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A NON-TZD PPARα/γ DUAL AGONIST CG301360 ALLEVIATES INSULIN RESISTANCE AND LIPID DYSREGULATION IN *db/db* MICE

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Running title: Anti-diabetic effect of CG301360

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776 words in the Discussion

Abbreviations

PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; LBD, ligand binding domain

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Abstract

Activation of peroxisome proliferator-activated receptors (PPARs) have been implicated in the treatment of metabolic disorders with different mechanisms; PPARα agonists promote fatty acid oxidation and reduce hyperlipidemia, while PPARγ 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 PPARα/γ dual agonists have been developed. However, several adverse effects such as weight gain and organ failure of PPARα/γ dual agonists have been reported. By use of virtual ligand screening, we identified and characterized a novel PPARα/γ dual agonist, CG301360, exhibiting the improvement in insulin sensitivity and lipid metabolism. CG301360 selectively stimulated transcriptional activities of PPARα and PPARγ and induced expression of their target genes in a PPARα- and PPARγ-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 PPARα/γ agonist, which might be a potential lead compound to develop against insulin resistance and hyperlipidemia.

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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 last several decades, numerous drugs or therapeutic agents targeting against above disorders have been developed. For instance, fibrates have been prescribed to treat hyperlipidemia and lipid dysregulation (Guerin et al., 1996), while 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) α and γ, respectively, which are key transcriptional regulators of glucose and lipid metabolism (Issemann and Green, 1990; Lehmann et al., 1995).

Although TZDs potently 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 (Hirsch et al., 1999; Shim et al., 2006; Tang et al., 2003; Watkins and Whitcomb, 1998). Unlike TZD, PPARα 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

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PPARα and PPARγ enhance lipid and glucose metabolism, respectively (Carmona et al., 2005; Chaput et al., 2000), combination of PPARα and PPARγ agonists would be expected to achieve the beneficial effects on restoring metabolic disorders. Hence, a number of PPARα/γ dual agonists have been designed and developed (Goldstein et al., 2006; Harrity et al., 2006; Murakami et al., 1998). However, recently identified PPARα/γ dual agonists were futile due to undesirable side effects during pre-clinical or clinical trials. For example, muraglitazar, a synthetic PPARα/γ dual agonist, was aborted during clinical trials due to 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 been recently 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 PPARα/γ dual agonists to overcome and/or minimize previously reported side effects of PPARα/γ dual agonists. To screen and identify novel PPARα/γ dual agonists, we utilized virtual ligand screening and identified several candidate compounds which might fit into ligand binding domain (LBD) of PPARα.

In the present study, we characterized a novel PPARα/γ dual agonist CG301360 and examined its effects on insulin sensitivity and lipid metabolism through *in vitro* and *in vivo* studies. As a modest PPARα/γ dual agonist, CG301360 selectively activated the transcriptional activities of both PPARα and PPARγ. 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 with insulin sensitivity. Together, our findings suggest that CG301360 would be a useful chemical for the treatment of insulin resistance and lipid dysregulation.

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Materials and Methods

Reagents

CG301360 [(R)-1-(4-(2-(5-methyl-2-p-tolyloxazol-4-yl)ethoxy)benzyl)piperidine-2-carboxylic acid] was designed in Crystal Genomics (Seoul, Korea) and synthesized by Korea Research Institute of Chemical Technology (Daejeon, Korea). Rosiglitazone and WY14643 were obtained from Cayman Chemicals (Ann Arbor, MI, USA), and GW501516 was kindly provided by Dr. J. B. Seo (Boram Pharmaceutical Co., Seoul, Korea). Tumor necrosis factor (TNF)α and lipopolysaccharide (LPS) were acquired from Sigma (St. Louis, MO, USA). Antibodies for adiponectin and GAPDH were purchased from Cell Signaling (Danver, MA, USA) and Ab Frontier (Seoul, Korea), respectively. All media and additives for cell culture were purchased from Hyclone (Logan, UT, USA). Adiponectin-ELISA kit was acquired from Adipogen (Incheon, Korea).

Cell culture and glucose uptake assay

All cells [human embryonic kidney (HEK) 293, FAO, C_2C_{12}, 3T3-L1, and RAW 264.7] used in this study were obtained from American Type Culture Collection (Manassas, VA, USA). HEK 293 and 3T3-L1 cells were cultured in DMEM supplemented with 10% bovine calf serum and 1 %

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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_2. C_2C_{12} and 3T3-L1 cells were differentiated as described earlier (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 previously described (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 pulsed once at 1400 V for 20 ms, 3T3-L1 cells pulsed twice at 1300V 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. Co-crystal structures of the ligand-bound ligand binding domains of human PPARα and human

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PPARγ (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.

Quantitative RT-PCR analysis

Total RNA was prepared using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) and cDNA was synthesized by M-MuLV reverse transcriptase kit (Fermentas, Glen Burnie, MD, USA) following to manufacturer's instruction. qRT-PCR was performed using My-IQ thermocycler (Bio-Rad, Hercules, CA, USA). The amplifying reaction was conducted as follows; 40 repetitive thermal cycles (94°C for 20 sec, 60°C for 20 sec, 72°C for 20 sec) with SYBR green (Invitrogen, Carlsbad, CA, USA). 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*^{db}/*Lepr*^{db} male mice were purchased from Central Lab. Animal Inc

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(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*^{db}/*Lepr*^{db} male mice, at 10 weeks of age were daily administered 50 mg/kg of WY14643, rosiglitazone, or CG301360, and an equivalent volume of vehicle (D.W.) by oral gavage for 30 days.

For pharmacokinetic analysis of CG301360, male, ICR mice were injected with CG301360 (5 mpk dose) or vehicle (D.W.) 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 LC/MS/MS system. Pharmacokinetic parameters; area of under the time-concentration (AUC_{last}), time reach to maximum concentration (T_{max}), maximum plasma concentration (C_{max}) and mean residence time (MRT) were calculated.

Measurement of blood lipid profiles

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

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Glucose tolerance test (GTT) and insulin tolerance test (ITT)

For GTT, PPAR agonist-administered mice were fasted for 24 h. 5% glucose (Sigma) solution was orally injected and the blood glucose level was measured with Freestyle blood glucose meter (Therasense, Indianapolis, IN, USA). For ITT, mice were fasted for 6 h before injection of 1 U of human insulin (Lilly, Indianapolis, IN, USA). The blood glucose level was measured from the mice by Freestyle blood glucose meter (Therasense).

Western blotting

Western blot analysis was performed as previously described (Jeong et al., 2009) with minor modifications. Briefly, an equal amount of protein separated on SDS-PAGE gels was transferred to nitrocellulose membrane. Blots were blocked with Tris Buffered Saline with 0.1% Tween-20 (TBST) containing 0.5% non-fat milk at room temperature (RT) 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 RT for 2 h and followed by washing with TBST additional three times. The membrane was incubated with enhanced chemiluminescence reagents and exposed to X-ray film (Fujifilm, Tokyo, Japan).

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Fatty acid oxidation assay

Cells were incubated in palmitate (Sigma)-saturated α-MEM containing 0.1 mmol/l of 9,10-[^{3}H]palmitate (5 μCi/ml, PerkinElmer Life, Boston, MA, USA) and 2% bovine serum albumin for 24 h. 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 water and incubated at 55°C for overnight. After removal of the open microcentrifuge tubes, the content of ^3H_2O content was measured in a scintillation counter.

Statistical analysis

Results are representative data from multiple, at least three times independent experiments. Error bars represent standard deviations and the *P* value calculated from ANOVA (followed by Student-Newman-Keuls comparison test) < 0.05 is interpreted statistically significant.

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Results

Transcriptional activity of PPARα and PPARγ is upregulated by CG301360 through their ligand binding domains.

To design novel PPARα/γ dual agonists, we investigated to analyze 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. Similar to other PPAR agonists, we adopted a phenoxy as a linker and 5-methyl-2-phenyl-oxazole as a hydrophobic tail. 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 PPARα/γ dual agonists, compound CG301360 was selected after evaluating various cellular effects. As shown in Fig. 1A, CG301360 shows the non-TZD based structure. To confirm the specificity and sensitivity of CG301360 in the regulation of transcriptional activities of PPARs, luciferase reporter assays were examined. CG301360 effectively activated PPARα (Fig. 1B) and PPARγ (Fig. 1C), whereas CG301360 did not significantly activate PPARδ (Supplementary Fig. 1). Dose-

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response analyses showed that CG301360 acted as an effective agonist for PPARα and PPARγ when CG301360 was compared with the known PPAR α and PPAR γ agonists, WY 14643 and rosiglitazone, respectively (Supplementary Fig., 2A and B). Moreover, CG301360 enhanced the transactivation of GAL4-PPARα and GAL4-PPARγ LBD constructs in a dose-dependent manner (data not shown; EC_{50} of CG301360 is denoted in Supplementary Table 1), suggesting CG301360 as a modest and selective dual agonist for PPARα and PPARγ through the interaction with their LBD domains. However, CG301360 barely stimulated the transcriptional activity of PPARδ up to1 μM (Supplementary Fig. 2C). Also, when pharmacokinetic properties of CG301360 were evaluated, T_{max} was 0.167 h implying that CG301360 appears to be rapidly absorbed after administration (Supplementary Table 2).

In order to investigate whether CG301360 is able to indeed interact with the LBDs of PPARα and PPARγ, we simulated the docking study of CG301360 with the reported structures of PPARα LBD and PPARγ LBD (PDB ID: 3FEI and 3FEJ, respectively) (Grether et al., 2009). As shown in Fig. 1, D and E, overall structures of LBDs of PPARα and PPARγ containing CG301360 were similar to those of human PPARα and PPARγ with previously reported PPARα/γ dual agonist, phenylthiazole derivative (Grether et al., 2009). The electrostatic potential surface (molecular volume = 346.7 $Å^3$) of CG301360 nicely fits into the LBDs of PPARα and PPARγ (Supplementary Fig. 3). Particularly, the typical four hydrogen bonds linking carboxylate of CG301360 and the Ser, His, and Tyr residues of PPARα/γ are

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within distance ≤ 3 Å. Moreover, CG301360 appears to dock into the hydrophobic pockets of PPARα/γ that are composed of Met, Cys, and Gly residues. In addition, when we calculated the docking scores of CG301360 toward PPARα/γ by comparison with known PPAR ligands such as WY14643 and rosiglitazone (Supplementary Table 3), obtained data clearly suggest that CG301360 would be properly accommodated into the LBD of PPARα and γ as a potential dual ligand.

CG301360 stimulates expression of PPARα and PPARγ target genes.

As CG301360 effectively stimulated the transactivation of both PPARα and PPARγ, we next examined the effects of CG301360 on the expression of PPARα and PPARγ target genes. In hepatoma (FAO) and myocytes (C_2C_{12}), CG301360 augmented the expression of PPARα target genes such as acyl-CoA oxidase (ACO), carnitine-palmitoyl transferase (CPT), middle-chain acyl-CoA dehydrogenase (mCAD), and PPARα itself in a dose-dependent manner (Fig. 2, A and B). Furthermore, CG301360 stimulated the expression of PPARα 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. Expectedly in cultured adipocytes, CG301360 significantly augmented the expression of PPARγ target genes such as adipocyte fatty acid binding protein (aP2), adiponectin (Acrp30), lipoprotein lipase (LPL), and CD36 in a dose-dependent manner (Fig. 2D). These data indicate that CG301360 could act as a dual

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activator of PPARα and PPARγ in cultured cells.

CG301360 restores dysregulated glucose and lipid metabolism in diabetic mice.

To assess the *in vivo* effect of CG301360, obese and diabetic *db/db* mice were orally injected with CG301360 every day for one 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 PPARγ 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 is able to modulate metabolic abnormalities in obese and diabetic subjects. In *db/db* mice, CG301360 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 PPARγ agonist, and WY14643, a PPARα 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.

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As CG301360 greatly reduced plasma glucose and lipid levels in *db/db* mice (Fig. 3, C-F), we next conducted GTT and ITT assays 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 PPARγ 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 as 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 PPARγ activator.

CG301360 suppresses pro-inflammatory responses.

Because pro-inflammatory response is one of causal factors 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-

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inflammatory genes such as TNFα, interleukin-1β (IL-1β), IL-6, inducible NO synthase (iNOS), monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase 2 (COX2), and matrix metalloprotease 9 (MMP 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), CG301360 effectively reduced expression level of macrophage marker genes including CD68, F4/80, CD11b, and CD11c and pro-inflammatory genes such as TNFα, iNOS, and MCP-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 PPARα 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 β-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 β-oxidation appears to be

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mainly through PPARα activation because rosiglitazone rarely, promoted expression of fatty acid oxidation genes (Fig. 6, D- F). Interestingly, in CG301360-treated *db/db* mice, the expression of fibroblast growth factor 21 (FGF21), which induces β-oxidation and reduces the levels of glucose and TG in diabetic animals (Xu et al., 2009), was significantly upregulated in liver (Fig. 6G), providing further supporting evidence that CG301360 is capable of improving hyperlipidemia by facilitating lipid consumption.

CG301360 selectively potentiates PPARα and PPARγ 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 PPARα and/or PPARγ activation, we decided to utilize PPAR knockdown with siRNAs. As a control experiment, we examined whether CG301360 might influence activity of PPARδ, which shares several target genes and some features in lipid catabolism with PPARα (Evans et al., 2004). As shown in Fig. 7A, GW501516, a synthetic PPARδ ligand, failed to increase the expression of β-oxidation genes in PPARδ suppressed FAO cells. Unlike GW501516, CG301360 still stimulated the expression of genes involved in fatty acid oxidation (Fig. 7A, and average fold of induction is denoted in Supplementary Table 4). In contrast, cells transfected with PPARα siRNA evidently blunted the

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expression of genes related with β-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 PPARα rather than PPARδ. Additionally, knockdown of PPARγ also reduced the expression of both of basal and CG301360-induced PPARγ target genes in differentiated adipocytes (Fig. 7C).

To further confirm the repressing effects of PPARα and/or PPARγ activity via each knockdown, we assessed β-oxidation (for PPARα) and adipogenesis (for PPARγ). PPARα siRNA remarkably inhibited fatty acid oxidation in FAO cells (Fig. 7D) and knockdown of PPARγ reduced CG301360-mediated adipogenesis (Fig. 7E and Supplementary 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 for activation of both PPARα and PPARγ.

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Discussion

The importance of PPARs on metabolic diseases has been well established by genetic studies. PPAR$\alpha^{-/-}$ knockout mice fail to meet energy demands during fasting by exhibiting hypoketonemia, hyperlipidemia and fatty liver (Kersten et al., 1999). Tissue-specific PPARγ null mice are severely insulin resistant (He et al., 2003; Hevener et al., 2003), while whole-body PPAR$\gamma^{-/-}$ knockout mice are embryonic lethal(Barak et al., 1999), implying the importance of PPARγ in adipose tissue during development. On the other hands, PPARδ overexpressing transgenic mice enhance lipid metabolism and are resistant to obesity and tissue steatosis, whereas PPARδ 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 PPARα and PPARγ, PPARα/γ dual agonists have been expected to alleviate obesity and diabetes (Al-Salman et al., 2000; Hirsch et al., 1999; Shim et al., 2006; Tang et al., 2003; Watkins and Whitcomb, 1998). However, manifesting side effects of PPARα/γ dual agonists still make them unable to be used in the treatment for obesity and insulin resistance (Hellmold et al., 2007; Mittra et al., 2007; Tannehill-Gregg et al., 2007). In this regard, the failure of former PPARα/γ dual agonists prompted us to develop novel PPARα/γ dual agonists without (or with less) deleterious effects by screening different backbone structures of PPAR ligands.

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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 PPARα. After screening, we isolated non-TZD based CG301360, which is capable to activate both PPARα and PPARγ. In the current study, we demonstrate that this novel PPARα/γ dual agonist effectively and selectively activates PPARα and PPARγ and shows favorable effects on insulin sensitivity and lipid metabolism in *db/db* mice. Unlike many of previous PPARα/γ dual agonists (Hellmold et al., 2007; Mittra et al., 2007), CG301360 moderately activates PPARα and PPARγ. Furthermore, given that CG301360 action may not be primarily mediated through PPARδ propose that CG301360 might be free from PPARδ-mediated side effects such as tumorigenesis and cancer invasion (Zeng et al., 2008).

As a PPARα activator, CG301360 is able to alleviate hyperlipidemia by enhancing lipid catabolism. In fact, CG301360 increased the expression of PPARα target genes as well as FGF21, a PPARα-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 PPARγ. The possible mechanisms responsible for CG301360-dependent glucose sensitivity are probably by 1) enhancing insulin sensitivity and 2) stimulating adiponectin production and secretion,

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which are not mediated by PPARα activation. Collectively, it is feasible to speculate that CG301360 might increase insulin sensitivity as a PPARγ activator, while it relieves lipid dysregulation by primarily activating PPARα.

One of side effects of TZDs is tissue damage, which is often resulted from fluid retention and edema (Hirsch et al., 1999; Mittra et al., 2007; Tang et al., 2003). While some TZDs induce liver failure (Al-Salman et al., 2000; Hirsch et al., 1999; Watkins and Whitcomb, 1998), 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, (Supplementary Fig. 5). Although experiments of long-term administration are needed to clearly elucidate whether CG301360 might confer fluid retention or not, this notion reflects that CG301360 would relieve insulin resistance without acute organ failure in diabetic mice (at least within a month administration).

One of important features of CG301360 is an anti-inflammatory property. Since pro-inflammatory responses play key roles in insulin resistance, it is important to suppress pro-inflammatory responses to rescue insulin resistance. In fact, there are several anti-inflammatory reagents that relieve insulin resistance (Jeong et al., 2009). Both *in vitro* and *in vivo*, CG301360 showed anti-inflammatory effects, probably by activating PPARα and PPARγ.

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In conclusion, we identified a novel PPARα/γ dual agonist CG301360 which provides favorable effects on insulin sensitivity and lipid homeostasis. CG301360, a modest activator of PPARα and PPARγ, 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, we suggest that CG301360 would be a potential agent targeting insulin resistance and related metabolic disorders through activation of both PPARα and PPARγ.

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Footnotes

***** H. W. J., and J.-W. L., were equally contributed to this work

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Figure legends

Fig. 1 Trans-activation of PPAR α and PPARγ by CG301360. **A**. Chemical structure of CG301360 [(R)-1-(4-(2-(5-methyl-2-p-tolyloxazol-4-yl)ethoxy)benzyl)piperidine-2-carboxylic acid]. HEK293 cells overexpressing mouse PPARα (**B**) or PPARγ (**C**) were treated with 1 μM of WY14643 (WY), rosiglitazone (Rosi), GW501516 (GW), or CG301360 (CG360) for 24 h. Cells were subjected to reporter assays as described in Materials and Methods. Each bar represents mean ± S.D. of duplicates (**B, C**). ** *P* <0.01 *vs.* negative control. ** *P* <0.01 *vs.* negative control. **D** and **E.** Binding of CG301360 to LBDs of PPARs. (**D**) PPARα-LBD (PDB ID: 3FEI, binding site volume = 329.5 $Å^3$), (**E**) PPARγ-LBD (PDB ID: 3FEJ, binding site volume = 362.4 $Å^3$). All protein residues displayed a ligand contact distance ≤ 4.5Å. Green color indicates the four hydrogen bonds of the carboxylate head group with PPARα/γ. CG301360 docking simulation was performed as described in Materials and Methods. Blue cloud; human PPARα, yellow cloud; human PPARγ, red cloud; binding site volume of PPARα/γ dual agonist.

Fig. 2 Increased expression of PPARα and PPARγ target genes by CG301360. FAO rat hepatoma cells (**A**), C_2C_{12} myotubes (**B**), 3T3-L1 adipocytes (**C** and **D**) were incubated with WY (1 μM), Rosi (1 μM), or CG360 (0.5, 1, and 5 μM) for 24 h. Relative mRNA level of each gene was analyzed by use of qRT-PCR and normalized by GAPDH. Each bar represents mean ± S.D. of duplicates. Similar

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results were obtained from at least three independent experiments. * P <0.05 *vs.* negative control; ** P <0.01 *vs.* negative control. □, (-); ◘, WY 1 μM; ▨, Rosi 1 μM; ▩, CG360 0.5 μM; ▥, CG360 1 μM; ■, CG360 5 μM.

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 in Materials and Methods. Body weight change (**A**), tissue weight (**B**), plasma contents of glucose (**C**), insulin (**D**), TG (**E**), FFA (**F**), ALT (**G**), and AST (**H**) of CG301360-treated obese *db/db* mice were shown. Each bar represents mean ± S.D of each group of mice (n = 6). * P <0.05 *vs.* vehicle; ** P <0.01 *vs.* vehicle; *** P <0.001 *vs.* vehicle; $^{\#}P$ <0.05 *vs.* Rosi; $^{\#\#}P$ <0.01 *vs.* Rosi. ◇, vehicle; □, WY; △, Rosi; ○, CG360 (panel **A**). □, vehicle; ◘, WY; ▨, Rosi; ■, CG360 (panel B).

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 in Materials and Methods. Plasma glucose and insulin levels were measured from the blood samples which were drawn at baseline (t = 0 min) and indicated time points (as shown in Figure). ◇, vehicle; □, WY; △, Rosi; ○, CG360. **C.** Differentiated adipocytes were starved with low glucose DMEM media without serum for overnight. During starvation,

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each chemical (1 μM of WY, Rosi, or CG360) was added. Glucose uptake assay was performed as described in Materials and Methods. Relative fold of glucose uptake was shown in graph. Each bar represents mean ± S.D. of triplicates. **D.** Differentiated 3T3-L1 cells were treated with 1 μM of WY, Rosi, CG360 and 10 ng/ml of TNFα in serum-free DMEM media for 12 h. Cells were harvested and total cell lysates and media were blotted with anti-adiponectin antibody. GAPDH was used as loading control. **E.** Plasma adiponectin levels from mice of each group determined by adiponectin ELISA kit. * P <0.05 *vs.* vehicle; ** P <0.01 *vs.* vehicle; *** P <0.001 *vs.* vehicle.

Fig. 5 CG301360 suppresses pro-inflammatory gene expression. RAW 264.7 macrophages (**A**) and 3T3-L1 adipocytes (**B**) were incubated with WY (1 μM), Rosi (1 μM), or CG360 (1, and 5 μM) for overnight and were treated with 10 ng/ml of LPS for 6 h (**A**) and 1 ng/ml of TNFα (**B**) for 3 h. Relative mRNA level of each gene was examined as described in Fig. 2. Each bar represents mean ± S.D. of duplicates. Data are representative of at least three independent experiments. (**A** and **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**). ☐, (-); ▤, LPS (**A**) or TNFα (**B**); ◘, WY/LPS (A) or WY/TNFα (**B**); ▨, Rosi/LPS (**A**) or Rosi/TNFα (**B**); ▥, CG360 1 μM/LPS (**A**) or CG360 1 μM/TNFα (**B**); ■, CG360 5 μM/LPS (**A**) or CG360 5 μM/TNFα (**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 ± S.D. * P <0.05 *vs.* vehicle; ** P <0.01 *vs.* vehicle. ☐, vehicle ; ◘, WY; ▨, Rosi; ■, CG360.

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_2C_{12} (**E**), and 3T3-L1 (**F**) cells were treated with 10 μM of WY, Rosi, and CG360 for 24 h and fatty acid oxidation assay was performed. **G.** Relative mRNA level of FGF21 in liver from CG360-treated *db/db* mice was examined by use of qRT-PCR and normalized by GAPDH. Each bar represents mean ± S.D. * P <0.05 *vs.* vehicle; ** P <0.01 *vs.* vehicle; $^{\#}P$ <0.05 *vs.* Rosi. ☐, vehicle; ◘, WY; ▨, Rosi; ■, CG360.

Fig. 7 The effects of PPAR siRNAs on CG301360 action. FAO (**A, B,** and **D**) and 3T3-L1 (**C**) cells were transfected with GFP siRNA (**A**-**D**), PPARδ (**A**), PPARα (**B** and **D**), and/or PPARγ (**C** and **E**) siRNA with microporator and were treated with PPAR agonists (1 μM) 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 ± S.D. of triplicates. Obtained results were similar, at least, in three independent experiments. **D**. PPARα-suppressed FAO cells were performed fatty acid oxidation assay as described in

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Materials and Methods. Each bar represents mean ± S.D. of triplicates. * P <0.05 *vs.* GFP siRNA (-); ** P <0.01 *vs.* GFP siRNA (-); $^{\#}$ P <0.05 *vs.* GFP siRNA + GW (panel **A**), WY (panel **B)**, and Rosi (panel **C)**, respectively. ☐, GFP siRNA; ■, PPARδ (**A**)/PPARα (**B** and **D**)/PPARγ siRNA (**C**). **E.** 3T3-L1 cells were transfected with GFP or PPARγ siRNAs at 24 h after DMI treatment and differentiated as described in Materials and Methods. Cells were differentiated for 9 days further and stained with Oil-red O staining solution. Microscope pictures are representative of triplicates.

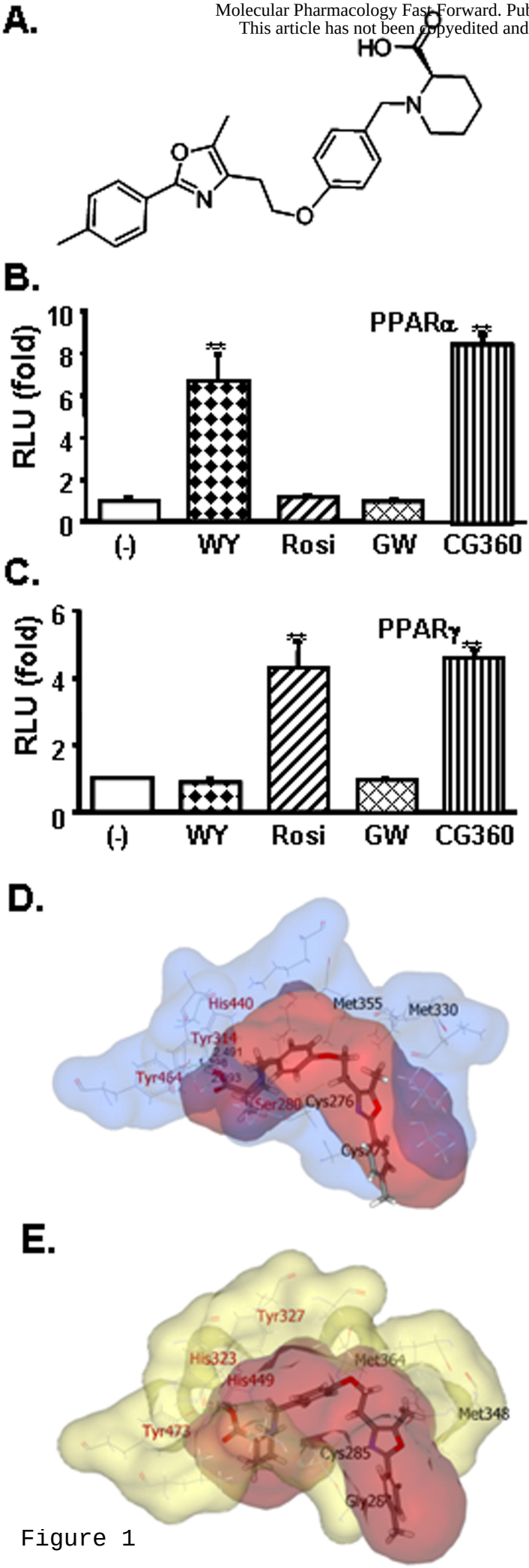

Figure 1

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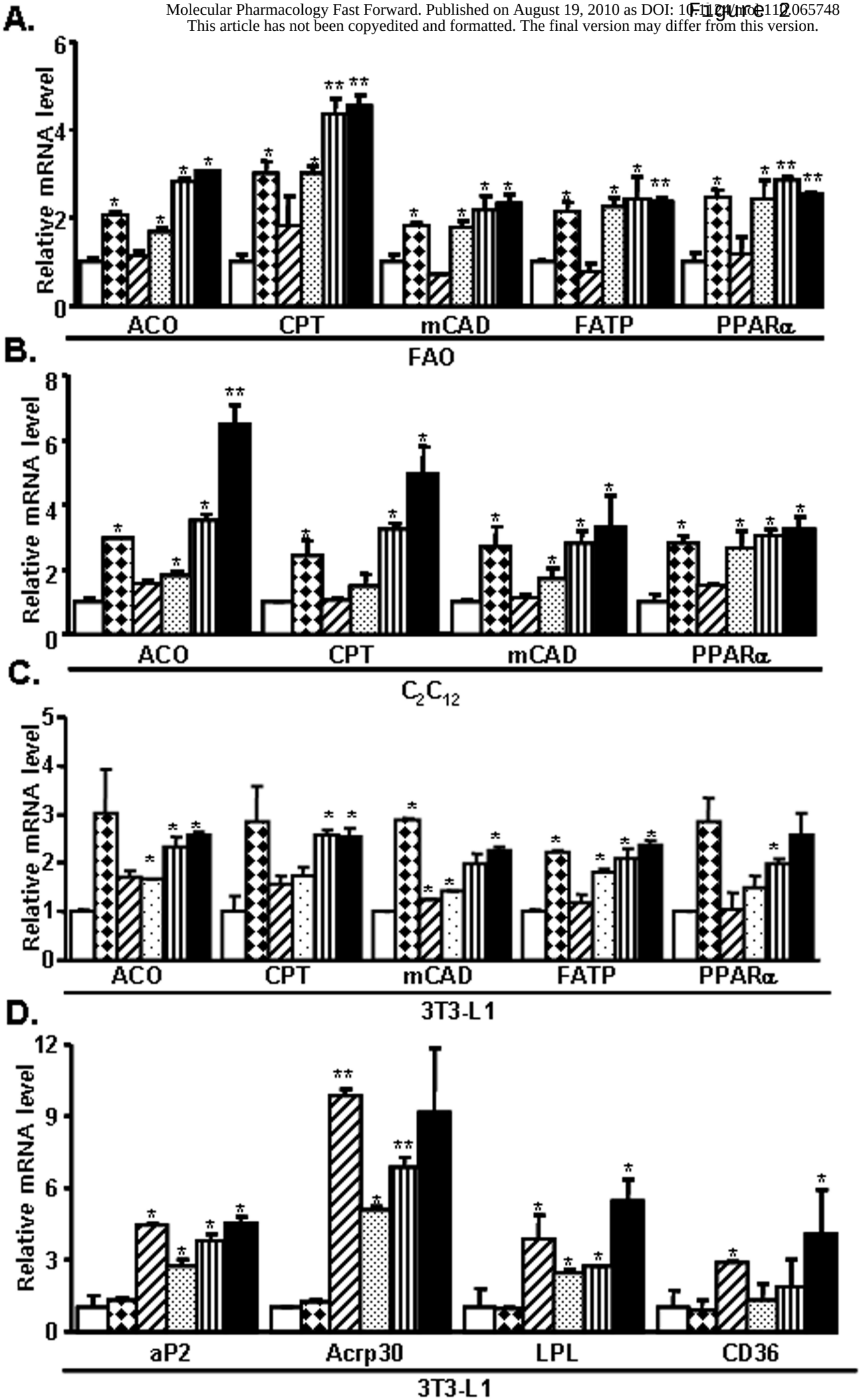
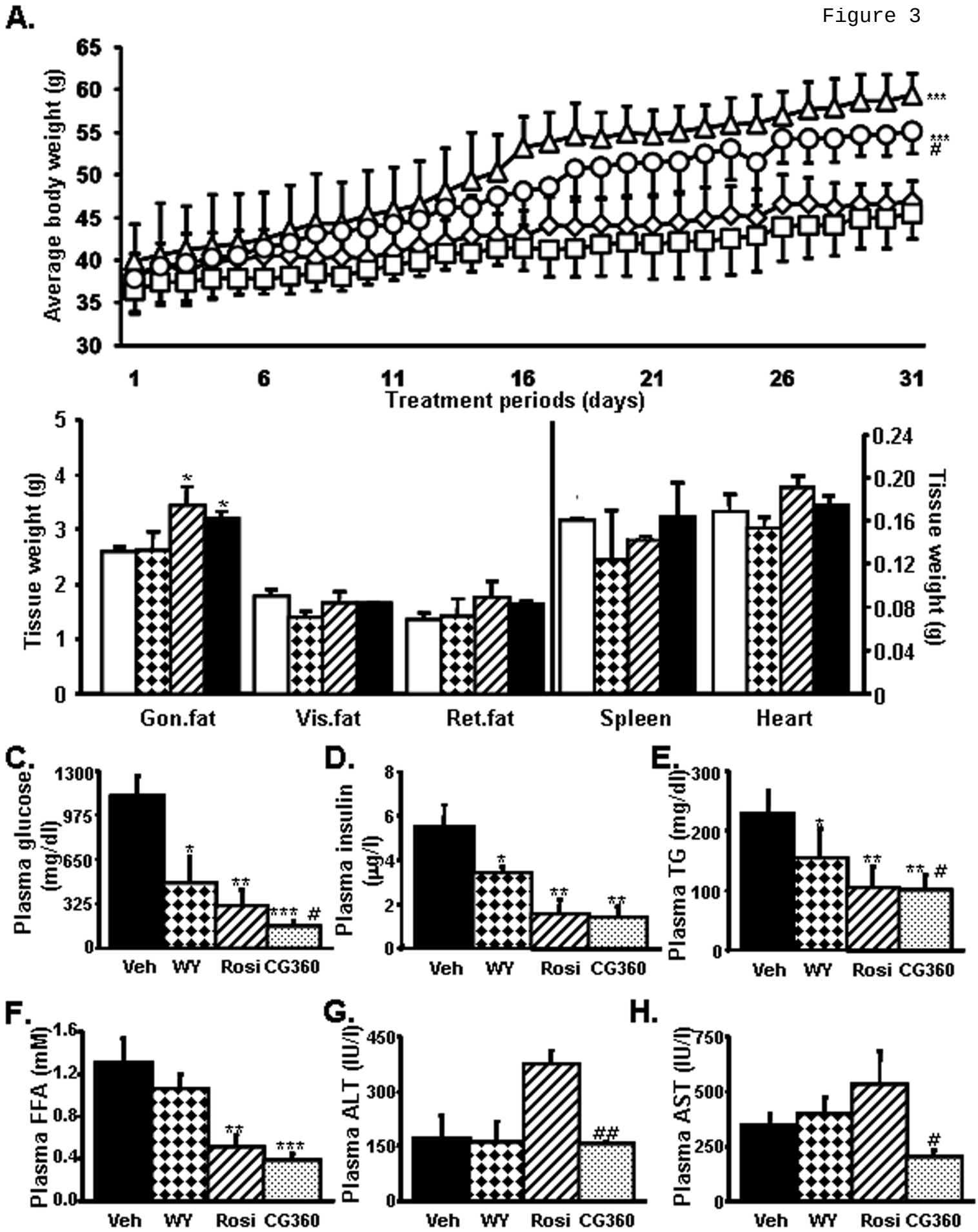
Figure 2.




A.
Average body weight (g)
65
60
55
50
45
40
35
30
***
***
#
1
6
11
16
21
26
31
Treatment periods (days)
Tissue weight (g)
5
4
3
2
1
0
*
*
0.24
0.20
0.16
0.12
0.08
0.04
0
Tissue weight (g)
Gon.fat
Vis.fat
Ret.fat
Spleen
Heart
C.
Plasma glucose (mg/dl)
1300
975
650
325
0
*
**
***
#
Veh
WY
Rosi
CG360
D.
Plasma insulin (µg/l)
8
6
4
2
0
*
**
**
Veh
WY
Rosi
CG360
E.
Plasma TG (mg/dl)
300
200
100
0
*
**
**
#
Veh
WY
Rosi
CG360
F.
Plasma FFA (mM)
1.6
1.2
0.8
0.4
0.0
**
***
Veh
WY
Rosi
CG360
G.
Plasma ALT (IU/l)
450
300
150
0
##
Veh
WY
Rosi
CG360
H.
Plasma AST (IU/l)
750
500
250
0
#
Veh
WY
Rosi
CG360

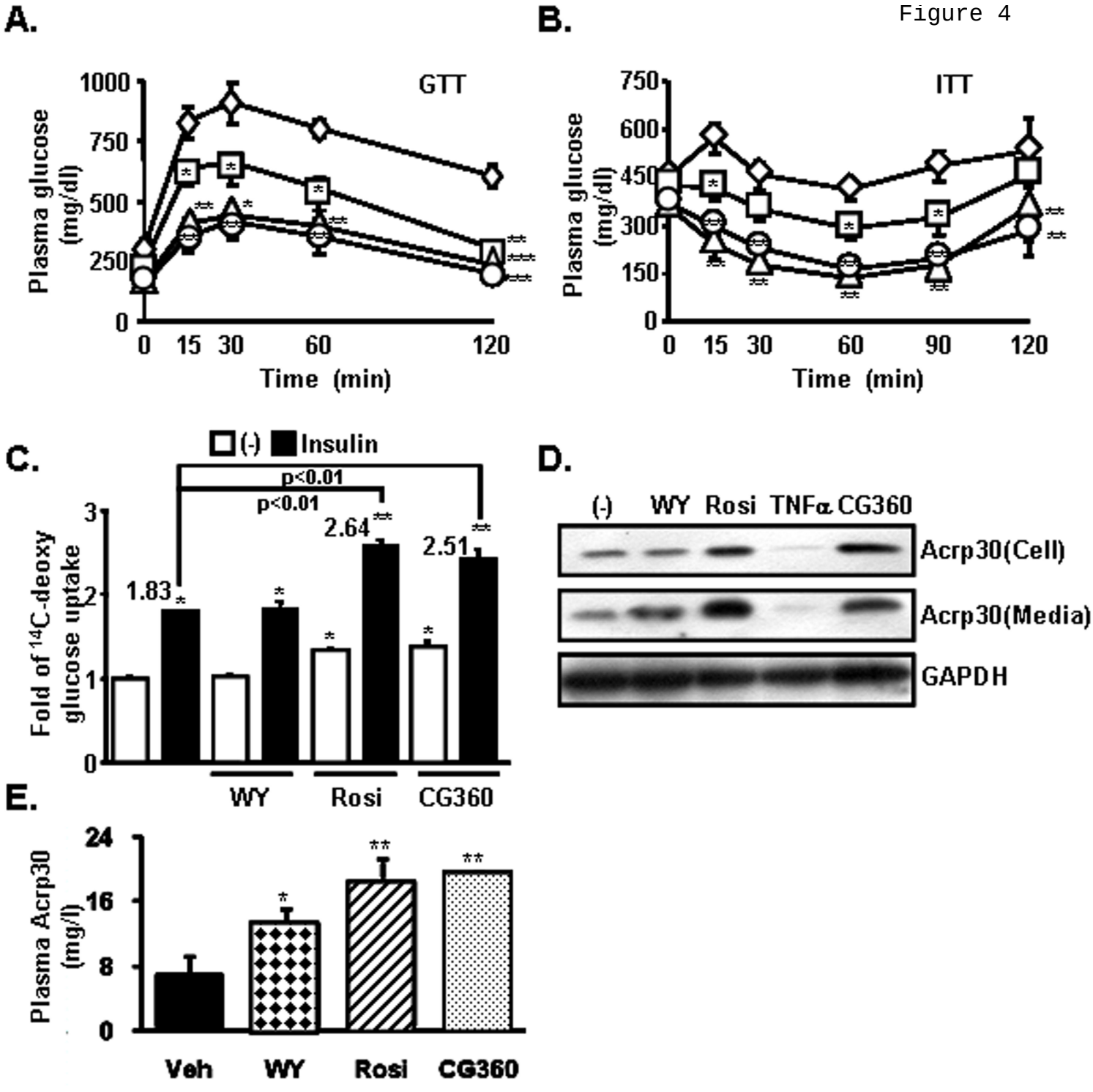

A.
GTT
Plasma glucose (mg/dl)
1000
750
500
250
0
0 15 30 60 120
Time (min)
B.
ITT
Plasma glucose (mg/dl)
750
600
450
300
150
0
0 15 30 60 90 120
Time (min)
C.
(-) Insulin
p<0.01
p<0.01
Fold of 14C-deoxy glucose uptake
1.83
2.64
2.51
WY
Rosi
CG360
D.
(-) WY Rosi TNFα CG360
Acrp30(Cell)
Acrp30(Media)
GAPDH
E.
Plasma Acrp30 (mg/l)
24
16
8
0
Veh
WY
Rosi
CG360

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Figure 5

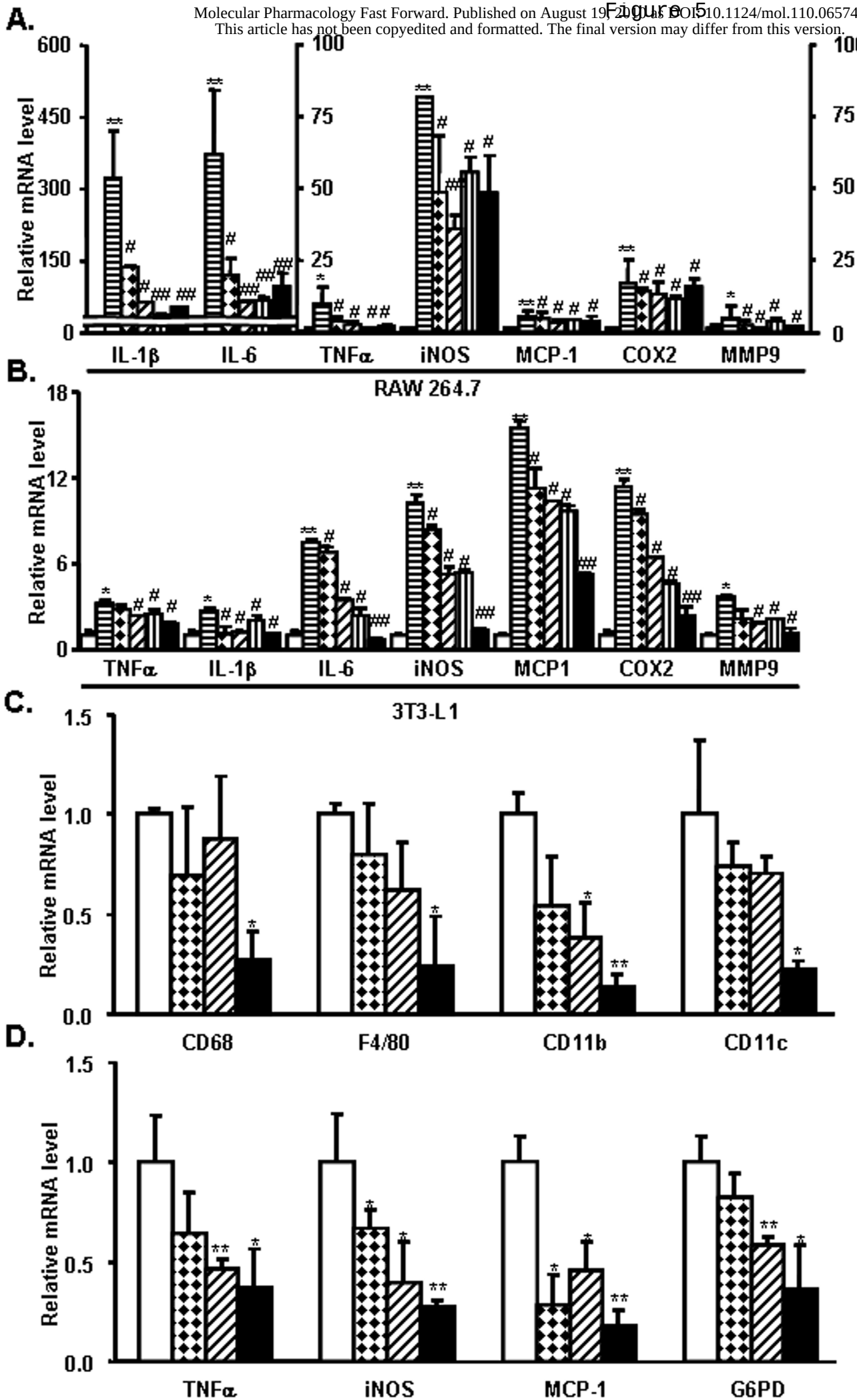

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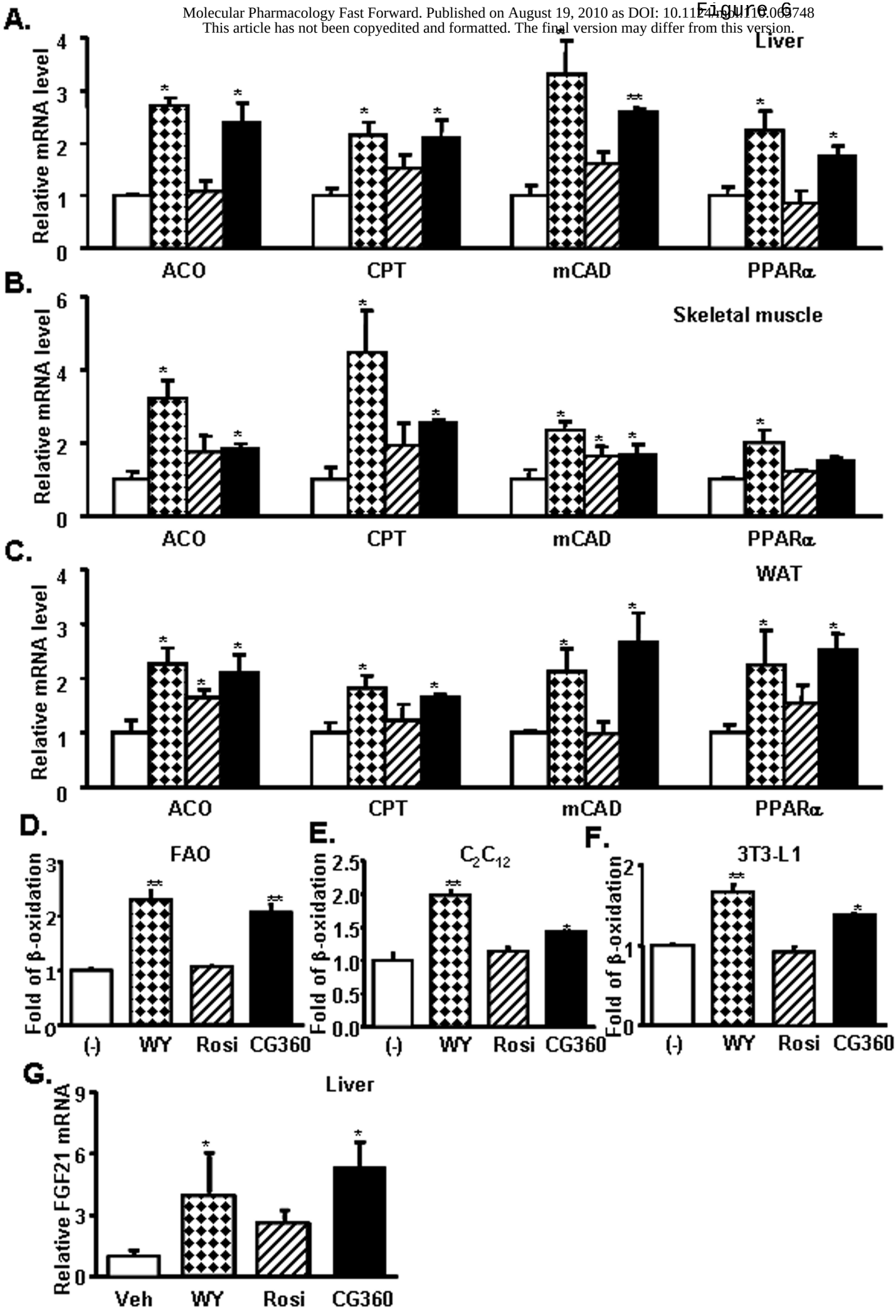
Figure 6



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