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Bezafibrate at Clinically Relevant Doses Decreases Serum/Liver Triglycerides via Down-Regulation of Sterol Regulatory Element-Binding Protein-1c in Mice: A Novel Peroxisome Proliferator-Activated Receptor α-Independent Mechanism

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ABSTRACT

The triglyceride-lowering effect of bezafibrate in humans has been attributed to peroxisome proliferator-activated receptor (PPAR) α activation based on results from rodent studies. However, the bezafibrate dosages used in conventional rodent experiments are typically higher than those in clinical use ($\geq$50 versus $\leq$10 mg/kg/day), and thus it remains unclear whether such data can be translated to humans. Furthermore, because bezafibrate is a pan-PPAR activator, the actual contribution of PPARα to its triglyceride-lowering properties remains undetermined. To address these issues, bezafibrate at clinically relevant doses (10 mg/kg/day; low) was administered to wild-type and *Ppara*-null mice, and its effects were compared with those from conventionally used doses (100 mg/kg/day; high). Pharmacokinetic analyses showed that maximum plasma concentration and area under the concentration-time curve in bezafibrate-treated mice were similar to those in humans at low doses, but not at high doses. Low-dose bezafibrate decreased serum/liver triglycerides in a PPARα-independent manner by attenuation of hepatic lipogenesis and triglyceride secretion. It is noteworthy that instead of PPAR activation, down-regulation of sterol regulatory element-binding protein (SREBP)-1c was observed in mice undergoing low-dose treatment. High-dose bezafibrate decreased serum/liver triglycerides by enhancement of hepatic fatty acid uptake and β-oxidation via PPARα activation, as expected. In conclusion, clinically relevant doses of bezafibrate exert a triglyceride-lowering effect by suppression of the SREBP-1c-regulated pathway in mice and not by PPARα activation. Our results may provide novel information about the pharmacological mechanism of bezafibrate action and new insights into the treatment of disorders involving SREBP-1c.

Bezafibrate and other fibrate drugs are clinically used as hypolipidemic agents to preferentially lower serum triglyceride (TG) levels. Several large-scale clinical trials have demonstrated a relationship between the TG-lowering effect of fibrates and a reduction in the risk of cardiovascular events in patients with dyslipidemia, type 2 diabetes mellitus, and metabolic syndrome (BIP Study Group, 2000; Keech et al., 2005; Tenenbaum et al., 2005). The mechanisms accounting for the hypolipidemic effect of fibrates in humans are explained mainly as an increase in the lipolysis of TG-rich lipoproteins, such as very-low-density lipoprotein (VLDL),

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ABBREVIATIONS: TG, triglyceride; Apo, apolipoprotein; ACC, acetyl-CoA carboxylase; AUC, area under the plasma concentration-time curve; C_{max}, maximum plasma concentration; CPT, carnitine palmitoyl-CoA transferase; FAS, fatty acid synthase; FAT, fatty acid translocase; FATP, fatty acid transport protein; FFA, free fatty acid; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; LC/MS/MS, high-performance liquid chromatography/tandem mass spectrometry; LPL, lipoprotein lipase; LXR, liver X receptor; MCAD, medium-chain acyl-CoA dehydrogenase; MTP, microsomal TG transfer protein; PCR, polymerase chain reaction; PDK, pyruvate dehydrogenase kinase; PMP70, 70-kDa peroxisomal membrane protein; PPAR, peroxisome proliferator-activated receptor; SCAP, SREBP cleavage-activating protein; SCD, stearoyl-CoA desaturase; SHP, short heterodimer partner; SREBP, sterol regulatory element-binding protein; T_{max}, time to reach C_{max}; VLDL, very-low-density lipoprotein.

and a decrease in VLDL secretion from the liver (Staels et al., 1998). These effects are believed to be mediated by activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR) α.

PPARα is highly expressed in the liver, heart, and kidney and functions as a central regulator of fatty acid and TG metabolism (Aoyama et al., 1998; Nakajima et al., 2004; Kamijo et al., 2007). A number of studies using mice lacking the PPARα gene (*Ppara*-null mice) have revealed the direct contribution of PPARα to the TG-lowering effect of fibrates; for example, PPARα activation by bezafibrate suppresses the transcriptional expression of apolipoprotein (Apo) CIII (Peters et al., 2003), an endogenous inhibitor of lipoprotein lipase (LPL) and hepatic TG lipase, leading to an enhancement of lipoprotein lipolysis. Fibrate-induced PPARα activation also increases hepatic expression of fatty acid transporters and β-oxidation enzymes (Aoyama et al., 1998; Motojima et al., 1998). These experimental results from rodent models have helped to understand the possible mechanisms of fibrate action in humans.

However, two important issues arise when rodent data on the TG-reducing effect of bezafibrate are used to explain the mechanism of this drug in humans. One is the uncertainty about the actual contribution of PPARα to its effect, because bezafibrate can activate other PPAR subtypes [β (δ) and γ] (Brown et al., 1999) and lower serum TG levels even in *Ppara*-null mice (Peters et al., 2003). Another issue is the dosage discrepancy between previous rodent studies (typically ≥50 mg/kg/day) and human clinical practice (≤10 mg/kg/day) (Peters et al., 2003; Hays et al., 2005; Nagasawa et al., 2006). As such, it remains unclear whether the earlier experimental conditions used in rodents accurately reflect the conditions in bezafibrate-treated humans.

In the present study, the contribution of PPARα to the TG-lowering effect of bezafibrate was examined using wild-type and *Ppara*-null mice administered the drug at clinically relevant doses (10 mg/kg/day), and its effects were compared with those at conventional experimental doses (100 mg/kg/day). We were surprised to find that the mechanism of bezafibrate TG reduction differed according to dose and at low doses was independent of PPARα.

Materials and Methods

Mice and Bezafibrate Treatment. All experiments were conducted in accordance with animal care guidelines approved by the Shinshu University School of Medicine. *Ppara*-null mice on a Sv/129 genetic background were described elsewhere (Lee et al., 1995; Akiyama et al., 2001). 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.

Sixteen-week-old male Sv/129 wild-type and *Ppara*-null mice (25–30 g b.wt.) were each randomly assigned to one of three groups [control group, $n = 6$ for each genotype; low-dose bezafibrate (10 mg/kg/day) group, $n = 24$ for each genotype; and high-dose bezafibrate (100 mg/kg/day) group, $n = 24$ for each genotype]. Bezafibrate (2-[4-[2-[4-chlorobenzamido]ethyl]phenoxy]-2-methylpropionic acid; Kissei Pharmaceutical, Matsumoto, Japan) was suspended in 1%

TABLE 1

Primers used for quantitative real-time PCR

Gene	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)	NCBI GenBank
ACC1	GGGCACAGACCGTGGTAGTT	CAGGATCAGCTGGGATACTGAGT	XM_193604
ACC2	CCTCGGGACCACCTATGTGTAC	TCCAACACCAGCTCTGTGTATGT	BC022940
ADRP	GAAGAGAAGCATCGGCTACGA	CGTGACTCGATGTGCTCAACA	NM_007408
aP2	TTTCCTTCAAACTGGGCGTG	AGGGTTATGATGCTCTTCACCTTC	NM_024406
ApoB	TCACCCCCGGGATCAAG	TCCAAGGACACAGAGGGCTTT	XM_137955
ApoCIII	CCTGAAAGGCTACTGGAGCAA	TGGTTGGTCCTCAGGGTTAGA	NM_023114
CPT-I	TGGCATCATCACTGGTGTGTT	GGTCCGATTGATCTTTGCAATC	NM_013495
FAS	ATCCTGGAACGAGAACACGATCT	AGAGACGTGTCACTCCTGGACTT	XM_126624
FAT	CCAAATGAAGATGAGCATAGGACAT	GTTGACCTGCAGTCGTTTTGC	NM_007643
FATP	ACCACCGGGCTTCCTAAGG	CTGTAGGAATGGTGGCCAAAG	NM_011977
FXR	GATTTGGAATCGTACTCCCCATAC	GAAGCCCAGGTTGGAATAGTAAGA	NM_009108
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTCTG	M32599
GPAT	GGCTACGTCCGAGTGGATTTT	AACATCATTCGGTCTTGAAGGAA	NM_008149
HTGL	ACGGGAAGAACAAGATTGGAAG	CGTTCCCTCAAACATAGGGC	NM_008280
Insig1	CACGCCAGTGCCAAATTAGA	CGATCAAATGTCCACCACAAAC	NM_153526
Insig2	GCTTTCTTAGCAACCGTTGTCA	CATCGTTATGCCTCCAGCAAA	NM_133748
LACS	TCCTACGGCAGTGATCTGGTG	GGTTGCCTGTAGTTCCACTTGTG	NM_007981
LPL	CGCTCCATTCATCTCTTCATT	GGCAGAGCCCTTTCTCAAAGG	M63335
LXRα	GCGTCCATTCAGAGCAAGTGT	TCACTCGTGGACATCCCAGAT	AF085745
MCAD	TGCTTTTGATAGAACCAGACCTACAGT	CTTGGTGCTCCACTAGCAGCTT	NM_007382
MTP	GAGCGGTCTGGATTTACAACG	GTAGGTAGTGACAGATGTGGCTTTTG	NM_008642
PDK2	CCGTTGTCCATGAAGCAGTTT	CCTGCCGGAGGAAAGTGAA	NM_133667
PDK4	CACGTACTCCACTGCTCCAACA	TTGGCGTAGAGACGAGAAATTG	NM_013743
PGC-1β	CCTCTCCAGGCAGGTTCAAC	CTTGCTAACATCACAGAGGATATCTTG	NM_133249
PH	CGATACTCTTCCCCCACTACCA	CAGTTACCAACAACGACTCCAATC	NM_023737
PPARα	CCTCAGGGTACCACTACGGAGT	GCCGAATAGTTCGCCGAA	NM_011144
PPARβ	TCAACATGGAATGTCGGGTG	ATACTCGAGCTTCATGCGGATT	NM_011145
PPARγ	TTCCACTATGGAGTTCATGCTTGT	TCCGGCAGTTAAGATCACACCTA	NM_011146
PT	TCTACGGTCAACAGACAGTGTTCA	GGCCATGCCAATGTCATAAGA	NM_130864
SCAP	CCCATACCTGGTGGTCGTTATT	ACTACTCAAGCCTTGTGCAATCC	NM_001103162
SCD-1	AGATCTCCAGTTCTTACACGACCAC	CTTTCATTTCAGGACGGATGTCT	NM_009127
SHP	TGGCCTCTACCCTCAAGAACA	CATGTCTTCAAGGAGTTCAGTGATG	NM_011850
SREBP-1c	GCCCACAATGCCATTGAGA	GCAAGAAGCGGATGTAGTCGAT	AB017337
VLACS	CAACGTCACGGTCATTCAGTACA	GGTCCCGGTCATTTGGTTT	NM_011978

ADRP, adipose differentiation-related protein; aP2, adipocyte fatty acid-binding protein; HTGL, hepatic TG lipase; Insig, insulin-induced gene; LACS, long-chain acyl-CoA synthase; PGC, PPARγ coactivator; PH, peroxisomal bifunctional protein; PT, peroxisomal thiolase; VLACS, very-long-chain acyl-CoA synthase.

(w/v) carboxymethylcellulose (Wako Pure Chemical Industries, Osaka, Japan) at final concentrations of 1.5 and 15 mg/ml for 10 and 100 mg/kg/day treatments, respectively, and 0.2 ml of suspension was administered by gavage once daily (10:00 AM) for 7 days. The same amount of 1% (w/v) carboxymethylcellulose without bezafibrate was administered to control mice in a similar manner.

In an additional experiment, body weight-matched 16-week-old male Sv/129 wild-type and *Ppara*-null mice were prepared and randomly divided into two groups ($n = 24$ in each group). Bezafibrate was suspended in 1% (w/v) carboxymethylcellulose at final concentrations of 4.5 and 9 mg/ml for 30 and 60 mg/kg/day treatments, respectively, and was administered for 7 days. The procedure of bezafibrate administration was identical to that mentioned above.

At day 7 of treatment, 18 mice in each bezafibrate treatment group were used for pharmacokinetic analysis and the remaining mice were subjected to biochemical and histological assays. At the end of treatment, all mice were fasted overnight and sacrificed under anesthesia for collection of blood and liver.

Treatment with Other Fibrates. For the purpose of comparing the effects of bezafibrate with those of other fibrates, 16-week-old male Sv/129 wild-type mice (25–30 g b.wt.) were randomly divided into one of three groups [control group, fenofibrate (5 mg/kg/day) group, and clofibrate (15 mg/kg/day) group; $n = 6$ in each]. Fenofibrate (isopropyl 2-[4-[4-chlorobenzoyl]phenoxy]-2-methylpropionate) and clofibrate (ethyl 2-[4-chlorophenoxy]-2-methylpropionate) were purchased from Wako and administered by gavage for 7 days. The preparation and administration of these agents and biochemical examination were performed in a manner similar to those of bezafibrate.

Pharmacokinetic Study of Bezafibrate. Wild-type and *Ppara*-null mice that had undergone 7-day bezafibrate treatment at doses of 10, 30, 60, or 100 mg/kg/day ($n = 18$ in each) were used. After the final treatment, three mice in each group were sacrificed at time points of 0 (before the final treatment), 0.25, 0.5, 1, 2, and 8 h. Bezafibrate was extracted from plasma (50 μl) by acidification with 0.1 M hydrochloric acid followed by absorption onto an Oasis MAX solid-phase cartridge (Waters Corporation, Milford, MA). The extract was dissolved in a methanol and 0.1% acetic acid solution (65:35, v/v), then subjected to LC/MS/MS analysis. Chromatographic separation was carried out on a liquid chromatograph (LC-10A; Shimadzu, Kyoto, Japan) using a Luna C18 (2) column (5 μm, 2 × 150 mm; Phenomenex, Torrance, CA) under the following conditions: mobile phase, methanol and 0.1% acetic acid (65:35, v/v); flow rate, 0.2 ml/min; column temperature, 40°C; and injection volume, 10 μl. Tandem mass spectrometry detection of bezafibrate was performed on an LC/MS/MS system (API 3000; Applied Biosystems/MDS Sciex, Concord, ON, Canada) using negative electrospray ionization in multiple reaction monitoring mode, where precursor and product ions for bezafibrate were monitored at *m/z* 360.2 and 274.1, respectively. Ketoprofen (Sigma-Aldrich Corporation, St. Louis, MO) was used as an internal standard, and its precursor and product ions were monitored at *m/z* 253.0 and 209.2, respectively. Plasma concentration of bezafibrate was determined by Analyst software 1.4.1 (Applied Biosystems/MDS Sciex), and its reliability was confirmed using quality control samples. Key pharmacokinetic parameters, including maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), and area under the plasma concentration-time curve (AUC), were calculated by noncompartmental analysis with WinNonlin Professional 5.0 (Pharsight Corporation, Mountain View, CA).

Analysis of mRNA Expression. Total liver RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and mRNA was reverse-transcribed using oligo-dT primers with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). 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, Foster City, CA). Specific primers were designed by Primer Express software (Applied Biosystems; Table 1). Each mRNA level was first normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then normalized to that of control wild-type mice.

Immunoblot Analysis. Whole-liver lysates and hepatic nuclear fractions were prepared as described previously (Aoyama et al., 1993, 1995; Tanaka et al., 2008a). Protein concentration was measured using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) (Aoyama et al., 1989). Whole-liver lysates and hepatic nuclear fractions (50 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

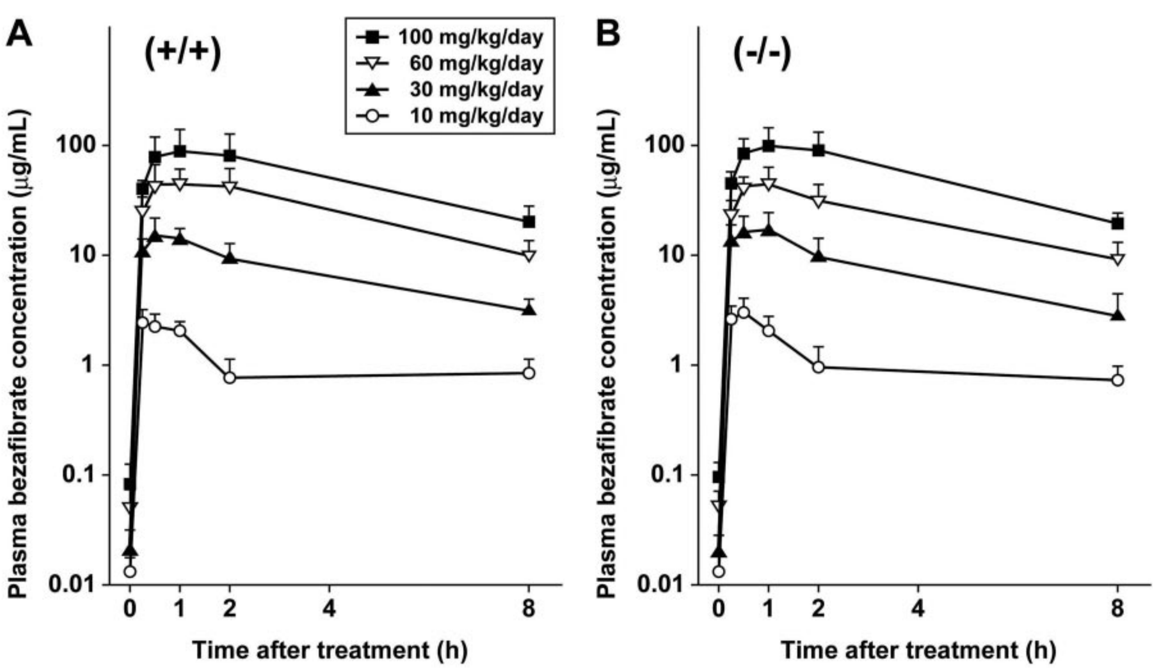

Fig. 1. Plasma pharmacokinetics of bezafibrate in wild-type (+/+) and *Ppara*-null (−/−) mice. Seven-day pharmacokinetics of bezafibrate at 10, 30, 60, or 100 mg/kg/day in wild-type (A) and *Ppara*-null (B) mice. Plasma concentration of bezafibrate was measured using the LC/MS/MS technique. Data are expressed as mean ± S.D. ($n = 3$ at each time point).

After blocking, the membranes were incubated with primary antibodies followed by alkaline phosphatase-conjugated secondary antibodies. Rabbit polyclonal primary antibodies against acetyl-CoA carboxylase (ACC) 1, sterol regulatory element-binding protein (SREBP)-1c, actin, and histone H1, as well as goat polyclonal primary antibodies against fatty acid synthase (FAS), microsomal TG transfer protein (MTP), and SREBP cleavage-activating protein (SCAP), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse polyclonal primary antibodies against glycerol-3-phosphate acyltransferase (GPAT) were obtained from Abnova Corporation (Taipei, Taiwan). Rabbit polyclonal primary antibodies against the 70-kDa peroxisomal membrane protein (PMP70) were described elsewhere (Hashimoto et al., 1986). Anti-rabbit, anti-goat, and anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The position of the immunostained bands was determined by coelectrophoresis of molecular weight standards (Bio-Rad Laboratories, Hercules, CA). The band intensity was quantified densitometrically, normalized to that of actin or histone H1, and then normalized to that of control wild-type mice.

Histopathological Analysis. Small blocks of liver tissue from each mouse were fixed in 4% paraformaldehyde (Wako) in sodium phosphate buffer and embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin for histopathological examination under light microscopy. Cytochemical staining of hepatic peroxisomes was performed using the 3,3′-diaminobenzidine technique, and the morphometry of peroxisomes was determined as described elsewhere (Zhang et al., 2006; Tanaka et al., 2008a).

Other Methods. Serum concentrations of TG and free fatty acid (FFA) were measured using a Triglyceride E-test kit and NEFA C-test kit (Wako), respectively. Serum levels of glucose, insulin, and adiponectin were determined using a Glucose CII-test kit (Wako), mouse insulin ELISA kit (AKRIN-011T; Shibayagi, Gunma, Japan), and mouse/rat adiponectin ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan), respectively. Serum aspartate aminotransferase and alanine aminotransferase levels were measured by a Transaminase CII-test kit (Wako). To determine hepatic lipid content, total lipid in liver tissue (50 mg) was extracted using the hexane/isopropanol method (Hara and Radin, 1978). The lipid extract was solubilized in distilled water by addition of Triton X-100 (Wako) as described previously (Carr et al., 1993) with minor modifications, and then hepatic TG and FFA levels were measured. For analysis of hepatic fatty acid composition, total lipid in liver tissue (50 mg) was extracted in a similar manner in the presence of butylated hydroxytoluene (Sigma-Aldrich) to avoid oxidation of unsaturated fatty acids, and then subjected to gas chromatography assays (Mitsubishi Chemical Medience Corporation, Tokyo, Japan).

Statistical Analysis. Results are expressed as mean ± S.D. Statistical analysis was performed using the Student's t test with

TABLE 2

Pharmacokinetic parameters of bezafibrate in mice and humans

Data are expressed as mean values. Pharmacokinetic parameters of bezafibrate-treated wild-type (+/+) and *Ppara*-null (−/−) mice were calculated from the data shown in Fig. 1 (n = 3 at each time point). The pharmacokinetic data for bezafibrate-treated humans were reported by three groups: Kajosaari et al. (2004) determined C_{max} and AUC from 0 to 8 h in 12 healthy male volunteers after 5-day oral administration of a 400-mg slow-release tablet; Abshagen et al. (1979) calculated those from 0 to 10 h in 10 healthy male subjects after a single intake of 300-mg tablets; and Ali et al. (2002) measured those from 0 to 9 h in 14 healthy male volunteers after single oral administration of a 200-mg tablet.

Species and Dose	T_{max} (+/+)	T_{max} (−/−)	C_{max} (+/+)	C_{max} (−/−)	AUC (+/+)	AUC (−/−)	Reference
	h		*μg/ml*		*μg · h/ml*		
Mouse							
100 mg/kg/day	1.00	1.00	84.4	93.3	396.0	419.9	
60 mg/kg/day	1.00	1.00	45.9	43.2	188.3	168.3	
30 mg/kg/day	0.50	1.00	15.2	16.8	55.3	58.9	
10 mg/kg/day	0.25	0.50	2.4	2.8	8.0	9.1	
Human							
400 mg/day			4.9		18.1		Kajosaari et al. (2004)
300 mg/day			10.5		39.0		Abshagen et al. (1979)
200 mg/day			1.7		5.2		Ali et al. (2002)

TABLE 3

General effects of bezafibrate in wild-type (+/+) and *Ppara*-null (−/−) mice

Results are expressed as mean ± S.D. (n = 6 in each group).

Bezafibrate	*Ppara*(+/+) 0 mg/kg/day	*Ppara*(+/+) 10 mg/kg/day	*Ppara*(+/+) 100 mg/kg/day	*Ppara*(−/−) 0 mg/kg/day	*Ppara*(−/−) 10 mg/kg/day	*Ppara*(−/−) 100 mg/kg/day
Body weight						
Beginning (g)	27.3 ± 1.8	28.1 ± 1.4	28.2 ± 1.7	27.3 ± 2.1	28.1 ± 1.4	28.2 ± 1.9
Ending (g)	27.4 ± 2.4	27.5 ± 1.6	27.4 ± 1.8	27.8 ± 2.0	27.5 ± 1.9	27.6 ± 1.5
Change (%)	100.3 ± 2.1	97.8 ± 1.3	97.3 ± 1.8	101.8 ± 3.0	97.9 ± 2.1	98.1 ± 1.9
Liver weight/body weight (%)	4.3 ± 0.4	4.5 ± 0.2	5.4 ± 0.3*	4.4 ± 0.3	4.5 ± 0.3	4.7 ± 0.2
Serum data						
TG (mg/dl)	131 ± 21	95 ± 12*	79 ± 11*	99 ± 12	73 ± 10*	92 ± 12
FFA (mEq/l)	0.63 ± 0.11	0.57 ± 0.06	0.74 ± 0.09	0.46 ± 0.04	0.43 ± 0.05	0.52 ± 0.05
Glucose (mg/dl)	122 ± 11	76 ± 10*	108 ± 12	89 ± 7	53 ± 16*	91 ± 6
Insulin (ng/ml)	0.33 ± 0.12	0.33 ± 0.07	0.30 ± 0.13	0.31 ± 0.19	0.31 ± 0.22	0.32 ± 0.23
Adiponectin (μg/ml)	19 ± 2	20 ± 2	22 ± 3	16 ± 2	17 ± 2	18 ± 2
Aspartate aminotransferase (IU/l)	61 ± 7	62 ± 5	77 ± 11	71 ± 6	70 ± 10	72 ± 9
Alanine aminotransferase (IU/l)	15 ± 2	15 ± 1	18 ± 2	13 ± 2	14 ± 2	15 ± 1
Liver data						
TG (mg/g liver)	16 ± 3	8 ± 2*	6 ± 2*	39 ± 6	22 ± 9*	60 ± 14
FFA (μEq/g liver)	15 ± 1	14 ± 2	17 ± 2	23 ± 4	20 ± 4	33 ± 8

* $P < 0.05$, between treated and untreated mice of the same genotype.

SPSS software (ver. 11.5J for Windows; SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

Pharmacokinetics of Bezafibrate. The time course changes in plasma concentration of bezafibrate were first determined to evaluate the validity of our experimental conditions. Bezafibrate concentrations at time 0 in wild-type mice after 7-day administration of 100 mg/kg/day doses were approximately 0.1 μg/ml; in the 10 mg/kg/day administration group, however, bezafibrate was almost undetectable (Fig. 1A), suggesting a rapid turnover of circulating bezafibrate in mice. There were no significant differences in T_{max}, C_{max}, and AUC between the genotypes at each dose (Fig. 1B and Table 2). The mean values of C_{max} and AUC increased in both genotypes in a dose-dependent manner, and these parameters at the 100 mg/kg/day dose were approximately 35- and 50-fold greater than those at the 10 mg/kg/day dose, respectively (Table 2). It is noteworthy that C_{max} and AUC of the 10 mg/kg/day groups were comparable with those of bezafibrate-treated humans shown in two recent reports (Ali et al., 2002; Kajosaari et al., 2004) (Table 2). These findings confirmed that the conditions in mice given 10 mg/kg/day of bezafibrate, but not 100 mg/kg/day, are pharmacokinetically comparable with human clinical conditions.

General Effects of Bezafibrate. Next, the effects of bezafibrate were compared in clinically relevant low doses (10 mg/kg/day) and conventional high doses (100 mg/kg/day). All mice appeared healthy throughout the experiment, and no significant differences in body weight were noted among the groups at the end of the study. Liver/body weight ratio was increased in wild-type mice treated with high-dose bezafibrate only. Serum/liver TG levels were decreased by high-dose bezafibrate in wild-type mice and by low doses of bezafibrate in both genotypes, whereas serum/liver FFA levels remained similar in both groups (Table 3). As demonstrated in Table 4, hepatic fatty acid composition was altered to some degree in wild-type and *Ppara*-null mice given high-dose bezafibrate but not in low-dose mice. Fasting serum glucose levels were decreased by low-dose bezafibrate in both genotypes, but serum levels of insulin and adiponectin remained constant (Table 3). Serum aspartate aminotransferase and alanine aminotransferase levels were not significantly altered by bezafibrate administration; however, these values tended to be elevated in wild-type mice administered high

TABLE 4

Fatty acid composition in the livers of bezafibrate-treated wild-type (+/+) and *Ppara*-null (−/−) mice

Results are expressed as mean ± S.D. (n = 6 in each group).

Bezafibrate	*Ppara*(+/+)			*Ppara*(−/−)		
	0 mg/kg/day	10 mg/kg/day	100 mg/kg/day	0 mg/kg/day	10 mg/kg/day	100 mg/kg/day
C12:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
C14:0	0.8 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.2
C14:1 n-5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C16:0	27.6 ± 2.0	29.2 ± 4.0	28.0 ± 0.1	20.9 ± 0.3	23.6 ± 4.0	20.8 ± 0.2
C16:1 n-7	2.5 ± 0.4	2.0 ± 0.5	3.4 ± 1.1	2.0 ± 0.2	1.6 ± 0.1	2.0 ± 0.8
C18:0	12.6 ± 0.4	12.5 ± 2.0	11.1 ± 1.1	12.8 ± 0.3	14.0 ± 1.5	8.5 ± 0.3*
C18:1 n-9	11.9 ± 0.4	10.5 ± 3.4	15.6 ± 1.1*	13.9 ± 0.3	11.0 ± 3.0	18.4 ± 3.5
C18:2 n-6	21.9 ± 0.4	23.3 ± 3.7	15.9 ± 0.3*	25.8 ± 0.3	25.8 ± 1.8	27.6 ± 3.5
C18:3 n-6	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0*
C18:3 n-3	1.0 ± 0.1	0.7 ± 0.2	0.3 ± 0.1*	1.4 ± 0.2	0.7 ± 0.2	0.9 ± 0.1
C20:0	0.5 ± 0.2	0.4 ± 0.1	0.2 ± 0.0	0.7 ± 0.0	0.5 ± 0.2	0.5 ± 0.0
C20:1 n-9	0.7 ± 0.1	0.7 ± 0.4	0.6 ± 0.0	1.1 ± 0.0	0.9 ± 0.3	1.4 ± 0.2
C20:2 n-6	0.5 ± 0.0	0.5 ± 0.1	0.4 ± 0.0*	0.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
C20:3 n-9	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1*	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C20:3 n-6	2.0 ± 0.1	2.0 ± 0.1	4.0 ± 0.1*	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.0
C20:4 n-6	6.2 ± 0.4	6.5 ± 0.5	7.4 ± 0.8	5.6 ± 0.1	6.7 ± 0.7	5.2 ± 0.4
C20:5 n-3	0.8 ± 0.2	0.8 ± 0.3	1.2 ± 0.3	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.3
C22:0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C22:1 n-9	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
C22:4 n-6	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.6 ± 0.1*
C22:5 n-3	0.7 ± 0.2	0.7 ± 0.0	1.0 ± 0.2	1.1 ± 0.0	1.1 ± 0.2	1.2 ± 0.3
C22:6 n-3	9.0 ± 0.7	8.5 ± 1.9	9.1 ± 0.1	9.3 ± 0.2	8.9 ± 0.2	8.1 ± 0.3*
C24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C24:1 n-9	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

* $P < 0.05$, between treated and untreated mice of the same genotype.

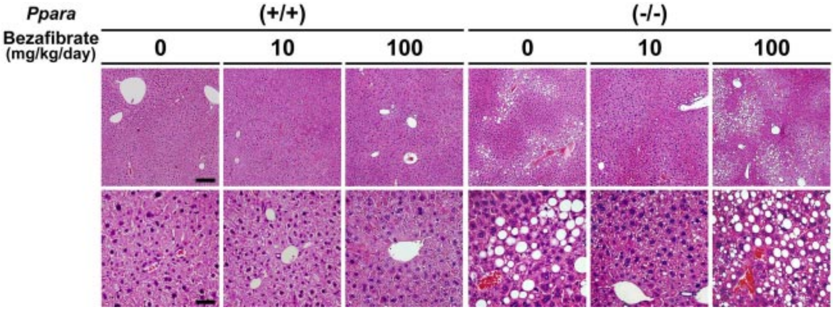

Fig. 2. Histological appearance of hematoxylin-and-eosin-stained liver sections from bezafibrate-treated wild-type (+/+) and *Ppara*-null (−/−) mice. Bezafibrate at high (100 mg/kg/day) or low (10 mg/kg/day) doses was orally administered to wild-type and *Ppara*-null mice for 7 days. No steatosis was found in the livers of wild-type mice. Hepatic steatosis in *Ppara*-null mice was markedly ameliorated by low-dose bezafibrate. Hepatic inflammation or hepatocyte degeneration was not detected in any group. Scale bars in the upper and lower panels of control wild-type mice indicate 200 and 50 μm, respectively.

doses. Histological examination showed that hepatic steatosis in *Ppara*-null mice was markedly ameliorated by low-dose bezafibrate, and apparent hepatic inflammation or hepatocyte degeneration was not detected in any group (Fig. 2). These results indicate that bezafibrate-induced TG reduction by low-dose treatment occurs via a PPARα-independent mechanism.

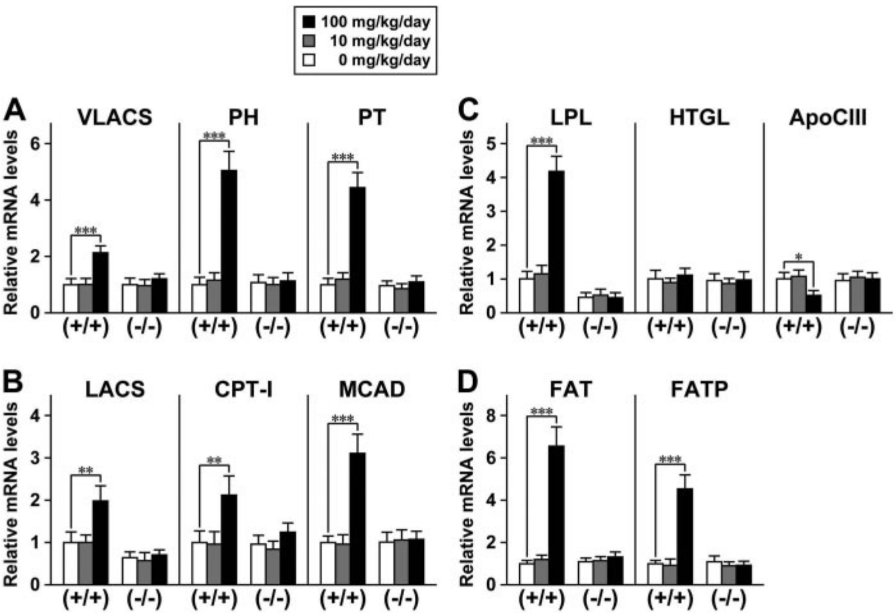

Fig. 3. Effects of bezafibrate on fatty acid β-oxidation and uptake in the livers of wild-type (+/+) and *Ppara*-null (−/−) mice. Hepatic expression of mRNAs encoding peroxisomal (A) and mitochondrial (B) fatty acid β-oxidation enzymes, proteins related to lipoprotein lipolysis (C), and fatty acid transporters (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 ± S.D. (n = 6 in each group). White bar, control group; gray bar, low-dose bezafibrate (10 mg/kg/day) group; black bar, high-dose bezafibrate (100 mg/kg/day) group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, between treated and untreated mice of the same genotype.

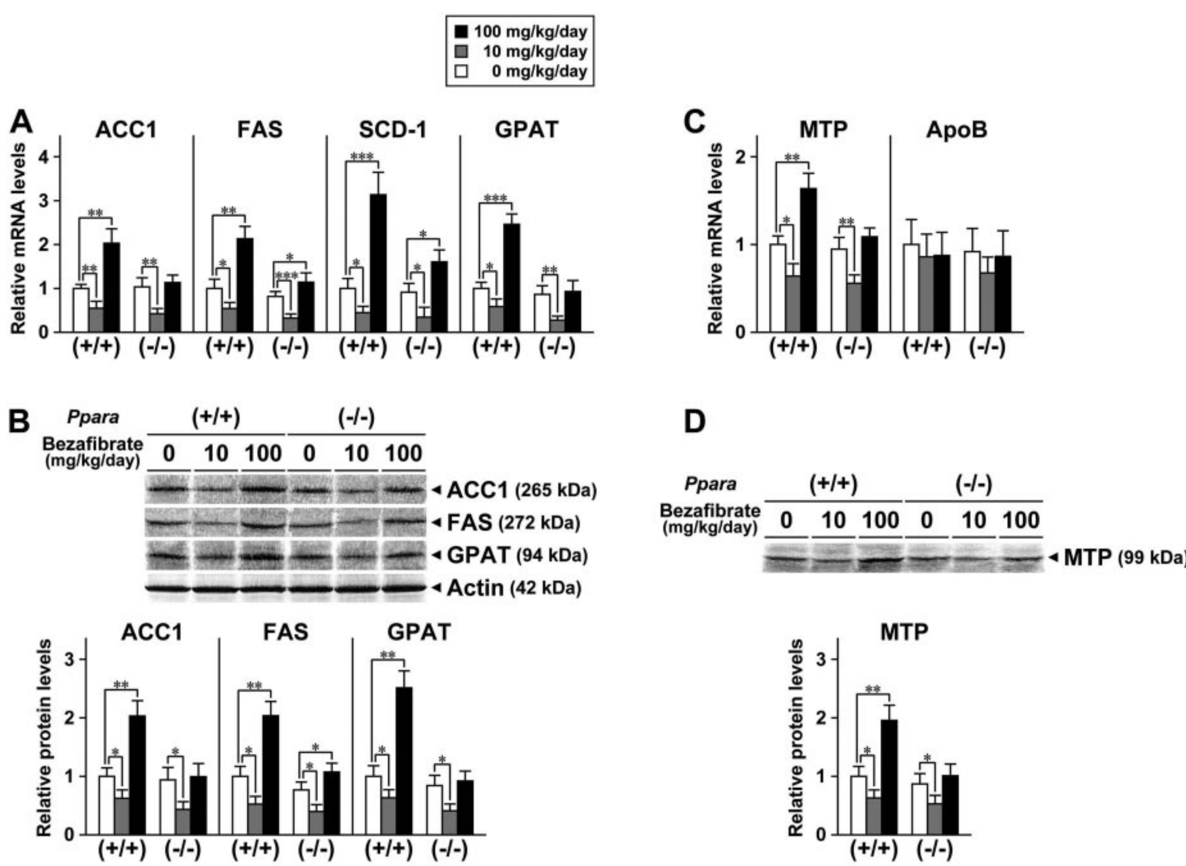

Fig. 4. Effects of bezafibrate on de novo lipogenesis and TG secretion in the livers of wild-type (+/+) and *Ppara*-null (−/−) mice. A and C, hepatic expression of mRNAs encoding lipogenic enzymes (A) and proteins associated with VLDL secretion (C) was analyzed by quantitative real-time PCR and normalized 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 ± S.D. (n = 6 in each group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ between treated and untreated mice of the same genotype. B and D, immunoblot analysis of lipogenic enzymes (B) and MTP (D). Fifty micrograms of whole-liver lysate proteins from each mouse were loaded into each well. Actin was used as a loading control. 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 ± S.D. (n = 6 in each group). *, $P < 0.05$; **, $P < 0.01$, between treated and untreated mice of the same genotype. White bars, control group; gray bars, low-dose bezafibrate (10 mg/kg/day) group; black bars, high-dose bezafibrate (100 mg/kg/day) group.

Effects of Bezafibrate on Fatty Acid β-Oxidation. To elucidate the mechanism of the PPARα-independent TG-lowering effect of low-dose bezafibrate, hepatic expression of mRNAs encoding proteins involved in fatty acid and TG metabolism was measured. High-dose bezafibrate administration significantly increased the mRNAs encoding peroxisomal fatty acid β-oxidation enzymes (very-long-chain acyl-CoA synthase, peroxisomal bifunctional protein, and peroxisomal thiolase) and mitochondrial β-oxidation enzymes [long-chain acyl-CoA synthase, carnitine palmitoyl-CoA transferase (CPT)-I, and medium-chain acyl-CoA dehydrogenase (MCAD)] in wild-type mice only (Fig. 3, A and B). In contrast, low-dose administration did not increase the mRNAs encoding any of these enzymes (Fig. 3, A and B), suggesting that the TG-lowering effect of low-dose bezafibrate is not due to stimulation of the fatty acid β-oxidation pathway.

Effects of Bezafibrate on Circulating Lipid Clearance. High-dose bezafibrate administration to wild-type mice resulted in a marked increase in LPL mRNA expression and a decrease in ApoCIII mRNA expression (Fig. 3C). High-dose treatment also elevated the mRNA levels of the fatty acid transporters fatty acid translocase (FAT) and fatty acid transport protein (FATP) in wild-type mice (Fig. 3D). In contrast, low-dose administration did not induce these changes (Fig. 3, C and D).

Effects of Bezafibrate on De Novo Lipogenesis and TG Secretion. High-dose bezafibrate administration markedly increased the expression of enzymes involved in de novo lipogenesis, ACC1, FAS, stearoyl-CoA desaturase (SCD)-1, and GPAT in wild-type mice, whereas their *Ppara*-null counterparts showed only slight increases in FAS and SCD-1 expression (Fig. 4, A and B). On the other hand, low-dose bezafibrate treatment significantly decreased the expression of these lipogenic enzymes in both genotypes (Fig. 4, A and B). Moreover, low-dose treatment reduced the expression of MTP, which acts on the assembly and secretion of VLDL particles, in both mouse lines but did not influence ApoB expression (Fig. 4, C and D). These data show that attenuation of hepatic de novo lipogenesis and VLDL secretion is associated with serum/liver TG reduction observed in mice given low-dose bezafibrate.

Effects of Bezafibrate on SREBP-1c. To investigate the effects of bezafibrate on lipogenesis more closely, the hepatic expression of transcription factors regulating lipogenic enzymes was examined. High-dose bezafibrate administration increased the expression of the mRNAs encoding SREBP-1c, liver X receptor (LXR) α, farnesoid X receptor (FXR), and short heterodimer partner (SHP) in wild-type mice (Fig. 5A). It is noteworthy that low-dose administration significantly reduced SREBP-1c mRNA level in both genotypes but did not affect the mRNA levels of PPARγ coactivator-1β, LXRα, FXR, or SHP (Fig. 5A). Immunoblot analysis revealed that the decreases in nuclear SREBP-1c expression by low-dose bezafibrate were in accordance with mRNA findings (Fig. 5B) and were well correlated with expression of the SREBP-1c target genes, ACC1, FAS, SCD-1, and GPAT (Fig. 4, A and B). In addition, high-dose bezafibrate treatment elevated the ex-

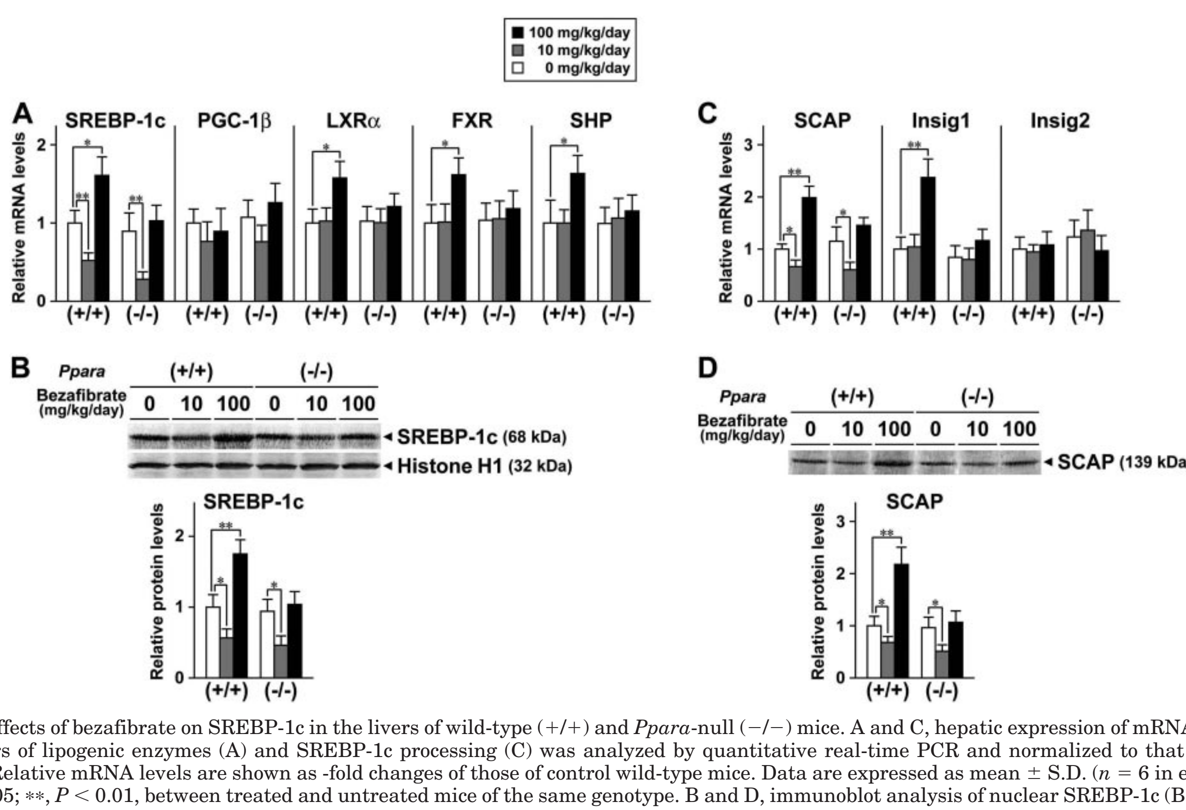

Fig. 5. Effects of bezafibrate on SREBP-1c in the livers of wild-type (+/+) and *Ppara*-null (−/−) mice. A and C, hepatic expression of mRNAs encoding regulators of lipogenic enzymes (A) and SREBP-1c processing (C) was analyzed by quantitative real-time PCR and normalized to that of GAPDH mRNA. Relative mRNA levels are shown as -fold changes of those of control wild-type mice. Data are expressed as mean ± S.D. ($n = 6$ in each group). *, $P < 0.05$; **, $P < 0.01$, between treated and untreated mice of the same genotype. B and D, immunoblot analysis of nuclear SREBP-1c (B) and SCAP (D). For detection of nuclear SREBP-1c, hepatic nuclear fractions were prepared from each mouse, and 50 μg of nuclear protein was loaded into each well. For SCAP detection, 50 μg of whole-liver lysate proteins from each mouse were loaded into each well. Histone H1 and actin were used as loading controls for nuclear SREBP-1c and SCAP, respectively. Band intensity was quantified densitometrically and normalized to that of histone H1 or actin. Relative protein levels are shown as -fold changes of those of control wild-type mice. Data are expressed as mean ± S.D. ($n = 6$ in each group). *, $P < 0.05$; **, $P < 0.01$, between treated and untreated mice of the same genotype. White bars, control group; gray bars, low-dose bezafibrate (10 mg/kg/day) group; black bars, high-dose bezafibrate (100 mg/kg/day) group.

pression of SCAP and insulin-induced gene 1, which influences the post-translational processing of SREBPs, in wild-type mice, whereas low-dose treatment reduced SCAP expression in both mouse lines (Fig. 5, C and D). The expression of insulin-induced gene 2, another regulator of SREBP processing, remained unchanged. These results demonstrate that attenuation of lipogenesis by low-dose bezafibrate is attributable to down-regulation of SREBP-1c.

Effects of Bezafibrate on PPARs. High-dose bezafibrate administration significantly increased the expression of PPARα and its target genes encoding β-oxidation enzymes, LPL, FAT, FATP, and PMP70 (Mandard et al., 2004) and caused marked peroxisome proliferation in wild-type mice only (Fig. 6, A–C), reflecting the presence of potent PPARα activation (Lee et al., 1995; Tanaka et al., 2003, 2008b). On the other hand, low-dose bezafibrate treatment did not induce these changes (Fig. 6, A–C). Low-dose treatment decreased PPARβ mRNA expression in both genotypes, whereas the treatment did not increase its target genes ACC2, pyruvate dehydrogenase kinase (PDK) 4, PDK2, and adipose differentiation-related protein (Chawla et al., 2003; Lee et al., 2006; Degenhardt et al., 2007) (Fig. 6, A and D). Furthermore, low-dose bezafibrate did not affect the expression of PPARγ, its specific target gene encoding adipocyte fatty acid-binding protein (Tontonoz et al., 1994), or PPARα/γ dual targets LPL, FAT, and FATP (Motojima et al., 1998) (Fig. 6, A and D). These findings confirmed that low doses of bezafibrate do not activate any murine PPARs.

Effects of Bezafibrate on PPARα and SREBP-1c at Intermediate Doses. We found that bezafibrate action was completely different between clinically comparable doses and conventional high ones. To explore in which doses this difference appeared, we analyzed the hepatic expression of PPARα, SREBP-1c, and their target genes in wild-type mice given 30 or 60 mg/kg/day intermediate doses of bezafibrate. Although bezafibrate treatment at 30 mg/kg/day did not activate PPARα at all, it significantly inhibited the SREBP-1c-regulated pathway (Fig. 7), in agreement with the results of the low-dose (10 mg/kg/day) treatments (Fig. 4 and 5). On the other hand, bezafibrate at 60 mg/kg/day caused activation of PPARα without any impact on SREBP-1c (Fig. 7). The degree of PPARα activation detected in this group was less than that with high doses (100 mg/kg/day), however. Down-regulation of SREBP-1c at a dose of 30 mg/kg/day was also detected in *Ppara*-null mice but was not observed at a dose of 60 mg/kg/day (data not shown). Considering these findings, the target of bezafibrate appears to vary between PPARα and SREBP-1c according to dose.

Effects of Fenofibrate and Clofibrate on PPARα and SREBP-1c at Clinically Relevant Doses. Finally, we evaluated whether clinically relevant low doses of other fibrates, such as fenofibrate and clofibrate, had SREBP-1c-suppressive potential as well. Both agents decreased hepatic TG content and activated PPARα but did not lower the mRNA levels of SREBP-1c or its targets (Fig. 8). Thus, the effect on SREBP-1c is considered to be unique to bezafibrate.

Discussion

Until now, the mechanism of the TG-lowering effect of bezafibrate has been explained mainly by PPARα activation according to rodent data from suprapharmacologic dosing regimens. In the present study, we demonstrated that major pharmacokinetic parameters in bezafibrate-treated humans, such as C_{max} and AUC, were similar to those in mice treated at clinically relevant low doses (10 mg/kg/day) but not in mice at conventionally used high doses (100 mg/kg/day). Furthermore, we found that low doses of bezafibrate exerted a serum/liver TG-lowering effect via down-regulation of SREBP-1c, not by PPAR activation. Thus, these findings provide a novel explanation for the pharmacological mechanism of bezafibrate action.

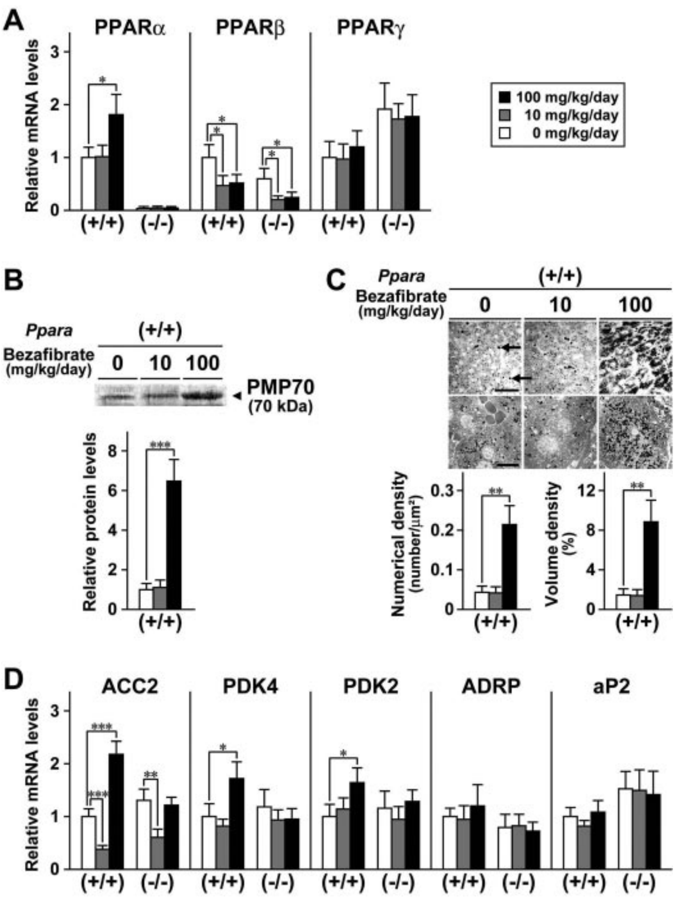

Fig. 6. Effects of bezafibrate on PPARs in the livers of wild-type (+/+) and *Ppara*-null (−/−) mice. A and D, hepatic expression of mRNAs encoding PPARs (A) and PPARβ target genes (ACC2, PDK4, PDK2, and adipose differentiation-related protein) and PPARγ target gene (adipocyte fatty acid-binding protein) (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 ± S.D. (n = 6 in each group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, between treated and untreated mice of the same genotype. B, immunoblot analysis of PMP70. Fifty micrograms of whole-liver lysate proteins from each mouse were loaded into each well. Actin was used as a loading control. 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 ± S.D. (n = 6 in each group). ***, $P < 0.001$, between treated and untreated wild-type mice. C, cytochemical staining and morphometry of hepatic peroxisomes. Peroxisomes were detected as dark particles, and marked peroxisome proliferation was found under high-dose bezafibrate treatment only. Scale bars in the light (upper) and electron (lower) photomicrographs of control mice indicate 50 and 10 μm, respectively. Arrows indicate erythrocytes. The number of peroxisomes and area of each individual peroxisomal profile were measured in 10 electron photomicrographs from each mouse, and numerical and volume densities were calculated. Data are expressed as mean ± S.D. (n = 6 in each group). **, $P < 0.01$, between treated and untreated wild-type mice. White bars, control group; gray bars, low-dose bezafibrate (10 mg/kg/day) group; black bars, high-dose bezafibrate (100 mg/kg/day) group.

The discrepancy in bezafibrate dosage between rodents and humans was previously thought to be due to species differences in pharmacokinetics. The present study uncovered a resemblance in bezafibrate pharmacokinetics between mice and humans at comparable clinical dosages. This suggests that the mechanism of TG reduction in mice by high-dose bezafibrate via the enhancement of hepatic fatty acid uptake and degradation induced by PPARα activation may not accurately reflect its clinical effect in humans. In contrast, mice receiving low-dose bezafibrate showed pharmacokinetics and marked decreases in serum TG and glucose levels similar to those in humans (Tenenbaum et al., 2005). Considering these close similarities, the mechanism of low-dose bezafibrate found in this study may be better translated to humans.

Human pharmacokinetic data that correspond to our mouse data from 10 mg/kg/day bezafibrate treatment have been reported (Ali et al., 2002; Kajosaari et al., 2004) but differ somewhat from those of an earlier report (Abshagen et al., 1979). This disagreement might have derived from differences in determination procedure of serum bezafibrate concentrations (high-performance liquid chromatography versus gas chromatography) and racial and/or anthropometric differences of the subjects. However, the data from Abshagen et al. (1979) were similar to ours obtained from mice receiving 30 mg/kg/day of bezafibrate, and SREBP-1c down-regulation without PPARα activation occurred in these mice as well. Taken together, these observations support the notion that mice with low-dose bezafibrate administration are superior to those with conventional high-dose administration as a model to evaluate drug action in humans.

Low-dose bezafibrate treatment significantly attenuated hepatic lipogenesis via down-regulation of SREBP-1c and decreased VLDL secretion via MTP reduction, while maintaining fatty acid and TG catabolism at a constitutive level. These effects were distinct from the effects of high doses of bezafibrate, indicating the importance of understanding bezafibrate dosage on its mode of action. Previous studies have suggested an involvement of SREBP-1c in the regulation of VLDL production and secretion (Horton et al., 2002;

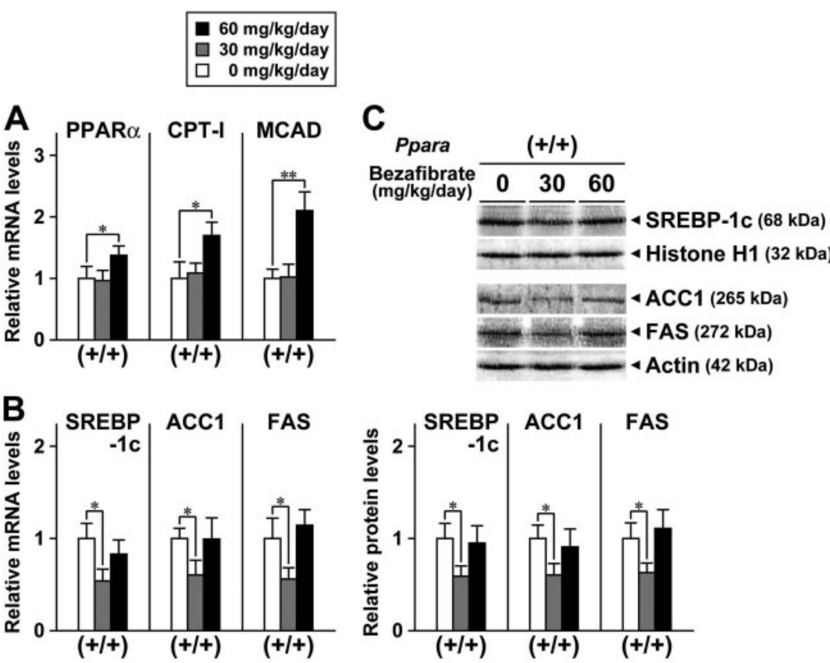

Fig. 7. Effects of bezafibrate on PPARα and SREBP-1c at two intermediate doses in the livers of wild-type (+/+) mice. A and B, hepatic expression of mRNAs encoding PPARα and its targets (CPT-I and MCAD) (A) and SREBP-1c and its targets (ACC1 and FAS) (B) was analyzed by quantitative real-time PCR and normalized to that of GAPDH mRNA. Relative mRNA levels are shown as -fold changes of those of control mice. Data are expressed as mean ± S.D. ($n = 6$ in each group). C, immunoblot analysis of nuclear SREBP-1c, ACC1, and FAS. For detection of nuclear SREBP-1c, hepatic nuclear fractions (50 μg of protein) were loaded into each well. For analysis of ACC1 and FAS, whole liver lysates (50 μg of protein) were adopted. Histone H1 and actin were used as loading controls. Band intensity was quantified densitometrically and normalized to that of histone H1 or actin. Relative protein levels are shown as -fold changes of those of control mice. Data are expressed as mean ± S.D. ($n = 6$ in each group). *, $P < 0.05$, between treated and untreated mice. White bar, control group; gray bar, 30 mg/kg/day bezafibrate group; black bar, 60 mg/kg/day bezafibrate group. *$P < 0.05$, **$P < 0.01$, between treated and untreated mice.

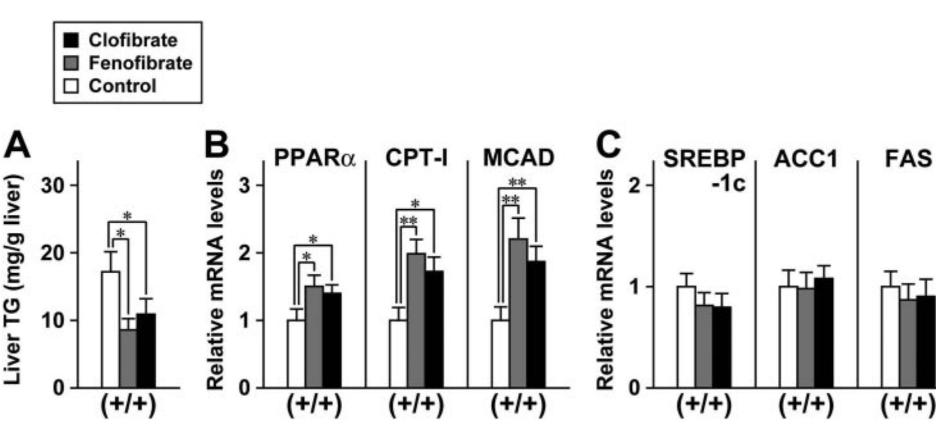

Fig. 8. Effects of fenofibrate and clofibrate at clinically relevant low doses on hepatic TG, PPARα, and SREBP-1c in wild-type (+/+) mice. A, hepatic TG content. Data are expressed as mean ± S.D. ($n = 6$ in each group). White bar, control group; gray bar, fenofibrate (5 mg/kg/day) group; black bar, clofibrate (15 mg/kg/day) group. *$P < 0.05$, between treated and untreated mice. B and C, hepatic expression of mRNAs encoding PPARα and its targets (CPT-I and MCAD) (B) and SREBP-1c and its targets (ACC1 and FAS) (C) 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 ± S.D. ($n = 6$ in each group). Bars are identical to those in Fig. 8A. *, $P < 0.05$; **, $P < 0.01$, between treated and untreated mice.

Watanabe et al., 2004), and others have found a similar expression pattern between SREBP-1 and MTP (Wang et al., 1997). Therefore, the decreased expression of MTP observed in mice administered low-dose bezafibrate might be linked to down-regulation of SREBP-1c. Suppression of lipid synthesis and secretion from the liver is presumed to be central to the hypolipidemic effects of low-dose bezafibrate.

A striking finding in this study is that clinically comparable doses of bezafibrate down-regulated SREBP-1c at the transcriptional level. The reduction of SREBP-1c mRNA levels is caused by decreases in insulin (Shimomura et al., 1999) and LXRα levels (Repa et al., 2000) and by increases in FXR-regulated SHP expression (Watanabe et al., 2004) and polyunsaturated fatty acids (Mater et al., 1999). Serum insulin concentrations and hepatic LXRα and FXR/SHP mRNA levels all remained constant in our study. Although fibrates have been reported to antagonize LXRα, such action has been limited to esterified fibrates, including fenofibrate and clofibrate, not bezafibrate (Thomas et al., 2003). Indeed, the mRNA level of LPL, a typical LXRα target gene (Zhang et al., 2001), did not change from low-dose bezafibrate treatment. Furthermore, the hepatic composition of fatty acids, especially polyunsaturated ones, was not altered in mice treated with low-dose bezafibrate. Judging from these findings, other mechanisms may be responsible for the down-regulation of SREBP-1c transcription. The decreased expression of SCAP, a protein critical for production of nuclear SREBPs (Matsuda et al., 2001), might indirectly contribute to a decrease in SREBP-1c mRNA to some degree because nuclear SREBPs activate the transcription of their own genes (Horton et al., 2002), and a disruption of SCAP leads to substantial decreases in nuclear SREBPs followed by their mRNAs (Matsuda et al., 2001). We previously reported that low doses of bezafibrate down-regulated SREBP-2 at both the mRNA and protein levels in a PPARα-independent manner (Nakajima et al., 2008), which might be also associated with SCAP reduction. As far as we know, there are no medical agents to date that can significantly decrease SREBP expression at relatively low concentrations. Thus, it is tempting to speculate that bezafibrate might become a new tool to suppress SREBP-regulated pathways.

Unlike bezafibrate, neither fenofibrate nor clofibrate could suppress SREBP-1c function without induction of PPARα activation at clinically relevant doses. Many studies have noted marked induction of SREBP-1c in several fatty liver diseases (Horton et al., 2002; Moriishi et al., 2007). It is also suggested that persistent and strong PPARα activation is sometimes harmful in such conditions (Tanaka et al., 2008b). Therefore, bezafibrate might be beneficial for treatment of fatty liver diseases than other fibrate drugs.

Finally, it is noteworthy that low-dose bezafibrate reduced serum glucose levels in a PPARα-independent manner without affecting serum insulin/adiponetin levels. The hypoglycemic effect of bezafibrate has been documented in patients with and without diabetes and is believed to be due to PPARα and PPARγ activation (Tenenbaum et al., 2005), the latter of which is associated with increased production of adiponectin (Yamauchi et al., 2001). However, a recent study showed that SREBP-1c directly suppresses the transcription of insulin receptor substrate-2 and inhibits insulin action in the liver (Ide et al., 2004). In addition, overexpression of hepatic SREBP-1c has been observed in many insulin-resistant animal models, including *ob/ob* and PPARα(+/−):low-density lipoprotein receptor(+/−) mice (Horton et al., 2002; Li et al., 2008). Thus, the glucose-lowering effect found in low-dose bezafibrate treatment might instead stem from enhanced hepatic insulin sensitivity through a decrease in SREBP-1c expression. To clarify the mechanism of bezafibrate-induced hypoglycemic effects, further studies using insulin-resistant animals are necessary.

Several points raised in this study require further investigation. First, although the absence of PPAR activation was based on the noninduction of various PPAR target genes and peroxisome proliferation, we cannot rule out the possibility that the above-mentioned effects of low-dose bezafibrate were nonetheless somehow mediated by PPARβ and/or PPARγ. Similar experiments using mice lacking all PPAR subtypes might address this issue. Second, our experiments were conducted under normal conditions; the actual efficacy of low-dose bezafibrate needs to be evaluated using diseased mouse models as well. Finally, it is difficult to explain why the SREBP-1c-down-regulating effect disappears at higher doses of bezafibrate; its precise molecular mechanism requires further investigation.

In conclusion, this study demonstrated for the first time that the TG-lowering effect of bezafibrate at clinically relevant doses in mice is independent of PPAR activation. Moreover, our findings revealed that this action is associated with suppression of the SREBP-1c-regulated pathway in the liver. Because up-regulation of hepatic SREBP-1c is closely related to the development of various metabolic disorders, such as dyslipidemia, diabetes, and fatty liver diseases, our results may offer a new mechanism of bezafibrate efficacy against these diseases.

Acknowledgments

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