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

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The recent discovery that the nuclear peroxisome proliferator-activated receptor (PPARγ) and PPARα potentially provides beneficial effects over existing PPARγ and α preferential drugs, respectively, in treatment of type 2 diabetes. We examined the effects of the dual PPARα/γ agonist ragaglitazar on hyperglycemia and whole body insulin sensitivity in early and late diabetes stages in Zucker diabetic fatty (ZDF) rats and compared them with treatment with the PPARγ preferential agonist rosiglitazone. Despite normalization of hyperglycemia and Hb A1c and reduction of plasma triglycerides by both compounds in both prevention and early intervention studies, ragaglitazar treatment resulted in overall reduced circulating insulin and improved insulin sensitivity to a greater extent than after treatment with rosiglitazone. In late intervention therapy, ragaglitazar reduced Hb A1c by 2.3% compared with 1.1% by rosiglitazone. Improvement of insulin sensitivity caused by the dual PPARα/γ agonist ragaglitazar seemed to have beneficial impact over that of the PPARγ-preferential activator rosiglitazone on glycemic control in frankly diabetic ZDF rats.

euglycemic clamp; peroxisome proliferator-activated receptor; ragaglitazar; rosiglitazone; type 2 diabetes; Zucker diabetic fatty

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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-phenoxyazin-10-yloxy)-phenyl]-propionic acid (28), structurally distinct from ligands, including TZDs, N-(2-benzoylphenyl)-1-tyrosine derivatives, and fibrates (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-preferring 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-HL, ID: 0.016 in.; OD: 0.031 in.; Norton 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 (Streptocil; Boehringer Ingelheim) and analgesic treatments (Anorphin; Jansen 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 μU·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-[14C]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.

Ra 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′-AACCCGGACCGCTGCGAC-3′ and reverse, 5′-TGCAAGTGTCCTCGGTTG-3′; adipocyte lipid-binding protein (aP2): forward, 5′-ATGCTTTGTGGGAACCTGG-3′ and reverse, 5′-CCAGTTGGAAGGAACTCCTGG-3′; apolipoprotein CIII (ApoCIII): forward, 5′-TCAGTGATCGAGTGGCAT-3′ and reverse, 5′-TCCATGAGCCCTGGACACAGG-3′; acyl-Coa oxidase (ACO): forward, 5′-TAATCTGCTGTCGGTCG-3′ and reverse, 5′-GCTGCTTACATAATCCCTTAAGG-3′; enoyl-Coa hydratase-3-hydroxyacyl-Coa dehydrogenase bifunctional enzyme (BIFEZ): forward, 5′-GACATGAGTATCGAGCGCAGG-3′ and reverse, 5′-CT-GGGATTATTACGTACGGG-3′; lipoprotein lipase (LPL): forward, 5′-GAAACACCTCACACAGGCAAAG-3′ and reverse, 5′-GATAACGATACGGCCGTTGC-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′-TAAAGACTGGAGA-3′ and reverse, 5′-AGAAGCGGAGGT-CAGGGC-3′.

**Analytical Assays**

Blood (BG) and plasma glucose concentrations were analyzed by the glucose oxidase method using either an EBIO 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 Diagnostik 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 Somogy 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 Ra (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 (GP ${\alpha}$ + GP ${\beta}$) 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 in TBS-T. For the double staining of -cells, 4% swine, 4% goat, and 3% rat serum in TBS-T was used for dilutions. Paraffin sections, 3 ${\mu}$m thick, were deparaffinized, endogenous peroxidase was blocked with 0.5% H 2O 2 in ethanol, and antigen retrieval treatment was carried out in 0.01 M citrate buffer (pH 6.0) preheated to 90°C for 3 × 5 min at 80% in a microwave oven (Polar Patent, Umeå, Sweden). Sections were cooled, rinsed, and incubated with avidin- and biotin-blocking solutions. Bromodeoxyuridine (BrDU; Sigma) and insulin double staining, to render BrDU-containing nuclei black and the -cell cytoplasm reddish-brown, was carried out as described earlier (38). -Cell and non- -cell double staining on neighbor sections was carried out essentially as described earlier (38) but with insulin in -cells developed with diaminobenzidine (DAB) plus NiSO 4 (black) and non- -cells developed with glucagon, somatostatin, and pancreatic polypeptide stained reddish-brown with Vector Nova Red. For GLUT2 and glucokinase staining, antigen retrieval was omitted; the sections were just treated for 20 min with 10% normal rabbit serum. For GLUT2, sections were incubated overnight with monoclonal mouse anti-GLUT2, 0.3 mg/ml, for 30 min with peroxidase-labeled rabbit anti-mouse IgG, and the peroxidase reaction was developed with DAB. For gluco kinase staining, sections were incubated for 30 min with goat anti-gluco kinase, 30 min with biotinylated rabbit anti-goat IgG, with the VectaStain Elite Kit and developed with DAB according to the manufacturer's instructions. Finally, after the immune stainings, the sections were lightly counterstained with hematoxylin, dehydrated, and mounted with Pertex.

**Stereological procedures.** Volume fractions for the -cell and non- -cell were analyzed on an Olympus BX-50 microscope (Olympus, Copenhagen, Denmark) with a video camera and monitor, a PC-controlled motorized stage, and the CAST-GRID software (Olympus). The -cell proliferation rate was estimated by the proportion of BrDU-positive -cells from a total of 1,000–1,500 cells counted in two to three sections cut 250 ${\mu}$m apart (38). The volume fractions of - or non- -cells were estimated by point counting at a total on-screen magnification of ×960 by random systematic scanning of the tissue sections controlled by the CAST-GRID software (27, 38). The sections were examined with the observer blinded to the origin of the sections.

**Statistics**

Data are presented as means ± SE. One-way ANOVA with (time course) or without (single time points) repeated measures and with Tukey's post hoc test for pairwise group comparisons were employed. The total area under the curve was calculated using the trapezoidal rule. Differences between groups with P values <0.05 were considered significant.

**Protocol 1: Prevention and Early Intervention Studies**

In two independent studies, initiated in 7-wk-old prediabetic (prevention study) and in 11-wk-old moderately diabetic (early-intervention study) ZDF male rats, animals were given either vehicle, ragaglitazar (3 mg·kg$^{-1}$·day$^{-1}$), or rosiglitazone (6 mg·kg$^{-1}$·day$^{-1}$) for a total of 7 (prevention study) or 4 (early-intervention study) wk (n = 14–16 rats/group; Fig. 1). The dose of ragaglitazar was based on preliminary dose-response studies in rodents and was chosen as the maximal efficacious dose (data not shown). The dose of rosiglitazone was based on a recent study (41) but increased to 6 mg·kg$^{-1}$·day$^{-1}$ to ensure maximal efficacy in the comparison with ragaglitazar. Compounds were administered two times daily by gavage to ensure continuous exposure. BG, PI, Hb A1c, FFA, and TG were measured at time points indicated in Tables 1–6 and Figs. 1–7. Body weight as well as communal (3–4 rats/cage, 4 cages/group) food and water consumption were measured weekly. After 3 (early intervention) or 6 (prevention) wk of treatment, 24-h profiles of BG and PI were obtained. Subgroups of rats were subjected to clamp studies for assessment of whole body insulin sensitivity. At the end of the treatment period, clamped and subgroups of nonclamped (nonfasted) rats were killed, and various tissues were excised and stored for further biochemical and histological examination. Nonclamped rats (n = 4/group) were administered BrDU (100 mg/kg ip) for determination of -cell proliferation index.

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

Overtly diabetic, 16-wk-old male ZDF rats were used for a dose-response study with ragaglitazar (0.75, 1.50, and 3.00 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 enable direct comparison with protocol 1. An additional group was given ragaglitazar one time daily at 0800 at the highest dose level (3.00 mg·kg$^{-1}$·day$^{-1}$) for comparison of two times daily vs. one time daily dosing regimens. Sixty-four out of 90 ZDF rats with the highest Hb A1c levels were assigned to eight treatment groups (8 rats/group) with matching Hb A1c levels. Nonfasting BG, PI, and body weight were measured weekly. At the end of treatment, 24-h BG and PI profiles and Hb A1c 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...
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

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

<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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretreatment</td>
<td>Posttreatment</td>
<td>Change</td>
</tr>
<tr>
<td><strong>Prevention study</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<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 ± 10</td>
<td>304 ± 8</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>206 ± 5</td>
<td>512 ± 8</td>
<td>304 ± 5</td>
</tr>
<tr>
<td><strong>Early-intervention study</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<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 ± 10</td>
<td>184 ± 5</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>351 ± 4</td>
<td>534 ± 6</td>
<td>185 ± 3</td>
</tr>
</tbody>
</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 Hb A1c compared with the vehicle-treated rats (Table 2). In the early intervention study, Hb A1c increased in the vehicle-treated group, whereas both drug treatments almost normalized Hb A1c 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>
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<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>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>6.39 ± 0.16</td>
<td>8.89 ± 0.20</td>
<td>2.50 ± 0.17</td>
</tr>
<tr>
<td>Ragaglitazar</td>
<td>6.22 ± 0.20</td>
<td>4.51 ± 0.06*</td>
<td>-1.71 ± 0.16*</td>
</tr>
<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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</tbody>
</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 \( \beta \)-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). HbA1c 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 HbA1c caused by rosiglitazone was \( \approx 1\% \) at all three dose levels, suggesting that the maximal response to this compound was reached. Ragaglitazar reduced HbA1c 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 HbA1c 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 HbA1c 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 HbA1c 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 eu-glycemic 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/)

### Table 3. Pre- and posttreatment plasma lipid concentrations obtained in the prevention and early-intervention studies (protocol 1)

<table>
<thead>
<tr>
<th></th>
<th>Pre-FFA</th>
<th>Post-FFA</th>
<th>ΔFFA</th>
<th>Pre-TG</th>
<th>Post-TG</th>
<th>ΔTG</th>
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<td><strong>Prevention study</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>ND</td>
<td>1.19 ± 0.09</td>
<td></td>
<td>ND</td>
<td>8.19 ± 0.38</td>
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<tr>
<td>Ragaglitazar</td>
<td>ND</td>
<td>0.94 ± 0.06*</td>
<td></td>
<td>ND</td>
<td>1.55 ± 0.07*</td>
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<tr>
<td>Rosiglitazone</td>
<td>ND</td>
<td>1.09 ± 0.05</td>
<td></td>
<td>ND</td>
<td>1.88 ± 0.11*</td>
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<tr>
<td><strong>Early-intervention study</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>0.70 ± 0.04</td>
<td>0.69 ± 0.04</td>
<td>−0.02 ± 0.05</td>
<td>6.73 ± 0.51</td>
<td>8.46 ± 0.45</td>
<td>1.74 ± 0.47</td>
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<tr>
<td>Ragaglitazar</td>
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<td>0.73 ± 0.03*</td>
<td>0.06 ± 0.05*</td>
<td>7.22 ± 0.40</td>
<td>2.10 ± 0.12*</td>
<td>−5.62 ± 0.36*</td>
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<tr>
<td>Rosiglitazone</td>
<td>0.70 ± 0.04</td>
<td>0.93 ± 0.04*</td>
<td>0.23 ± 0.05*</td>
<td>7.45 ± 0.37</td>
<td>2.46 ± 0.14*</td>
<td>−5.00 ± 0.32*</td>
</tr>
</tbody>
</table>

Data are means ± SE; \( n = 14–16 \) rats/group. Units are mmol/l. FFA, free fatty acid; TG, triglyceride; ND, not determined. *\( P < 0.05 \) vs. vehicle in the same study. †\( P < 0.05 \) vs. rosiglitazone in the same study.
Body composition (DEXA) and correlations with 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 BIFEZ 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, adipin, 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-
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.
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 such as muscle and adipose tissue (4, 49). It is known that activation of PPARα mediates catabolism of fatty acids mainly through hepatic fatty acid oxidation. Activation of PPARγ, by contrast, predominantly causes direct effects on adipose tissue, supposedly leading to secondary beneficial effects on muscle and/or liver. The fact that PPARγ agonists lower circulating fatty acids, presumably via increased sequestration of fatty acids into adipose tissue and decreased lipolysis, is consistent with this notion (32). In any respect, activation of both PPARα and PPARγ affects fatty acid homeostasis, which, in turn, may result in beneficial changes of glucose homeostasis as originally proposed by Randle et al. (36).

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 (adipsin, 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>31 ± 7</td>
</tr>
<tr>
<td>Ragaglitazar (3 mg·kg⁻¹·day⁻¹)</td>
<td>491 ± 7†</td>
<td>280 ± 6†</td>
<td>59 ± 1</td>
</tr>
<tr>
<td>Rosiglitazone (6 mg·kg⁻¹·day⁻¹)</td>
<td>444 ± 15‡</td>
<td>204 ± 26‡</td>
<td>45 ± 4</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. vehicle in the same study. ‡P < 0.05 vs. vehicle in the same study.

![Graph](image-url)
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, non-diabetic 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 Hb A1c in the ZDF rat occurred concomitantly with a decrease in body weight, particularly in total body fat mass. This is generally thought to be because of increased lipid storage in fat tissue caused by increased clearance of circulating lipids along with PPARγ-mediated stimulation of adipogenesis, as indicated by an increase in aP2 gene expression in adipose tissue compared with that in untreated rats. In addition, however, the increased fat mass could also be caused by preventing loss of calories through glucosuria when glycemic control is improved. In fact, pilot studies performed in our laboratory suggest that 20–30% of the calories consumed are lost via glucosuria in severely diabetic ZDF rats (Brand CL, unpublished observations). Therefore, unless food intake is decreased and/or energy expenditure is increased to counterbalance the increased amount of retained calories caused by preventing glucosuria, an increase in body weight would obviously be expected. Hence, by restoring glycemic control with ragaglitazar or rosiglitazone, this animal model seems to revert to its original phenotype, which is the obese Zucker rat. A similar association between body weight gain and treatment of 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) Rg 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 Rg in white gastrocnemius muscle, which represents ~70% of the hindlimb musculature, whereas only ragaglitazar improved Rg 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 phosphorlase 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.

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REFERENCES


34. Petersen JS, Shalmi M, Lam HR, and Christensen S. Rilmenic and lipid modulating activity. *Phenoxazin-10-yl)ethoxy[phenyl]-2-ethoxypropanoic acid*.

35. Petersen JS, Shalmi M, Lam HR, and Christensen S. Rilmenic and lipid modulating activity. *Phenoxazin-10-yl)ethoxy[phenyl]-2-ethoxypropanoic acid*.

36. Petersen JS, Shalmi M, Lam HR, and Christensen S. Rilmenic and lipid modulating activity. *Phenoxazin-10-yl)ethoxy[phenyl]-2-ethoxypropanoic acid*.

37. Petersen JS, Shalmi M, Lam HR, and Christensen S. Rilmenic and lipid modulating activity. *Phenoxazin-10-yl)ethoxy[phenyl]-2-ethoxypropanoic acid*.

38. Petersen JS, Shalmi M, Lam HR, and Christensen S. Rilmenic and lipid modulating activity. *Phenoxazin-10-yl)ethoxy[phenyl]-2-ethoxypropanoic acid*.


