Antidiabetic and Adipogenic Properties in a Newly Synthesized Thiazolidine Derivative, FPFS-410

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We report here a newly synthesized cyanoimino-oxothiazolidine derivative, FPFS-410, which has properties to ameliorate both hyperglycemia and dyslipidemia. Treatment of genetically obese-diabetic *db/db* mice with FPFS-410 markedly ameliorates severe hyperglycemia and hypertriglyceridemia. Although the oxothiazolidine ring of FPFS-410 shares a structural similarity with other thiazolidinedione derivatives, reporter assays showed that FPFS-410 was much less potent to activate peroxisome proliferators-activated receptor γ (PPARγ) as compared with pioglitazone. When 3T3-L1 preadipocytes were treated with FPFS-410, intracellular accumulation of lipids was facilitated in a similar fashion to pioglitazone. Moreover, treatment with FPFS-410 throughout the differentiation course resulted in a significant increase in glucose transport. These results suggest that FPFS-410 may provide a useful therapeutic candidate for diabetes mellitus and dyslipidemia.

ADIPOSE TISSUE plays a pivotal role in systemic energy homeostasis not only by lipid accumulation, but also by releasing humoral bioactive substances, such as nonesterified fatty acid, leptin,[1] tumor necrosis factor-α (TNF-α),[2] and adiponectin.[3] Peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor highly expressed in adipocytes, plays a critical role in adipocyte differentiation through regulating a line of adipocyte genes.[4] Thiazolidinedione class of drugs (TZDs) have proven to be efficacious for the treatment of diabetes and insulin resistance.[5] It is generally believed that the activation of PPARγ is linked to the function of TZDs.[6] Although TZDs are widely used in clinical practice, in some cases, TZDs cause weight gain, plasma volume expansion, edema or hepatotoxicity.[7,8] These unwelcome effects of TZDs are also supposed to be due to activation of PPARγ.

Recently, some compounds, which can ameliorate fuel dyshomeostasis with less affinity for PPARγ, have been reported.[9-11] For example, the affinity for PPARγ of CLX-0921, a TZD analogue, was 6.5-fold less than that of rosiglitazone.[9] FMOC-L-leucine, a non-TZD class compound, has 400-fold less affinity for PPARγ than rosiglitazone.[10] These compounds are capable of ameliorating hyperglycemia in diabetic rodent models with a similar potency to rosiglitazone.[9-11] Noteworthy is that these compounds do not cause body weight gain in rodents.

Here we report a novel thiazolidine derivative, FPFS-410, which shows strong antidiabetic and antidyslipidemic properties. Treatment of genetically obese-diabetic *db/db* mice with FPFS-410 markedly ameliorates hyperglycemia and dyslipidemia. Despite strong structural similarities to pioglitazone, FPFS-410 has much less potency for PPARγ activation compared with pioglitazone. With a similar potency to pioglitazone, FPFS-410 enhances adipogenesis in 3T3-L1 preadipocytes. Treatment of 3T3-L1 cells with FPFS-410 resulted in a significant increase in 2-deoxy-D-glucose (2-DG) transport. These results raise the possibility that FPFS-410 is a potent therapeutic agent for diabetes mellitus and dyslipidemia.

MATERIALS AND METHODS

Materials

FPFS-410[12,13] and pioglitazone (AD-4833) were provided by Fujimoto Pharmaceutical (Osaka, Japan) and Takeda Chemical (Osaka, Japan), respectively. Insulin (INS) was purchased from Boehringer Mannheim (Mannheim, Germany). 3-Isobutyl-1-methylxanthine (IB) and dexamethasone (DX) were purchased from Nacalai Tesque (Kyoto, Japan). Polyclonal antibodies recognizing CCAAT/enhancer binding protein (C/EBP) β, C/EBPδ and PPARγ, and the horseradish peroxidase (HRP)-conjugated antigoat IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated antirabbit IgG antibody and ECL plus Western detecting kit were purchased from Amersham Bioscience (Piscataway, NJ). Dulbecco's modified Eagle medium (DMEM), calf serum (CS), and fetal bovine serum (FBS) were acquired from Invitrogen (Carlsbad, CA).

Animals

Animals were housed in temperature-controlled animal facility with 12-hour light and dark cycle under standard diet (MF, Oriental Yeast, Tokyo, Japan). Male C57BL/KsJ-*db/db* mice were obtained from CLEA Japan (Tokyo, Japan) at 7 weeks of age. Mice were divided into 3 groups matched for their fasting plasma glucose levels at 9 weeks of age. Then, animals were treated with 75 mg/kg FPFS-410, 10 mg/kg pioglitazone, or vehicle (3% Arabic gum solution) orally once daily by gavage for 14 days. Blood samples in the fasted state were collected at 24 hours after the final dosing. For measurement of plasma glucose, blood samples were collected from tail vein in heparin treated hematocrit-capillary and transferred to plastic tubes. Plasma was separated by centrifuge. For measurement of serum triglyceride, blood samples were collected from the femoral artery in plastic tubes. Serum was separated by centrifuge. Plasma glucose and serum triglyceride levels were measured using N-test Glu-T (Nittobo, Fukushima, Japan) and L type Wako TG H (Wako, Osaka, Japan), respectively. All animal experi-

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ments were in accordance with the Institutional Guideline of Care and Use of Laboratory Animals and approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Plasmids

The full-length mouse PPARγ cDNA coding sequence was isolated from mouse epididymal adipose tissue total RNA by reverse transcription-polymerase chain reaction. PPARγ expression plasmid was constructed by inserting PPARγ cDNA into pCMX eukaryotic vector[14] (kindly provided by Dr K. Umesono). The reporter plasmid contains triplet repeat of PPAR response element located in the rat acyl-CoA oxidase promoter.

Adipocyte Differentiation and Oil Red O Staining

3T3-L1 cells were cultured and differentiated as described previously.[15] Briefly, 3T3-L1 preadipocytes were grown in a 12-well plate until confluent and maintained another 2 days (defined as day 0). Three agents required for adipogenesis (ie, IB, DX, and INS) were added alone, in combination of 2, or complete set. FPFS-410 and pioglitazone were dissolved in dimethyl sulfoxide followed by dilution into culture medium at indicated period. Culture medium was changed every other day. At day 8, cells were washed with phosphate-buffered saline (PBS) twice, fixed in 3.7% formaldehyde for 1 hour, and then stained with 0.6% (wt/vol) Oil Red O solution (60% isopropanol, 40% water) for 2 hours at room temperature. Cells were then washed with water to remove unbound dye. Oil Red O was eluted with isopropanol and quantified by measuring the optical absorbance at 510 nm.[16]

Transfection

CV-1 cells were maintained in DMEM supplemented with 10% CS, at 37 °C in 10% CO_2. Cells were plated to 50% to 80% confluence 1 day prior to transfection in DMEM supplemented with 10% dialyzed FBS following transfection via lipofection using DOTAP reagent (Roche Diagnostics, Tokyo, Japan) according to manufacturer's instruction. After 2 hours, medium containing liposomes was removed and added fresh one containing vehicle or drug at indicated concentrations for 40 hours. Cells were harvested using Reporter Lysis Buffer (Promega, Tokyo, Japan) and assayed for luciferase and β-galactosidase activity.

Preparation of Total Cell Lysates and Immunoblot Assays

Total cell lysates were prepared from pair cultured cells in 10-cm plates at day 2. One pair was provided for protein quantification by the Bradford method after washing with PBS (pH 7.4), disrupting with 1 mL of 1 mol/L NaOH and neutralizing with 1mL of 1 mol/L HCl. The other pair was prepared for subject by disrupting in Laemmli's sample buffer in 5 μg/mL after washing cells with PBS twice. All samples were then boiled for 10 minutes. For immunoblotting, 50 μg of lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to polyvinylidene fluoride membrane (NEN Life Science Products, Boston, MA). Membranes were blocked with Block Ace (Yukijirushi Nyugyo, Sapporo, Japan) and then incubated with the appropriate antibody, washed, reacted with antirabbit or antigoat IgG coupled to HRP, and developed with ECL plus as instructed by the manufacturer. The signals on the blot were quantified by densitometry.

2-DG Transport Assay

3T3-L1 cells were treated with or without FPFS-410 or pioglitazone throughout the course of differentiation. Glucose transport by monolayers of 3T3-Ll adipocytes was determined by measuring the uptake of 2-deoxy-D-[3H]glucose (100 μmol/L) as described previously.[17]

Statistical Analysis

The data are presented as means ± SEM. Dunnett's multiple comparison test or Student's *t* test was used to compare the data between the control and treated groups. Differences were accepted as significant at $P < .05$ level.

RESULTS

FPFS-410 Treatment Drastically Ameliorates Hyperglycemia and Dyslipidemia in Genetically Obese-Diabetic db/db Mice

A series of 2-(N-cyanoimino)-4-oxothiazolidine derivatives was designed and synthesized at Fujimoto Pharmaceutical Corporation.[12,13] One of these structurally novel compounds, FPFS-410 (Fig 1A) was selected for experimental use based on pharmacologic and preliminary toxicologic studies. Oxothiazolidine ring of FPFS-410 shares a structural similarity with TZDs, such as pioglitazone (Fig 1A). To explore a potential effect of FPFS-410 on metabolic disorders, such as diabetes or dyslipidemia, genetically diabetic *db/db* mice were treated with the compound. Oral administration with 75 mg/kg of FPFS-410 for 2 weeks significantly reduced plasma glucose levels in the fasting state comparable to the administration with 10 mg/kg of pioglitazone (Fig 1B). Moreover, compared with the vehicle group, serum triglyceride levels significantly decreased by 63% with FPFS-410 treatment, but not with pioglitazone (Fig 1B). Body weight gain during the experimental period in pioglitazone-treated *db/db* mice was significantly higher than that in the vehicle-treated *db/db* mice (8.1 g ± 0.2 g *v* 4.6 g ± 0.4 g, $P < .001$). On the other hand, body weight gain of FPFS-410-treated mice moderately increased during the 2-week administration (5.6 g ± 0.4 g).

FPFS-410 Has Subtle Potency for PPARγ Activation

We assessed the potency of FPFS-410 for PPARγ activation using transiently transfected CV-1 cells. Although addition of 1 μmol/L pioglitazone resulted in the maximal activation of PPARγ, treatment with 30 μmol/L FPFS-410 exerted the activation less than treatment with 0.1 μmol/L pioglitazone (Fig 1C). A higher concentration of FPFS-410 (>30 μmol/L) could not be examined because of its solubility limit in aqueous solution.

FPFS-410 Facilitates Adipogenesis

3T3-L1 preadipocytes require hormonal stimulation by inducers, such as IB, DX, and INS to differentiate into adipocytes. To elucidate the condition in which FPFS-410 affects 3T3-L1 differentiation, FPFS-410 was added to differentiation medium in a defined period (2 days) together with the designated inducers (none, alone, combination of 2 inducers or complete set of inducers). The addition of FPFS-410 in any stages without inducers or with any single inducer did not change the morphology of 3T3-L1 preadipocytes (data not shown). Figure 2A shows a microscopic view of cells stained with Oil red O at day 8. Without addition of FPFS-410 or pioglitazone (control group), any combination of 2 inducers led only a small part of 3T3-L1 preadipocytes into adipocytes, however, addition of FPFS-410 or pioglitazone from day −2 to day 0 (pretreatment group) followed by supplement of any combination of 2 inducers enabled cells to facilitate lipid accumulation (Fig 2A). Figure 2B shows the amounts of lipids

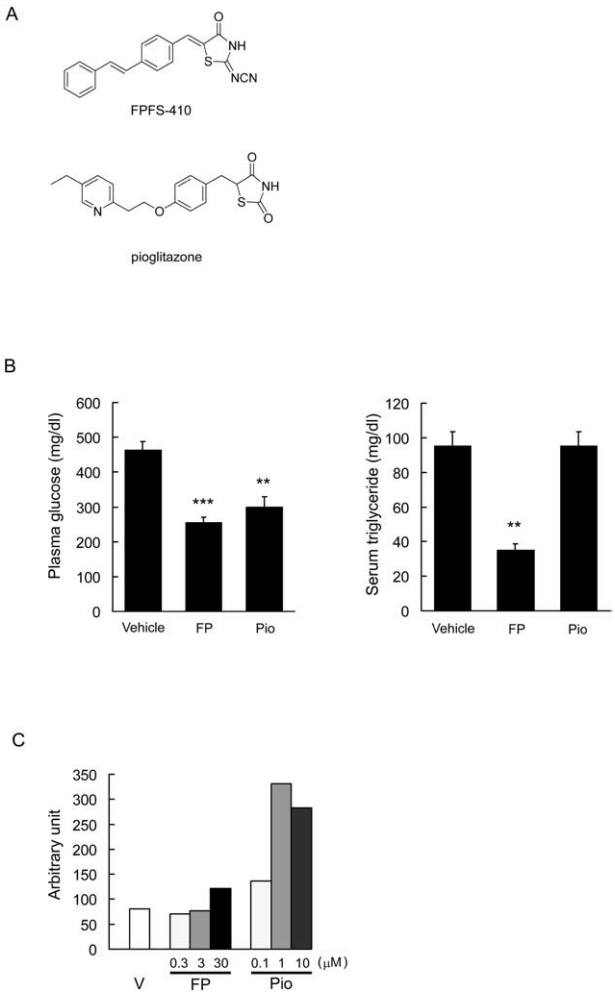

Fig 1. (A) Comparison of chemical structure between FPFS-410 and pioglitazone. (B) In vivo effects of FPFS-410 in *db/db* mice. Male *db/db* mice at 9 weeks of age were divided into 3 groups matched for their plasma glucose levels. FPFS-410 or pioglitazone was suspended in 3% Arabic gum solution and administered orally for 14 days. Twenty-four hours after final dosing, levels of plasma glucose and serum triglyceride were measured. Each group was composed of 8 to 10 mice. Data are presented as mean ± SEM. **$P < .01$, ***$P < .001$ (Dunnett's multiple comparison test) compared with vehicle-treated group. V, vehicle; FP, FPFS-410; Pio, pioglitazone. (C) The effect of FPFS-410 on PPARγ activation in luciferase assay. Expression vector carrying PPARγ, reporter gene and β-galactosidase control gene were cotransfected into CV-1 cells. Luciferase activity was justified by transfection efficacy measured by galactosidase activity. V, vehicle; FP, FPFS-410; Pio, pioglitazone.

measured by eluted Oil red O. When cells were differentiated with the combination of 2 inducers in the control group, lipid accumulation was observed about 20% of differentiation with complete set of inducers (IB, DX, and INS). In the pretreatment (FPFS-410 or pioglitazone) group, however, cells that were differentiated with 2 inducers showed lipid accumulation about 40% of those with the complete set. Simultaneous addition of FPFS-410 or pioglitazone and 2 inducers from day 0 to day 2 also facilitated adipogenesis, especially the combination of DX + INS. In this case (treatment with FPFS-410 or pioglitazone and combination of DX + INS), lipid accumulation reached 80% of that treated with complete set of inducers. Treatment with FPFS-410 or pioglitazone could not accelerate further accumulation of lipids when cells were differentiated with the complete set of inducers (Fig 2B). Addition of compounds after day 2 in the course of differentiation did not facilitate lipid accumulation compared with vehicle control (data not shown).

Effects on Expression of Transcription Factors in 3T3-L1 Preadipocytes

Because FPFS-410 was shown to enhance adipogenesis when cells were treated in an early stage of the differentiation course, the effect of simultaneous addition of FPFS-410 with 2 inducers on the expression of transcription factors was examined (Fig 2C). The stimulation with IB + DX is sufficient to induce C/EBPβ, C/EBPδ and PPARγ, (Fig 2C, left panels). The treatment with FPFS-410 from day 0 to day 2 in IB + DX condition increased the expression of C/EBPβ and C/EBPδ, but the expression of PPARγ did not alter compared with vehicle treatment. When FPFS-410 was added simultaneously with DX + INS, expression of C/EBPβ, C/EBPδ, and PPARγ was increased (Fig 2C, left panels). As the expression of PPARγ is induced by C/EBPβ and C/EBPδ in the course of adipose differentiation, increased expression of PPARγ that is induced by FPFS-410 might be an indirect effect. We also examined the effect of pioglitazone on adipogenesis in 3T3-L1 cells in various stages stimulated with IB + DX or DX + INS. When cells were stimulated with combinations of 2 inducers, the addition of pioglitazone from day −2 to day 0 was the most effective in lipid accumulation (Fig 2B). Based on this result, the effect of pioglitazone treatment from day −2 to day 0 on expression of transcription factors at day 2 was examined (Fig 2C, right panels). Increased expression of PPARγ occurred in pioglitazone-treated cells in IB + DX condition. When pioglitazone was added in DX + INS condition, expression of C/EBPβ, C/EBPδ, and PPARγ were increased (Fig 2C, right panels).

Treatment With FPFS-410 Increases 2-DG Uptake in 3T3-L1 Adipocytes

To examine the effect of FPFS-410 on glucose transport in 3T3-L1 cells, 2-DG uptake assay was performed when cells were treated with FPFS-410 in the course of differentiation (Fig 3). Although treatment with FPFS-410 increased 2-DG uptake (2.3-fold to 2.5-fold and 1.4-fold to 1.6-fold in the absence and presence of insulin, respectively) to a smaller extent than treatment with pioglitazone (7.6-fold to 8.0-fold and 2.4-fold to 3.8-fold in the absence and presence of insulin, respectively), the increase treated with the higher dose (10 μmol/L) of FPFS-410 was significant (Fig 3).

DISCUSSION

We here reported that a newly synthesized cyanoimino-oxothiazolidine derivative, FPFS-410, had strong antidiabetic and lipid-lowering properties. Administration with 75 mg/kg FPFS-410 orally to *db/db* mice reduced plasma glucose level comparable to the administration with 10 mg/kg pioglitazone. Of note, serum triglyceride levels were also markedly reduced in mice by treatment with FPFS-410, but not in mice treated with pioglitazone.

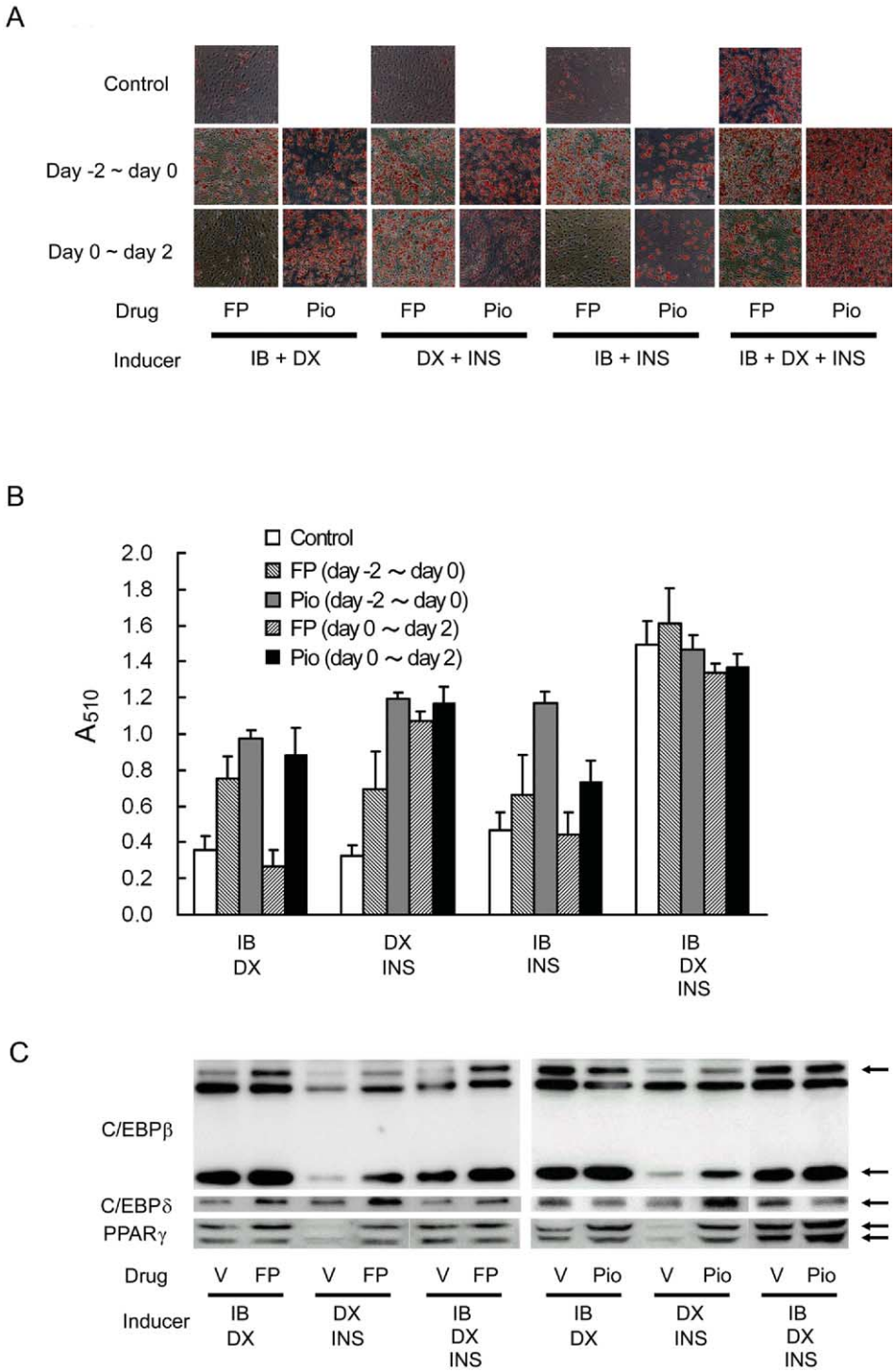

Fig 2. (A) The effect of FPFS-410 on adipogenesis. FPFS-410 (30 μmol/L) was added to post-confluent 3T3-L1 cells for 2 days at indicated periods with stimulation by inducers indicated. All cells were fixed and stained with Oil red O at day 8. (B) Oil red O binding to lipid was extracted with 2-propanol followed by quantification by measuring absorbance at 510 nm. Data are presented as mean ± SEM. (C) The effects of FPFS-410 on the expression of transcription factors regulating adipogenesis in 3T3-L1 preadipocytes. 3T3-L1 cells were treated with vehicle or 30 μmol/L FPFS-410 from day 0 to day 2 (left panels) or with vehicle or 100 μmol/L pioglitazone from day −2 to day 0 (right panels) in IB + DX, DX + INS or IB + DX + INS conditions. Cells were harvested at day 2 and subjected to SDS-PAGE followed by immunoblot to analyze the expression levels of transcription factors. V, vehicle; FP, FPFS-410; Pio, pioglitazone.

Although the administration with 10 mg/kg pioglitazone should decrease serum triglyceride levels of mice when fed ad libitum, it is possible that overnight fasting would minimize the effect of pioglitazone. TZDs sometimes cause unwelcome effects, such as edema, weight gain, and hepatotoxicity, resulting in discontinuance of administration.[18] Consistent with this notion, body weight in pioglitazone-treated *db/db* mice was further elevated in our experiments, while body weight gain in FPFS-410-treated *db/db* mice was marginal. Although further studies using normal weight rodents and various kind of genetically-obese models are required to test the hypothesis, it is tempting to speculate that FPFS-410 could be a useful therapeutic alternative for diabetic and dyslipidemic subjects in which such adverse effects occur by already-released TZDs.[18]

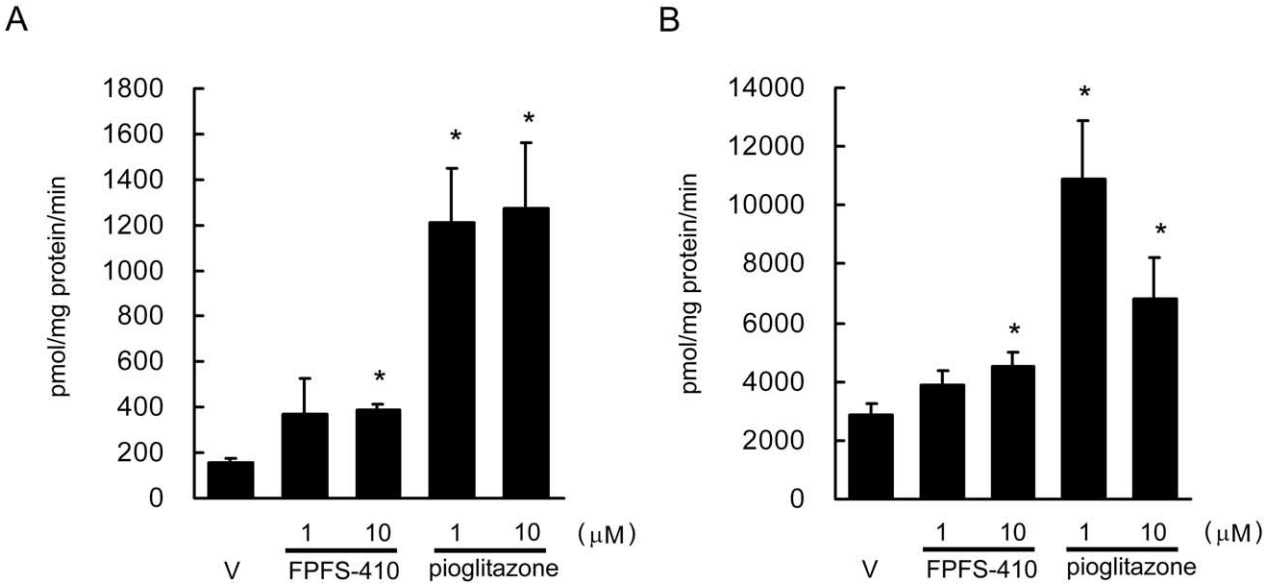

Fig 3. The treatment with FPFS-410 increases glucose transport of 3T3-L1 cells. 3T3-L1 cells were treated with compounds throughout the course of differentiation (ie, day 0 ~ day 8). 2-DG uptake was measured as described in Materials and Methods in the absence (A) or presence (B) of 1 μmol/L insulin. Data are presented as mean ± SEM of quadruplicate experiments. *P < .05 (Student's *t* test) compared with vehicle-treated group. V, vehicle; FP, FPFS-410; Pio, pioglitazone.

In the present study, we demonstrated that FPFS-410 enhanced adipogenesis in 3T3-L1 preadipocytes. Because the potency for PPARγ activation and glucose-lowering effect are correlated,[19] it is considered that the antidiabetic effect of TZDs is largely attributed to PPARγ activation. However, recently reported PPARγ agonists with antidiabetic potency are not necessarily subject to this correlation.[9-11] It is possible that differences in such ligand-mediated activities are determined by recruited coactivators.[9,10] Although reporter assays suggest that FPFS-410 is less potent to activate PPARγ, this is not enough to rule out the possibility that FPFS-410 has a potential to recruit PPARγ cofactors.

In the course of adipocyte differentiation, expression levels of C/EBPβ and C/EBPδ are increased immediately after hormonal stimulation, resulting in upregulation of C/EBPα and PPARγ and subsequent adipogenic gene expressions.[20] We thus evaluated the potential role of FPFS-410 on C/EBPβ and C/EBPδ protein expressions. Noteworthy is the finding that FPFS-410-induced expression of C/EBPβ and C/EBPδ precedes PPARγ expression, suggesting that mechanism whereby FPFS-410 induces adipogenesis is, at least in part, independent of PPARγ activation. Nevertheless a substantial amount of expression of PPARγ was induced by cotreatment with FPFS-410, IB, and DX, cells failed to differentiate. This finding suggests that the combination treatment with FPFS-410 under IB and DX condition induces PPARγ, but the treatment does not fully activate PPARγ. Thus, the adipogenic effect of FPFS-410 may be attributed, at least in part, to the increased expression of C/EBPβ and C/EBPδ.

Fat pad weight in adult C/EBPβ and C/EBPδ double-deficient mice was significantly reduced compared with wild-type mice.[21] Yamamoto et al[22] demonstrated that insulin-responsive 2-DG-uptake was lower in adipocytes from C/EBPβ/δ double-deficient mouse as compared with wild-types. These results indicate that C/EBPβ and C/EBPδ play a role not only in adipogenesis, but also in developing insulin responsiveness, leading us to speculate that cells treated with FPFS-410 enhance insulin responsiveness. In the present study, we demonstrated that treatment of 3T3-L1 cells with FPFS-410 resulted in significant increase in glucose transport. Therefore, increased expression of C/EBPβ and C/EBPδ by FPFS-410 may contribute to improve systemic energy homeostasis via enhancing adipogenesis and insulin responsiveness in adipocytes.

Many investigators are intensively engaged in developing ligands for orphan nuclear receptors as therapeutic agents against metabolic disorders. Synthetic ligands for PPARα are clinically employed for the treatment of hyperlipidemia.[23] Recent works have demonstrated that activation of PPARα also ameliorates hyperglycemia and insulin resistance.[24,25] Synthetic ligands for PPARδ are effective for the treatment of metabolic syndrome in mice via enhancing fatty acid β-oxidation in skeletal muscle.[26] In this context, potential effects of FPFS-410 on other organs, such as skeletal muscle and liver, or on PPARα and PPARδ must await further investigation.

In summary, we report a newly synthesized oxothiazolidine-derivative, FPFS-410. With lesser potency in PPARγ activation, this compound can strongly ameliorate severe diabetes and dyslipidemia in *db/db* mice without increasing body weight. Our results show that FPFS-410 facilitate adipogenesis and, to some extent, glucose uptake in 3T3-L1 adipocytes. Thus, FPFS-410 may provide a useful therapeutic candidate for diabetes and dyslipidemia.

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