

# Peroxisome Proliferator–Activated Receptor $\alpha/\gamma$ Dual Agonist Tesaglitazar Attenuates Diabetic Nephropathy in *db/db* Mice

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Peroxisome proliferator–activated receptors (PPARs) are nuclear transcription factors and play a central role in insulin sensitivity, lipid metabolism, and inflammation. Both PPAR $\alpha$  and  $\gamma$  are expressed in the kidney, and their agonists exhibit renoprotective effects in type 2 diabetes. In the present studies, we investigated the effect of the PPAR $\alpha/\gamma$  dual agonist tesaglitazar on diabetic nephropathy in type 2 diabetic *db/db* mice. Treatment of *db/db* mice with tesaglitazar for 3 months significantly lowered fasting plasma glucose and homeostasis model assessment of insulin resistance levels but had little effect on body weight, adiposity, or cardiac function. Treatment with tesaglitazar was associated with reduced plasma insulin and total triglyceride levels and increased plasma adiponectin levels. Notably, tesaglitazar markedly attenuated albuminuria and significantly lowered glomerulofibrosis, collagen deposition, and transforming growth factor- $\beta$ 1 expression in renal tissues of *db/db* mice. In cultured mesangial cells and proximal tubule cells, where both PPAR $\alpha$  and  $\gamma$  were expressed, tesaglitazar treatment abolished high glucose–induced total collagen protein production and type I and IV collagen gene expression. Collectively, tesaglitazar treatment not only improved insulin resistance, glycemic control, and lipid profile but also markedly attenuated albuminuria and renal glomerular fibrosis in *db/db* mice. These findings support the utility of dual PPAR $\alpha/\gamma$  agonists in treating type 2 diabetes and diabetic nephropathy. *Diabetes* 56:2036–2045, 2007

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Received for publication 14 August 2006 and accepted in revised form 16 May 2007.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 29 May 2007. DOI: 10.2337/db06-1134.

Additional information for this article can be found in an online appendix at <http://dx.doi.org/10.2337/db06-1134>.

Y.G. has received grant support from AstraZeneca.

BUN, blood urea nitrogen; HOMA-IR, homeostasis model assessment of insulin resistance; MC, mesangial cell; PAS, periodic acid schiff; PPAR, peroxisome proliferator–activated receptor; PPRE, peroxisome proliferator–response element; PTC, proximal tubule cell; TGF, transforming growth factor; TZD, thiazolidinedione.

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Type 2 diabetes is characterized by hyperglycemia, insulin resistance, and progressive loss of pancreatic  $\beta$ -cell function during the disease process (1). In recent years, synthetic ligands of  $\alpha$  and  $\gamma$  subtypes of peroxisome proliferator–activated receptors (PPARs) have been reported to have beneficial effects in type 2 diabetes (2–4). The fibrate class of PPAR $\alpha$  agonists are hypolipidemic agents and also suppress atherogenic processes, including vascular inflammation, plaque instability, and thrombosis (5). On the other hand, PPAR $\gamma$  agonists, including various thiazolidinediones, improve glycemic control by enhancing insulin sensitivity in peripheral insulin-sensitive tissues and also have been shown to be potent antidiabetes, antifibrotic, and anti-inflammatory agents in patients with type 2 diabetes (6). Importantly, both PPAR $\alpha$  and  $\gamma$  agonists exert renal protective effects in type 2 diabetic animals (7–10). These beneficial actions of PPAR $\alpha$  and  $\gamma$  agonists support the idea that the combination of PPAR $\alpha$  and  $\gamma$  agonists may have additive or synergistic benefits on metabolic control and vascular complications in type 2 diabetes. Moreover, several studies (11,12) report that PPAR $\alpha$  agonists may mitigate the weight gain in rodent models of insulin resistance without affecting food intake. These observations suggest the possibility that dual activation of PPAR $\alpha$  and  $\gamma$  may eliminate the undesirable side effects of PPAR $\gamma$  agonists, especially weight gain.

Recently, a number of PPAR $\alpha/\gamma$  dual agonists have been or remain under evaluation for their efficacy in animal models of insulin resistance and in type 2 diabetic patients (13–18). However, most studies have focused on the lipid-lowering effect and metabolic alterations, and little is known about the renal protective effect of PPAR  $\alpha/\gamma$  dual agonists in diabetic nephropathy. In present studies, we examined the effects of a PPAR  $\alpha/\gamma$  dual agonist, tesaglitazar, on renal function and histological changes in a type 2 diabetic *db/db* mouse model.

## RESEARCH DESIGN AND METHODS

Eight-week-old male C57BKS/J *db/db* mice were purchased from the Jackson Laboratory. Mice were maintained on a standard rodent diet until 5 months of age, when they were treated with tesaglitazar. Because the primary aim of the study was to evaluate the efficacy of tesaglitazar in fully developed diabetic nephropathy instead of the preventive role of the drug in the progression of diabetic nephropathy, we started to administer drugs at 5 months of age. One group ( $n = 5$ ) of mice was gavaged with tesaglitazar (a gift from Dr. Eric Lundholm; AstraZeneca) at  $1 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  suspended in 0.5% methylcellulose. This dose of tesaglitazar is sufficient for normalization of hypergly-

TABLE 1  
Summary of physical parameters in experimental animals

	Body weight (g)		Food intake (g/day)		Water intake (g/day)		Urine volume (ml/day)	
	Control	Tesaglitazar	Control	Tesaglitazar	Control	Tesaglitazar	Control	Tesaglitazar
Baseline	51.8 ± 1.2	54.0 ± 1.0	6.0 ± 2.0	5.1 ± 0.7	17.0 ± 4.0	15.0 ± 3.5	11.1 ± 3.1	8.5 ± 2.6
1 month	55.0 ± 1.8	54.3 ± 1.3	4.0 ± 0.9	5.0 ± 1.2	17.0 ± 4.0	2.8 ± 0.3*	12.2 ± 3.7	1.7 ± 0.1†
2 months	55.8 ± 2.1	54.6 ± 1.1	6.0 ± 1.8	6.4 ± 2.8	10.0 ± 2.6	2.2 ± 0.4*	7.8 ± 1.6	0.8 ± 0.1*
3 months	55.6 ± 2.4	55.1 ± 2.0	3.6 ± 0.9	4.2 ± 1.2	9.6 ± 2.2	2.9 ± 0.5*	5.6 ± 1.8	0.6 ± 0.2*

Data are means ± SE. Statistical analysis was performed between groups at the same time periods. \* $P < 0.05$  control vs. tesaglitazar; † $P < 0.01$  control vs. tesaglitazar.

emia and hypertriglyceridemia in *ob/ob* mice and increased the whole-body insulin sensitivity in obese Zucker rats (13). The second group (control group,  $n = 5$ ) was gavaged with vehicle alone. During the experiment, food consumption, water intake, urine volume, and plasma insulin levels were measured every month. Body weight, fasting plasma glucose concentrations, and A1C levels were measured every 2 weeks. A1C; plasma glucose and insulin levels; blood urea nitrogen (BUN); electrolytes; hematocrit; and serum creatinine, triglyceride, and cholesterol levels were analyzed as previously reported (8,19,20). Lipoprotein profile was measured by a fast-protein liquid chromatography system at the Lipid/Lipid Peroxidation Core at Vanderbilt University. Plasma adiponectin levels were measured by a radioimmunoassay kit (Linco Research). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5. Urinary albumin concentration was measured using an enzyme-linked immunosorbent assay method (Albuwell kit; Exocell).

#### Light microscopy, immunohistochemistry, and islet area measurement.

The kidney tissues embedded in paraffin were cut into 4- $\mu$ m-thick slices and were stained with periodic acid schiff (PAS). Immunohistochemistry was performed for type IV collagen. A semiquantitative score for a sclerosis index was used to evaluate the degree of glomerulosclerosis on PAS-stained sections. Sclerosis was defined as collapse and/or obliteration of the glomerular capillary tuft, accompanied by hyaline material and/or an increase in the matrix. The severity of sclerosis for each glomerulus was graded from 0 to 4+ as follows: 0, no lesion; 1+, sclerosis of <25% of the glomerulus; and 2+, 3+, and 4+, sclerosis of 25–50%, 51–75%, and >75% of the glomerulus, respectively. A whole-kidney average sclerosis index was obtained from specimens by averaging scores from all glomeruli on each section in each group. Histologic examination was carried out by a pathologist in a blinded manner, according to previously described methods (21). More than 100 glomeruli were analyzed in kidney sections of each mouse.

For immunohistochemical staining, renal tissues were cut into 4- $\mu$ m-thick sections and the slides were then incubated overnight at 4°C with primary antibody against rabbit polyclonal anti-type IV collagen antibody (1:150; Santa Cruz). For the evaluation of type IV collagen staining, glomerular fields were graded semiquantitatively. Briefly, each score reflects both changes in the extent and the intensity of staining and is graded on a five-scale basis: grade 0, very weak or absent staining and no localized increases of staining; grade 1, diffuse weak staining with 1–25% of the glomerulus showing focally increased staining; grade 2, 26–50% of the glomerulus demonstrating focal strong staining; grade 3, 51–75% of the glomerulus stained strongly in a focal manner; and grade 4, >75% of the glomerulus stained strongly. From >60 glomeruli were counted under high power ( $\times 400$ ), and an average score was calculated. Each slide was scored by a pathologist blinded to the experimental conditions.

Total islet area was measured in PAS-stained sections using a National Institutes of Health image analysis system (Scion Image Beta 4.02 for Windows) equipped with a digital camera through an Olympus microscope (IX 81; Olympus America). More than 10 fields were examined under high power ( $\times 400$ ), and islet area was traced and total islet area calculated and expressed as the average score.

**Culture of mouse mesangial and proximal tubule cells.** Mesangial cells (MCs) were isolated from PPAR $\alpha$  gene-deficient ( $^{-/-}$ ) mice and *db/db* mice using a standard sieving method, and MCT cells, a murine line of proximal tubule cells (PTCs), were cultured as previously reported (22,23). To evaluate the effect of tesaglitazar on collagen synthesis under high-glucose conditions, subconfluent MCs and MCTs were serum starved for 24 h, and tesaglitazar was administered at a final concentration of 30  $\mu$ mol/l to the culture media containing 30 mmol/l of glucose concentration. All experimental groups were cultured in triplicate and harvested at 24 h for extraction of total RNA and protein.

**Expression of PPAR $\alpha$  and  $\gamma$  in cultured renal cells.** Expression of PPAR $\alpha$  and  $\gamma$  in MCs and PTCs was determined using RT-PCR and immunoblots.

Messenger RNA (mRNA) levels were detected using RT-PCR using the following primers: 5' GAATTTGCCAAGGCTATCCCA3' (sense primer for PPAR $\alpha$ ), 5' ATGATGTCACAGAACGGCTTC 3' (antisense primer for PPAR $\alpha$ ), 5' CCGAAGAACCATCCGATTGA 3' (sense primer for PPAR $\gamma$ ), and 5' CGG-GAAGGACTTTATGTATGA 3' (antisense primer for PPAR $\gamma$ ). Immunoblots were utilized to determine protein expression levels of PPAR $\alpha$  and  $\gamma$  using a rabbit anti-PPAR $\alpha$  and a rabbit anti-PPAR $\gamma$  antibody (SC-9000 and SC-7273; Santa Cruz), respectively. To determine the endogenous PPAR transcriptional activity and effect of tesaglitazar on transforming growth factor (TGF)  $\beta$ -induced transcriptional activation, peroxisome proliferator-response element (PPRE)-luciferase reporter and 3TP-luciferase reporter activity were measured in cultured MCs and PTCs, as previously reported (24).

**Analysis of gene expression by real-time quantitative PCR.** Primers were designed from the respective gene sequences using the Primer 3 software, and template secondary structures were examined and excluded using an mfold software program. The sequence of each primer was as follows: procollagen  $\alpha 1$  chain of type I collagen, forward: 5' CCA AAG GTG CTG ATG GTT CT 3' and reverse: 5' ACC AGC TTC ACC CTT GTC AC 3'; procollagen  $\alpha 1$  chain of type IV collagen, forward: 5' GCT CTG GCT GTG GAA AAT GT 3' and reverse: 5' CTT GCA TCC CGG GAA ATC 3'; TGF $\beta 1$ , forward: 5' AGC CCG AAG CGG ACT ACT AT 3' and reverse: 5' CTG TGT GAG ATG TCT TTG GTT TTC 3'; adiponectin, forward: 5' TGT TGG AAT GAC AGG AGC TGA A 3' and reverse: 5' CAC ACT GAA GCC TGA GCG ATA C 3'; and  $\beta$ -actin, forward: 5' GGA CTC CTA TGT GGG TGA CG 3' and reverse: 5' CTT CTC CAT GTC GTC CCA GT 3'. The amplicon length of each gene was as follows: procollagen  $\alpha 1$  chain of type I collagen, 107 basepairs (bp); procollagen  $\alpha 1$  chain of type IV collagen, 102 bp; TGF $\beta 1$ , 96 bp; adiponectin, 121 bp; and  $\beta$ -actin, 103 bp. Quantitative gene expression was performed on a Bio-Rad iCycler system (Bio-Rad) using SYBR green technology. Total mRNA was reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad). Real-time RT-PCR was performed, and the mRNA level of each sample was normalized to that of  $\beta$ -actin mRNA. The ratio of each gene to  $\beta$ -actin level (relative gene expression number) was calculated by subtracting the threshold cycle number ( $C_t$ ) of the target gene from that of  $\beta$ -actin and raising two to the power of this difference.  $C_t$  values are defined as the number of PCR cycles in which the fluorescent signal during the PCR reaches a fixed threshold.

**Measurement of secreted collagen in cultured MCs and PTCs.** Total soluble collagen was measured in culture supernatants by the Sircol soluble collagen assay kit (Biocolor, Belfast, Northern Ireland), following the manufacturer's instructions. The calibration curve was set up using the collagen standard provided by the manufacturer.

**Statistical analysis.** We used nonparametric analysis due to few sample numbers. Results were expressed as means ± SE. A Kruskal-Wallis test was used for comparison of more than two groups, followed by a Mann-Whitney *U* test for comparison using a microcomputer-assisted program with SPSS for Windows 10.0 (SPSS).  $P < 0.05$  was considered statistically significant.

## RESULTS

**Physical parameters in experimental animals.** Physical parameters between the tesaglitazar-treated and the untreated group were compared (Table 1). Although water intake and urine volume were initially not different between the two groups, their levels were decreased in the tesaglitazar-treated group following 1 month of treatment. Interestingly, food intake and body weight did not differ throughout the study.

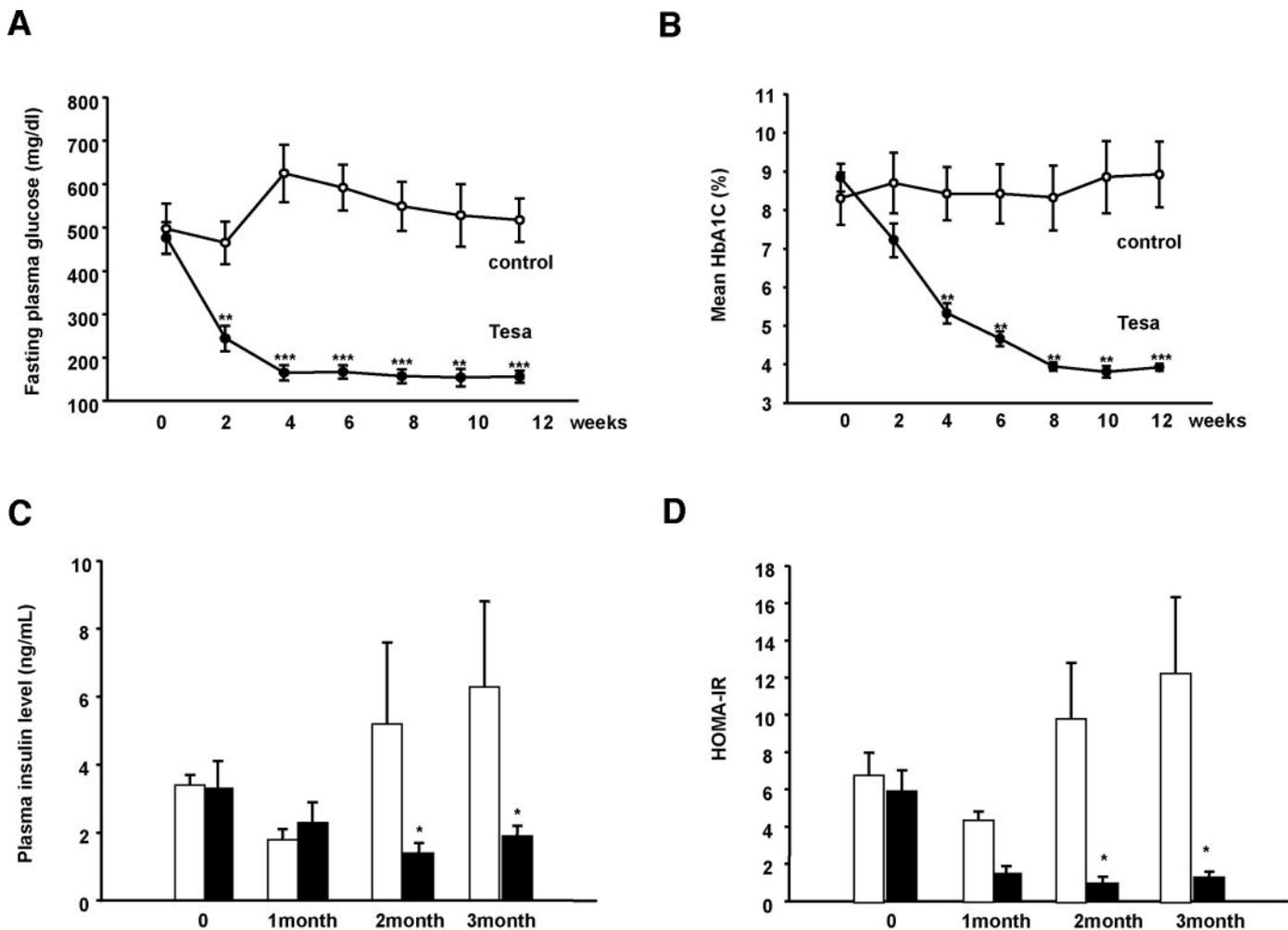


FIG. 1. Effects of tesaglitazar on metabolic parameters in experimental animals. Tesaglitazar was administered for 3 months in *db/db* mice, and various metabolic parameters including fasting plasma glucose concentrations (A), A1C levels (B), plasma insulin concentrations (C), and HOMA-IR (D) were serially measured. Data are shown as means  $\pm$  SE. HOMA-IR was calculated using fasting glucose (mmol/l)  $\times$  fasting insulin (mU/l)/22.5. Comparison was performed between the control group and the tesaglitazar treatment group at same time point. \* $P < 0.05$ , control vs. tesaglitazar; \*\* $P < 0.01$ , control vs. tesaglitazar; \*\*\* $P < 0.001$ , control vs. tesaglitazar.  $\square$ , control group;  $\blacksquare$ , tesaglitazar group.

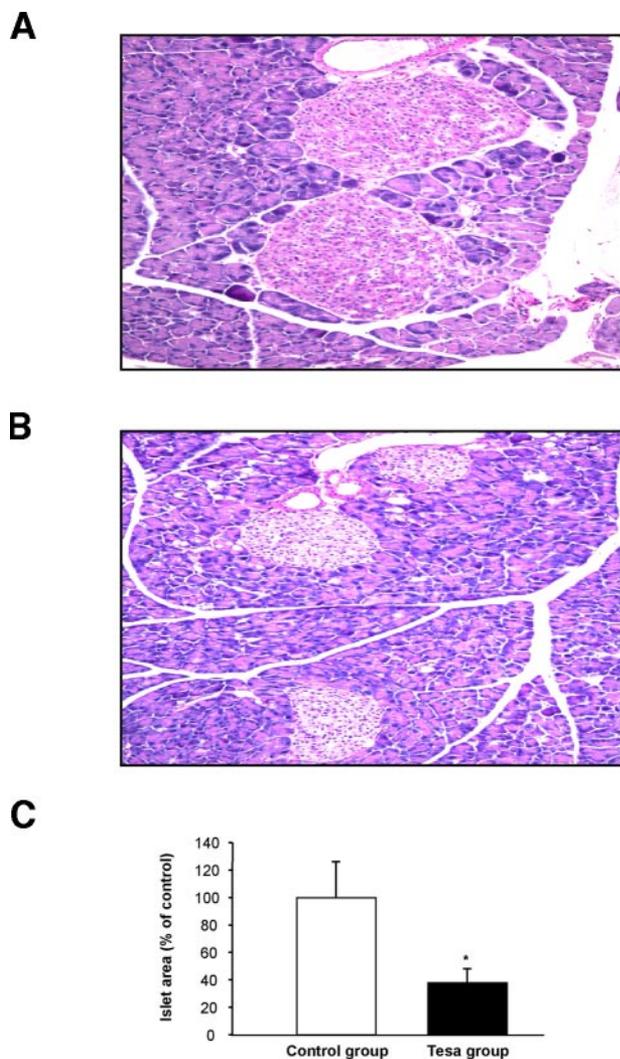
**Effect of tesaglitazar on metabolic parameters in experimental animals.** Tesaglitazar treatment markedly improved glycemic control. Within 2 weeks of treatment, fasting plasma glucose levels were significantly decreased, and this hypoglycemic effect persisted throughout the study (Fig. 1A). In accordance with plasma glucose levels, A1C levels were also markedly improved in the tesaglitazar-treated group ( $3.92 \pm 0.09$  vs.  $8.84 \pm 0.36\%$ ,  $P < 0.01$ ) (Fig. 1B). In addition, at the end of the studies, plasma insulin levels were markedly decreased in the tesaglitazar-treated group compared with the control group (tesaglitazar group,  $1.98 \pm 0.33$  vs. control group,  $6.37 \pm 4.4$   $\mu$ g/ml,  $P < 0.05$ ) (Fig. 1C). Similarly, HOMA-IR indexes were dramatically improved after tesaglitazar treatment (Fig. 1D).

Tesaglitazar treatment markedly decreased triglyceride levels, whereas HDL cholesterol levels were significantly increased compared with the untreated control group (online appendix Table 1 [available at <http://dx.doi.org/10.2337/db06-1134>]). It was also noticed that total and LDL cholesterol levels were significantly increased following tesaglitazar treatment. Tesaglitazar treatment did not change heart and kidney weight (online appendix Table 2). As expected, liver was significantly heavier in the tesagli-

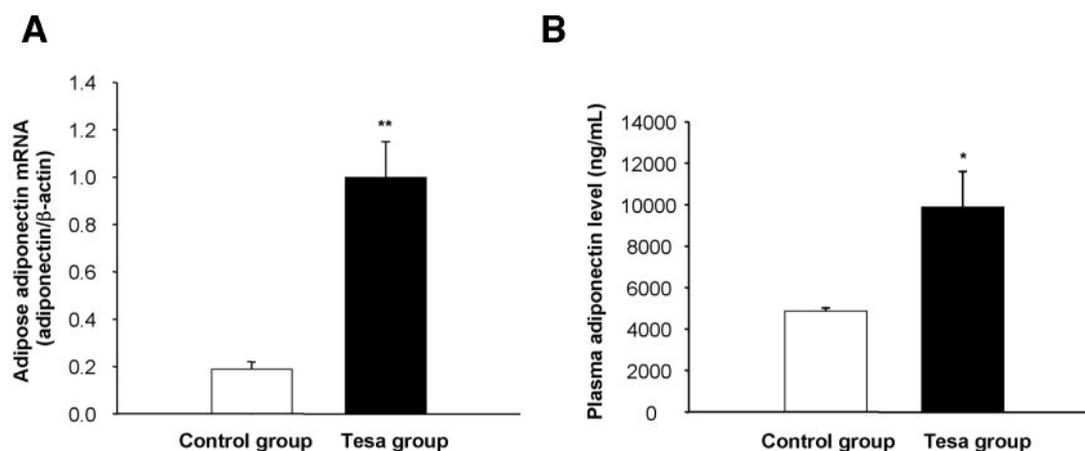
tazar-treated group without significant histological change (online appendix Fig. 1), consistent with the effect of hepatic PPAR $\alpha$  activation. Surprisingly, epididymal fat mass did not differ between the two groups, whereas pancreatic weight was significantly reduced in the tesaglitazar-treated group compared with the control group (online appendix Table 2).

Tesaglitazar did not alter plasma K<sup>+</sup> concentration, BUN, creatinine, or hematocrit levels between the two groups at the end of the study (online appendix Table 3). However, plasma sodium (Na<sup>+</sup>) and chloride (Cl<sup>-</sup>) levels were significantly higher in the tesaglitazar-treated group. **Effect of tesaglitazar on pancreatic islet hypertrophy in *db/db* mice.** Tesaglitazar treatment significantly reduced islet hypertrophy in C57BKS *db/db* mice (control group,  $100 \pm 26.3$  vs. tesaglitazar group,  $38.4 \pm 10.2$ ,  $P < 0.05$ ) (Fig. 2).

**Effect of tesaglitazar on adipose expression and plasma levels of adiponectin in *db/db* mice.** To elucidate the possible mechanism by which tesaglitazar improves insulin resistance, adiponectin mRNA levels in adipose tissues and plasma adiponectin levels were measured. As shown in Fig. 3, adiponectin gene expression was dramatically upregulated by tesaglitazar treatment in



**FIG. 2.** Representative pancreatic morphologic findings in experimental animals. Animals were treated with tesaglitazar for 3 months, and PAS and immunohistochemical staining was performed in pancreas tissues. **A:** Control group, PAS staining. **B:** Tesaglitazar treatment group, PAS staining. Original magnification  $\times 400$ . **C:** Quantitative analysis of islet mass. Islet mass in the tesaglitazar-treated group is expressed as percent value relative to control group. \* $P < 0.05$ , control vs. tesaglitazar.



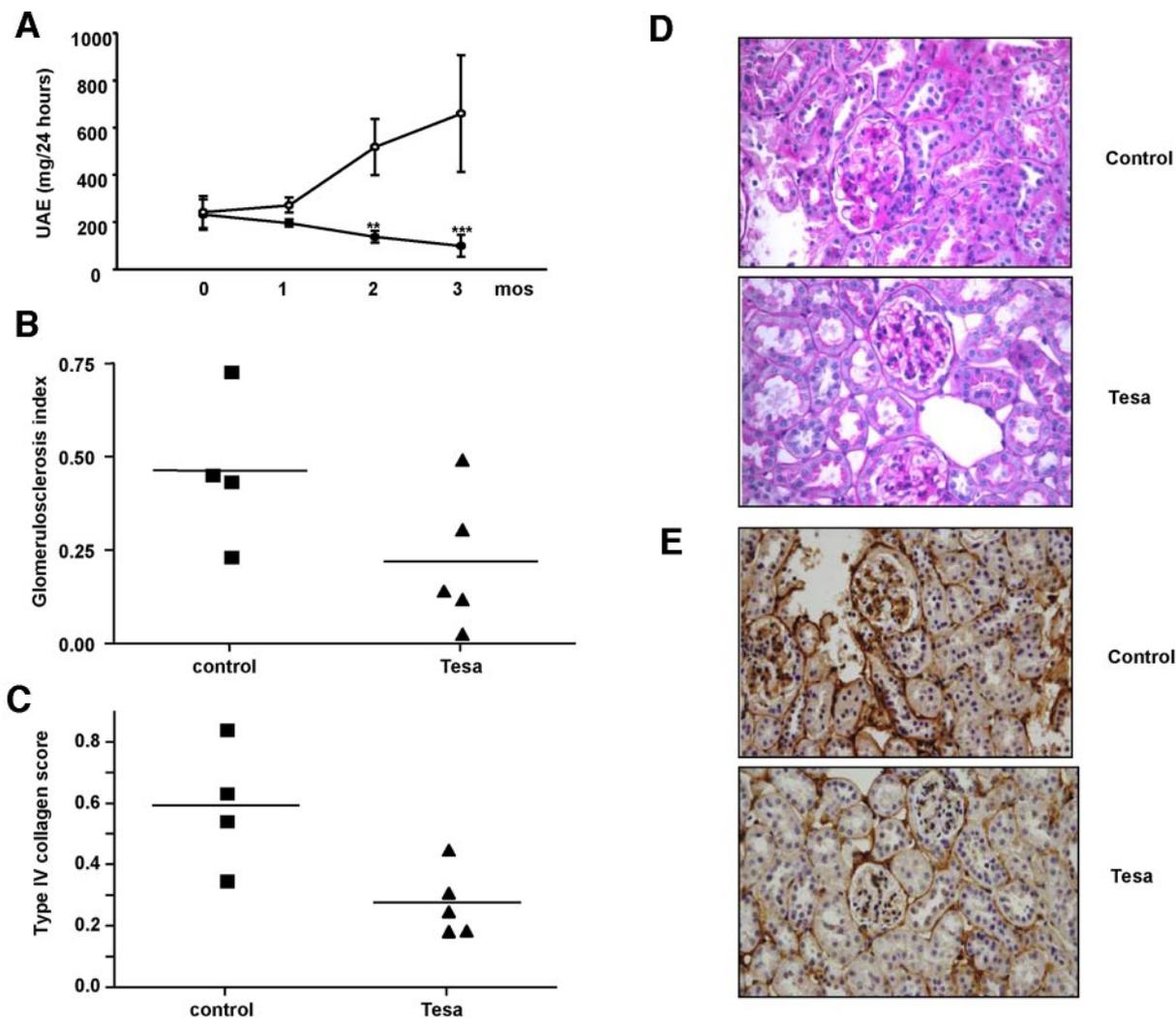
**FIG. 3.** Effect of tesaglitazar on adiponectin gene expression in epididymal adipose tissues (**A**) and plasma levels of adiponectin in *db/db* mice (**B**). Data are shown as means  $\pm$  SE. Comparison was performed between control group and tesaglitazar treatment group. \* $P < 0.05$ , control vs. tesaglitazar; \*\* $P < 0.01$ , control vs. tesaglitazar.

the adipose tissues (Fig. 3A), which was in agreement with significantly increased plasma levels of adiponectin (Fig. 3B).

**Effect of tesaglitazar on renal function and glomerulosclerosis.** Baseline daily urinary albumin excretion was markedly increased in C57 BKS *db/db* mice at 5 months of age in both groups (control group,  $241 \pm 67$  vs. pretesaglitazar group,  $230 \pm 65$   $\mu\text{g/day}$ ). In the control group, albuminuria was persistently increased, and at the end of the study albuminuria reached  $659 \pm 247$   $\mu\text{g/day}$ . However, tesaglitazar treatment markedly reduced urinary albumin excretion from  $230 \pm 65$  to  $98 \pm 47$   $\mu\text{g/day}$  (Fig. 4A). In terms of renal function, the tesaglitazar group tended to have slightly higher levels of both BUN and serum creatinine than the control group, but no statistical difference in serum creatinine levels was observed (control group,  $0.52 \pm 0.11$  vs. tesaglitazar group,  $0.92 \pm 0.28$  mg/dl,  $P > 0.05$ ) (online appendix Table 3).

Consistent with the marked attenuation of albuminuria, glomerulosclerosis was significantly reduced in the tesaglitazar-treated group (Fig. 4B and D). Furthermore, the immunostaining score for type IV collagen, a major extracellular matrix component of fibrotic glomeruli, also demonstrated dramatic improvement in the tesaglitazar-treated group (Fig. 4C and E). To further characterize the molecular mechanism of the beneficial effect of tesaglitazar on the kidney, we quantified mRNA levels for profibrotic factors including type I collagen, type IV collagen, and TGF $\beta$ 1. Gene expression of all of these profibrotic molecules was downregulated by tesaglitazar treatment (Fig. 5).

**Endogenous PPAR $\alpha$  and - $\gamma$  transcriptional activity in cultured renal cells.** To test the cytotoxic effect of tesaglitazar, an MTT assay was performed. Surprisingly, tesaglitazar did not affect cell viability even at a concentration of 100  $\mu\text{mol/l}$  in either cultured mouse MCs or PTCs (data not shown). Both MCs and PTCs had constitutive PPAR $\alpha$  and - $\gamma$  gene expression (Fig. 6A and B). Tesaglitazar treatment resulted in a dose-dependent induction of PPRE-driven luciferase activity (Fig. 6C). At a concentration of 30  $\mu\text{mol/l}$ , tesaglitazar increased PPRE transcriptional activity by  $286 \pm 36.6\%$  in *db/db* MCs ( $P < 0.001$ ) and by  $252 \pm 14.0\%$  in PTCs ( $P < 0.001$ ). In cultured PPAR $\alpha$  knockout MCs, tesaglitazar treatment increased PPRE activity by  $155.0 \pm 8.0$ ,  $215 \pm 26.0$ , and  $252 \pm 14.0\%$  at doses of 0.5, 5, and 30  $\mu\text{mol/l}$ , respectively. Due to the



**FIG. 4.** Effects of tesaglitazar on renal function and morphologic changes. Twenty-four hour urine was collected at monthly intervals after tesaglitazar administration. After 3 months' treatment, urine albumin excretion, glomerulosclerosis index, and type IV collagen were examined. **A:** Twenty-four hour urinary albumin excretion. **B:** Glomerulosclerosis index. **C:** Glomerular immunostaining score for type IV collagen. **D:** Representative renal PAS staining of *db/db* mice treated with (lower panel) or without (upper panel) tesaglitazar. **E:** Representative renal type IV collagen staining of *db/db* mice treated with (lower panel) or without (upper panel) tesaglitazar. Data are shown as means  $\pm$  SE. Comparison was performed between the control group and the tesaglitazar-treated group at the same time point. \* $P < 0.05$ , control vs. tesaglitazar. \*\* $P < 0.01$ , control vs. tesaglitazar. \*\*\* $P < 0.001$ , control vs. tesaglitazar. Original magnification  $\times 100$ . PAS stain and type IV collagen staining.

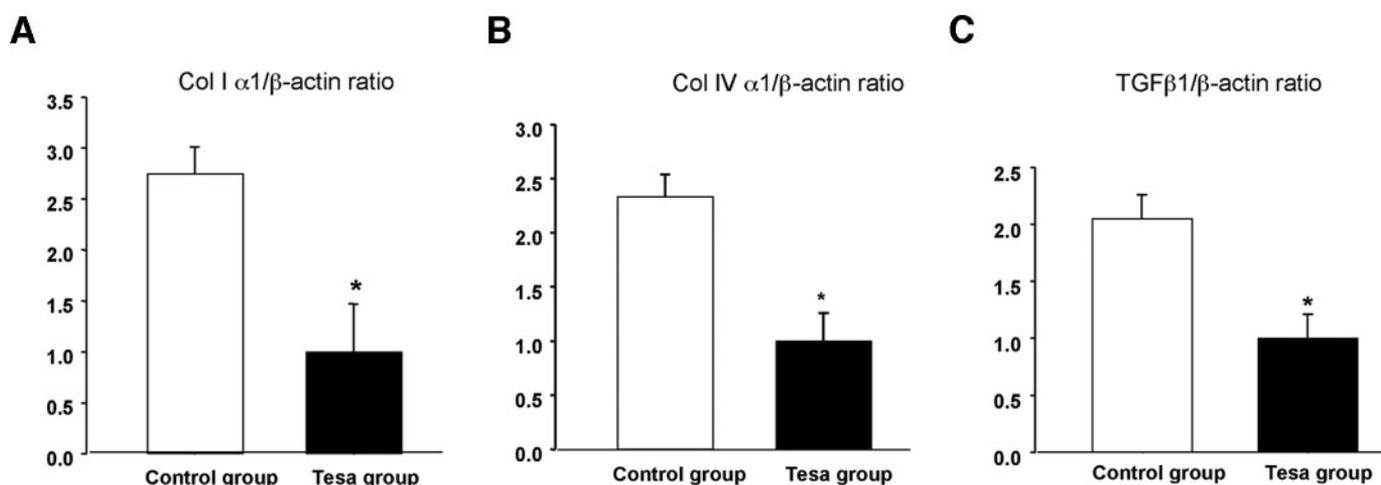
lack of a PPAR $\alpha$  gene in the MCs, these results suggest that tesaglitazar-induced PPRE transcriptional activity was mediated by endogenous PPAR $\gamma$  activation. Interestingly, after cotransfection with a human PPAR $\alpha$  expression vector, PPRE activity was further increased by  $219 \pm 17.0$ ,  $248 \pm 41.0$ , and  $400 \pm 59.0\%$  at doses of 0.5, 5, and 30  $\mu\text{mol/l}$ , respectively (Fig. 6D). Taken together, these results point to the fact that tesaglitazar indeed activates both endogenous PPAR $\alpha$  and  $\gamma$  pathway in cultured MCs and PTCs.

**Effect of tesaglitazar on collagen production in cultured renal cells.** High-glucose treatment increased type I and type IV collagen mRNA expression in both MCs and PTCs, which was markedly blocked by tesaglitazar treatment (Fig. 7A, B, D, and E). Similarly, increased secreted total collagen induced by high-glucose treatment was almost completely abolished by tesaglitazar treatment in both cell types (Fig. 7C and F). Interestingly, tesaglitazar treatment also markedly inhibited the luciferase activity of the TGF $\beta$  responsive reporter 3TP-Luc by  $\sim 2.5$ -fold in

cultured MCs under normal glucose conditions (online appendix Fig. 2A). Similarly, tesaglitazar reduced basal levels of collagen biosynthesis under normal glucose treatment in both MCs and PTCs (online appendix Fig. 2B and C).

## DISCUSSION

The present studies demonstrated that a PPAR $\alpha/\gamma$  dual activator tesaglitazar markedly improved metabolic parameters, including hyperglycemia, hyperinsulinemia, and dyslipidemia, in *db/db* mice. Tesaglitazar treatment also decreased urinary albumin excretion and ameliorated glomerulosclerosis in these animals. The renoprotective effects of tesaglitazar were associated with suppression of the profibrotic molecule TGF $\beta$ 1 and reduction of collagen synthesis in the diabetic kidneys. We also provided evidence that in addition to metabolic improvement, tesaglitazar could directly activate endogenous renal PPAR $\alpha$  and  $\gamma$ , resulting in downregulation of collagen mRNA and



**FIG. 5.** Quantitative PCR analysis showing mRNA expression levels of  $\alpha$ 1 chain of type I collagen (A),  $\alpha$ 1 chain of type IV collagen (B), and TGF $\beta$ 1 (C) in renal cortical tissues. Animals were treated with tesaglitazar for 3 months, and mRNA expression was measured by real-time quantitative PCR. Data are shown as means  $\pm$  SE. Comparison was performed between the control group and the tesaglitazar treatment group. \* $P < 0.05$ , control vs. tesaglitazar.

protein expression in cultured mesangial cells and proximal tubule cells.

PPAR $\alpha$ , the first identified PPAR, has been implicated in the regulation of lipid and glucose metabolism through regulation of various proteins involved in the uptake, binding, and oxidation of fatty acids (25–27). In addition, increasing evidence suggests that PPAR $\alpha$  plays an important role in the pathogenesis of obesity and insulin resistance (28). Activation of PPAR $\alpha$  reduces weight gain in rodents (11,29,30) and improves glycemic control via increasing insulin sensitivity in type 2 diabetes (8). Collectively, studies over the past few years have suggested that PPAR $\alpha$  agonists may improve dyslipidemia, adiposity, and insulin sensitivity in type 2 diabetes.

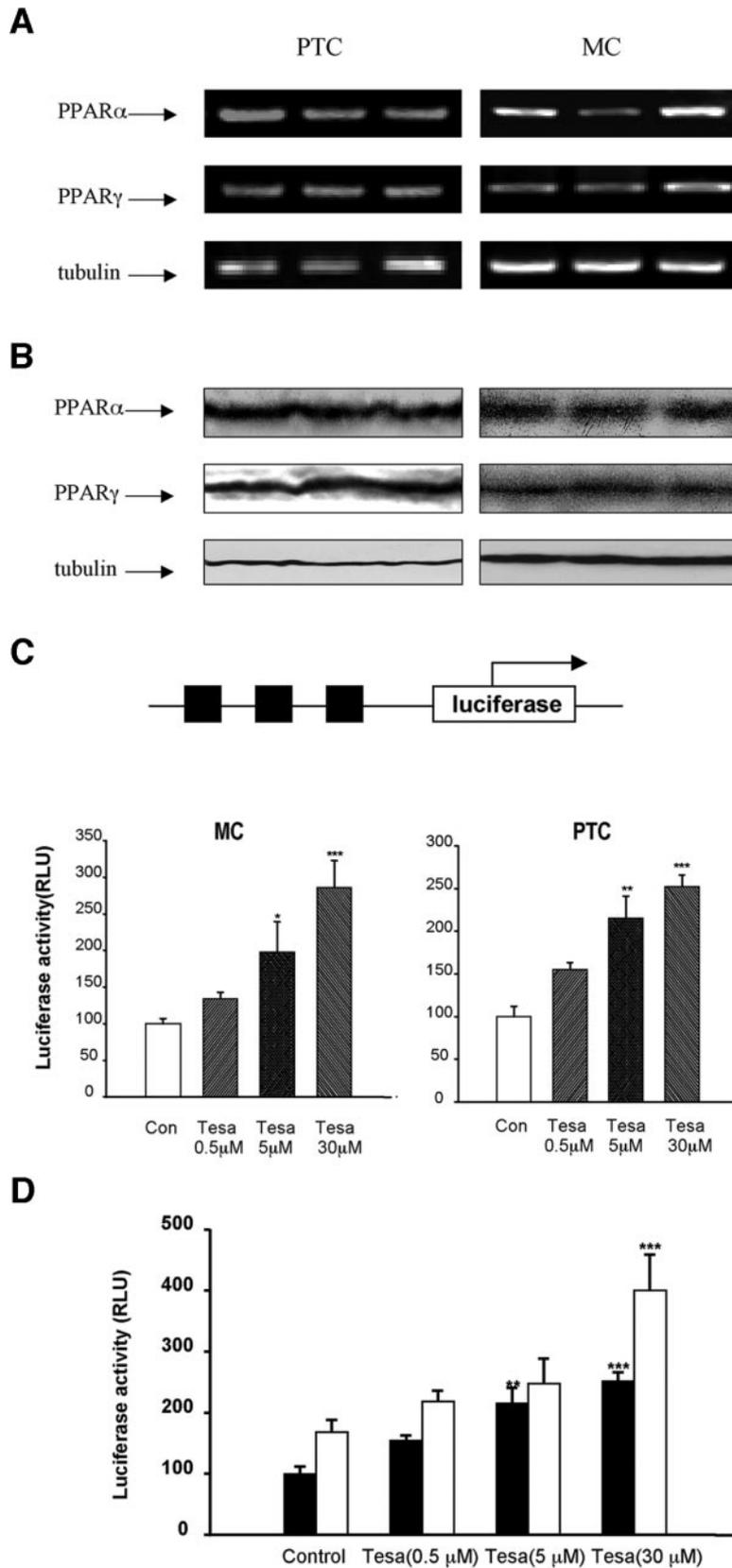
On the other hand, antidiabetes PPAR $\gamma$  agonists have been found to increase peripheral glucose utilization, reduce hepatic glucose output, and improve lipid metabolism (31). In addition, PPAR $\gamma$  activation leads to an antihypertensive effect via direct vascular and antiatherogenic effects through the modulation of macrophage biology (32–34). Nevertheless, experimental studies in rodents and clinical observations in type 2 diabetes all raise a serious concern regarding the obesity-induced side effect after thiazolidinedione (TZD) PPAR $\gamma$  agonist treatment (35). Based on the biological effects of PPAR $\alpha$  and  $\gamma$ , it is very likely that combined treatment of PPAR $\alpha$  and  $\gamma$  agonists may not only improve metabolic abnormalities but also alleviate undesirable effects, especially weight gain following TZD treatment in type 2 diabetic patients. An agent that simultaneously activates both PPAR isoforms therefore holds a great promise in acting as an antidiabetes medicine. In the present study, we utilized a new PPAR $\alpha$  and  $\gamma$  dual activator, tesaglitazar, and examined its effect on metabolic parameters in a murine model of type 2 diabetes, the *db/db* mice. Similar to previous reports on another PPAR $\alpha$ / $\gamma$  dual activator, muraglitazar (36), tesaglitazar treatment also markedly improved glycemic control and reduced A1C levels and HOMA-IR index in these overtly diabetic insulin-resistant mice. Tesaglitazar-treated animals also exhibited much better lipid profiles, including a decrease in triglyceride and an increase in HDL cholesterol. The antidiabetes and lipid-lowering effects of tesaglitazar were probably attributed to the activation of

both PPAR $\alpha$  and  $\gamma$ , since both isoforms have been shown to possess potential insulin-sensitizing and lipid-modulating activity.

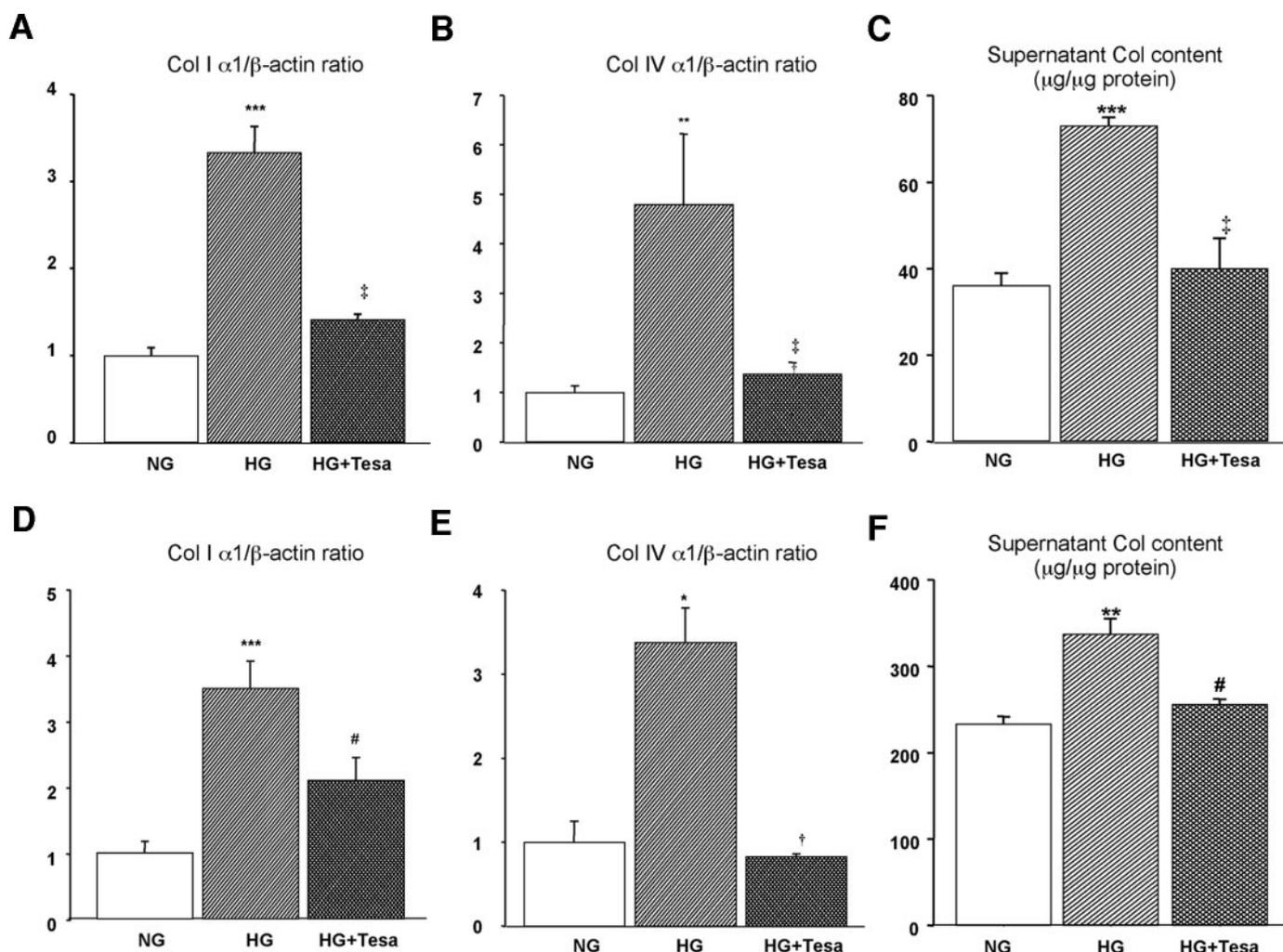
Importantly, strikingly different from TZD PPAR $\gamma$  agonists, tesaglitazar treatment did not increase body weight and affect food intake in *db/db* mice. These findings are in agreement with previous reports that KRP-297 and muraglitazar, two other PPAR $\alpha$ / $\gamma$  dual agonists, improve insulin resistance without weight gain (18,36). Although great concern has been recently raised about the undesirable cardiovascular side effect of muraglitazar (37), the present studies did not show any significant cardiotoxic and hemodynamic effects of tesaglitazar (data not shown).

Multiple mechanisms may be involved in the insulin-sensitizing effects of tesaglitazar. Pancreatic mass and islet size were significantly attenuated in tesaglitazar-treated *db/db* mice, accompanied by a marked decrease in serum insulin levels. The beneficial effect of tesaglitazar on insulin resistance may be through increasing the production of insulin-sensitizing adipokines. In support of this, in the present studies we found that adipose expression of adiponectin mRNA and plasma adiponectin levels were markedly increased following the tesaglitazar treatment. The increase of adiponectin expression may be attributed to the PPAR $\gamma$  activation, since a large body of evidence demonstrates that PPAR $\gamma$  agonists modulate the expression of many adipocytokines in adipose tissue, including upregulation of adiponectin transcription (38,39).

Both PPAR $\alpha$  and  $\gamma$  agonists have been shown to be potentially therapeutic agents in treating diabetic nephropathy (7–10). However, little is known about the efficacy of PPAR $\alpha$  and  $\gamma$  dual activators on renal complications in type 2 diabetes. In the present study, we investigated the effect of tesaglitazar on the progression of diabetic nephropathy in type 2 diabetic mice. Treatment of *db/db* mice with tesaglitazar dramatically reduced the urinary albumin excretion after 3 months. Although the present study did not attempt to compare the efficacy of tesaglitazar with the PPAR $\alpha$  and  $\gamma$  agonists alone, it seems that the albuminuria-attenuating effect of tesaglitazar was less effective than a PPAR $\alpha$  agonist, fenofibrate (8), and a TZD PPAR $\gamma$  agonist, troglitazone (data not shown). Consistent with a decrease in albuminuria, glomerulosclerosis and



**FIG. 6.** Expression of PPAR $\alpha$  and  $\gamma$  and effect of tesaglitazar on endogenous PPAR activity in cultured mesangial cells and proximal tubule cells. **A:** RT-PCR analysis showing PPAR $\alpha$  and  $\gamma$  mRNA were detected in three preparations of mouse MCs and PTCs. PCR products with expected sizes were indicated by arrows. **B:** Immunoblot assay demonstrating PPAR $\alpha$  and  $\gamma$  protein expression in three preparations of murine MCs and PTCs. The arrows indicated PPAR $\alpha$  and  $\gamma$  bands recognized by specific PPAR $\alpha$  and  $\gamma$  antibodies in three preparations of MCs and PTCs. Tubulin was utilized as internal loading control. **C:** Effect of tesaglitazar on PPRE-luciferase activity in cultured MCs and PTCs. **D:** Effect of tesaglitazar on PPRE-luciferase activity in PPAR $\alpha$  ( $^{-/-}$ ) MCs. In some wells, the PPAR $\alpha$  overexpression vector was cotransfected with PPRE-luciferase reporter. Cells were then treated with tesaglitazar at various concentrations of tesaglitazar. \* $P < 0.05$ , control vs. tesaglitazar. \*\* $P < 0.01$ , control vs. tesaglitazar. \*\*\* $P < 0.001$ , control vs. tesaglitazar. ■, PPRE; □, PPRE + PPAR $\alpha$ .



**FIG. 7.** Effect of tesaglitazar on mRNA expression of  $\alpha 1$  chain of type I collagen or type IV collagen (A, B, D, and E) and total collagen protein secretion (C and F) in cultured MCs (A–C) and PTCs (D–F). Cells were cultured under a high-glucose medium (30 mmol/l of D-glucose) with or without tesaglitazar at a concentration of 30  $\mu\text{mol/l}$ . Total collagen secreted in the supernatant was measured by a collagen assay kit. Supernatant collagen content was corrected by protein content of cells. **A:** Real-time PCR analysis showing high-glucose-induced  $\alpha 1$  chain of type I collagen mRNA expression was blocked by tesaglitazar treatment in MCs. **B:** Quantitative PCR analysis demonstrating high-glucose-stimulated  $\alpha 1$  chain of type IV collagen mRNA expression was abolished by tesaglitazar in MCs. **C:** Total collagen amount in culture supernatant. Note that tesaglitazar treatment significantly inhibited high-glucose-induced collagen protein production in MCs. **D:** Quantitative analysis of  $\alpha 1$  chain of type I collagen mRNA levels in PTCs. Incubation of PTCs with tesaglitazar significantly blocked high-glucose-induced  $\alpha 1$  chain of collagen I expression. **E:** Induction of type IV collagen  $\alpha 1$  chain mRNA expression was almost completely abolished by tesaglitazar treatment in PTCs. **F:** Enhanced production of total collagen production by high glucose was inhibited by tesaglitazar treatment in PTCs. Data are shown as means  $\pm$  SE. HG, high glucose (30 mmol/l); NG, normal glucose (5 mmol/l). \* $P < 0.05$ , NG vs. HG; \*\* $P < 0.01$ , NG vs. HG; \*\*\* $P < 0.001$ , NG vs. HG; # $P < 0.05$ , HG vs. tesaglitazar; † $P < 0.01$ , HG vs. tesaglitazar; ‡ $P < 0.001$ , HG vs. tesaglitazar.

type IV collagen deposition were also markedly improved after tesaglitazar treatment. Furthermore, tesaglitazar therapy was associated with the decreased gene expression of TGF $\beta$ 1 and extracellular matrix proteins, including type I and type IV collagen in the renal cortex, all of which have been considered as important contributors to renal disease progression in diabetic nephropathy.

The above-mentioned tesaglitazar-induced improvement in renal function is further supported by in vitro experiments that demonstrated the direct inhibitory effects of tesaglitazar on the synthesis of collagen MCs and PTCs cultured under both normal and high-glucose conditions. The PPRE reporter assays clearly showed that tesaglitazar activated endogenous PPAR $\alpha$  and  $\gamma$  transcriptional activity in these cells. While PPAR $\alpha$  versus PPAR $\gamma$  effects could not be clearly differentiated, our in vitro findings suggest that tesaglitazar may contribute to the observed effects in *db/db* mice via both PPAR $\alpha$  and  $\gamma$  activation.

Although tesaglitazar appears to be a very promising therapeutic agent in treating diabetic nephropathy in type 2 diabetes, its treatment may also result in some unexpected side effects. For example, muraglitazar-treated patients exhibited increased cardiovascular risk (37), and tesaglitazar has been recently reported to increase serum creatinine levels in insulin-resistant patients (14). Several mechanisms by which tesaglitazar raises serum creatinine levels have been proposed including the following: 1) PPAR $\alpha$  activation mediates skeletal myopathy such as acute rhabdomyolysis (40), 2) direct vascular action of PPAR $\gamma$  might decrease the glomerular perfusion and ultimately elevate creatinine levels through hemodynamic mechanism (41), and 3) PPAR $\gamma$  may have a direct effect on efferent arteriolar tone via downregulation of the AT1 receptor, as described previously (42). Although in the present studies there was no statistical difference between the two groups, we indeed observed slightly higher serum

creatinine levels in tesaglitazar-treated *db/db* mice. However, it seems unlikely that skeletal myopathy played a role, since both BUN and creatinine were found to be slightly elevated. Moreover, systemic hemodynamic effect does not appear to be involved, since no systemic hypotensive effect was observed following tesaglitazar treatment in *db/db* mice (data not shown). However, increased serum Na<sup>+</sup> and Cl<sup>-</sup> levels found in the present studies imply that tesaglitazar may cause intravascular volume depletion, thereby contributing to elevated BUN and Scr. To date, although it remains unclear whether underlying mechanism(s) contribute to these changes, these important clinically relevant issues warrant further investigation.

In conclusion, the present studies show that the dual PPAR $\alpha/\gamma$  agonist tesaglitazar improved metabolic abnormalities including hyperglycemia, hyperinsulinemia, and dyslipidemia without weight gain in type 2 diabetic *db/db* mice. More importantly, we have shown, for the first time, that tesaglitazar treatment decreased urinary albumin excretion and ameliorated glomerulosclerosis through suppression of profibrotic molecules, including TGF $\beta$ 1 and collagen synthesis in the diabetic kidney. In addition, we provided in vitro evidence that tesaglitazar could directly affect intrinsic renal cells to downregulate collagen synthesis. These findings suggest that agonism of PPAR $\alpha$  and  $\gamma$  may represent a potential therapeutic approach in the treatment of type 2 diabetes and diabetic nephropathy. However, the benefit-risk profile for the PPAR $\alpha/\gamma$  dual agonists needs to be carefully demonstrated.

#### ACKNOWLEDGMENTS

These studies were supported by DK065074-03 and DK38226 (to Y.G.) and P01-DK 38226 (to M.D.B.), funding from the China National Natural Science Foundation (30271521 and 30530340), and the National Basic Research Program of China (2006CB503900) (to Y.G.).

We thank Drs. Kumar Sharma and Steve Dunn and the Animal Models of Diabetic Complications Consortium U01 for help in measuring serum creatinine.

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