirm our results.

In conclusion, we demonstrated that the cholesterol-reducing effect of bezafibrate in mice at the clinically-relevant dose stems from suppression of cholesterol biosynthesis and enhancement of catabolism without significant PPAR activation. Additionally, our results indicated remarkable differences in the mechanisms involved in bezafibrate action by comparing high-dose and low-dose treatments. These findings may lead to better identification of the mechanisms underlying the beneficial effects of bezafibrate in patients with dyslipidemia.

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## Figure Legends

### **Fig. 1. Phenotype changes by bezafibrate treatment in wild-type (+/+) and**

#### ***Ppara*-null (-/-) mice**

Liver/body weight ratio (**A**) and total cholesterol (TC) level in serum (**B**) and liver (**C**) were measured. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). White bar, control group; gray bar, low-dose (10 mg/kg/day) bezafibrate group; black bar, high-dose (100 mg/kg/day) bezafibrate group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , between treated and untreated mice of the same genotype.

### **Fig. 2. Effects of bezafibrate on cholesterol biosynthesis in wild-type (+/+) and**

#### ***Ppara*-null (-/-) mice**

(**A**) Hepatic expression of mRNA encoding cholesterol biosynthetic enzymes was analyzed by quantitative real-time PCR and normalized to that of GAPDH mRNA. Relative mRNA levels are shown as fold-changes of those of control wild-type mice. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). White bar, control group; gray bar, low-dose (10 mg/kg/day) bezafibrate group; black bar, high-dose (100 mg/kg/day) bezafibrate group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , between treated and untreated mice of the same genotype.

(**B**) Immunoblot analysis of HMGR and SQS. Fifty microgram of whole liver lysate protein was loaded into each well. The actin band was used as the loading control. Results are representative of three independent experiments. Doses of bezafibrate are indicated above the bands of each treatment group.

(**C**) Quantification of HMGR and SQS protein levels. Band intensity was quantified densitometrically and normalized to that of actin. Relative protein levels are shown as

fold-changes of those of control wild-type mice. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in panel **A**.  $*P < 0.05$ ,  $***P < 0.001$ , between treated and untreated mice of the same genotype.

**Fig. 3. Effects of bezafibrate on cholesterol catabolism in wild-type (+/+) and *Ppara*-null (-/-) mice**

(**A**) Hepatic expression of mRNA encoding cholesterol-catabolizing enzymes was analyzed by quantitative real-time PCR and normalized to that of GAPDH mRNA. Relative mRNA levels are shown as fold-changes of those of control wild-type mice. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in **Fig. 2A**.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , between treated and untreated mice of the same genotype.

(**B**) Immunoblot analysis of CYP7A1 and CYP7B1. The same samples in **Fig. 2B** were used. Protein sample of 50  $\mu$ g was loaded into each well.

(**C**) Quantification of CYP7A1 and CYP7B1 protein levels. Band intensity was quantified densitometrically and normalized to that of actin. Relative protein levels are shown as fold-changes of those of control wild-type mice. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in **Fig. 2A**.  $*P < 0.05$ ,  $**P < 0.01$ , between treated and untreated mice of the same genotype.

**Fig. 4. Effects of bezafibrate on cholesterol influx, efflux, and intracellular sterol transport in wild-type (+/+) and *Ppara*-null (-/-) mice**

Hepatic expression of mRNA encoding apolipoproteins (**A**), cholesterol transporters and receptors (**B**), intracellular sterol transporters (**C**), and acyltransferases (**D**) was

analyzed by quantitative real-time PCR and normalized to that of GAPDH mRNA. Relative mRNA levels are shown as fold-changes of those of control wild-type mice. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in **Fig. 2A**. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , between treated and untreated mice of the same genotype.

**Fig. 5. Effects of bezafibrate on bile secretion and bile acid reabsorption in wild-type (+/+) and *Ppara*-null (-/-) mice**

Hepatic expression of mRNA encoding transporters related to bile secretion (**A**) and bile acid reabsorption (**B**) was analyzed by quantitative real-time PCR and normalized to that of GAPDH mRNA. Relative mRNA levels are shown as fold-changes of those of control wild-type mice. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in **Fig. 2A**. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , between treated and untreated mice of the same genotype.

**Fig. 6. Effects of bezafibrate on transcription factor expression in wild-type (+/+) and *Ppara*-null (-/-) mice**

(**A**) Hepatic expression of mRNA encoding transcription factors was analyzed by quantitative real-time PCR and normalized to that of GAPDH mRNA. Relative mRNA levels are shown as fold-changes of those of control wild-type mice. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in **Fig. 2A**. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , between treated and untreated mice of the same genotype.

(**B**) Immunoblot analysis of nuclear SREBP2. Hepatic nuclear fraction was prepared for each mouse, and 50  $\mu$ g of protein was loaded into each well. The histone H1 band was

used as the loading control. Results are representative of three independent experiments.

Doses of bezafibrate are indicated above the bands of each treatment group.

(C) Quantification of nuclear SREBP2 levels. Band intensity was quantified densitometrically and normalized to that of histone H1. Relative protein levels are shown as fold-changes of those of control wild-type mice. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in **Fig. 2A**.  $*P < 0.05$ , between treated and untreated mice of the same genotype.

**Fig. 7. Effects of bezafibrate on PPAR $\alpha$  target gene expression and peroxisome proliferation in wild-type (+/+) mice**

(A) Hepatic expression of mRNA encoding PPAR $\alpha$  target genes, AOX and CTE, was analyzed by quantitative real-time PCR and normalized to that of GAPDH mRNA. Relative mRNA levels are shown as fold-changes of those of control mice. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in **Fig. 2A**.  $***P < 0.001$ , between treated and untreated mice.

(B) Immunoblot analysis of AOX and CTE. The same samples in **Fig. 2B** were used. Protein sample of 50  $\mu$ g was loaded into each well. Results are representative of three independent experiments. Doses of bezafibrate are indicated above the bands of each treatment group. A and B in AOX bands indicate full-length and truncated AOX, respectively.

(C) Quantification of AOX and CTE protein levels. Band intensity was quantified densitometrically and normalized to that of actin. Relative protein levels are shown as fold-changes of those of control mice. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in **Fig. 2A**.  $***P < 0.001$ , between treated and

untreated mice.

(D) Cytochemical staining of hepatic peroxisomes. Peroxisomes are detectable as dark particles, and marked peroxisome proliferation can be observed only in mice with high-dose bezafibrate treatment. The bars in the light (upper) and electron (lower) photomicrographs of control mice indicate 40 and 10  $\mu\text{m}$ , respectively. The arrows indicate erythrocytes.

(E) Morphometric analysis of hepatic peroxisomes. The number of peroxisomes and area of each individual peroxisomal profile were measured in ten electron photomicrographs from each mouse, and numerical and volume densities were calculated. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in **Fig. 2A**.  $**P < 0.01$ , between treated and untreated mice.

**Fig. 8. Effects of bezafibrate on PPAR $\beta$  or PPAR $\gamma$  target gene expression in wild-type (+/+) and *Ppara*-null (-/-) mice**

Hepatic expression of PPAR $\beta$  target genes (ACC2 and PDK4) (A-C) and PPAR $\gamma$  target genes (CD36 and aP2) (D-F) was examined using quantitative real-time PCR and immunoblot analysis. The same samples in **Fig. 2A and B** were used. For immunoblot analysis, 50  $\mu\text{g}$  of protein was loaded into each well. Data are expressed as mean  $\pm$  S.D. ( $n = 6$  in each group). Bars are identical to those in **Fig. 2A**.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , between treated and untreated mice of the same genotype.