

including inflammation and oxidative stress (18). Ceramide is generated by *de novo* synthesis as well as hydrolysis of membrane sphingomyelin by sphingomyelinase (SMase) (18, 19). Ceramide and its metabolites, sphingosine and sphingosine 1-phosphate (S1P), mediate a variety of biological events such as apoptosis, cell growth, and the stress response (18, 19). In contrast, AMP-activated protein kinase (AMPK) is another mediator of metabolic stress that responds to the negative energy balance within cells (20–22). AMPK is activated by stresses that increase intracellular AMP level such as local hypoxia, glucose deprivation, and ischemia (21). Whereas the function of AMPK in liver and muscle has been well investigated, its role in adipose tissue still remains obscure (23, 24). In the present study, using murine 3T3-L1 preadipocytes and human preadipocytes, we investigated the effect of ceramide- and AMPK-mediated signaling pathways on the expression of 11 β -HSD1.

Materials and Methods

Reagents and chemicals

5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) was obtained from Toronto Research Chemicals (Toronto, Canada). A selective AMPK inhibitor, Compound C, and C₂ ceramide (*N*-acetyl-sphingosine) were purchased from Carbiochem (San Diego, CA). Bacterial SMase and S1P were from Sigma Chemicals (St. Louis, MO). Antibodies against phospho (Ser 79) acetyl-CoA carboxylase (ACC), CCAAT/enhancer-binding protein (C/EBP) β , and β -actin were from Upstate Biotechnology (Lake Placid, NY), Affinity BioReagents Inc. (Golden, CO), and Sigma Chemicals.

Cell culture and treatment

3T3-L1 fibroblasts (kindly provided from Dr. H. Green and Dr. M. Morikawa, Harvard Medical School, Boston, MA) were maintained in DMEM containing 10% (vol/vol) calf serum at 37 C in 10% CO₂. Human perirenal preadipocytes were purchased from Cambrex (Walkersville, MD) (25, 26) and maintained according to the manufacturer's instructions. For differentiation of 3T3-L1 preadipocytes into mature adipocytes, cells (2 d postconfluence) were incubated with DMEM containing 10% (vol/vol) fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 μ M dexamethasone, and 1 μ g/ml insulin for 2 d, followed by another 2-d incubation with DMEM containing FBS and insulin (27). Additional incubation with DMEM containing FBS for 4 d completes the differentiation. Compound C and C₂ ceramide were dissolved in DMSO and added to the media within 0.1% of volume. S1P was dissolved in water containing 0.4% BSA and added to the media within 1.0% of volume.

RNA preparation and quantitative real-time PCR

Total RNA was extracted from cultured cells using TRIzol Reagent (Invitrogen, Carlsbad, CA), and cDNA was then synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. To determine the mRNA levels, probes and primers were employed as follows: probe (5'-FAM-tccgagttcaaggcagc-gagacactacc-TAMRA-3'), forward (5'-ccaggctcggaggaggtctc-3'), and reverse (5'-ccagcaatgtagttagcagagg-3') for murine 11 β -HSD1; probe (5'-FAM-cccactcactctgctactcattca-TAMRA-3'), forward (5'-ttggct-cagccagatgca-3'), and reverse (5'-ccagcctactcattggatca-3') for murine monocyte chemoattractant protein-1 (MCP-1); probe (5'-FAM-acattgt-cagcctgcagaatccactactg-TAMRA-3'), forward (5'-atgcgacccacctgtgac-3'), and reverse (5'-gacctccagccaacatg-3') for murine preadipocyte factor-1 (Pref-1); and probe (5'-cattgttgctctcctctctgctggg-3'), forward (5'-ttgccatgctgaagcagag-3'), and reverse (5'-gcaaccattggataagccactt-3') for human 11 β -HSD1. TaqMan PCR was performed using ABI Prism 7700 Sequence Detection System as instructed by the manufacturer (Applied Biosystems, Foster City, CA). Each value of mRNA level was normalized to that of 18S rRNA.

Western blot analysis

Cells were washed twice with ice-cold PBS, and harvested in lysis buffer [40 mM HEPES, 10 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na₃VO₄, 0.1 mg/ml aprotinin, 1 mM PMSF, 50 mM okadaic acid, and 1% (vol/vol) Nonidet P-40 at pH 7.5]. For analysis of C/EBP β , cells were harvested in lysis buffer [1% (wt/vol) SDS, 60 mM Tris-HCl, 1 mM Na₃VO₄, 0.1 mg/ml aprotinin, 1 mM PMSF and 50 mM okadaic acid at pH 6.8], and boiled at 100 C for 10 min. After centrifugation, supernatants were normalized for protein concentration via Bradford method and then equal amounts of protein were subjected to SDS-PAGE and immunoblot.

Measurement of AMPK activity

Activation of AMPK is assessed by the immunoblot of Thr172-phosphorylated AMPK α . Antibodies against AMPK α and phosphorylated (Thr172) AMPK (Cell Signaling Technology, Beverly, MA) can detect both α 1 and α 2 isoform of the catalytic subunit (28). In 3T3-L1 adipocytes, AMPK activity is largely attributable to the α 1 isoform (29), and both α 1 and α 2 catalytic subunit isoforms are activated by AICAR (29). The phosphorylation at Thr172 parallels the degree of AMPK activation, thus making it possible to estimate the activity of both isoform complexes (28).

Measurement of 11 β -HSD1 activity

Assays for 11 β -HSD1 activity were performed by incubating viable cells with 250 nM corticosteroids with appropriate tritium-labeled tracer. In assays for oxo-reductase activity, cells were incubated in serum-free DMEM containing 250 nM cortisone that includes 6.4 μ Ci/ml tritium-labeled tracer [1,2-³H]₂ cortisone (Muromachi Yakuhin LTD, Kyoto, Japan). In assays for dehydrogenase activity, cells were incubated in serum-free DMEM containing 250 nM cortisol with 6.4 μ Ci/ml tritium-labeled tracer [1,2,6,7-³H]₄ cortisol (Muromachi Yakuhin LTD). After the incubation at 37 C for indicated time, corticosteroids were extracted by ethyl acetate, separated by thin-layer chromatography in chloroform:methanol (95:5), and quantified by autoradiography. As a control (indicated as ref.), serum-free DMEM with tritium-labeled cortisone or cortisol was incubated without cells.

Chromatin immunoprecipitation (ChIP) analysis

ChIP analysis was performed using an assay kit (Upstate Biotechnology) according to the manufacturer's protocol. Anti-C/EBP β antibody used for immunoprecipitation is from Santa Cruz Biotechnology (Santa Cruz, CA). Forward primer (5'-ctggaagttgctcttactc-3') and reverse primer (5'-cctgtaggacacacgaaga-3') were used to amplify the DNA fragment between -170 and +71 (relative to the transcription start site) of mouse 11 β -HSD1 genomic DNA, which contains two putative C/EBP binding sites (30).

RNA interference (RNAi)

Two Stealth RNAi for mouse C/EBP β were obtained (Invitrogen). Each small interfering RNA (siRNA), termed RNAi154 and RNAi168, was designed as a 25-bp duplex oligoribonucleotide with a sense strand corresponding to nucleotides 154–173 or 168–192 of the reported mouse C/EBP β coding sequence (GenBank accession no. NM009883), respectively. The Stealth RNAi negative control duplex (Invitrogen) was used as a control oligoribonucleotide. According to the manufacturer's protocol, 3T3-L1 preadipocytes were transfected with 10 nM siRNA in antibiotic-free medium using Lipofectamine RNAiMAX (Invitrogen). The transfected cells were cultured overnight and applied to the experiments.

Statistical analysis

The data are presented as means \pm SEM from independent three or four experiments. Student's *t* test was used to compare the data when the variances of two groups were regarded equal (within 5%). When variances of two groups were apparently different, Welch's *t* test was used. Differences were accepted as significant at *P* < 0.05 level.

Results

When 3T3-L1 preadipocytes were differentiated into mature adipocytes, the level of 11 β -HSD1 mRNA was increased by 150-fold (Fig. 1). Based on this result, we first analyzed the effect of ceramide signaling on the expression of 11 β -HSD1 in both 3T3-L1 preadipocytes and differentiated adipocytes.

Effect of ceramide on the expression of 11 β -HSD1 in 3T3-L1 preadipocytes

When 3T3-L1 preadipocytes were treated with a short chain ceramide analog, C₂ ceramide (10–100 μ M), for 24 h, mRNA of Pref-1, which is known to express abundantly in preadipocytes (31, 32), was not altered (Fig. 2A). In contrast, expression of MCP-1 and 11 β -HSD1 was induced dose-dependently, reaching to 2.0 \pm 0.1-fold ($P < 0.01$) and 2.2 \pm 0.1-fold ($P < 0.01$), respectively (Fig. 2, B and C). To evaluate the effect of C₂ ceramide on the 11 β -HSD1 enzyme activity, cells were incubated with tritium-labeled cortisone or cortisol. C₂ ceramide significantly increased the conversion of cortisone to cortisol by 1.7 \pm 0.1-fold ($P < 0.05$) (Fig. 2D, left). This indicates that 11 β -HSD1 oxo-reductase activity was increased in response to C₂ ceramide. In contrast, 11 β -HSD1 dehydrogenase activity was neither detected nor induced during a 24-h incubation period (Fig. 2D, right). These results indicate that 11 β -HSD1 in 3T3-L1 preadipocytes acts solely as an oxo-reductase.

Hydrolysis of membrane sphingomyelin by SMase provoke intracellular accumulation of ceramide (19). Ceramide is subsequently metabolized to sphingosine and S1P, both of which mediate biological events including cell growth and apoptosis (19). We thus treated 3T3-L1 preadipocytes with bacterial SMase and S1P. When cells were treated with bacterial SMase (25–200 mU/ml) and S1P (1.0–10 μ M), 11 β -HSD1 mRNA level was significantly induced, reaching to 1.7 \pm 0.2-fold ($P < 0.05$) and 3.0 \pm 0.5-fold ($P < 0.05$), respectively (Fig. 2, E and F). To further investigate whether ceramide signaling would be involved in the induction of 11 β -HSD1 by inflammatory cytokines, inhibitors of ceramide [fumonisins B1, an inhibitor of ceramide synthase (33); myriocin, an inhibitor of serine palmitoyltransferase (34); desi-

pramine, an inhibitor of SMase (35)] were cotreated with 3T3-L1 preadipocytes. Desipramine (10 and 20 μ M) dose-dependently attenuated the expression of 11 β -HSD1 mRNA induced by TNF α or IL-1 β [46 \pm 10% ($P = 0.07$) and 41 \pm 2% ($P < 0.01$), respectively]. This result indicates that induction of 11 β -HSD1 by TNF α or IL-1 β , at least in part, is attributable to the activation of SMase. Such an effect was not observed when treated with fumonisins B1 or myriocin (data not shown). These results raise a possibility that ceramide-mediated signaling pathway is involved in the regulation of 11 β -HSD1 in preadipocytes.

Effect of ceramide on the expression of 11 β -HSD1 in 3T3-L1 differentiated adipocytes

Induction of 11 β -HSD1 mRNA by C₂ ceramide (10–100 μ M) was not observed in 3T3-L1 differentiated adipocytes during 24-h incubation periods (Fig. 3A). Similarly, SMase (25–200 mU/ml) and S1P (1.0–10 μ M) did not induce the expression of 11 β -HSD1 (Fig. 3, B and C). These results are in agreement with our data that a robust induction of 11 β -HSD1 expression by TNF α and IL-1 β was observed only in 3T3-L1 preadipocytes but not in differentiated adipocytes (data not shown). Altogether, our data suggest that the effect of C₂ ceramide, SMase, and S1P on 11 β -HSD1 expression is restricted in preadipocytes.

Effect of AICAR on the expression of 11 β -HSD1 in 3T3-L1 preadipocytes

AMPK is activated by cellular stresses that interfere with ATP production (20–22). In contrast, 11 β -HSD1 is a NADPH-dependent enzyme and its oxo-reductase activity requires NADPH supply from a glucose metabolizing pathway (36). This scheme tempted us to speculate that 11 β -HSD1 would be involved in an AMPK-mediated fuel sensing mechanism. To test this hypothesis, we examined the effect of AMPK on the expression of 11 β -HSD1 using a cell permeable AMP analog, AICAR (37).

When 3T3-L1 preadipocytes were treated with AICAR (0.1–0.5 mM) for 24 h, AMPK phosphorylation was increased pronouncedly (Fig. 4A). MCP-1 mRNA level was not altered even after the treatment with AICAR (Fig. 4B). In contrast, 11 β -HSD1 mRNA level was increased in a dose-dependent manner, reaching to 8.7 \pm 0.8-fold ($P < 0.05$) (Fig. 4C). Oxo-reductase activity of 11 β -HSD1 was concomitantly increased by 3.0 \pm 0.2-fold ($P < 0.01$) after the treatment with 0.5 mM AICAR (Fig. 4D).

To further evaluate the involvement of AMPK in the regulation of 11 β -HSD1, cells were cotreated with AICAR and a selective AMPK inhibitor, compound C (38). Western blot analyses showed that augmented phosphorylation of AMPK and ACC by the treatment with 0.5 mM AICAR were attenuated when cells were cotreated with 10 μ M compound C (Fig. 5A). Compound C completely blocked the effect of 0.5 mM AICAR on its augmentation of 11 β -HSD1 (80 \pm 2.4% decrease compared with AICAR-treated group, $P < 0.01$) (Fig. 5B). These results indicate that augmented expression of 11 β -HSD1 by AICAR largely is attributable to the activation of AMPK. In contrast, mRNA of glucocorticoid receptor, which is known to express considerably in preadipocytes (39), was not affected by AICAR or compound C (data not shown).

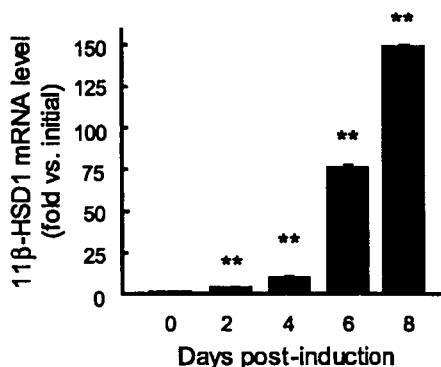


FIG. 1. Expression profile of 11 β -HSD1 mRNA during the course of differentiation in 3T3-L1 cells. Postconfluent 3T3-L1 preadipocytes were induced to differentiate as described in *Materials and Methods*. mRNA level of 11 β -HSD1 was determined. Results are means \pm SEM from three experiments. **, $P < 0.01$ compared with 2 d postconfluent preadipocytes (initial).

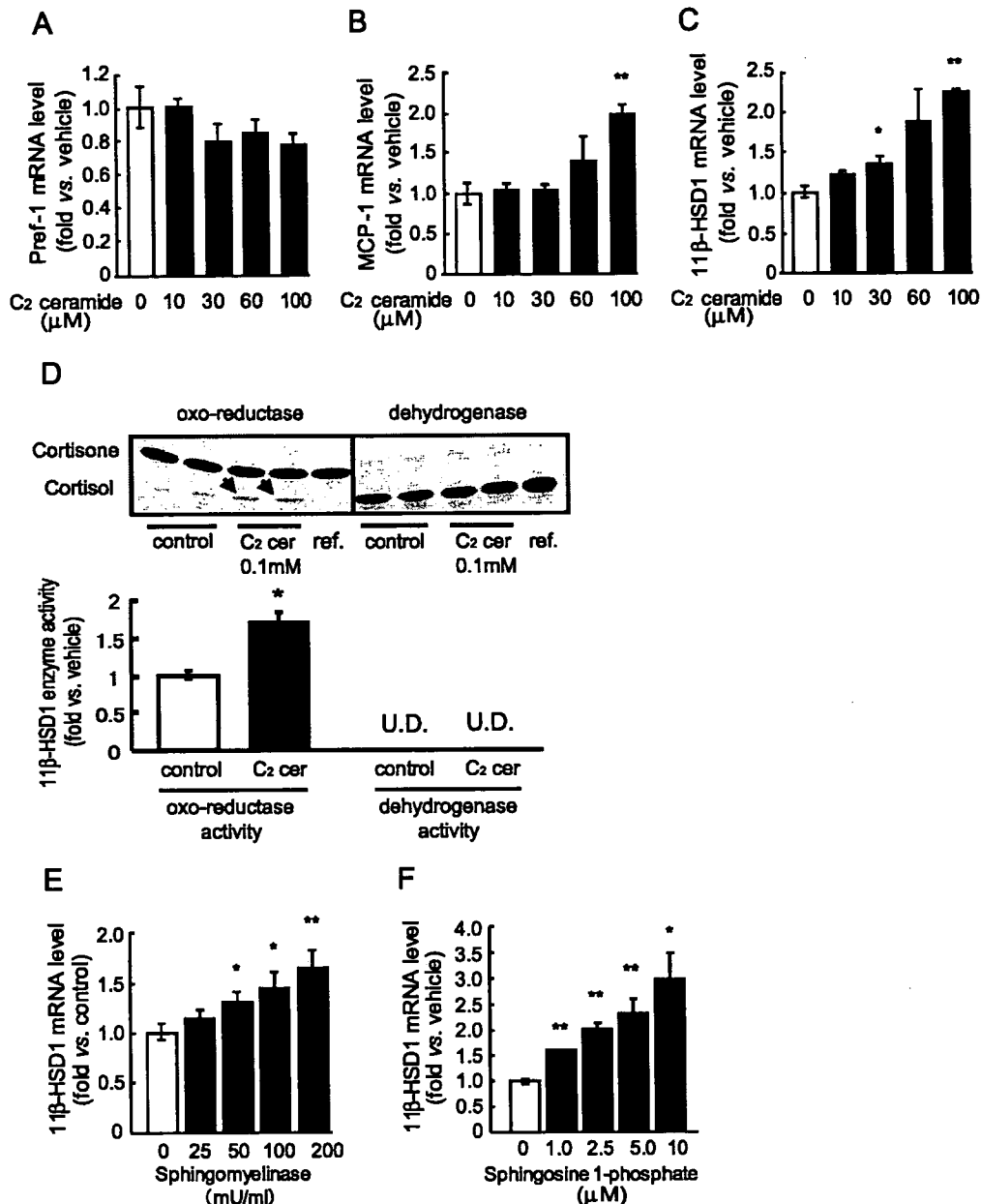


FIG. 2. Ceramide induces the expression of 11 β -HSD1 in 3T3-L1 preadipocytes. A–D, Cells were treated with C₂ ceramide (10–100 μ M) for 24 h. mRNA level of Pref-1 (A), MCP-1 (B), and 11 β -HSD1 (C). D, Assay for 11 β -HSD1 enzyme activity. Cells treated with C₂ ceramide (100 μ M) for 24 h were subsequently incubated in serum-free media containing 250 nM of cortisone or cortisol with tritium-labeled tracer (6.4 μ Ci/ml of [1,2-³H]₂ cortisone or [1,2,6,7-³H]₄ cortisol) for 12 h. Emerged spots of cortisol are indicated by arrows. E and F, mRNA level of 11 β -HSD1 after treatment with bacterial SMase (25–200 mU/ml) (E) and S1P (1.0–10 μ M) (F) for 24 h. Results are means \pm SEM from three experiments. *, $P < 0.05$, **, $P < 0.01$ compared with vehicle-treated group. U.D., Under detectable.

Effects of ceramide and AMPK signaling on the expression of 11 β -HSD1 in human preadipocytes

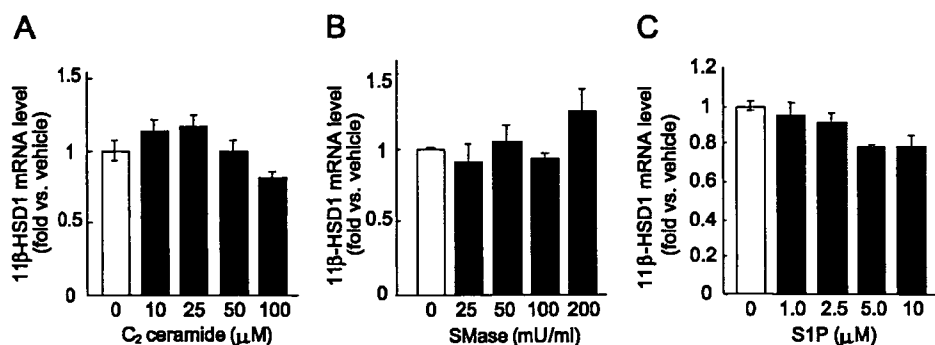
To explore whether potent effects of ceramide and AMPK on 11 β -HSD1 expression are reproduced in human preadipocytes (25, 26), impact of C₂ ceramide, S1P, and AICAR on 11 β -HSD1 expression and enzyme activity were similarly analyzed. When cells were treated with C₂ ceramide (25–100 μ M) for 24 h, 11 β -HSD1 mRNA level was increased dose dependently, reaching to 5.3 ± 0.3 -fold ($P < 0.01$) (Fig. 6A). In contrast, the effect of S1P (2.5–10 μ M) was marginal (Fig. 6A). AICAR (0.3–1.0 mM) induced

the phosphorylation of AMPK (Fig. 6B) and augmented 11 β -HSD1 mRNA level by 2.8 ± 0.1 -fold ($P < 0.01$) (Fig. 6A). C₂ ceramide (50 μ M) and AICAR (1.0 mM) significantly increased the oxo-reductase activity of 11 β -HSD1 by 2.9 ± 0.3 -fold ($P < 0.01$) and 2.1 ± 0.04 -fold ($P < 0.05$) (Fig. 6C).

Induction of C/EBP β by ceramide and AMPK signaling in 3T3-L1 preadipocytes

11 β -HSD1 promoter contains a couple of C/EBP binding sites, and C/EBP mediated regulation of 11 β -HSD1 has long

FIG. 3. Ceramide does not influence the expression of 11 β -HSD1 in differentiated 3T3-L1 adipocytes. mRNA level of 11 β -HSD1 after treatment with C₂ ceramide (10–100 μ M) (A), SMase (25–200 mU/ml) (B), and S1P (1.0–10 μ M) (C) for 24 h. Results are means \pm SEM from three independent experiments.



been investigated exclusively in hepatocytes (40). Because C/EBP α is not expressed in preadipocytes (41), we tested a possibility that C/EBP β would be involved in the regulation of 11 β -HSD1 by ceramide or AICAR. Western blot analysis showed that C/EBP β expression was substantially increased after the treatment with C₂ ceramide (0.1 mM) or AICAR (0.5 mM), culminating in 1.8- and 6.5-fold increase within 3 h, respectively (Fig. 7, A and B). Expression of C/EBP β by C₂ ceramide or AICAR was increased in a dose-dependent manner (data not shown). In contrast, 11 β -HSD1 expression was induced 12 h after the treatment with C₂ ceramide or AICAR (data not shown).

To explore a possible involvement of C/EBP β in 11 β -HSD1 regulation, ChIP analysis was performed with primers spanning putative C/EBP binding sites in mouse 11 β -HSD1 promoter (30). No amplified band was observed in samples processed without antibody (No antibody control), excluding a possibility of nonspecific binding between DNA fragments and protein A-agarose (Fig. 7C). When treated with C₂

ceramide or AICAR for 6 h, association of C/EBP β with the promoter of 11 β -HSD1 gene was substantially induced (Fig. 7C). Our data demonstrated that the activation of ceramide or AMPK pathways induced the expression of C/EBP β and its binding to the 11 β -HSD1 promoter.

Effect of C/EBP β knockdown on the expression of 11 β -HSD1 induced by ceramide or AMPK signaling in 3T3-L1 preadipocytes

To further validate a role of C/EBP β in the control of 11 β -HSD1 by ceramide or AICAR, C/EBP β protein was transiently knocked down by siRNA. When 3T3-L1 preadipocytes were transfected with siRNA, C/EBP β protein expression induced by C₂ ceramide or AICAR was markedly attenuated, demonstrating effective silencing of C/EBP β (Fig. 8, A and B). Notably, augmented expression of 11 β -HSD1 induced by C₂ ceramide or AICAR was significantly attenuated in cells transfected with C/EBP β siRNA (Fig. 8,

FIG. 4. AMPK activation enhances the expression of 11 β -HSD1 in 3T3-L1 preadipocytes. Cells were treated with AICAR (0.1–0.5 mM) for 24 h. A, Western blot of AMPK protein and phosphorylated AMPK (p-AMPK). mRNA levels of MCP-1 (B) and 11 β -HSD1 (C). D, Assay for 11 β -HSD1 activity. Cells treated with AICAR (0.5 mM) for 24 h were incubated in serum-free media containing 250 nM of cortisone or cortisol with tritium-labeled tracer (6.4 μ Ci/ml of [1,2-³H]₂ cortisone or [1,2,6,7-³H]₄ cortisol) for 12 h. Emerged spots of cortisol were indicated by arrows. Results are means \pm SEM from three or four experiments. *, $P < 0.05$, **, $P < 0.01$ compared with control (cells without AICAR treatment) group. U.D., Under detectable.

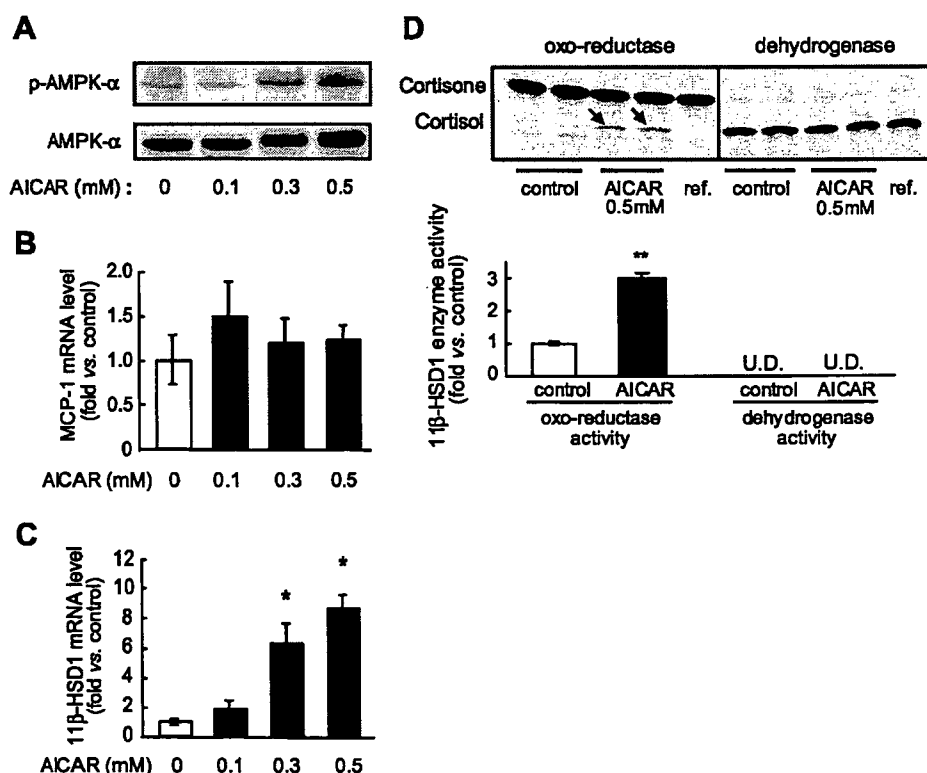
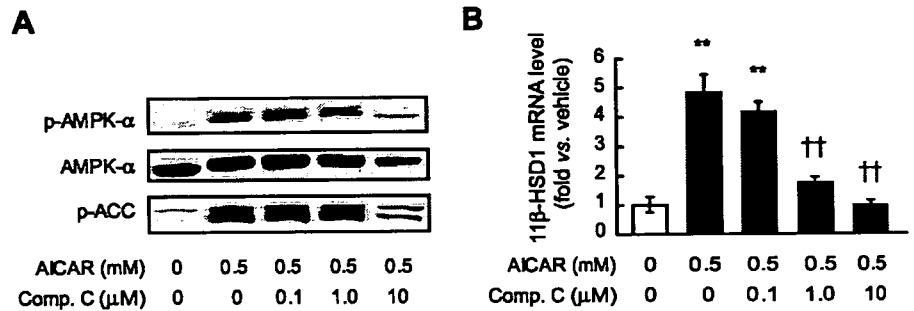


FIG. 5. AICAR-induced expression of 11 β -HSD1 is attenuated by an AMPK inhibitor. 3T3-L1 preadipocytes were cotreated with AICAR (0.5 mM) and indicated concentrations of compound C (Comp. C) for 24 h. A, Western blot of total AMPK protein, phosphorylated AMPK (p-AMPK) and phosphorylated ACC (p-ACC). B, mRNA level of 11 β -HSD1. Results are means \pm SEM from three experiments. **, $P < 0.01$ compared with vehicle-treated group. ††, $P < 0.01$ compared with AICAR-treated group.



C and D). In contrast, negative control Stealth RNAi treatment had no impact on the expression of C/EBP β or 11 β -HSD1. These results suggest that C/EBP β is involved critically in the induction of 11 β -HSD1 by ceramide- or AMPK-mediated signaling pathways.

Discussion

The major finding of the present study is that ceramide- and AMPK-mediated signaling pathways augment the expression and enzyme activity of 11 β -HSD1 in both murine and human preadipocytes. We provide novel evidence that activation of ceramide and AMPK pathways induce the expression of C/EBP β . ChIP analyses demonstrate the DNA binding of C/EBP β to 11 β -HSD1 promoter, and transient knockdown of C/EBP β protein by siRNA further support the notion that C/EBP β is critically involved in the expression of 11 β -HSD1 induced by C₂ ceramide or AICAR. Taken together, the present study highlights a novel mechanism that metabolic stress-related signaling pathways mediated by ceramide and AMPK regulate the expression of 11 β -HSD1 in preadipocytes.

Ceramide acts as a lipid mediator of metabolic stress response (18, 42). Fatty acids, proinflammatory cytokines, glucocorticoids, and serum deprivation in cultured cells are known to induce intracellular accumulation of ceramide (43, 44). Importantly, aberrant accumulation of ceramide in insulin target tissues appreciably contributes to local insulin resistance and underlies, at least partly, the molecular mechanism of lipotoxicity (43, 44). A recent study demonstrated that mRNA level of enzymes involved in sphingolipid metabolism in adipose tissue as well as plasma level of SMase, ceramide, sphingosine, and S1P were increased in *ob/ob* mice (45). Thus we tested a possibility whether 11 β -HSD1 would be induced by ceramide signals in preadipocytes. In this context, the present study is the first to demonstrate that the expression of 11 β -HSD1 was induced by C₂ ceramide, bacterial SMase and S1P in preadipocytes (Fig. 2). In contrast, induction of 11 β -HSD1 expression by C₂ ceramide, SMase, and S1P was not observed in differentiated adipocytes (Fig. 3), suggesting that such effects are restricted in preadipocytes.

SMase catalyzes the hydrolysis of sphingomyelin in outer side of the plasma membrane, leading to the production of ceramide (19). Ceramide is subsequently metabolized to S1P, which functions through S1P receptors (19). In the present study, we found that inhibition of SMase by desipramine attenuated the TNF α - or IL-1 β -induced expression of 11 β -HSD1. This observation is in agreement with previous reports that TNF α and IL-1 β promptly induced the hydrolysis

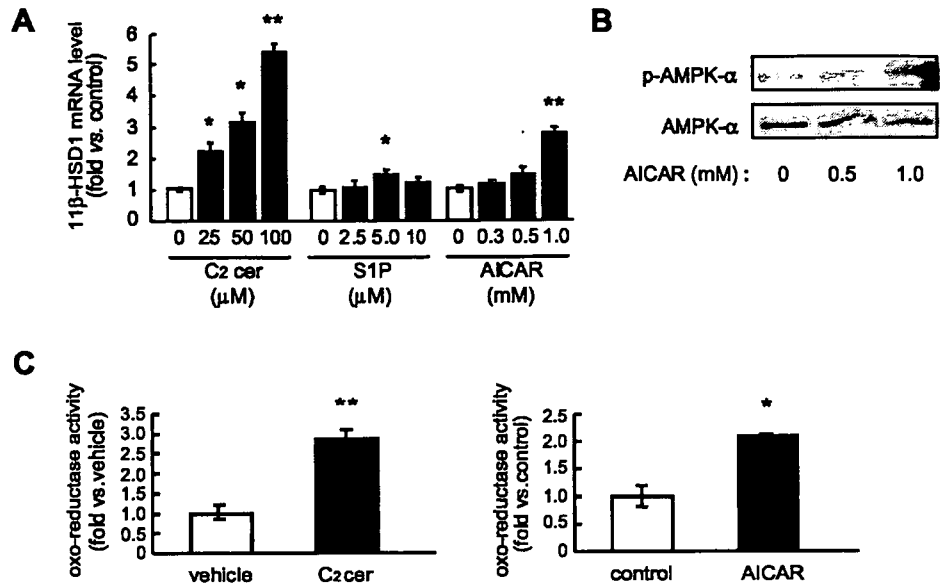
of sphingomyelin to generate ceramide (46). Assays in the present study are validated by the finding that expression of MCP-1 was induced by C₂ ceramide (Fig. 2), consistent with the notion that NF κ B and MAPK signaling pathways are involved in the regulation of MCP-1 (47) and that ceramide and S1P potentially mediate NF κ B and MAPK pathways (18, 48).

In the present study, C₂ ceramide induced the expression of 11 β -HSD1 at 50–100 μ M (Fig. 2). It has been reported that the physiological concentration of ceramide within cells are approximately 1–5 μ M (49). Accordingly, C₂ ceramide does induce biological effects including differentiation and growth inhibition at 1–5 μ M in serum-free media. However, higher concentration (50–100 μ M) of C₂ ceramide are required to induce equipotent effects in serum-containing medium, because serum proteins bind C₂ ceramide and reduce its potency (50), and for this, 50–100 μ M C₂ ceramide has been commonly used in many previous studies (51, 52). It should also be noted that, because ceramide resides in “lipid rafts” in the membrane (53), local concentration of ceramide within cells must be much higher than 5 μ M. In this context, the concentration of C₂ ceramide used in the present study is appropriate for analyzing the responsiveness of ceramide in adipocytes.

AMPK, activated by the increase in intracellular AMP level, plays a crucial role in mediating cellular stress such as hypoxia, glucose deprivation, and ischemia (20–22). Recent studies highlighted a potential role of AMPK in regulating energy balance and mass of adipose tissue (23, 24). ATP level of adipose tissue is decreased in obese rodent models (54), supporting the notion that local hypoxia and inflammation is associated with defective energy metabolism in obese adipose tissue (14, 54). In this context, the present study demonstrates for the first time that AICAR markedly augmented the expression of 11 β -HSD1 in preadipocytes (Fig. 4). Furthermore, a potent AMPK inhibitor compound C completely suppressed the induction of 11 β -HSD1 (Fig. 5), verifying that AMPK signaling pathway is involved in the regulation of 11 β -HSD1. Based on the present study in 3T3-L1 preadipocytes (Figs. 2, 4, and 5), a potential interaction between ceramide and AMPK in terms of the effect on 11 β -HSD1 expression would be of considerable interest. In 3T3-L1 preadipocytes, AMPK was not activated when treated with C₂ ceramide (data not shown). Although further studies are required, these results suggest that ceramide signal is not directly involved in AMPK-mediated induction of 11 β -HSD1.

The present study is the first to demonstrate that C₂ cer-

FIG. 6. Ceramide and AMPK signaling induces the expression of 11 β -HSD1 in human preadipocytes. Human preadipocytes were treated with C₂ ceramide (C₂cer, 25–100 μ M), S1P (2.5–10 μ M) and AICAR (0.3–1.0 mM) for 24 h. **A**, mRNA levels of 11 β -HSD1 were determined. **B**, Western blot of AMPK protein and phosphorylated AMPK (p-AMPK) after treatment with AICAR for 3 h. **C**, Assay for 11 β -HSD1 oxo-reductase activity. Cells were treated with C₂ ceramide (50 μ M, left) or AICAR (1.0 mM, right) for 24 h, and subsequently incubated with tritium-labeled cortisone for 12 h. Results are means \pm SEM from three experiments. *, $P < 0.05$, **, $P < 0.01$ compared with control or vehicle-treated group.

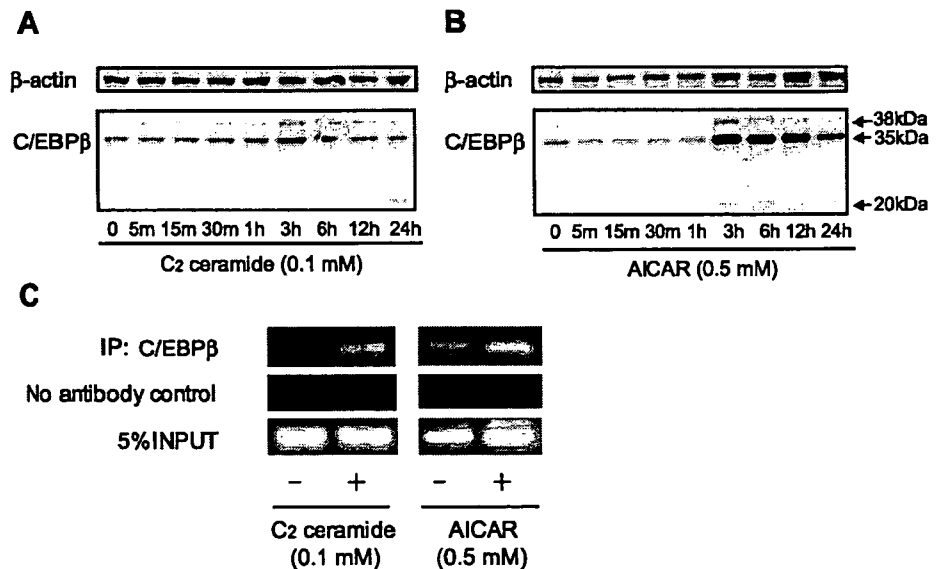


amide and AICAR augmented the expression of C/EBP β in 3T3-L1 preadipocytes (Fig. 7). This result is consistent with a line of previous reports showing that S1P induced phosphorylation of cAMP-responsive-element-binding protein (CREB) (48), and CREB potentially controls the expression of C/EBP β in adipocytes (55). Furthermore, the present study is the first demonstration that the activation of AMPK induced the expression of C/EBP β in any kinds of cells. It is well-characterized that transient expression of C/EBP β is essential for the induction of PPAR γ and C/EBP α in the early phase of adipogenesis (41). A recent report raised a possibility that AICAR inhibited adipogenesis by interfering induction of PPAR γ and C/EBP α (56). Therefore, it is tempting to speculate that AMPK-induced augmentation and sustenance of C/EBP β and resultant suppression of adipogenesis may be a facet of adaptation to nutritional threat.

Compared with murine adipocytes, the mechanism responsible for adipogenesis and adipokine secretion is poorly

understood in humans (57). For example, human preadipocytes do not require the process of clonal expansion in the course of adipogenesis (57). Therefore, we examined the effect of ceramide and AMPK signaling on the expression of 11 β -HSD1 using human preadipocytes. The present study demonstrates that sphingolipids (C₂ ceramide and S1P) and AICAR induced the expression of 11 β -HSD1 also in human preadipocytes (Fig. 6). The effect of C₂ ceramide was exaggerated in human preadipocytes compared with 3T3-L1 preadipocytes, whereas the effect of S1P was mild in human preadipocytes. Treatment of C₂ ceramide did not affect 11 β -HSD1 mRNA level, but AICAR substantially reduced the expression in a dose-dependent manner (data not shown), representing a contrast to the data in 3T3-L1 preadipocytes (Figs. 2 and 4). Even considering differences in cell types or species (58), our data provide novel evidence that ceramide and AMPK signals induce the expression of 11 β -HSD1 in both rodent and human cultured preadipocytes. Recent

FIG. 7. Ceramide and AICAR induce the expression of C/EBP β in 3T3-L1 preadipocytes. Western blot of C/EBP β after the treatment with 0.1 mM C₂ ceramide (A) or 0.5 mM AICAR (B). β -Actin was used as a loading control. ChIP analysis after the treatment with 0.1 mM C₂ ceramide or 0.5 mM AICAR for 6 h (C). Chromatin-associated DNA was immunoprecipitated with an antibody against C/EBP β . The immunoprecipitated DNA, samples processed without antibody (indicated as No antibody control), and 5% amount of sonicated DNA (indicated as 5% INPUT) were subjected to PCR using specific primers for 11 β -HSD1 promoter region. Amplified DNA indicates the binding of C/EBP β to the 11 β -HSD1 promoter.



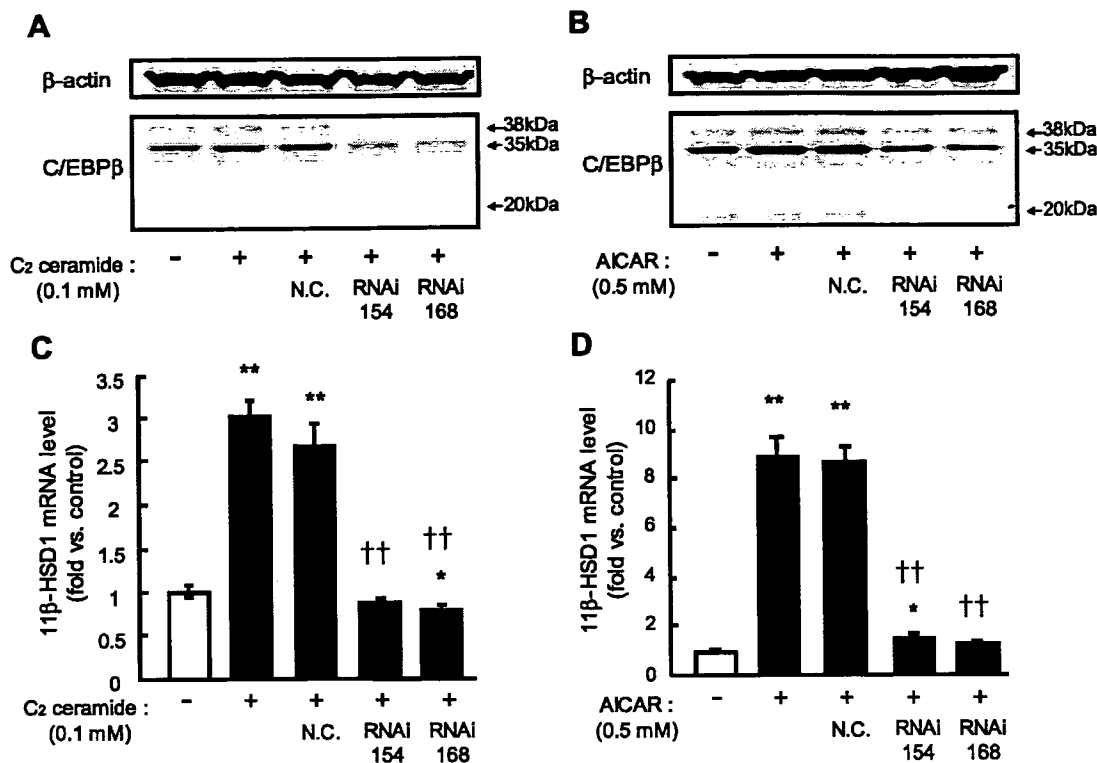


FIG. 8. Effect of C/EBP β knockdown on C₂ ceramide- or AICAR-induced expression of 11 β -HSD1 in 3T3-L1 preadipocytes. Cells were transfected with either Stealth RNAi negative control (N.C.) or C/EBP β Stealth RNAi (RNai154, RNai168). After 12 h of incubation, cells were treated with C₂ ceramide or AICAR. Western blot of C/EBP β after the treatment with 0.1 mM C₂ ceramide (A) or 0.5 mM AICAR (B) for 3 h. β -Actin was used as a loading control. mRNA level of 11 β -HSD1 after the treatment with 0.1 mM C₂ ceramide (C) or 0.5 mM AICAR (D) for 24 h. Results are means \pm SEM from four experiments. *, $P < 0.05$, **, $P < 0.01$ compared with control group. ††, $P < 0.01$ compared with C₂ ceramide or AICAR-treated group.

works demonstrated that human adipose tissue contained a considerable amount of preadipocytes (59, 60), which may be involved in some aspects of adipose tissue function (15, 60). In this context, further *in vivo* studies are warranted to validate the possible involvement of ceramide and AMPK signals in 11 β -HSD1 regulation in human preadipocytes.

C/EBP family of transcription factors (C/EBPs) in adipocytes serve as master regulators of a variety of cellular response (61), and expression of C/EBPs is regulated by a variety of hormones, cytokines, and nutrients (61, 62). 11 β -HSD1 promoter contains a couple of C/EBP binding sites, and previous studies demonstrated that the expression of 11 β -HSD1 was controlled by C/EBPs (40, 63). In this context, the present study demonstrates, for the first time, that induction of C/EBP β in preadipocytes was observed around 3 h after the treatment with C₂ ceramide or AICAR (Fig. 7), which preceded the robust induction of 11 β -HSD1. Our data of ChIP analyses and C/EBP β knockdown experiments further reinforced the notion that C/EBP β is involved in ceramide- and AMPK-mediated augmentation of 11 β -HSD1 in preadipocytes (Figs. 7 and 8).

It should be noted that glucocorticoid is known to increase the expression and activity of SMase, resulting in intracellular ceramide accumulation and local insulin resistance (43). This notion prompts us to speculate a vicious cycle within cells where ceramide and 11 β -HSD1-derived active glucocorticoid reciprocally aggravate preadipocyte dysfunction.

Unexpected regulation of 11 β -HSD1 by AMPK pathway may also provide a novel clue to better understand molecular pathophysiology of adipose dysfunction. Collectively, the present study is the first demonstration that ceramide and AMPK signaling pathways augment the expression and enzyme activity of 11 β -HSD1 in human and rodent preadipocytes, thereby highlighting a metabolic stress-related regulation of 11 β -HSD1 in a cell-specific manner.

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Bezafibrate regulates the expression and enzyme activity of 11 β -hydroxysteroid dehydrogenase type 1 in murine adipose tissue and 3T3-L1 adipocytes

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Nakano S, Inada Y, Masuzaki H, Tanaka T, Yasue S, Ishii T, Arai N, Ebihara K, Hosoda K, Maruyama K, Yamazaki Y, Shibata N, Nakao K. Bezafibrate regulates the expression and enzyme activity of 11 β -hydroxysteroid dehydrogenase type 1 in murine adipose tissue and 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab* 292: E1213–E1222, 2007. First published December 26, 2006; doi:10.1152/ajpendo.00340.2006.—A clinically employed antihyperlipidemic drug, bezafibrate, has been characterized as a PPAR(α , γ , and δ) pan-agonist in vitro. Recent extended trials have highlighted its antidiabetic properties in humans. However, the underlying molecular mechanism is not fully elucidated. The present study was designed to explore potential regulatory mechanisms of intracellular glucocorticoid reactivating enzyme, 11 β -HSD1 and anti-diabetic hormone, adiponectin by bezafibrate in murine adipose tissue, and cultured adipocytes. Treatment of *db/db* mice with bezafibrate significantly ameliorated hyperglycemia and insulin resistance, accompanied by a marked reduction of triglyceride and nonesterified fatty acids. Despite equipotent in lipid-lowering effects, another fibrate, fenofibrate, did not show such beneficial effects on glycemic control. Treatment of bezafibrate caused a marked decrease in the mRNA level of 11 β -HSD1 preferentially in adipose tissue of *db/db* mice (-47% , $P < 0.05$), concomitant with a significant increase in plasma adiponectin level ($+37\%$, $P < 0.01$). Notably, treatment of bezafibrate caused a marked decrease in the mRNA level (-34% , $P < 0.01$) and enzyme activity (-32% , $P < 0.01$) of 11 β -HSD1, whereas the treatment substantially augmented the expression ($+71\%$, $P < 0.01$) and secretion ($+27\%$, $P < 0.01$) of adiponectin in 3T3-L1 adipocytes. Knockdown of 11 β -HSD1 by siRNA confirmed that 11 β -HSD1 acts as a distinct oreductase in adipocytes and validated the enzyme activity assays in the present study. Effects of bezafibrate on regulation of 11 β -HSD1 and adiponectin in murine adipocytes were comparable with those in thiazolidinediones. This is the first demonstration that bezafibrate directly regulates 11 β -HSD1 and adiponectin in murine adipocytes, both of which may contribute to metabolically-beneficial effects by bezafibrate.

metabolic syndrome; adiponectin

FAMILY GENES of peroxisome proliferator-activated receptors (PPARs) are profoundly relevant to fuel homeostasis (14, 41). Agonists for PPAR α and PPAR γ have been widely used for the treatment of dyslipidemia and type 2 diabetes (46). Recent research progress (3) has highlighted the potential usefulness

of PPAR α / γ dual agonists or PPAR(α , γ , and δ) pan-agonists for metabolic diseases. However, some of these compounds are reported to cause adverse effects, including carcinogenesis, edema, hepatotoxicity, and increase in body weight in rodent experiments (45). Bezafibrate has been widely used for the treatment of dyslipidemia in human clinics (11). Recent in vitro experiments have shown that bezafibrate serves as a pan-agonist for PPAR α , γ , and δ (9, 48). Furthermore, recent extended clinical trials (15, 43) have highlighted its antidiabetic properties. However, the underlying mechanism is not fully clarified. To explore unidentified mechanisms whereby bezafibrate ameliorates metabolic derangement, using genetically obese diabetic BKS.Cg-*Lepr^{db}/+*Lept^{db} (*db/db*) mice, which represent a cluster of detrimental metabolic sequelae such as morbid obesity, diabetes, dyslipidemia, and liver steatosis (39), we examined metabolic response to bezafibrate.

Intracellular glucocorticoid reactivating enzyme 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) is abundantly expressed in liver, adipose tissue, skeletal muscle, and central nervous system (38). Targeted overexpression of 11 β -HSD1 in adipose tissue in mice results in visceral fat obesity, insulin resistance, and hypertension, suggesting that increased level of adipose 11 β -HSD1 plays a critical role in metabolic derangements by providing local glucocorticoid excess within fat cells (29, 30). On the other hand, 11 β -HSD1 knockout mice (32), as well as adipose-specific 11 β -HSD2 overexpressors, which mimicked adipose-specific 11 β -HSD1 knockout mice (18), are protected against the metabolic syndrome under overnutrition. Furthermore, recent studies (36, 47) in rodents suggest that local glucocorticoid excess mediated by 11 β -HSD1 in liver or skeletal muscle is also involved in the pathophysiology of the metabolic diseases.

Here, we show that bezafibrate potently decreases the mRNA level of 11 β -HSD1 (28) in adipose tissue of *db/db* mice. We also demonstrate for the first time that bezafibrate inhibits the expression and enzyme activity of 11 β -HSD1 in 3T3-L1 adipocytes. Our data show that bezafibrate potently increases the plasma level of antidiabetic adiponectin (26) in mice and augments substantially the expression and secretion of adiponectin in 3T3-L1 adipocytes. To our knowledge, this is the first study to demonstrate that bezafibrate potently regulates 11 β -HSD1 and adiponectin in murine fat cells.

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MATERIALS AND METHODS

Materials. Bezafibrate was obtained from Chugai Pharmaceutical (Tokyo, Japan). Fenofibrate and WY-14,643 were purchased from Sigma-Aldrich (St. Louis, MO). Pioglitazone hydrochloride (pioglitazone), fenofibric acid, an active metabolite of fenofibrate, and rosiglitazone were synthesized by Kissei Pharmaceutical (Matsumoto, Japan). WY-14,643 and fenofibric acid were used for *in vitro* experiments.

Animal experiments. Animal experiments in the present study were conducted in accordance with the guidelines for animal experiments of Kissei Pharmaceutical and the Animal Research Committee, Graduate School of Medicine, Kyoto University, and were approved by the Japanese Pharmacological Society. Six-week-old male BKS.Cg-m/+Lept^{db}/Jcl (lean) mice and their obese littermates, BKS.Cg-m/+Lept^{db}/Jcl (db/db) mice, were purchased from Clea Japan (Tokyo, Japan). Mice were housed individually with free access to tap water and commercial pellet food (CE-2; Clea Japan) and kept at 23°C with a humidity of 55% under a 12:12-h light-dark cycle. Mice were randomly divided into groups before each experiment. Bezafibrate (100 or 300 mg·kg⁻¹·day⁻¹), fenofibrate (300 mg·kg⁻¹·day⁻¹), pioglitazone (30 mg·kg⁻¹·day⁻¹), rosiglitazone (10 mg·kg⁻¹·day⁻¹), or vehicle (1% methylcellulose) were orally administered to db/db mice once per day at ~10:00 AM for 6 or 8 wk. Simultaneously, vehicle was administered to lean mice for 6 or 8 wk. Body weight and food consumption were monitored twice per week.

Measurement of metabolic parameters in plasma. Blood samples were collected from tail vein with heparinized hematocrit tube into the collection tube with 50 U aprotinin (Trasylol; Bayer Yakuhin, Osaka, Japan) when fed ad libitum at around 10:00 AM. Plasma glucose, triglyceride (TG), and nonesterified fatty acids (NEFA) were measured via commercially-available kits (Glucose CII-test, Triglyceride E-test, and NEFA C test, respectively; Wako Pure Chemical Industries, Osaka, Japan). Plasma LDL cholesterol and HDL cholesterol were measured by Cholestest LDL and Cholestest N HDL (Daiichi Pure Chemicals, Tokyo, Japan) with a biochemistry automatic analyzer (Clinical analyzer 7150; Hitachi High-Technologies, Tokyo, Japan). Plasma insulin, leptin, adiponectin, and corticosterone concentrations were determined using Insulin ELISA kit, Mouse Leptin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan), Mouse/Rat Adiponectin ELISA Kit (Otsuka Pharmaceutical, Tokushima, Japan), and AssayMax Corticosterone ELISA kit (Assay-Pro, Winfield, MO), respectively.

Oral glucose tolerance test. At 4 wk after the treatments were initiated, db/db and lean mice fasted for 12 h were orally administered 2 g/kg body wt glucose (Wako Pure Chemical Industries). Blood

sample was collected from tail vein at 0.5, 1, 1.5, and 2 h after glucose load. The areas under the curve (AUC) of glucose levels (0–0.5 and 0–1 h) were calculated to assess the status of glucose homeostasis by the linear trapezoidal equation (35).

Intravenous insulin tolerance test. At 2 wk after the treatments were initiated, db/db and lean mice fasted for 12 h were injected 0.8 U/kg body wt insulin (Novo Nordisk Pharma, Tokyo, Japan) via the tail vein. Blood was collected from tail vein at 0.5, 1, 1.5, and 2 h after insulin injection. The AUC of glucose levels (0–0.5 and 0–1 h) were calculated to assess the status of insulin resistance by the equation described above.

TG and total cholesterol contents in liver. At 8 wk after the treatments were initiated, mice were anesthetized with intraperitoneal injection of 20% chloral hydrate (Wako Pure Chemical Industries). Removed liver tissue was quickly weighed, and one part was homogenized and subjected to lipid extraction according to the method of Folch et al. (7). Hepatic TG and cholesterol contents were measured using commercially available kits (TG E-test and cholesterol E-test; Wako Pure Chemical Industries).

Determination of mRNA levels. Total RNA was isolated from liver, mesenteric fat, subcutaneous fat, triceps surae muscle, and 3T3-L1 adipocytes by Isogen (Nippon Gene, Tokyo, Japan). The mRNA level corresponding to a couple of target genes was determined by real-time quantitative RT-PCR using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and double-dye probes for carnitine palmitoyltransferase (CPT) I, acyl-CoA oxidase (ACO), uncoupling protein (UCP)3, and adiponectin were designed using Primer Express 2.0 (Applied Biosystems; Table 1). Primers and double-dye probes for 11 β -HSD1 and UCP2 were designed as described previously (1, 16). Results were normalized to 18S ribosomal RNA concentration determined by PreDeveloped TaqMan Assay Reagents (Applied Biosystems).

Cell culture and treatment. 3T3-L1 preadipocytes (passages 3–10; American Type Culture Collection, Manassas, VA) were grown and differentiated into adipocytes, as described previously (8). Briefly, differentiation was induced by incubating the cells in DMEM that included 10% FBS with 0.5 mmol/l IBMX, 1 μ mol/l dexamethazone, and 10 μ g/ml insulin for 2 days, followed by another 2-day incubation in DMEM that included 10% FBS with 10 μ g/ml insulin. The cells were further incubated in DMEM with 10% FBS for an additional 4 days to complete the adipocyte conversion. At day 8 following the initiation of differentiation, vehicle (1% DMSO), bezafibrate (1–300 μ mol/l), rosiglitazone (3 μ mol/l), pioglitazone (10 μ mol/l), fenofibric acid (1–300 μ mol/l), or WY-14,643 (1–10 μ mol/l) were replenished to cultured cells and coincubated from 8 to 48 h at 37°C in 5% CO₂.

Table 1. Primers and double-dye probes used for real-time quantitative PCR

Name	Oligonucleotide Sequence	Amplicon Size, bp	Accession No.
ACO			
Forward:	5'-TGCACCCGCCCATGAC-3'	72	AF006688
Reverse:	5'-GGCAACGGCCGAAAGC-3'		
Probe:	FAM5'-TCCTGACACAGCTAAGTTGCTTCTTTACCTCC-3' TAM		
CPT I			
Forward:	5'-CCTCCCTGGGCATGATTG-3'	87	AF017175
Reverse:	5'-ACGCCACTCAGATGTTCTTC-3'		
Probe:	FAM5'-CACCACCTGGCCCATGTCAAGC-3' TAM		
UCP3			
Forward:	5'-TGGCCCAACATCACAAGAAA-3'	79	NM_009464
Reverse:	5'-TCCAGCAACTTCTCCTTGATGA-3'		
Probe:	FAM5'-TGTCACCTGTGCTGAGATGGTGACCTACGA-3' TAM		
Adiponectin			
Forward:	5'-CAGTGGATCTGACGACAC-3'	74	U37222
Reverse:	5'-TGGGCAGGATTAAGAGGAACA-3'		
Probe:	FAM5'-AGGGCTCAGGATGCTACTGTTGCAAGCT-3' TAM		

ACO, acyl-CoA oxidase; CPT I, carnitine palmitoyltransferase I; UCP3, uncoupling protein 3.

Suppression of 11 β -HSD1 expression by RNA interference. 11 β -HSD1 expression in 3T3-L1 differentiated adipocytes was suppressed by a small interfering RNA (siRNA) duplex oligonucleotide-targeted 11 β -HSD1 mRNA sequence. Individual siRNA duplex was designed using a siRNA Design Support System (TaKaRa Bio, Shiga, Japan). 3T3-L1 differentiated adipocytes (day 8) were transfected with the siRNA duplex (10 nmol/l; sense: 5'-GAAAUGGCAUAUCAUCUGUTT-3' and antisense: 3'-TTCUUUACCGUAUAGUAGACA-5') using TransIT-TKO Transfection Reagent (Mirus Bio, Madison, WI) for 48 h. Negative Control siRNA (Qiagen, Tokyo, Japan) was used as a nonsilencing control.

Measurement of 11 β -HSD1 enzyme activity. Oxoreductase enzyme activity of 11 β -HSD1 was assessed according to a previous report (5), with slight modification. Assays for 11 β -HSD1 activity were performed by incubating intact cells with corticosteroids with appropriate tritiated tracer. In assays for oxoreductase activity, cells were incubated in serum-free DMEM containing 250 nmol/l cortisone with appropriate tritium-labeled tracer [1,2-³H₂]cortisone (50 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO). After the incubation at 37°C for indicated time, corticosteroids were extracted using ethyl acetate, separated by thin layer chromatography (TLC) in chloroform-methanol (95:5), and quantified by autoradiography. 11 β -HSD1 oxoreductase activities were expressed as pmol product ([1,2-³H₂]cortisol)·min⁻¹·well⁻¹ after correction for apparent conversion in reactions without cells.

Western blot analysis. 3T3-L1 pre- or differentiated adipocytes were coincubated with drugs or vehicle for 24 h. Proteins in cultured media (25 ng/lane) were separated by standard Laemmli's method (23) and subjected to immunoblotting. Western blot analyses were performed using anti-mouse adiponectin antibody (MAB3608; Chemicon International, Temecula, CA).

Statistical analyses. All data were expressed as means \pm SE. All statistical analyses were performed using SAS System Version 8.2 (SAS Institute, Cary, NC) and its interlocking movement program, Clinical Package Version 5.0. Student's *t*-test was performed between 1) vehicle-treated lean mice and vehicle-treated *db/db* mice, 2) vehicle-treated 3T3-L1 differentiated adipocytes and vehicle-treated 3T3-L1 preadipocytes, and 3) nontransfected control and nonsilencing control or siRNA transfected for RNA interference experiments. In case of multiple comparisons, one-way analysis of variance (ANOVA) followed by post hoc analysis of Dunnett's multiple comparison test was performed. Statistical analysis between vehicle-

treated *db/db* mice and other groups in oral glucose tolerance test (OGTT) and intravenous insulin tolerance test (IVITT) were performed using Student's *t*-test or Dunnett's multiple comparison test, followed by univariate repeated-measures ANOVA, taking pretreatment values as covariate. Differences were considered significant at *P* < 0.05.

RESULTS

Body weight and food consumption. Increment in body weight and food consumption in *db/db* mice was exaggerated compared with lean mice during the course of the experiment (*P* < 0.01). Both bezafibrate and fenofibrate tended to increase body weight compared with vehicle-treated *db/db* mice (*P* = 0.055 by ANOVA; Table 2).

Liver and mesenteric fat weight. Liver and mesenteric fat weight in vehicle-treated *db/db* mice was significantly elevated compared with vehicle-treated lean mice (*P* < 0.01; Table 2). Treatments of both bezafibrate and fenofibrate significantly increased liver weight compared with vehicle-treated *db/db* mice, whereas increase in liver weight in fenofibrate treatment was exaggerated compared with that in bezafibrate treatment (Table 2). It should be noted that both treatments did not significantly increase mesenteric fat weight (*P* = 0.63 by ANOVA; Table 2).

Plasma lipid parameters. Plasma levels of TG, NEFA, HDL cholesterol, and LDL cholesterol in vehicle-treated *db/db* mice at 15 wk of age were significantly higher than those in vehicle-treated lean mice (*P* < 0.01; Table 2). Both bezafibrate and fenofibrate markedly lowered plasma TG levels to around normal range at 8 wk after the treatments were initiated (Table 2). In addition, both treatments significantly lowered plasma level of NEFA (Table 2). Furthermore, both compounds significantly increased HDL cholesterol level in plasma (Table 2). In contrast, both compounds did not alter LDL cholesterol level in plasma (Table 2).

Hepatic TG and total cholesterol contents. Hepatic TG content in vehicle-treated *db/db* mice at 15 wk of age was significantly higher than that in vehicle-treated lean mice (*P* <

Table 2. Body weight, food consumption, liver weight, mesenteric fat weight, plasma lipid level, and hepatic lipid content in *db/db* and vehicle-treated lean mice

	Lean	Vehicle	Bezafibrate		Fenofibrate
			100	300	300
Physical parameters					
Body weight, g (15 wk of age)	29 \pm 0.7##	34 \pm 1	39 \pm 1	38 \pm 3	41 \pm 1
Average food consumption, g (7–15 wk of age)	5.1 \pm 0.2##	7.9 \pm 0.1	7.7 \pm 0.1	7.1 \pm 0.1	7.0 \pm 0.1
Liver weight, g	1.7 \pm 0.1##	2.3 \pm 0.1	3.3 \pm 0.1**	3.7 \pm 0.4**	4.8 \pm 0.1**
Mesenteric fat weight, mg	264 \pm 29##	598 \pm 70	771 \pm 91	729 \pm 160	738 \pm 71
Plasma lipid parameters					
Triglyceride, mg/dl	113 \pm 10##	346 \pm 35	201 \pm 9**	135 \pm 15**	119 \pm 12**
NEFA, mEq/l	0.7 \pm 0.1##	2.1 \pm 0.2	1.4 \pm 0.2*	1.4 \pm 0.2*	1.3 \pm 0.2**
HDL cholesterol, mg/dl	40 \pm 2##	76 \pm 5	96 \pm 4**	98 \pm 6**	94 \pm 4*
LDL cholesterol, mg/dl	8 \pm 0##	15 \pm 1	14 \pm 2	14 \pm 2	14 \pm 1
Hepatic lipid contents					
Triglyceride, mg/g liver	4.9 \pm 0.8##	12.2 \pm 0.7	10.3 \pm 0.4	8.4 \pm 0.5*	8.6 \pm 1.4*
Total cholesterol, mg/g liver	1.2 \pm 0.2	1.6 \pm 0.1	1.1 \pm 0.1**	0.9 \pm 0.1**	0.9 \pm 0.2**

Data are presented as means \pm SE (*n* = 7–8 in each). Values for bezafibrate and fenofibrate are given in mg·kg⁻¹·day⁻¹. NEFA, nonesterified fatty acids. ##*P* < 0.01 vs. vehicle-treated *db/db* mice (Student's *t*-test). **P* < 0.05; ***P* < 0.01 vs. vehicle-treated *db/db* mice (1 way ANOVA followed by Dunnett's multiple comparison test).

0.01; Table 2). Bezafibrate and fenofibrate significantly reduced hepatic TG and total cholesterol contents compared with vehicle-treated *db/db* mice (Table 2).

Plasma glucose and insulin levels. Plasma glucose levels in vehicle-treated *db/db* mice at 15 wk of age were significantly higher than those in vehicle-treated lean mice ($P < 0.01$; Fig. 1A). Bezafibrate significantly reduced plasma glucose levels at 8 wk after the treatments were initiated. In contrast, fenofibrate did not show beneficial effects on plasma glucose levels (Fig. 1A). Plasma insulin levels in vehicle-treated *db/db* mice at 15 wk of age tended to be higher than those in vehicle-treated lean mice ($P = 0.12$; Fig. 1B). Both bezafibrate and fenofibrate showed a trend toward reducing plasma insulin levels at 8 wk after the treatments were initiated ($P = 0.78$ by ANOVA; Fig. 1B).

OGTT and IVITT. To further assess the potential impact of drug administration on glucose tolerance and insulin sensitivity, OGTT and IVITT were performed in *db/db* mice and lean mice. At 4 wk after the treatments were initiated, bezafibrate, pioglitazone, and rosiglitazone significantly lowered plasma glucose levels compared with vehicle-treated *db/db* mice, but not fenofibrate (bezafibrate: 117 ± 21 mg/dl, $P = 0.0004$; fenofibrate: 197 ± 23 mg/dl, $P = 0.24$; pioglitazone: 143 ± 21 mg/dl, $P = 0.006$; rosiglitazone: 147 ± 27 mg/dl, $P = 0.007$ vs. vehicle-treated *db/db* mice; Fig. 2A). Glucose levels at each time point were significantly elevated in vehicle-treated *db/db* mice compared with vehicle-treated lean mice (Fig. 2A). $AUC_{0-0.5 h}$ and $AUC_{0-1 h}$ in vehicle-treated *db/db* mice were significantly higher than those in vehicle-treated lean mice (Table 3). Bezafibrate, pioglitazone, rosiglitazone and fenofibrate significantly reduced $AUC_{0-0.5 h}$ and $AUC_{0-1 h}$ (Table 3).

Reduction of $AUC_{0-0.5 h}$ and $AUC_{0-1 h}$ in bezafibrate was exaggerated compared with fenofibrate.

At 2 wk after the treatments were initiated, bezafibrate and rosiglitazone significantly lowered plasma glucose levels compared with vehicle-treated *db/db* mice, but not pioglitazone and fenofibrate (bezafibrate: 197 ± 22 mg/dl, $P = 0.005$; fenofibrate: 253 ± 21 mg/dl, $P = 0.20$; pioglitazone: 258 ± 35 mg/dl, $P = 0.31$; rosiglitazone: 216 ± 28 mg/dl, $P = 0.021$ vs. vehicle-treated *db/db* mice; Fig. 2B). At 0.5 h after the insulin injection in IVITT, bezafibrate, pioglitazone, and rosiglitazone significantly reduced plasma glucose levels ($P < 0.05$). On the other hand, glucose-lowering effects in fenofibrate-treated mice were marginal ($P = 0.29$; Fig. 2B). $AUC_{0-0.5 h}$ and $AUC_{0-1 h}$ in vehicle-treated *db/db* mice were significantly higher than those in vehicle-treated lean mice (Table 3). Bezafibrate, pioglitazone, and rosiglitazone significantly reduced the value of $AUC_{0-0.5 h}$, and reduction of $AUC_{0-1 h}$ in bezafibrate was equipotent to rosiglitazone (Table 3). In contrast, fenofibrate represented no significant effects on AUCs.

Plasma leptin, adiponectin, and corticosterone levels. Plasma leptin levels in vehicle-treated *db/db* mice were markedly elevated compared with vehicle-treated lean mice ($P < 0.01$). Treatments of bezafibrate and fenofibrate did not change plasma leptin levels significantly in *db/db* mice ($P = 0.08$ by ANOVA), whereas pioglitazone significantly decreased the levels (Fig. 1C).

Plasma adiponectin levels in vehicle-treated *db/db* mice were significantly lower than those in vehicle-treated lean mice ($P < 0.01$; Fig. 1D). Treatments of bezafibrate, pioglitazone, and rosiglitazone caused a significant rise in plasma adiponec-

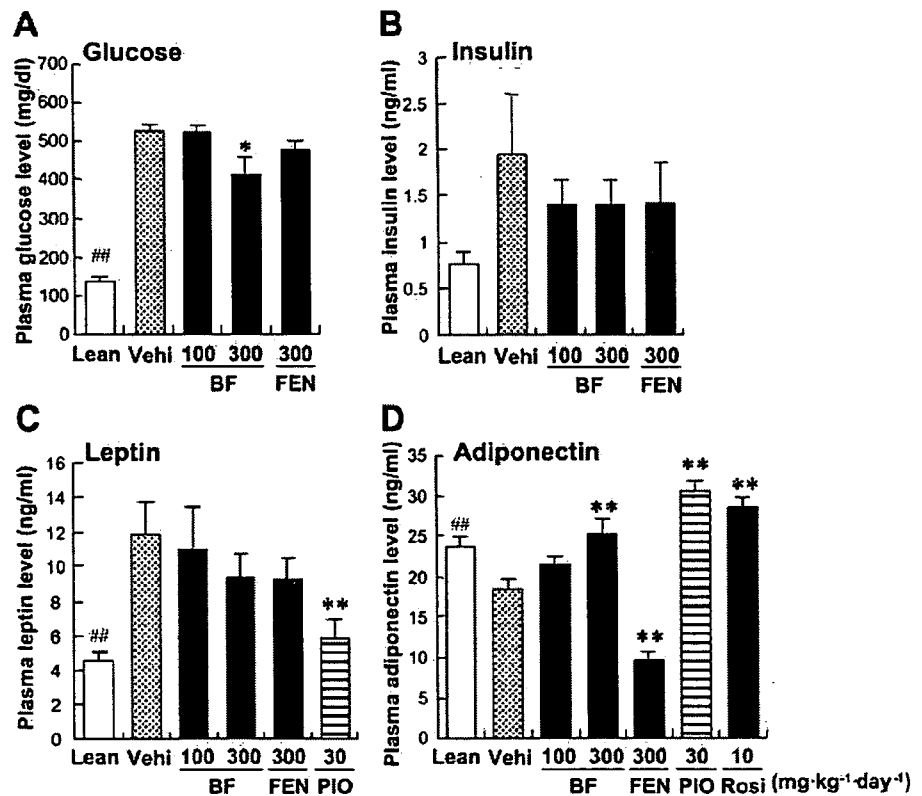


Fig. 1. Plasma glucose (A), plasma insulin (B), plasma leptin (C), and plasma adiponectin levels (D) in *db/db* mice treated with bezafibrate (BF; 100 or 300 mg·kg⁻¹·day⁻¹), fenofibrate (FEN; 300 mg·kg⁻¹·day⁻¹), pioglitazone (PIO; 30 mg·kg⁻¹·day⁻¹), rosiglitazone (Rosi; 10 mg·kg⁻¹·day⁻¹), or vehicle (Vehi) and vehicle-treated lean mice ($n = 7-8$ in each). Each column shows plasma glucose, insulin, and leptin levels at 8 wk and adiponectin levels in mice at 6 wk after the treatments were initiated. Data are presented as means \pm SE. * $P < 0.05$; ** $P < 0.01$ vs. vehicle-treated *db/db* mice; ## $P < 0.01$ vs. vehicle-treated lean mice.

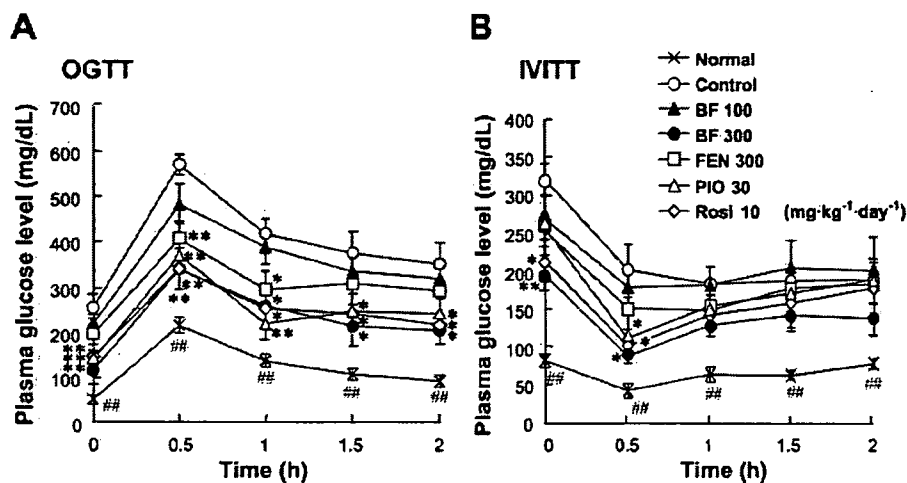


Fig. 2. Oral glucose tolerance test (OGTT; A) and intravenous insulin tolerance test (IVITT; B) in *db/db* mice treated with BF [100 (Δ) or 300 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (\bullet)], FEN [300 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (\square)], PIO [30 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (\triangle)], Rosi [10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (\diamond)], or vehicle (\circ) and vehicle-treated lean mice (\times) at 4 or 2 wk after initiating the treatments, respectively ($n = 8-10$ in each). Data of plasma glucose levels are presented as means \pm SE. * $P < 0.05$; ** $P < 0.01$ vs. vehicle-treated *db/db* analyzed by univariate repeated-measures ANOVA; ## $P < 0.01$ vs. vehicle-treated *db/db* mice.

tin levels. Conversely, fenofibrate rather decreased the levels compared with vehicle-treated *db/db* mice (Fig. 1D).

Plasma corticosterone level in vehicle-treated *db/db* mice (133.7 ± 21.3 ng/ml) was markedly elevated compared with vehicle-treated lean mice (37.4 ± 14.5 ng/ml, $P < 0.01$). Treatments of bezafibrate, fenofibrate, and pioglitazone did not significantly change plasma corticosterone levels in *db/db* mice (bezafibrate: 122.7 ± 14.2 ng/ml; fenofibrate: 121.2 ± 8.5 ng/ml; pioglitazone: 132.6 ± 14.7 ng/ml).

Expression of genes related to fuel metabolism in liver. It is well known (2) that the activation of PPAR α induces mRNA expression of a series of genes involved in lipid handling and fatty acid β -oxidation. In liver, there were no significant differences in mRNA level of CPT I, ACO, UCP2, or UCP3 between vehicle-treated *db/db* and vehicle-treated lean mice (Table 4). On the other hand, both bezafibrate and fenofibrate markedly increased the levels for CPT I ($P = 0.30$ by ANOVA), ACO, UCP2, and UCP3 (bezafibrate, $P = 0.23$) compared with vehicle-treated *db/db* mice (Table 4).

Expression of 11 β -HSD1 mRNA. In liver, triceps surae muscle, mesenteric fat, and subcutaneous fat, 11 β -HSD1 mRNA levels in vehicle-treated *db/db* mice were significantly higher than those in vehicle-treated lean mice (Fig. 3). In liver,

bezafibrate, fenofibrate, and pioglitazone significantly decreased the 11 β -HSD1 mRNA levels (Fig. 3A). In triceps surae muscle, bezafibrate and fenofibrate also showed a trend toward a decrease in the 11 β -HSD1 mRNA levels, but these effects were marginal (Fig. 3A).

Of note, in mesenteric fat, bezafibrate and pioglitazone substantially decreased the 11 β -HSD1 expression levels, whereas fenofibrate did not affect the levels (Fig. 3B). In subcutaneous fat, bezafibrate also showed a trend toward lower levels of 11 β -HSD1, which were comparable to those in pioglitazone. In contrast, fenofibrate had no effects (Fig. 3B).

mRNA expression levels and enzyme activity of 11 β -HSD1 in bezafibrate-treated 3T3-L1 adipocytes. The mRNA levels of 11 β -HSD1 in 3T3-L1 differentiated adipocytes were apparently higher than those in vehicle-treated 3T3-L1 preadipocytes. Importantly, bezafibrate, rosiglitazone, and pioglitazone markedly reduced the 11 β -HSD1 mRNA levels in 3T3-L1 differentiated adipocytes at 48 h after the treatments. In contrast, fenofibrate and WY-14,643 had no effect on 11 β -HSD1 mRNA levels (Fig. 4A).

To further explore the impact on enzyme activity of 11 β -HSD1, cells were incubated with tritium-labeled cortisone. Assay for 11 β -HSD1 activity clearly showed that 11 β -HSD1 acted as distinct oxoreductase in 3T3-L1 differentiated adipocytes (Vehi; Fig. 4B). Bezafibrate, rosiglitazone, and pioglitazone considerably inhibited cortisone-to-cortisol conversion for 48-h treatment (Fig. 4B). Actually, bezafibrate, rosiglitazone, and pioglitazone markedly reduced 11 β -HSD1 oxoreductase ac-

Table 3. Effects of bezafibrate, fenofibrate, pioglitazone, rosiglitazone, or vehicle on AUC of plasma glucose level in OGTT or IVITT

Dose, $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$	OGTT		IVITT	
	AUC _{0-0.5 h}	AUC _{0-1 h}	AUC _{0-0.5 h}	AUC _{0-1 h} ($\text{mg}\cdot\text{h}\cdot\text{dl}^{-1}$)
Lean	68 \pm 4##	246 \pm 17##	32 \pm 3##	87 \pm 11##
Vehicle	207 \pm 7	702 \pm 28	131 \pm 13	228 \pm 24
Bezafibrate 100	176 \pm 14	612 \pm 53*	113 \pm 13	204 \pm 26
Bezafibrate 300	115 \pm 15**	415 \pm 59**	72 \pm 7**	127 \pm 12*
Fenofibrate 300	152 \pm 14*	504 \pm 48*	102 \pm 11	178 \pm 21
Pioglitazone 30	128 \pm 11**	424 \pm 38**	93 \pm 12*	160 \pm 20
Rosiglitazone 10	122 \pm 15**	420 \pm 59**	80 \pm 11**	127 \pm 23*

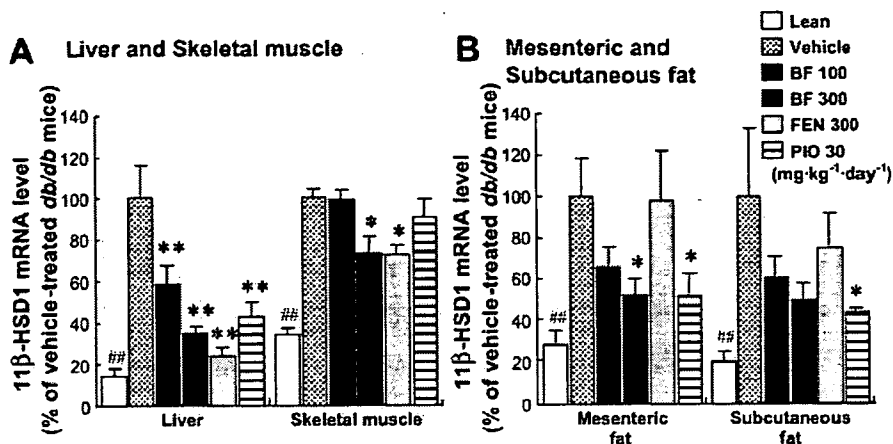
Data are presented as means \pm SE ($n = 8-10$ in each). OGTT, oral glucose tolerance test; IVITT, intravenous insulin tolerance test. ## $P < 0.01$ vs. vehicle-treated *db/db* mice (Student's *t*-test); * $P < 0.05$; ** $P < 0.01$ vs. vehicle-treated *db/db* mice (1-way ANOVA followed by Dunnett's multiple comparison test).

Table 4. Hepatic mRNA levels related to fuel metabolism in *db/db* and vehicle-treated lean mice

	Lean	Vehicle	Bezafibrate		Fenofibrate
			100	300	300
CPT I	72 \pm 5	100 \pm 22	125 \pm 16	149 \pm 30	152 \pm 17
ACO	88 \pm 12	100 \pm 18	227 \pm 29*	279 \pm 26**	401 \pm 48**
UCP2	79 \pm 5	100 \pm 7	181 \pm 19	250 \pm 28**	352 \pm 51**
UCP3	203 \pm 73	100 \pm 26	1,273 \pm 380	3,782 \pm 2,639	7,777 \pm 1,441**

Data are presented as means \pm SE ($n = 7-8$ in each). Values for bezafibrate and fenofibrate are given in $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$. * $P < 0.05$; ** $P < 0.01$ vs. vehicle-treated *db/db* mice (1-way ANOVA followed by Dunnett's multiple comparison test).

Fig. 3. 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) mRNA levels in insulin target tissues of *db/db* mice treated with BF (100 or 300 mg·kg⁻¹·day⁻¹), FEN (300 mg·kg⁻¹·day⁻¹), PIO (30 mg·kg⁻¹·day⁻¹), or vehicle and vehicle-treated lean mice (*n* = 8–10 in each). Each column shows 11 β -HSD1 mRNA levels in liver, triceps surae muscle (skeletal muscle; A), mesenteric fat, and subcutaneous fat (B) at 8 wk after the treatments were initiated. Each column represents %11 β -HSD1 mRNA levels compared with vehicle-treated *db/db* mice (defined as 100%). Data are presented as means \pm SE. **P* < 0.05; ***P* < 0.01 vs. vehicle-treated *db/db* mice; ##*P* < 0.01 vs. vehicle-treated *db/db* mice.



tivities expressed as pmol product (³H)cortisol·min⁻¹·well⁻¹ (Fig. 4C).

Suppression of 11 β -HSD1 expression and enzyme activity by transfection with a siRNA duplex. To validate the enzyme activity assay in the present study, 3T3-L1 differentiated adipocytes were transfected with siRNA duplex targeted to 11 β -HSD1 mRNA sequence. The mRNA levels of 11 β -HSD1 were markedly reduced by transfection of siRNA duplex (*P* < 0.01; Fig. 4D). Consequently, the enzyme activity of 11 β -HSD1 was also reduced (*P* < 0.05; Fig. 4E).

Adiponectin level in cultured media of bezafibrate-treated 3T3-L1 adipocytes. Adiponectin levels in cultured media of 3T3-L1 adipocytes were determined by Western blot analysis. Adiponectin protein was detected as a single band at ~35 kDa (Fig. 5A). Adiponectin protein from vehicle-treated 3T3-L1 preadipocytes was under detectable range. Of note, bezafibrate and rosiglitazone markedly increased adiponectin level in cultured media. In contrast, adiponectin levels in media of cells treated with fenofibric acid did not increase at all (Fig. 5B).

The mRNA levels of adiponectin in 3T3-L1 adipocytes were determined at the point of 8, 16, and 24 h after the each treatment. Bezafibrate and rosiglitazone increased mRNA levels of adiponectin from 8 to 24 h in a time-dependent manner, with significant increases at 16 and 24 h (Fig. 5C). Consequently, at 24 h after the treatment, bezafibrate, rosiglitazone, and pioglitazone significantly increased the adiponectin mRNA levels (Fig. 5D).

DISCUSSION

Recent extensive clinical trials (43) provided compelling evidence that bezafibrate reduces the incidence of type 2 diabetes in patients with coronary artery diseases. To our knowledge, however, the underlying molecular mechanism of its metabolically beneficial effects has not been fully elucidated. The present study confirmed that repeated administration of bezafibrate significantly ameliorated hyperglycemia and insulin resistance in *db/db* mice. Nevertheless, antihyperlipidemic effects of fenofibrate were equipotent to bezafibrate (Table 2), and improvement of glucose homeostasis by fenofibrate was marginal compared with bezafibrate. In the present study, 100 or 300 mg·kg⁻¹·day⁻¹ of bezafibrate and 300 mg·kg⁻¹·day⁻¹ of fenofibrate were administered. Because the sensitivity of PPAR α agonists in terms of fuel homeostasis in

rodents is known to be much lower than in humans, higher doses have been commonly used for rodent experiments (12, 22, 49). Administered doses of compounds were decided mainly on the basis of their plasma TG-lowering effects. In our pilot study, treatment of bezafibrate or fenofibrate (100 mg·kg⁻¹·day⁻¹) in *db/db* mice for 8 wk did not significantly lower plasma levels of TG. In the next pilot study, treatment of bezafibrate (300 mg·kg⁻¹·day⁻¹) or fenofibrate (300 mg·kg⁻¹·day⁻¹) in *db/db* mice for 6 wk equipotently lowered plasma TG level (Vehi: 204 \pm 18 mg/dl; bezafibrate: 95 \pm 7, *P* < 0.01 mg/dl; fenofibrate: 85 \pm 5 mg/dl, *P* < 0.01 vs. vehicle-treated *db/db* mice). Notably, treatment of bezafibrate (300 mg·kg⁻¹·day⁻¹) significantly lowered plasma glucose level at 6 wk with an equipotency to rosiglitazone (Vehi: 473 \pm 24 mg/dl; bezafibrate: 216 \pm 40 mg/dl, *P* < 0.01; fenofibrate: 408 \pm 30 mg/dl, *P* = 0.10; rosiglitazone: 197 \pm 23 mg/dl, *P* < 0.01 vs. vehicle-treated *db/db* mice). In the present study, we evaluated antidiabetic properties of bezafibrate and fenofibrate with the dose showing equipotent TG-lowering profile. In this context, TG-lowering profile in bezafibrate and fenofibrate in the present study indicates that the doses of drugs used were appropriate for assessing antidiabetic effects. Because we focused our special attention on PPAR γ agonistic properties of bezafibrate, fenofibrate was employed as a "selective" PPAR α agonist. Therefore, we evaluated two doses (100 or 300 mg·kg⁻¹·day⁻¹) of bezafibrate and a single dose (300 mg·kg⁻¹·day⁻¹) of fenofibrate.

Our results raise a couple of possibilities responsible for metabolically beneficial effects of bezafibrate. Decline of adiponectin levels is known (17, 33, 50) to associate with insulin resistance, hepatic fibrosis, and atherosclerosis in humans and rodents. In accordance with previous reports (10), plasma levels of adiponectin in *db/db* mice were significantly lower than those in vehicle-treated lean littermates (Fig. 1). Notably, bezafibrate significantly increased plasma adiponectin levels in *db/db* mice, whereas fenofibrate did not provoke such effects. More importantly, we demonstrated for the first time that bezafibrate pronouncedly augmented the expression and secretion of adiponectin in 3T3-L1 differentiated adipocytes (Fig. 5). Activation of PPAR γ is known to increase plasma adiponectin levels in humans and rodents (27, 52). First, the present study confirmed that pioglitazone and rosiglitazone increased plasma adiponectin levels in *db/db* mice (Fig. 1D),

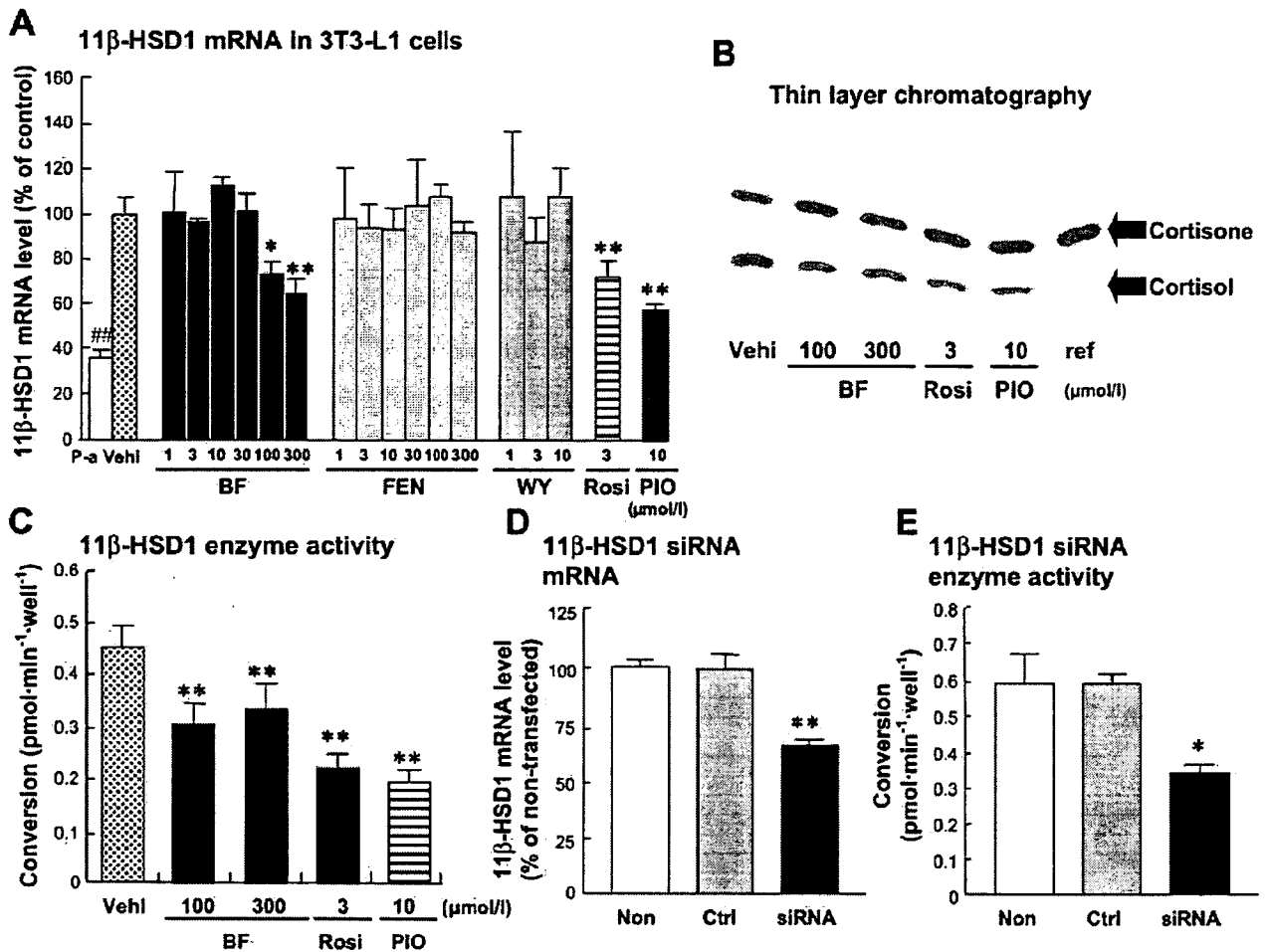


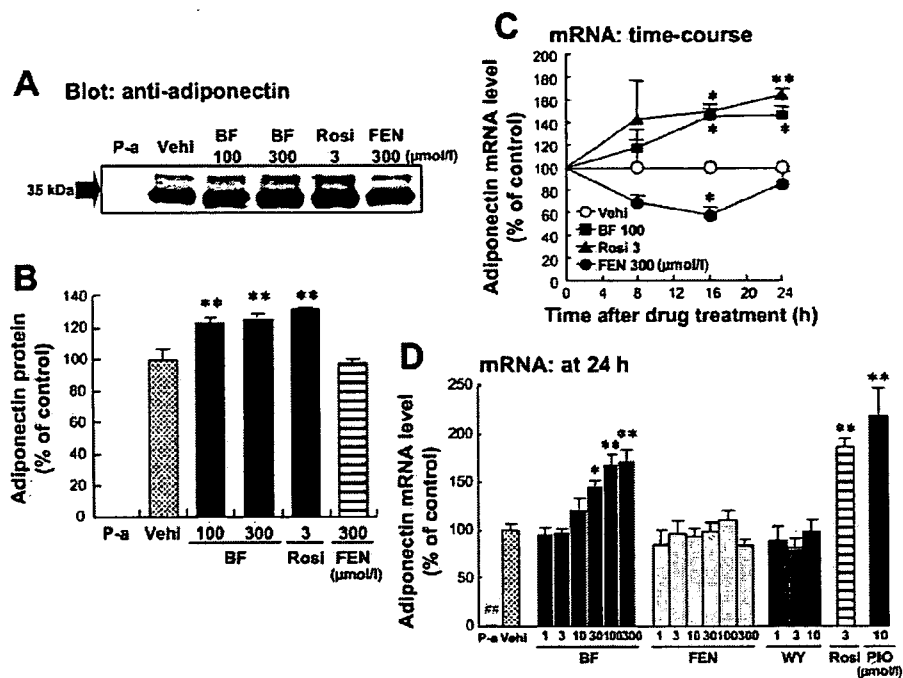
Fig. 4. 11 β -HSD1 mRNA levels (A and D) and oxoreductase activity (B, C, and E) in 3T3-L1 adipocytes. A–C: 3T3-L1 adipocytes were treated with BF (1–300 $\mu\text{mol/l}$), Rosi (3 $\mu\text{mol/l}$), PIO (10 $\mu\text{mol/l}$), FEN (1–300 $\mu\text{mol/l}$), WY-14,643 (WY; 1–10 $\mu\text{mol/l}$), or Vehi and 3T3-L1 preadipocytes (P-a) treated with vehicle for 48 h. A: each column represents %11 β -HSD1 mRNA levels compared with vehicle-treated 3T3-L1 differentiated adipocytes (defined as 100%; $n = 6$). B: representative autoradiograph image of TLC; ref, cell-free control. C: each column shows 11 β -HSD1 oxoreductase activity ($n = 3$). Data are presented as means \pm SE. * $P < 0.05$; ** $P < 0.01$ vs. vehicle-treated 3T3-L1 differentiated adipocytes; ### $P < 0.01$ vs. vehicle-treated 3T3-L1 differentiated adipocytes. D and E: 3T3-L1 differentiated adipocytes were transfected with reagent only [nontransfected control (Non)], nonsilencing control small interfering (si)RNA (Ctrl), or siRNA duplex-targeted 11 β -HSD1 mRNA sequence. D: each column represents %11 β -HSD1 mRNA levels compared with Non (defined as 100%; $n = 3$). E: each column shows 11 β -HSD1 oxoreductase activity ($n = 3$). Data are presented as means \pm SE. * $P < 0.05$; ** $P < 0.01$ vs. nontransfected control.

and rosiglitazone induced adiponectin expression and secretion in 3T3-L1 differentiated adipocytes (Fig. 5). To date, there has been only one report (31) that bezafibrate significantly increased plasma adiponectin levels in obese-diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Moreover, there were some reports (20, 21) showing that fenofibrate increased circulating adiponectin levels in humans. On the other hand, there was only one report (6) that fenofibrate increased mRNA levels of adiponectin in fat depots but did not increase serum levels in OLETF rats. A recent report (44) showed that WY-14,643, a selective PPAR α agonist, did not increase serum adiponectin levels in obese-diabetic KKA y mice. Collectively, the mechanistic link between PPAR α activation and elevation of circulating adiponectin still remains unclear. In the present study, fenofibrate and WY-14,643 did not change adiponectin secretion in 3T3-L1 adipocytes, suggesting that PPAR α activation by fenofibrate and WY-14,643 does not exert direct effects on adiponectin secretion. On the other hand, our data

support the notion that bezafibrate directly influences adiponectin secretion in 3T3-L1 adipocytes. In contrast, several previous works (20, 21) have suggested that fenofibrate indirectly increased circulating adiponectin, presumably as a reflection of systemic improvement of fuel homeostasis. Our data suggest that bezafibrate-induced increase in plasma adiponectin levels is independent of PPAR α activation. Thus, it is likely that a significant increase in adiponectin level induced by bezafibrate may be attributable to its PPAR γ agonistic activity (48) and thus contribute, at least partly, to the improvement of glucose metabolism in *db/db* mice. Future investigations to see whether bezafibrate could increase circulating adiponectin level in humans will be of great interest.

A previous report (19) demonstrated that fenofibrate significantly reduced plasma leptin levels in OLETF rats. In the present study, bezafibrate and fenofibrate did not significantly change plasma leptin levels. In contrast, consistent with previous reports (34, 37, 51), pioglitazone decreased plasma leptin

Fig. 5. Adiponectin protein and mRNA levels in 3T3-L1 preadipocytes (P-a) or differentiated adipocytes. **A**: representative Western blot. **B**: each column represents %adiponectin protein in cultured media quantified from Western blot compared with vehicle-treated 3T3-L1 differentiated adipocytes (defined as 100%; $n = 3$). **C** and **D**: adiponectin mRNA levels in 3T3-L1 adipocytes. Time course of adiponectin mRNA levels (**C**) in 3T3-L1 adipocytes treated with BF (100 μ mol/l; \blacksquare), Rosi (3 μ mol/l; \blacktriangle), FEN (300 μ mol/l; \bullet), or Vehi (\circ) ($n = 3$) and adiponectin mRNA levels at 24 h ($n = 6$) (**D**). Data are presented as means \pm SE or means \pm SE. * $P < 0.05$; ** $P < 0.01$ vs. vehicle-treated 3T3-L1 differentiated adipocytes; ## $P < 0.01$ vs. vehicle-treated 3T3-L1 differentiated adipocytes.



levels, presumably via PPAR γ activation in adipocytes (Fig. 1C). Taken together, a possible mechanistic link between PPAR α activation and leptin regulation remains obscure. There were a couple of studies (6, 19) showing that fenofibrate decreased body weight, as well as adipose tissue weight, in OLETF rats. In the present study, both bezafibrate and fenofibrate treatments tended to increase body weight and mesenteric fat weight (Table 2). Because body weight increase was almost parallel to the increment in liver weight (Table 2), it is likely that weight gain in both groups was largely attributable to liver hypertrophy caused by PPAR α activation.

Notably, with an equipotency to pioglitazone, bezafibrate markedly lowered the expression level of 11 β -HSD1 in mesenteric fat (Fig. 3B). With a sharp contrast, fenofibrate did not change the expression of 11 β -HSD1. Importantly, bezafibrate markedly lowered the expression and enzyme activity of 11 β -HSD1 in 3T3-L1 differentiated adipocytes, equipotent to rosiglitazone and pioglitazone (Fig. 4). To our knowledge, this is the first demonstration that bezafibrate potently reduces mRNA expression and enzyme activity of 11 β -HSD1 in cultured adipocytes. Our data clearly demonstrated that a knockdown of 11 β -HSD1 by transfection of siRNA duplex significantly reduced mRNA expression and enzyme activity of 11 β -HSD1 in 3T3-L1 differentiated adipocytes (Fig. 4, D and E), confirming that 11 β -HSD1 acts as a distinct oxidoreductase and activates glucocorticoid from inactive form. This result also validates the enzyme activity assays in the present study. It has been reported (4) that PPAR γ agonists suppressed the expression levels of 11 β -HSD1 mRNA exclusively in adipocytes. In accordance with this notion, in the present study, pioglitazone markedly suppressed the 11 β -HSD1 mRNA in mesenteric and subcutaneous fat depots. Taken together, it is reasonable to speculate that bezafibrate suppresses 11 β -HSD1 in fat depots via its agonistic property for PPAR γ , thereby contributing, at least partly, to metabolically beneficial effects by bezafibrate.

It is important to note that the treatment of bezafibrate did not change circulating corticosterone levels in *db/db* mice in the present study. This indicates that, via the regulation of 11 β -HSD1, bezafibrate modifies intracellular glucocorticoid metabolism in a tissue- or cell-specific manner but does not affect systemic glucocorticoid homeostasis. The present study demonstrated that bezafibrate, fenofibrate, and pioglitazone significantly decreased 11 β -HSD1 mRNA levels in liver (Fig. 3A). These effects may also explain unidentified, metabolically beneficial properties of fibrates (13, 25), because transgenic mice overexpressing 11 β -HSD1 exclusively in liver exemplify fatty liver, glucose intolerance, dyslipidemia, and severe hypertension (36). In this context, the potential pathophysiological role of 11 β -HSD1 in liver must await further investigation.

Bezafibrate has been characterized as a PPAR(α , γ , and δ) pan-agonist in vitro (9, 48). It was reported (24, 40, 42) that GW501516, known as a PPAR δ agonist, exerts beneficial effects on insulin resistance. Thus, the activation of PPAR δ by bezafibrate may also contribute to its metabolically beneficial effects. Although physiological relevance and an entire picture of the mechanism whereby bezafibrate ameliorates fuel dyshomeostasis in humans must await further investigation, our results presented here provide novel evidence that bezafibrate directly regulates 11 β -HSD1 and adiponectin in murine adipocytes. Further investigations on other rodent models and human clinics should validate its accuracy and may open a fresh avenue for therapeutic strategies that might target convergence of multiple risk factors.

GRANTS

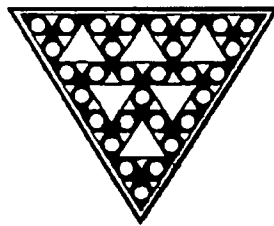
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Effect of acute activation of 5'-AMP-activated protein kinase on glycogen regulation in isolated rat skeletal muscle

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Miyamoto L, Toyoda T, Hayashi T, Yonemitsu S, Nakano M, Tanaka S, Ebihara K, Masuzaki H, Hosoda K, Ogawa Y, Inoue G, Fushiki T, Nakao K. Effect of acute activation of 5'-AMP-activated protein kinase on glycogen regulation in isolated rat skeletal muscle. *J Appl Physiol* 102: 1007–1013, 2007. First published November 22, 2006; doi:10.1152/jappphysiol.01034.2006.—5'-AMP-activated protein kinase (AMPK) has been implicated in glycogen metabolism in skeletal muscle. However, the physiological relevance of increased AMPK activity during exercise has not been fully clarified. This study was performed to determine the direct effects of acute AMPK activation on muscle glycogen regulation. For this purpose, we used an isolated rat muscle preparation and pharmacologically activated AMPK with 5-aminoimidazole-4-carboxamide-1- β -D-ribose nucleoside (AICAR). Tetanic contraction *in vitro* markedly activated the α_1 - and α_2 -isoforms of AMPK, with a corresponding increase in the rate of 3-O-methylglucose uptake. Incubation with AICAR elicited similar enhancement of AMPK activity and 3-O-methylglucose uptake in rat epitrochlearis muscle. In contrast, whereas contraction stimulated glycogen synthase (GS), AICAR treatment decreased GS activity. Insulin-stimulated GS activity also decreased after AICAR treatment. Whereas contraction activated glycogen phosphorylase (GP), AICAR did not alter GP activity. The muscle glycogen content decreased in response to contraction but was unchanged by AICAR. Lactate release was markedly increased when muscles were stimulated with AICAR in buffer containing glucose, indicating that the glucose taken up into the muscle was catabolized via glycolysis. Our results suggest that AMPK does not mediate contraction-stimulated glycogen synthesis or glycogenolysis in skeletal muscle and also that acute AMPK activation leads to an increased glycolytic flux by antagonizing contraction-stimulated glycogen synthesis.

contraction; glycogen synthase; glycogen phosphorylase; epitrochlearis muscle; glycolysis

EXERCISE PROFOUNDLY AFFECTS glycogen metabolism by stimulating glycogenolysis during exercise, which is followed by the resynthesis of glycogen after exercise. Intracellular glycogen plays a major role as a fuel for acute muscle contraction, and its concentration is dramatically reduced in response to a single bout of exercise (6, 14, 25, 38). The facilitation of glycogen resynthesis after exercise causes a marked accumulation of glycogen, i.e., the well-known phenomenon of glycogen supercompensation. Similar to exercise, insulin causes strong glucose uptake and glycogen synthesis. Therefore, the increase in insulin sensitivity in skeletal muscle after exercise also plays

an important role in glucose metabolism (11, 25, 36). In contrast to the physiological relevance of muscle glycogen, the signaling mechanisms leading to the regulation of glycogen synthesis and glycogenolysis in contracting muscle have not been fully clarified.

Several aspects of the contraction-evoked metabolic events in skeletal muscle have been shown to be related to 5'-AMP-activated protein kinase (AMPK), including GLUT4 translocation and glucose transport (1, 4, 18, 19, 27, 31, 32), fatty acid oxidation via the inactivation of acetyl-CoA carboxylase (23, 32, 41, 43), GLUT4 expression (7, 21, 22, 32, 35, 44, 48), and insulin sensitivity (12, 24, 32). AMPK, a heterotrimeric serine/threonine protein kinase, consists of a catalytic α -subunit and regulatory β - and γ -subunits. Two distinct α -isoforms, α_1 and α_2 , are expressed in skeletal muscle (39), and both isoforms can be activated in response to muscle contraction (40). AMPK is allosterically activated in response to an elevation in AMP concentration or in the AMP-to-ATP ratio. It is also activated when phosphorylated by upstream kinases (42, 46). Therefore, it has been hypothesized that AMPK acts as an intracellular energy sensor that plays a key role in regulating cellular metabolism in skeletal muscle (9, 13, 18, 19, 23, 37, 41, 43).

Recent studies of natural and manipulated mutations in AMPK suggest that chronic changes in AMPK action have substantial effects on glycogen metabolism in skeletal muscle. The glycogen concentration in the gastrocnemius muscles of mice that muscle-specifically express a dominant-negative kinase-dead form of the α -isoform is about half that observed in nontransgenic mice (31), although glycogen synthase (GS) activity and glycogen phosphorylase (GP) activity are not significantly different between the transgenic and nontransgenic animals under basal conditions or after contraction (30). The AMPK R225Q mutation in the *PRKAG3* gene, which encodes the muscle-specific isoform of the γ_3 -subunit of AMPK, leads to increased glycogen accumulation in Hampshire pig skeletal muscle (29). The amount of glycogen in the gastrocnemius muscle is twofold higher in the R225Q *PRKAG3* transgenic mouse than in the wild-type mouse but is unaltered in the *PRKAG3*-null mouse (5). In contrast, another mutation has been identified in the pig *PRKAG3* gene (V224I) that is associated with low muscle glycogen (10). It has also been suggested that mutations in *PRKAG2*, the gene for the γ_2 -subunit of AMPK, cause glycogen-associated vacuoles to

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