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Pharmacological characteristics of a novel nonthiazolidinedione insulin sensitizer, FK614

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Abstract

We evaluated antidiabetic effects of 3-(2,4-dichlorobenzyl)-2-methyl-*N*-(pentylsulfonyl)-3*H*-benzimidazole-5-carboxamide (FK614), a benzimidazole derivative without a thiazolidinedione structure, which was obtained using C57BL/KsJ-*db*/*db* mice (*db*/*db* mice). In *db*/*db* mice, the potency of FK614 for hypoglycemic effect was comparable to that of rosiglitazone and approximately 15-fold greater than that of pioglitazone. FK614 also showed a potent attenuating effect on hypertriglyceridemia in *db*/*db* mice, as well as rosiglitazone and pioglitazone. In C57BL/6J-*ob*/*ob* mice (*ob*/*ob* mice), ED_{50} values of FK614 and pioglitazone for hypoinsulinemic effect were 1.3 and 11.8 mg/kg, respectively. FK614 also improved the impaired glucose tolerance in *ob*/*ob* mice. In normal rats, FK614 did not influence plasma glucose and insulin levels but significantly decreased both plasma triglyceride and nonesterified fatty acid levels. FK614 was found to activate peroxisome proliferator-activated receptor (PPAR)γ-mediated transcriptional activity in the reporter gene assay as well as thiazolidinedione derivatives, although its maximum effect was less than that of thiazolidinedione derivatives. In rat toxicity studies, hemodilution effects for FK614 were less than that for rosiglitazone. Overall, these studies suggest that FK614 improves insulin resistance in such animal models through activation of PPARγ-mediated transcriptional activity and that it would be a new therapeutic candidate with potential for the treatment of type 2 diabetic patients.

Keywords: FK614; Nonthiazolidinedione; Insulin sensitizer; *db*/*db,* mouse; *ob*/*ob,* mouse; Peroxisome proliferator-activated receptor (PPAR)γ

1. Introduction

Insulin resistance underlies the pathogenesis of type 2 diabetes (DeFronzo, 1992). Also, the combination of insulin resistance and compensatory hyperinsulinemia appears to contribute to the development of many other disorders such as dyslipidemia, hypertension, and coronary heart disease (Reaven, 1995). Thus, amelioration of insulin resistance might be efficacious for the treatment of type 2 diabetes and the prevention of other complex diseases closely related to insulin resistance. Recently, several thiazolidinedione derivatives have been shown to improve glucose and lipid metabolism by ameliorating insulin resistance in various animal models of type 2 diabetes and in type 2 diabetic patients (Arakawa et al., 1998; Barman Balfour and Plosker, 1999; Buckingham et al., 1998; Fujiwara et al., 1988; Ikeda et al., 1990; Gillies and Dunn, 2000; Murakami et al., 1998; Plosker and Faulds, 1999; Reginato et al., 1998; Young et al., 1995). However, besides its therapeutic effects, side effects of thiazolidinedione derivatives, such as hemodilution, weight gain, and hepatic failure, have been reported clinically.

Thiazolidinedione derivatives, such as troglitazone, rosiglitazone, and pioglitazone are peroxisome proliferator-activated receptor (PPAR)γ agonists (Berger et al., 1996; Lehmann et al., 1995). PPARγ is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors, predominantly expressed in adipose tissues and

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regulating the expression of genes closely related to adipocyte differentiation, glucose, and lipid metabolism (Spiegelman, 1998). In addition, Willson et al. (1996) reported that there was a close relationship between in vitro PPARγ-mediated transcriptional activities and in vivo hypoglycemic effects of thiazolidinedione derivatives. Thus, it is thought that PPARγ is a predominant target molecule for thiazolidinedione derivatives to function as insulin-sensitizing agents. Recently, Brown et al. (1999) reported that a nonthiazolidinedione compound identified as a high-affinity ligand for PPARγ had antidiabetic effects in Zucker diabetic fatty rats. In addition, other nonthiazolidinedione insulin sensitizers were reported (Berger et al., 1999; Etgen et al., 2002; Ljung et al., 2002; Pill and Kühnle, 1999; Shibata et al., 1999; Ye et al., 2003), and most of these compounds also had the ability to activate PPARγ-mediated transcriptional activity.

We previously found a compound with a nonthiazolidinedione chemical structure, which slightly improved hyperglycemia in C57BL/KsJ-*db*/*db* mice (*db*/*db* mice), and then we evaluated antidiabetic activities of the related compounds using *db*/*db* mice. Eventually, we discovered a nonthiazolidinedione-type oral antidiabetic benzimidazole derivative, 3-(2,4-dichlorobenzyl)-2-methyl-*N*-(pentylsulfonyl)-3*H*-benzimidazole-5-carboxamide (FK614), which alleviates insulin resistance potently (Minoura et al., 2001; Uchino et al., 2001). Because FK614 was selected using *db*/*db* mice, it is interesting to learn its pharmacological and toxicological characteristics as well as its action mechanism in comparison with the existing thiazolidinedione agents. In this report, we have shown the pharmacological and toxicological characteristics of FK614 and its effects on PPARγ and α-mediated transcriptional activity.

2. Materials and methods

2.1. Compounds

FK614, rosiglitazone, pioglitazone, and troglitazone were synthesized at Daicel Chemical Industries or the Exploratory Research Laboratories of Fujisawa Pharmaceutical. The compounds were dissolved in dimethyl sulfoxide (DMSO) and added to medium to a final DMSO concentration of 0.1% for in vitro studies and were suspended in 0.5% methylcellulose solution for in vivo studies.

2.2. PPARs transactivation assay

2.2.1. Plasmids

Expression and reporter plasmids used in this study were all constructed at the Molecular Biological Research Laboratories of Fujisawa Pharmaceutical. Full-length cDNAs for mouse PPARγ2, mouse PPARα, human PPARγ1, and human PPARα were inserted into the mammalian expression vector pCDM8 (Invitrogen, Carlsbad, CA, USA) to generate pCDM8-mPPARγ2, pCDM8-mPPARα, pCDM8-hPPARγ1, and pCDM8-hPPARα, respectively. The reporter plasmid pGV-PPRE-Luc was constructed by inserting three copies of a 33-bp long PPAR-responsive element identical to that of rat acyl-CoA oxidase (Osumi et al., 1991; Kliewer et al., 1992) into the pGV-P2 vector (Wako, Osaka, Japan) containing firefly (*Photimus pyralis*) luciferase cDNA. As a control transfection plasmid, pRL-CMV (Promega, Madison, WI, USA) was used. It uses a sea pansy (*Renilla reniformis*) luciferase cDNA driven by the cytomegalovirus promoter.

2.2.2. Transient transfection and transcription assay

CV-1 cells, purchased from RIKEN Cell Bank (Tsukuba, Japan), were inoculated in 6-well plates (2×10^5 cells/well) and cultured for approximately 24 hours in Dulbecco's modified Eagle's essential medium (DMEM) supplemented with 10% delipidated and charcoal-treated fetal calf serum in 5% CO_2 at 37 °C. The cells were transfected with each PPAR expression plasmid, pGV-PPRE-Luc and pRL-CMV (each 0.34 μg in 1 ml/well) by lipofection, using Lipofectamine (Gibco BRL, Rockville, MD, USA). Cells without PPAR expression plasmid were treated with calf thymus DNA instead of each PPAR expression plasmid. After transfection, the cells were harvested and seeded into a 96-well plate (ViewPlate, Packard Instrument, Meriden, CT, USA) at a density of 1.8×10^4 cells/well in 50 μl of DMEM with 10% delipidated and charcoal-treated fetal calf serum. After the cells had adhered, 50 μl of DMEM with 10% delipidated and charcoal-treated fetal calf serum containing test compound was added. No drug control cells were added with DMSO concentration of 0.1% as final. After 2 days, cells were lysed and the luciferase activity was measured in a luminometer (MLX Microtiter Plate Luminometer, Dynatech Laboratories, VA, USA) using the Dual-Luciferase Reporter Assay System (Promeg, Madison, WI, USA).

2.2.3. Calculation of transcriptional activity

The firefly luciferase activity was divided by the sea pansy luciferase activity to adjust for transfection efficiency, and the value was defined as the normalized luciferase activity. As CV-1 cells are known to express PPARγ (Forman et al., 1995), PPAR-mediated transcriptional activity was calculated by subtracting the normalized luciferase activity without exogenous PPAR expression plasmid from that with exogenous PPAR expression plasmid. Eventually, fold increase was calculated as the ratio of PPAR-mediated transcriptional activity in cells treated with each drug to that in DMSO-treated control cells.

2.3. Animals and treatment

2.3.1. Animals

Female C57BL/KsJ-*db*/*db* mice (*db*/*db* mice) and C57BL/KsJ-+*m*/+*m* mice (lean mice) were purchased from CLEA Japan (Tokyo, Japan). Male C57BL/6J-*ob*/*ob* mice (*ob*/*ob* mice) and C57BL/6J-?/+ mice (lean mice) were purchased from Jackson Laboratories (Bar Harbor, ME,

USA). Sprague–Dawley rats were purchased from Charles River Japan (Yokohama, Japan) or CLEA Japan. Animals were maintained on standard laboratory chow and water ad libitum.

All experimental procedures using animals were performed under the guidelines of the Animal Experiment Committee of Fujisawa Pharmaceutical.

2.3.2. Drug administration and blood sampling

Drugs were administered orally once a day at a volume of 5 ml/kg of body weight for 14 days. Administration of the drug was started at 10, 9–10, and 6–7 weeks of age for *db*/*db* mice, *ob*/*ob* mice, and Sprague–Dawley rats, respectively. Blood sampling was performed on the day after the final treatment. Blood was taken from the retro-orbital sinus by capillary pipette under feeding condition for *db*/*db* mice or after overnight fasting for *ob*/*ob* mice. For the measurement of plasma glucose, insulin, and lipids in Sprague–Dawley rats, blood was taken from the abdominal aorta under anesthesia with pentobarbital Na (50 mg/kg, i.p. injection) under feeding condition (using heparin as an anticoagulant). To evaluate subacute toxicity of drugs in Sprague–Dawley rats, blood sample was collected from the abdominal aorta under light ether anesthesia for the measurement of hematological parameters (using EDTA-2K as an anticoagulant) and blood parameters, followed by autopsy and histopathological examination.

2.3.3. Glucose tolerance test

For the glucose tolerance test, *ob*/*ob* and lean mice received 2 g/kg glucose (5 ml/kg) orally, after overnight fasting, on the day after final drug treatment, and blood samples were taken before and 30, 60, and 120 min after glucose loading, as described above.

2.4. Analytical methods

Plasma glucose, triglyceride, and nonesterified fatty acid levels were determined by a mutarotase·glucose oxidase method or a glucokinase·glucose-6-phosphate dehydrogenase method, a glycerol-3-phosphate oxidase·3,5-dimethoxy-*N*-ethyl-*N*-(2′-hydroxy-3′-sulfopropyl)-aniline natrium method, and an acyl-CoA synthase·acyl-CoA oxidase method using commercial kits (Wako), respectively. Plasma immunoreactive insulin was measured using an enzyme-linked immunosorbent assay kit (Morinaga Institute of Biological Science, Yokohama, Japan). Hematological parameters were measured using a Technicon H*1 hematology autoanalyzer (Bayer–Sankyo, Tokyo, Japan), and blood chemistry was analyzed using a HITACHI 7150 autoanalyzer (Hitachi, Tokyo, Japan).

2.5. Statistical analysis

Data were presented as mean ± S.E.M. except for subacute toxicity studies, where data were presented as mean ± S.D. Student's *t*- or Aspin–Welch test was used to determine the significance of differences between nondiabetic (lean mice) and diabetic (*db*/*db* mice or *ob*/*ob* mice) mice. Dunnett's multiple comparisons were used between diabetic control and drug-treated diabetic mice or between control and drug-treated rats. $P < 0.05$ was considered statistically significant. ED_{50} values of each compound were calculated as a concentration required to reduce the levels of blood parameters in diabetic mice by 50%, considering the levels of these in diabetic control mice as 0% and in nondiabetic mice as 100% by a linear-regression analysis.

3. Results

3.1. Antidiabetic activity of FK614 in db/db and ob/ob mice

Plasma glucose level in diabetic control, *db*/*db* mice, was significantly higher than that in normal control, lean mice. Both FK614 and rosiglitazone dose-dependently reduced plasma glucose level in *db*/*db* mice (Fig. 1A). ED_{50} values of FK614 and rosiglitazone for hypoglycemic effect were 2.3 and 1.3 mg/kg, respectively, and therefore, the potency of FK614 was taken to be comparable to that of rosiglitazone. When the lowering activity of FK614 on plasma glucose level was compared with that of pioglitazone (Fig. 1B), ED_{50} values of FK614 and pioglitazone were 1.8 and 28.1 mg/kg, respectively. Thus, FK614 was approximately 15-fold more potent than pioglitazone with regard to reducing activity on plasma glucose levels. FK614, as well as rosiglitazone and pioglitazone, dose-dependently reduced plasma triglyceride levels in *db*/*db* mice (Fig. 2). FK614 was approximately 10-fold more potent than pioglitazone (Fig. 2B; ED_{50}: FK614, 0.29 mg/kg; pioglitazone, 2.9 mg/kg).

In *ob*/*ob* mice, another representative animal model of type 2 diabetes, FK614 and pioglitazone lowered the plasma levels of glucose and insulin (Fig. 3). ED_{50} values of FK614 and pioglitazone for hypoglycemic effect were 1.1 and 8.8 mg/kg, respectively, and therefore, FK614 was approximately 8-fold more potent than pioglitazone. ED_{50} values for plasma insulin levels were 1.3 and 11.8 mg/kg, respectively. Thus, FK614 had antidiabetic effects with higher potency than pioglitazone in *ob*/*ob* mice. Furthermore, FK614 improved the impaired glucose tolerance, a characteristic of type 2 diabetes, in *ob*/*ob* mice as shown in Fig. 4. After glucose loading, plasma glucose level at each time point in *ob*/*ob* mice was higher than that of lean mice, indicating glucose tolerance was impaired in *ob*/*ob* mice. In addition, plasma insulin level at each time point in *ob*/*ob* mice was significantly higher than that of lean mice. FK614 dose-dependently reduced plasma glucose and insulin levels in *ob*/*ob* mice during the glucose tolerance test. In the 10 mg/kg-treated group, plasma insulin levels were significantly reduced compared with the diabetic control group, and plasma glucose levels were decreased to the levels observed in lean mice.

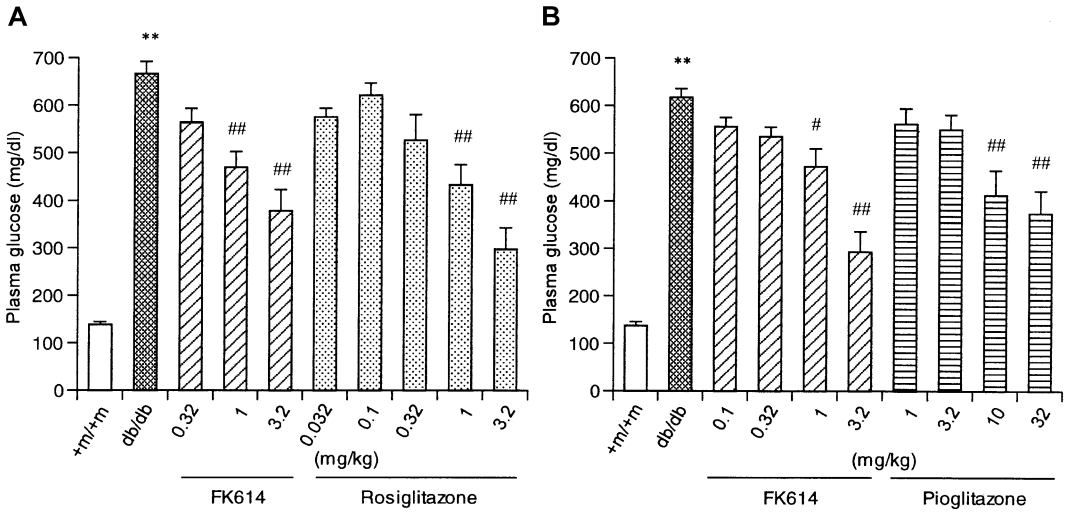

Fig. 1. Hypoglycemic effects of FK614 in *db*/*db* mice compared with that of rosiglitazone (A) or pioglitazone (B). Drugs were orally administrated for 14 days. Data are shown as mean ± S.E.M. ($n=8$). $^{**}P<0.01$ versus normal control (+*m*/+*m*). $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus diabetic control (*db*/*db*). In (A), ED_{50} values for FK614 and rosiglitazone were 2.3 and 1.3 mg/kg, respectively. In (B), ED_{50} values for FK614 and pioglitazone were 1.8 and 28.1 mg/kg, respectively.

3.2. Effects of FK614 on plasma glucose, insulin, and lipids in normal Sprague–Dawley rats

We examined the effects of FK614 on plasma glucose, insulin, and lipids in nondiabetic rats. As shown in Table 1, FK614 dose-dependently reduced plasma triglyceride and nonesterified fatty acid levels in male Sprague–Dawley rats. At doses of 1 mg/kg or more, effects of FK614 on plasma triglyceride and nonesterified fatty acid levels were significant. However, plasma glucose and insulin levels were not affected by repeated (Table 1) and single (data not shown) administrations of FK614.

3.3. Effects of FK614, rosiglitazone, pioglitazone, and troglitazone on PPARγ- and α-mediated transcriptional activity

FK614 dose-dependently stimulated mouse PPARγ2- and human PPARγ1-mediated transcriptional activity as well as three thiazolidinedione derivatives, rosiglitazone, pioglitazone, and troglitazone (Fig. 5A,C). The order of the potency for increasing PPARγ-mediated transcriptional activity was as follows: troglitazone≒FK614≒pioglitazone < rosiglitazone. However, the maximum effect of FK614 on mouse or human PPARγ activation was rather

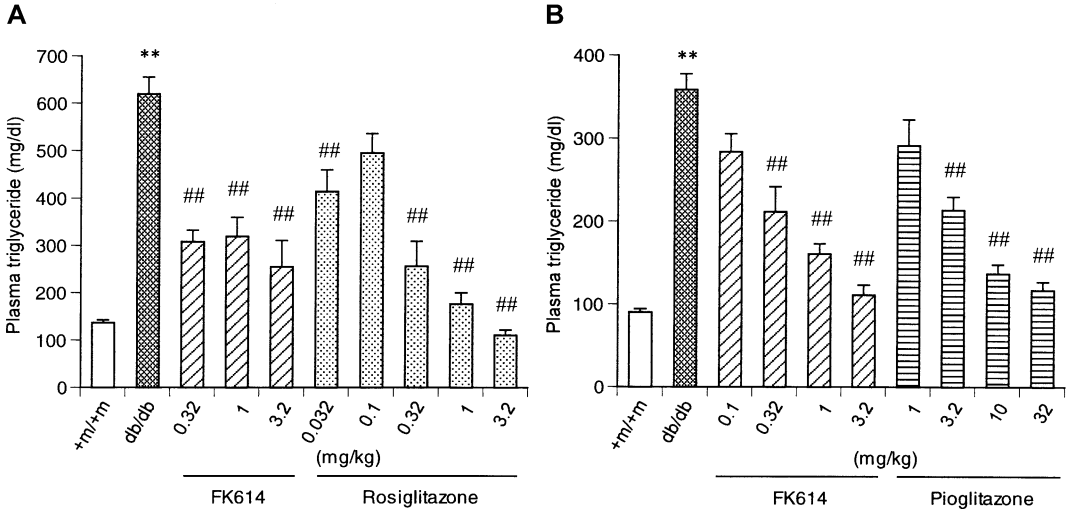

Fig. 2. Effects of FK614 on plasma triglyceride level in *db*/*db* mice compared with that of rosiglitazone (A) or pioglitazone (B). Drugs were orally administrated for 14 days. Data are shown as mean ± S.E.M. ($n=8$). $^{**}P<0.01$ versus normal control (+*m*/+*m*). $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus diabetic control (*db*/*db*). In (A), ED_{50} values for FK614 and rosiglitazone were <0.32 and 0.11 mg/kg, respectively. In (B), ED_{50} values for FK614 and pioglitazone were 0.29 and 2.9 mg/kg, respectively.

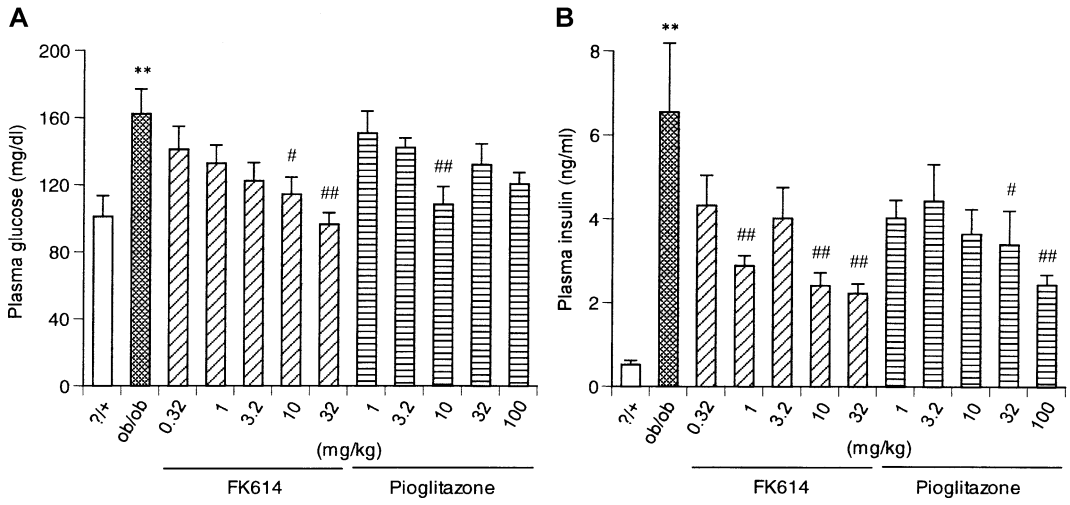

Fig. 3. Effects of FK614 and pioglitazone on plasma glucose (A) and insulin (B) level in *ob/ob* mice. Drugs were orally administrated for 14 days. Data are shown as mean ± S.E.M. ($n = 9–10$). $^{**}P < 0.01$ versus normal control (?/+). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus diabetic control (*ob/ob*). ED_{50} values for FK614 and pioglitazone for blood glucose levels were 1.1 and 8.8 mg/kg, respectively. ED_{50} values for blood insulin levels were 1.3 and 11.8 mg/kg, respectively.

lower than that of thiazolidinedione derivatives. FK614 showed only about 3.8- and 10.5-fold activation as the maximum activity for mouse PPARγ2 and human PPARγ1, respectively, while thiazolidinedione derivatives showed about 5- and 14-fold activation. For PPARα-mediated transcriptional activity, FK614 had almost no effect for both mouse and human PPARα at concentrations of 10^{-5} M or lower. However, all thiazolidinedione derivatives stimulated PPARα-mediated transcriptional activity at 10^{-5} M, the maximum concentration tested (rosiglitazone: 2.7- and 2.5-fold; pioglitazone: 2.5- and 2.2-fold; troglitazone: 1.9- and 1.7-fold, for mouse and human, respectively; Fig. 5B,D).

3.4. Subacute toxicity of FK614 and rosiglitazone

We also performed subacute toxicity studies in Sprague–Dawley rats for FK614 (10, 32, 100, and 320 mg/kg) and rosiglitazone (3.2, 10, 32, and 100 mg/kg). As shown in Table 2, FK614 decreased hemoglobin and red blood cells, with statistically significant levels only at a dose of 320 mg/kg in female rats. Significant decreases of hematocrit, hemoglobin, and red blood cells were observed in male and female rats treated with rosiglitazone at 32 mg/kg or more, which means that the effect of FK614 on hematology was 10-fold less than that of rosiglitazone. Looking at the effects on cardiac mass as another index of hemodilution, results were comparable to

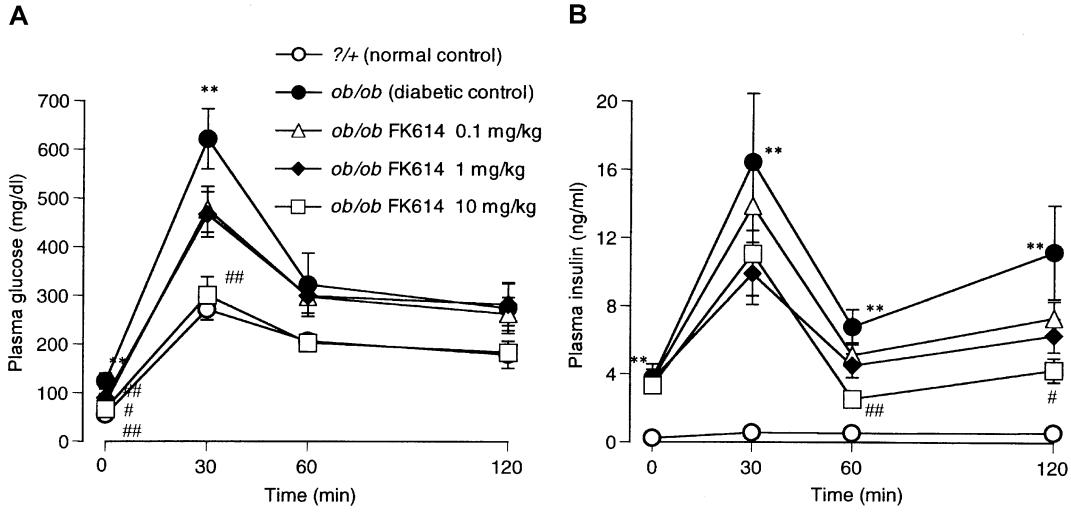

Fig. 4. Effects of FK614 on impaired glucose tolerance in *ob/ob* mice. FK614 was orally administrated for 14 days. One day after the final drug treatment, mice orally received 2 g/kg glucose (5 ml/kg). Data are shown as mean ± S.E.M. [$n = 7$, normal control (?/+); $n = 10$, the other groups]. $^{**}P < 0.01$ versus normal control (?/+). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus diabetic control (*ob/ob*).

Table 1
Effects of FK614 on plasma glucose, insulin, and lipids in Sprague–Dawley rats

	Dose (mg/kg)	N	Plasma glucose (mg/dl)	Plasma insulin (ng/ml)	Plasma triglyceride (mg/dl)	Plasma NEFA (mEq/l)
Control		10	N.D.	N.D.	149 ± 20	0.35 ± 0.03
FK614	0.1	10	N.D.	N.D.	145 ± 17	0.33 ± 0.05
	0.32	10	N.D.	N.D.	105 ± 12	0.27 ± 0.02
	1	10	N.D.	N.D.	64 ± 7[a]	0.17 ± 0.02[a]
Control		10	178 ± 11	1.28 ± 0.06	146 ± 22	0.74 ± 0.04
FK614	1	10	180 ± 8	1.24 ± 0.07	81 ± 13[a]	0.56 ± 0.06[b]
	3.2	10	179 ± 6	1.17 ± 0.05	86 ± 11[b]	0.36 ± 0.03[a]
	10	10	187 ± 7	1.20 ± 0.04	66 ± 5[a]	0.39 ± 0.02[a]

FK614 was orally administrated to male Sprague–Dawley rats (7 weeks of age) for 14 days. Blood was taken one day after the final drug treatment. Data are shown as mean ± S.E.M. NEFA, nonesterified fatty acid; N.D., not determined.
[a] $P < 0.01$ versus control.
[b] $P < 0.05$.

the hematological changes. Increases in cardiac mass were observed in male rats treated with FK614 at a dose of 320 mg/kg (control: 1059 ± 78 mg; FK614: 1212 ± 86 mg, $p < 0.01$ vs. control) and female rats treated with rosiglitazone at doses of 32 and 100 mg/kg (control: 703 ± 54 mg; 32 mg/kg: 847 ± 105 mg, $p < 0.05$ vs. control; 100 mg/kg: 902 ± 107 mg, $p < 0.01$ vs. control). No change in liver function enzymes, including glutamic pyruvic transaminase and glu-

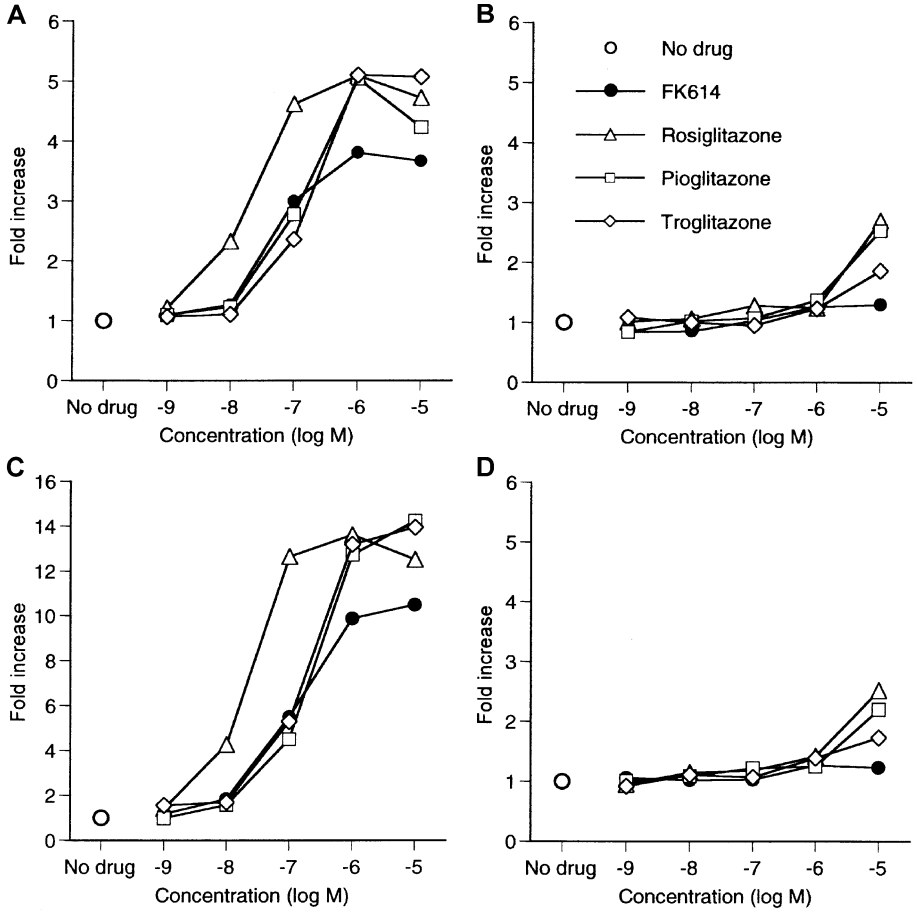

Fig. 5. Effects of FK614, rosiglitazone, pioglitazone, and troglitazone on mouse PPARγ2- (A), mouse PPARα- (B), human PPARγ1- (C), and human PPARα- (D) mediated transcriptional activities. CV-1 cells were transfected with or without expression plasmid for each PPAR isoform, with a PPRE luciferase reporter plasmid plus internal control plasmid. After 2 days of treatment at the indicated concentrations of each drug or 0.1% DMSO (No drug), cells were subsequently assayed for luciferase activity. Transient transfection assays were carried out in triplicate. Relative stimulation is given as fold increase compared to DMSO-treated (No drug) control cells, as described in Materials and methods.

Table 2
Effects of FK614 and rosiglitazone on hematology in Sprague–Dawley rats

Drug	Sex	*N*	HCT (%)	Hb (g/dl)	RBC (10^6/μl)	Sex	*N*	HCT (%)	Hb (g/dl)	RBC (10^6/μl)
Dose (mg/kg)										
FK614										
Control	Male	5	41 ± 1.9	14.4 ± 0.52	7.0 ± 0.30	Female	5	40 ± 1.5	14.4 ± 0.60	7.0 ± 0.18
10		5	38 ± 1.5	13.7 ± 0.66	6.7 ± 0.40		5	39 ± 1.3	14.2 ± 0.30	6.8 ± 0.11
32		5	41 ± 1.7	14.3 ± 0.57	6.8 ± 0.48		5	39 ± 1.3	14.3 ± 0.29	6.9 ± 0.17
100		5	40 ± 0.7	13.9 ± 0.36	6.8 ± 0.29		5	40 ± 0.8	14.4 ± 0.21	6.9 ± 0.07
320		5	39 ± 0.4	13.7 ± 0.20	6.5 ± 0.13		5	38 ± 0.8	13.7 ± 0.23[a]	6.6 ± 0.12[b]
Rosiglitazone										
Control	Male	5	44 ± 0.4	14.6 ± 0.26	7.4 ± 0.19	Female	5	42 ± 1.1	14.5 ± 0.56	7.3 ± 0.32
3.2		5	43 ± 0.9	14.5 ± 0.48	7.2 ± 0.28		5	41 ± 2.2	14.1 ± 0.60	7.0 ± 0.27
10		5	42 ± 1.0	14.0 ± 0.35	7.1 ± 0.64		5	41 ± 1.3	13.9 ± 0.54	7.0 ± 0.22
32		5	41 ± 0.6[b]	13.6 ± 0.28[b]	6.7 ± 0.21[b]		5	40 ± 1.9	13.5 ± 0.58[a]	6.6 ± 0.28[b]
100		5	40 ± 1.4[b]	13.1 ± 0.43[b]	6.4 ± 0.13[b]		5	39 ± 1.6[b]	13.2 ± 0.36[b]	6.6 ± 0.26[b]

Each drug was orally administrated to male and female Sprague–Dawley rats (6 weeks of age) for 14 days. Blood was taken one day after the final drug treatment. Data are shown as mean ± S.D.
HCT, hematocrit; Hb, hemoglobin; RBC, red blood cells.
[a] $P<0.05$.
[b] $P<0.01$ versus control.

tamic oxaloacetate transaminase, was observed in rats treated with these drugs at all doses.

4. Discussion

In this report, we evaluated the antidiabetic effects of FK614, a novel insulin sensitizer with a distinctly different chemical structure from thiazolidinedione derivatives. In *db*/*db* mice, FK614 dose-dependently reduced plasma glucose level. In addition, FK614 lowered plasma glucose and insulin levels in *ob*/*ob* mice. Because FK614 did not influence plasma insulin level in normal rats from repeated or single administrations, these results suggest that FK614 reduces plasma glucose level by ameliorating insulin resistance. This speculation was supported by the results that FK614 improved the impaired glucose tolerance in *ob*/*ob* mice whose insulin level after glucose loading was significantly decreased by FK614 treatment. The area under the curve of plasma concentrations at the ED_{50} value of hypoglycemic activity for each drug in *db*/*db* mice estimated from data of plasma concentrations at multiple doses was as follows: 12.59 or 16.34 μg·h/ml for FK614, 18.06 μg·h/ml for rosiglitazone, and 117.1 μg·h/ml for pioglitazone. Therefore, the potency of FK614 for improvement of insulin resistance in those animal models was almost similar to that of rosiglitazone and much higher than that of pioglitazone.

Thiazolidinedione insulin sensitizers are well known as PPARγ agonists and PPARγ activation appears to contribute to the hypoglycemic effect in vivo (Willson et al., 1996). Although a detailed mechanism by which thiazolidinedione derivatives ameliorate insulin resistance is not fully characterized, it is likely that adipocyte differentiation or the regulation of PPARγ-responsive genes through the stimulation of PPARγ by thiazolidinedione derivatives might contribute to the improvement of insulin sensitivity (Hallakou et al., 1997; Okuno et al., 1998; Spiegelman, 1998). Those informations prompted us to evaluate the effects of FK614 on PPARγ-mediated transcriptional activity. Of interest is that FK614 had the ability to activate both mouse and human PPARγ-mediated transcriptional activities at more than 10^{-7} M, but the maximum effect of FK614 at 10^{-6} and 10^{-5} M was not as high as thiazolidinedione derivatives. Thus, FK614 is assumed to be a partial PPARγ agonist and the mode of PPARγ activation by FK614 seems to be different from thiazolidinedione derivatives. Further studies including molecular interaction of FK614 and PPARγ will be necessary to elucidate the different mode of PPARγ activation from thiazolidinedione derivatives.

In mammals, two other types of PPAR have been identified: PPARα and PPARδ (Schoonjans et al., 1996b). PPARα is expressed at high levels in the liver and plays an important role in lipid metabolism in the liver, including β-oxidation and lipoprotein metabolism. Recently, Pill and Kühnle (1999) reported that a compound structurally unrelated to thiazolidinedione showed antihyperglycemic and antihyperinsulinemic effects in various animal models of type 2 diabetes, and this compound activated PPARα but not PPARγ (Meyer et al., 1999). In addition, PPARγ and α dual-agonists function as antidiabetic agents or insulin-sensitizers in rodent models (Etgen et al., 2002; Ljung et al., 2002; Murakami et al., 1998; Shibata et al., 1999; Ye et al., 2003). Although PPARδ is ubiquitously expressed and its physiological role has not been determined, it is reported that PPARδ activation does not seem to modulate plasma glucose or triglyceride levels (Berger et al., 1999). Because FK614 had no effect on PPARα-mediated transcriptional activities at 10^{-5} M or lower, this suggests that the antidiabetic effect of FK614 is principally due to stimulating PPARγ. These results indicated that FK614 activated both mouse and

human PPARγ-mediated transcriptional activities and showed antidiabetic effects in type 2 diabetic models of mice. According to these data, it is expected that FK614 may be at least as effective as pioglitazone or rosiglitazone in clinical use for the treatment of type 2 diabetes.

In addition to its antidiabetic effects, FK614, as well as rosiglitazone and pioglitazone, had reducing activity against plasma triglyceride levels in diabetic *db*/*db* mice. Thus, FK614 may be beneficial in the treatment of type 2 diabetic patients associated with dyslipidemia. FK614 dose-dependently reduced plasma triglyceride and nonesterified fatty acid levels in normal Sprague–Dawley rats. In the reporter gene assay, it was evident that FK614 activated PPARγ but not PPARα at concentrations tested. Furthermore, hypotriglyceridemic effects of thiazolidinedione derivatives and fibrates are likely to be due to the increase of lipoprotein lipase expression through activation of PPARγ in adipose tissue and PPARα in the liver, respectively (Schoonjans et al., 1996a). Therefore, it seems reasonable to assume that FK614 reduces plasma triglyceride and nonesterified fatty acid levels by increasing LPL activity in adipose tissue through, in part, PPARγ stimulation. On the other hand, as it seems likely that resistance to insulin-stimulated glucose uptake leads to a compensatory hyperinsulinemia, enhanced hepatic very-low-density lipoprotein-triglyceride secretion and hypertriglyceridemia (Reaven, 1995), the possibility that decreases in plasma triglyceride levels by FK614 may result from ameliorating insulin resistance cannot be ruled out.

The influences of thiazolidinedione derivatives on hematology, for example, small reductions of hemoglobin and hematocrit, have been reported in clinical use and animal experiments (Barman Balfour and Plosker, 1999; Gillies and Dunn, 2000; Pickavance et al., 2000; Plosker and Faulds, 1999). Indeed, it was ascertained that rosiglitazone reduced hematocrit, hemoglobin, and red blood cells and further increased cardiac mass in rat toxicity studies. FK614 also reduced hematocrit, hemoglobin, and red blood cells and increased cardiac mass although the minimum dose of FK614 at which these effects were significant was 10-fold larger than that of rosiglitazone. Although these effects are likely to be a kind of class effects for insulin sensitizers and may be due to increasing plasma volume, FK614 has a larger safety margin than rosiglitazone in animal studies. The ratios of the minimum effective dose on hematology and cardiac mass to ED_{50} for hypoglycemic effect in *db*/*db* mice are 139.1 and 24.6 for FK614 and rosiglitazone, respectively. If this observation is also true for humans, it is expected that FK614 would be a safer drug for the treatment of type 2 diabetes than rosiglitazone.

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