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The Novel Lipid-Lowering Drug D-47 Ameliorates Hepatic Steatosis and Promotes Brown/Beige-Like Change of White Adipose Tissue in db/db Mice

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D-47 is a newly developed solid dispersion of the arginine salt of (S)-(+)-4-[1-(4-tert-butylphenyl)-2-oxo-pyrrolidin-4-yl]methoxybenzoic acid (S-2E), which inhibits sterol and fatty acid synthesis. D-47 was recently shown to lower the serum level and hepatic content of both triglyceride and cholesterol in a rabbit model of familial hypercholesterolemia. We here investigated the effects of D-47 on dyslipidemia and hepatic steatosis in comparison with those of bezafibrate in the db/db mouse model of obesity. Treatment of db/db mice with D-47 or bezafibrate for 14 days lowered the serum triglyceride concentration without affecting that of cholesterol. D-47, but not bezafibrate, almost completely eliminated lipid droplets in hepatocytes and markedly lowered the triglyceride content of the liver in these mice. The two agents induced similar changes in the hepatic expression of genes including those related to β-oxidation or fatty acid synthesis. D-47 however significantly reduced the mass of white adipose tissue and up-regulated the expression of genes related to energy expenditure, mitochondrial function, fatty acid oxidation or lipolysis in this tissue, indicating that D-47 induced the brown/beige adipocyte-like change in white adipose tissue, whereas bezafibrate had no such effects. Treatment of 3T3-L1 adipocytes with D-47 provoked the expression of genes related to mitochondrial function, fatty acid oxidation or lipolysis. Our data have thus shown that D-47 ameliorated hypertriglyceridemia and hepatic steatosis in an animal model of obesity, and they suggest that this latter effect might be mediated through the change of adipose tissue characteristics.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. It is triggered by metabolic disorders associated with obesity or overnutrition, and it manifests at various levels of severity ranging from simple hepatic steatosis to nonalcoholic steatohepatitis, the latter of which may progress further to more serious conditions such as hepatic cirrhosis or hepatic cancer (1). Although the mechanisms underlying its development are not fully understood, NAFLD is often associated with dyslipidemia (2), suggesting that the two conditions may result from common pathological disorders of lipid metabolism. Consistent with this notion, drugs administered for dyslipidemia—which include statins, fibrates, ezetimibe, and niacin—have been found to have favorable effects on NAFLD (2), although these effects are not sufficient for clinical application.

D-47 is a newly developed solid dispersion of the arginine salt of (S)-(+)-4-[1-(4-tert-butylphenyl)-2-oxo-pyrrolidin-4-yl]methoxybenzoic acid (S-2E) (3), which inhibits the biosynthesis of sterols and fatty acids (4). Administration of D-47 was recently shown to lower serum triglyceride and lipoprotein(a) levels in transgenic mice that overproduce lipoprotein(a) (3). It was also shown to ameliorate hypertriglyceridemia and hypercholesterolemia in WHHLMI rabbits (5), which develop dyslipidemia as the result of a spontaneous mutation in the gene for the low density lipoprotein receptor (6). Moreover, whereas WHHLMI rabbits do not show exaggerated accumulation of lipids in the liver, D-47 reduced the liver content of triglyceride and cholesterol in these animals. Although these findings suggest that D-47 may prove beneficial for the treatment of dyslipidemia and NAFLD, they were obtained with animal models in which the disorders of lipid metabolism were induced

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directly by genetic alterations (3, 5). The effects of the drug on dyslipidemia and hepatic steatosis in obese model animals have thus remained undetermined.

To provide further insight into the pharmacological properties of D-47, we have now investigated the effects of this compound on dyslipidemia and hepatic steatosis in db/db mice, a widely studied model of obesity-related metabolic disorders. The effects of D-47 were compared with those of the lipid-lowering drug bezafibrate. We found that D-47 ameliorates hypertriglyceridemia and hepatic steatosis in db/db mice, and that the latter effect may be mediated at least in part through an action in adipose tissue.

MATERIALS AND METHODS

Animals, cell culture and experimental protocol

This study was performed in accordance with the guidelines for the care and use of laboratory animals of Kobe University, and the protocol was approved by the Committee on Animal Research of Kobe University Graduate School of Medicine (approval no. P160308). BKS.Cg-+ Lepr^{db}/+ Lepr^{db}/Jcl (db/db) male mice were obtained from CLEA Japan (Tokyo, Japan). At 6 weeks of age, the mice were divided into three groups (n = 8 per group): control, D-47, and bezafibrate. Those in the control group were fed a control diet (CE-2, CLEA Japan), whereas those in the D-47 and bezafibrate groups were fed CE-2 supplemented with 0.4% (w/w) D-47 or 0.1% (w/w) bezafibrate (Wako, Osaka, Japan), respectively. D-47 was produced in Tokushima-Bunri University and is a solid dispersion of the S-2E-arginine salt (C28H38N5O6, MW: 540, PubChem CID: 9864020) (3). 3T3-L1 fibroblasts were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Adipogenesis was induced by treatment of the cells with insulin, dexamethasone, and isobutylmethylxanthine as described previously (7). The cells were treated with D-47 at 50 μM and 500 μM for 48 hours at day 8 after the induction of differentiation. The concentration of D-47 (500 μM) in the treatment of 3T3-L1 adipocytes was determined to be equivalent to the serum concentration assumed in mice fed a diet supplemented with 0.4% D-47 (w/w) (5).

Biochemical analysis

Serum triglyceride, total cholesterol, and nonesterified fatty acid levels were measured with the use of Triglyceride E-Test, Cholesterol E-Test, and NEFA C-Test kits (Wako, Osaka, Japan), respectively. Blood glucose was measured with the use of a glucometer (Glutest Ace R; Sanwa Kagaku Kenkyusho, Aichi, Japan), and serum insulin levels were determined with a Mouse Insulin ELISA kit (Shibayagi, Shibukawa, Japan). For the measurement of hepatic triglyceride content, total lipids were extracted from the liver with the use of isopropanol, and the triglyceride content of the extract was determined with a Triglyceride E-Test kit. Collection of blood samples and measurement of hepatic triglyceride content were performed in a fed state.

Histological analysis

The liver was fixed in 10% formaldehyde and embedded in paraffin. The tissue was then sectioned and stained with hematoxylin-eosin.

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from the liver and epididymal adipose tissue with the use of an RNeasy kit (Qiagen). The RNA samples were subjected to reverse transcription (RT) with the use of a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and the resulting cDNA was subjected to real-time polymerase chain reaction (PCR) analysis with a StepOne Plus Real-Time PCR System (Applied Biosystems), Fast SYBR Green Master Mix Reagent (Thermo Fisher Scientific), and specific primers (sequences available on request). The amounts of target mRNAs were normalized by the corresponding amount of 36B4 mRNA.

Statistical analysis

All quantitative data are presented as means ± SE and were analyzed with the Tukey's test. A P value of <0.05 was considered statistically significant.

RESULTS

Effects of D-47 on serum lipid and insulin as well as blood glucose levels

Administration of D-47 or bezafibrate for 14 days resulted in a significant lowering of serum triglyceride levels by similar extents in db/db mice which genetically lack leptin receptor and develop obesity, hyperglycemia and dyslipidemia (Fig. 1A). The serum concentrations of total cholesterol and free fatty acids (FFAs) were not significantly altered by the administration of either agent, whereas FFAs was tended to be lowered by bezafibrate. Previous studies found that fibrates reduced blood glucose levels in obese diabetic animals, possibly as a result of

the amelioration of lipotoxicity (8, 9). Both D-47 and bezafibrate lowered blood glucose levels of db/db mice (Fig. 1B), whereas serum insulin levels were not significantly altered by either drug.

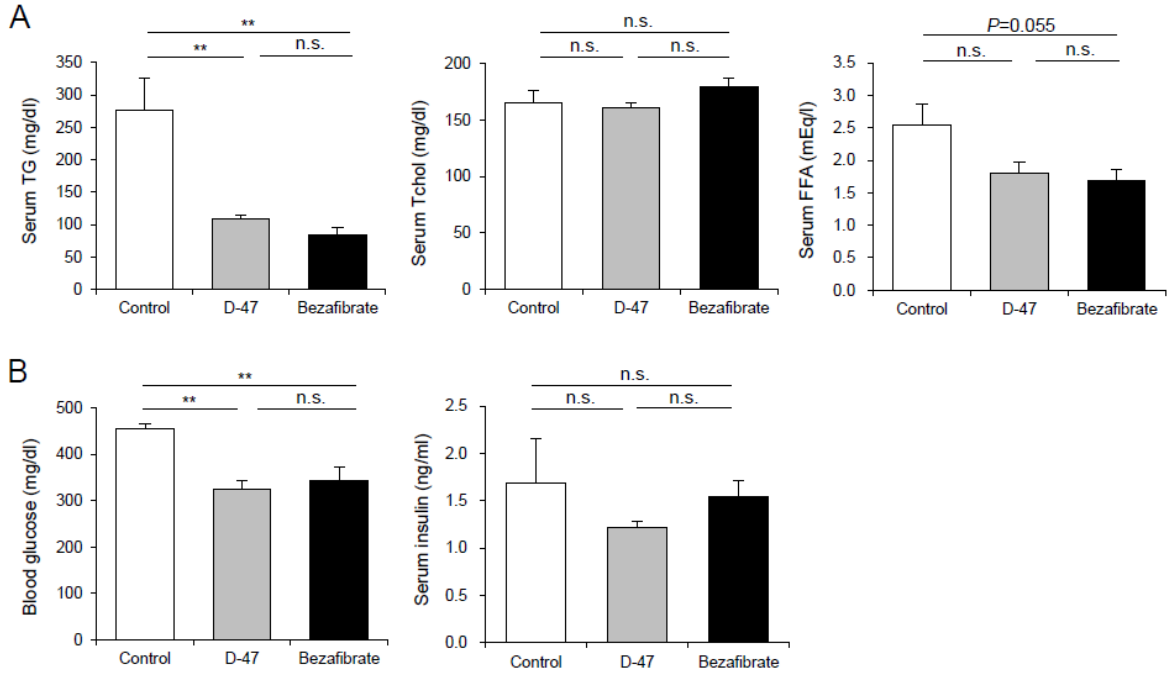

Fig. 1. Effects of D-47 on serum lipid, blood glucose, and serum insulin levels in db/db mice.
Six-week-old male db/db mice were left untreated (control) or treated with D-47 or bezafibrate for 14 days, after which serum triglyceride (TG), total cholesterol (Tchol), and FFA concentrations (A) as well as blood glucose and serum insulin levels (B) were measured. Data are means ± SE (n = 8 mice per group). *P < 0.05, **P < 0.01 (Tukey's test); n.s., not significant.

Effects of D-47 on hepatic steatosis, liver mass, and fat mass

Fat accumulation in hepatocytes as assessed by histological analysis was markedly diminished (Fig. 2A) and hepatic triglyceride content was significantly reduced (Fig. 2B) in db/db mice treated with D-47 compared with those in the control group. Although fat droplets in hepatocytes tended to be smaller in mice treated with bezafibrate than in control animals (Fig. 2A), hepatic triglyceride content was not affected by this drug (Fig. 2B). Liver mass was significantly increased in mice treated with bezafibrate (Fig. 2C), consistent with previous findings (10, 11), as well as in those treated with D-47 (Fig. 2C). However, administration of D-47 induced a significant reduction in the mass of epididymal adipose tissue, whereas bezafibrate had no such effect (Fig. 2D). Body mass was similar in the three groups of mice both before (control, 31.6 ± 0.3 g; D-47, 31.4 ± 0.3 g; bezafibrate, 31.1 ± 0.7 g) and after (control, 38.9 ± 0.6 g; D-47, 38.7 ± 0.6 g; bezafibrate, 40.4 ± 0.9 g) the 14-day period of drug administration.

Effects of D-47 on gene expression in the liver

We next examined the effects of D-47 and bezafibrate on gene expression in the liver. Expression of the gene for peroxisome proliferator–activated receptor α (PPARα) showed a tendency to be increased by the administration of D-47, whereas it was not affected by bezafibrate (Fig. 2E). Genes regulated by PPARα including those for medium-chain acyl-CoA dehydrogenase (MCAD), and lipoprotein lipase (LPL) were up-regulated by D-47 or bezafibrate (Fig. 2E). However, expression of the gene for carnitine palmitoyltransferase 1a (CPT1a), which is also regulated by PPARα, was not affected by either agent. The expression of genes related to fatty acid synthesis including those for PPARγ, fatty acid synthase (FAS), and acetyl-CoA carboxylase 1 (ACC1) was up-regulated by the administration of bezafibrate, consistent with previous observations (11, 12). D-47 also increased the expression of genes related to fatty acid synthesis including those for sterol response element binding protein 1c (SREBP1c), FAS, and ACC1. These results thus suggested that D-47 and bezafibrate exerted similar effects on the expression of genes related to lipid metabolism in the liver.

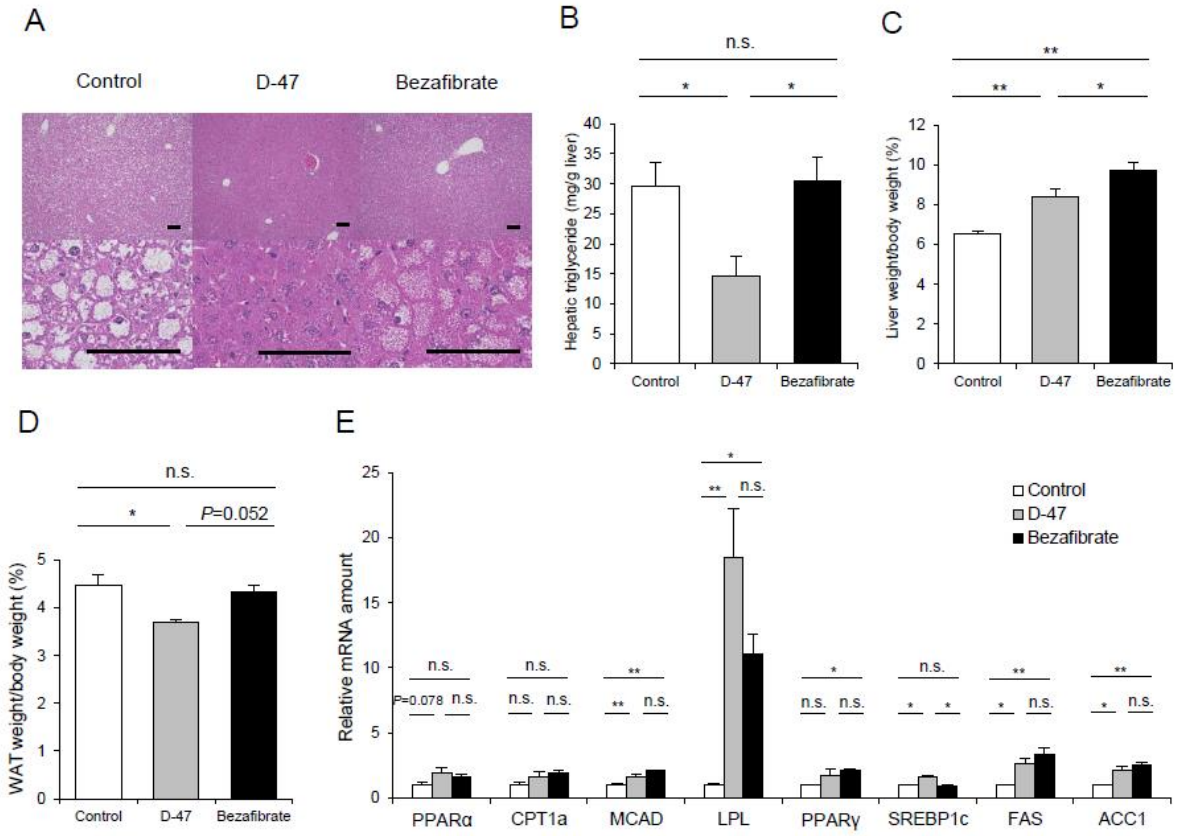

Fig. 2. Effects of D-47 on hepatic steatosis and gene expressions.
Six-week-old male db/db mice were left untreated (control) or treated with D-47 or bezafibrate for 14 days, after which sections of the liver were stained with hematoxylin-eosin (A) and the hepatic triglyceride content (B) as well as the ratio of liver weight to body weight (C) and the ratio of epididymal white adipose tissue (WAT) weight to body weight (D) were determined. Scale bars in (A), 100 μm. Quantitative data are means ± SE (n = 4 mice per group). (E) The abundance of the indicated mRNAs in the liver was determined by RT and real-time PCR analysis. Data are expressed relative to the value for the control group and are means ± SE (n = 4 mice per group). *P < 0.05, **P < 0.01 (Tukey's test); n.s., not significant.

Effects of D-47 on gene expression in adipose tissue

Given that the changes in gene expression in the liver appeared not to explain the differential effects of bezafibrate and D-47 on hepatic steatosis and that treatment with D-47 reduced adipose tissue mass, we investigated the effects of the two drugs on the expression of genes related to energy metabolism in adipose tissue of db/db mice. Unexpectedly, administration of D-47 markedly increased expression of the gene for uncoupling protein 1 (UCP1), a key regulator of heat production and a marker for brown or beige adipocytes (13, 14), in epididymal adipose tissue, a representative white adipose tissue (Fig. 3). Treatment with D-47 also up-regulated expression of the gene for the lipid droplet binding protein Cidea, another marker for brown or beige adipocytes (15). Expression of the genes for the transcriptional coactivator PGC1α (PPARγ coactivator 1α) and the transcription factor PRDM16 (PRD1-BF1-RIZ1 homologous domain–containing 16), both of which play important roles in the function of brown adipose tissue (16–18) were tended to be up-regulated by administration of D-47. Brown/beige adipocytes are rich in mitochondria (13), and the transcription factor ERRα (estrogen-related receptor α) contributes to mitochondrial biogenesis (19). Expression of the gene for ERRα as well as that of those for the mitochondrial proteins cytochrome c and cytochrome c oxidase subunit II (COXII) were significantly increased by D-47 treatment. Expression of the gene for glucose transporter 4 (GLUT4), which is regulated by PGC1α (20), was also significantly up-regulated by D-47. Expression of the gene for PPARα, that of PPARα target genes including those for CPT1b and MCAD which are involved in fatty acid oxidation, and that of lipolysis-related genes including those for adipocyte triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) were increased by the administration of D-47 (Fig. 3). However, administration of bezafibrate did not affect the expression of any of these genes in adipose tissue (Fig. 3).

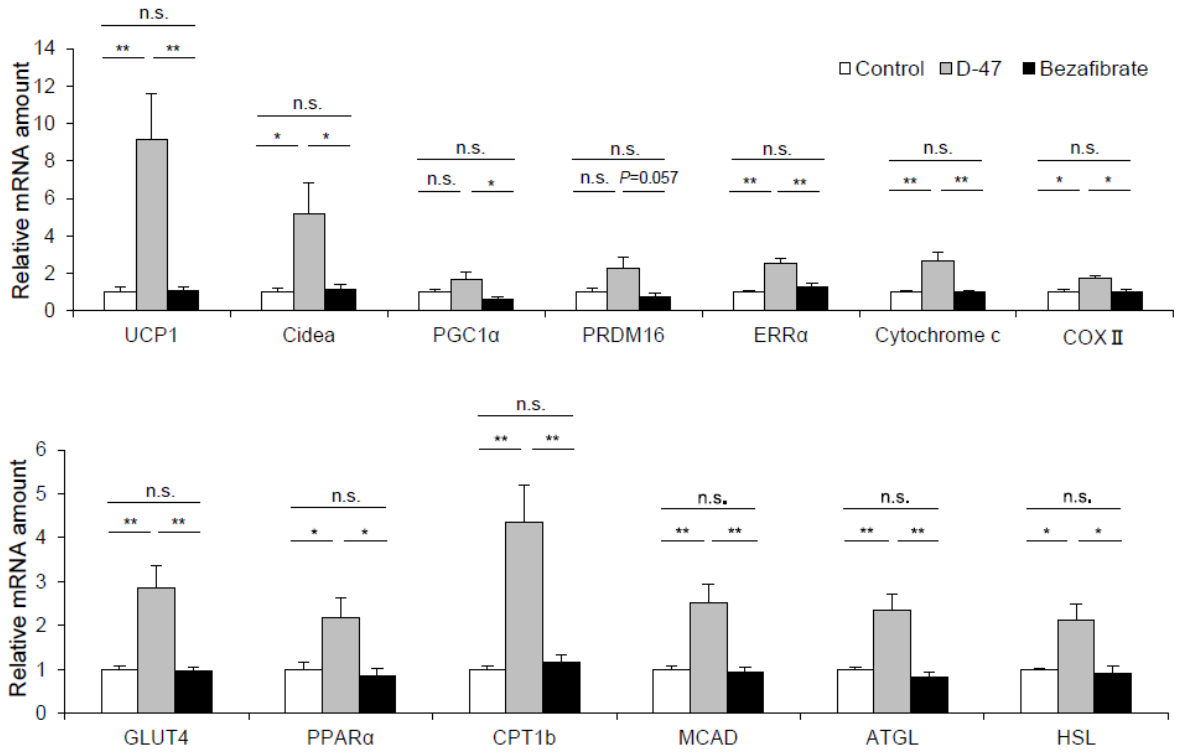

Fig. 3. Effects of D-47 on gene expression in adipose tissue.
(A) Six-week-old male db/db mice were left untreated (control) or treated with D-47 or bezafibrate for 14 days, after which the abundance of the indicated mRNAs in epididymal adipose tissue was determined by RT and real-time PCR analysis. Data are expressed relative to the value for the control group and are means ± SE (n = 4 mice per group). *P < 0.05, **P < 0.01 (Tukey's test); n.s., not significant.

Effects of D-47 on gene expression in cultured 3T3-L1 adipocytes

To investigate whether the induction of genes related to brown/beige adipocytes by D-47 are triggered by its direct effects on adipocytes, we examined the effects of the drug on the expression of the genes in cultured 3T3-L1 adipocytes. We treated mature 3T3-L1 adipocytes with D-47 at the concentrations of 50μM or 500μM for 48 h; the latter concentration is equivalent to the serum concentration assumed in mice fed a diet supplemented with 0.4% D-47 (w/w) (5). D-47 at the concentration of 500μM significantly increased the expression of the genes for cytochrome c, GLUT4, PPARα, CPT1b, MCAD, ATGL and HSL (Fig. 4) in 3T3-L1 adipocytes. The gene for UCP-1 was tended to be upregulated by this treatment, but the induction did not reach to the statistically significant (P=0.050). Among the genes upregulated by the D-47 treatment in adipose tissues of db/db mice, Cidea, ERRα and COXII were not induced by D-47 in 3T3L1 adipocytes (Fig. 4). These results suggest that the induction of genes related to brown/beige adipocytes by D-47 in db/db mice was at least partly attributable to the direct effect of the drug to white adipose tissue whereas the change in some genes likely occurred through an indirect effect.

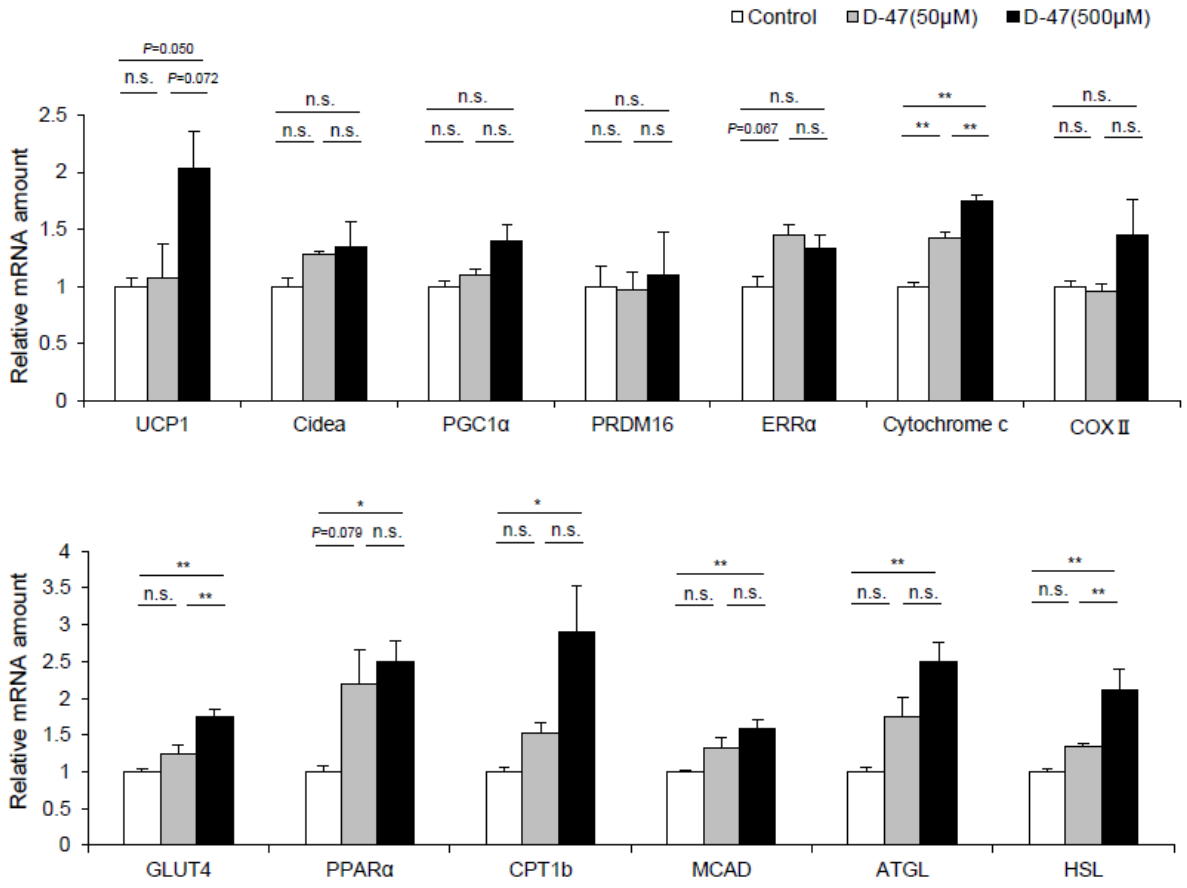

Fig. 4. Effects of D-47 on gene expression in cultured 3T3-L1 adipocytes.
3T3-L1 adipocytes were incubated with D-47 at 50μM or 500μM for 48 h at day 8 after the induction of differentiation. The abundance of the indicated mRNAs was determined by RT and real-time PCR analysis. Data are expressed relative to the value for the control group and are means ± SE (n = 4 per group). *P < 0.05, **P < 0.01 (Tukey's test); n.s., not significant.

DISUCUSSION

We have shown here that D-47 not only lowered the serum level of triglyceride but markedly ameliorated hepatic steatosis in db/db mice. D-47 thus exerted beneficial effects on both hypertriglyceridemia and hepatic steatosis in an animal model of obesity.

Although previous studies have shown that fibrates attenuated hepatic steatosis in rodent models of obesity (10, 11, 21), bezafibrate did not reduce the hepatic content of triglyceride in the present study. The difference between these previous and our present findings may be attributable to differences in experimental conditions: in a previous study (10), the period of bezafibrate administration was 6-8 weeks, although it was 2 weeks in ours. In other two studies (11, 21), the used model mice (diet-induced obese mice and TallyHo mice) were different from db/db mice. Our histological analysis revealed that the size of fat droplets in hepatocytes was reduced by treatment with bezafibrate, however, suggesting that this drug may influence some aspect of fat accumulation in hepatocytes but that this effect does not result in a reduction in the overall triglyceride content of the liver.

The triglyceride-lowering action of fibrates is thought to be mediated via stimulation of the PPARα pathway and subsequent up-regulation of fatty acid oxidation in the liver (22, 23). Given that treatment with D-47 induced changes in hepatic gene expression similar to those elicited by bezafibrate, the triglyceride-lowering effect of D-47 may also be achieved through stimulation of the PPARα pathway. The expression of genes related to fatty acid synthesis was also up-regulated in the liver of db/db mice treated with bezafibrate. Although the mechanism of this paradoxical phenomenon remains unclear, similar effects of fibrates have been described previously (11, 12). D-47 had similar effects on the expression of genes related to fatty acid synthesis in the liver of db/db mice.

In contrast to their similar effects on gene expression in the liver, the effects of D-47 and bezafibrate on the expression of genes in adipose tissue differed markedly. Whereas D-47 increased the expression of various genes

related to energy metabolism, bezafibrate had no effect on the expression of such genes. Adipocytes have been classically categorized into two types: white and brown. White adipocytes store energy in the form of triacylglycerol, whereas brown adipocytes consume stored energy for heat production in a cold environment. In addition, recent evidence has shown that white adipocytes can transform into brown-like, or beige, adipocytes under certain conditions (24). Increase in energy consumption by brown and beige adipocytes are thought to ameliorate obesity and metabolic disease. Evidence suggests that impairment of the transformation of white adipocytes into brown/beige cells appears to be related to the pathogenesis of hepatic steatosis (25), and, vice versa, the stimulation of the transformation of white adipocytes into brown/beige cells ameliorates hepatic steatosis (26). Given that D-47 up-regulated the expression of marker genes for brown or beige adipocytes, the beneficial effect of D-47 on hepatic steatosis might be achieved through promotion of such transformation.

In conclusion, we have shown that D-47 had beneficial effects on hypertriglyceridemia and hepatic steatosis in a mouse model of obesity, and that the latter effect might be mediated through an action in adipose tissue. Further studies with different models and treatment protocols should provide further insight into the pharmacological properties of this novel compound.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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