Potent Antidiabetic Effects of Rivoglitazone, a Novel Peroxisome Proliferator–Activated Receptor-γ Agonist, in Obese Diabetic Rodent Models

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Abstract. The pharmacological effects of rivoglitazone, a novel thiazolidinedione-derivative peroxisome proliferator–activated receptor (PPAR)-γ agonist, were characterized in vitro and in vivo. Rivoglitazone activated human PPARγ more potently compared with rosiglitazone and pioglitazone and had little effect on PPARα and PPARδ activity in luciferase reporter assays. In Zucker diabetic fatty (ZDF) rats, 14-day administration of rivoglitazone decreased the plasma glucose and triglyceride (TG) levels in a dose-dependent manner. The glucose-lowering effect of rivoglitazone was much more potent than those of pioglitazone (ED\textsubscript{50}: 0.19 vs. 34 mg/kg) and rosiglitazone (ED\textsubscript{50}: 0.20 vs. 28 mg/kg). In addition, rivoglitazone showed potent antidiabetic effects in diabetic \textit{db/db} mice. In Zucker fatty rats, rivoglitazone at a dose of 0.1 mg/kg clearly ameliorated insulin resistance and lowered plasma TG levels by accelerating the clearance of plasma TG. Gene expression analysis in the liver and heart of ZDF rats treated with rivoglitazone for 14 days suggested that rivoglitazone may reduce hepatic glucose production and modulate the balance of the cardiac glucose/fatty acid metabolism in diabetic animals. In summary, we showed that rivoglitazone is a potent and selective PPARγ agonist and has a potent glucose-lowering effect via improvement of the insulin resistance in diabetic animal models.

Keywords: thiazolidinedione, peroxisome proliferator–activated receptor (PPAR)-γ, diabetes mellitus, insulin resistance, gene expression

Introduction

The major causes of type 2 diabetes (T2DM) are impaired insulin secretion, increased hepatic glucose production, and decreased insulin-stimulated glucose uptake to the peripheral tissues (1). Increased hepatic glucose production and decreased insulin-stimulated glucose uptake are characterized as insulin resistance in which insulin cannot cause sufficient effects on the target organs. Moreover, it is often accompanied by compensatory hyperinsulinemia. Insulin resistance with hyperinsulinemia is a common abnormality in obesity as well as T2DM and may increase susceptibility to other disorders such as hyperlipidemia and hypertension and lead to cardiovascular disease (2).

Pioglitazone and rosiglitazone are thiazolidinedione (TZD) derivative antidiabetic agents available in clinical practice. There is a great deal of evidence showing that these drugs ameliorate insulin resistance in the liver and skeletal muscle and preserve pancreatic β-cell function, which makes them one of the most important drug classes for the treatment of T2DM (3).

Pioglitazone and rosiglitazone have been shown to act as activators of peroxisome proliferator–activated receptor (PPAR)-γ and are thought to exert their anti-diabetic effects via the activation of PPARγ (4). PPARs are ligand-activated nuclear transcription factors that modulate the expression of a large number of genes associated with lipid and glucose metabolism and consist of three subtypes, namely, PPARα, PPARγ, and
PPARδ (5, 6). In general, PPARγ regulates genes involved in fatty acid uptake and storage and glucose homeostasis in adipose tissues, whereas PPARα regulates genes involved in fatty acid uptake and oxidation in the liver. PPARδ regulates genes involved in fatty acid metabolism and lipid homeostasis.

Rivoglitazone, formerly CS-011, is a newly synthesized TZD-derivative. In this study, we examined the effect of rivoglitazone on the transactivation of human PPARs (hPPARs) in vitro in a cell-based reporter assay and in the in vivo antidiabetic effects in diabetic animal models in comparison with pioglitazone and rosiglitazone. In addition, the effects on triglyceride (TG) metabolism and gene expression in the liver and heart were also examined and compared with the effects of a PPARα agonist.

Materials and Methods

Materials

Rivoglitazone hydrochloride, pioglitazone hydrochloride, and rosiglitazone maleate were synthesized at Sankyo Co., Ltd. (Tokyo) and, hereafter, are indicated as rivoglitazone, pioglitazone and rosiglitazone, respectively. GW7647 was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). GW501516 was purchased from ALEXIS Corporation (Lausen, Switzerland). Wy-14643 was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). The compounds were dissolved in DMSO and added to the medium to the final DMSO concentration of 0.03% (v/v) for the in vitro studies. For the in vivo studies, rivoglitazone and rosiglitazone were suspended in 0.5% (w/v) carboxymethylcellulose (CMC) solution, pioglitazone was suspended in distilled water, and Wy-14643 was suspended in 0.5% (w/v) methylcellulose solution.

Cell culture and transfection

HT-1080 cells (American Type Culture Collection, Manassas, VA, USA), derived from human fibrosarcoma, were cultured in the Dulbecco’s Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 25 mM HEPES. Passages of the cells were performed every 2 to 3 days. The GAL4-hPPAR chimera receptor expression vectors pM-hPPARα, pM-hPPARγ, or pM-hPPARδ, which express the ligand-binding domain of each PPAR as a fusion protein with the DNA-binding domain of GAL4, were constructed by insertion of cDNA encoding amino acids H168-Y468 of hPPARα, H175-Y475 of hPPARγ2, or H140-Y441 of hPPARδ, respectively (GenBank accession numbers: S74349, X90563, and L07592, respectively), into a plasmid pM (Invitrogen Corporation, Carlsbad, CA, USA). Each expression vector was cotransfected with the pG5Luc vector (Promega Corporation, Madison, WI, USA), which codes firefly luciferase driven by GAL4, to the HT-1080 cells using Lipofectamine 2000 (Invitrogen Corporation).

Luciferase assay

HT-1080 cells cultured for about 6 h after transfection were harvested, resuspended in DMEM without phenol red containing 10% FBS, and seeded in 96-well plates. After 22 – 23 h, the cells were treated with rivoglitazone, pioglitazone, or rosiglitazone at 0.001, 0.01, 0.1, 1, and 10 μM and incubated for another 24 h until the luciferase activity was determined. As a vehicle control, cells were also treated with 0.03% of DMSO. The luciferase activity was determined using a Dual-Glo Luciferase Assay System (Promega Corporation). The fold-increase of the light intensity relative to the vehicle control was calculated and then normalized by that of the positive control (10 μM rosiglitazone for the PPARγ activity, 10 μM GW7647 for the PPARα activity, and 1 μM GW501516 for the PPARδ activity) for the calculation of the relative activity.

Animals

Male Zucker diabetic fatty (ZDF) rats (ZDF/Crl-Leprdb/db) were purchased at 6 weeks of age from Charles River Japan, Inc. (Kanagawa) and acclimatized until they were 9-weeks-old. Male Zucker fatty (ZF) rats [Crlj:ZUC-Leprdb/db (Leprdb/db)] were purchased at 7 weeks of age from Charles River Japan, Inc. and acclimatized until they were 8-week-old. Male db/db mice (BKS.Cg-Leprdb/db) were purchased at 7 weeks of age from CLEA Japan, Inc. (Tokyo) and acclimatized until they were 8-week-old. Rodent chow (FR-2; Funabashi Farm Co., Ltd., Chiba) and water were given ad libitum. The animals were housed 3 or 4 animals per cage for rats and 5 or 6 animals per cage for mice at a room temperature of 23°C – 24°C, humidity of 50% – 75%, and a 12-h light/dark cycle (lighting: 7:00 – 19:00). All experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Sankyo Co., Ltd.

Administration and blood sampling

The suspensions of the compounds were orally administered once a day for 7 – 15 days at a volume of 1 mL/kg for rats and 10 mL/kg for mice. The animals in the control group were treated with 0.5% CMC solution. The day the drug administration started was defined as day 0. Blood was collected from the tip of the tails of fed-state animals using capillary tubes before and
after repeated drug administration. The collected blood was centrifuged to obtain plasma. The plasma samples were stored at −20°C for later measurement of the biochemical parameters.

**Measurement of pancreatic insulin content**

The ZDF rats were sacrificed on day 15 by decapitation and the pancreases were excised. The pancreases were put in the 1.5% hydrochloric acid / 75% ethanol solution and homogenized on ice. After overnight incubation at 4°C, the homogenates were centrifuged at 3000 rpm for 15 min at 4°C. The insulin levels in the supernatants were measured and the pancreatic insulin content was calculated in μg/pancreas.

**Hyperinsulinemic-euglycemic clamp**

The hyperinsulinemic-euglycemic clamp study was performed after 8-day administration of rivoglitazone at a dose of 0.1 mg/kg to ZF rats. To infuse the insulin and the glucose solution, an infusion catheter was inserted into the jugular vein 2 days before the clamp study.

On day 8, under overnight fasting, the clamp study was initiated by infusing the insulin solution at 20 mU⋅kg⁻¹.min⁻¹. The blood glucose levels in the whole blood were monitored every 5 min and the 20% (w/v) glucose solution was infused at variable rates to keep the blood glucose levels around 100 mg/dL. The blood samples were collected for insulin measurement at 80, 90, and 100 min after the initiation of the insulin infusion. The blood glucose levels, plasma insulin levels, and glucose infusion rate (GIR) at steady-state was calculated as the mean values at 80, 90, and 100 min.

**Whole-body TG elimination and production**

ZF rats were treated for 7 days with rivoglitazone or Wy-14643 at doses of 1 or 100 mg/kg, respectively. On Day 7, the TG elimination and the TG production were examined after 4-h fasting.

To examine the TG elimination, lipid emersion (Intralipos 20%; Otsuka Pharmaceutical Factory, Inc., Tokushima) was injected into the tail vein at the volume of 4 mL/kg. The blood was collected just before (0 min) and at 10, 20, 30, 45, 60, and 90 min after the injection. The 0 min value was subtracted from the value at each time point and the area under the curve of the plasma TG from 0 – 90 min (AUC₁₀⁻⁹₀ min, h⋅mg/dL) was then calculated using these values. The TG elimination rate constant (min⁻¹) was calculated using the plasma TG levels at 20, 30, and 45 min assuming that the plasma TG elimination rate at these time points follows first-order kinetics.

To examine the TG production, tyloxapol (lipoprotein lipase inhibitor) solution in saline (200 mg/mL, Sigma-Aldrich Corporation) was injected into the tail vein at a volume of 1 mL/kg. The blood was collected just before (0 min) and at 30, 60, 90, 120, and 150 min after the injection. The TG production rate (mg⋅dL⁻¹⋅min⁻¹) was calculated using the plasma TG levels from 30 – 150 min by linear regression.

**Gene expression analysis in the liver and heart**

ZDF rats were treated with rivoglitazone, pioglitazone, or Wy-14643 for 14 days at doses of 1, 100, or 100 mg/kg, respectively. The rats were decapitated 6 h after the final administration on day 14 and the liver and heart were rapidly removed. The total RNA was extracted with TRIZol reagent (Invitrogen Corporation) and purified using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and the cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). The expression levels of the following genes were analyzed by real time PCR: acyl-coenzyme A oxidase 1 (ACO), carnitine palmitoyltransferase 1b (CPT1b), enoyl-coenzyme A hydratase / 3-hydroxyacyl coenzyme A dehydrogenase (ECH/HAD), pyruvate dehydrogenase kinase isoenzyme 4 (PDK4), apolipoprotein C-III (ApoCIII), glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxy-kinase 1 (PEPCK), muscle pyruvate kinase [PK (muscle)], glucose transporter 4 (GLUT4), medium chain acyl-coenzyme A dehydrogenase (MCAD), myosin heavy chain alpha (MHCα), myosin heavy chain beta (MHCβ), sarco/endoplasmic reticulum Ca²⁺ ATPase 2 (SERCA2), and acidic ribosomal phosphoprotein P0 (ARBP). The primer and probe sets used in this study are shown in Table 1. The real-time PCR was performed and the data was analyzed using an ABI Prism 7900HT Sequence Detection System with SDS 2.1.1 software (Applied Biosystems). The relative expression levels were calculated by dividing the expression levels by that of the ARBP.

**Analytical methods for biochemical parameters**

The blood glucose levels were measured with a blood glucose test meter (Glutest PRO R; Sanwa Kagaku Kenkyusho Co., Ltd., Aichi). The plasma glucose levels were measured by an automatic glucose analyzer (Glucoroder-GXT; A&T Corporation, Kanagawa). The plasma TG levels were measured with a colorimetric assay kit (Triglyceride E-Test or L-Type Triglyceride H; Wako Pure Chemical Industries, Ltd., Osaka). The plasma nonesterified fatty acid (NEFA) levels were measured with a colorimetric assay kit (NEFA C-Test; Wako Pure Chemical Industries, Ltd.). The insulin and adiponectin levels were measured by using radio-
immunoassay kits (Rat Insulin RIA Kit and Mouse Adiponectin RIA Kit, respectively; Linco Research, Inc., St. Charles, MO, USA).

**Table 1.** Primers and probes used for analyzing gene expression

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank accession No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acyl-coenzyme A oxidase 1 (ACO)</strong></td>
<td>NM_017340</td>
<td>Probe: CACCACTGTCAGCAGGAAAAATGGA Primer 1: TGTGGCCTCAAATTCACTCATG Primer 2: AGATGGTCCGATGCCCACATC</td>
</tr>
<tr>
<td><strong>Carnitine palmitoyltransferase 1b (CPT1b)</strong></td>
<td>NM_013200</td>
<td>Probe: TACCTGGATTGAGCTGTCCTGACAA Primer 1: AGTGGCGAGGCGACAAATGACAC Primer 2: CCAGAATCGGAAATAGCGTCATC</td>
</tr>
<tr>
<td><strong>Enoyl-coenzyme A hydratase / 3-hydroxyacyl coenzyme A dehydrogenase</strong></td>
<td>NM_133606</td>
<td>Probe: TCGCAACGGTATATGACCTGTCGTTGCCC Primer 1: TAGCGGTAATCTCCCATCTCTAC Primer 2: CCACCTACTCATCTTCCGTCATC</td>
</tr>
<tr>
<td><strong>Pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4)</strong></td>
<td>NM_053551</td>
<td>Probe: TCGAGCATCAAGAAAACGCGCTTT Primer 1: TGCCATGAGGCGCATC Primer 2: TGGGCTCGAGACTGCAG</td>
</tr>
<tr>
<td><strong>Apolipoprotein C-III (ApoCIII)</strong></td>
<td>NM_012501</td>
<td>Probe: AGAGGGATCCTGCTGCTGGGACAGTAA Primer 1: CTGCCAGAGTCGAAGGAG 3' Primer 2: TTGCTCGAGACTGTCAG</td>
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<td><strong>Glucose-6-phosphatase (G6P)</strong></td>
<td>NM_013098</td>
<td>Probe: GGCTGAAACTTCTAACTAGC Primer 1: AACGTCTGTCCGACATTCACTGA Primer 2: AGAAGGATGGACAGTACCTTGCATC</td>
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<td><strong>Phosphoenolpyruvate carboxykinase 1 (PEPCK)</strong></td>
<td>NM_198780</td>
<td>Probe: CCACTCAACTCCCATGCTGACCC Primer 1: TGAATCAAAGCAGGATGACAT Primer 2: CGATGGACTCTGCAATC</td>
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<td><strong>Liver pyruvate kinase (lPK)</strong></td>
<td>NM_012624</td>
<td>Probe: CGACTCAACTTCCCATGCTGACCC Primer 1: TGAATCAAAGCAGGATGACAT Primer 2: CGATGGACTCTGCAATC</td>
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<td><strong>Muscle pyruvate kinase (mPK)</strong></td>
<td>NM_053297</td>
<td>Probe: AGGAGGTGCTGCTTCGACCAAGTACCA Primer 1: TTCGCAATCGACACCTGATA Primer 2: CCGCAGACTCTTCATC</td>
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<tr>
<td><strong>Glucose transporter 4 (GLUT4)</strong></td>
<td>NM_012751</td>
<td>Probe: CCGGATGCAACTCCCATGCTGACCC Primer 1: TGAATCAAAGCAGGATGACAT Primer 2: CGATGGACTCTGCAATC</td>
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<td><strong>Medium chain acyl-coenzyme A dehydrogenase (MCAD)</strong></td>
<td>NM_016986</td>
<td>Probe: CCCAGTCGCCAGAAGCATCAGA Primer 1: GGAAGGTGGCCGAGGAGAAATAA Primer 2: CGGGATATCCCTCCGCTTTT</td>
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<tr>
<td><strong>Myosin heavy chain alpha (MHCα)</strong></td>
<td>NM_017239</td>
<td>Probe: CCGAGGCTGGAATCTGCTGCAATC Primer 1: TGTGAAGGGGATTTAACCAGGTAGTTAA Primer 2: TCTGAGCTTGGCAGGATAG</td>
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<tr>
<td><strong>Myosin heavy chain beta (MHCβ)</strong></td>
<td>NM_017240</td>
<td>Probe: CATCAGCTCCAGATGTGGCAGAACAA Primer 1: AGTCCAGCTCCAGCAACCTACTG Primer 2: TGTGCTGCTCAGTCCTGCTG</td>
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<tr>
<td><strong>Sarco/endoplasmic reticulum Ca2+ ATPase 2 (SERCA2)</strong></td>
<td>NM_017290</td>
<td>Probe: AACCTTGCCCTACTATTCCCATGACAA Primer 1: GTCCAAGTCCCTCAATCTCAGT Primer 2: CATCAGGCTCAGCAGACAA</td>
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<tr>
<td><strong>Acidic ribosomal phosphoprotein P0 (ARBP)</strong></td>
<td>NM_022402</td>
<td>Probe: CAAGAACCACTGATGCAGGAAAGG Primer 1: GGTCTCCAGACAGATGACCA Primer 2: CCGGATGGAAGGACAGAGCA</td>
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From left to right, the 5' to 3' end of the oligonucleotide sequences were expressed.

**Statistical analysis**

All data were expressed as the mean ± standard error of the mean (S.E.M.). A multiple comparison between
Antidiabetic Effects of Rivoglitazone

the control group and drug-treated groups was performed by a Dunnett test and the simple comparison between the control group and a drug-treated group was performed by a t-test. A P value less than 0.05 was considered statistically significant.

The EC\textsubscript{50} for activation of hPPAR\textsubscript{γ} was estimated as the concentration required to increase the relative activity to 50\% by linear regression. The ED\textsubscript{50} for the plasma glucose or TG-lowering effect was defined as the dose required to decrease the plasma glucose or TG levels to half of the corresponding value of the control group and was calculated by linear regression. SAS System Release 8.2 (SAS Insitute Inc., Cary, NC, USA) was used for these calculations.

Results

Activation of PPARs in vitro

To investigate the selectivity for PPARs, we performed a luciferase reporter assay using GAL4-fusion protein of the ligand-binding domain of hPPAR\textsubscript{α}, hPPAR\textsubscript{γ}, and hPPAR\textsubscript{δ}. Rivoglitazone as well as rosiglitazone and pioglitazone increased the hPPAR\textsubscript{γ} reporter activity in a concentration-dependent manner and the EC\textsubscript{50}s were 0.22, 0.79, and 3.6 \(\mu\text{M}\), respectively (Fig. 1a). Rivoglitzzone, rosiglitazone, and pioglitazone increased the hPPAR\textsubscript{α} activity by 29.8\%, 8.0\%, and 16.9\% of the positive control, GW7647, at 10 \(\mu\text{M}\) (Fig. 1b). These compounds did not affect the hPPAR\textsubscript{δ} activity at any concentrations examined, whereas GW501516 increased the activity concentration-dependently (Fig. 1c).

Antidiabetic effects of rivoglitazone in diabetic animal models

Rivoglitazone was orally administered to male ZDF rats, a severe insulin-resistant diabetic model, for 15 days. As shown in Fig. 2a, rivoglitazone as well as pioglitazone decreased the plasma glucose levels in a dose-dependent manner on day 14. The ED\textsubscript{50}s of the hypoglycemic effect of rivoglitazone and pioglitazone were 0.19 and 34 mg/kg, respectively. Rivoglitazone and pioglitazone also decreased the plasma TG dose-dependently and the ED\textsubscript{50}s were 0.21 and 17 mg/kg (Fig. 2c). There was little change in the plasma insulin levels, and the body weights were dose-dependently increased by both rivoglitazone and pioglitazone. The pancreases were excised on day 15 and the insulin content was measured. Rivoglitazone as well as pioglitazone increased the pancreatic insulin content in parallel with the decrease of the plasma glucose (Fig. 2e).

In comparison with rosiglitazone, rivoglitazone also showed much more potent effects on the plasma glucose- and plasma TG-lowering and preservation of the pancreatic insulin content in ZDF rats (Fig. 2: b, d, f). The ED\textsubscript{50}s of the plasma glucose-lowering effect were 0.20 and 28 mg/kg and those of the plasma TG-lowering effect were 0.22 and 18 mg/kg for rivoglitzzone and rosiglitazone, respectively.

Rivoglitazone also showed a potent glucose-lowering effect after 14-day treatment in diabetic male db/db mice compared with pioglitazone (Fig. 3a). The ED\textsubscript{50}s of the hypoglycemic effect for rivoglitazone and pioglitazone were 0.47 and 70 mg/kg, respectively. In this model, the plasma adiponectin levels were strikingly increased by rivoglitazone in comparison with pioglitazone (Fig. 3b).

Fig. 1. Effects of rivoglitazone, rosiglitazone, and pioglitazone on hPPAR\textsubscript{γ} (a), hPPAR\textsubscript{α} (b), and hPPAR\textsubscript{δ} (c) activity. HT-1080 cells were cotransfected with an expression plasmid coding the ligand-binding domain of hPPAR\textsubscript{γ}, hPPAR\textsubscript{α}, or hPPAR\textsubscript{δ} fused to a GAL4 DNA-binding domain and a luciferase reporter plasmid. The luciferase activity was measured after 24-h treatment with rivoglitazone, pioglitazone, or rosiglitazone and was expressed as percentage of the positive controls (PPAR\textsubscript{γ} activity: 10 \(\mu\text{M}\) rosiglitazone; PPAR\textsubscript{α} activity: 10 \(\mu\text{M}\) GW7647; PPAR\textsubscript{δ} activity: 1 \(\mu\text{M}\) GW501516). Data are reported as the mean ± S.E.M. (n = 4).
Rivoglitazone improved the insulin resistance in obese ZF rats

To clarify the improvement of insulin resistance more directly, rivoglitazone was administered to obese insulin-resistant ZF rats at a dose of 0.1 mg/kg for 8 days and a hyperinsulinemic-euglycemic clamp study was performed. As shown in Table 2, rivoglitazone significantly increased the GIR in the steady-state period. Rivoglitazone did not affect the basal levels of blood glucose and plasma insulin, but decreased the plasma insulin levels at the steady-state.

Effects of rivoglitazone on the whole-body elimination and production of TG

To evaluate the effects of rivoglitazone on TG metabolism, rivoglitazone was administered to male ZF rats exhibiting dyslipidemia as well as insulin resistance, and the effects were compared with those of Wy-14643, a PPARα agonist. Seven days treatment with rivoglitazone or Wy-14643 at doses of 0.1 or 100 mg/kg, respectively, decreased the basal plasma TG levels to similar levels (Table 3). The TG-eliminating ability from the blood was accessed by injecting an emulsion of lipids (20% Intralipos, 4 mL/kg), and the TG production rate was examined by an injection of tyloxapol (a lipoprotein lipase inhibitor, 200 mg/kg). The AUC TG after lipid load was similar between the rivoglitazone-treated group and the Wy-14643–treated group. The TG elimination rate constant was decreased by rivoglitazone, but the TG production rate was not changed. In contrast, Wy-14643 decreased the TG production rate without affecting the TG elimination rate.

Fig. 2. Effects of rivoglitazone on plasma glucose and TG levels and pancreatic insulin content in comparison with pioglitazone (a, c, e) or rosiglitazone (b, d, f) in ZDF rats. Male ZDF rats were orally treated with rivoglitazone and pioglitazone or rosiglitazone at the indicated doses once a day for 15 days. The control group was treated with 0.5% CMC solution. The plasma glucose and TG levels on day 14 are shown. The pancreases were excised on day 15 and the insulin content of each was measured. Data are reported as the mean ± S.E.M. (n = 6). *P<0.05, compared with the control group by a Dunnett test.
Gene expression analysis in the liver and heart

The effects of rivoglitazone on the gene expression in the liver and heart were examined and compared with pioglitazone and Wy-14643 after 14 days treatment in male ZDF rats. As shown in Table 4, rivoglitazone and pioglitazone at doses of 1 and 100 mg/kg, respectively, significantly decreased the plasma glucose, TG, and NEFA as expected, but Wy-14643 at a dose of 100 mg/kg decreased only the plasma TG and NEFA.

The relative gene expression levels in the liver are shown in Fig. 4. Rivoglitazone significantly decreased the expression of ACO, CPT1b, ECH/HAD, and MCAD, which are involved in fatty acid oxidation. However, significant decrease of the gene expression caused by pioglitazone was observed only for the MCAD gene. In contrast, Wy-14643 strikingly increased

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**Table 2.** The effect of rivoglitazone on blood glucose, plasma insulin, and glucose-infusion rate in the basal and steady-state period under euglycemic-hyperinsulinemic condition

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Steady-state</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Rivoglitazone (0.1 mg/kg)</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>79 ± 3</td>
<td>77 ± 3</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>9.1 ± 0.8</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>Glucose infusion rate (mg⋅kg⁻¹⋅min⁻¹)</td>
<td>—</td>
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</tbody>
</table>

Data are expressed as the mean ± S.E.M. (n = 7). *P<0.05, compared with the control group by a t-test.

**Table 3.** The effect of rivoglitazone on the TG elimination and TG production in ZF rats

<table>
<thead>
<tr>
<th></th>
<th>Basal TG (mg/dL, n = 12)</th>
<th>AUCTG after lipid load (h⋅mg/dL, n = 6)</th>
<th>TG elimination rate constant (min⁻¹, n = 6)</th>
<th>TG production rate (mg⋅dL⁻¹⋅min⁻¹, n = 6)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>671 ± 62</td>
<td>1724 ± 114</td>
<td>0.0236 ± 0.0019</td>
<td>18.1 ± 0.7</td>
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<tr>
<td>Rivoglitazone (0.1 mg/kg)</td>
<td>227 ± 19*</td>
<td>939 ± 101*</td>
<td>0.0689 ± 0.0133*</td>
<td>16.3 ± 0.9</td>
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<tr>
<td>Wy-14643 (100 mg/kg)</td>
<td>275 ± 29*</td>
<td>1158 ± 87*</td>
<td>0.0269 ± 0.0023</td>
<td>10.4 ± 0.4*</td>
</tr>
</tbody>
</table>

*P<0.05, compared with the control group by a t-test.

**Table 4.** Effect of rivoglitazone on the plasma glucose, TG, and NEFA levels in ZDF rats

<table>
<thead>
<tr>
<th></th>
<th>Plasma glucose (mg/dL)</th>
<th>Plasma TG (mg/dL)</th>
<th>Plasma NEFA (mEq/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>504 ± 21</td>
<td>790 ± 48</td>
<td>1.761 ± 0.196</td>
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<tr>
<td>Rivoglitazone (1 mg/kg)</td>
<td>162 ± 19*</td>
<td>140 ± 16*</td>
<td>0.311 ± 0.023*</td>
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<tr>
<td>Pioglitazone (100 mg/kg)</td>
<td>184 ± 19*</td>
<td>221 ± 43*</td>
<td>0.428 ± 0.081*</td>
</tr>
<tr>
<td>Wy-14643 (100 mg/kg)</td>
<td>469 ± 29</td>
<td>301 ± 30*</td>
<td>1.003 ± 0.074*</td>
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</tbody>
</table>

Data are expressed as the mean ± S.E.M. (n = 5). *P<0.05, compared with the control group by a t-test.
the expressions of ACO, CPT1b, and ECH/HAD and decreased the expression of ApoCIII. All of the compounds decreased the expression of the gluconeogenic enzymes, G6Pase and PEPCK. Rivoglitazone and pioglitazone did not affect the expression levels of PK (liver) and PDK4, which are related to glucose utilization, but they were decreased and increased, respectively, by Wy-14643.

In the heart, rivoglitazone decreased the expression levels of genes involved in β-oxidation, ACO, CPT1b, and MCAD (Fig. 5: a, b, c). As for the genes that participate in glucose utilization, rivoglitazone increased the expression levels of GLUT4, remarkably decreased those of PDK4, and did not affect those of PK (muscle) (Fig. 5: d, e, f). On the contrary, Wy-14643 increased the expression of ACO and PDK4 similarly in the liver. Rivoglitazone did not affect the expression of the genes involved in cardiac contractile function, MHCα, MHCβ, and SERCA2 (Fig. 5: g, h, i). The effect of pioglitazone on cardiac gene expression was almost the same as that of rivoglitazone.

**Discussion**

In this study, we evaluated the characteristics of rivoglitazone, a novel TZD-derivative PPARγ agonist, in vivo and in vitro as an antidiabetic agent.

We used a GAL4-driven reporter assay system to
investigate the capabilities of rivoglitazone to activate hPPARα, hPPARγ, and hPPARδ. Rivoglitazone increased the hPPARγ activity with the lowest EC₅₀ (0.22 μM) compared with pioglitazone and rosiglitazone. Based on the EC₅₀s, the potency of rivoglitazone to activate hPPARγ is estimated to be about 16.4- and 3.6-fold higher than those of pioglitazone (EC₅₀: 3.6 μM) and rosiglitazone (EC₅₀: 0.79 μM), respectively. Rivoglitazone slightly increased hPPARα activity at the highest concentration (29.8%), but the concentration (10 μM) was much higher than its own EC₅₀ to activate PPARγ. Therefore, we considered that rivoglitazone has little effect on hPPARα activity. Rivoglitazone did not activate hPPARδ at all up to 10 μM. In mouse PPAR reporter assays, we confirmed that rivoglitazone also potently activates the PPARγ and has similar selectivity for PPARγ over PPARα and PPARδ (data not shown). These results suggest that rivoglitazone is a potent and selective PPARγ agonist.

In male ZDF rats and db/db mice exhibiting severe hyperglycemia, rivoglitazone had highly potent antidiabetic effects compared with the clinically available PPARγ agonists. Based on the ED₅₀s, the glucose-lowering effect of rivoglitazone is 179- and 140-fold more potent than that of pioglitazone and rosiglitazone, respectively, in ZDF rats. Similarly, rivoglitazone had about 80-fold more potent plasma TG-lowering effect compared with both pioglitazone and rosiglitazone. In ZDF rats, we determined the plasma concentrations of rivoglitazone and pioglitazone after 14-day administra-
tion (data not shown). We found that the plasma concentrations of rivoglitazone linearly increased with the doses examined in this study. According to the pharmacokinetics and pharmacodynamics analysis, the EC$_{50}$ of the hypoglycemic effect of rivoglitazone was 123 ng/mL and that of pioglitazone was 1490 ng/mL in the mean plasma concentration. Accordingly, rivoglitazone is 12 times more potent than pioglitazone in the plasma concentration, which is consistent with the PPAR$_{y}$-activating potency estimated in vitro as discussed above (16.4-fold).

We confirmed the insulin-sensitizing effect of rivoglitazone by an euglycemic-hyperinsulinemic clamp study in insulin-resistant obese ZF rats. Because the plasma insulin levels in the rivoglitazone-treated group were lower than those in the control group in the steady-state period, the GIRs in the rivoglitazone-treated group might have been underestimated. Nonetheless, rivoglitazone increased the GIR by about 40% compared with the control at a dose of 0.1 mg/kg, which is about half of the ED$_{50}$ of the plasma glucose–lowering effect in ZDF rats. These results clearly indicate that amelioration of whole-body insulin resistance contributes to the antidiabetic effect of rivoglitazone.

It has been reported that the plasma insulin levels in ZDF rats decrease with aging because of pancreatic dysfunction and that pioglitazone increases pancreatic insulin content in diabetic mice as a result of a reduction in insulin demand by improving insulin sensitivity (7–9). The increase in pancreatic insulin content induced by rivoglitazone as well as by pioglitazone and rosiglitazone is thought to result from the preservation of the pancreatic β-cells, which is one of the important features of TZDs, especially in clinical practice, and is thought to be the basis for the durability of the glycemic control by these agents (10, 11). It has been reported that improvement of insulin sensitivity and/or relief of glucotoxicity caused by decreased plasma glucose levels may contribute to the preservation of the pancreatic β-cells induced by TZDs (9, 12). The potent insulin-sensitizing and glucose-lowering effect of rivoglitazone may result in effective preservation of pancreatic function. In addition, it has been suggested that amelioration of lipotoxicity and the anti-inflammatory effects of TZDs also contribute to the protection of pancreatic β-cells (13, 14). The mechanism of this increase in the pancreatic insulin content should be clarified in future studies.

In db/db mice, rivoglitazone strikingly increased the plasma adiponectin in comparison with pioglitazone. It has been reported that adiponectin, which is secreted from adipocytes, is increased by treatment with TZDs and that adiponectin has beneficial effects on diabetes and cardiovascular disease (15–17). The potent plasma adiponectin–increasing effect of rivoglitazone may contribute to the higher efficacy of the antidiabetic effect of this compound.

In the study examining the whole-body TG metabolism, the AUC$_{TG}$ after lipid load as well as the basal plasma TG levels is considered to be affected by both the TG elimination and production abilities. Rivoglitazone and Wy-14643 lowered the basal TG and the AUC$_{TG}$ to similar extents at doses of 0.1 and 100 mg/kg, respectively, which suggests that they exhibited similar net plasma TG–lowering effects. The TG elimination rate constant in the rats treated with rivoglitazone was significantly higher than that of the control group, although the TG production was not significantly different from that in the control group. In contrast, Wy-14643 only significantly increased the TG production. Together, it is suggested that rivoglitazone lowers the plasma TG levels primarily via an increase in the plasma TG elimination rate, and this effect of rivoglitazone is quite different from that of a PPAR$_{x}$ agonist, which strongly suppressed hepatic TG production. On the other hand, at a dose of 1 mg/kg, rivoglitazone showed a more pronounced TG-lowering effect and decreased the TG production in addition to the acceleration of the TG elimination (data not shown). The decrease of TG production by a higher dose of rivoglitazone may be a secondary effect because PPAR$_{y}$ agonists have been reported to reduce liver TG content, which is a major source of plasma TG at least in part due to lipolysis inhibition and augmentation of fatty acid uptake in the adipose tissue (18–20).

In the gene expression analysis in ZDF rats, the expression levels of ACO, CPT1b, and ECH/HAD were remarkably increased by Wy-14643 in the liver. These results are consistent with many reports showing that the PPAR$_{x}$ agonists increase fatty acid β-oxidation through the increase of its related genes, including ACO, CPT1b, and ECH/HAD, in the liver (21–23). In contrast, rivoglitazone reduced the expression of ACO, CPT1b, and ECH/HAD. Together with the reduction of MCAD expression, both mitochondrial and peroxisomal β-oxidation of fatty acids may not be increased but rather would be decreased in the livers of ZDF rats treated with rivoglitazone. On the other hand, pioglitazone did not cause any significant effect on the expression of ACO, CPT1b, and ECH/HAD. The difference between the effects of rivoglitazone and pioglitazone on these genes could be a secondary effect caused by the somewhat greater lipid-lowering effect of rivoglitazone compared with pioglitazone in this study and/or some possible differences in the target gene profiles between rivoglitazone and pioglitazone (24). Rivoglitazone as well as
pioglitazone decreased the expression levels of G6Pase and PEPCK, which are rate-limiting enzymes of hepatic gluconeogenesis. This finding is consistent with the previous reports in db/db mice (25) and ZDF rats (26) treated with PPARγ agonists and suggests that rivoglitazone may reduce hepatic glucose production. The increased expression of ACO, CPT1b, ECH/HAD, and PDK4 and the decreased expression of PK (liver) induced by Wy-14643 may result in an increase of fatty acid utilization as an energy source. Wy-14643 also decreased the expression of ApoCIII, which inhibits lipoprotein lipase activity. These results suggest, consistent with the TG elimination and production study described above and with previous reports (27, 28), that the plasma lipid–lowering effect of Wy-14643 may be mediated by the reduction of hepatic TG-rich lipoprotein production through the enhancement of fatty acid utilization in the liver.

It has been reported that in diabetes and insulin-resistant states, myocardial fatty acid utilization is increased and glucose uptake and subsequent oxidation are decreased, which leads to cardiac dysfunction due to reduced backup energy sources other than fatty acids and increased formation of oxygen radicals through up-regulated fatty acid β-oxidation (29–31). It has also been reported that the expression of GLUT4, a glucose transporter that plays a major role in insulin-dependent glucose uptake, was decreased and the expression of MCAD was increased in the hearts of ZDF rats compared with lean rats (32). Accordingly, the increased expression of GLUT4 and the decreased expression of ACO, CPT1b, and MCAD induced by rivoglitazone may contribute to improve cardiac metabolic abnormalities through promoting glucose utilization and reducing fatty acid β-oxidation in the hearts of ZDF rats. MHCs are contractile proteins and the shift from the adult isoform (MHCα) toward a fetal isoform (MHCβ) is implicated in cardiac dysfunction (33). The decreased expression of SERCA2 is also related to cardiac dysfunction because of dysregulation of Ca2+ handling in the myoplasma (34). Rivoglitazone had no effects on the expression levels of MHCs and SERCA2. It has been reported that GL-262570, a non-TZD PPARγ agonist, reduced the cardiac expression of ACO and PDK4 and that it improved cardiac contractile function with an increased rate of glucose oxidation in ZDF rats (32). Therefore, rivoglitazone may cause beneficial effects on cardiac function by modulating cardiac energy homeostasis.

In summary, we showed that rivoglitazone is a potent and selective PPARγ agonist and has a potent glucose-lowering effect via the improvement of insulin resistance in diabetic animal models. Rivoglitazone also reduced the plasma TG primarily through accelerating the TG clearance from the plasma. Gene expression analysis suggested that rivoglitazone may reduce hepatic glucose production and fatty acid β-oxidation in the liver and modulate the balance of the cardiac glucose/fatty acid metabolism, which is shifted toward fatty acid oxidation in diabetic states in ZDF rats. These superior antidiabetic characteristics of rivoglitazone observed in this study may lead to superior effectiveness in clinical practice.

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