A Newly Identified CG301269 Improves Lipid and Glucose Metabolism Without Body Weight Gain Through Activation of Peroxisome Proliferator–Activated Receptor α and γ

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OBJECTIVE—Peroxisome proliferator–activated receptor (PPAR)-α/γ dual agonists have been developed to alleviate metabolic disorders. However, several PPARα/γ dual agonists are accompanied with unwanted side effects, including body weight gain, edema, and tissue failure. This study investigated the effects of a novel PPARα/γ dual agonist, CG301269, on metabolic disorders both in vitro and in vivo.

RESEARCH DESIGN AND METHODS—Function of CG301269 as a PPARα/γ dual agonist was assessed in vitro by luciferase reporter assay, mammalian one-hybrid assay, and analyses of PPAR target genes. In vitro profiles on fatty acid oxidation and inflammatory responses were acquired by fatty acid oxidation assay and quantitative (q)RT-PCR of proinflammatory genes. In vivo effect of CG301269 was examined in *db/db* mice. Total body weight and various tissue weights were measured, and hepatic lipid profiles were analyzed. Systemic glucose and insulin tolerance were measured, and the in vivo effect of CG301269 on metabolic genes and proinflammatory genes was examined by qRT-PCR.

RESULTS—CG301269 selectively stimulated the transcriptional activities of PPARα and PPARγ. CG301269 enhanced fatty acid oxidation in vitro and ameliorated insulin resistance and hyperlipidemia in vivo. In *db/db* mice, CG301269 reduced inflammatory responses and fatty liver, without body weight gain.

CONCLUSIONS—We demonstrate that CG301269 exhibits beneficial effects on glucose and lipid metabolism by simultaneous activation of both PPARα and PPARγ. Our data suggest that CG301269 would be a potential lead compound against obesity and related metabolic disorders. ***Diabetes*** **60:496–506, 2011**

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Energy homeostasis is regulated by metabolic organs such as adipose tissue, liver, and muscle. Excess energy produced by surplus nutrient intake or reduced energy expenditure, or both, is mostly stored in the form of triglyceride (TG) in adipose tissue (1). When adipose tissue fails to accommodate lipid storage, excess energy is accumulated in other peripheral tissues, including liver and skeletal muscle (2). Because the increase of free fatty acids (FFAs) produced by TG breakdown from peripheral tissues provokes insulin resistance through diverse signaling pathways, including c-Jun NH_2-terminal kinase and protein kinase C (3,4), abnormal lipid metabolism is closely associated with many metabolic disorders, including obesity, insulin resistance, type 2 diabetes, hyperglycemia, hyperlipidemia, hypercholesterolemia, fatty liver, atherosclerosis, and cardiovascular diseases (1). Therefore, numerous chemicals and therapeutic agents targeting lipid metabolism have been developed to treat lipid dysregulation and its related complications.

Peroxisome proliferator–activated receptors (PPARs), members of the nuclear hormone receptors, are important regulators of lipid metabolism. The PPAR family consists of three isoforms, PPARα, PPARβ/δ, and PPARγ (5). Emerging evidence indicates that activation of PPARα or PPARβ/δ would stimulate lipid consumption by enhancing the expression of fatty acid oxidation genes, resulting in amelioration of hyperlipidemia (6,7). PPARγ, however, controls lipid mobilization into adipocytes by promoting adipogenesis and inducing the expression of lipid transport genes such as adipocyte fatty acid binding protein (*aP2*) and *CD36*, thereby reducing lipotoxicity (5,8).

By controlling lipid metabolism through distinct mechanisms, PPAR agonists have been widely used to rescue lipid dysregulation and related metabolic disorders (9,10). Thiazolidinedione, a synthetic PPARγ ligand, greatly improves insulin action, hyperglycemia, and lipotoxicity. However, several concerns such as body weight gain associated with excess increase of fat mass arise in overt type 2 diabetic patients (11). In contrast, fibrate, a selective agonist for PPARα, relieves lipid dysregulation greatly and insulin resistance mildly by the induction of lipid catabolism in hyperlipidemic patients, with a minor reduction of body weight (6,12).

Because some fibrates also have several side effects, such as hepatotoxicity and gallstone formation (13–15), it

has been proposed that more desirable effects with few side effects (e.g., extra body weight gain and hepatotoxicity) would be achieved by simultaneous activation of PPARα and PPARγ in a single chemical compound. In fact, numerous PPARα/γ dual agonists have been identified and tested in obese and insulin-resistant individuals (16–18). However, most of the current PPARα/γ dual agonists that have been developed have shown unexpected side effects such as stroke and heart failure as well as body weight gain (19–22). Therefore, the development of novel PPARα/γ dual agonists with little adverse effects is urgently needed.

In the current study, we characterized a novel PPARα/γ dual agonist, CG301269, which exhibits potent agonist activity more toward PPARα rather than to PPARγ. CG301269 selectively stimulated the activities of PPARα and PPARγ. Biochemical and computational analyses revealed that CG301269 is able to directly activate PPARα and PPARγ. In various cultured cell lines, CG301269 enhanced fatty acid oxidation while it suppressed proinflammatory gene expression. Furthermore, in obese and diabetic *db/db* mice, CG301269 improved the abnormalities of lipid metabolism and insulin resistance without the previously reported side effects of PPARα or PPARγ single agonists such as body weight gain, fatty liver, hepatomegaly, and hepatotoxicity. Collectively, these data suggest that CG301269 would be a potential therapeutic agent for obesity and lipid dysregulation.

RESEARCH DESIGN AND METHODS

Materials. CG301269 was designed by Crystal Genomics (Seoul, Korea) and synthesized by Korea Research Institute of Chemical Technology (Daejeon, Korea). Rosiglitazone and WY14643 were purchased from Cayman Chemicals (Ann Arbor, MI). Oil-red O, tumor necrosis factor α (TNFα), and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO). GW501516 was provided by Dr. J.B. Seo.

Cell culture. Human embryonic kidney (HEK) 293 fibroblasts, FAO rat hepatoma cells, C_2C_{12} murine myocytes, 3T3-L1 murine adipocytes, and RAW 264.7 macrophages were purchased from the American Type Culture Collection (Manassas, VA). Media were supplemented with penicillin (100 units/mL) and streptomycin (100 mg/mL). HEK 293 and 3T3-L1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (both Hyclone, Logan, UT). FAO, C_2C_{12}, and RAW 264.7 cells were grown in DMEM supplemented with 10% fetal bovine serum (Hyclone). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. C_2C_{12} cells were differentiated as described previously (23), and differentiated adipocytes were prepared as described earlier (24).

Transient transfection and gene suppression. Transactivation reporter assay in HEK 293 cells was performed as previously described (23). Briefly, cells were transiently transfected with murine *PPARα* or *PPARγ* expression vector, *RXRα* expression vector, β-galactosidase (*β-gal*) expression vector, and *DR-1 luciferase* reporter vector. At 6 h after transfection, the transfection mixture was replaced with fresh medium containing the appropriate agonist. Luciferase assays were performed after 24 h. Mammalian one-hybrid assay was performed by use of *UAS-luciferase* reporter vector and *GAL4-PPARα* ligand-binding domain (LBD) or *PPARγ* LBD fusion construct. For *PPARα* or *PPARγ* gene suppression, specific small interfering (si)RNA (Bioneer, Daejeon, Korea) was transfected into cells with a MicroPorator (Digital Bio, Seoul, Korea). After electroporation, cells were maintained overnight in growth medium without antibiotics.

Docking simulation. Computer-aided docking simulation was conducted with the Discovery Studio 1.7 program (Accelrys, Inc., San Diego, CA). The LigandFit module implemented in the receptor-ligand interaction protocol was used for detailed calculation. We used the crystal structure of the ligand-bound human PPARα and PPARγ LBD (PDB ID: 3FEI and 3FEJ, respectively) (25). The potential of mean force (26) was used as a scoring function to compare the binding affinity of the each molecule (Supplementary Table 1).

Quantitative RT-PCR analysis. Total RNA was isolated from cells or tissues and cDNA was synthesized. For quantitive (q)RT-PCR reactions, the amplification was performed for 40 repetitive thermal cycles (94°C for 20 s, 60°C for 20 s, and 72°C for 20 s) with SYBR green (Invitrogen, Carlsbad, CA) in a My-IQ thermocycler (Bio-Rad, Hercules, CA). The primers were designed and synthesized from Bioneer (Daejeon, Korea). The primer sequences are listed in Supplementary Table 2.

Metabolic assays. Fatty acid oxidation assays were performed as described previously (23). Briefly, cells were incubated in α-minimum essential medium (Hyclone) containing 0.1 mmol/L palmitate (9,10-[^{3}H]palmitate, 5 μCi/mL; PerkinElmer Life, Boston, MA) and 1% bovine serum albumin for 24 h. After incubation, medium was precipitated with an equal volume of 10% trichloroacetic acid (Sigma), vigorously mixed, and centrifuged at 4°C for 10 min. The supernatants were transferred to capless 1.5-mL microcentrifuge tubes, placed in a scintillation vial containing 0.5 mL of water and incubated overnight at 55°C. Then tubes were removed and ^3H_2O contents were measured in a scintillation counter.

Animal experiments. All animal experiments were approved by the Seoul National University Animal Experiment Ethics Committee. Ten-week-old C57BLKS/J-*Lepr^{db}*/*Lepr^{db}* or 7-week-old C57BL/6J male mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea). C57BLKS/J-*Lepr^{db}*/*Lepr^{db}* mice were orally injected with vehicle (distilled water), WY14643, rosiglitazone, or CG301269 at the concentration of 50 mg/kg once a day for 1 month. PPAR ligands were dissolved into distilled water. Animal experiments were conducted twice. For myocardial ischemia/reperfusion experiments, C57BL/6J mice underwent surgically-induced myocardial infarction and were orally administered with various treatments, as described previously. Detailed experimental procedures are described in the Fig. 8 legend.

For the oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (IPITT), mice were fasted for 24 h (for OGTT) or 6 h (for IPITT) and were injected with glucose (5 g/kg, for OGTT; Sigma) or human insulin (1 unit/kg, for IPITT; Lilly, Indianapolis, IN). The blood glucose level was measured at indicated time point with Freestyle blood glucose meter (Therasense, Uppsala, Sweden).

Biochemical analysis. Blood samples and liver lysates were collected from each group of mice. Plasma and liver TG, FFA, and total cholesterol were measured using Infinity reagents (Thermo, Melbourne, Australia). Plasma concentrations of LDL and HDL were determined as previously described (27). Plasma insulin level was assessed with an insulin ELISA kit (Mercodia, Uppsala, Sweden). Hepatotoxicity was measured by plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

Tissue section and staining. Frozen tissue dissects were prepared as described previously (28). After mounting on glass slides and fixation, dissected samples were stained with Oil-red O solution and photographed at original magnification ×100.

Myocardial ischemia/reperfusion surgery. C57BL/6J mice were anesthetized. Reperfused myocardial infarction surgery was performed as previously described (29). Briefly, after anesthesia, mice were orally intubated and connected to a murine ventilator (Harvard Apparatus, Holliston, MA). A median sternotomy was performed, and the left anterior descending coronary artery was ligated, occluded for 30 min, and then reperfused for 1 h. After reperfusion, the chest wound was reapproximated and mice were extubated and allowed to recover with supplemental oxygen until mobile.

Statistical analysis. Results were representative data from at least three independent experiments. Mean data are presented with standard deviations and shown as error bars in the graphs. *P* values were calculated from ANOVA (Student-Newman-Keuls comparison test as post hoc). $P < 0.05$ was interpreted as being statistically significant.

RESULTS

CG301269 increases transcriptional activities of PPARα and PPARγ. For the specific interactions between nuclear hormone receptors and their ligands, one of the key chemical bonds is the hydrogen bond, which often links between ligands and amino acid residues in the ligand-binding domain of nuclear hormone receptors. To develop a novel PPARα/γ dual agonist, we used the HipHop process (Catalyst software, Accelrys, Inc.) (30), which provides potential accommodation statuses of ligands and receptors to predict hypothetic three-dimensional interactions based on the structures. According to the HipHop process, thiazolidine-4-carboxylic acid and 5-methyl-2-phenyl-oxazole were linked with a phenoxy to provide numerous hydrophobic interactions in the binding pocket.

A number of modified compounds were synthesized and evaluated by transactivation reporter assays with both

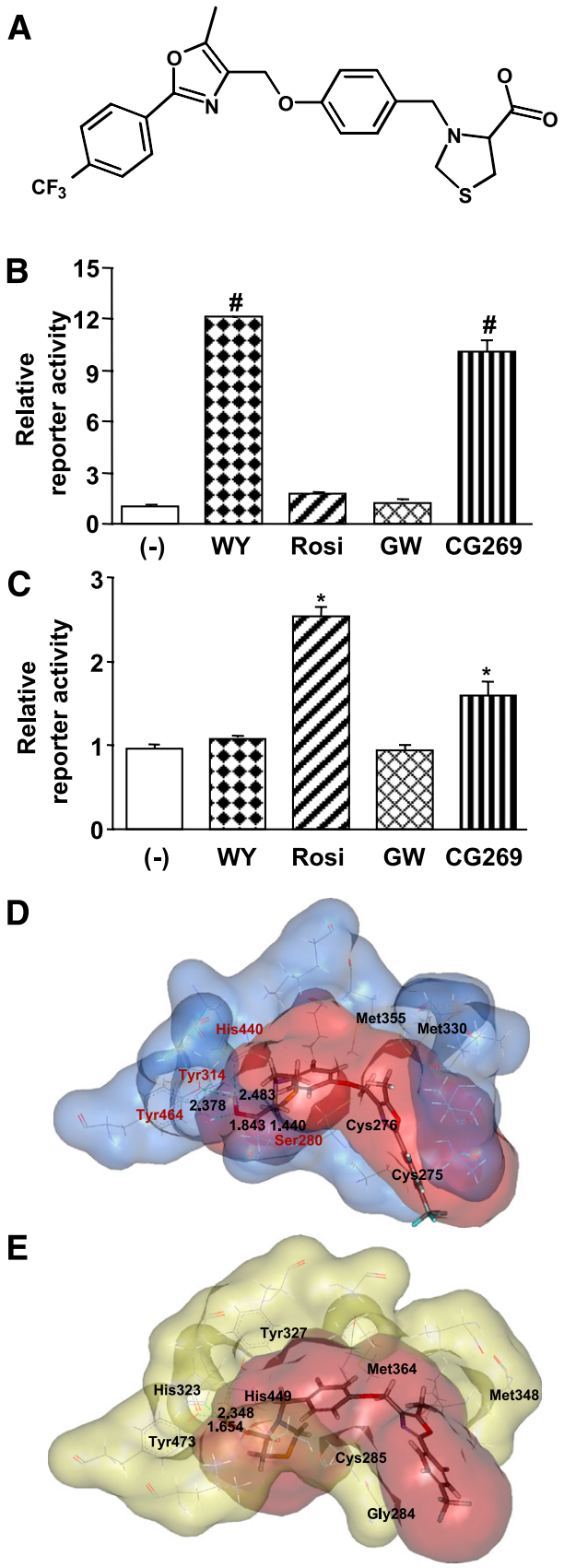

FIG. 1. CG301269 selectively activates PPARα and PPARγ in vitro. *A*: Chemical structure of CG301269. HEK293 cells were transiently cotransfected with murine *PPARα* (*B*) or *PPARγ* (*C*) expression vector, *RXRα* expression vector, and *DR1-luciferase* reporter construct and treated for 24 h with 1 μmol/L WY14643 (WY), rosiglitazone (Rosi), GW501516 (GW), or CG301269 (CG269). Harvested cells were analyzed by reporter assay as described in RESEARCH DESIGN AND METHODS. Each bar represents mean ± SD of duplicates. *$P < 0.05$ vs. negative control; #$P < 0.01$ vs. negative control. *D* and *E*: Docking modes of CG301269 toward the LBD of human PPARα or γ. *D*: PPARα-LBD (PDB ID: 3FEI, binding site volume = 329.5 Å^3). *E*: PPARγ-LBD (PDB ID: 3FEJ, binding site volume = 362.4 Å^3). Displayed amino acids are all protein residues with a ligand contact distance ≤4.5 Å. The docking protocol is described in RESEARCH DESIGN AND METHODS. CG301269 (stick model) has similar binding sites and binding volume compared with known PPARα/γ dual agonist (red cloud); in particular, typical four hydrogen bonds between the carboxylate of CG301269 and the Ser, His, and Tyr residues of PPARα/γ within hydrogen bond distance ≤3 Å. CG301269 can be accommodated at the hydrophobic pockets composed of Met, Cys, and Gly residues of PPARα/γ. Blue cloud, human PPARα; yellow cloud, human PPARγ; red cloud, binding site volume of recently uploaded PPARα/γ dual agonist.

PPARα and PPARγ, and among them, CG301269 (Fig. 1*A*) was selected for further evaluation. As shown in Fig. 1*B* and *C*, CG301269 enhanced both PPARα-and PPARγ-dependent reporter activities in HEK 293 cells to an extent comparable with those of the known agonists WY14643 and rosiglitazone, respectively. CG301269 stimulated the transcriptional activities of murine and human PPARα in a dose-dependent manner (Supplementary Fig. 1*A* and *B*) but failed to activate other nuclear hormone receptors, including PPARδ and LXRα (Supplementary Fig. 1*C* and *D*).

In mammalian one-hybrid assays, CG301269 activated PPARα and PPARγ with half-maximal effective concentration (EC_{50}) of 39 nmol/L and 2.69 μmol/L, respectively (Supplementary Fig. 1*E* and *F*). Under the same condition, EC_{50s} for WY14643 (for murine PPARα) and rosiglitazone (for murine PPARγ) were 81 nmol/L and 14 nmol/L, respectively. On the other hand, CG301269 transactivated human PPARα and PPARγ with a EC_{50} of 29 nmol/L and 5.3 μmol/L, respectively. These data suggest that CG301269 would be a potent PPARα activator but would be a relatively weak PPARγ activator.

CG301269 interacts with the LBD of PPARα and PPARγ. To further address whether CG301269 is able to interact with the LBD of PPARα and PPARγ, CG301269 was subjected to the docking study by comparison with previously reported ternary structures of human PPARα LBD (PDB ID: 3FEI) and human PPARγ LBD (PDB ID: 3FEJ; Fig. 1*D* and *E*) (25). Similar to co-crystal structures of human PPARα and PPARγ with known PPARα/γ dual agonist (31,32), the electrostatic potential surface (molecular volume = 337.5 Å^3) and the docking score of CG301269 toward PPARα/γ implied that CG301269 might fit at the LBD of PPARα and PPARγ with proper binding mode (Supplementary Fig. 2 and Supplementary Table 1). In addition, CG301269 altered trypsin digestion patterns, probably due to changing conformation of liganded PPARα and PPARγ (Supplementary Fig. 3). These data suggest that CG301269 would interact with PPARα and PPARγ through their LBD domains.

CG301269 promotes fatty acid oxidation. Because CG301269 appeared to be a more potent agonist toward PPARα than PPARγ, the effect of CG301269 on fatty acid oxidation was tested. In FAO murine hepatoma cells, various PPARα target genes involved in β-oxidation were induced by CG301269 in a dose-dependent manner (Fig. 2*A*). CG301269-induced genes were acyl-CoA oxidase (*ACO*), carnitine-palmitoyl transferase (*CPT*), fatty acid transporter protein (*FATP*), and middle-chain acyl-CoA dehydrogenase

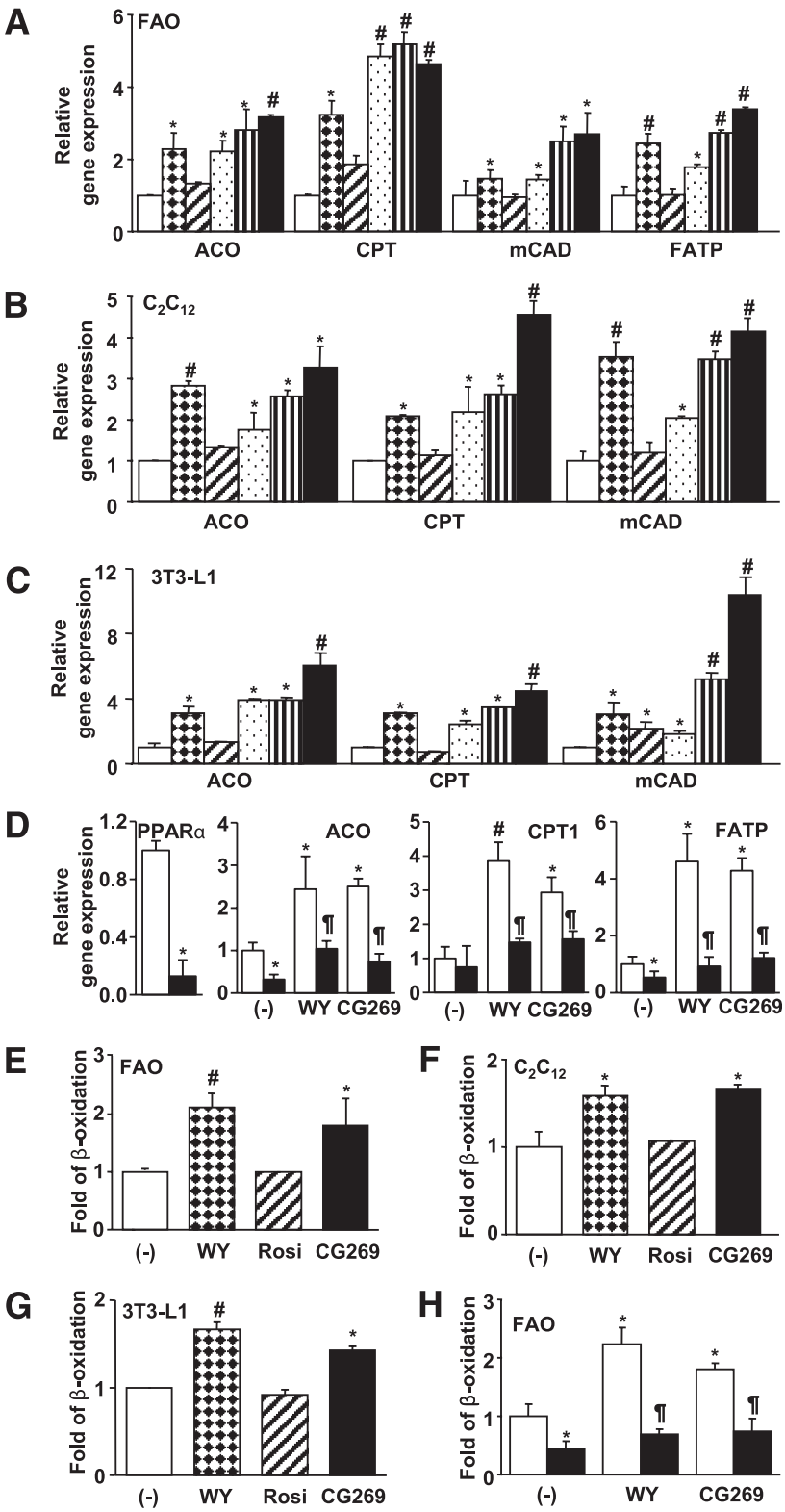

FIG. 2. CG301269 enhances in vitro fatty acid oxidation. FAO rat hepatoma cells (*A*), C_2C_{12} myotubes (*B*), and 3T3-L1 adipocytes (*C*) were incubated with indicated doses of WY, rosiglitazone (Rosi), or CG269 for 24 h. Relative gene expression was analyzed by use of qRT-PCR and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each bar represents mean ± SD of duplicates. □, (−); ◘, WY (1 μmol/L); ▨, Rosi (1 μmol/L); ▩, CG269 (0.5 μmol/L); ▥, CG269 (1 μmol/L); ■, CG269 (5 μmol/L). *$P < 0.05$ vs. (−); #$P < 0.01$ vs. (−). *D*: FAO cells were transfected with *GFP* siRNA (□) or *PPARα* siRNA (■) and treated with indicated PPAR agonists (1 μmol/L) for 24 h. Relative gene expression was measured by qRT-PCR and normalized by GAPDH. Each bar represents mean ± SD of triplicates. *$P < 0.05$ vs. *GFP* siRNA (−); #$P < 0.01$ vs. *GFP* siRNA (−); ¶$P < 0.05$ vs. *GFP* siRNA + WY or CG269. FAO (*E*), C_2C_{12} (*F*), and 3T3-L1 (*G*) cells were incubated with 10 μmol/L of WY, Rosi, or CG269 for 24 h. Fatty acid oxidation assay was performed as described in RESEARCH DESIGN AND METHODS. Each bar represents mean ± SD of triplicates. *$P < 0.05$ vs. negative control; #$P < 0.01$ vs. negative control. *H*: FAO cells were transfected with *GFP* siRNA (□) or *PPARα* siRNA (■) and were subjected to fatty acid oxidation assay. Each bar represents mean ± SD of triplicates. *$P < 0.05$ vs. *GFP* siRNA; #$P < 0.01$ vs. *GFP* siRNA; ¶$P < 0.05$ vs. *GFP* siRNA + WY or CG269.

(*mCAD*). Similar results were observed in C_2C_{12} (Fig. 2*B*), 3T3-L1 (Fig. 2*C*), and 3T3-F442A cells (Supplementary Fig. 4*A*). However, knockdown of PPARα (Fig. 2*D*) with siRNA, but not PPARδ (data not shown), attenuated the induction of these fatty acid oxidation genes upon CG301269, supporting the idea that CG301269 would selectively act as a PPARα agonist.

Fatty acid oxidation assays were performed to further examine whether CG301269 would indeed promote β-oxidation. In various cell lines, CG301269 and WY14643 significantly elevated fatty acid oxidation (Fig. 2*E–G* and Supplementary Fig. 4*B*), which was required for activated PPARα (Fig. 2*H*). Taken together, these findings indicate that CG301269 would enhance lipid catabolism by induction of fatty acid oxidation genes by way of PPARα activation.

CG301269 suppresses proinflammatory gene expression in vitro. As a dual activator of PPARα and PPARγ, CG301269 stimulated the expression of PPARγ target genes including *aP2*, *CD36*, adiponectin (*Acrp30*), and lipoprotein lipase (*LPL*) in fully differentiated adipocytes (3T3-L1 and 3T3-F442A cells) and white adipose tissue (Supplementary Fig. 5*A–C*). Moreover, the expression of PPARγ target genes by CG301269 was prevented by knockdown of PPARγ with siRNA (Supplementary Fig. 5*D*). In differentiated 3T3-L1 adipocytes, certain genes regulated by CG301269 were also modulated by rosiglitazone, implying that CG301269 appears to alter the expression of certain sets of genes by partial activation of PPARγ (Supplementary Fig. 6).

The fact that activation of PPARs with their ligands drastically downregulates proinflammatory responses (33–35) led us to investigate whether CG301269 would repress proinflammatory gene expression. In RAW 264.7 macrophages and 3T3-L1 adipocytes, LPS and TNFα, respectively, greatly stimulated the expression of proinflammatory genes, including *TNFα*, interleukin-1β (*IL-1β*), matrix metalloprotease 9 (*MMP9*), inducible nitric oxide synthase (*iNOS*), monocyte chemoattractant protein-1 (*MCP-1*), chemokine (*C-C motif*) receptor 2 (*CCR2*), cyclooxygenase-2 (*COX2*), and *IL-6* (Fig. 3). Notably, preincubation with CG301269 suppressed LPS- and TNFα-induced inflammatory responses in a dose-dependent manner (Fig. 3). Similar results were obtained in TNFα-induced 3T3-F442A adipocytes (Supplementary Fig. 7). The inhibitory effect of CG301269 on proinflammatory gene expression was comparable or even better than known PPARα and PPARγ ligands, WY14643, and rosiglitazone (Fig. 3 and Supplementary Fig. 7). These data suggest that CG301269 would decrease proinflammatory responses through activation of PPARα or PPARγ.

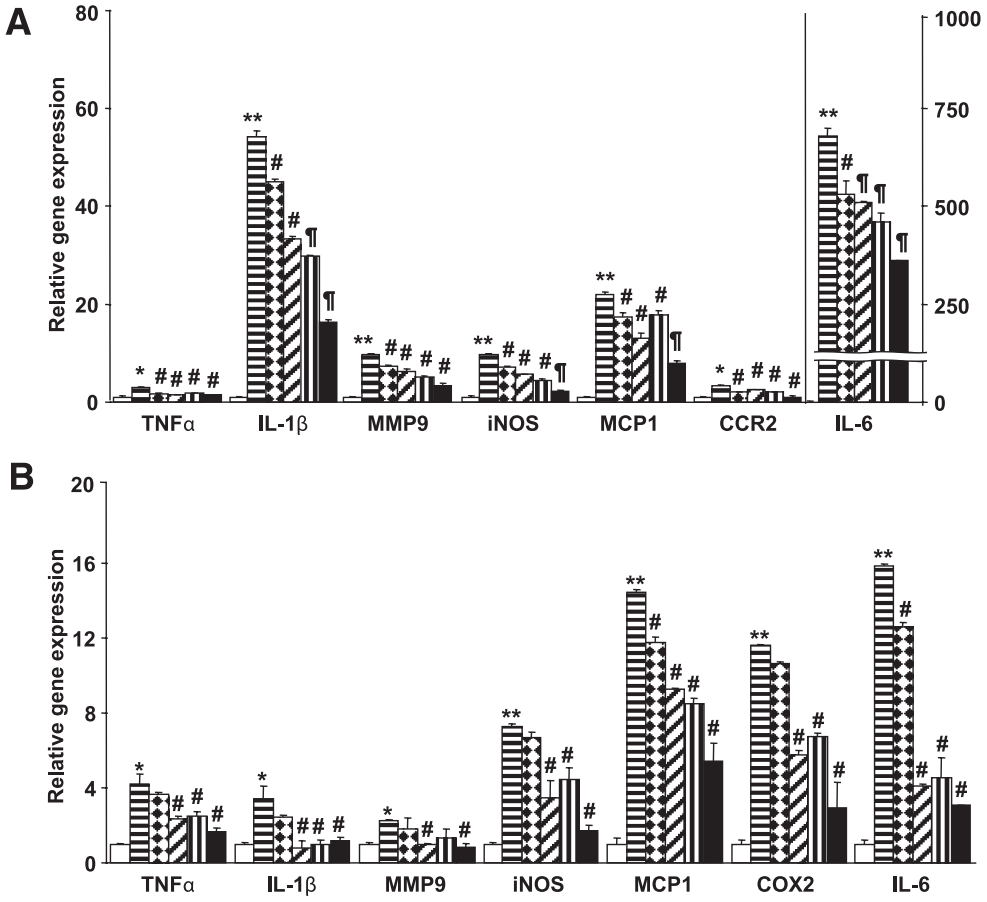

FIG. 3. CG301269 suppresses proinflammatory gene expression in vitro. RAW 264.7 macrophages (*A*) and 3T3-L1 adipocytes (*B*) were preincubated with indicated agonists for 12 h and were treated with LPS (10 ng/mL; *A*) for 6 h and TNFα (1 ng/mL; *B*) for 3 h. Relative gene expression was determined by qRT-PCR and normalized by glyceraldehyde-3-phosphate dehydrogenase. Each bar represents mean ± SD of duplicates. □, (-); ▤, LPS 10 ng/mL (*A*) or TNFα 1 ng/mL (*B*); ◘, WY 1 μmol/L/LPS (*A*) or WY 1 μmol/L/TNFα (*B*); ▨ Rosi 1 μmol/L/LPS (*A*) or Rosi 1 μmol/L/TNFα (*B*); ▥, CG269 1 μmol/L/LPS (*A*) or CG269 1 μmol/L/TNFα (*B*); ■, CG269 5 μmol/L/LPS (*A*) or CG269 5 μmol/L/TNFα (*B*). *$P < 0.05$ vs. negative control; **$P < 0.01$ vs. negative control; #$P < 0.05$ vs. LPS (*A*) or TNFα (*B*); ¶$P < 0.01$ vs. LPS (*A*) or TNFα (*B*).

CG301269 attenuates body weight gain and improves plasma lipid and glucose profiles in *db/db* mice. To explore the effects of CG301269 in vivo, we administered CG301269 to obese and diabetic *db/db* mice. Unlike rosiglitazone, a strong PPARγ ligand that yielded significant body weight gain as previously reported (11,36), CG301269 prevented body weight gain of *db/db* mice (Fig. 4*A*), without food intake change (Supplementary Fig. 8). Activation of PPARα or PPARγ with their ligands has been reported to lower plasma glucose, insulin, and lipid contents in diabetic individuals (9,10). Thus, we next examined whether CG301269 is able to achieve such beneficial effects on lipid and glucose metabolism. Consistent with previous reports (10,12), WY14643 and rosiglitazone effectively improved hyperglycemia and lipid profiles in *db/db* mice (Fig. 4*B*). As a PPARα/γ dual agonist, CG301269 also remarkably diminished blood glucose, insulin, and lipid contents (Fig. 4*B*). Additionally, CG301269 decreased plasma LDL and increased plasma HDL levels (Fig. 4*B*), implying that CG301269 appears to rescue cholesterol metabolism in obese animals. Moreover, CG301269 decreased plasma ALT and AST levels by 25% in *db/db* mice (Fig. 4*B*), indicating that CG301269 would not induce hepatotoxicity, unlike pure PPARγ agonists (37,38). Furthermore, compared with known PPARα or PPARγ ligands, CG301269 did not increase liver weight or fat tissue masses (Fig. 4*C*). These data strongly propose that CG301269 would efficiently improve plasma glucose and lipid contents without a significant body weight gain (with increased adiposity) or hepatotoxicity, or both.

CG301269 ameliorates glucose and insulin tolerance in *db/db* mice. To assess whether CG301269 might affect glucose and insulin tolerance in obese and diabetic animals, OGTT and IPITT assays were conducted in *db/db* mice. Like WY14643 and rosiglitazone, CG301269 ameliorated glucose and insulin tolerance in obese *db/db* mice (Fig. 5*A* and *B*). The effect of CG301269 on glucose and insulin sensitivity was stronger than that of WY14643 and weaker than that of rosiglitazone, which appeared to correlate with the results that CG301269 is a strong PPARα agonist but is a weak PPARγ agonist.

In diabetic animals, expression of hepatic gluconeogenic enzyme genes is greatly increased, contributing to hyperglycemia (39). Because we observed that CG301269

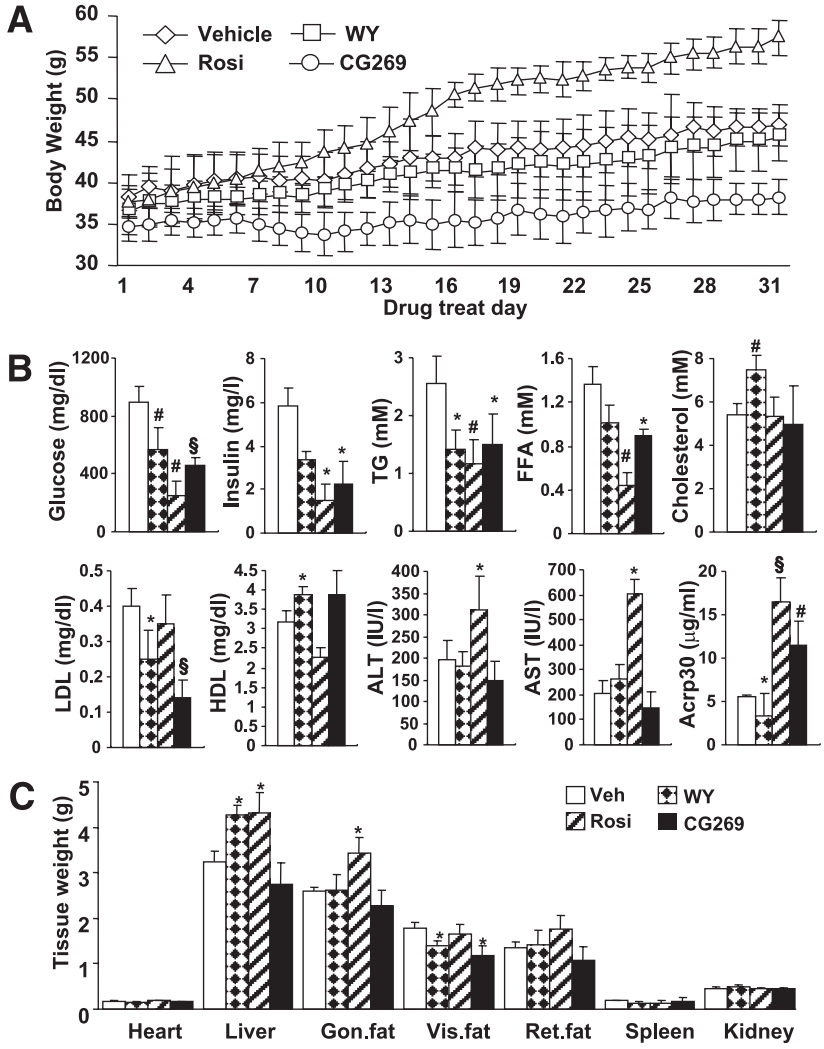

FIG. 4. CG301269 attenuates body weight gain in *db/db* mice. *A*: Body weight changes of PPAR agonists-administered obese and diabetic *db/db* mice. Data (n = 6 for each group and n = 7 for CG269) were shown as symbols; ◇, vehicle (Veh); □, WY; △, Rosi; ○, CG269. Each bar represents mean ± SD. *B*: A series of plasma profiles from PPAR agonists-administered *db/db* mice. Data are means ± SD. *P < 0.05 vs. Veh; #P < 0.01 vs. Veh; §P < 0.001 vs. Veh. *C*: Average tissue weights of PPAR agonist-treated *db/db* mice. Each bar represents mean ± SD. □, Veh; ⊡, WY; ▨, Rosi; ■, CG269. *P < 0.05 vs, Veh; #P < 0.01 vs, Veh. Gon, gonadal; Vis, visceral; Ret, retroperitoneal.

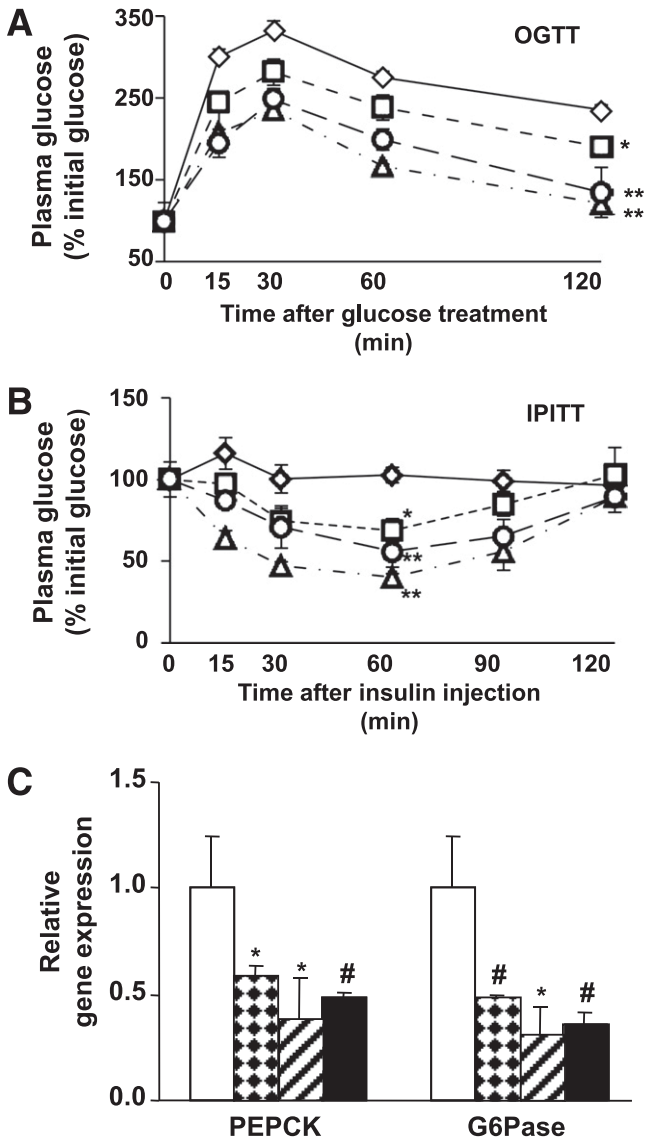

FIG. 5. CG301269 ameliorates glucose and insulin tolerance in *db/db* mice. Mice were fasted and injected with glucose (*A*) or insulin (*B*) as described in RESEARCH DESIGN AND METHODS. Plasma glucose and insulin levels were measured from the blood samples that were drawn at baseline (t = 0 min) and indicated time points. ◇, vehicle; □, WY14643; △, rosiglitazone; ○, CG301269. *C*: Relative gene expression of gluconeogenic genes from the liver of indicated drug-treated obese *db/db* mice were determined by use of qRT-PCR and normalized with glyceraldehyde-3-phosphate dehydrogenase. Data represent means ± SD. □, Veh; ⊡, WY; ▨, Rosi; ■, CG269. *P < 0.05 vs. Veh, #P < 0.01 vs. Veh.

recovered insulin sensitivity and plasma glucose level in *db/db* mice, the effect of CG301269 on gluconeogenic gene expression was tested. As shown in Fig. 5*C*, CG301269 decreased the expression of key gluconeogenic genes, such as phosphoenolpyruvate carboxykinase (*PEPCK*) and glucose-6-phosphatase (*G6Pase*) in the liver of *db/db* mice.

CG301269 improves hepatic steatosis in *db/db* mice. Fatty liver is one common complication of obesity, insulin resistance, and type 2 diabetes. Activation of PPARα has been reported to alleviate fatty liver by increase of lipid consumption and decrease of lipogenesis, whereas the effect of activated PPARγ on fatty liver is controversial (40,41). Because CG301269 was developed and identified as a PPARα/γ dual agonist, we asked whether CG301269 might relieve hepatic steatosis in *db/db* mice. In accordance with previous reports (40,41), rosiglitazone failed to decrease hepatic steatosis, whereas WY14643 evidently alleviated fatty liver (Fig. 6*A* and *B*). Similar to WY14643, CG301269 greatly decreased hepatic TG, FFA, and cholesterol accumulation in *db/db* mice (Fig. 6*A*). Reduced hepatic lipid contents upon CG301269 were further monitored through Oil-red O staining (Fig. 6*B*). As expected, CG301269 increased the expression of fatty acid oxidation genes in several metabolic tissues, including liver, skeletal muscle, and white adipose tissue (Fig. 6*C*), but decreased the expression of hepatic lipogenic genes (Supplementary Fig. 9). CG301269 also elevated the plasma content of adiponectin (Acrp30) (Fig. 4*B*), which enhances lipid metabolism and alleviates metabolic disorders including fatty liver (42). Although WY14643 induced hepatomegaly, a pathologically enlarged liver induced by aberrant PPARα activation and peroxisome proliferation, CG301269 did not increase liver weight in *db/db* mice (Fig. 4*C* and Supplementary Fig. 10). These results suggest that CG301269 would improve fatty liver without severe hepatomegaly, a fatal side effect of potent PPARα activators.

CG301269 reduces fat cell size and proinflammatory gene expression in *db/db* mice. Chronic and low-grade inflammation in hypertrophic adipocytes containing large amounts of lipid contents is reported to play a key role in the progress of obesity-induced insulin resistance (43). Because adipocyte size has been considered one of the indicators of metabolic stresses, including insulin resistance, inflammation, and hyperlipidemia, we measured the fat cell size in CG301269-treated obese animals. In white adipose tissue, CG301269 not only decreased fat cell size but also downregulated the expression of inflammatory genes, including *TNFα*, *iNOS*, and *MCP-1*, and glucose-6-phosphate dehydrogenase (*G6PD*; Fig. 7*A* and *B*). Furthermore, the expression levels of macrophage-specific marker genes (i.e., *CD68*, *F4/80*, *CD11b*, and *CD11c*) were reduced by CG301269 in white adipose tissue of obese *db/db* mice (Fig. 7*C*), implying that CG301269 may effectively decrease hypertrophic adipocytes and repress proinflammatory responses in obese animals.

CG301269 does not deteriorate heart failure. Previous research indicated that PPARα/γ dual agonists often caused cardiovascular dysfunction (20,44,45). To test this upon CG301269, we have conducted myocardial ischemia/reperfusion surgical procedures with C57BL/6J mice and *db/db* mice. Because of the low survival rate of *db/db* mice after surgery, probably due to hyperglycemia (29), C57BL/6J mice have been extensively analyzed. Compared with the sham group, mice treated with vehicle or CG301269 revealed the characteristics of heart failure, such as hypokinetic wall motion abnormality in the septum, dilated left ventricle (LV), altered LV function, and LV fibrosis at 4 weeks after myocardial ischemia/reperfusion surgery (Fig. 8*A*–*C*). More importantly, CG301269 treatment did not further deteriorate these parameters, implying that CG301269 may not aggravate heart failure. The body weight, heart weight/body weight, and wet lung weight/body weight were not changed by CG301269 administration (Supplementary Table 3). Together, these data proposed that CG301269 might not exacerbate heart failure or related cardiomyopathy, at least in C57BL/6J mice.

DISCUSSION

Type 2 diabetes and related complications are rising as serious health problems in westernized societies. The

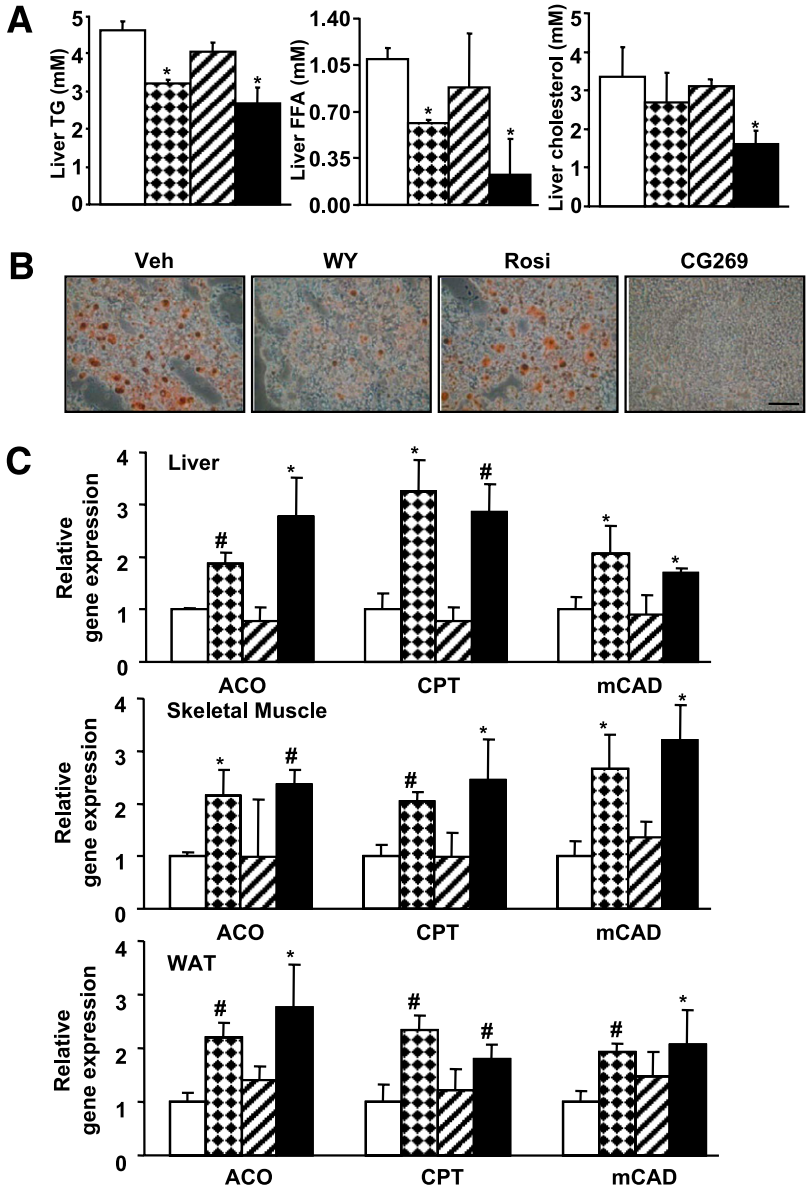

FIG. 6. CG301269 improves hepatic steatosis in *db/db* mice. *A*: Accumulated lipids in liver of PPAR agonists-treated *db/db* mice. Liver TG, FFA, and cholesterol levels were measured as described in RESEARCH DESIGN AND METHODS. Each bar represents mean ± SD. □, Veh; ⊡, WY; ▨, Rosi; ■, CG269. **P* < 0.05 vs. Veh. *B*: Oil-red O staining of liver tissue from PPAR agonist-treated *db/db* mice. Scale bar = 50 μm. *C*: Relative gene expression of each gene in liver, skeletal muscle, and white adipose tissue (WAT) from *db/db* mice was determined by use of qRT-PCR and normalized by glyceraldehyde-3-phosphate dehydrogenase. Each bar represents mean ± SD. □, Veh; ⊡, WY; ▨, Rosi; ■, CG269. **P* < 0.05 vs. Veh; #*P* < 0.01 vs. Veh. (A high-quality digital representation of this figure is available in the online issue.)

therapeutic advantages of PPARα and/or PPARγ agonists in metabolic disorders have prompted development of PPARα/γ dual activators in a single compound. In this study, we identified CG301269 through virtual screening for PPARα/γ dual agonists and characterized its specificity to activation of PPARα and PPARγ. Compared with earlier PPARα/γ dual agonists, CG301269 seemed to exhibit favorable effects on obesity and hyperlipidemia, without the concerns of weight gain, fatty liver, hepatotoxicity, tissue hypertrophy, and fluid retention.

CG301269 shows dual agonist activities for both PPARα and PPARγ, but shows higher affinity to PPARα than PPARγ. Therefore, it seems likely that the mechanisms responsible for CG301269-mediated regulation of lipid homeostasis would be distinct from thiazolidinedione. Even though CG301269 was able to enhance adipogenesis as a partial PPARγ agonist, the adipogenic effect of CG301269 was much milder than that of rosiglitazone in vitro with 3T3-L1 cells (data not shown). Rather, the findings that CG301269 effectively stimulates PPARα activities, even at a low dose, proposed the idea that CG301269 would primarily govern lipid metabolism by induction of fatty acid oxidation. Indeed, consistent with in vitro experiments, CG301269 increased expression of PPARα target genes in peripheral tissues of obese *db/db* mice, suggesting that CG301269 would potently reduce extra lipid contents by enhancing lipid catabolism, and thereby result in the prevention of body weight gain, at least in part.

Fatty liver is a common complication in obesity and in type 2 diabetes and is closely associated with insulin

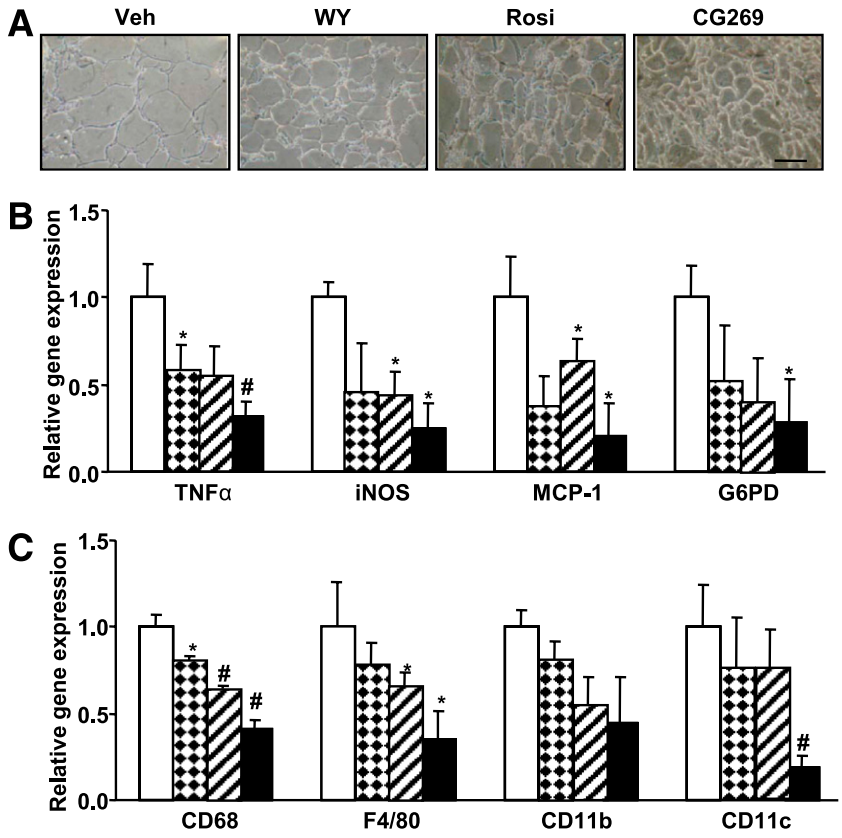

FIG. 7. CG301269 reduces average fat cell size and improves inflammation in WAT of *db/db* mice. *A*: WAT histology of CG301269-treated *db/db* mice. WAT of each group of *db/db* mice was frozen-sectioned as described in RESEARCH DESIGN AND METHODS. Scale bar = 100 μm. The mRNA expression of proinflammatory genes (*B*) and macrophage marker genes (*C*) in WAT of PPAR agonists-treated *db/db* mice was determined by use of qRT-PCR and normalized by glyceraldehyde-3-phosphate dehydrogenase. Data represent mean ± SD. □, Veh; ⊡, WY; ▨, Rosi; ■, CG269. **P* < 0.05 vs. Veh; #*P* < 0.01 vs. Veh. (A high-quality digital representation of this figure is available in the online issue.)

resistance (46). CG301269 improved hepatic steatosis by decreasing hepatic TG and FFA contents in obese *db/db* mice, an effect probably due to higher efficacy of CG301269 toward PPARα over PPARγ. Intriguingly, CG301269-treated obese *db/db* mice did not show hepatomegaly, a common side effect of potent PPARα agonists in rodents (5,47). The beneficial effect against fatty liver without hepatomegaly is not exclusive to CG301269, because several PPARα/γ dual agonists are reported to do so (18,48), However, its exact molecular mechanism remains to be elucidated.

Low-grade and chronic inflammation is closely associated with metabolic disorders, including obesity, insulin resistance, type 2 diabetes, and atherosclerosis. Enlarged, hypertrophic fat cells secrete a number of proinflammatory cytokines (43), and blockade of proinflammatory responses ameliorates insulin resistance (49,50). In obese and diabetic *db/db* mice, CG301269 reduced fat cell size and repressed the expression of proinflammatory genes, implying that CG301269 might contribute to anti-inflammatory responses in the adipose tissue of obese individuals. Although it is yet unclear whether reduced expression of proinflammatory genes is a cause or effect of CG301269 in obese animals, it is likely that the effects of antiadiposity and the anti-inflammatory property of CG301269 would contribute to improve insulin sensitivity.

We found it interesting that CG301269 potently prevented the increase of body fat mass. Because many type 2 diabetic patients have associated overweight or obesity, induction of extra fat accumulation could be a serious side effect. Unfortunately, most of the PPARα/γ dual agonists developed to date have failed to solve this problem because they have preferential affinity to PPARγ (17,19). Unlike previously synthesized PPARα/γ dual agonists, CG301269 partially activates PPARγ. Compared with other known PPARα/γ dual agonists, the finding that CG301269 did not induce body weight gain proposes therapeutic advantages of CG301269. Detailed molecular mechanism (s) by which CG301269 would protect increase in adiposity still remains to be elucidated.

We examined the effects of CG301269 on cardiac complications because previously developed PPARα/γ dual agonists often deteriorate cardiovascular complications (20,44,45). Although our experimental approaches were not perfect to exclude the possibility that CG301269 is safe for cardiovascular diseases, we observed that administration of CG301269 into myocardial ischemia-induced C57BL/6J mice for 4 weeks did not deteriorate heart failure (Fig. 8 and Supplementary Table 3).

In summary, we have demonstrated that CG301269 has beneficial effects on glucose and lipid metabolism to relieve metabolic disorders by selectively activating PPARα and PPARγ without several adverse effects, including body weight gain, fatty liver, hepatotoxicity, and hepatomegaly, which have been observed in previously identified PPAR agonists, including PPARα/γ dual agonists (36,37). CG301269 ameliorates lipid dysregulation by consumption of excess lipids in peripheral tissues rather than redistribution of them into adipose tissue to alleviate fatty liver and chronic

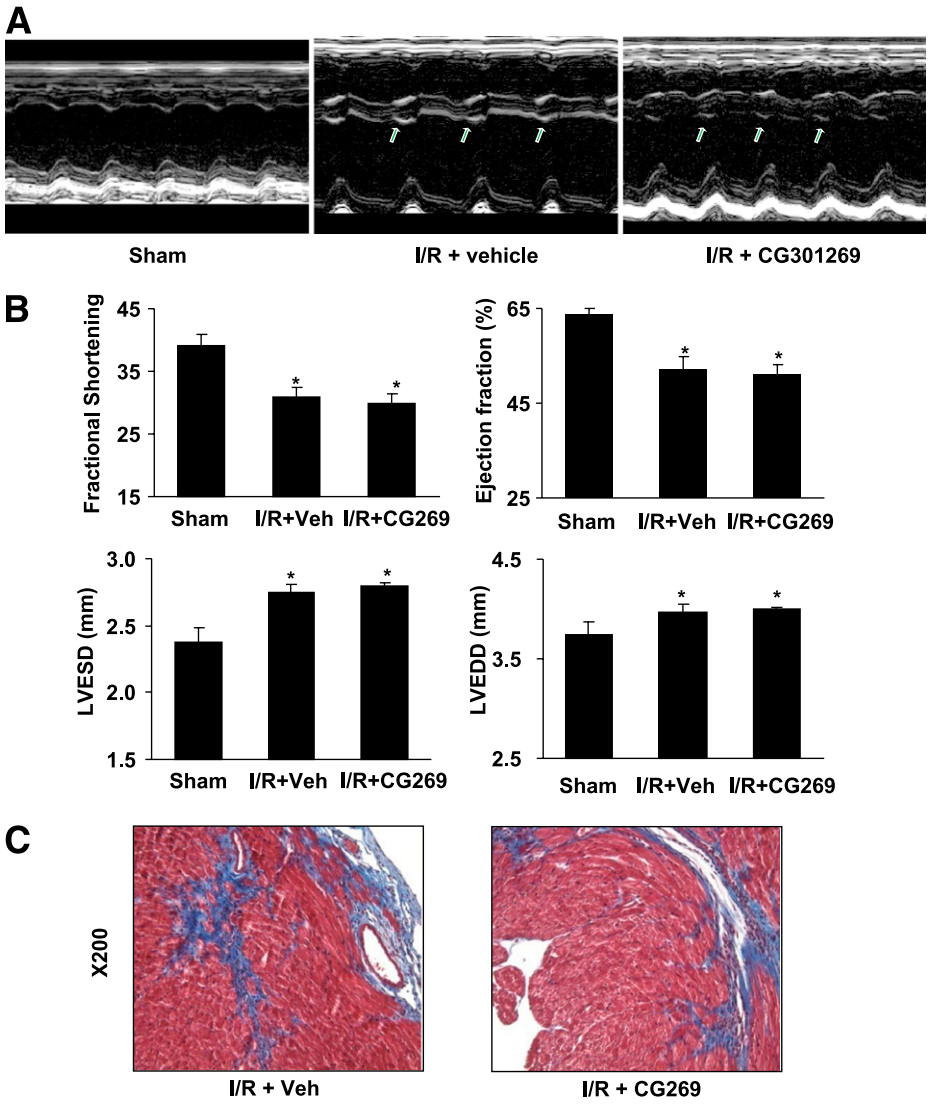

FIG. 8. CG301269 does not aggravate heart failure after myocardial ischemia/reperfusion (I/R) in C57BL/6J mice. Surgical reperfused myocardial infarction was induced in mice as described in RESEARCH DESIGN AND METHODS. Three groups of mice—sham, myocardial I/R with carboxymethyl cellulose (CMC) as vehicle, and myocardial I/R with CG269 dissolved with CMC—were monitored for 4 weeks. *A*: Echocardiography was performed at baseline and at 4 weeks after myocardial I/R with a 14-MHz linear probe (Toshiba, Japan). White arrow indicates hypokinetic wall motion abnormality in the septum area. *B*: Heart function was analyzed by measuring fractional shortening (FS = [LV end-diastolic diameter (LVEDD) − LV end-systolic diameter (LVESD)] × 100/LVEDD) and LV ejection fraction (LVEF = [$LVEDD^2 - LVESD^2$]/$LVEDD^2$). *C*: Histologic analysis. Heart tissue was sectioned in to 4 μm slices and stained with Masson trichrome for evaluation of fibrosis. *$P < 0.05$ vs. sham. LV, left ventricle. (A high-quality digital representation of this figure is available in the online issue.)

inflammation. Therefore, we suggest that CG301269 would be a potential lead compound against obesity and insulin resistance.

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H.W.J. and J.-W.L researched data, wrote the manuscript, and reviewed and edited the manuscript. W.S.K. and S.K.C. researched data. K.-H.K. researched data and contributed to discussion. H.S.P., H.J.S., and G.Y.L. researched data. D.S. researched data and contributed to discussion. H.L. researched data. J.H.L. researched data and contributed to discussion. E.B.C. and H.K.L. researched data. H.C. researched data and reviewed and edited the manuscript; S.B.P., K.S.P., H.-S.K., and S.R. contributed to discussion and wrote the manuscript. J.B.K. contributed to discussion, wrote the manuscript, and reviewed and edited the manuscript.

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