Original Article

Accelerated Diabetic Nephropathy in Mice Lacking the Peroxisome Proliferator–Activated Receptor α

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Peroxisome proliferator–activated receptor (PPAR)α, a member of the ligand-activated nuclear receptor superfamily, plays an important role in lipid metabolism and glucose homeostasis and is highly expressed in the kidney. The present studies were aimed at determining the role of PPARα in the pathogenesis of diabetic nephropathy using PPARα-knockout mice and cultured murine mesangial cells. Diabetes was induced using a low-dose streptozotocin protocol in 8-week-old male 129 SvJ PPARα-knockout and wild-type mice. Diabetic PPARα-knockout and wild-type mice developed elevated fasting blood glucose ($P < 0.001$) and HbA$_{1c}$ levels ($P < 0.001$). Renal functional and histopathological changes in diabetic and nondiabetic PPARα-knockout and wild-type mice were evaluated after 16 weeks of hyperglycemia. PPARα immunostaining of the cortical tubules of diabetic wild-type mice was elevated by hyperglycemia. In diabetic PPARα-knockout mice, renal disease with accompanying albuminuria, glomerular sclerosis, and mesangial area expansion was more severe than in diabetic wild-type mice ($P < 0.05$) and was accompanied by increased levels of serum free fatty acids and triglycerides ($P < 0.01$). Furthermore, they exhibited increased renal immunostaining for type IV collagen and osteopontin, which was associated with increased macrophage infiltration and glomerular apoptosis. There were no significant differences in these indexes of renal disease between nondiabetic PPARα-knockout and wild-type mice and diabetic PPARα wild-type mice. In vitro studies demonstrated that high glucose levels markedly increased the expression of type IV collagen, transforming growth factor-β1, and the number of leukocytes adherent to cultured mesangial cells. Adherence of leukocytes was inhibited by the PPARα agonist fenofibrate. Taken together, PPARα deficiency appears to aggravate the severity of diabetic nephropathy through an increase in extracellular matrix formation, inflammation, and circulating free fatty acid and triglyceride concentrations. PPARα agonists may serve as useful therapeutic agents for type 1 diabetic nephropathy. *Diabetes* 55:885–893, 2006

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Peroxisome proliferator–activated receptor (PPAR)α is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors and plays an important role in lipid metabolism (1). The role of PPARα expression in the kidney remains poorly characterized. PPARα expression predominates in the proximal tubule and the thick limb, which could contribute to dietary lipid–induced gene expression of peroxisomal and mitochondrial β-oxidation proteins in these segments (2–4). Interestingly, the PPARα agonist clofibrate reduces blood pressure in Dahl salt-sensitive rats (5,6). PPARα has also been shown to play an important role in sustaining the balance between energy production and utilization in the kidney by regulating the expression of genes involved in fatty acid β-oxidation (7). This effect may explain the observation that PPARα ligands protect the kidney from ischemia/reperfusion injury in rats. Mice lacking the PPARα gene exhibit more severe cortical necrosis and worse kidney function than wild-type control mice (7).

PPARα is relatively abundant in tissues with a high oxidative capacity, such as the liver, kidney, and heart. Recent reports demonstrate that altered fatty acid metabolism is associated with cardiomyopathy and nephropathy (8,9). Accumulation of excess lipids in nonadipose tissues leads to cell dysfunction or cell death (8,9). This phenomenon, known as lipotoxicity, may play an important role in the pathogenesis of diabetes and heart failure in humans (8). Several mechanisms have been suggested to underlie lipotoxicity, including a direct toxic effect of fatty acids or products of their metabolism, increased production of reactive oxygen species, ATP deficiency, and fatty acid–induced apoptosis (8,9).

The generation of PPARα-knockout mice has helped to confirm several of the proposed actions of this nuclear receptor and has identified previously unknown actions. These mice are viable, fertile, and exhibit no gross phenotypic defects (10); however, they exhibit profound metabolic abnormalities of the liver and heart. These mice fail to exhibit peroxisome proliferation or activation of fatty acid oxidation target genes when exposed to PPARα agonists (11). They accumulate increased hepatic triglycerides in response to feeding and during fasting (11). PPARα-knockout mice develop severe hypoglycemia when fasted (12) because of impaired fatty acid oxidation and increased reliance on glucose as an energy source. In

this study, we used PPARα-knockout mice to investigate the role of PPARα in diabetic nephropathy associated with type 1 diabetes.

RESEARCH DESIGN AND METHODS

Male PPARα-knockout and wild-type background 129SvJ mice were used for all studies. Mice were housed in a sterile environment with 12:12-h light-dark cycles and had free access to food and water. To measure 24-h albumin excretion and creatinine clearance, mice were placed in individual mouse metabolic cages (Nalgene, Rochester, NY) with access to water and food for 24 h. Eight-week-old homozygous PPARα-knockout mice ($n = 12$) and 129SvJ wild-type mice ($n = 12$) were treated with or without streptozotocin by intraperitoneal injection ($50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 5 days). After 2 weeks, fasting blood glucose was monitored using Accu-Chek meter (Roche Diagnostics, St. Louis, MO) and hyperglycemia confirmed. Mouse body weight was measured weekly. Blood glucose was measured every 2 weeks, and HbA$_{1c}$ (A1C) and 24-h urinary albumin and creatinine were measured every 4 weeks. Systolic blood pressure was assessed by noninvasive tail cuff system in conscious mice at the end of the study (IITC Life Science, Woodland Hills, CA). Animals were habituated to the tail cuff device before measuring the blood pressure for 5 days over 15 min to reduce variability of the measurements.

After 16 weeks, animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (55 mg/kg body wt; Nembutal; Boehringer Ingelheim, Artanrmon, NSW, Australia). The kidneys were rapidly dissected and stored in buffered formalin (10%) for subsequent immunohistochemical analyses. Blood was collected from the left ventricle and centrifuged, and plasma was stored at −70°C for subsequent analyses. A1C was determined on red cell lysates by high-pressure liquid chromatography (Bio-Rad, Richmond, CA). Total cholesterol and triglyceride concentrations were measured by autoanalyzer (Hitachi 917; Tokyo, Japan). The free fatty acid levels were measured with the JCA-BM1250 automatic analyzer (JEOL, Tokyo, Japan).

Assessment of renal function. At week 16, the animals were housed in metabolic cages for 24 h to collect urine for subsequent measurements of albumin concentration by an immunoassay (Bayer, Elkhart, IN). Plasma creatinine and urea concentrations were measured using autoanalyzer (Beckman Instruments, Fullerton, CA). Serum triglyceride and cholesterol levels were measured using commercial kits (nos. 33740A and 33710B and 402-20, respectively) purchased from Sigma-Aldrich.

Light microscopic study. Kidney samples were collected following systemic perfusion with PBS and then fixed in 4% paraformaldehyde. Fixed tissues were embedded in paraffin. Histology was assessed following hematoxylin, eosin, and periodic acid Schiff staining. To examine the effect of PPARα deletion on glomerular area and mesangial matrix area, glomerular analysis was performed on periodic acid Schiff–stained kidney sections. Fifteen consecutive glomerular cross sections were photographed by an examiner, blinded to the source of the tissue, using a digital camera (Olympus DP11; Olympus America, Melville, NY). Mesangial matrix area and glomerular tuft area were quantified for each glomerular cross section as previously reported (13). More than 30 glomeruli that were cut through the vascular pole were counted per kidney, and the average was used for analysis.

Immunohistochemistry for PPARα, osteopontin, F4/80, transforming growth factor-β1, type IV collagen, and caspase-3. We performed immunohistochemistry for PPARα, type IV collagen, transforming growth factor (TGF)-β1, osteopontin, F4/80, and caspase-3. Briefly, small blocks of kidney were immediately fixed in 10% buffered formalin for 24 h before embedded in paraffin. Five micrometer–thick sections were deparaffinized, washed with PBS, and incubated with 1.5% H$_2$O$_2$ in methanol to block endogenous peroxidase activity. Nonspecific binding was blocked with 10% normal goat serum in PBS. Sections were incubated overnight with the anti-PPARα (1:1,500 in blocking solution), anti-osteopontin (1:1,500), anti–type IV collagen (1:150; Biodesign International, Saco, ME), anti–TGF-β1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-F4/80 (1:100; Serotek, Oxford, U.K.), and anti–caspase-3 (1:50; SantaCruz Biotechnology) in a humidified chamber at 4°C. For immunohistochemistry, tissue sections were treated with an antigen unmasking solution consisting of 10 mmol/l Na citrate, pH 6.0, and 0.05% Tween 20. Antibodies were localized with the ABC technique (Vector Labs, Buringame, CA) and 3,3-diamninobenzidine substrate solution with nickel chloride enhancement. Sections were then dehydrated in ethanol, cleared in xylene, and mounted without counterstaining.

All of these sections were examined in a masked manner using light microscopy (Olympus BX-50; Olympus Optical, Tokyo, Japan). For the quantification of proportional area of staining, ~20 views (×400 magnification) were randomly located in the renal cortex and corticomedullary junction of each slide (Scion Image Beta 4.0.2; Scion, Frederick, MD).

Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling assay. Detection of apoptotic cells in formalin-fixed, paraffin-embedded tissue was performed by in situ terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL), using a commercially available kit (Apoptag Plus; Intergen Company, New York, NY). Sections were deparaffinized, digested with proteinase K (20 μg/ml), and incubated with hydrogen peroxyde to block endogenous peroxidase activity. After washing, slides were incubated with the TdT enzyme and then with anti–digoxigenin-peroxidase and developed using a substrate containing diaminobenzidine. Negative controls included omission of TdT. Positive apoptotic cells exhibited an intense brown nuclear colorimetric reaction product. The number of cells positive for TUNEL reaction was determined in the whole glomeruli biopsy under ×400 magnification.

Cell culture. Mouse mesangial cells were obtained from primary cell culture of 129SvJ mice using sieving methods. Mouse mesangial cells cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5 mmol/l D-glucose and 10% FBS with penicillin 100 units/ml and streptomycin 100 μg/ml at 37°C in atmosphere containing 5% CO$_2$ with 95% humidity. Experiments were carried out in DMEM containing 0.2% FBS using cells between the third and sixth passages. At these passages mesangial cells showed normal morphology and function. Cell viability was measured with tryptan blue exclusion and lactate dehydrogenase assay (Sigma Chemicals, St. Louis, MO). These results showed no loss of viability after 4 days of incubation and various concentrations of medium used (data not shown).

Effects of fenofibrate on TGF-β1 and type IV collagen expression and effect of osteopontin on type IV collagen expression in cultured mouse mesangial cells. To examine the effects of fenofibrate on TGF-β1 and type IV collagen expression in 30 mmol/l high-glucose media, mesangial cells (2×10^4) were seeded in each well of 24-well plates. After washing cells three times with 1× PBS, normal (5 mmol/l) or high-glucose (30 mmol/l) medium containing 0.1% FBS was added to the cells in the presence of or absence of fenofibrate (10 μmol). Total TGF-β1 and type IV collagen were measured in cell culture supernatants from mesangial cells with specific enzyme-linked immunosorbent assay (ELISA) kits (Predicta TGF-β1 Kit and Genzyme; Biodesign International, Saco, MA) according to the manufactures' instructions. The absorbance was measured at 450 nm in an ELISA reader (14).

Leukocyte adhesion assay. To examine the effect of high glucose on leukocyte adhesion on mouse mesangial cells, mesangial cells were cultured for 24 h with normal (5 mmol/l) or high glucose (30 mmol/l). For adhesion assay, we also used fenofibrate (10 μmol) or anti-osteopontin antibody on normal- or high-glucose–induced leukocyte adhesion to mesangial cells. Leukocyte adhesion was assayed by modified methods from another study (15). Briefly, confluent mesangial cell monolayers (1×10^5) on 12-mm coverslips were coincubated for 30 min at room temperature under static condition in six-well cell culture plates with human leukocyte (1×10^5) in 1 ml of DMEM plus 1% FCS. In this assay, we used human leukocytes obtained from healthy male volunteers. To remove unattached leukocytes, each coverslip was washed by immersion three times in DMEM plus 1% FCS, then once in PBS, and fixed in 2% paraformaldehyde at 4°C. After staining with hematoxylin, the adherent leukocytes were counted in 20 selected at random high power field (×100) on each of the coverslips and the results expressed as the mean number of leukocytes for each field.

Statistical analysis. Data are expressed as means ± SD. Differences between the groups were examined for statistical significance using ANOVA with Bonferroni correction (SPSS 11.5; SPSS, Chicago, IL). A P value of <0.05 was considered a statistically significant difference. In mesangial cell culture studies, the average of the six experimental measurements was used for statistical analysis.

RESULTS

Body weight and levels of glucose and A1C. The body weight of diabetic PPARα-knockout mice was less than that of nondiabetic wild-type mice throughout the entire period of experimentation ($P < 0.05$; Table 1). Although diabetic wild-type mice had slightly reduced body weight compared with nondiabetic wild-type mice, the difference was not significant. Diabetic PPARα-knockout and wild-type mice exhibited dramatically increased blood glucose and A1C levels throughout the entire period of experimentation ($P < 0.001$) compared with those of nondiabetic PPARα-knockout and wild-type mice. However, there were no significant differences in blood glucose and A1C between diabetic PPARα-knockout and diabetic wild-type mice.

TABLE 1
Metabolic and physiologic parameters

Parameters	Wild-type control	Wild-type diabetic	Knockout control	Knockout diabetic
Body weight (g)	29.2 ± 0.8	27.4 ± 1.6	27.5 ± 1.0	25.8 ± 2.3*
Hematocrit (%)	42.4 ± 4.0	43.8 ± 1.7	44.8 ± 1.7	44.5 ± 3.7
Serum glucose (mmol/l)	5.2 ± 0.3	21.5 ± 2.9†	5.4 ± 0.9	19.5 ± 2.3†
A1C (%)	2.9 ± 0.1	5.5 ± 0.4‡	2.9 ± 0.1	5.4 ± 0.5‡
Mean systolic blood pressure (mmHg)	98 ± 10	99 ± 7	101 ± 8	99 ± 10
Plasma cholesterol (mmol/l)	2.31 ± 0.38	2.92 ± 0.54	2.50 ± 0.49	3.23 ± 0.31
Plasma triglycerides (mmol/l)	1.00 ± 0.29	1.18 ± 0.27	1.15 ± 0.15	1.77 ± 0.28*
Free fatty acids (mmol/l)	0.74 ± 0.05	0.80 ± 0.17	0.92 ± 0.11	1.77 ± 0.23*

Data are means ± SD. *$P < 0.05$, †$P < 0.001$, ‡$P < 0.01$ compared with other groups.

Blood chemistry and serum lipid levels. Na^+, K^+, Cl^-, urea nitrogen, and creatinine concentration in blood and hematocrit did not differ significantly between experimental groups (Table 1). In contrast, serum free fatty acid and triglyceride levels were significantly increased in diabetic PPARα-knockout mice compared with those of other groups ($P < 0.05$; Table 1). There were no differences in total cholesterol concentrations between groups.

Kidney weight, albuminuria, creatinine, and systolic blood pressure. As expected, diabetic mice exhibited increased 24-h urine volumes and kidney weights compared with those of nondiabetic PPARα-knockout and wild-type mice ($P < 0.05$; Table 2). Interestingly, the kidney weight of diabetic PPARα-knockout mice was heavier than that of diabetic wild mice ($P < 0.05$; Table 2). Diabetic PPARα-knockout mice also exhibited a persistent increase in urine albumin excretion, whereas albuminuria in diabetic wild-type mice, although initially elevated, declined toward baseline after 3–4 months of hyperglycemia ($P < 0.01$; Fig. 1). No significant differences in systolic blood pressure (Table 1) were observed among the groups.

Expression of PPARα and renal histological examination. Immunohistochemistry revealed significantly increased PPARα expression in diabetic wild-type kidneys compared with that in nondiabetic wild-type kidneys. As expected, PPARα was not detected in kidneys of diabetic or nondiabetic PPARα-knockout mice (Fig. 2C and D). The 2.9-fold increase in renal PPARα immunostaining in the wild-type diabetic kidney compared with the nondiabetic kidney occurred primarily in the proximal tubules and thick ascending tubules (Fig. 2E). Glomerular injury in diabetic PPARα-knockout mice was characterized by mesangial extracellular matrix expansion and glomerulosclerosis (Fig. 3A–D). There was no significant difference in glomerular area between diabetic PPARα-knockout and wild-type mice. Glomerular fractional mesangial areas were increased in diabetic PPARα-knockout mice compared with diabetic wild-type mice and nondiabetic controls ($P < 0.01$, Fig. 3E).

Expression of type IV collagen, TGF-β, osteopontin, and macrophage infiltration. Diabetes was associated with an increase in type IV collagen protein expression in the glomeruli (Fig. 4A–D). The increase in type IV collagen in the glomeruli was more prominent in diabetic PPARα-knockout mice than in diabetic wild-type mice ($P < 0.01$, Fig. 4E). In the kidneys of nondiabetic PPARα-knockout and wild-type mice and diabetic wild-type mice, positive staining for TGF-β was sparse and localized to the glomeruli (Fig. 4F–I). In contrast, a prominent increase in TGF-β expression was detected in diabetic PPARα-knockout kidneys (Fig. 4J).

In the kidneys of nondiabetic PPARα-knockout and wild-type mice, sparsely positive osteopontin was observed in the interstitium and glomeruli (Fig. 4K–N). In the kidneys of diabetic PPARα-knockout mice, osteopontin expression was significantly increased in the glomeruli compared with that of nondiabetic PPARα-knockout and wild-type mice and diabetic wild-type mice ($P < 0.05$; Fig. 4O). Only modest macrophage infiltration, as assessed by F4/80-positive staining, was observed in the glomeruli of nondiabetic and diabetic wild-type mice (Fig. 4P–S). In contrast, F4/80 immunostaining was markedly increased in the glomeruli of PPARα-knockout mice. F4/80 immunostaining was strongly increased within the glomeruli of diabetic PPARα-knockout mice compared with that of nondiabetic PPARα-knockout mice (Fig. 4T).

Expression of TUNEL and caspase 3. In the kidneys of nondiabetic PPARα-knockout and wild-type mice as well as in diabetic wild-type mice, a few TUNEL-positive cells were observed within the glomeruli. In the kidneys of diabetic PPARα-knockout mice, diabetes was associated with a markedly increased number of glomerular TUNEL-positive cells ($P < 0.001$; Fig. 5). Consistent with TUNEL staining reflecting increased apoptosis, caspase 3 immunostaining was also markedly increased in glomerular

TABLE 2
Renal functional, structural parameters

Parameters	Wild-type control	Wild-type diabetic	Knockout control	Knockout diabetic
Plasma creatinine (μ mol/l)	60.0 ± 8.8	61.8 ± 8.8	57.4 ± 15.8	51.3 ± 15.0
Plasma urea (mmol/l)	10.1 ± 0.8	10.4 ± 0.5	10.7 ± 0.9	11.4 ± 1.0
Urine volume (ml)	0.36 ± 0.28	1.81 ± 1.37*	0.20 ± 0.13	2.32 ± 0.97*
Kidney/body wt (%)	0.72 ± 0.03	0.87 ± 0.14†	0.67 ± 0.03	1.07 ± 0.08*
Glomerular size ($\times 10^3$ μm^2)	3.4 ± 0.4	5.8 ± 1.5†	3.3 ± 1.0	5.7 ± 1.3†

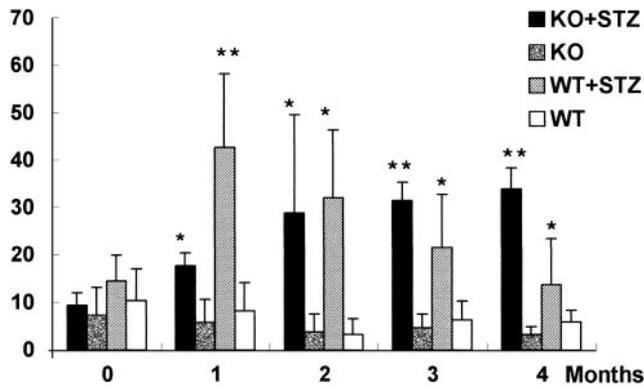Data are means ± SD. *$P < 0.01$, †$P < 0.05$ compared with other groups.

FIG. 1. Twenty-four–hour urinary albumin concentration in diabetic and nondiabetic PPARα wild-type (WT) and knockout (KO) mice. *$P <$ 0.01, **$P <$ 0.001 vs. nondiabetic PPARα wild-type and knockout mice. STZ, streptozotocin.
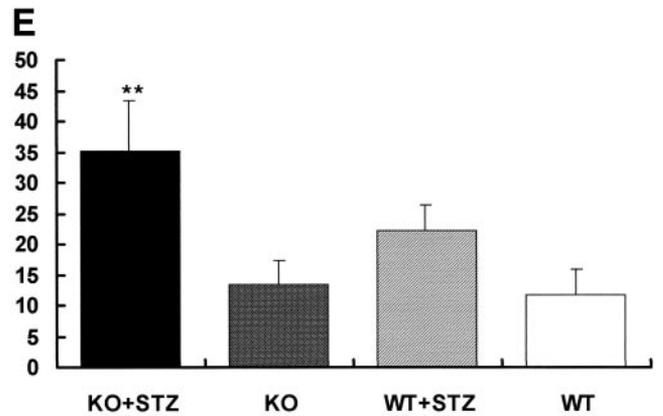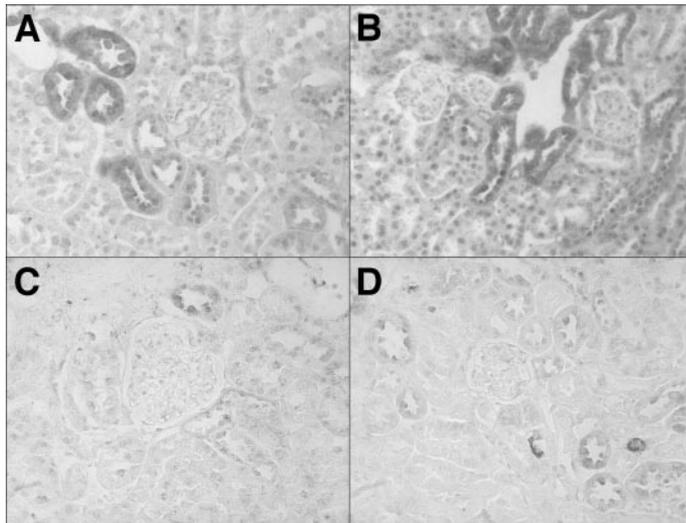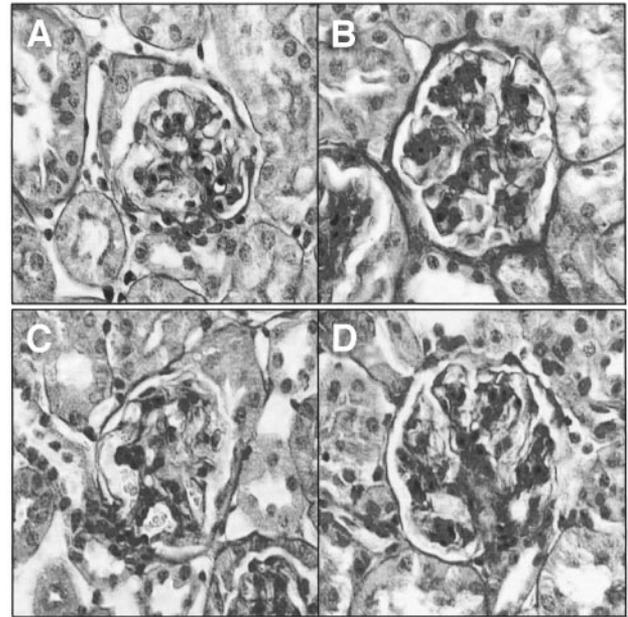

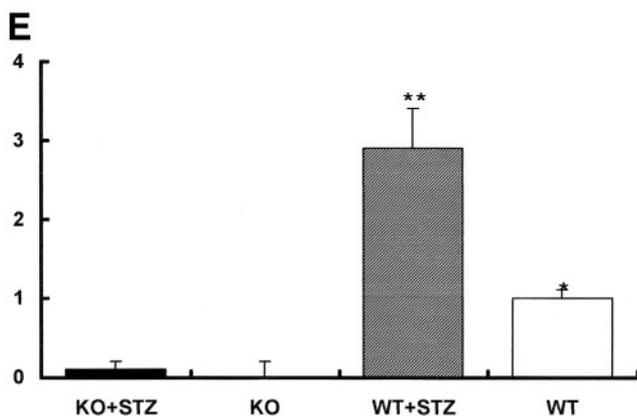FIG. 2. Immunohistochemical expression of PPARα protein in PPARα wild-type (WT) and knockout (KO) kidney cortical tubules. Elevated PPARα protein expression in diabetic PPARα wild-type mice. *A*: Representative immunohistochemical staining for PPARα in nondiabetic wild-type mice. *B*: Abundant PPARα protein localized to the nucleus and cytoplasm of the cortical tubules in diabetic PPARα wild-type mice. *C*: Sparsely staining for PPARα in nondiabetic PPARα knockout mice. *D*: Sparse staining for PPARα in diabetic PPARα knockout mice. Quantitative assessment of PPARα (*E*) immunoreactivity in diabetic and nondiabetic PPARα wild-type and knockout kidney cortical tubules. Statistical significance was calculated using Mann-Whitney test. *$P <$ 0.01, **$P <$ 0.001 vs. diabetic and nondiabetic knockout mice. STZ, streptozotocin.

FIG. 3. Renal histopathology in normal and diabetic PPARα wild-type (WT) and knockout (KO) mice. A representative photomicrograph of mesangial matrix accumulation in periodic acid Schiff–stained nondiabetic (*A* and *C*) and diabetic PPARα wild-type and knockout (*B* and *D*) kidneys, respectively. *E*: Quantitative assessment (%) of mesangial matrix fraction in diabetic and nondiabetic PPARα wild-type and knockout kidneys. Statistical significance was calculated using Mann-Whitney test. **$P <$ 0.01 vs. diabetic PPARα wild-type mice and nondiabetic PPARα wild-type and knockout mice. STZ, streptozotocin.

areas of diabetic PPARα knockout kidneys compared with those of other groups ($P <$ 0.001; Fig. 6).

**Effect of PPARα agonist on glucose-induced type IV collagen and TGF-β1 production in cultured mouse mesangial cells and leukocyte adhesion to mesangial cells.** To determine whether PPARα activation directly modulates glomerular extracellular matrix production and inflammatory cell adhesion to mesangial cells, mesangial cells from a 129SvJ mouse were cultured in normal- (5 mmol/l) or high-glucose (30 mmol/l) media in the presence or absence of the PPARα agonist fenofibrate. Treatment with high glucose concentrations markedly increased type IV collagen and TGF-β1 in these cells as determined by ELISA, while treatment with fenofibrate (10 μmol/l) significantly suppressed the production of type IV collagen and TGF-β1 (52.2 ± 8.8 vs. 39.8 ± 9.2 ng/ml, $P <$ 0.01, Fig. 7*A*; 3.14 ± 0.19 vs. 2.65 ± 0.17 pg/10^4 cells, $P <$ 0.05, Fig. 7*B*, respectively). In addition, the high-glucose treatment also increased the number of leukocytes adherent to mesangial
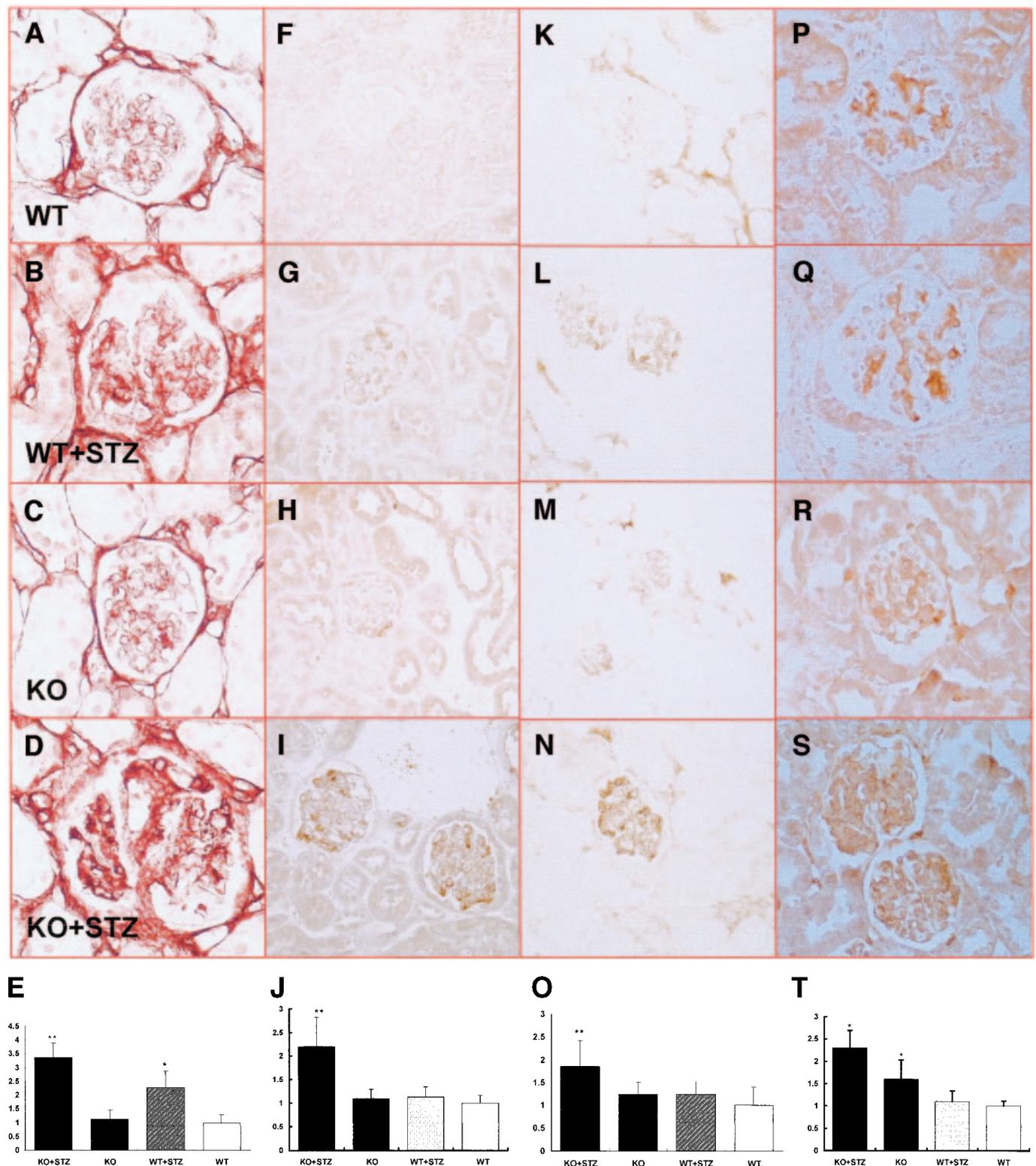
FIG. 4. Immunohistochemical staining for type IV collagen, TGF-β, osteopontin, and F4/80 in kidney cortical glomeruli. Representative immunostains for type IV collagen (*A–D*), TGF-β (*F–I*), osteopontin (*K–N*), and F4/80 (*P–S*) in diabetic and nondiabetic PPARα wild-type and knockout kidneys. Quantitative assessment of type IV collagen (*E*), TGF-β (*J*), osteopontin (*O*), and F4/80 (*T*) immunoreactivity in diabetic and nondiabetic PPARα wild-type and knockout kidneys. Statistical significance was calculated using Mann-Whitney test. *$P < 0.05$, **$P < 0.01$ compared with other groups. STZ, streptozotocin.

cells (42 ± 11 vs. 119 ± 15 cells per high-power field, $P < 0.05$). When mesangial cells cultured in high-glucose media were pretreated with fenofibrate (10 μmol/l) or an anti-osteopontin antibody, high-glucose–induced leukocyte ad-

hesion to mesangial cells was inhibited (63 ± 11 and 68 ± 15 cells per high-power field, respectively, $P < 0.05$, Fig. 8). In contrast, when mesangial cells were pretreated with fenofibrate and cultured in normal glucose, leukocyte
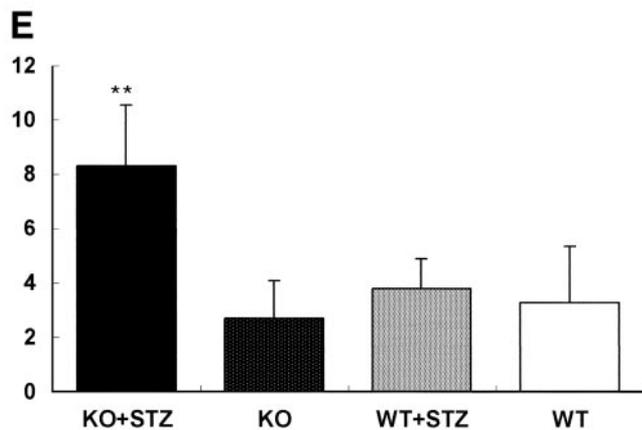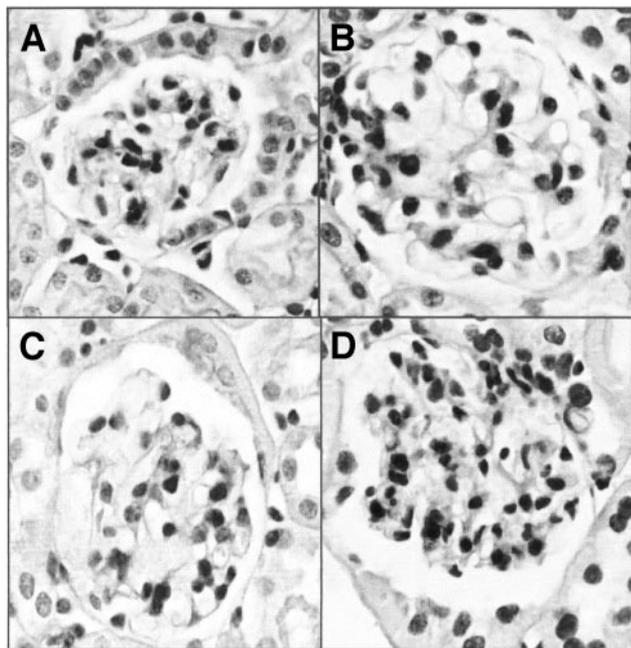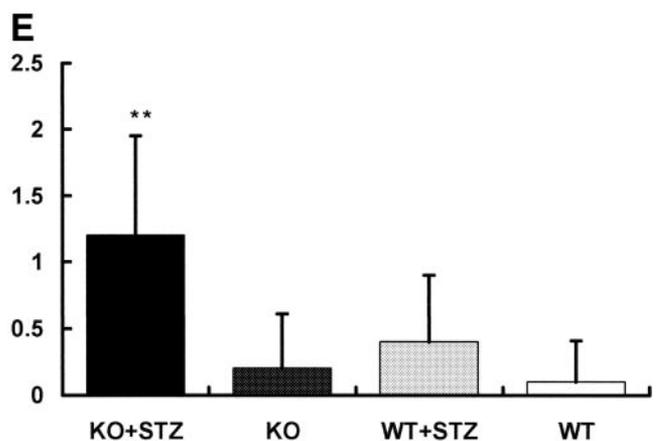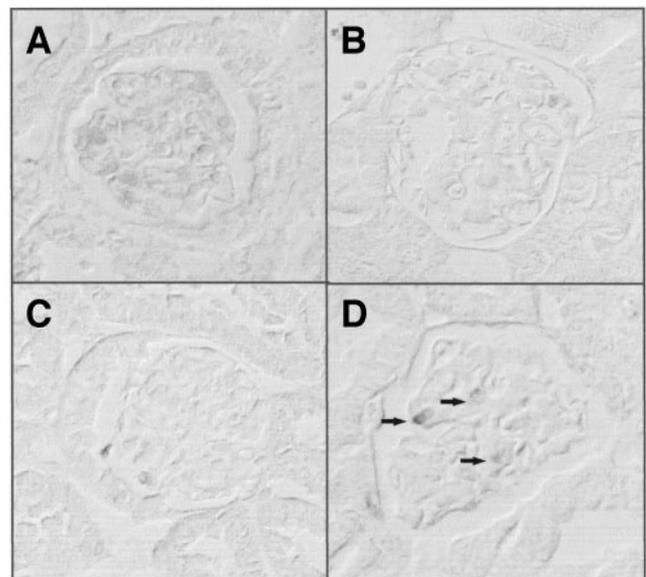
FIG. 5. TUNEL staining in mesanigal areas in nondiabetic (*A* and *C*) and diabetic (*B* and *D*) PPARα wild-type (WT) and knockout (KO) cortical kidneys. Increased number of TUNEL-positive cells were seen in diabetic PPARα knockout mice (*D*). *E*: Number of TUNEL-positive cells per glomerulus in diabetic and nondiabetic PPARα wild-type and knockout kidneys. ** $P < 0.01$ vs. diabetic PPARα wild-type and nondiabetic PPARα wild-type and knockout mice. STZ, streptozotocin.

FIG. 6. Immunohistochemical staining for caspase-3 in cortical glomeruli. Immunoreactivity for caspase-3 and representative pictures (*A–D*) expression in nondiabetic (*A* and *C*) and diabetic (*B* and *D*) PPARα wild-type (WT) and knockout (KO) cortical kidneys. Increased caspase-3 expression was seen in diabetic PPARα knockout mice. *E*: Number of caspase-3–positive cells per glomerulus in diabetic and nondiabetic PPARα kidneys. Statistical significance was calculated using Mann-Whitney test. ** $P < 0.001$ vs. diabetic PPARα wild-type mice and nondiabetic PPARα wild-type and knockout mice. STZ, streptozotocin.

adhesion to mesangial cells tended to decrease (34 ± 7 cells per high-power field), but the difference was not statistically significant.
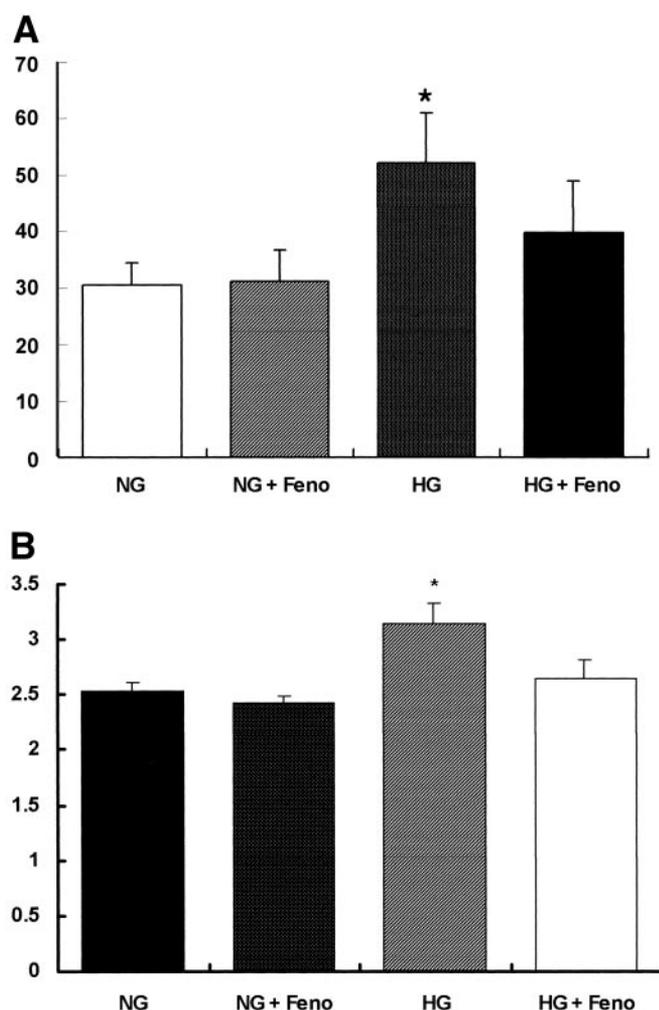
DISCUSSION

We evaluated the role of PPARα activation in the development of diabetic nephropathy. PPARα immunostaining in the kidney was increased in diabetic wild-type mice compared with control mice. Genetic disruption of PPARα expression was associated with abnormal lipid metabolism, including increased circulating free fatty acid and triglyceride concentrations and exacerbation of diabetic nephropathy. More severe structural changes (glomerulosclerosis and mesangial area expansion) as well as functional changes (albuminuria) were noted in diabetic PPARα-knockout mice and were associated with an increase in profibrotic, proinflammatory, and proapoptotic pathways implicated in renal extracellular matrix accumu-
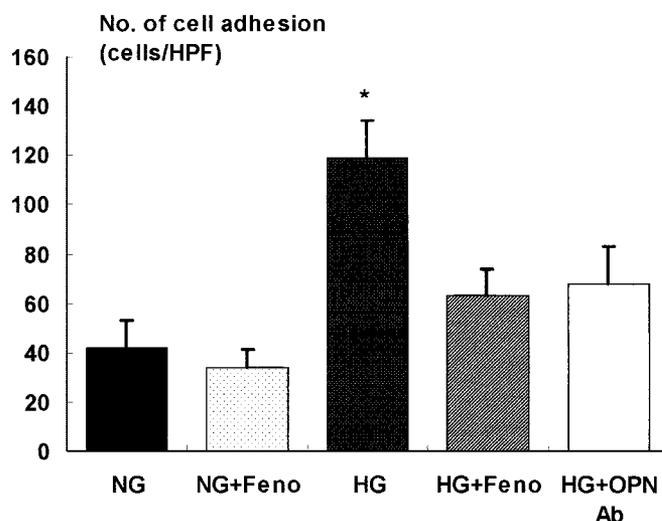
lation. No differences in systolic blood pressure were detected.

PPARα-knockout mice are reported to develop massive accumulations of myocardial lipids under conditions that increase fatty acid flux (16). Later, Campbell et al. (17) demonstrated that the marked increase in malonyl-CoA, a potent inhibitor of fatty acid oxidation, in the hearts of PPARα-knockout mice is due to decreased expression of malonyl-CoA carboxylase. It is well known that PPARα agonists lower triglyceride levels by increasing lipoprotein lipase gene expression via a PPAR response element (PPRE) in the LPL promoter (18) and decreasing apo C-III levels (19), thus resulting in an enhanced lipolytic activity. In our study, we found that diabetic PPARα-knockout mice exhibit profound metabolic abnormalities, including increased circulating free fatty acid and triglyceride concentrations. Taken together, these results indicate that a lack of PPARα activity in diabetic PPARα-knockout mice is associated with increased circulating free fatty acid and

No. of cell adhesion (cells/HPF)

FIG. 8. Activation of PPARα or blocking of osteopontin suppresses high-glucose (HG)-mediated leukocyte adhesion to mesangial cells. Fenofibrate and anti-osteopontin antibody suppressed the high-glucose (30 mmol/l)–mediated increase in leukocyte adhesion to mesangial cells. *P < 0.01 compared with normal glucose (NG), high glucose + fenofibrate (HG+Feno) or high glucose + anti-osteopontin antibody (HG+OPN Ab).

FIG. 7. Activation of PPARα suppresses high-glucose (HG)-mediated type IV collagen expression (A). Treatment of primary cultured 129JsV mouse mesangial cells with high glucose (30 mmol/l) significantly increased type IV collagen production as assessed by ELISA. Fenofibrate, PPARα agonist markedly suppressed the high-glucose–mediated increase in type IV collagen protein production. Activation of PPARα suppresses high-glucose–induced TGF-β1 protein expression in mesangial cells (B). Fenofibrate suppressed the high-glucose (30 mmol/l)–mediated increase in TGF-β1 protein production in mesangial cells. *P < 0.05 vs. cells with normal glucose (NG), cells treated with normal glucose + fenofibrate (NG+Feno), or cells with high glucose + fenofibrate (HG+Feno). Values are means ± SD. n = 6 in a single experiment representative of two independent experiments.

triglyceride concentrations, which could contribute to lipotoxicity in the kidney.

Recently, Mishra et al. (20) demonstrated that PPARα is a diabetes-induced transcription factor that helps control the kidney's response to lipids. Cardiac-specific PPARα overexpression increases fatty acid oxidation, elevates lipid storage droplets, and exacerbates cardiomyopathy in diabetes compared with wild-type controls (21). On the other hand, oral fibrates, PPARα agonists that lower serum triglycerides, reduced proteinuria in a subset of diabetic patients (22,23). Our study consistently showed that diabetes increased PPARα expression in diabetic wild-type mice and in vitro PPARα activation in mesangial cells by fenofibrate-attenuated extracellular matrix production associated with reduced TGF-β1 levels and leukocyte adherence to mesangial cells. Therefore, the net effect of PPARα activation may be beneficial because of reduced systemic and intrarenal free fatty acid and triglyceride levels. This may underlie the milder renal damage in diabetic wild-type mice compared with diabetic PPARα-knockout mice.

PPARα also exerts direct and indirect anti-inflammatory activities. Because PPARα enhances the activity of the β-oxidation pathway, the consequent degradation of arachidonic acid and leukotrienes directly results in the termination of inflammation (24). In the vascular wall, PPARs interfere with chemo attraction, including monocyte-chemoattractant protein 1 and RANTES (regulated on activation normal T-cell expressed and secreted) and cell adhesion of monocytes, T-cells, and eosinophils (25–27). Furthermore, PPAR activators inhibit endothelin 1 expression and cytokine-induced vascular cell adhesion molecule 1 (27). In our study, genetic deletion of PPARα, in the presence or absence of diabetes, was associated with increased F4/80 immunostaining in the glomeruli. Furthermore, in the kidneys of diabetic PPARα-knockout mice, F4/80 and osteopontin immunostaining within the glomeruli was increased over and above that of nondiabetic PPARα-knockout mice. These results indicate that both the deletion of the PPARα gene as well as diabetes might be associated with activation of the proinflammatory status. These findings demonstrate that PPARα plays a role in impeding inflammatory cell infiltration into the glomeruli in diabetes by blunting osteopontin expression. This finding is also consistent with a previous in vivo study using db/db mice (28) and with our in vitro primary mesangial cell culture study, which showed that high glucose levels resulted in leukocyte adhesion to mesangial cells and that the PPARα agonist fenofibrate blocked high-glucose–induced leukocyte adhesion to mesangial cells. Therefore, it appears plausible that fenofibrate exerts renoprotective effects in addition to its effect of lowering plasma free fatty acid and triglyceride levels.

In early diabetic nephropathy, increased amounts of type IV collagen and fibronectin in the mesangium accompany increased mesangial cell proliferation (29). In concord with this association, the glomerular lesions in PPARα-knockout mice exhibited increased type IV collagen and TGF-β expression in diabetic kidney disease. The

mechanisms involved in the direct anti-fibrotic effects of PPARα agonists on mesangial cells remain to be defined. Interestingly, Wilmer et al. (30) demonstrated that TGF-β1 expression mediated by oxidant stress is suppressed by PPARα activation, which inhibits the *TRE* gene. In our study, the production of the type IV collagen and TGF-β1 by mesangial cells pretreated with fenofibrate cultured under high-glucose media was also inhibited. These findings suggest that this PPARα agonist is an endogenous regulator of high-glucose–induced TGF-β1 expression.

Recent studies demonstrated that saturated free fatty acids and hyperglycemia activate the intrinsic pathway of apoptosis in mesangial cells. Apoptosis of glomerular cells correlated with greater expansion of the mesangial matrix and with more severe albuminuria (31,32) associated with increased in TGF-β levels in *db/db* mice. In contrast to nondiabetic PPARα-knockout or diabetic wild-type mice, diabetic PPARα-knockout mice developed significantly more TUNEL-positive glomerular cells. Taken together, reduction of PPARα activity in the diabetic status increases glomerular cell apoptosis and aggravates mesangial matrix expansion.

Proteinuria in diabetes is considered to have both a hemodynamic (glomerular capillary hypertension and hyperfiltration) and a structural/cellular (alterations in basement membrane, mesangial cell matrix, and podocyte function) basis (33). In our study, diabetic PPARα-knockout mice developed profound proteinuria compared with diabetic wild-type mice. Therefore, we speculate that PPARα agonists or activity may similarly attenuate diabetic renal injury by preventing an increase in extracellular matrix formation in the mesangium and apoptosis in the glomeruli, not by the induction of hemodynamic changes. Accumulating evidence suggests that diabetic nephropathy can be viewed as an inflammatory disease triggered by disordered glucose metabolism (34,35).

In another experiment with *db/db* mice, we demonstrated that PPARα activation by fenofibrate significantly increased PPARα immunostaining in the tubule and decreased the glomerular and mesangial surface areas (36). We also demonstrated that fenofibrate treatment markedly increased PPRE-luciferase activity in mesangial cells from *db/db* mice as determined by PPRE3X luciferase reporter analysis, which is consistent with the existence of endogenous PPARα activity in these cells. Therefore, we suggest that increased PPARα activity in the tubule may exert anti-inflammatory and anti-fibrotic effects via paracrine action resulting from increased PPRE activity in the glomeruli without significant differences in PPARα immunostaining in the glomerular areas.

In conclusion, deficiency of PPARα in a model of type 1 diabetic nephropathy is associated with increased circulating free fatty acids and triglycerides. This likely contributes to the aggravation of proteinuria and the pathologic changes observed in this model. Activation of PPARα with fenofibrate, a ligand agonist, may effectively prevent the glomerular matrix expansion that accompanied apoptosis and inflammatory cell infiltration in the glomerulus.

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