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PPARα/γ ragaglitazar eliminates fatty liver and enhances insulin action in fat-fed rats in the absence of hepatomegaly

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peroxisome proliferator-activated receptor subtypes; adipokines; tissue lipids; insulin resistance

INSULIN RESISTANCE IS A FUNDAMENTAL DEFECT of type 2 diabetes. It is central to the insulin resistance syndrome characterized by hyperglycemia, hyperinsulinemia, dyslipidemia, obesity, and hypertension. There is increasing evidence to suggest that central adiposity (8) and fatty liver (30) are also important features of this syndrome. It is clear that a lipid accumulation in muscle and liver can cause the development of insulin resistance (4, 26, 41). Realization of the role of excess lipids in the pathogenesis of insulin resistance has led to various strategies to improve insulin sensitivity by lowering excess lipid accumulation in liver and muscle (32).

Peroxisome proliferator-activated receptors (PPAR) are nuclear transcription factors that include three subtypes: α, δ(β), and γ. PPARγ agonists, such as thiazolidinediones (TZDs), improve insulin action in peripheral tissues, attenuate hyperinsulinemia, and lower circulating levels of lipids. PPARγ agonists are highly expressed in adipocytes and mediate their differentiation. A major mechanism of the insulin-sensitizing action of PPARγ agonists results from the lowering of lipid supply to muscle and liver through a "lipid-stealing" by PPARγ-mediated effects in adipose tissue (22, 40). However, some concerns also arise over increases in fat mass and body weight associated with adipocyte proliferation (10, 14, 48).

Unlike PPARγ, PPARα mediates expression of genes regulating lipid oxidation (22). PPARα agonists, such as fibrates, have been used to treat hypertriglyceridemia and reduce cardiovascular risk (24). A number of studies in insulin-resistant animal models have shown marked decreases in liver triglyceride content and adiposity by PPARα agonists (6, 10, 16, 37). Using a euglycemic hyperinsulemic clamp technique, our recent studies have clearly shown that, while exerting these effects, the PPARα agonist Wy-14643 can also lessen insulin resistance and muscle lipid accumulation in high-fat-fed rats. However, compared with a PPARγ agonist, the improvement of muscle insulin sensitivity by Wy-14643 was much smaller for similar reductions in muscle lipids (49). There are also opposite reports that PPARα deficiency may even protect

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insulin sensitivity (43). These data suggest that other factors, such as PPARγ-responsive adipokines, may be involved in the insulin-sensitizing action of PPARγ agonists. Currently, there is enormous interest in the potential of combined PPARα/γ agonists for enhancement of insulin action together with reductions in tissue lipid accumulation and central adiposity (22, 32).

Although possible benefits of combined PPARα/γ agonists have been suggested in earlier reports in genetically obese insulin-resistant models (13, 29, 34), their effects on insulin action in liver and muscle in relation to adipokines in a nutritional model of insulin resistance have not been demonstrated. Thus the aim of the present study was to investigate whether a dual PPARα/γ compound would exert additional beneficial effects on liver steatosis, adiposity, and insulin sensitivity compared with selective activation of PPARγ or PPARα. Lipid metabolism was examined in parallel, with particular focus on liver and muscle lipid content and central adiposity, and their relationship with leptin and adiponectin was investigated.

RESEARCH DESIGN AND METHODS

Study design. All experimental procedures were approved by the Animal Experimentation Ethics Committee (Garvan Institute/St Vincent's Hospital, Sydney) and were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation. Ragaglitazar [also known as NNC 61–0029 or DRF(−)2725] is a 2-ethoxy-3-[4-(2-phenoxazin-10-yl-ethoxy)-phenyl]-propionic acid, structurally different from other PPARγ and PPARα ligands including TZDs, *N*-(2-benzoylphenyl)-L-tyrosine derivatives, α-alkoxy-β-phenylpropanoic acids, or fibrates (38). It is a full agonist of both PPARγ and PPARα. In vitro transactivation assay showed that the maximal activation of ragaglitazar on PPARγ was 117% (EC_{50}: 0.57 μM) compared with rosiglitazone (100% with EC_{50} of 0.16 μM). For the stimulation of PPARα, ragaglitazar produces 97% (EC: 3.2 μM) of the maximal response induced by Wy-14643 (100% and EC_{50}: 12.6 μM) (38).

Male Wistar rats supplied from the Animal Resources Centre (Perth, Australia) were conditioned at 22 ± 0.5°C with a 12:12-h day-night cycle (lights on 0600) for 1 wk in communal cages. They were fed ad libitum a standard chow diet containing 69% carbohydrate, 21% protein, 5% fat, fiber, vitamins, and minerals. After the acclimatization period, rats (∼300 g) were fed the same chow diet or an isocaloric high-fat diet (350 kJ/day given at 1600) for 3 wk, as described previously (41). They were divided into five groups (n = 6–9/group unless otherwise indicated): insulin-sensitive chow-fed normal rats (CH-Con), and insulin-resistant high-fat-fed rats treated with vehicle (HF-Con), ragaglitazar (HF-Raga), rosiglitazone (HF-Rosi), or Wy-14643 (HF-WY). The nutrient composition of the fat diet, expressed as a percentage of energy, was 59% fat, 21% protein, 20% carbohydrate, with quantities of fibers, vitamins, and minerals equal to those in the chow-fed group. Previous studies showed that rats started to develop hepatic insulin resistance after 3 days of high-fat feeding (26). High-fat-fed rats were administered ragaglitazar, rosiglitazone, or Wy-14643 in the diet (each at 3 $mg \cdot kg^{-1} \cdot day^{-1}$) for 2 wk. Body weight and food intake were recorded daily, and no appetite-averting effect was observed for any of the compounds.

Assessment of PPARα and PPARγ activation in vivo. The mRNA expression levels of liver peroxisome bifunctional enzyme (PBE) and acyl-CoA oxidase (ACO) were determined to assess activation of PPARα. Total RNA was extracted from ∼100 mg of liver using Tri-Reagent (Sigma, St. Louis, MO). Real-time LightCycler RT-PCR was employed to quantify ACO and PBE mRNA levels by use of the LightCycler Fast-Start DNA Master SYBR Green 1 kit (Roche Molecular Biochemicals, Mannheim, Germany) in a similar way as recently described (44). The primer combinations were: 5′-GAT TCA AGA CAA AGC CGT CCA AG-3′ and 5′-TCC ACC AGA GCA ACA GCA TTG-3′ for ACO, 5′-CGC ACT TGA CAC ATT CCA GCT-3′ and 5′- GGG CTA CTC ATC TAT GTT GTC CAC-3′ for PBE. Expression levels were normalized to the expression levels of cyclophylin. Expression levels of phospho*enol*pyruvate carboxykinase (PEPCK) mRNA in retroperitoneal white adipose tissue, an indicator of PPARγ activation in adipocytes (42, 46), were determined by semiquantitative RT-PCR, as described previously (20). Two primer sets (5 pmol of each oligo) were used simultaneously, one specific for the gene in question and the other specific for an internal standard (elongation factor 1a). For PEPCK, forward primer 5′-ACAGGATGAGGAACCGTGC-3′ and reverse primer 5′-CCTTGCCCTTATGCTCTGC-3′ were used. For adipocyte protein-2 (aP2), forward primer 5′-AAGACAGCTCCTCCTCGAAGGTT-3′ and reverse primer 5′-TGACCAAATCCCCATTTACGC-3′ were employed. PCR products were separated on a 6% polyacrylamide-7 M urea gel and analyzed using a phosphorimager and ImageQuant (Molecular Dynamics). Results are expressed as gene per internal standard. Activation of PPARγ in vivo was also assessed by plasma concentration of adiponectin as a marker (11) with a commercial radioimmunoassay kit (Linco, St. Louis, MO).

Experimental protocol. A week before the study, the left carotid artery and right jugular vein of rats were cannulated, and cannulas were exteriorized in the back of the neck under ketamine-xylazine (90:10 mg/kg ip) anesthesia. Rats were handled daily to minimize stress. On the study day between 0900 and 1000, after animals had been fasted for 5 h, the cannulas were connected to infusion apparatus (via the carotid line) and a blood-sampling syringe (via the jugular line). The sampling line was filled with sodium citrate (20.6 mM) to prevent clotting. After a period (50–60 min) of settling, two basal blood samples were collected for measurement of plasma parameters, as described previously (49). Rats were allowed to settle for ∼1–2 h and then euthanized by pentobarbital sodium (∼180 mg/kg). Muscles (red and white quadriceps) and heart were immediately freeze-clamped with aluminum tongs precooled in liquid nitrogen. Visceral (epididymal and retroperitoneal) fat and liver were weighed before being frozen in liquid nitrogen. Tissues were stored at −80°C for subsequent analyses for basal metabolites. The hyperinsulinemic euglycemic clamp was performed at an insulin infusion rate of 0.25 $U \cdot kg^{-1} \cdot h^{-1}$ to elevate circulating insulin levels to approximately a half-maximal physiological concentration (25), with glucose infused at variable rates (GIR) to maintain euglycemia. After plasma glucose levels reached the steady state, a bolus of 2-deoxy-D-[2,6-^{3}H]glucose and D-[U-^{14}C]glucose was injected (iv) to determine glucose disappearance rate (R_d) and hepatic glucose output rate (HGO). After the clamp, tissues of interest were freeze-clamped to determine glucose uptake ($R_{g'}$), glycogen, and lipid synthesis. Details of these measurements have been described previously (25, 41, 49).

Metabolite measurements. Glucose concentrations were determined using a glucose analyzer (YSI 2300, Yellow Springs, OH). Plasma free fatty acid (FFA) and triglyceride levels

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were determined using enzymatic colorimetric methods by commercial kits (Sigma). Plasma insulin and leptin levels were determined with radioimmunoassay kits (Linco). Tissue triglycerides were extracted by the method of Bligh and Dyer (7), and content was determined by a Peridochrom Triglyceride GPO-PAP kit (Boehringer Mannheim, Mannheim, Germany). Tissue content of glycogen (9) and long-chain acyl (LCA)-CoAs (2) was determined as previously described.

Statistical analyses. All results are presented as means ± SE. A one-way analysis of variance (ANOVA) was used to assess the statistical significance across all groups. When tested as significant, a post hoc (Fisher paired least significant difference) test was used to establish differences between groups. All data were processed in Excel 5.0, and statistical analyses were performed using the Statview SE+Graphic Program (Abacus Concepts-Brain Power).

RESULTS

Activation of PPARα and PPARγ by ragaglitazar. Activation of PPARα is known to upregulate the expression of liver PBE, as shown in the HF-WY group (Fig. 1*A*). Although to a lesser extent, ragaglitazar treatment also led to a dramatic increase (3.1-fold) in PBE mRNA expression level in liver. Similarly, liver ACO mRNA levels were significantly increased ($P < 0.01$) in both HF-WY (35.8 ± 4.2) and HF-Raga (21.5 ± 5.9) groups but not in the HF-Rosi group (8.7 ± 1.4) compared with the CH-Con (8.9 ± 0.8) or HF-Con (7.3 ± 1.4) group. The expression levels of PEPCK mRNA in white adipose tissue were increased more than twofold in the HF-Raga group (Fig. 1*B*) in a pattern similar to the HF-Rosi group. These data indicated that both PPARα and PPARγ were activated by ragaglitazar, an observation that was also supported by a substantial (60%) reduction in plasma leptin levels in all treated groups (Fig. 1*C*). There was a positive correlation between plasma leptin concentrations and visceral fat weight among all five groups ($r = 0.75$, $P < 0.001$, data not shown). Recently, elevated plasma adiponectin concentration has been suggested as a specific indicator of PPARγ but not PPARα stimulation (11). Figure 1*D* shows that plasma adiponectin levels were raised approximately threefold in both the HF-Raga group and the HF-Rosi group. In addition, aP2 mRNA expression levels in retroperitoneal white adipose tissue were significantly higher in the HF-Raga group compared with the HF-Con or CH-Con group [2.9 ± 0.16 vs. 1.5 ± 0.14 or 1.2 ± 0.04 arbitrary units (AU), respectively, $P < 0.01$].

Effects on basal lipid and glucose metabolism. Table 1 shows basal metabolic parameters in all groups. Except for a small decrease in body weight gain in the HF-WY group (15% vs. HF-Con), there were no differences in weight gain among the groups. Compared with CH-Con rats, visceral fat mass was increased by 55% in the HF-Con group. This increase was prevented in HF-WY and HF-Raga groups but not in HF-Rosi rats. Thus the visceral adiposity of both the HF-Raga and the HF-WY groups was significantly ($P < 0.01$) less (32 and 21%, respectively) than that of the HF-Rosi group. There was severe hepatomegaly in HF-WY (58% increase in liver weight vs. HF-Con), whereas neither ragaglitazar nor rosiglitazone altered liver weight. Associated with a small reduction in plasma glucose levels, elevated plasma insulin levels (34% vs. CH-Con) in HF-Con were abolished by all drugs. A similar pattern of decreases in plasma triglyceride levels by the three agonists (16–28%) was found despite their relatively

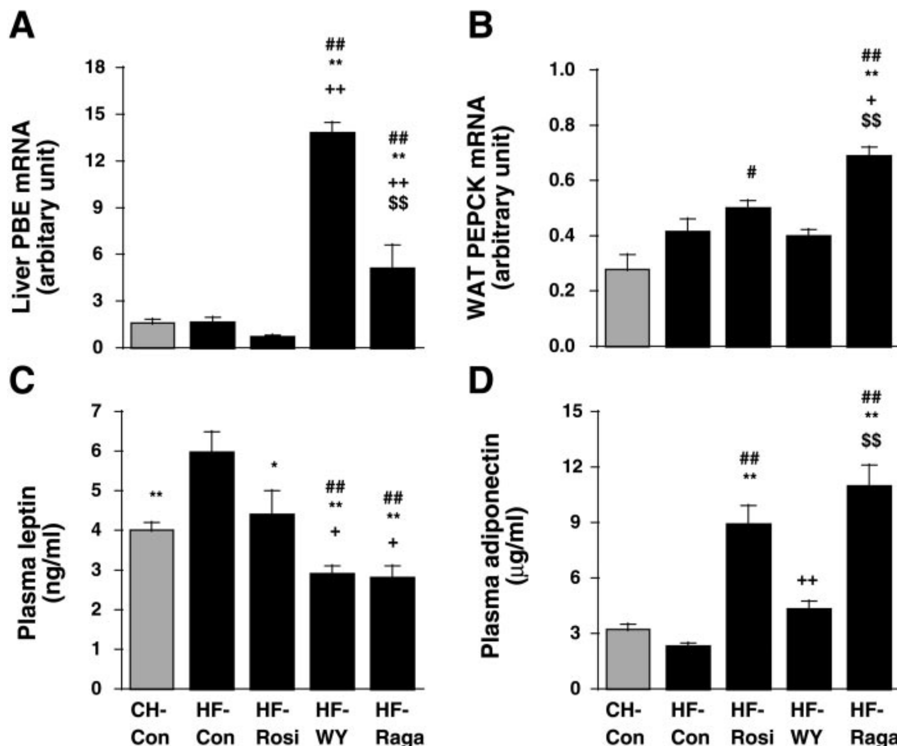

Fig. 1. Indicators of in vivo activation of peroxisome proliferator-activated receptor (PPAR)α and PPARγ. *A*: expression mRNA levels of peroxisomal bifunctional enzyme (PBE) in liver. Expression levels were normalized to expression levels of cyclophylin. *B*: expression mRNA levels of phospho*enol*pyruvate carboxykinase (PEPCK) in retroperitoneal white adipose tissue (WAT). Expression levels were normalized to the same internal standard. *C*: plasma levels of leptin. *D*: plasma levels of adiponectin. Data were obtained in the basal state (means ± SE). ##$P < 0.01$ vs. chow normal rats (CH-Con); *$P < 0.05$, **$P < 0.01$ vs. high-fat, vehicle (HF-Con); +$P < 0.05$, ++$P < 0.01$ vs. HF-rosiglitazone (Rosi); $$$P < 0.01$ vs. HF-Wy-14643 (WY). Raga, ragaglitazar.

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Table 1. *Effects on basal metabolic parameters*

	CH-Con	HF-Con	HF-Rosi (PPARγ)	HF-WY (PPARα)	HF-Raga (PPARα/γ)
Initial body weight, g	307 ± 6	311 ± 3	312 ± 3	295 ± 4[c,e]	315 ± 4[g]
Final body weight, g	367 ± 3	368 ± 3	373 ± 4	347 ± 4[a,c,e]	375 ± 4[g]
Visceral fat wt, g	8.0 ± 0.6[d]	12.4 ± 0.4	11.6 ± 0.4[b]	7.9 ± 0.3[d,f]	9.2 ± 0.4[d,f]
Liver wt, g	14.7 ± 0.5	14.4 ± 0.3	13.9 ± 0.4	22.7 ± 0.4[d,f]	15.2 ± 0.2[h]
Plasma glucose, mM	7.9 ± 0.3[d]	8.9 ± 0.2	8.5 ± 0.2[b]	8.4 ± 0.1[b,d]	8.3 ± 0.1[b,d]
Plasma insulin, mU/l	38 ± 5[d]	51 ± 5	24 ± 2[b,d]	31 ± 3[d]	21 ± 2[b,d,g]
Plasma triglyceride, mM	1.10 ± 0.10[d]	0.79 ± 0.04	0.57 ± 0.03[b,d]	0.66 ± 0.04[a,e]	0.59 ± 0.03[b,d]
Plasma FFAs, mM	0.60 ± 0.06	0.52 ± 0.04	0.45 ± 0.05	0.45 ± 0.02	0.48 ± 0.05

Data from the basal (CH-Con) and clamp subgroup were pooled together for body weight ($n \geq 13$ group). HF-Con, high fat-vehicle; HF-Rosi, HF-WY, and HF-Raga, HF + peroxisome proliferator-activated receptors (PPAR)γ; rosiglitazone; α, Wy-14643; and α/γ, ragaglitazar; FFA, free fatty acids. Plasma parameters were obtained from 5 to 7-h-fasted cannulated rats (n = 6–9/group). [a]$P < 0.05$, [b]$P < 0.01$ vs. CH-Con; [c]$P < 0.05$, [d]$P < 0.01$ vs. HF-Con, [e]$P < 0.05$, [f]$P < 0.01$ vs. HF-Rosi; [g]$P < 0.05$, [h]$P < 0.01$ vs. HF-WY.

lower levels (vs. CH-Con) in HF-Con, an adaptive response due to increased lipid clearance (18). There was no significant effect on FFA levels in any of the treated groups.

High-fat feeding led to a threefold increase in liver triglyceride content compared with CH-Con (Fig. 2*A*). This was completely prevented by ragaglitazar (CH-Con: 7.0 ± 0.3 vs. HF-Raga: 7.0 ± 0.3 μmol/g, $P >$

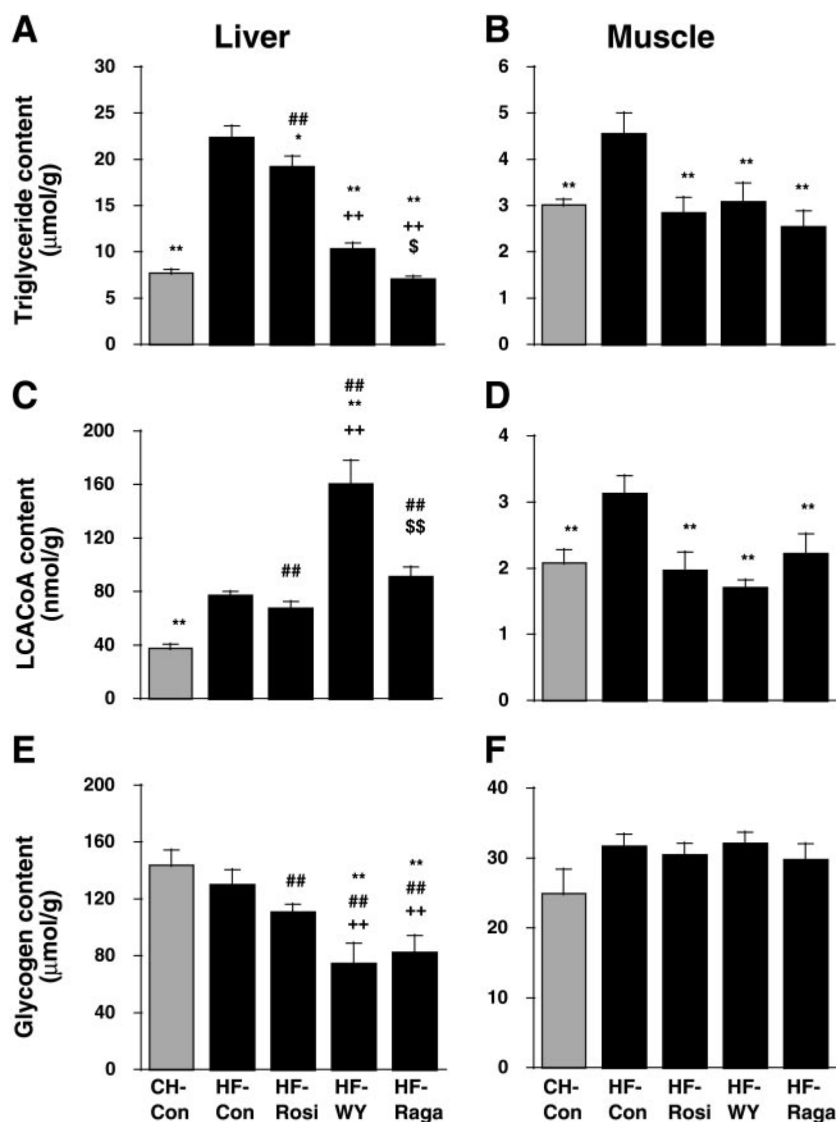

Fig. 2. Effects on basal triglyceride (*A* and *B*), long-chain acyl (LCA)-CoA (*C* and *D*), and glycogen content (*E* and *F*) in liver (*A, C,* and *E*) and muscle (*B, D,* and *F*). #$P < 0.05$, ##$P < 0.01$ vs. CH-Con, *$P < 0.05$, **$P < 0.01$ vs. HF-Con, ++$P < 0.01$ vs. HF-Rosi; $$P < 0.05$, $$$P < 0.01$ vs. HF-WY.

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0.05). Although liver triglyceride content in the HF-Rosi group was marginally lower (by 14% vs. HF-Con), its level (19.2 ± 1.2 μmol/g) was approximately 2.6-fold higher ($P < 0.001$) than that of the normal CH-Con group or ragaglitazar-treated rats. The effect of ragaglitazar on lowering liver triglyceride content of high-fat-fed rats was also significantly greater than that of Wy-14643 ($P < 0.05$). As expected, liver LCA-CoA content (Fig. 2*B*) was also increased (by 108%) with high-fat feeding. Its levels in HF rats were unaltered by rosiglitazone or ragaglitazar but further increased in HF-WY by 108%. All three agonists decreased liver glycogen content, and the effect of Wy-14643 and ragaglitazar was greater than that of rosiglitazone (Fig. 2*C*). In contrast, all three PPAR agonists similarly affected these parameters in muscle. Compared with CH-Con, muscle levels of triglyceride (Fig. 2*D*) and LCA-CoA (Fig. 2*E*) were increased in HF-Con by 63 and 50%, respectively. None of the treatments altered muscle glycogen content (Fig. 2*F*).

Whole body insulin sensitivity and lipids during hyperinsulinemic euglycemic clamp. High-fat feeding induced insulin resistance, as evidenced by 50% of the clamp GIR (Fig. 3*A*). The underlying cause of insulin resistance was a combination of inhibited insulin-mediated glucose disposal rate (R_d) in peripheral tissues and impaired suppression of HGO. Compared with HF-Con, all three PPAR agonists increased the GIR (HF-Rosi: 51%, HF-WY: 39%, and HF-Raga: 66%) and R_d (15–22%). Increases in GIR and R_d values were not significantly different among the treated groups. Ragaglitazar substantially enhanced insulin's suppressibility of HGO by 79%, whereas the apparent reduction of HGO induced by rosiglitazone or Wy-14643 did not reach statistical significance.

During the clamp, plasma triglyceride levels were also significantly lower in all treated rats compared with the HF-Con group, but only the HF-Raga group had lower FFA levels than the HF-Con group (Table 2). Plasma adiponectin levels were markedly higher ($P < 0.01$) in both HF-Rosi and HF-Raga groups than in HF-Con rats. These levels were most elevated in the HF-Raga group; they were 43% higher than those of the HF-Rosi group ($P < 0.01$).

Insulin action in liver and associated changes in lipids. As shown in Fig. 4*A*, the inhibition of insulin-stimulated glycogen synthesis by high-fat feeding was completely overcome by rosiglitazone and ragaglitazar, but not by Wy-14643. Although insulin-mediated de novo triglyceride synthesis from glucose remained largely inhibited in all high-fat-fed groups compared with CH-Con, there was a 41% increase in HF-Raga that was also significantly higher than in HF-Rosi or HF-WY ($P < 0.05$, Fig. 4*B*). As in the basal state, triglyceride content was reduced by all three drugs after the clamp (Table 2). However, its reduction in rosiglitazone-treated rats was relatively small (24%) and significantly less ($P < 0.01$) than the reduction in liver triglyceride content in groups treated with Wy-14643 (45%) and ragaglitazar (88%). Indeed, the effect of ragaglitazar was even greater than that of

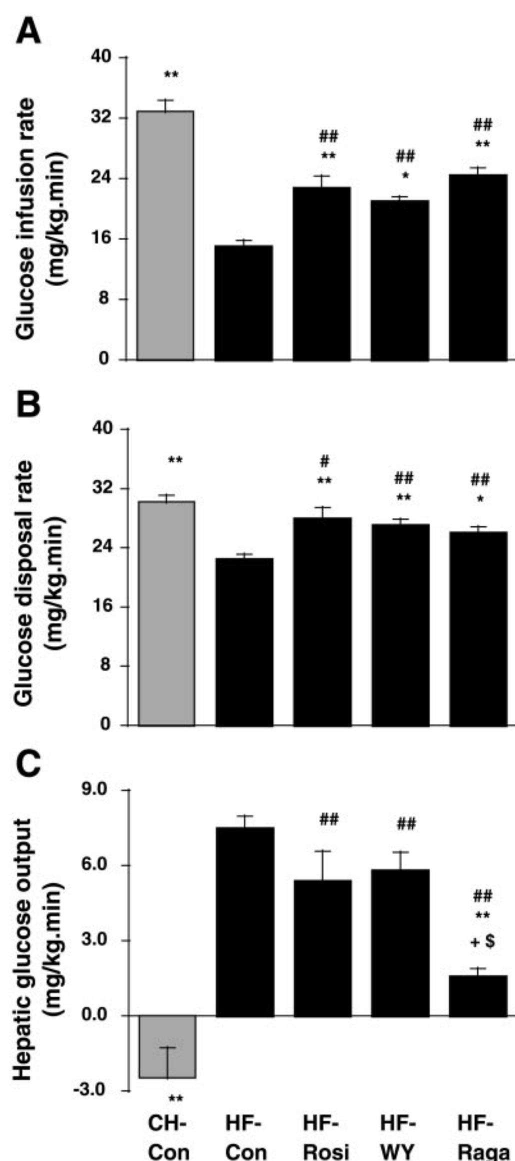

Fig. 3. Insulin-stimulated whole body glucose fluxes during the hyperinsulinemic euglycemic clamp. *A*: glucose infusion rate required to maintain euglycemia. *B*: glucose disposal rate. *C*: hepatic glucose output. #$P < 0.05$, ##$P < 0.01$ vs. CH-Con; *$P < 0.05$, **$P < 0.01$ vs. HF-Con; +$P < 0.05$ vs. HF-Rosi; $$P < 0.05$ vs. HF-WY.

Wy-14643 ($P < 0.05$) and reverted liver triglyceride content to the normal level of chow-fed rats. When expressed as total hepatic triglyceride content per liver, the levels in the high-fat-fed groups (354 ± 32, 245 ± 25, 239 ± 37, and 75 ± 13 μmol per liver in HF-Con, HF-Rosi, HF-WY, and HF-Raga, respectively) were positively correlated with HGO (Fig. 5*A*). Both liver total triglyceride content and HGO were negatively correlated with plasma adiponectin levels (Fig. 5, *B* and *C*).

Insulin action in peripheral tissues and associated changes in lipids. As illustrated in Table 2, rosiglitazone and ragaglitazar enhanced insulin-mediated $R_{g'}$ in both red (65 and 150%) and white (168 and 113%) muscles, whereas improvement of $R_{g'}$ in HF-WY oc-

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Table 2. *Plasma and tissue metabolites during the hyperinsulinemic euglycemic clamp*

	CH-Con	HF-Con	HF-Rosi (PPARγ)	HF-WY (PPARα)	HF-Raga (PPARα/γ)
Plasma triglyceride, mM	0.52 ± 0.09	0.50 ± 0.04	0.40 ± 0.04[a]	0.41 ± 0.03[a]	0.31 ± 0.02[b,d,g]
Plasma FFAs, mM	0.33 ± 0.06[c]	0.21 ± 0.03	0.27 ± 0.04	0.23 ± 0.02	0.13 ± 0.03[b,f,g]
Plasma adiponectin, μg/ml	3.8 ± 0.3	3.7 ± 0.5	6.5 ± 0.8[b,c]	4.1 ± 0.5[e]	9.3 ± 0.6[b,d,f,h]
Muscle LCA-CoAs, nmol/g	3.8 ± 0.3[d]	11.8 ± 1.7	7.7 ± 0.8[b,c]	8.9 ± 0.6[b,c]	7.0 ± 1.3[b,d,g]
Liver TG, μmol/g	7.3 ± 1.2[d]	23.3 ± 1.6	17.8 ± 2.1[b,c]	10.4 ± 1.6[d,f]	5.0 ± 0.8[d,f,g]
Red muscle $R_{g'}$, μmol·100 g^{-1}·min^{-1}	34 ± 4[d]	20 ± 2	33 ± 3[d]	24 ± 2[b,e]	43 ± 4[a,d,e,h]
White muscle $R_{g'}$, μmol·100 g^{-1}·min^{-1}	6.0 ± 0.5	4.1 ± 0.5	11.0 ± 1.7[b,d]	6.6 ± 0.6[c,e]	8.7 ± 1.0[d]
WAT $R_{g'}$, μmol·100 g^{-1}·min^{-1}	2.9 ± 0.4[c]	1.9 ± 0.2	3.4 ± 0.4[d]	3.3 ± 0.2[d]	4.7 ± 0.4[b,d,e,g]

Plasma values were averaged of 2 samples during steady state during the clamp. LCA-CoAs, long-chain acyl-CoAs; TG, triglycerides; $R_{g'}$, glucose uptake; WAT, white adipose tissue. [a]$P < 0.05$, [b]$P < 0.01$ vs. CH-Con; [c]$P < 0.05$, [d]$P < 0.01$ vs. HF-Con, [e]$P < 0.05$, [f]$P < 0.01$ vs. HF-Rosi; [g]$P < 0.05$, [h]$P < 0.01$ vs. HF-WY (n = 6–9/group).

curred mainly in white muscle (61%). All three agonists increased the $R_{g'}$ in white adipose tissue (WAT), with the greatest improvement observed in HF-Raga (HF-Rosi: 79%, HF-WY: 74%, and HF-Raga: 147% above HF-Con). Compared with the HF-Rosi group, the $R_{g'}$ values in the HF-Raga group were 30 and 38% higher in red muscle and WAT, respectively ($P < 0.05$), whereas white muscle showed similarly enhanced $R_{g'}$ in these two groups. Compared with Wy-14643, ragaglitazar was clearly much more effective in enhancing insulin-mediated $R_{g'}$ in muscle (79% vs. red quadriceps, $P < 0.01$, and 32% vs. white quadriceps, $P < 0.08$) and WAT (42%, $P < 0.05$). Because red muscle is the major muscle type for insulin-mediated $R_{g'}$, we further investigated changes in glycogen and triglyceride synthesis in the red quadriceps. Glucose incorporation into glycogen (Fig. 4*C*) showed similar improvements to those shown for $R_{g'}$, suggesting that the glycogen synthesis pathway has an important role in muscle insulin sensitivity enhanced by rosiglitazone and ragaglitazar. In comparison, there was only a small improvement of glucose incorporation into triglyceride by the three agonists (Fig. 4*D*), suggesting that de novo lipogenesis from glucose contributes very little to the enhanced insulin action in muscle. After the clamp, muscle LCA-CoA content was markedly decreased in all groups ($P < 0.01$ vs. basal values), and their levels remained significantly lower than the level of HF-Con (Table 2). There was a negative correlation between muscle LCA-CoA content and insulin-stimulated muscle $R_{g'}$ (Fig. 5*D*) and plasma adiponectin levels (Fig. 5*E*). In contrast, insulin-stimulated muscle $R_{g'}$ was positively correlated with plasma adiponectin levels (Fig. 5*F*).

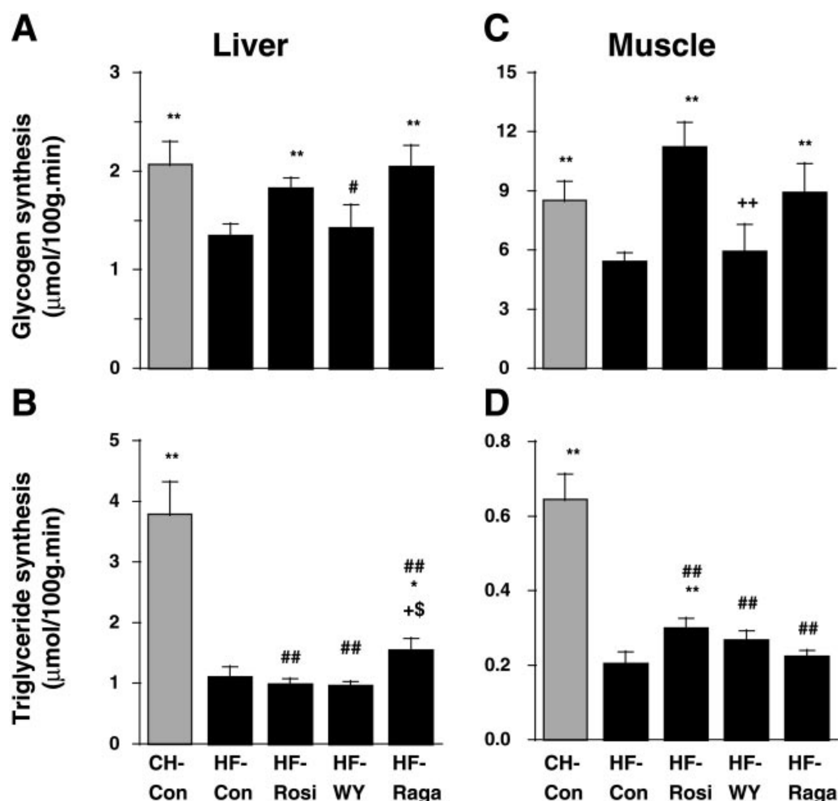

Fig. 4. Insulin-stimulated glycogen and triglyceride synthesis in liver (*A* and *B*) and muscle (*C* and *D*) during the hyperinsulinemic euglycemic clamp. *A* and *C*: glycogen synthesis measured by [^{14}C]glucose incorporation into glycogen; *B* and *D*: triglyceride synthesis measured by [^{14}C]glucose incorporation into triglyceride. #$P < 0.05$, ##$P < 0.01$ vs. CH-Con; *$P < 0.05$, **$P < 0.01$ vs. HF-Con; +$P < 0.05$, ++$P < 0.01$ vs. HF-Rosi; \$$P < 0.05$ vs. HF-WY.

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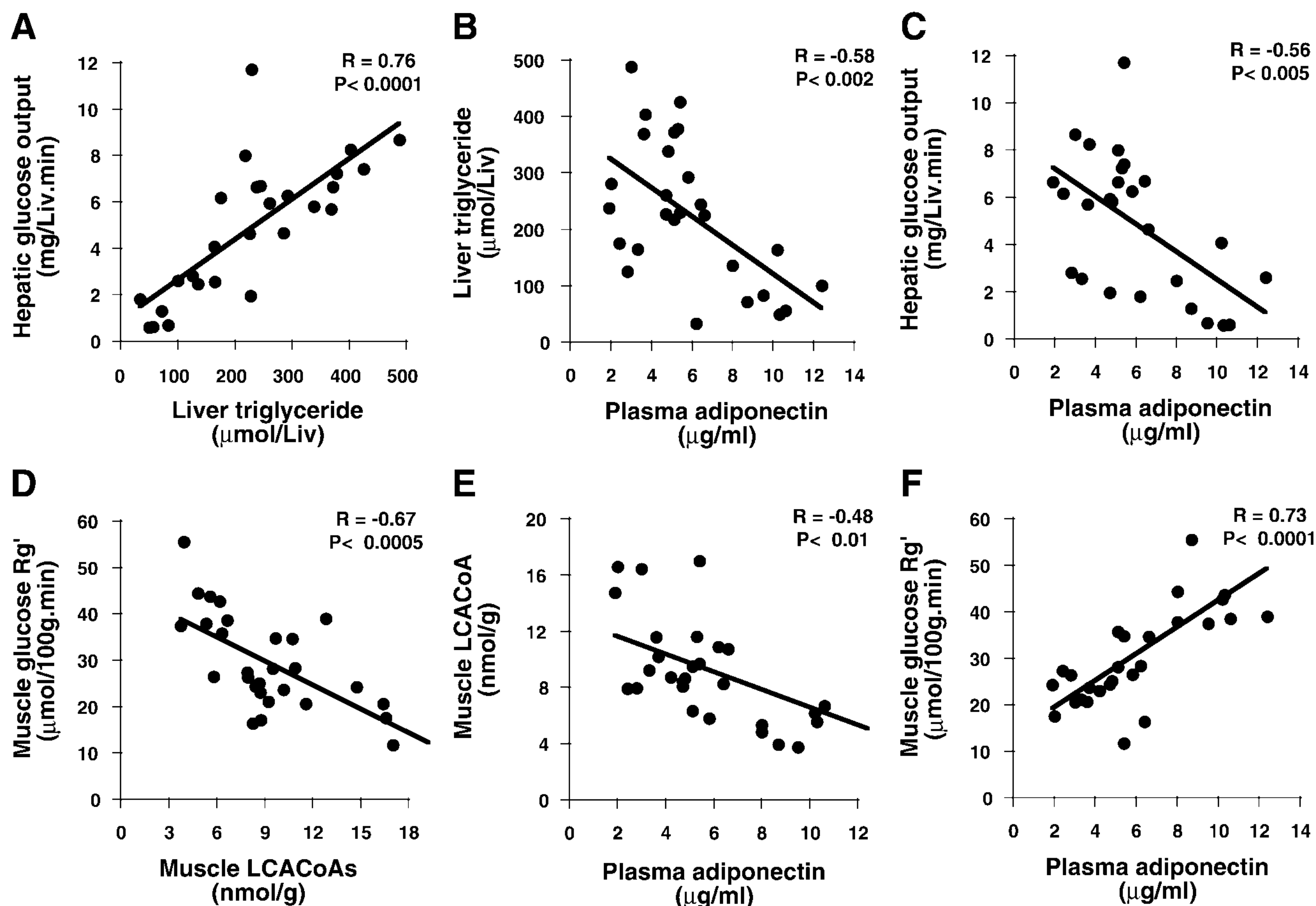

Fig. 5. Relationship among plasma adiponectin levels, tissue lipids, and insulin action in liver and muscle. Both insulin-mediated hepatic glucose output and muscle (red quadriceps) glucose uptake ($R_{g'}$) were steady-state values during hyperinsulinemic euglycemic clamp. Results for liver triglyceride content and muscle LCA-CoAs at the end of the hyperinsulinemic euglycemic clamp and results in all high-fat-fed rats were pooled for correlation analysis.

DISCUSSION

The present study demonstrates that combined activation of PPARα/γ markedly counteracts high-fat feeding-induced hepatic steatosis and visceral adiposity in combination with a marked improvement of insulin action. With use of a hyperinsulinemic euglycemic clamp technique and double glucose tracers, in parallel with determinants of lipid metabolism, we have clearly demonstrated additional benefits on liver insulin sensitivity of dual activation of PPARα/γ compared with selective activation of PPARγ. Interestingly, although stimulating PPARα, the PPARα/γ agonist ragaglitazar did not cause hepatomegaly. As seen for selective PPARγ agonists, dual PPARα/γ activation substantially increased circulating levels of adiponectin.

Liver steatosis is a characteristic in patients with type 2 diabetes and obesity (39) and is closely associated with the insulin resistance syndrome (30). Our first important finding was the complete prevention of high-fat feeding-induced liver steatosis by the dual PPARα/γ activator ragaglitazar and associated substantial enhancement of insulin's suppressibility of HGO. It appears that the PPARα agonist ragaglitazar decreased liver triglyceride content primarily by activating PPARα, as indicated by increased expression levels of liver PBE and ACO mRNA, in a similar way to Wy-14643. These results are consistent with the finding in obese Zucker rats that the PPARα/γ coligand KRP-297 reduces liver triglyceride content more effectively than rosiglitazone because it stimulates FFA oxidation and inhibits lipogenesis (34). There is evidence that rats treated with PPARα activators have a sustained increase in uncoupling protein (UCP)2 mRNA expression in the liver and particularly the small intestine, where UCP2 is abundantly expressed (35). Further studies are required to investigate whether this may provide a plausible mechanism to facilitate energy depletion of the increased fatty acid oxidation mediated by PPARα or combined PPARα/γ activation. In addition to PPARα, PPARγ-mediated responses also appear to contribute to the effects of ragaglitazar on liver. Like rosiglitazone, ragaglitazar improved insulin-mediated hepatic glycogen synthesis. More importantly, both ragaglitazar and rosiglitazone dramatically increased plasma levels of adiponectin, a specific PPARγ-induced adipokine (11) that has recently been shown to enhance insulin action in liver (5). Although adiponectin has been shown to promote

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muscle lipid oxidation (15), this effect has not been described in liver. Our results clearly demonstrated a close association of plasma adiponectin levels with triglyceride content and insulin action in liver. PPARγ agonists have been reported to downregulate gluconeogenic enzymes (3, 46, 48). However, their relationship with adiponectin and lipids needs to be further investigated.

The second important finding of this study was that ragaglitazar almost completely counteracted a high-fat feeding-induced increase in visceral fat mass, an effect clearly different from that of rosiglitazone. Although subcutaneous fat depots have been shown to be more responsive to PPARγ activation than visceral depots in humans (1) and to potentially influence fat distribution, a recent study showed that ragaglitazar does not alter subcutaneous fat weight (27). This finding is of potential significance because of body weight gain induced by PPARγ agonists alone (10, 14, 48). Additionally, insulin resistance is closely correlated with visceral fat mass in rats (23) and humans (8), and reduction of visceral fat can ameliorate insulin resistance, particularly in the liver (4, 16, 17). PPARα activators, such as fibrates and GW-9578, have been shown to reduce adiposity in obese rodents (10, 16). In keeping with these previous findings, the present study also found a reduction in visceral adiposity by the selective PPARα agonist Wy-14643. Because dual PPARα/γ agonists (such as KRP-297) can increase FFA oxidation (34), like selective PPARα agonists, it is highly likely that ragaglitazar reduces visceral adiposity development via stimulating PPARα-mediated FFA oxidation. It may be argued that the lack of overall increased visceral fat mass upon combined PPARα/γ activation is associated with a lack of de novo adipogenesis. However, like other dual PPARα/γ agonists (34), ragaglitazar stimulated, rather than inhibited, adipogenesis, as indicated by an increase in aP2 expression in adipose tissue compared with untreated high-fat-fed rats. This suggests that the less visceral adiposity in the ragaglitazar-treated group was not associated with reduced adipogenesis. Although PPARγ-responsive adipokines, such as leptin and adiponectin, may potentially influence body adiposity (15), they do not seem to contribute to the reduced visceral adiposity by ragaglitazar, because rosiglitazone exerted similar effects on these adipokine plasma concentrations but without altering visceral adiposity.

Despite apparent responses mediated by PPARα, ragaglitazar did not cause hepatomegaly. Most pure PPARα agonists cause hepatomegaly in rodents in a species-specific manner due to peroxisome proliferation (19). However, it is not clear how important hepatomegaly is for PPARα-mediated response in rodents. Our results from ragaglitazar indicate that PPARα-mediated effects in the liver do not rely on enlargement of liver size (or peroxisome proliferation). This interpretation is also supported by the effects of fish oil in rats, where PPARα is activated in the absence of hepatomegaly (Cooney GJ and Kraegen EW, unpublished observations). There are also reports that coactivation of PPARα/δ (33) or PPARα/γ (34) does not induce hepatomegaly. Consistent with these observations, PPARα-mediated lipid oxidation in mitochondria has been shown to be independent of peroxisome proliferation (28). It is possible that activation of PPARγ interacts to prevent PPARα-induced hepatomegaly or, alternatively, the stimulation of PPARα by ragaglitazar was not strong enough to produce hepatomegaly. Regardless of the mechanism, the observed effects of ragaglitazar may have significant implications, because PPARα agonists such as fibrates also lower lipids in the absence of hepatomegaly in humans.

Like rosiglitazone, ragaglitazar strongly enhanced insulin action in muscle, a result similar to that previously reported in other insulin-resistant models by dual PPARα/γ agonists such as JTT-501 (29) and LY-465608 (13). These data suggest that PPARγ-mediated effects may be mainly responsible for the insulin-sensitizing action in muscle induced by ragaglitazar, because the effects of PPARα agonist Wy-14643 upon insulin-mediated $R_{g'}$ were relatively small and mainly seen in white muscle. We have previously postulated that PPARγ activation may also improve muscle insulin action by mechanisms independent of lipid stealing (49). PPARγ agonists can alter adipokines, which may affect insulin sensitivity (21). Because leptin and adiponectin have been shown to improve insulin action and increase lipid oxidation in muscle (15, 31, 45), we examined the effects of their circulating levels. The suppressed plasma leptin levels in all treated groups indicate that leptin is unlikely to be involved in PPARα-mediated insulin sensitization in muscle. Whereas PPARγ activation is known to suppress leptin expression (12), the decreased plasma leptin levels in the HF-WY group may result from reduced visceral fat mass in a way similar to surgical removal (4). In contrast, plasma adiponectin concentrations were threefold higher in HF-Rosi and HF-Raga rats, and there was a strong correlation of plasma adiponectin levels with muscle LCA-CoA content, and even more so with insulin-mediated $R_{g'}$. These results strongly suggest an important role of adiponectin in PPARγ-mediated lipid metabolism and insulin sensitization, as recently proposed (15). However, the distinct effects of ragaglitazar and rosiglitazone on liver triglyceride content, visceral adiposity, and HGO during the hyperinsulinemic clamp suggest that these improvements could not be entirely explainable by the elevated systemic adiponectin concentrations. Furthermore, as these effects were substantially similar to those caused by Wy-14643 without a significant increase in plasma adiponectin concentrations, PPARα-mediating changes may be more likely to play an important role in reducing liver triglyceride content and visceral adiposity.

We recognized that limitations might occur for comparisons based on a single dose and that it could be argued that greater responses might be obtained at higher doses of rosiglitazone and Wy-14643. However, rosiglitazone-induced responses in this study were almost the same as those obtained by our laboratory under similar conditions at a 3.8-fold high dose admin-

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istered by oral gavage (36). In terms of Wy-14643, our pilot study showed that higher doses (e.g., 10 $mg \cdot kg^{-1} \cdot day^{-1}$) caused appetite-averting effects with no further improvement of insulin sensitivity (clamp GIR: 19.6 ± 4.2 $mg \cdot kg^{-1} \cdot min^{-1}$, $n = 3$). These data led us to believe that the responses produced by both rosiglitazone and Wy-14643 had reached their plateau. Similar degrees of activation of PPARγ by rosiglitazone and ragaglitazar in the present study were suggested by similar plasma adiponectin levels. It was also noticed that there was no reduction in liver LCA-CoAs in any of the drug-treated rats, a response contradictory to that in muscle. The reason for this finding is not known, but PPAR ligands are known to increase liver fatty acid-binding protein, which has a strong affinity to bind LCA-CoAs and interacts directly with PPARα compounds (47).

In summary, the present study compared three types of PPAR agonists at the levels of whole body and tissues. The results demonstrated that combined activation of PPARα/γ in the insulin-resistant high-fat-fed rat exerts additional benefits to counteract fatty liver, hepatic insulin resistance, and visceral adiposity while maintaining at least equivalent effectiveness to enhance muscle insulin action and elevate circulating adiponectin concentrations to selective PPARγ stimulation. There is a close association between elevated plasma adiponectin levels and improved insulin action in liver and muscle induced by PPARα/γ (and PPARγ) activation. Our results suggest that the PPARα/γ agonist ragaglitazar may have significant therapeutic potential in insulin-resistant states, with additional benefits of ameliorating fatty liver, hepatic insulin resistance, and adiposity without any involvement of increased liver mass compared with PPARγ activation alone.

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