Role of Altered Renal Lipid Metabolism in the Development of Renal Injury Induced by a High-Fat Diet

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ABSTRACT
Metabolic syndrome is associated with increased risk of chronic kidney disease, and the renal injury in patients with metabolic syndrome may be a result of altered renal lipid metabolism. We fed wild-type or insulin-sensitive heterozygous peroxisome proliferator–activated receptor γ–deficient (PPARγ+/−) mice a high-fat diet for 16 weeks. In wild-type mice, this diet induced core features of metabolic syndrome, subsequent renal lipid accumulation, and renal injury including glomerulosclerosis, interstitial fibrosis, and albuminuria. Renal lipogenesis accelerated, determined by increased renal mRNA expression of the lipogenic enzymes fatty acid synthase and acetyl-CoA carboxylase (ACC) and by increased ACC activity. In addition, renal lipolysis was suppressed, determined by reduced mRNA expression of the lipolytic enzyme carnitine palmitoyl acyl-CoA transferase 1 and by reduced activity of AMP-activated protein kinase. In PPARγ+/− mice, renal injury, systemic metabolic abnormalities, renal accumulation of lipids, and the changes in renal lipid metabolism were attenuated. Thus, a high-fat diet leads to an altered balance between renal lipogenesis and lipolysis, subsequent renal accumulation of lipid, and renal injury. We suggest that renal lipid metabolism could serve as a new therapeutic target to prevent chronic kidney disease in patients with metabolic syndrome.


Metabolic syndrome, which is characterized by concurrent existence of obesity, dyslipidemia, hyperinsulinemia, hyperglycemia, and hypertension, is increasingly common because of increased prevalence of obesity. This syndrome is a growing health problem because of the associated increased risk for cardiovascular disease and premature death.1,2 Furthermore, a recent report suggested that individuals with metabolic syndrome are also at increased risk for developing chronic kidney diseases (CKD).3 Several pathomechanisms underlying the development of renal injury in metabolic syndrome have been proposed.4–8 Among them, renal lipid accumulation, lipotoxicity, has been reported to play an important role in the pathogenesis of renal injury in metabolic syndrome, although the precise mechanism of renal lipid accumulation has not been fully

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elucidated. Excess energy intake, including high-fat diet (HFD), contributes to the development of metabolic syndrome. HFD also causes renal lipid accumulation and renal injury. Therefore, elucidation of precise mechanisms that are responsible for renal lipid accumulation under an HFD could suggest the possible mechanisms underlying the development of renal injury in metabolic syndrome and thus enhance the design of novel therapeutic strategies against this renal injury.

Various intracellular molecules regulate local lipid metabolism in several tissues, such as skeletal muscle and liver. Under an altered systemic glucose and lipid metabolism, the imbalance between lipogenesis and lipolysis in such tissues contributes to the local lipid accumulation and subsequent pathophysiological changes. However, in the kidney, the role of local lipid metabolism in lipid accumulation and subsequent renal injury in metabolic syndrome has not been fully determined.

The purpose of this study was to clarify further the role of renal lipid metabolism in the development of renal injury in metabolic syndrome. We first examined how HFD could affect renal lipid metabolism. We especially focused on the balance of renal lipid metabolism in the development of renal injury in metabolic syndrome has not been fully determined.

RESULTS

Systemic Metabolic Abnormalities

The characteristics of the four groups at 16 wk of experimental period are presented in Table 1. PPAR-γ+/+ mice on an HFD were significantly heavier than PPAR-γ+/+ mice on a low-fat diet (LFD). These obese mice showed significantly high plasma triglycerides, cholesterol, TNF-α and monocyte chemoattractant protein-1 (MCP-1) levels, compared with their counterparts on an LFD. Plasma adiponectin levels in PPAR-γ+/+ mice on an HFD were significantly lower than in PPAR-γ+/+ mice on an LFD. Moreover, PPAR-γ+/+ mice on an HFD showed hyperinsulinemia during 4 wk of HFD (Figure 1A) and hyperglycemia during 8 wk of HFD (Figure 1B). In contrast, PPAR-γ−/− mice were significantly protected against obesity, insulin resistance, and the altered adipokine secretions during the 16-wk HFD, although no differences in food intake were observed between PPAR-γ+/+ and PPAR-γ−/− mice (Table 1, Figure 1, A and B). Glucose intolerance (determined by intraperitoneal glucose tolerance test) and insulin resistance (determined by intraperitoneal insulin tolerance test) at 16 wk of HFD in PPAR-γ−/− mice were attenuated in PPAR-γ+/+ mice (Figure 1, C and D). PPAR-γ+/+ mice showed features of metabolic syndrome from the early stage of HFD, whereas these alterations under an HFD were attenuated in insulin-sensitive PPAR-γ−/− mice, as previously reported.

Renal Injuries

We confirmed the significant downregulation of mRNA expression of PPAR-γ in the kidneys of PPAR-γ+/+ mice on both diets, compared with PPAR-γ+/+ mice (Table 2). Under an HFD, PPAR-γ+/+ mice exhibited a significant rise in urinary albumin excretion at 16 wk, although no significant differences were observed among the four groups at 4 and 8 wk (Figure 2). The increase in urinary albumin excretion at 16 wk was significantly inhibited in PPAR-γ−/− mice on an HFD (Figure 2). Examination of renal histopathologic changes with periodic acid-Schiff (PAS) in four groups revealed that HFD induced mesangial expansion in PPAR-γ+/+ mice (Figure 3, B and M). The expression of fibronectin was significantly increased in both the glomeruli and interstitium of PPAR-γ+/+ mice on an HFD (Figure 3, F and N, and J and O). In contrast, these HFD-induced glomerular and interstitial lesions were not observed under an LFD (Figure 3, A, C, E, G, I, and K). Furthermore, under an HFD, the mRNA expression levels of fibronectin, type IV collagen, plasminogen activator-1, and MCP-1 were significantly increased in the renal cortex of PPAR-γ+/+ mice, and these changes were significantly attenuated in PPAR-γ−/− mice (Table 2).

Table 1. Characteristics of the four groups of mice at the end of the 16-wk experimental period

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PPAR-γ+/+ Mice</th>
<th>PPAR-γ−/− Mice</th>
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<tbody>
<tr>
<td></td>
<td>LFD</td>
<td>HFD</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>32.6 ± 2.9</td>
<td>45.2 ± 3.1b,c</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>2.71 ± 0.17</td>
<td>2.57 ± 0.19</td>
</tr>
<tr>
<td>BP (mmHg)</td>
<td>91.0 ± 11.3</td>
<td>95.2 ± 8.7</td>
</tr>
<tr>
<td>Plasma triglyceride (mg/dl)</td>
<td>60.5 ± 14.5</td>
<td>156.0 ± 33.1b</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dl)</td>
<td>122.3 ± 13.8</td>
<td>221.7 ± 18.9b</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>4.56 ± 1.11</td>
<td>10.1 ± 3.11b,c</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>8.21 ± 0.78</td>
<td>6.1 ± 0.31b,c</td>
</tr>
<tr>
<td>MCP-1 (ng/ml)</td>
<td>45.2 ± 11.1</td>
<td>154.0 ± 21.2b,c</td>
</tr>
<tr>
<td>TNF-α (ng/ml)</td>
<td>9.50 ± 4.0</td>
<td>24.6 ± 4.6b</td>
</tr>
</tbody>
</table>

aData are means ± SEM; n = 11 in each group.
bP < 0.05 versus PPAR-γ+/+ mice fed LFD.
cP < 0.05 versus PPAR-γ+/+ mice fed HFD.
Renal Lipid Accumulation

Increased renal triglyceride content was observed in PPAR-γ+/+ mice at 8 and 16 wk of HFD, although no significant increase was observed at 4 wk of HFD (Figure 4A). Furthermore, HFD-induced increases in renal triglyceride content at 8 and 16 wk of HFD were significantly reduced in PPAR-γ+/− mice (Figure 4A). During the experimental period, no significant differences in renal cholesterol content were observed among the four groups (Figure 4B). Oil-Red O staining of kidney sections in the four groups revealed that HFD caused marked neutral lipid accumulations in both the glomerular and tubulointerstitial lesion (Figure 5, B and F). These accumulations were markedly decreased in PPAR-γ+/− mice (Figure 5, D and H). In both PPAR-γ+/+ and PPAR-γ+/− on an

Table 2. Levels of mRNA expression in the renal cortex at the end of 16-wk experimental period

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PPAR-γ+/+ Mice</th>
<th>PPAR-γ+/− Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LFD</td>
<td>HFD</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>0.95 ± 0.29</td>
<td>1.21 ± 0.23b,c</td>
</tr>
<tr>
<td>Fibrosis and inflammation</td>
<td></td>
<td></td>
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<tr>
<td>fibronectin</td>
<td>0.85 ± 0.16</td>
<td>1.31 ± 0.22h,c</td>
</tr>
<tr>
<td>type IV collagen</td>
<td>1.47 ± 0.18</td>
<td>2.10 ± 0.35h,c</td>
</tr>
<tr>
<td>PAI-1</td>
<td>0.81 ± 0.65</td>
<td>2.06 ± 0.37h,c</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2.95 ± 1.22</td>
<td>5.73 ± 0.80h,c</td>
</tr>
<tr>
<td>Fatty acid synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>1.66 ± 0.80</td>
<td>2.77 ± 0.62h,c</td>
</tr>
<tr>
<td>FAS</td>
<td>1.56 ± 0.56</td>
<td>5.22 ± 2.13h,c</td>
</tr>
<tr>
<td>ACC</td>
<td>0.44 ± 0.19</td>
<td>3.70 ± 1.33h,c</td>
</tr>
<tr>
<td>Fatty acid oxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>1.81 ± 0.99</td>
<td>3.04 ± 0.45</td>
</tr>
<tr>
<td>CPT-1</td>
<td>2.82 ± 0.98</td>
<td>2.11 ± 0.87h,c</td>
</tr>
<tr>
<td>ACO</td>
<td>0.84 ± 0.15</td>
<td>1.02 ± 0.15</td>
</tr>
<tr>
<td>MCAD</td>
<td>3.81 ± 0.79</td>
<td>3.01 ± 0.55</td>
</tr>
</tbody>
</table>

aData are means ± SEM; n = 11 in each group. PAI-1, plasminogen activator-1.

bP < 0.05 versus PPAR-γ+/− mice fed HFD.

cP < 0.05 versus PPAR-γ+/− mice fed LFD.

Figure 1. (A) Plasma insulin levels during 16-wk experimental period in each group of mice. Data are means ± SEM for five to 11 mice in each group. (B) Fasting blood glucose during 16-wk experimental period in each group of mice. Data are means ± SEM for 11 mice in each group. (C) Glucose tolerance test at the 16-wk experimental period in each group of mice. Data are means ± SEM for seven mice in each group. (D) Insulin tolerance test at the 16-wk experimental period in each group of mice. Data are means ± SEM for seven mice in each group. *P < 0.05 versus PPAR-γ+/− mice on an LFD; †P < 0.05 versus PPAR-γ+/− mice on an HFD.
LFD, these renal neutral lipid accumulations were not observed (Figure 5, A, C, E, and G).

Renal Lipid Metabolism
Sterol regulatory element-binding protein-1c (SREBP-1c) is a transcriptional factor that regulates the transcriptional activity of the enzymes that are involved in lipogenesis, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). In the kidneys from all four groups, we measured the mRNA expressions of SREBP-1c, FAS, and ACC at 4 and 16 wk. The mRNA expression levels of these molecules were increased in the kidneys of PPAR-γ+/+ mice on an HFD at both time points (Tables 2 and 3). However, these changes were not observed in PPAR-γ−/− mice (Tables 2 and 3). Furthermore, under an HFD, ACC protein content was increased in the kidneys of PPAR-γ+/+ mice at 16 wk but not in PPAR-γ−/− mice (Figure 6, A and B).

We next measured the mRNA expression levels of the molecules that are involved in lipolysis. At both 4 and 16 wk, we did not observe any differences in mRNA expression levels of PPAR-α, acyl-CoA oxidase, (ACO), and acyl-CoA dehydrogenase (MCAD) in the kidneys among the four groups (Tables 2 and 3). However, at both 4 and 16 wk of HFD, a significant decrease in mRNA expression of carnitine palmitoyl transferase-1 (CPT-1) in the kidney of PPAR-γ+/+ mice was observed, although this was not found in PPAR-γ−/− mice (Tables 2 and 3).

The 5′ AMP-activated protein kinase (AMPK) phosphorylates and inactivates ACC, resulting in a decrease in intracellular level of malonyl-CoA, thereby relieving inhibition of CPT-1 activity and accelerating lipolysis. Phosphorylation of both AMPKa(Thr172) (Figure 6, A and C) and ACC(Ser79) (Figure 6, A and D) were significantly decreased in the kidneys of PPAR-γ+/+ mice on an HFD at 16 wk. In contrast, these HFD-induced decreases in phosphorylation of AMPKa(Thr172) (Figure 6, A and C) and ACC(Ser79) (Figure 6, A and D) were not observed in the PPAR-γ−/− mice.

DISCUSSION
Here, we show that HFD induces the alteration of renal lipid metabolism by an imbalance between lipogenesis and lipolysis in the kidney per se, as well as systemic metabolic abnormalities and subsequent renal lipid accumulation and renal injury. Furthermore, these renal involvements under an HFD are ameliorated in insulin-sensitive PPAR-γ+/+ mice.

Recent study has reported to induce renal injury, although the exact mechanisms have not been fully clarified. Several reports have suggested that renal lipid accumulation, lipotoxicity, is associated with the development of such renal injury. It is interesting that our results show that HFD induces systemic metabolic abnormalities such as insulin resis-
tance during 4 wk of HFD and subsequent renal lipid accumulation during 8 wk of HFD and finally renal injury at 16 wk of HFD. Furthermore, these HFD-induced renal involvements are ameliorated in insulin-sensitive PPAR-α mice. These results suggest that lipotoxicity in the kidney could be one of the important mechanisms for the development of renal injury associated with metabolic syndrome.

To date, the precise mechanisms for renal lipid accumulation have not been fully determined. However, there is growing evidence that the increased renal lipogenesis plays a role in the pathogenesis of renal injury.11,13,25,26 Therefore, we investigated whether HFD increases renal mRNA expression levels of SREBP-1c, FAS, and ACC, which are involved in lipogenesis. Similar to previous reports,11,13,25,26 mRNA expression levels of these molecules were increased in the kidneys of PPAR-γ+/+ mice during 4 wk of HFD, whereas these were not observed in the kidneys of insulin-sensitive PPAR-γ−/− mice. Therefore, we can show that the increase in renal lipogenesis is observed from the early stage of HFD, before neutral lipid accumulation in the kidney. These observations provide further evidence that the accelerated renal lipogenesis contributes to the development of renal lipid accumulation under insulin resistance.

In addition to renal lipogenesis, we examined the effects of HFD on renal lipolysis to determine its role in the development of renal lipid accumulation. Our results showed that mRNA expression levels of CPT-1, which is one of the key enzymes involved in lipolysis, were significantly decreased during the 4 wk of HFD but not in PPAR-γ+/− mice. These results suggest that renal lipolysis decreases under insulin resistance, which may contribute to renal lipid accumulation. PPAR-α also regulates lipolysis in various tissues.27 However, we failed to find significant differences of renal mRNA expression levels of PPAR-α among the four groups. Furthermore, we could not observe differences of renal mRNA expression of ACO and MCAD, which are transcriptional target molecules of PPAR-α. These results suggest that HFD might not affect mRNA expression of PPAR-α or activity of PPAR-α in this mouse model of metabolic syndrome.

In this study, we found decreased renal mRNA expression levels of CPT-1, although those of ACO, MCAD, and PPAR-α were not changed, in PPAR-γ+/− mice on an HFD. We therefore focused on the activity of the AMPK pathway to explore this discrepancy, because this pathway is a key regulator of intracellular lipid metabolism in other tissues23 and because activated AMPK inactivates ACC, resulting in a decrease in malonyl-CoA, with subsequent release of inhibition of CPT-1 expression levels and acceleration of lipolysis.23 Under an HFD, phosphorylation of AMPKα(Thr172) and ACC(Ser79) was significantly decreased in the kidneys of PPAR-γ+/+ mice but not in the kidneys of PPAR-γ−/− mice. These results suggest that decreased AMPKα activity in the kidney under an HFD could increase the activity of ACC and intracellular malonyl-CoA content, resulting in the decreases in renal mRNA expression of CPT-1. These results could provide new evidence that a decrease in lipolysis via inhibiting the AMPK–CPT-1 pathway but not PPAR-α could contribute to renal lipid accumulation under an HFD. Furthermore, these results suggest that posttranslational activation of ACC by inhibiting AMPK activity under an HFD might contribute to the acceleration of renal lipogenesis, as well as increased renal expression of ACC.

Figure 4. Triglyceride (A) and cholesterol (B) contents in the kidneys of mice in each group. Data are means ± SEM for five to 11 mice in each group. *P < 0.05 versus PPAR-γ+/+ mice on an LFD; †P < 0.05 versus PPAR-γ+−/− mice on an HFD.

Figure 5. (A through H) Representative photomicrographs of Oil-Red O–stained kidney sections in each group of mice. Magnifications: ×200 in A through D; ×400 in E through H.
In this study, we show that the improvements of systemic metabolic abnormalities result in the attenuation of HFD-induced renal lipid accumulation and renal injury with the improvement of renal lipid metabolism in PPAR-γ−/− mice. We previously reported that moderate reduction of PPAR-γ activity could decrease local lipid accumulation in the liver and skeletal muscle in PPAR-γ−/− mice. These results raise the question of whether the reduction of PPAR-γ activity could directly affect the improvement of renal lipid metabolism in the kidneys of PPAR-γ−/− mice on an HFD. Liver-specific PPAR-γ disruption could attenuate steatohepatitis with the reduction of lipid accumulation in leptin-deficient mice. Also, deletion of PPAR-γ in adipose tissues of mice protects against HFD-induced adipocyte hypertrophy, which inhibits obesity and insulin resistance. These reports suggest that a reduction of PPAR-γ activity may inhibit various diseases that are associated with local lipid accumulation in various peripheral tissues, including kidney. However, our study does not provide enough evidence to clarify whether PPAR-γ deficiency in the kidney directly regulates renal lipid metabolism, as well as other peripheral tissues. Further studies are required to determine the direct effects of PPAR-γ activity on renal lipid metabolism.

Several investigators have reported that PPAR-γ agonists can protect against the various types of renal injury through their anti-inflammatory and antifibrotic effects. In contrast, our results showed that systemic reduction of PPAR-γ expression could improve HFD-induced renal injury. We therefore suggest that both PPAR-γ agonists and PPAR-γ insufficiency in the absence of ligands can protect against renal injury that is associated with glucose and lipid metabolism abnormalities, at least in part, through the attenuation of both systemic and renal lipid metabolism. Furthermore, several reports show that PPAR-γ recruits other transcriptional co-repressor complexes in the absence of ligand and that these co-repressors are capable of down-regulating PPAR-γ-mediated transcriptional activity.

This might be another mechanism through which both ligand binding to PPAR-γ and ligand-free PPAR-γ deficiency could promote renal protection.

Here, we present evidence that HFD causes renal lipid accumulation and renal injury with increased renal lipogenesis and decreased renal lipolysis, whereas these abnormalities are attenuated in insulin-sensitive PPAR-γ−/− mice. These results suggest that the improvement of an imbalance between renal lipogenesis and lipolysis results in a reduction of renal lipid accumulation and subsequent attenuation of renal injury under insulin resistance. Therefore, we propose that attenuation of renal lipid metabolism could serve as a new therapeutic strategy to prevent the development of CKD in metabolic syndrome.

**CONCISE METHODS**

**Animal Models**
PPAR-γ−/− mice were generated as described previously. Six-week-old mice were housed in box cages, maintained on a 12-h light/12-h dark cycle, and fed an LFD (10% of kilocalories from fat) or HFD (45% of kilocalories from fat) obtained from Research Diets (New Brunswick, NJ) for 16 wk. At the end of 16-wk period, body weight, BP, and blood glucose were measured. BP of conscious mice was measured at a steady state with a programmable tail-cuff sphygmomanometer (BP98-A; Softron, Tokyo, Japan). Mice were placed in metabolic balance cages for 24-h urine collection to measure albumin concentration. Mice were anesthetized and perfused as described previously. The right kidney was embedded in paraffin for PAS staining and immunohistochemistry or was frozen for Oil-Red O staining. Total RNA and protein were extracted from the remaining renal cortex of the left kidney. The Research Center for Animal Life Science of Shiga University of Medical Science approved all experiments.

**Antibodies**
Anti-phospho-acetyl CoA carboxylase(Ser79) was obtained from Upstate Cell Signaling (Lake Placid, NY). Anti-phospho-AMPKα(Thr172), anti-AMPKα(23A3), and anti-ACC were from Cell Signaling Technology (Beverly, MA).

**Table 3. Levels of mRNA expression in the renal cortex at 4 wka**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PPAR-γ−/− Mice LFD</th>
<th>PPAR-γ−/− Mice HFD</th>
<th>PPAR-γ−/− Mice LFD</th>
<th>PPAR-γ−/− Mice HFD</th>
</tr>
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<tbody>
<tr>
<td>Fatty acid synthesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>1.21 ± 0.10</td>
<td>2.93 ± 0.31b,c</td>
<td>1.29 ± 0.10</td>
<td>1.69 ± 0.16</td>
</tr>
<tr>
<td>FAS</td>
<td>1.54 ± 0.15</td>
<td>3.38 ± 0.67b,c</td>
<td>1.63 ± 0.17</td>
<td>1.72 ± 0.15</td>
</tr>
<tr>
<td>ACC</td>
<td>0.57 ± 0.07</td>
<td>1.06 ± 0.17b,c</td>
<td>0.59 ± 0.04</td>
<td>0.69 ± 0.13</td>
</tr>
<tr>
<td>Fatty acid oxidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>2.13 ± 0.61</td>
<td>3.13 ± 0.60</td>
<td>2.18 ± 0.24</td>
<td>2.78 ± 0.34</td>
</tr>
<tr>
<td>CPT-1</td>
<td>3.20 ± 0.29</td>
<td>2.03 ± 0.47b,c</td>
<td>3.04 ± 0.35</td>
<td>2.77 ± 0.20</td>
</tr>
<tr>
<td>ACO</td>
<td>0.91 ± 0.22</td>
<td>1.09 ± 0.22</td>
<td>1.02 ± 0.28</td>
<td>0.90 ± 0.13</td>
</tr>
<tr>
<td>MCAD</td>
<td>3.56 ± 1.89</td>
<td>3.89 ± 1.10</td>
<td>4.45 ± 2.14</td>
<td>3.95 ± 1.50</td>
</tr>
</tbody>
</table>

*aData are means ± SEM; n = 5 to 6 in each group.
bP < 0.05 versus PPAR-γ−/− mice fed LFD.
cP < 0.05 versus PPAR-γ−/− mice fed HFD.
Blood and Urine Analysis
Cholesterol or triglycerides were measured using the cholesterol CII kit or L type TG H kit (Wako Chemicals, Richmond, VA). Plasma insulin was determined using an ELISA (Exocell, Philadelphia, PA). Plasma leptin, MCP-1, and TNF-α were assayed with the immunoassay kit (R&D Systems, Minneapolis, MN). Plasma adiponectin was determined with a mouse-specific ELISA kit (Linco Research, St. Charles, MO). Urinary albumin excretion was measured with a mouse-specific sandwich ELISA system (Albuwell; Exocell) and was expressed as total amount excreted in 24 h.

Protein Extraction and Western Blot Analysis
The renal cortex was homogenized in an ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1% SDS, 1% Nonidet P-40, and protease inhibitor cocktail (Boehringer Mannheim, Lewes, UK). These samples were resolved by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon, Bedford, MA). The membranes were incubated with the appropriate antibodies, washed, and incubated with horseradish peroxidase-coupled secondary antibodies (Amersham, Buckinghamshire, UK). The blots were visualized by using an enhanced chemiluminescence detection system (Perkin Elmer Life Science, Boston, MA).

RNA Extraction and Quantitative Real-Time PCR
Total RNA was isolated from the renal cortex based on the TRIzol protocol (Invitrogen Life Technologies, Carlsbad, CA). cDNA was synthesized using reverse transcript reagents (Takara, Otsu, Japan). iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) was used for real-time PCR (ABI Prism 7500 Sequence Detection System; Perkin-Elmer Applied Biosystems). The levels of mRNA expression of these molecules were quantified using standard curve method. Standard curves were constructed using serially diluted standard template. 

Lipid Extraction and Analysis
Total lipid was extracted from the renal cortex by the method of Bligh and Dyer. 

Morphologic Analysis
Fixed kidneys were embedded in paraffin, sectioned (3-μm thick), and then stained with PAS reagent as described previously. From each mouse, 20 glomeruli cut at their vascular poles were used for morphometric analysis. The extent of the mesangial matrix (defined as mesangial area) was determined by assessment of the PAS-positive and nucleus-free area in the mesangium using a computer-assisted color image analyzer (LUZEX F; Nikon, Tokyo, Japan). Immunohistochemical staining was performed with fibronectin-specific polyclonal anti-mouse antibody (A852/R5H; Biogenesis, Poole, UK). For evaluation of immunostaining for fibronectin, the percentages of area stained for fibronectin were graded as follows: 0, staining absent to 5%; 1, 5 to 25%; 2, 25 to 50%; 3, 50 to 75%; and 4, >75%. An investigator who was masked to sample identity performed the image analysis. Frozen sections were used for Oil-Red O staining, as previously reported.

Blood and Urine Analysis
Cholesterol or triglycerides were measured using the cholesterol CII kit or L type TG H kit (Wako Chemicals, Richmond, VA). Plasma
Glucose Tolerance Test and Insulin Tolerance Test
For glucose tolerance tests, mice were fasted overnight for 14 h followed by intraperitoneal glucose injection (1 g/kg body wt). Blood glucose was measured using tail blood collected at 0, 15, 30, 60, and 120 min after the injection.37 For insulin tolerance tests, mice were administered an injection of human regular insulin (Novolin R; Novo Nordisk, Clayton, NC) at 0.75 U/kg body wt intraperitoneally after a 6-h fast, and blood glucose was measured at 0, 15, 30, and 60 min.37

Statistical Analyses
Results are expressed as means ± SEM. ANOVA with subsequent Scheffe test was used to determine the significance of differences in multiple comparisons. P < 0.05 was considered statistically significant.

ACKNOWLEDGMENTS
We are highly appreciative of Makiko Sera for excellent technical assistance.

DISCLOSURES
None.

REFERENCES


