Dual PPARα/γ activation provides enhanced improvement of insulin sensitivity and glycemic control in ZDF rats

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TYPE 2 DIABETES is a heterogeneous, progressive disorder initially characterized by impaired glucose tolerance and compensatory hyperinsulinemia and, in the later stages, by severe insulin resistance and impaired β-cell function. The syndrome is characterized by an imbalanced interplay between endocrine pancreatic function, insulin sensitivity of liver, muscle, and adipose tissues, and neural activity. This is associated with hyperglycemia, dyslipidemia, hypertension, and obesity and, in the long term, micro- and macrovascular complications, leading to impaired life quality and increased mortality (10, 12, 13, 17, 28, 37, 48). Unfortunately, none of the available drugs for clinical use has proved sufficiently efficacious in restoring normal glucose metabolism alone or in combination therapy as the disease progresses (35).

Previously, two classes of compounds, the thiazolidinediones (TZDs) and the fibrates, were empirically discovered by their ability to improve insulin sensitivity and lipidemia, respectively, in rodents. In type 2 diabetic patients, the TZDs reduce both hyperglycemia and the compensatory hyperinsulinemia but exert only marginal effects on plasma lipid parameters (3). In contrast, the fibrates are effective at lowering plasma triglycerides (TG) and free fatty acids (FFA) and increasing favorable high-density lipoprotein cholesterol via increased clearance and decreased synthesis of very low density lipoprotein (VLDL; see Ref. 45). In addition, they have been shown also to improve glycemic control in type 2 diabetic patients (23, 26).

The recent discovery that the nuclear peroxisome proliferator-activated receptor (PPAR)γ and PPARα potentially provides beneficial effects over existing PPARγ and α preferential drugs, respectively (7, 51), has provided the opportunity for application of target-directed approaches in the optimization of drug candidates for the treatment of type 2 diabetes. Consequently, agonists with dual PPARγ and α activity would potentially have beneficial effects superior to those obtained with drugs activating either of the PPAR subtypes alone.

Here we report on the effects on carbohydrate metabolism invoked by ragaglitazar, which is a non-TZD compound that was recently identified as a combined PPARγ and α agonist with a good pharmacokinetic profile and full PPARγ and α agonistic property both in vitro and in vivo (28, 54). We have investigated the effects of ragaglitazar treatment on glycemic control and insulin sensitivity in prediabetic and moderately diabetic Zucker diabetic fatty (ZDF) rats in comparison with the preferential PPARγ agonist rosiglitazone. TZDs have previously been shown capable of preventing or delaying the onset of diabetes (44, 47) but unable...
to reverse fully established diabetes in the ZDF rat (41). Therefore, we also conducted a late-intervention dose-response study with ragaglitazar and rosiglitazone in severely diabetic ZDF rats to explore the extent to which reversal of frank diabetes is feasible by dual activation of PPARγ and α.

**EXPERIMENTAL PROCEDURES**

**Animals**

ZDF (ZDF-fa/fa; genetic model) rats were housed under controlled ambient conditions after a 12:12-h light-dark cycle with light on at 6:00 AM and were fed Purina 5008 diet ad libitum with free access to water. All procedures were approved by the Danish Animal Experiments Inspectorate.

**Test Compounds**

Ragaglitazar [NNC 61-0029, (−)DRF2725] is an (S)-2-ethoxy-3-[4-((2-phenoxazin-10-yloxy)-phenyl)-propionic acid (28), structurally distinct from ligands, including TZDs, N-2-benzoylphenyl-1,tyrosine derivatives, and fbrates (50). Ragaglitazar is a full agonist of both PPARα and -γ receptors and has a potency and efficacy on PPARγ comparable to that of rosiglitazone (39), which was synthesized (8) and used as a PPARγ-preferential agonist reference compound. Control ZDF rats received the vehicle (0.2% carboxymethylcellulose + 0.4% Tween 80).

**Anesthesia and Surgical Preparation of Rats**

Catheters (Tygon S-50-5L, ID: 0.016 in.; OD: 0.031 in.; Portex Performance Plastics) were inserted (halothane anesthesia) in the left carotid artery (blood sampling) and jugular vein (infusions) and exteriorized on the back of the neck, as described (34). Prophylactic antibiotic (Streptocillin; Nordisk) on the day of surgery and the following day to improve their postsurgical recovery. Rats recovered for 5–7 days after the clamp study. Rats subjected to dual-energy X-ray absorptiometry (DEXA) were anesthetized using a mixture of fentanyl (0.05 mg/kg), fluanizone (2.5 mg/kg; Hypnorm; Janssen Pharma, Copenhagen, Denmark), and midazolam (1.25 mg/kg; Dormicum; Roche, Basel, Switzerland).

**Hyperinsulinemic Euglycemic Clamp Studies**

After an overnight fast (18 h), catheters were connected to the infusion system, and the rats were placed in clamp cages and allowed to settle for 45–60 min. A primed (80 μCi) continuous (0.8 μCi·kg⁻¹·min⁻¹) infusion of HPLC-purified [3,3H]glucose was given from 90 to 120 min for assessment of whole body glucose disappearance and appearance (Ra) using Steele’s non-steady-state equations (46). The hyperinsulinemic euglycemic clamp (0–120 min) was performed by a primed (65 μCi/kg over 3 min) constant infusion (10 μCi·kg⁻¹·min⁻¹) of insulin (Actrapid; Novo Nordisk) with 20% glucose (containing 10 μCi [3,3H]glucose/ml) infused at variable rates (GIR) to maintain euglycemia. At plasma glucose steady state (75 min), a bolus (40 μCi/rat) of 2-deoxy-[2,3H]glucose (2-DG) was injected (iv) to measure tissue-specific glucose utilization index (IRg; see Ref. 15). At the end of the clamp, rats were given a lethal dose of pentobarbital sodium, and tissues were excised and snap-frozen.

Endogenous glucose production rates (EGP) were calculated as tracer-determined Ra, minus GIR.

Rg was calculated as described (21). Values were not corrected by a “discrimination constant” for 2-DG in the glucose metabolic pathways; therefore, results represent the index of glucose utilization.

**DEXA**

Body composition was determined by DEXA (pDEXA Sabre, Stratec Medizintechnic; Norland Medical Systems, Pforzheim, Germany). The coefficient of variation, as assessed by 10 repeated measurements (repositioning of the rat between each measurement), was 3.73% for the fat tissue mass.

**In Vivo Expression of Various PPAR-Regulated Genes**

RNA was isolated by Trizol (Invitrogen) according to the manufacturer’s instructions. Total RNA was DNase treated, and reverse transcription reactions were performed using SuperScript II RT (GIBCO-BRL) following the manufacturer’s instructions. mRNA expression levels were determined using real-time fluorescent detection in a Lightcycler instrument (Roche) and the following primer combinations: adipin: forward, 5′-AACCCGGCACGCTTGACGAC-3′ and reverse, 5′-CCAGTTGAAATCTCAGG-3′; apolipoprotein CIII (ApoCIII): forward, 5′-TCCAGTTCATGAGTGTCATG-3′ and reverse, 5′-TCCAGTTCATGAGTGATGCTC-3′; apolipoprotein A1 (apoA1): forward, 5′-AAACCTGACTGATGGTGGCTG-3′ and reverse, 5′-AAACCTGACTGATGGTGGCTG-3′; apoCIII: forward, 5′-TCCAGTTCATGAGTGTCATG-3′ and reverse, 5′-TCCAGTTCATGAGTGATGCTC-3′; adipsin: forward, 5′-ATGCTTCATGAGTGATGCTC-3′ and reverse, 5′-ATGCTTCATGAGTGATGCTC-3′. mRNA expression levels were determined two times in each first-strand synthesis reaction and normalized to the expression levels of 36B4 mRNA using the following primers: forward, 5′-TAAATGCTCGTGGTGGTGGTGG-3′; and reverse, 5′-TAAATGCTCGTGGTGGTGGTGG-3′.

**Analytical Assays**

Blood (BG) and plasma glucose concentrations were analyzed by the glucose oxidase method using either an EBO Plus autoanalyzer (Eppendorf) or, during clamp studies, a YSI 2500 STAT (Yellow Spring Instruments).

Plasma concentrations of FFA (Wako Chemicals) and TG (Roche, Hvidovre, Denmark), as well as glycolated hemoglobin A1c (Hb A1c; Roche), were measured using a COBAS MIRA Plus autoanalyzer (Roche Diagnostic Systems). Plasma insulin (PI) concentration was measured by ELISA, as previously described (22).

3H and 14C counts in neutralized supernatants of deproteinized plasma samples and 2-[14C]DG and phosphorylated 2-[14C]DG [2,14C]DG-P counts in digested tissue samples before and after the Somogy extraction procedure (42) were measured in a scintillation counter. The Somogyi extraction procedure employed on the tissue samples removes both free intracellular 2-DG-P and any 2-DG incorporated in glycogen, and the value thus provides an estimate of the total Rg (33).

For determination of [3H]- and [14C]glucose specific activities, glucose concentrations in subfractions of the deproteinized plasma supernatants were measured spectrophotometrically using a glucose oxidase-based colorimetric method (Boehringer Mannheim).
Glycogen content in muscle tissues was measured by a hexokinase method (19). Liver TG were extracted and quantified by a Peridochrom Triglyceride CPO-PAP kit (Boehringer Mannheim; see Ref. 6). Liver glycogen was measured (Bio-Rad, Copenhagen, Denmark) as previously described (1). Liver glycogen phosphorylase (GPα + GPβ) activity and total protein content (Bio-Rad) was assayed in supernatants of homogenates of tissue samples, and glycogen phosphorylase activity was measured in the direction of glycogen breakdown from the photometric determination of the rate of NADPH formation in an assay coupled to phosphoglucomutase and glucose-6-phosphate dehydrogenase (29).

**Immunohistochemistry**

Immunohistochemical reagents were from Dako except guinea pig anti-insulin (ICN), goat anti-glucokinase (Santa Cruz), VectaStain Elite Kit and Vector Nova Red (Vector Laboratories), and monoclonal mouse anti-glucagon and anti-GLUT2 antibodies (Novo Nordisk). Sections were washed two times with Tris-buffered saline (TBS) plus Triton X-100 (TBS-T) and then one time with TBS if not mentioned otherwise. Antisera were diluted in 7% goat plus 3% rat serum (TBS-T) and then one time with TBS if not mentioned, two times with Tris-buffered saline (TBS) plus Triton X-100.

For GLUT2 staining, sections were incubated for 30 min with monoclonal mouse anti-GLUT2, 0.3 mg/ml, and stored for further biochemical and histological examination. Nonclamped rats (n = 4/group) were administered BrDU (100 mg/kg ip) for determination of β-cell proliferation index.

**Protocol 2: Late-Intervention Dose-Response Study**

Overtly diabetic, 16-kg-old male ZDF rats were used for a dose-response study with rosiglitazone (1.50, 3.00, and 6.00 mg·kg−1·day−1) and rosiglitazone (1.50, 3.00, and 6.00 mg·kg−1·day−1) lasting for 3 wk (Fig. 1). Compounds were administered two times daily by gavage to ensure continuous exposure. BG, PI, and HbA1c were measured. Total fat mass was determined by DEXA in rats. In one time daily dosing regimens. Sixty-four out of 90 ZDF rats with the highest HbA1c levels were assigned to eight treatment groups (8 rats/group) with matching HbA1c levels. Nonfasting BG, PI, and body weight were measured weekly. At the end of treatment, 24-h BG and PI profiles and HbA1c were measured. Total fat mass was determined by DEXA in the groups of rats treated with vehicle, ragaglitazar, and rosiglitazone at 3 and 6 mg·kg−1·day−1 (similar dosing regime as employed in protocol 1). Tissues of interest were isolated for gene expression analyses.

**RESULTS**

**Prevention and Early Intervention of Diabetes (Protocol 1)**

Body weight and food intake. Body weight and food and water intake data are shown in Table 1. Ragaglitazar and rosiglitazone increased body weight and 24-h

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communal food intake to the same extent as with vehicle. Water intake increased gradually during the treatment period in the vehicle-treated rats (data not shown), and, during the last week of the treatments, 24-h communal water intake was ~50% higher in control rats compared with both drug-treated groups.

**Glycemic control.** BG and PI levels during the treatments are shown in Fig. 2. In the prevention study, pretreatment BG levels were ~6 mmol/l in all groups. This level of BG remained unchanged in both the ragaglitazar- and rosiglitazone-treated rats in contrast to a gradual increase observed in the vehicle-treated rats, in which BG reached 16 mmol/l at the end of the study (Fig. 2A). After 10 days of treatment, a marked compensatory hyperinsulinemia was observed in the vehicle-treated control group. After an additional 4 wk of vehicle treatment, -cells were exhausted, as evidenced by reduced hyperinsulinemia despite a marked

![Fig. 1. Schematic outline of the two protocols employed in the present investigation. In protocol 1, two individual studies were initiated in prediabetic (prevention study) and moderately diabetic (early intervention study) Zucker diabetic fatty (ZDF) male rats. In both studies, three groups of rats were dosed orally by gavage with either vehicle, ragaglitazar (1.5 mg/kg two times daily), or rosiglitazone (3 mg/kg two times daily) for a total of 7 (prevention study) or 4 (early intervention study) wk. At the end of the treatment periods, subgroups of nonfasted rats were either killed for tissue harvest or clamped and subsequently killed for tissue harvest. In protocol 2, overtly diabetic male ZDF rats were used for a late intervention dose-response study with ragaglitazar (0.37, 0.75, and 1.50 mg/kg two times daily) and rosiglitazone (0.75, 1.50, and 3.00 mg/kg two times daily). Control animals received vehicle. Compounds were administered by gavage two times daily to ensure 24-h exposure and to enable an easy comparison with protocol 1. An additional group of rats was dosed only one time daily with ragaglitazar at the highest daily dose level (3.00 mg/kg one time daily) for comparison of once vs. twice daily dosing regimens. At the end of the study, total fat mass was determined by dual-energy X-ray absorptiometry (DEXA) in rats treated with vehicle and ragaglitazar at 1.5 and rosiglitazone at 3.0 mg/kg two times daily (similar dosing regime as employed in protocol 1). After 3 wk of treatment, tissues were isolated for gene expression studies.](image)

![Table 1. Body weight and food and water intake in the prevention and early-intervention studies (protocol 1)](table)

<table>
<thead>
<tr>
<th></th>
<th>Body Wt, g</th>
<th>Food Intake, g·rat⁻¹·24 h⁻¹</th>
<th>Water Intake, g·rat⁻¹·24 h⁻¹</th>
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<tr>
<td></td>
<td>Pretreatment</td>
<td>Posttreatment</td>
<td>Change</td>
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<td><strong>Prevention study</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>204 ± 5</td>
<td>361 ± 5</td>
<td>156 ± 6</td>
</tr>
<tr>
<td>Ragaglitazar</td>
<td>196 ± 3</td>
<td>503 ± 10a</td>
<td>304 ± 8a</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>206 ± 5</td>
<td>512 ± 8a</td>
<td>304 ± 5a</td>
</tr>
<tr>
<td><strong>Early-intervention study</strong></td>
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<td></td>
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<tr>
<td>Vehicle</td>
<td>354 ± 3</td>
<td>391 ± 6</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>Ragaglitazar</td>
<td>353 ± 6</td>
<td>538 ± 10a</td>
<td>184 ± 5a</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>351 ± 4</td>
<td>534 ± 6a</td>
<td>185 ± 3a</td>
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</table>

Data are means ± SE; n = 14–16 rats/group. Communal food and water intake were measured during the last week of treatment. *P < 0.05 vs. vehicle in the same study.
hypoglycemia. Hyperinsulinemia seemed less pronounced in the ragaglitazar-treated animals compared with the rosiglitazone treatment during the entire course of the study (Fig. 2B).

In the early-intervention study, pretreatment BG levels were ~16 mmol/l in all groups and increased further to 22 mmol/l in the vehicle-treated group at the end of treatment. In contrast, both drug treatments restored euglycemia after 3 wk (Fig. 2C). Compensatory hyperinsulinemia was present in all groups before the treatment, and at the end of the study β-cells were exhausted in the vehicle-treated group, as evidenced by insufficient compensatory hyperinsulinemia despite marked hyperglycemia. In contrast, the compensatory hyperinsulinemia was reduced significantly by both drug treatments, with ragaglitazar causing the greater insulin-lowering effect (Fig. 2D).

A similar pattern regarding nonfasting BG and PI levels was observed among the three groups of animals during the 24-h profiles obtained at the end of both the prevention and intervention studies (Fig. 3). In both studies, euglycemia was maintained throughout the 24 h by both drug treatments (Fig. 3, A and C), but consistently lower insulin concentrations were observed in ragaglitazar- than in rosiglitazone-treated rats (Fig. 3, B and D).

In the prevention study, ragaglitazar and rosiglitazone both significantly reduced HbA1c compared with the vehicle-treated rats (Table 2). In the early intervention study, HbA1c increased in the vehicle-treated group, whereas both drug treatments almost normalized HbA1c compared with our historical values obtained in age-matched lean ZDF rats (3.8 ± 0.3%, n = 10).

**Plasma lipids.** Results are given in Table 3. In the prevention study, ragaglitazar reduced FFA by 20% compared with vehicle treatment, whereas rosiglitazone had no such effect. Treatment with either compound reduced plasma TG levels by 80% compared with vehicle. In the early-intervention study, rosiglitazone increased FFA by ~35% compared with vehicle, whereas both drugs reduced TG by ~70% compared with vehicle.

**Insulin sensitivity.** Results from the clamp studies are presented in Fig. 4 and Table 4. In both studies, the GIR required to maintain matching glycemic levels during the clamp were higher in the ragaglitazar-treated rats compared with both vehicle and rosiglitazone-treated rats. Basal EGP was reduced significantly by both drugs compared with vehicle (Table 4). In both studies, the insulin-mediated suppression of EGP during the clamp tended to be more pronounced in ragaglitazar- than in rosiglitazone-treated rats, although this did not reach statistical significance.

In the prevention study, both ragaglitazar and rosiglitazone treatment increased the insulin-stimulated $R_g$ 2.9- and 2.3-fold, respectively ($P < 0.05$ vs. vehicle), in the “fast-twitch white fiber” muscle tissue (white gastrocnemius). This effect was only inflicted by ragaglitazar in the early-intervention study (4.7-fold, $P < 0.05$ vs. vehicle). $R_g$ in the muscles of the fast (red gastrocnemius)- and slow (soleus)-twitch red fiber types were overall unaffected by either of the two drug treatments. In both studies, treatment with ragaglitazar increased $R_g$ in the white adipose tissue ~2.5-fold compared with vehicle treatment ($P < 0.05$) and 1.5-fold compared with rosiglitazone treatment ($P < 0.05$; data not shown).
Biochemical parameters in tissues. Results are shown in Table 5. Both compounds significantly decreased hepatic glycogen content in the clamped rats compared with vehicle, with ragaglitazar being the more efficacious. Liver glycogen phosphorylase activity of both clamped (prevention study) and nonclamped (early intervention study) rats was reduced by the ragaglitazar treatment compared with vehicle and rosiglitazone treatment. In both studies, rosiglitazone treatment increased liver TG content in clamped rats compared with vehicle treatment, whereas ragaglitazar either had no such effect (early-intervention study) or even significantly reduced (prevention study) the liver TG content. The drugs did not exert significant changes in muscle (extensor digitorum longus and soleus) glycogen content obtained from the clamped and nonclamped rats compared with vehicle. Likewise, there was no effect of either rosiglitazone or ragaglitazar treatment on hepatic glycogen content in the nonclamped rats compared with vehicle.

Histology. β-Cell proliferation was measured by BrDU incorporation. Figure 5A depicts representative fields of BrDU incorporation in insulin double-stained sections from rats included in the prevention study. In both studies, however, the labeling index of β-cells was low, and relatively more non-β-cells and exocrine cells contained BrDU. There were no statistically significant differences among the three groups, either in the prevention or in the early-intervention study. The staining intensity for insulin in β-cells was higher and less variable in ragaglitazar- and rosiglitazone-treated rats, and the islets were more regular with less ragged rims than in the vehicle-treated rats. The same trend was found in the early-intervention study (data not shown). Separate sections were stained for β- and non-β-cells, as shown in Fig. 5B (prevention study). The volume fractions of β- and non-β-cells in ragaglitazar- and rosiglitazone-treated rats were not different from the vehicle-treated rats. The appearance of the islets showed a more regular distribution of non-β-cells in or near the rim of the islets in the ragaglitazar- and rosiglitazone-treated rats. In the intervention study, the overall morphology of the islets was also more regular and rounded, although minor streaks of fibrosis revealed that the rats had developed diabetes before treatment was commenced (data not shown). GLUT2 staining intensity in β-cells from the ragaglitazar- and rosiglitazone-treated rats in the prevention study was higher than that of the vehicle-treated rats and more widely distributed over the plasma mem-

Table 2. Hb A1c levels obtained in the prevention and early intervention studies (protocol 1)

<table>
<thead>
<tr>
<th></th>
<th>Pre-Hb A1c</th>
<th>Post-Hb A1c</th>
<th>ΔHb A1c</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prevention study</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>ND</td>
<td>8.13 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>Ragaglitazar</td>
<td>ND</td>
<td>4.26 ± 0.23*</td>
<td></td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>ND</td>
<td>3.91 ± 0.03*</td>
<td></td>
</tr>
<tr>
<td><strong>Early-intervention study</strong></td>
<td></td>
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</tr>
<tr>
<td>Vehicle</td>
<td>6.39 ± 0.16</td>
<td>8.89 ± 0.20</td>
<td>2.50 ± 0.17</td>
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<tr>
<td>Ragaglitazar</td>
<td>6.22 ± 0.20</td>
<td>4.51 ± 0.06*</td>
<td>−1.71 ± 0.16*</td>
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<tr>
<td>Rosiglitazone</td>
<td>6.37 ± 0.15</td>
<td>4.95 ± 0.12*</td>
<td>−1.42 ± 0.09*</td>
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</table>

Data are means ± SE; n = 14–16 rats/group. Units are %. Δ, Change; ND, not determined. *P < 0.05 vs. vehicle in the same study.
brane (Fig. 5C). In both studies, GLUT2 staining intensity in hepatocytes from the rats treated with ragaglitazar and rosiglitazone was higher compared with vehicle-treated rats (data not shown). Improved glucokinase staining patterns in β-cells were found in ragaglitazar- but not in rosiglitazone-treated rats (data not shown). In both studies, the staining intensity of glucokinase in liver was equally low, and the distribution in liver cells was quite similar in all three groups of ZDF rats (data not shown).

Late-Intervention Dose-Response Study (Protocol 2)

Glycemic control and body weight. At the end of the 3-wk treatment period, ragaglitazar more efficaciously improved the 24-h BG profile at all dose levels employed compared with rosiglitazone, which only partly had this effect at the highest dose (Fig. 6A). The improvement of glycemic control was paralleled by a dose-related increase in body weight (Fig. 6B). PI concentrations did not differ between the groups at any time point during the treatment (data not shown). Hb A1c did not change in the control group, indicating that the diabetic state was stabilized during the course of this study (data not shown). The reduction in Hb A1c caused by rosiglitazone was 1% at all three dose levels, suggesting that the maximal response to this compound was reached. Ragaglitazar reduced Hb A1c by 0.7% at 0.75 mg·kg⁻¹·day⁻¹ and by 2.3% at 1.5 mg·kg⁻¹·day⁻¹. No further reduction in Hb A1c was observed at 3 mg·kg⁻¹·day⁻¹, indicating that the maximum response to the compound was reached at 1.5 mg·kg⁻¹·day⁻¹. The reduction of Hb A1c observed in the groups administered one time daily and two times daily with ragaglitazar at 3 mg·kg⁻¹·day⁻¹ was identical. The reduction in Hb A1c correlated positively with the increase in body weight in both ragaglitazar (r = 0.747, P < 0.000005) and rosiglitazone (r = 0.772, P < 0.00001)-treated rats.

![Fig. 4. Plasma glucose concentration and glucose infusion rates during euglycemic hyperinsulinemic clamp studies performed at the end of the prevention (A) and early-intervention (B) studies in vehicle (■), ragaglitazar (●), and rosiglitazone (○)-treated ZDF rats (protocol 1). Data are means ± SE; n = 4–6 rats/group.](http://ajpendo.physiology.org/)
comparable to that of the ragaglitazar-treated rats and correspondingly also had a relative body fat mass a reduction in Hb A1c by at least 1.75% and had a (Table 6). All ragaglitazar-treated rats responded with 1.5-fold in the ragaglitazar- and rosiglitazone-treated however, all responded with a reduction in Hb A1c.

The relative body fat mass was increased 2- and 1.5-fold in the ragaglitazar- and rosiglitazone-treated rats, respectively, compared with vehicle treatment (Table 6). All ragaglitazar-treated rats responded with a reduction in Hb A1c by at least 1.75% and had a relative body fat mass of at least 56%, whereas five out of eight rats treated with rosiglitazone showed no significant reduction in Hb A1c [−0.49 ± 0.40 vs. −0.05 ± 0.18% for vehicle, not significant (NS)] and correspondingly had a relative body fat mass comparable to that of the vehicle-treated rats (36 ± 1 vs. 31 ± 2% for vehicle, NS). The three remaining rosiglitazone-treated rats, however, all responded with a reduction in Hb A1c comparable to that of the ragaglitazar-treated rats (−2.08 ± 0.47 vs. −2.26 ± 0.23% for ragaglitazar, NS) and correspondingly also had a relative body fat mass comparable to that of the ragaglitazar-treated rats (60 ± 0.4 vs. 59 ± 1% for ragaglitazar, NS).

In vivo activation of PPARα and -γ. The PPARα-responsive gene BIF2E was induced in liver tissue up to 9.4-fold, whereas no response was detected in the animals treated with rosiglitazone (Fig. 7, top). Likewise, mRNA levels of ACO were induced to a maximum of 3.1-fold in liver in a dose-dependent manner by ragaglitazar, and treatment with rosiglitazone did not reveal any changes on this mRNA (data not shown). Hepatic ApoCIII gene expression was reduced 1.7-fold at the higher doses (1.5 mg/kg two times and 3 mg/kg one time daily) of ragaglitazar and was not affected by rosiglitazone, lending further support to different actions of the two compounds in liver tissue. A similar pattern of differential action by the two compounds in the liver were, although to lesser extent, noted on the levels of LPL mRNA, which was slightly upregulated (1.5-fold) by ragaglitazar and not regulated by rosiglitazone. In contrast, both ragaglitazar and rosiglitazone reduced the mRNA levels of liver phosphoenolpyruvate carboxykinase (PEPCK) approximately twofold at all doses. In the adipose tissue, adipins, aP2, and LPL levels were all dose-dependently increased by both ragaglitazar and rosiglitazone. Adipin mRNA levels were increased a maximum of 6.3- and 5-fold upon treatment with ragaglitazar and rosiglitazone, respectively (Fig. 7, bottom). aP2 gene expression was induced up to 2.3-fold by ragaglitazar and 2-fold by rosiglitazone, whereas the inductions for LPL reached 2.9- and 2.4-fold, respectively, for the two compounds. Adipocyte PEPCK mRNA was elevated 3.2- and 1.8-

Table 5. Tissue glycogen and triglyceride content and liver glycogen phosphorylase activity in samples obtained from clamped and nonclamped rats at the end of the prevention and intervention studies (protocol 1)

<table>
<thead>
<tr>
<th>Data From Clamped Rats (n = 4–6/group)</th>
<th>Data From Nonclamped, Nonfasted Rats (n = 4/group)</th>
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<tr>
<td>Study type</td>
<td>Liver TG, μmol/g</td>
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<tr>
<td></td>
<td>Liver GP&lt;sub&gt;act&lt;/sub&gt;, nmol/min·mg&lt;sup&gt;-1&lt;/sup&gt;</td>
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<td></td>
<td>Liver, Soleus, EDL</td>
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**Prevention study**

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<tr>
<th>Vehicle</th>
<th>14.5 ± 1.1</th>
<th>82 ± 12</th>
<th>176 ± 154</th>
<th>23.9 ± 3.0</th>
<th>37.1 ± 2.5</th>
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<tbody>
<tr>
<td>Ragaglitazar</td>
<td>8.8 ± 0.4&lt;sup&gt;††&lt;/sup&gt;</td>
<td>46 ± 5&lt;sup&gt;††&lt;/sup&gt;</td>
<td>36 ± 13&lt;sup&gt;††&lt;/sup&gt;</td>
<td>27.0 ± 3.0</td>
<td>37.5 ± 5.3</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>18.0 ± 1.5&lt;sup&gt;†&lt;/sup&gt;</td>
<td>71 ± 3</td>
<td>73 ± 12&lt;sup&gt;†&lt;/sup&gt;</td>
<td>29.2 ± 2.3</td>
<td>33.8 ± 2.4</td>
</tr>
</tbody>
</table>

**Early-intervention study**

<table>
<thead>
<tr>
<th>Truck</th>
<th>11.6 ± 0.7</th>
<th>35 ± 4</th>
<th>195 ± 29</th>
<th>30.3 ± 2.5</th>
<th>34.6 ± 5.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ragaglitazar</td>
<td>10.0 ± 1.6&lt;sup&gt;†&lt;/sup&gt;</td>
<td>27 ± 3</td>
<td>70 ± 7&lt;sup&gt;††&lt;/sup&gt;</td>
<td>29.2 ± 3.5</td>
<td>32.8 ± 1.5</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>16.7 ± 1.0&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>32 ± 3</td>
<td>107 ± 15&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>26.0 ± 1.6</td>
<td>32.7 ± 3.2</td>
</tr>
</tbody>
</table>

Data are means ± SE; n, no. of rats. GP<sub>act</sub>, glycogen phosphorylase activity; EDL, extensor digitorus longus. 1Data expressed per g wet tissue. 2Data expressed per mg protein. 3P < 0.05 vs. vehicle within study. 4P < 0.05 vs. rosiglitazone within same study. 5P < 0.05 vs. vehicle in the same study.
fold by treatment with ragaglitazar and rosiglitazone, respectively.

DISCUSSION

The present study demonstrates that combined activation of PPARα and -γ markedly prevents and ameliorates insulin resistance in the diabetic ZDF rat and with superior beneficial impact on glycemic control over that achieved upon activation of PPARγ only. Recently, a limited number of investigations have reported on the effects of dual PPARα/γ agonism in animal models of dyslipidemia, insulin resistance, and/or type 2 diabetes (5, 11, 14, 30, 40, 54). In those studies, the focus has been directed toward glycemic control and diabetic late complications in the ZDF rat or on insulin sensitivity and lipid metabolism in obese Zucker or high-fat-fed rats. In contrast, the present investigation aimed to explore the comparable effects of PPARγ and dual PPARα/γ agonism on both glycemic control and insulin sensitivity during different stages.
of diabetes in the ZDF rat, enabling an evaluation of the linkage between the two parameters within the same animal model that we consider relevant for the clinical situation.

Most importantly and in general for both the prevention and early-intervention studies (see protocol 1) is the finding that normalization of glycemic control was obtained with a generally reduced requirement for insulin in ragaglitazar-treated rats compared with that of rosiglitazone treatment. This suggests that dual PPARα/γ activation by ragaglitazar more effectively improves insulin sensitivity relative to that obtained upon PPARγ activation by rosiglitazone. In both studies, this observation was further substantiated by the fact that significantly increased GIR were required to maintain euglycemia in ragaglitazar-treated animals during the clamp studies. The importance hereof is that maintenance of euglycemia together with the improved insulin sensitivity brought about by the ragaglitazar treatment result in a reduced burden on the β-cells, as evidenced by reduced PI levels, which, in the long term, may delay or even prevent β-cell exhaustion and consequently the development of frank diabetes. Although not accessed directly, it is reasonable to assume that ragaglitazar treatment also more effectively improved insulin sensitivity in the late-intervention study (see protocol 2) and that this is the reason why further improvement of glycemic control was obtained compared with rosiglitazone treatment, since PI levels were indistinguishable between the study groups. The reason why PI levels were unaffected by the drugs is probably that the β-cells had irreversibly deteriorated already at the time of initiation of the late-intervention treatment. Therefore, in an attempt to compensate for the hyperglycemia present to various degrees in all groups, maximal insulin secretion was required.

The physiological consequences of simultaneous activation of both PPARα and PPARγ suggest that additional therapeutic benefit can be achieved on glycemic control using a dual PPARα/γ agonist compared with treatment with a preferential PPARγ agonist. This is supported by the recent findings of improved whole body and muscle insulin resistance and hyperinsulinemia.

Fig. 6. Twenty-four-hour blood glucose profiles obtained at the end of the treatment period and the corresponding area under the curve (AUC, inset; A) as well as changes in body weight (B) and reduction in hemoglobin (Hb) A1c (C) in the late-intervention dose-response study (protocol 2). Values are means ± SE for n = 8/group. x, Vehicle; Δ, ragaglitazar (0.37 mg/kg two times daily); ▼, ragaglitazar (0.75 mg/kg two times daily); ■, ragaglitazar (1.5 mg/kg two times daily); ●, ragaglitazar (3.0 mg/kg one time daily); ○, rosiglitazone (0.75 mg/kg two times daily); □, rosiglitazone (1.5 mg/kg two times daily); ○, rosiglitazone (3.0 mg/kg two times daily). Inset: open bar, vehicle; filled bars, ragaglitazar; hatched bars, ragaglitazar. Statistics based on repeated-measures ANOVA of 24-h blood glucose (BG) data. *P < 0.001 vs. b-e and h; b-P < 0.001 vs. c-e; c-P < 0.05 vs. e-h; d-P < 0.001 vs. f-h; e-P < 0.01 vs. b-h; F: P < 0.001 vs. b-h; b-P < 0.001 vs. d-e; c-P < 0.001 vs. f-g; d-P < 0.001 vs. f-h; e-P < 0.009 vs. f-h. C: ●, ragaglitazar two times daily; ▼, ragaglitazar one time daily; ○, rosiglitazone two times daily. Data are means ± SE; n = 8 rats/group. *P < 0.05 vs. all rosiglitazone dose levels and ragaglitazar at 0.37 mg/kg two times daily (ANOVA; vehicle group included).
emia in various models of insulin resistance and type 2 diabetes upon activation of PPARα (18, 31, 52, 53). The mechanisms underlying those findings are still unclear, but apparently they differ from that known for PPARγ agonists, which reduce hepatic glucose production and improve glucose disposal to peripheral tissue.

Ragaglitazar treatment altered gene expression in a PPARα- and γ-dependent manner, as evidenced by changes observed in the liver and in adipose tissue. In contrast, administration of rosiglitazone altered gene expression in a PPARγ-dependent manner, since changes in PPAR target gene levels could be found solely in the adipose tissue. Whereas treatment with ragaglitazar induced the expression of genes in the peroxisomal β-oxidation pathway such as liver ACO and BIFEZ, these genes were unaffected by rosiglitazone. This indicates that ragaglitazar, like the PPARα-activating fibrates, elicits augmented peroxisomal fatty acid oxidation. Similarly, as for fibrates and other PPARα agonists that have been reported to reduce the liver expression of ApoCIII, a major component of VLDL particles, it was observed that ragaglitazar but not rosiglitazone elicited this change. Both compounds reduced the expression of PEPCK in liver. This may, however, be a secondary response to the increase of insulin sensitivity in the animals, leading to a decrease in hepatic gluconeogenesis. In the adipose tissue, treatment with either ragaglitazar or rosiglitazone led to increased expression of known adipocyte PPAR target genes. For all genes tested (adipin, aP2, and LPL), the maximum degree of increase brought about by ragaglitazar was ~20% higher than that obtained with rosiglitazone, suggesting a quantitatively slightly stronger in vivo effect of ragaglitazar. Adipose tissue expression of PEPCK was also regulated by both compounds, which may offer a clue to the mechanism whereby increased reesterification of FFA, and thus sequestration into fat, is attained (16). In concert, the data clearly demonstrate that the two compounds exert qualitatively similar effects on the adipose tissue.

**Table 6.** Body composition as measured by DEXA and reduction in Hb A₁c, obtained in the late-intervention dose-response study (protocol 2)

<table>
<thead>
<tr>
<th>Late-Intervention Dose-Response Study</th>
<th>Body Wt, g</th>
<th>Fat Mass</th>
<th>Reduction in Hb A₁c, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>392 ± 7</td>
<td>122 ± 10</td>
<td>−0.05 ± 0.18</td>
</tr>
<tr>
<td>Ragaglitazar (3 mg·kg⁻¹·day⁻¹)</td>
<td>491 ± 7†</td>
<td>280 ± 6‡</td>
<td>−2.26 ± 0.23†</td>
</tr>
<tr>
<td>Rosiglitazone (6 mg·kg⁻¹·day⁻¹)</td>
<td>444 ± 15‡</td>
<td>204 ± 26‡</td>
<td>−1.09 ± 0.41‡</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 8 rats/group. *P < 0.0005 vs. vehicle in the same study. †P < 0.05 vs. rosiglitazone in the same study. ‡P < 0.05 vs. vehicle in the same study.
Recently, a study with the dual PPARα/γ agonist LY-465608 (14) demonstrated that complete restoration of euglycemia could be obtained in ZDF male rats during treatment in the early diabetic period (8–12 wk of age). That effect was reported along with a lack of augmentation of food consumption and an increase in body weight, as seen with rosiglitazone. Unfortunately, that study evaluated the effects of LY-465608 on insulin sensitivity in obese, nondiabetic Zucker rats only and not in the ZDF rat as well. Furthermore, the study did not include a rosiglitazone-treated group for comparison in the assessment of insulin sensitivity. Nevertheless, their first observation stands in contrast to the results obtained in the present study (protocol I) in which ragaglitazar and rosiglitazone both maintained normal glycemic control equally well, but at the same time both also augmented food consumption and increased body weight. The discrepancy between the results obtained in the two studies may well be explained by different binding and activation ratios between PPARα and γ brought about by ragaglitazar and LY-465608. For example, we have observed similar beneficial effects to those reported for LY-465608 on body weight, food consumption, and early glycemic control in ZDF rats during the characterization of another dual PPARα/γ agonist (NNC 61-4655), which, like LY-465608, has a higher α than γ binding and activation potency compared with ragaglitazar. However, when treatment with NNC 61-4655 was extended beyond the 12 wk of age in the ZDF rat (from 11 to 17 wk of age), the effect on glycemic control and insulin sensitivity ceased despite continued reduction in food intake and less increase in body weight compared with ragaglitazar (Brand CL, unpublished data). Therefore, it remains to be demonstrated how well dual PPARα/γ agonists with higher α than γ binding and activation potencies such as LY-465608 are suited for treatment of later stages of type 2 diabetes.

Independent of the type of PPAR agonist employed in the present study, it appeared that an improvement of HbA1c in the ZDF rat occurred concomitantly with treatment of the early stages of type 2 diabetes has previously also been demonstrated employing chronic insulin therapy in the ZDF rat (43), which is in support of this notion.

Insulin-stimulated (clamp) Rglc and glycogen content were both measured only in the soleus muscle, which, on the other hand, seemed unaffected by the drug treatments. However, both drugs increased Rglc in white gastrocnemius muscle, which represents ~70% of the hindlimb musculature, whereas only ragaglitazar improved Rglc in white adipose tissue (2). The differential effects of the two drugs on glucose uptake by muscle and fat tissues could therefore partly account for the extrahepatic effects observed.

Somewhat surprisingly, the liver glycogen content in all groups of nonfasted rats was near maximal, which is considered normal in healthy rats. However, despite an impaired glucose homeostasis in vehicle-treated rats, non-insulin-dependent (GLUT2) hepatic glucose uptake was probably markedly increased because of the mass action of glucose (BG >18 mmol/l). The consequent increase in intracellular glucose 6-phosphate concentrations would then activate glycogen synthase, which in turn will lead to the high glycogen content observed in the vehicle-treated rats.

Compared with vehicle-treated rats, postclamp liver glycogen stores were reduced significantly after an overnight fast in drug-treated animals from both studies, which is considered normal in fasted, healthy rats, since hepatic glycogen content is not expected to increase during a clamp (9). The reduced liver glycogen phosphorylase activity found in some but not all ragaglitazar-treated groups of rats would expectedly decrease hepatic glycogen breakdown and thereby reduce hepatic glucose production. This is also supported by the finding that insulin-mediated suppression of EGP seemed more pronounced by ragaglitazar than by rosiglitazone treatment. Whether this is a direct PPARα-mediated effect by the ragaglitazar treatment or caused indirectly via an increase in hepatic insulin sensitivity resulting from, e.g., marked reduction in hepatic TG levels, is not clear (54).

Histological analyses of the pancreas were only carried out on a small subset of rats (4 rats/group). We observed a partial restoration of both GLUT2 and glucokinase in β-cells from the drug-treated ZDF rats, but both were still far from being normal. Troglitazone has previously been shown to partially normalize the reduced GLUT2 in β-cells from ZDF rats (20). PPARγ is expressed in β-cells, and peroxisome proliferator response element is found in the GLUT2 (24) and in the glucokinase promoter region (25). We have at present no explanation for the lack of change for the GLUT2 and glucokinase expression in the liver, except that the indirect effect of the reduced insulin resistance and improved glucose metabolism in liver is less important than in the β-cells. In vehicle-treated ZDF rats, the islets showed substantial fibrosis, whereas there was little fibrosis in the ragaglitazar- and rosiglitazone-treated ZDF rats. In the early-intervention study, the
general picture from the immunostainings of β-cell markers and of the BrDU incorporation was quite similar to that of the prevention study. Because the animals were diabetic at the start of the experiment, there was, however, some fibrosis present in a number of the big islets.

In summary, effects of dual PPARα/γ (ragaglitazar) and only PPARγ (rosiglitazone) agonism on glycemic control and insulin sensitivity were studied during different stages of diabetes in the ZDF rat. In prevention and early-intervention settings, both drugs normalized glycemic control. Yet, ragaglitazar-treated rats generally required less insulin compared with rosiglitazone treatment, suggesting that dual PPARα/γ activation more effectively improves insulin sensitivity compared with activation of PPARγ alone. This was substantiated by the hyperinsulinemic euglycemic clamp technique for assessment of insulin sensitivity. During treatment of frank diabetes, ragaglitazar improved glycemic control more effectively than rosiglitazone without affecting circulating insulin, suggesting that improvement of insulin sensitivity by treatment with ragaglitazar, over the long term, may result in a reduced burden on the β-cells and consequently increase their likelihood of survival. Dual PPARα/γ activation by ragaglitazar may therefore represent a superior therapy over that of PPARγ activation alone by, e.g., rosiglitazone in early, intermediate, and late stages of type 2 diabetes.

We thank S. Gronemann, B. S. Hansen, M. B. Jappe, W. Listov-Saabye, S. Primdahl, P. Rothe, A. Seneca, and A. Vinterby for excellent technical assistance, and the staff at the animal unit for daily care, monitoring, and dosing of animals.

REFERENCES

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31. Sreenan S, Torsvik MV, Brand CL, Sturis J, Woldike 854 PPAR


