

# A Nonthiazolidinedione Peroxisome Proliferator-Activated Receptor $\alpha/\gamma$ Dual Agonist CG301360 Alleviates Insulin Resistance and Lipid Dysregulation in *db/db* Mice<sup>[S]</sup>

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## ABSTRACT

Activation of peroxisome proliferator-activated receptors (PPARs) have been implicated in the treatment of metabolic disorders with different mechanisms; PPAR $\alpha$  agonists promote fatty acid oxidation and reduce hyperlipidemia, whereas PPAR $\gamma$  agonists regulate lipid redistribution from visceral fat to subcutaneous fat and enhance insulin sensitivity. To achieve combined benefits from activated PPARs on lipid metabolism and insulin sensitivity, a number of PPAR $\alpha/\gamma$  dual agonists have been developed. However, several adverse effects such as weight gain and organ failure of PPAR $\alpha/\gamma$  dual agonists have been reported. By use of virtual ligand screening, we identified and characterized a novel PPAR $\alpha/\gamma$  dual agonist, (R)-1-(4-(2-(5-

methyl-2-*p*-tolylloxazol-4-yl)ethoxy)benzyl)piperidine-2-carboxylic acid (CG301360), exhibiting the improvement in insulin sensitivity and lipid metabolism. CG301360 selectively stimulated transcriptional activities of PPAR $\alpha$  and PPAR $\gamma$  and induced expression of their target genes in a PPAR $\alpha$ - and PPAR $\gamma$ -dependent manner. In cultured cells, CG301360 enhanced fatty acid oxidation and glucose uptake and it reduced pro-inflammatory gene expression. In *db/db* mice, CG301360 also restored insulin sensitivity and lipid homeostasis. Collectively, these data suggest that CG301360 would be a novel PPAR $\alpha/\gamma$  agonist, which might be a potential lead compound to develop against insulin resistance and hyperlipidemia.

## Introduction

Insulin resistance is an abnormal physiological phenomenon caused by defects in insulin action devoid of the deficiency of insulin (Kahn, 1994). Diminished insulin activity leads to elevated circulating glucose and free fatty acids (FFAs), which are crucial factors for various metabolic disorders, including type 2 diabetes, hyperlipidemia, hyperglycemia, atherosclerosis, and cardiovascular diseases (DeFronzo and Ferrannini, 1991). In the last several decades, numerous drugs or therapeutic agents targeting the above disorders

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**ABBREVIATIONS:** FFA, free fatty acid; TZD, thiazolidinedione; PPAR, peroxisome proliferator-activated receptor; LBD, ligand-binding domain; WY14643, pirinixic acid; GW501516, 2-[2-methyl-4-([4-methyl-2-(4-(trifluoromethyl)phenyl)-1,3-thiazol-5-yl]methylsulfanyl)phenoxy]acetic acid; CG301360, (R)-1-(4-(2-(5-methyl-2-*p*-tolylloxazol-4-yl)ethoxy)benzyl)piperidine-2-carboxylic acid; TNF, tumor necrosis factor; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PDB, Protein Data Bank; qRT-PCR, quantitative real-time polymerase chain reaction; TG, triacylglyceride; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBST, Tris-buffered saline with 0.1% Tween 20; WAT, white adipose tissue; FGF21, fibroblast growth factor 21; siRNA, small interfering RNA.

have been developed. For instance, fibrates have been prescribed to treat hyperlipidemia and lipid dysregulation (Guérin et al., 1996), whereas thiazolidinediones (TZDs) have been used to relieve insulin resistance and type 2 diabetes (Saltiel and Olefsky, 1996). Fibrates and TZDs act as ligands for peroxisome proliferator-activated receptor (PPAR)  $\alpha$  and  $\gamma$ , respectively, which are key transcriptional regulators of glucose and lipid metabolism (Issemann and Green, 1990; Lehmann et al., 1995).

Although TZDs potentially sensitize insulin action in peripheral tissues to ameliorate insulin resistance, several negative concerns, such as weight gain, hepatotoxicity, edema, and heart failure, have been reported (Watkins and Whitcomb, 1998; Hirsch et al., 1999; Tang et al., 2003; Shim et al., 2006). Unlike TZD, PPAR $\alpha$  agonists mediate fatty acid oxidation to alleviate hypertriglyceridemia and also mildly reduce body adiposity (Guerre-Millo et al., 2000). Given the facts that selective activation of PPAR $\alpha$  and PPAR $\gamma$  enhance lipid and glucose metabolism, respectively (Chaput et al., 2000; Carmona et al., 2005), combination of PPAR $\alpha$  and PPAR $\gamma$  agonists would be expected to achieve beneficial effects on restoring metabolic disorders. Hence, a number of PPAR $\alpha/\gamma$  dual agonists have been designed and developed (Murakami et al., 1998; Goldstein et al., 2006; Harrity et al., 2006). However, recently identified PPAR $\alpha/\gamma$  dual agonists were ineffective because of undesirable side effects during preclinical or clinical trials. For example, muraglitazar, a synthetic PPAR $\alpha/\gamma$  dual agonist, was aborted during clinical trials because of increased mortality, fluid retention, edema, and cancer (Mittra et al., 2007; Tannehill-Gregg et al., 2007), and tesaglitazar was reported to cause fibrosarcoma in subcutaneous tissues (Hellmold et al., 2007). Furthermore, it has recently been reported that several types of TZDs induce tissue toxicity (Lloyd et al., 2002; Nissen and Wolski, 2007). Therefore, we aimed to identify non-TZD-based PPAR $\alpha/\gamma$  dual agonists to overcome and/or minimize previously reported side effects of PPAR $\alpha/\gamma$  dual agonists. To screen and identify novel PPAR $\alpha/\gamma$  dual agonists, we used virtual ligand screening and identified several candidate compounds that might fit into the ligand-binding domain (LBD) of PPAR $\alpha$ .

In the present study, we characterized a novel PPAR $\alpha/\gamma$  dual agonist, (*R*)-1-(4-(2-(5-methyl-2-*p*-tolylloxazol-4-yl)ethoxy)benzyl)piperidine-2-carboxylic acid (CG301360), and examined its effects on insulin sensitivity and lipid metabolism through *in vitro* and *in vivo* studies. As a modest PPAR $\alpha/\gamma$  dual agonist, CG301360 selectively activated the transcriptional activities of both PPAR $\alpha$  and PPAR $\gamma$ . In obese and diabetic *db/db* mice, CG301360 rescued severe insulin resistance, hyperlipidemia, and hyperglycemia through stimulation of glucose uptake and fatty acid oxidation. Furthermore, CG301360 also suppressed pro-inflammatory responses, which are closely related to insulin sensitivity. Together, our findings suggest that CG301360 would be a useful chemical for the treatment of insulin resistance and lipid dysregulation.

## Materials and Methods

**Reagents.** CG301360 was designed in Crystal Genomics (Seoul, Korea) and synthesized by Korea Research Institute of Chemical Technology (Daejeon, Korea). Rosiglitazone and pirinixic acid (WY14643) were obtained from Cayman Chemical (Ann Arbor, MI), and 2-[2-methyl-4-([4-methyl-2-[4-(trifluoromethyl)phenyl]-1,3-thia-

zol-5-yl)methylsulfanyl]phenoxy]acetic acid (GW501516) was kindly provided by Dr. J. B. Seo (Boram Pharmaceutical Co., Seoul, Korea). Tumor necrosis factor (TNF)  $\alpha$  and lipopolysaccharide (LPS) were acquired from Sigma (St. Louis, MO). Antibodies for adiponectin and GAPDH were purchased from Cell Signaling Technology (Danvers, MA) and Ab Frontier (Seoul, Korea), respectively. All media and additives for cell culture were purchased from Hyclone (Logan, UT). Adiponectin-ELISA kit was acquired from Adipogen (Incheon, Korea).

**Cell Culture and Glucose Uptake Assay.** All cells [human embryonic kidney (HEK) 293, FAO, C<sub>2</sub>C<sub>12</sub>, 3T3-L1, and RAW 264.7] used in this study were obtained from American Type Culture Collection (Manassas, VA). HEK 293 and 3T3-L1 cells were cultured in DMEM supplemented with 10% bovine calf serum and 1% penicillin/streptomycin, and the other cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. C<sub>2</sub>C<sub>12</sub> and 3T3-L1 cells were differentiated as described previously (Yoon et al., 2006). Glucose uptake assay was performed in differentiated 3T3-L1 adipocytes as described previously (Jeong et al., 2009).

**Gene Transfer and Reporter Assay.** Transfection and reporter assay in HEK293 cells were performed as described previously (Seo et al., 2004). To suppress PPAR expression, specific siRNAs (Bioneer, Daejeon, Korea; the siRNA sequences are available upon request) were transfected with a Microporator (Digital Bio, Seoul, Korea) as follows: FAO cells were pulsed once at 1400 V for 20 ms and 3T3-L1 cells were pulsed twice at 1300 V for 20 ms. After transfection, cells were maintained with growth medium in the absence of antibiotics for overnight.

**Docking Simulation.** Docking simulation was performed with Discovery Studio 1.7 computer program. Docking calculation was constructed according to LigandFit module implemented in the receptor-ligand interaction protocol. Cocrystal structures of the ligand-bound ligand binding domains of human PPAR $\alpha$  and human PPAR $\gamma$  (PDB ID: 3FEI and 3FEJ, respectively) were employed, and the binding sites were defined from each receptor cavities. For estimating the binding affinity of each chemical, the potential of mean force (Muegge and Martin, 1999) was used as a docking score function. All parameters used in calculation are also provided.

**qRT-PCR Analysis.** Total RNA was prepared using Tri-Reagent (Molecular Research Center, Cincinnati, OH), and cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase kit (RevertAid; Fermentas, Glen Burnie, MD) following to manufacturer's instruction. qRT-PCR was performed using My-IQ thermocycler (Bio-Rad Laboratories, Hercules, CA). The amplifying reaction was conducted as follows; 40 repetitive thermal cycles (94°C for 20 s, 60°C for 20 s, 72°C for 20 s) with SYBR green (Invitrogen, Carlsbad, CA). The primers used in this study were synthesized from Bioneer. The sequences of qRT-PCR primers are available upon request.

**Animal Experiments.** All animal experiments were approved by the Seoul National University Animal Experiment Ethics Committee. C57BLKS/J-*Lepr<sup>db</sup>/Lepr<sup>db</sup>* male mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea). They were housed at 22 ± 2°C, 55 ± 5% relative humidity, with a light/dark cycle of 12 h, and they were fed *ad libitum*. C57BLKS/J-*Lepr<sup>db</sup>/Lepr<sup>db</sup>* male mice, at 10 weeks of age, were administered 50 mg/kg WY14643, rosiglitazone, or CG301360 daily, and an equivalent volume of vehicle (distilled water) by oral gavage for 30 days.

For pharmacokinetic analysis of CG301360, male ICR mice were injected with CG301360 (5 mg/kg body weight dose) or vehicle (distilled water) via oral gavage. Blood samples were collected at 0.167, 0.5, 1, 2, 4, 6, 8, and 24 h after injection. The quantification of the CG301360 compound in plasma was monitored using a liquid chromatography/tandem mass spectrometry system. Pharmacokinetic parameters (area of under the time-concentration, time to reach maximum concentration, maximum plasma concentration, and mean residence time) were calculated.

**Measurement of Blood Lipid Profiles.** Blood samples were used to determine triacylglyceride (TG), FFAs, and total cholesterol levels with Infinity reagents (Thermo Fisher Scientific, Melbourne, Australia). Plasma insulin level was measured with insulin enzyme-linked immunosorbent assay kit according to manufacturer's instruction (Mercodia, Uppsala, Sweden). The level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma were measured as described previously (Kim et al., 2009).

**Glucose Tolerance Test and Insulin Tolerance Test.** For the glucose tolerance test, PPAR agonist-administered mice were fasted for 24 h. Glucose (Sigma) solution (5%) was administered orally, and the blood glucose level was measured with Freestyle blood glucose meter (Therasense, Indianapolis, IN). For the insulin tolerance test, mice were fasted for 6 h before injection of 1 U of human insulin (Lilly, Indianapolis, IN). The blood glucose level was measured from the mice by Freestyle blood glucose meter (Therasense, Alameda, CA).

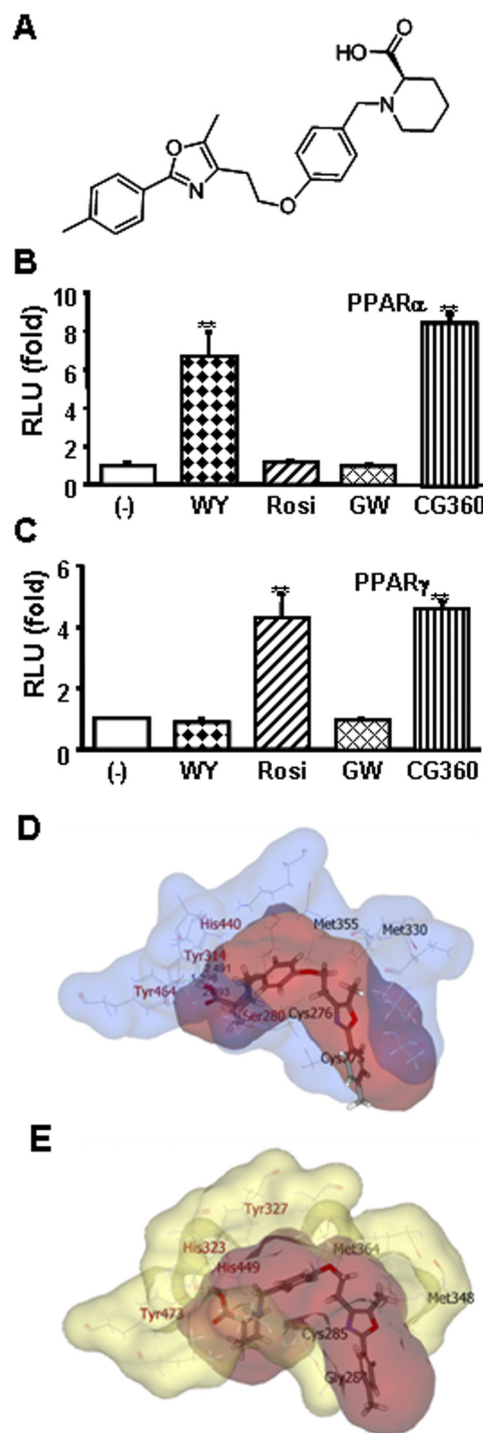
**Western Blot.** Western blot analysis was performed as described previously (Jeong et al., 2009) with minor modifications. In brief, an equal amount of protein separated on SDS-polyacrylamide gel electrophoresis gels was transferred to nitrocellulose membrane. Blots were blocked with Tris-buffered saline with 0.1% Tween 20 (TBST) containing 0.5% nonfat milk at room temperature for 15 min and incubated with primary antibodies at 4°C for overnight. After washing with TBST three times, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma) at room temperature for 2 h and followed by washing with TBST an additional three times. The membrane was incubated with enhanced chemiluminescence reagents and exposed to X-ray film (Fujifilm, Tokyo, Japan).

**Fatty Acid Oxidation Assay.** Cells were incubated for 24 h in palmitate (Sigma)-saturated  $\alpha$ -minimal essential medium containing 0.1 mM 9,10- $^3\text{H}$ palmitate (5  $\mu\text{Ci}/\text{ml}$ ; PerkinElmer Life and Analytical Sciences, Waltham, MA) and 2% bovine serum albumin. After incubation, the medium was precipitated with an equal volume of 10% trichloroacetic acid (Sigma). Then the supernatants were transferred to open 1.5-ml microcentrifuge tubes placed in a scintillation vial containing 0.5 ml of water and incubated at 55°C for overnight. After removal of the open microcentrifuge tubes, the content of  $^3\text{H}_2\text{O}$  content was measured in a scintillation counter.

**Statistical Analysis.** Results are representative data from independent experiments repeated at least three times. Error bars represent S.D. and  $P$  values  $< 0.05$  (calculated from analysis of variance followed by Student-Newman-Keuls comparison test) were considered statistically significant.

## Results

**Transcriptional Activity of PPAR $\alpha$  and PPAR $\gamma$  Is Up-Regulated by CG301360 through Their Ligand Binding Domains.** To design novel PPAR $\alpha/\gamma$  dual agonists, we analyzed the known PPAR agonists and their modes of action. For the specific interactions between ligands and nuclear receptors, the most important interaction is the hydrogen bond networks linking agonists and amino acid residues in the AF2 helix of nuclear receptors, which induce conformational change of the receptor. We adopted a phenoxy as a linker and 5-methyl-2-phenyl-oxazole as a hydrophobic tail, similar to other PPAR agonists. As a new acid head, piperidine-2-carboxylic acid was introduced to make an additional hydrophobic interaction in the binding pocket. Chemical compounds were synthesized on the basis of virtual information and evaluated through transactivation reporter assays with three PPAR isoforms. Among potential candidates for PPAR $\alpha/\gamma$  dual agonists, compound CG301360 was selected after evaluating various cellular effects. As shown in Fig. 1A, CG301360 has a non-TZD-based structure. To con-



**Fig. 1.** Trans-activation of PPAR  $\alpha$  and PPAR $\gamma$  by CG301360. A, Chemical structure of CG301360. B and C, HEK293 cells overexpressing mouse PPAR $\alpha$  (B) or PPAR $\gamma$  (C) were treated with 1  $\mu\text{M}$  WY14643 (WY), rosiglitazone (Rosi), GW501516 (GW), or CG301360 (CG360) for 24 h. Cells were subjected to reporter assays as described under *Materials and Methods*. Each bar represents mean  $\pm$  S.D. of duplicates (B and C). \*\*,  $P < 0.01$  versus negative control. D and E, binding of CG301360 to LBDs of PPARs. D, PPAR $\alpha$ -LBD (PDB ID: 3FEI, binding site volume = 329.5  $\text{\AA}^3$ ). E, PPAR $\gamma$ -LBD (PDB ID: 3FEJ, binding site volume = 362.4  $\text{\AA}^3$ ). All protein residues displayed a ligand contact distance  $\leq 4.5\text{\AA}$ . Green color indicates the four hydrogen bonds of the carboxylate head group with PPAR $\alpha/\gamma$ . CG301360 docking simulation was performed as described under *Materials and Methods*. Blue cloud; human PPAR $\alpha$ , yellow cloud; human PPAR $\gamma$ , red cloud; binding site volume of PPAR $\alpha/\gamma$  dual agonist.



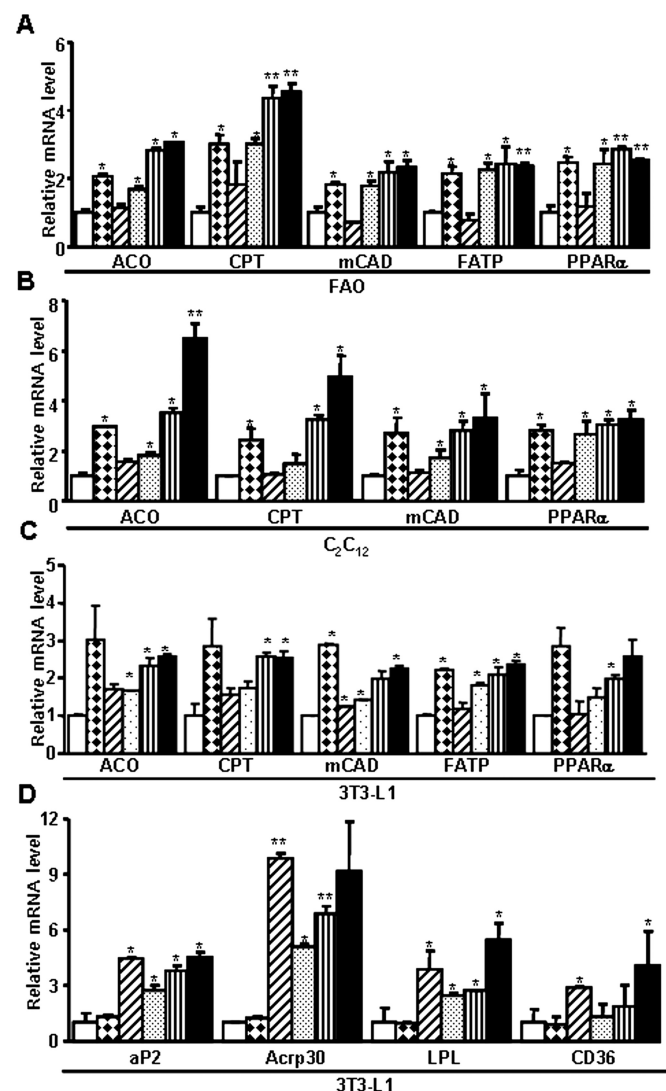
firm the specificity and sensitivity of CG301360 in the regulation of transcriptional activities of PPARs, luciferase reporter assays were performed. CG301360 effectively activated PPAR $\alpha$  (Fig. 1B) and PPAR $\gamma$  (Fig. 1C), whereas CG301360 did not significantly activate PPAR $\delta$  (Supplemental Fig. 1). Dose-response analyses showed that CG301360 acted as an effective agonist for PPAR $\alpha$  and PPAR $\gamma$  when CG301360 was compared with the known PPAR $\alpha$  and PPAR $\gamma$  agonists WY14643 and rosiglitazone, respectively (Supplemental Fig. 2, A and B). Moreover, CG301360 enhanced the transactivation of GAL4-PPAR $\alpha$  and GAL4-PPAR $\gamma$  LBD constructs in a dose-dependent manner (data not shown; EC<sub>50</sub> of CG301360 is given in Supplemental Table 1), suggesting CG301360 as a modest and selective dual agonist for PPAR $\alpha$  and PPAR $\gamma$  through the interaction with their LBD domains. However, CG301360 barely stimulated the transcriptional activity of PPAR $\delta$  up to 1  $\mu$ M (Supplemental Fig. 2C). In addition, when pharmacokinetic properties of CG301360 were evaluated, time to reach maximum concentration was 0.167 h, implying that CG301360 is rapidly absorbed after administration (Supplemental Table 2).

To investigate whether CG301360 is able to indeed interact with the LBDs of PPAR $\alpha$  and PPAR $\gamma$ , we simulated the docking study of CG301360 with the reported structures of PPAR $\alpha$  LBD and PPAR $\gamma$  LBD (PDB ID: 3FEI and 3FEJ, respectively) (Grether et al., 2009). As shown in Fig. 1, D and E, overall structures of LBDs of PPAR $\alpha$  and PPAR $\gamma$  containing CG301360 were similar to those of human PPAR $\alpha$  and PPAR $\gamma$  with previously reported PPAR $\alpha/\gamma$  dual agonist, phenylthiazole derivative (Grether et al., 2009). The electrostatic potential surface (molecular volume = 346.7 Å<sup>3</sup>) of CG301360 nicely fits into the LBDs of PPAR $\alpha$  and PPAR $\gamma$  (Supplemental Fig. 3). In particular, the typical four hydrogen bonds linking carboxylate of CG301360 and the Ser, His, and Tyr residues of PPAR $\alpha/\gamma$  are within a distance of  $\leq 3$  Å. Moreover, CG301360 seems to dock into the hydrophobic pockets of PPAR $\alpha/\gamma$ , which are composed of Met, Cys, and Gly residues. In addition, when we calculated the docking scores of CG301360 toward PPAR $\alpha/\gamma$  by comparison with known PPAR ligands such as WY14643 and rosiglitazone (Supplemental Table 3), the data obtained clearly suggest that CG301360 would be properly accommodated into the LBD of PPAR $\alpha$  and  $\gamma$  as a potential dual ligand.

**CG301360 Stimulates Expression of PPAR $\alpha$  and PPAR $\gamma$  Target Genes.** Because CG301360 effectively stimulated the transactivation of both PPAR $\alpha$  and PPAR $\gamma$ , we next examined the effects of CG301360 on the expression of PPAR $\alpha$  and PPAR $\gamma$  target genes. In hepatoma (FAO) and myocytes (C<sub>2</sub>C<sub>12</sub>), CG301360 augmented the expression of PPAR $\alpha$  target genes such as acyl-CoA oxidase, carnitine-palmitoyl transferase, medium-chain acyl-CoA dehydrogenase, and PPAR $\alpha$  itself in a dose-dependent manner (Fig. 2, A and B). Furthermore, CG301360 stimulated the expression of PPAR $\alpha$  target genes in differentiated 3T3-L1 (Fig. 2C) and 3T3-F442A adipocytes (data not shown), implying that CG301360 might promote fatty acid oxidation even in fat cells. As expected, in cultured adipocytes, CG301360 significantly augmented the expression of PPAR $\gamma$  target genes such as adipocyte fatty acid binding protein, adiponectin, lipoprotein lipase, and CD36 in a dose-dependent manner (Fig. 2D). These data indicate that CG301360 could act as a dual activator of PPAR $\alpha$  and PPAR $\gamma$  in cultured cells.

**CG301360 Restores Dysregulated Glucose and Lipid Metabolism in Diabetic Mice.** To assess the in vivo effect of CG301360, CG301360 was administered to obese and diabetic *db/db* mice every day for 1 month (from 10 to 14 weeks of age). Without significant change in food intake (data not shown), increase in body weight and white adipose tissue (WAT) weight was observed in CG301360-treated *db/db* mice, albeit to a lesser extent than by rosiglitazone, a well known PPAR $\gamma$  agonist (Fig. 3, A and B) (Chaput et al., 2000; Shim et al., 2006).

Next, plasma glucose and lipid profiles were analyzed to examine whether CG301360 was able to modulate metabolic abnormalities in obese and diabetic subjects. In *db/db* mice, CG301360



**Fig. 2.** Increased expression of PPAR $\alpha$  and PPAR $\gamma$  target genes by CG301360. FAO rat hepatoma cells (A), C<sub>2</sub>C<sub>12</sub> myotubes (B), and 3T3-L1 adipocytes (C and D) were incubated with WY14643 (WY; 1  $\mu$ M), rosiglitazone (Rosi; 1  $\mu$ M), or CG301360 (CG360; 0.5, 1, and 5  $\mu$ M) for 24 h. The relative mRNA level of each gene was analyzed by use of qRT-PCR and normalized by GAPDH. Each bar represents mean  $\pm$  S.D. of duplicates. Similar results were obtained from at least three independent experiments. \*,  $P < 0.05$  versus negative control; \*\*,  $P < 0.01$  versus negative control. □, vehicle; diamond-filled box, WY14643 (1  $\mu$ M); ▨, rosiglitazone (1  $\mu$ M); ▩, CG301360 (0.5  $\mu$ M); ■, CG301360 (1  $\mu$ M); ■, CG301360 (5  $\mu$ M). ACO, acyl-CoA oxidase; CPT, carnitine-palmitoyl transferase; mCAD, medium-chain acyl-CoA dehydrogenase; FATP, fatty acid transport protein; aP2, adipocyte fatty acid binding protein; Acrp30, adiponectin; LPL, lipoprotein lipase.

significantly reduced plasma glucose, insulin, TG, and FFA levels (Fig. 3, C–F). These effects of CG301360 on plasma profiles were more potent than those of rosiglitazone, a PPAR $\gamma$  agonist, and WY14643, a PPAR $\alpha$  agonist. Moreover, CG301360 even slightly decreased the level of plasma ALT and AST levels (Fig. 3, G and H) (Al-Salman et al., 2000). Collectively, these data suggest that CG301360 in *db/db* mice would ameliorate hyperglycemia and hyperlipidemia.

**CG301360 Mitigates Glucose and Insulin Intolerance in Diabetic Mice.** Because CG301360 greatly reduced plasma glucose and lipid levels in *db/db* mice (Fig. 3, C–F), we next conducted glucose tolerance and insulin tolerance tests to examine the effect of CG301360 on systemic glucose and insulin sensitivity. As illustrated in Fig. 4, A and B, CG301360 was as effective as rosiglitazone in alleviating glucose and insulin tolerance in *db/db* mice.

Increased glucose uptake through activation of PPAR $\gamma$  has been suggested as a mechanism of reducing circulating glucose level (Tamori et al., 2002). Next, we tested the effect of CG301360 on glucose uptake. In 3T3-L1 adipocytes, CG301360 significantly enhanced insulin-stimulated glucose uptake (Fig. 4C) as well as in 3T3-F442A adipocytes (data not shown). Moreover, expression of

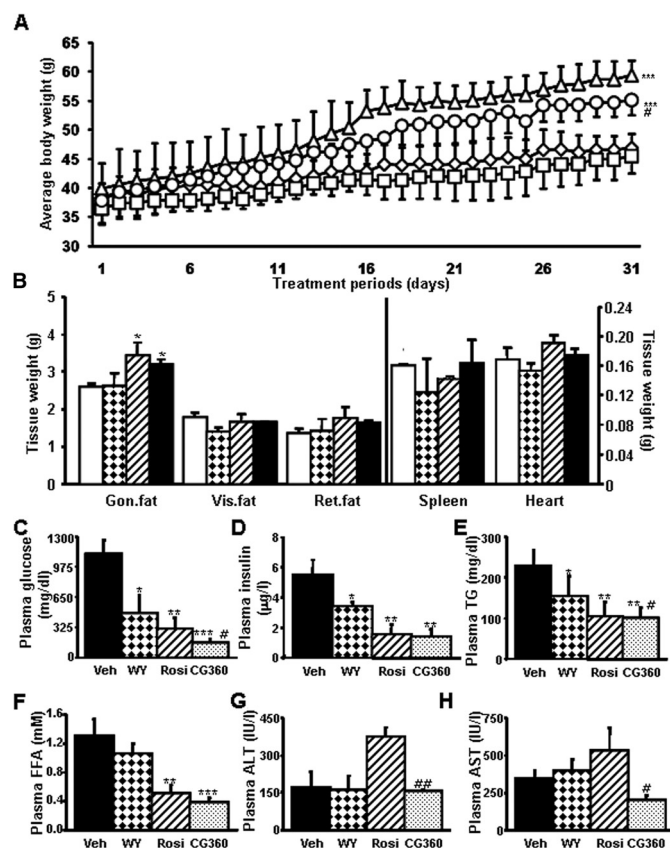
adiponectin was measured because it is known to enhance insulin sensitivity by promoting glucose uptake and fatty acid oxidation (Yamauchi et al., 2002). CG301360 elevated the level of adiponectin both in vitro and in vivo (Fig. 4, D and E). Taken together, these data suggest the role of CG301360 in improving insulin sensitivity and glucose metabolism as a PPAR $\gamma$  activator.

**CG301360 Suppresses Proinflammatory Responses.** Because pro-inflammatory response is a causal factor for insulin resistance (Shoelson et al., 2006), we examined the effect of CG301360 on the inflammatory responses of macrophages and adipocytes. In RAW 264.7 macrophages and 3T3-L1 adipocytes, enhanced expression of pro-inflammatory genes such as TNF $\alpha$ , interleukin-1 $\beta$ , interleukin-6, inducible NO synthase, monocyte chemoattractant protein-1, cyclooxygenase 2, and matrix metalloproteinase 9 upon inflammatory stimuli were markedly reduced in the presence of CG301360 (Fig. 5, A and B).

Although CG301360 slightly increased adipose tissue mass in vivo (Fig. 3B), it effectively reduced expression level of macrophage marker genes including *CD68*, *F4/80*, *CD11b*, and *CD11c* and pro-inflammatory genes such as TNF $\alpha$ , inducible NO synthase, and monocyte chemoattractant protein-1 in adipose tissue of *db/db* mice (Fig. 5, C and D). These data suggest that CG301360 would effectively suppress pro-inflammatory responses, eventually leading to improve insulin resistance in obese subjects.

**CG301360 Promotes Fatty Acid Oxidation.** As a PPAR $\alpha$  activator, CG301360 elevated the expression of fatty acid oxidation genes in various cell lines (Fig. 2). In *db/db* mice, CG301360 augmented the expression of several  $\beta$ -oxidation genes in peripheral tissues such as liver, skeletal muscle, and WAT (Fig. 6, A–C), indicating that CG301360 might stimulate fatty acid oxidation in peripheral tissues. Consistent with the results from gene expression profiles, CG301360 indeed promoted fatty acid oxidation in various cell types, including hepatoma, myocytes, and adipocytes (Fig. 6, D–F). The effect of CG301360 on  $\beta$ -oxidation seems to be mainly through PPAR $\alpha$  activation, because rosiglitazone rarely promoted expression of fatty acid oxidation genes (Fig. 6, D–F). It is noteworthy that in CG301360-treated *db/db* mice, the expression of fibroblast growth factor 21 (FGF21), which induces  $\beta$ -oxidation and reduces the levels of glucose and TG in diabetic animals (Xu et al., 2009), was significantly up-regulated in liver (Fig. 6G), providing further supporting evidence that CG301360 is capable of improving hyperlipidemia by facilitating lipid consumption.

**CG301360 Selectively Potentiates PPAR $\alpha$  and PPAR $\gamma$  Activities.** CG301360 exhibited several beneficial effects on both glucose and lipid metabolism in *db/db* mice. To further confirm whether these favorable effects of CG301360 on glucose and lipid metabolism are mediated by PPAR $\alpha$  and/or PPAR $\gamma$  activation, we decided to use PPAR knockdown with siRNAs. As a control experiment, we examined whether CG301360 might influence activity of PPAR $\delta$ , which shares several target genes and some features in lipid catabolism with PPAR $\alpha$  (Evans et al., 2004). As shown in Fig. 7A, GW501516, a synthetic PPAR $\delta$  ligand, failed to increase the expression of  $\beta$ -oxidation genes in PPAR $\delta$ -suppressed FAO cells. Unlike GW501516, CG301360 still stimulated the expression of genes involved in fatty acid oxidation (Fig. 7A, and average fold induction is denoted in Supplemental Table 4). In contrast, cells transfected with PPAR $\alpha$  siRNA evi-



**Fig. 3.** Body weight and plasma profiles of CG301360-treated obese and diabetic *db/db* mice. *db/db* mice were administered with several PPAR ligands by oral gavage as described under *Materials and Methods*. Body weight change (A), tissue weight (B), plasma content of glucose (C), insulin (D), TG (E), FFA (F), ALT (G), and AST (H) of CG301360-treated obese *db/db* mice are shown. Each bar represents mean  $\pm$  S.D. of each group of mice ( $n = 6$ ). \*,  $P < 0.05$  versus vehicle; \*\*,  $P < 0.01$  versus vehicle; \*\*\*,  $P < 0.001$  versus vehicle; #,  $P < 0.05$  versus rosiglitazone; ##,  $P < 0.01$  versus rosiglitazone. A,  $\diamond$ , vehicle;  $\square$ , WY14643;  $\triangle$ , rosiglitazone;  $\circ$ , CG301360. B,  $\square$ , vehicle; diamond-filled box, WY14643;  $\boxtimes$ , rosiglitazone;  $\boxplus$ , CG301360. C–H, Veh, vehicle; WY, WY14643; Rosi, rosiglitazone; CG360, CG301360; Gon.fat, gonadal fat; Vis.fat, visceral fat; Ret.fat, retroperitoneal fat.



dently blunted the expression of genes related to  $\beta$ -oxidation induced by CG301360 (Fig. 7B). These results propose that the effects of CG301360 on the expression of fatty acid oxidation genes would be primarily required for PPAR $\alpha$  rather than PPAR $\delta$ . In addition, knockdown of PPAR $\gamma$  also reduced the expression of both of basal and CG301360-induced PPAR $\gamma$  target genes in differentiated adipocytes (Fig. 7C).

To further confirm the suppressing effects of PPAR $\alpha$  and/or PPAR $\gamma$  activity via each knockdown, we assessed  $\beta$ -oxidation (for PPAR $\alpha$ ) and adipogenesis (for PPAR $\gamma$ ). PPAR $\alpha$  siRNA remarkably inhibited fatty acid oxidation in FAO cells (Fig. 7D), and knockdown of PPAR $\gamma$  reduced CG301360-mediated adipogenesis (Fig. 7E and Supplemental Fig. 4). Together, these data strongly support the idea that the effects of CG301360 on the regulation of insulin sensitivity and lipid metabolism are mediated by activation of both PPAR $\alpha$  and PPAR $\gamma$ .

## Discussion

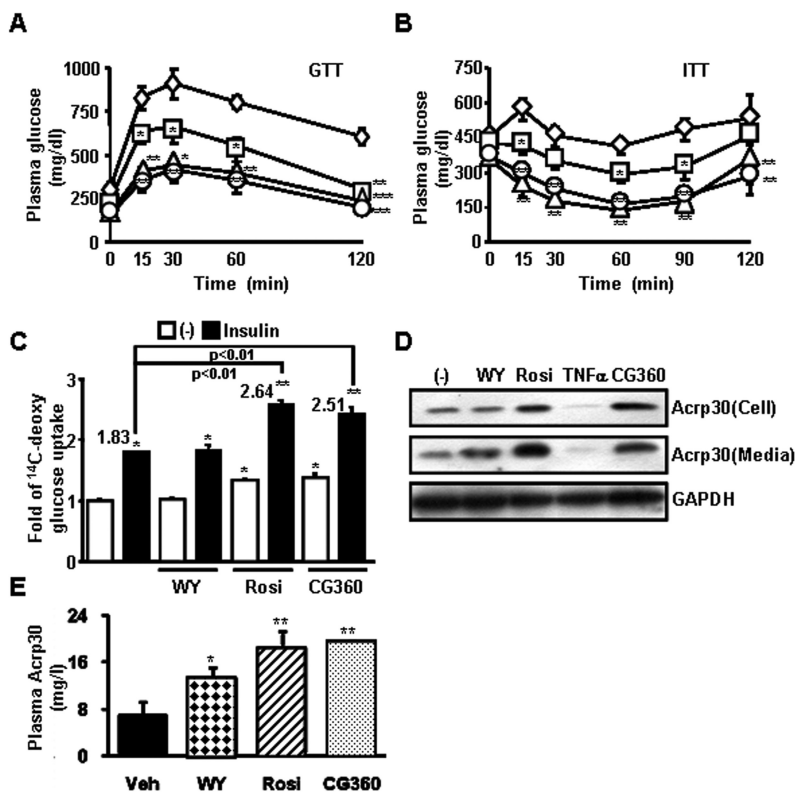
The importance of PPARs on metabolic diseases has been well established by genetic studies. PPAR $\alpha$ ( $-/-$ ) mice fail to meet energy demands during fasting by exhibiting hypoketonemia, hyperlipidemia, and fatty liver (Kersten et al., 1999). Tissue-specific PPAR $\gamma$ -null mice are severely insulin-resistant (He et al., 2003; Hevener et al., 2003), whereas whole-body PPAR $\gamma$ ( $-/-$ ) mice are embryonic lethal (Barak et al., 1999), implying the importance of PPAR $\gamma$  in adipose tissue during development. On the other hands, PPAR $\delta$ -over-expressing transgenic mice have enhanced lipid metabolism and are resistant to obesity and tissue steatosis, whereas PPAR $\delta$  deficiency exhibits obesity and causes defect in energy uncoupling (Wang et al., 2003). Thus, it is very likely that tight regulation of PPAR activity would be crucial to

treat metabolic diseases including obesity, hyperlipidemia, and insulin resistance.

Through simultaneous activation of both PPAR $\alpha$  and PPAR $\gamma$ , PPAR $\alpha/\gamma$  dual agonists have been expected to alleviate obesity and diabetes (Watkins and Whitcomb, 1998; Hirsch et al., 1999; Al-Salman et al., 2000; Tang et al., 2003; Shim et al., 2006). However, side effects of PPAR $\alpha/\gamma$  dual agonists still make them unsuitable for treatment of obesity and insulin resistance (Hellmold et al., 2007; Mittra et al., 2007; Tannehill-Gregg et al., 2007). In this regard, the failure of former PPAR $\alpha/\gamma$  dual agonists prompted us to develop novel PPAR $\alpha/\gamma$  dual agonists without (or with less) deleterious effects by screening different backbone structures of PPAR ligands.

The LBD of nuclear hormone receptors undergoes conformational change upon interaction with their ligands, leading to activate nuclear receptors (Bourguet et al., 2000). By using virtual screening, we found optimal chemicals to fit into the LBD of PPAR $\alpha$ . After screening, we isolated the non-TZD-based CG301360, which is capable of activating both PPAR $\alpha$  and PPAR $\gamma$ . In the current study, we demonstrate that this novel PPAR $\alpha/\gamma$  dual agonist effectively and selectively activates PPAR $\alpha$  and PPAR $\gamma$  and shows favorable effects on insulin sensitivity and lipid metabolism in *db/db* mice. Unlike many previous PPAR $\alpha/\gamma$  dual agonists (Hellmold et al., 2007; Mittra et al., 2007), CG301360 moderately activates PPAR $\alpha$  and PPAR $\gamma$ . Furthermore, given that CG301360 action may not be primarily mediated through PPAR $\delta$  propose that CG301360 might be free from PPAR $\delta$ -mediated side effects.

As a PPAR $\alpha$  activator, CG301360 is able to alleviate hyperlipidemia by enhancing lipid catabolism. In fact, CG301360 increased the expression of PPAR $\alpha$  target genes

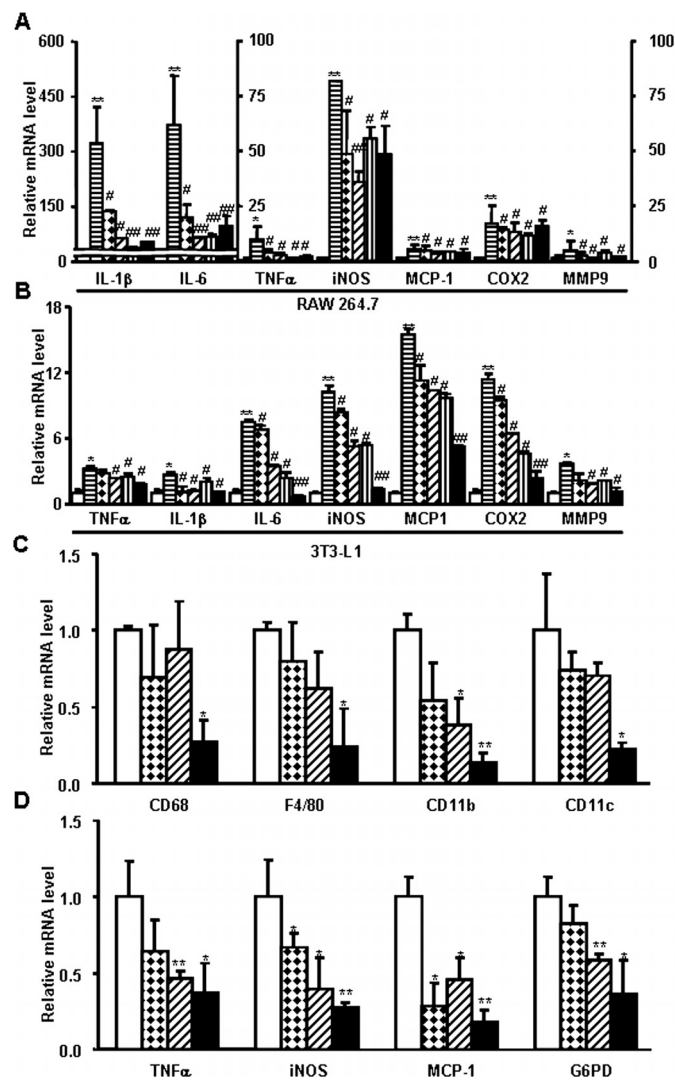


**Fig. 4.** CG301360 improves glucose and insulin sensitivity in *db/db* mice. *db/db* mice were fasted and injected with glucose (A) or insulin (B) as described under *Materials and Methods*. Plasma glucose and insulin levels were measured from the blood samples that were drawn at baseline ( $t = 0$  min) and at time points indicated on figure. ◇, vehicle; □, WY14643; △, rosiglitazone; ○, CG301360. C, differentiated adipocytes were starved with low-glucose DMEM without serum for overnight. During starvation, each chemical [1  $\mu$ M WY14643 (WY), rosiglitazone (Rosi), or CG301360 (CG360)] was added. Glucose uptake assay was performed as described under *Materials and Methods*. Relative fold of glucose uptake is shown in graph. Each bar represents mean  $\pm$  S.D. of triplicates. D, differentiated 3T3-L1 cells were treated with 1  $\mu$ M WY14643 (WY), rosiglitazone (Rosi), or CG301360 (CG360) and 10 ng/ml TNF $\alpha$  in serum-free DMEM for 12 h. Cells were harvested and total cell lysates and media were blotted with anti-adiponectin antibody. GAPDH was used as loading control. Acrp30, adiponectin. E, plasma adiponectin levels from mice of each group determined by adiponectin enzyme-linked immunosorbent assay kit. \*,  $P < 0.05$  versus vehicle; \*\*,  $P < 0.01$  versus vehicle; \*\*\*,  $P < 0.001$  versus vehicle. Veh, vehicle; WY, WY14643; Rosi, rosiglitazone; CG360, CG301360.

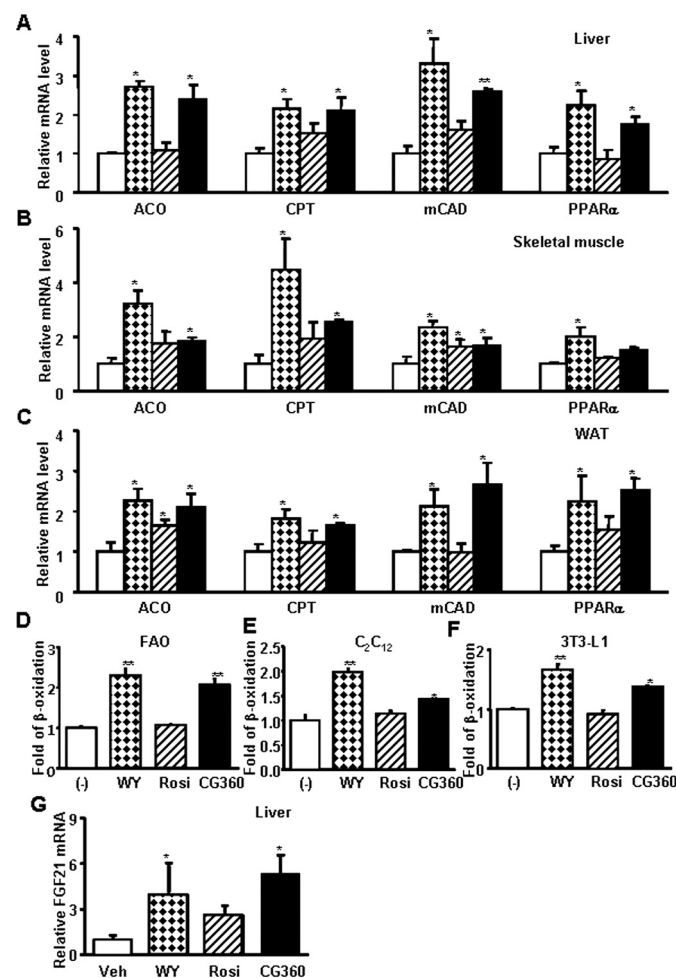
as well as FGF21, a PPAR $\alpha$ -responsive cytokine regulating lipid metabolism (Xu et al., 2009), and CG301360 augmented fatty acid oxidation, implying that CG301360 would promote lipid oxidation to improve hyperlipidemia in obese and/or diabetic animals. Furthermore, CG301360 also improves glucose metabolism by activation of PPAR $\gamma$ . The possible mechanisms responsible for CG301360-dependent glucose sensitivity are probably by 1) enhancing

insulin sensitivity and 2) stimulating adiponectin production and secretion, which are not mediated by PPAR $\alpha$  activation. Collectively, it is feasible to speculate that CG301360 might increase insulin sensitivity as a PPAR $\gamma$  activator, whereas it relieves lipid dysregulation by primarily activating PPAR $\alpha$ .

One of the side effects of TZDs is tissue damage, which often results from fluid retention and edema (Hirsch et al., 1999; Tang et al., 2003; Mittra et al., 2007). Although some TZDs induce liver failure (Watkins and Whitcomb, 1998; Hirsch et al., 1999; Al-Salman et al., 2000), CG301360 slightly but substantially decreased the level of ALT and AST in *db/db* mice. Furthermore, CG301360 is ascertained not to cause fluid retention, edema, and related tissue failures such as renal tubular damage and cardiac muscle damage in *db/db* mice, (Supplemental Fig. 5). Although experiments of long-term administration are needed to clearly elucidate whether CG301360 might confer fluid retention or not, this



**Fig. 5.** CG301360 suppresses pro-inflammatory gene expression. RAW 264.7 macrophages (A) and 3T3-L1 adipocytes (B) were incubated with WY14643 (1  $\mu$ M), rosiglitazone (1  $\mu$ M), or CG301360 (1 and 5  $\mu$ M) overnight and were treated with 10 ng/ml LPS for 6 h (A) and 1 ng/ml TNF $\alpha$  (B) for 3 h. Relative mRNA level of each gene was examined as described in Fig. 2. Each bar represents mean  $\pm$  S.D. of duplicates. Data are representative of at least three independent experiments. A and B, \*,  $P < 0.05$  versus negative control; \*\*,  $P < 0.01$  versus negative control; #,  $P < 0.05$  versus LPS (A) or TNF $\alpha$  (B); ##,  $P < 0.01$  versus LPS (A) or TNF $\alpha$  (B). □, vehicle; ▨, LPS (A) or TNF $\alpha$  (B); diamond-filled box, WY14643/LPS (A) or WY14643/TNF $\alpha$  (B); ▩, rosiglitazone/LPS (A) or rosiglitazone/TNF $\alpha$  (B); ■, CG301360 1  $\mu$ M/LPS (A) or CG301360 1  $\mu$ M/TNF $\alpha$  (B); ▤, CG301360 5  $\mu$ M/LPS (A) or CG301360 5  $\mu$ M/TNF $\alpha$  (B). Relative mRNA expression of macrophage genes (C) and pro-inflammatory genes (D) of WAT from CG301360-treated *db/db* mice was quantified by use of qRT-PCR and was normalized by GAPDH. Data represent mean  $\pm$  S.D. \*,  $P < 0.05$  versus vehicle; \*\*,  $P < 0.01$  versus vehicle. □, vehicle; diamond-filled box, WY14643; ▩, rosiglitazone; ■, CG301360. IL, interleukin; iNOS, inducible NO synthase; MCP1/MCP-1, monocyte chemoattractant protein 1; COX2, cyclooxygenase 2; MMP9, matrix metalloproteinase 9; G6PD, glucose-6-phosphate dehydrogenase.



**Fig. 6.** CG301360 stimulates fatty acid oxidation. Relative mRNA level of fatty acid oxidation genes in liver (A), skeletal muscle (B), and WAT (C) was examined by qRT-PCR analysis. FAO (D), C<sub>2</sub>C<sub>12</sub> (E), and 3T3-L1 (F) cells were treated with 10  $\mu$ M WY14643, rosiglitazone, and CG301360 for 24 h, and fatty acid oxidation assay was performed. G, relative mRNA level of FGF21 in liver from CG301360-treated *db/db* mice was examined by use of qRT-PCR and normalized by GAPDH. Each bar represents mean  $\pm$  S.D. \*,  $P < 0.05$  versus vehicle; \*\*,  $P < 0.01$  versus vehicle; #,  $P < 0.05$  versus rosiglitazone. □, vehicle (Veh); diamond-filled box, WY14643 (WY); ▩, rosiglitazone (Rosi); ■, CG301360 (CG360). ACO, acyl-CoA oxidase; CPT, carnitine-palmitoyl transferase; mCAD, medium-chain acyl-CoA dehydrogenase.



notion reflects that CG301360 would relieve insulin resistance without acute organ failure in diabetic mice (at least within a month of administration).

One of important features of CG301360 is its anti-inflammatory property. Because pro-inflammatory responses play key roles in insulin resistance, it is important to suppress pro-inflammatory responses to rescue insulin resistance. In fact, several anti-inflammatory reagents relieve insulin resistance (Jeong et al., 2009). Both in vitro and in vivo,

CG301360 showed anti-inflammatory effects, probably by activating PPAR $\alpha$  and PPAR $\gamma$ .

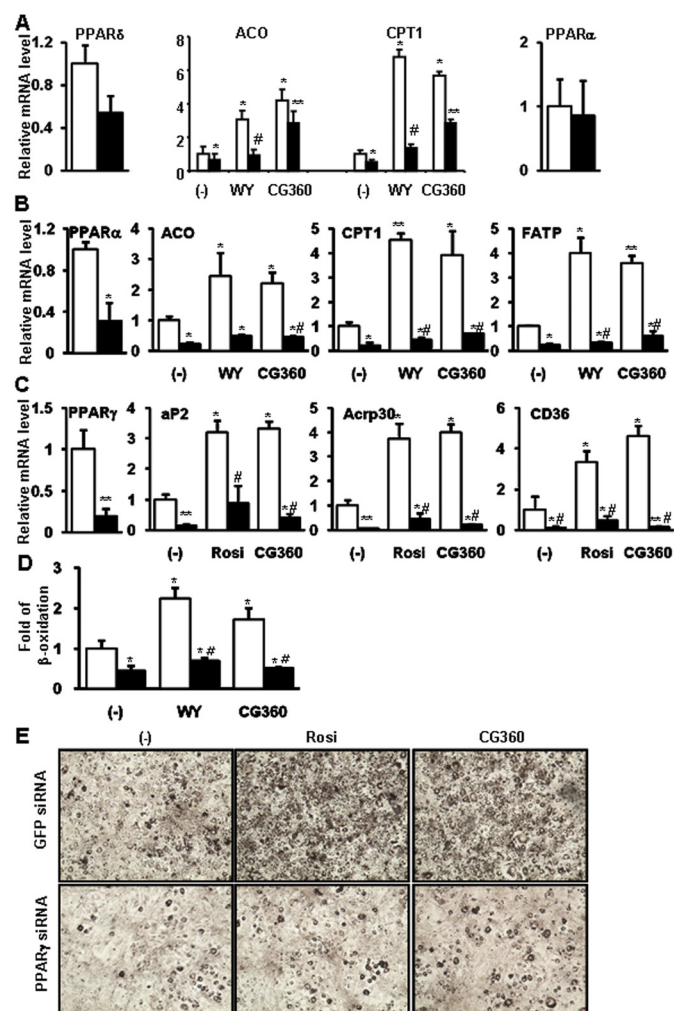
In conclusion, we identified a novel PPAR $\alpha$ / $\gamma$  dual agonist, CG301360, that provides favorable effects on insulin sensitivity and lipid homeostasis. CG301360, a modest activator of PPAR $\alpha$  and PPAR $\gamma$ , ameliorates hyperglycemia and hyperinsulinemia by enhancing glucose and lipid metabolism via increased glucose uptake and fatty acid oxidation. CG301360 also suppresses pro-inflammatory responses to restore insulin resistance in vitro and in vivo. Taken together, these data lead us to suggest that CG301360 would be a potential agent targeting insulin resistance and related metabolic disorders through activation of both PPAR $\alpha$  and PPAR $\gamma$ .

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#### References

- Al-Salman A, Arjomand H, Kemp DG, and Mittal M (2000) Hepatocellular injury in a patient receiving rosiglitazone. A case report. *Ann Intern Med* 132:121–124.
- Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, and Evans RM (1999) PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol Cell* 4:585–595.
- Bourguet W, Germain P, and Gronemeyer H (2000) Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications. *Trends Pharmacol Sci* 21:381–388.
- Carmona MC, Louche K, Nibbelink M, Prunet B, Bross A, Desbazeille M, Dacquet C, Renard P, Casteilla L, and Penicaud L (2005) Fenofibrate prevents rosiglitazone-induced body weight gain in ob/ob mice. *Int J Obes (Lond)* 29:864–871.
- Chaput E, Saladin R, Silvestre M, and Edgar AD (2000) Fenofibrate and rosiglitazone lower serum triglycerides with opposing effects on body weight. *Biochem Biophys Res Commun* 271:445–450.
- DeFronzo RA and Ferrannini E (1991) Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* 14:173–194.
- Evans RM, Barish GD, and Wang YX (2004) PPARs and the complex journey to obesity. *Nat Med* 10:355–361.
- Goldstein BJ, Rosenstock J, Anzalone D, Tou C, and Ohman KP (2006) Effect of tesaglitazar, a dual PPAR alpha/gamma agonist, on glucose and lipid abnormalities in patients with type 2 diabetes: a 12-week dose-ranging trial. *Curr Med Res Opin* 22:2575–2590.
- Grether U, Bénardeau A, Benz J, Binggeli A, Blum D, Hilpert H, Kuhn B, Märki HP, Meyer M, Mohr P, et al. (2009) Design and biological evaluation of novel, balanced dual PPARalpha/gamma agonists. *ChemMedChem* 4:951–956.
- Guérin M, Bruckert E, Dolphin PJ, Turpin G, and Chapman MJ (1996) Fenofibrate reduces plasma cholesteryl ester transfer from HDL to VLDL and normalizes the atherogenic, dense LDL profile in combined hyperlipidemia. *Arterioscler Thromb Vasc Biol* 16:763–772.
- Guerre-Millo M, Gervois P, Raspé E, Madsen L, Poulain P, Derudas B, Herbert JM, Winegar DA, Willson TM, Fruchart JC, et al. (2000) Peroxisome proliferator-activated receptor alpha activators improve insulin sensitivity and reduce adiposity. *J Biol Chem* 275:16638–16642.
- Harriy T, Farrelly D, Tieman A, Chu C, Kunselman L, Gu L, Ponticelli R, Cap M, Qu F, Shao C, et al. (2006) Muraglitazar, a novel dual (alpha/gamma) peroxisome proliferator-activated receptor activator, improves diabetes and other metabolic abnormalities and preserves beta-cell function in db/db mice. *Diabetes* 55:240–248.
- He W, Barak Y, Hevener A, Olson P, Liao D, Le J, Nelson M, Ong E, Olefsky JM, and Evans RM (2003) Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle. *Proc Natl Acad Sci USA* 100:15712–15717.
- Hellmold H, Zhang H, Andersson U, Blomgren B, Holland T, Berg AL, Elebring M, Sjögren N, Bamberg K, Dahl B, et al. (2007) Tesaglitazar, a PPARalpha/gamma agonist, induces interstitial mesenchymal cell DNA synthesis and fibroblastic in subcutaneous tissues in rats. *Toxicol Sci* 98:63–74.
- Hevener AL, He W, Barak Y, Le J, Bandyopadhyay G, Olson P, Wilkes J, Evans RM, and Olefsky J (2003) Muscle-specific Pparg deletion causes insulin resistance. *Nat Med* 9:1491–1497.
- Hirsch IB, Kelly J, and Cooper S (1999) Pulmonary edema associated with troglitazone therapy. *Arch Intern Med* 159:1811.
- Issemann I and Green S (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* 347:645–650.
- Jeong HW, Hsu KC, Lee JW, Ham M, Huh JY, Shin HJ, Kim WS, and Kim JB (2009) Berberine suppresses proinflammatory responses through AMPK activation in macrophages. *Am J Physiol Endocrinol Metab* 296:E955–E964.
- Kahn CR (1994) Banting Lecture. Insulin action, diabetogenesis, and the cause of type II diabetes. *Diabetes* 43:1066–1084.
- Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, and Wahli W (1999)



**Fig. 7.** The effects of PPAR siRNAs on CG301360 action. FAO (A, B, and D) and 3T3-L1 (C) cells were transfected with green fluorescent protein (GFP) siRNA (A–D), PPAR $\delta$  (A), PPAR $\alpha$  (B and D), and/or PPAR $\gamma$  (C and E) siRNA with a micropipettor and were treated with PPAR agonists (1  $\mu$ M) for 24 h. Relative mRNA levels of PPAR target genes were measured by use of qRT-PCR (A–C) and normalized by GAPDH. Each bar represents mean  $\pm$  S.D. of triplicates. Obtained results were similar, at least, in three independent experiments. D, fatty acid oxidation assay was performed in PPAR $\alpha$ -suppressed FAO cells as described under *Materials and Methods*. Each bar represents mean  $\pm$  S.D. of triplicates. \*,  $P < 0.05$  versus GFP siRNA + vehicle (–); \*\*,  $P < 0.01$  versus GFP siRNA + vehicle (–); #,  $P < 0.05$  versus GFP siRNA + GW501516 (GW) (A), WY14643 (WY) (B), and rosiglitazone (Rosi) (C). □, GFP siRNA; ■, PPAR $\delta$  (A)/PPAR $\alpha$  (B and D)/PPAR $\gamma$  siRNA (C). E, 3T3-L1 cells were transfected with GFP or PPAR $\gamma$  siRNAs at 24 h after DMI (dexamethasone/3-isobutyl 1-methylxanthine/insulin) mixture treatment and differentiated as described under *Materials and Methods*. Cells were differentiated for 9 days further and stained with Oil-red O staining solution. Microscope pictures are representative of triplicates. ACO, acyl-CoA oxidase; CPT1, carnitine-palmitoyl transferase1; mCAD, medium-chain acyl-CoA dehydrogenase; FATP, fatty acid transport protein; aP2, adipocyte fatty acid binding protein; Acip30, adiponectin.



- Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* **103**:1489–1498.
- Kim WS, Lee YS, Cha SH, Jeong HW, Choe SS, Lee MR, Oh GT, Park HS, Lee KU, Lane MD, et al. (2009) Berberine improves lipid dysregulation in obesity by controlling central and peripheral AMPK activity. *Am J Physiol Endocrinol Metab* **296**:E812–E819.
- Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, and Kliewer SA (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem* **270**:12953–12956.
- Lloyd S, Hayden MJ, Sakai Y, Fackett A, Silber PM, Hewitt NJ, and Li AP (2002) Differential in vitro hepatotoxicity of troglitazone and rosiglitazone among cryopreserved human hepatocytes from 37 donors. *Chem Biol Interact* **142**:57–71.
- Mitra S, Sangle G, Tandon R, Sharma S, Roy S, Khanna V, Gupta A, Sattigeri J, Sharma L, Priyadarsini P, et al. (2007) Increase in weight induced by muraglitazar, a dual PPARalpha/gamma agonist, in db/db mice: adipogenesis/or oedema? *Br J Pharmacol* **150**:480–487.
- Muegge I and Martin YC (1999) A general and fast scoring function for protein-ligand interactions: a simplified potential approach. *J Med Chem* **42**:791–804.
- Murakami K, Tobe K, Ide T, Mochizuki T, Ohashi M, Akanuma Y, Yazaki Y, and Kadowaki T (1998) A novel insulin sensitizer acts as a coligand for peroxisome proliferator-activated receptor-alpha (PPAR-alpha) and PPAR-gamma: effect of PPAR-alpha activation on abnormal lipid metabolism in liver of Zucker fatty rats. *Diabetes* **47**:1841–1847.
- Nissen SE and Wolski K (2007) Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N Engl J Med* **356**:2457–2471.
- Saltiel AR and Olefsky JM (1996) Thiazolidinediones in the treatment of insulin resistance and type II diabetes. *Diabetes* **45**:1661–1669.
- Seo JB, Moon HM, Kim WS, Lee YS, Jeong HW, Yoo EJ, Ham J, Kang H, Park MG, Steffensen KR, et al. (2004) Activated liver X receptors stimulate adipocyte differentiation through induction of peroxisome proliferator-activated receptor gamma expression. *Mol Cell Biol* **24**:3430–3444.
- Shim WS, Do MY, Kim SK, Kim HJ, Hur KY, Kang ES, Ahn CW, Lim SK, Lee HC, and Cha BS (2006) The long-term effects of rosiglitazone on serum lipid concentrations and body weight. *Clin Endocrinol (Oxf)* **65**:453–459.
- Shoelson SE, Lee J, and Goldfine AB (2006) Inflammation and insulin resistance. *J Clin Invest* **116**:1793–1801.
- Tamori Y, Masugi J, Nishino N, and Kasuga M (2002) Role of peroxisome proliferator-activated receptor-gamma in maintenance of the characteristics of mature 3T3–L1 adipocytes. *Diabetes* **51**:2045–2055.
- Tang WH, Francis GS, Hoogwerf BJ, and Young JB (2003) Fluid retention after initiation of thiazolidinedione therapy in diabetic patients with established chronic heart failure. *J Am Coll Cardiol* **41**:1394–1398.
- Tannehill-Gregg SH, Sanderson TP, Minnema D, Voelker R, Ulland B, Cohen SM, Arnold LL, Schilling BE, Waites CR, and Dominick MA (2007) Rodent carcinogenicity profile of the antidiabetic dual PPAR alpha and gamma agonist muraglitazar. *Toxicol Sci* **98**:258–270.
- Wang YX, Lee CH, Tiep S, Yu RT, Ham J, Kang H, and Evans RM (2003) Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. *Cell* **113**:159–170.
- Watkins PB and Whitcomb RW (1998) Hepatic dysfunction associated with troglitazone. *N Engl J Med* **338**:916–917.
- Xu J, Lloyd DJ, Hale C, Stanislaus S, Chen M, Sivits G, Vonderfecht S, Hecht R, Li YS, Lindberg RA, et al. (2009) Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. *Diabetes* **58**:250–259.
- Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, et al. (2002) Adiponectin stimulates glucose utilization and fatty acid oxidation by activating AMP-activated protein kinase. *Nat Med* **8**:1288–1295.
- Yoon MJ, Lee GY, Chung JJ, Ahn YH, Hong SH, and Kim JB (2006) Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha. *Diabetes* **55**:2562–2570.

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