Diabetic kidney disease has been associated with the presence of lipid deposits, but the mechanisms for the lipid accumulation have not been fully determined. In the present study, we found that db/db mice on the FVB genetic background with loss-of-function mutation of the leptin receptor (FVB-Leprdb mice or FVBdb/db) develop severe diabetic nephropathy, including glomerulosclerosis, tubulointerstitial fibrosis, increased expression of type IV collagen and fibronectin, and proteinuria, which is associated with increased renal mRNA abundance of transforming growth factor-β, plasminogen activator inhibitor-1, and vascular endothelial growth factor. Electron microscopy demonstrates increases in glomerular basement membrane thickness and foot process (podocyte) length. We found that there is a marked increase in neutral lipid deposits in glomeruli and tubules by oil red O staining and biochemical analysis for cholesterol and triglycerides. We also detected a significant increase in the renal expression of adipocyte differentiation-related protein (adipophilin), a marker of cytoplasmic lipid droplets. We examined the expression of sterol regulatory element–binding protein (SREBP)-1 and -2, transcriptional factors that play an important role in the regulation of fatty acid, triglyceride, and cholesterol synthesis. We found significant increases in SREBP-1 and -2 protein levels in nuclear extracts from the kidneys of FVBdb/db mice, with increases in the mRNA abundance of acetyl-CoA carboxylase, fatty acid synthase, and 3-hydroxy-3-methylglutaryl-CoA reductase, which mediates the increase in renal triglyceride and cholesterol content. Our results indicate that in FVBdb/db mice, renal triglyceride and cholesterol accumulation is mediated by increased activity of SREBP-1 and -2. Based on our previous results with transgenic mice overexpressing SREBP-1 in the kidney, we propose that increased expression of SREBPs plays an important role in causing renal lipid accumulation, glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria in mice with type 2 diabetes. Diabetes 54:2328–2335, 2005

There is growing evidence that abnormal lipid metabolism and renal accumulation of lipids play a role in the pathogenesis of diabetic nephropathy (1–4). Since the description by Kimmelstiel and Wilson (5) of nodular glomerulosclerosis and presence of lipid deposits in the diabetic kidney, several investigators have shown the presence of lipid accumulation in the kidneys of diabetic humans and experimental animals, and they have proposed that the lipids may play an important role in the pathogenesis of diabetic kidney disease (4,6,7). The major assumption has been that these lipid deposits originate solely from increased levels of serum lipids. Whether the accumulation of lipids per se may mediate diabetic renal disease is supported by increased abundance of transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) and changes of diabetic glomerulosclerosis and proteinuria in sterol regulatory element–binding protein-1a (SREBP-1a) transgenic mice with increased renal triglyceride content (4).

The SREBPs have been described as master regulators of both fatty acid and cholesterol metabolism (8–11). Three SREBP isoforms have been identified and characterized, SREBP-1a, -1c, and -2 (8). Studies in transgenic mice overexpressing each of the three SREBP isoforms in the liver have indicated that SREBP-1a and -1c isoforms play a preferential role in fatty acid synthesis, whereas SREBP-2 plays a preferential role in cholesterol synthesis (12,13). In a recent study, we found that in a rat model of type 1 diabetes, there is increased renal accumulation of...
lipids, which is mediated by increased expression of SREBP-1 (4). In cultured mesangial cells, high-glucose medium also stimulates increased expression of SREBP-1 (4). In SREBP-1a transgenic mice, in the absence of any changes in serum glucose or serum lipids, there is increased accumulation of triglyceride and cholesterol in the kidney, which is associated with increased expression of TGF-β, VEGF, and the extracellular matrix proteins type IV collagen and fibronectin, resulting in glomerular hypertrophy, glomerulosclerosis, and proteinuria (4). This study indicates that increased expression of SREBP-1 plays an important role in the pathogenesis of diabetic kidney disease in type 1 diabetes. However, whether SREBPs also play a role in the regulation of renal lipid metabolism and the development of diabetic nephropathy in type 2 diabetes has not been determined.

Three lines of evidence suggest that altered lipid metabolism is associated with diabetic and non-diabetic renal disease. First, there are a number of genetic abnormalities of lipid metabolism in humans and experimental animals, including Fabry’s disease (14), lecithin cholesterol acyltransferase deficiency (15), type IA glycogen storage disease (von Gierke’s disease) (16), genetic and acquired lipodystrophy (17,18), ATP-binding cassette transporter-1 (ABCA-1) knockout mice (a murine model of Tangier disease) and familial HDL deficiency, with defects in ABCA-1 and HDL-mediated reverse cholesterol transport (19,20), and ApoE knockout mice, where abnormalities in serum and tissue lipids, including renal lipid composition, are associated with glomerular disease and proteinuria (21,22). Second, increases in serum lipids have been associated with a faster decline of renal function (23,24). Third, there is also increasing evidence that inhibition of cholesterol synthesis by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors (statins), inhibition of triacylglyceride synthesis by peroxisome proliferator–activated receptor-α (PPAR-α) agonists (fibrates), or a decrease in LDL achieved by LDL apheresis protect against diabetic and non-diabetic renal disease (25–27). A meta-analysis of several small-scale interventional studies in diabetic and non-diabetic human subjects with glomerulosclerosis and proteinuria in fact indicates that long-term treatment with statins and/or fibrates significantly prevents the decline in glomerular filtration rate (25).

A recent study of obese db/db mice of the FVB/dan/dan congenic strain demonstrated the presence of long-term hyperglycemia, which is primarily caused by severe insulin resistance. The hyperglycemia of the obese mice in the fed state persists, despite escalating secretion of insulin and a massive increase of pancreatic β-cells (28). Obese FVB mice show evidence of mesangial matrix expansion, a hallmark of diabetic nephropathy. Therefore, FVB/db/db is an excellent animal model of type 2 diabetes for studying the pathogenesis of diabetic kidney disease. We tested our hypothesis that increased expression of SREBPs plays an important role in renal lipid accumulation and the development of diabetic nephropathy in this type 2 diabetic animal model.

RESEARCH DESIGN AND METHODS

A total of 12 female FVB/db/db mice and 12 control mice genotyped to be wild type at the db locus at age 3 months were transferred from Columbia University to the animal facility at the Denver Veterans Affairs Medical System. The mice had been maintained on a 12-h light/dark cycle and fed standard rodent chow (Rodent Chow 5015; Ralston Purina, St. Louis, MO) ad libitum for 3 more months until they were killed. The experimental animal committee of the University of Colorado Health Sciences Center and the Veterans Affairs Medical Center at Denver gave approval for all experiments involving animals.

Spot urine samples for measurement of albumin and creatinine were obtained on all mice. After the urine collection, eight mice in each age-group were killed by intraperitoneal injection of pentobarbital (Abbott Laboratories, Chicago, IL). We obtained 0.5 ml of blood in a heparinized syringe via heart puncture at the time of death. In addition, four mice in each age-group underwent in vivo perfusion fixation of the kidneys, and the kidneys were then processed for histological stains, immunofluorescence microscopy, and electron microscopy, as described below.

Urine chemistry. Urine albumin concentration was determined by competitive enzyme-linked immunosorbent assay via an Alburell M kit (Eexcult, Philadelphia, PA). Urine creatinine concentration was determined by Jaffé’s reaction of alkaline picate with creatinine via a Creatinine Companion kit (Eexcoll). Results were expressed as the urine albumin-to-creatinine ratio (μg/mg).

Homogenate and nuclei isolation. Kidneys were homogenized at 4°C in homogenization buffer, as previously described (4). Nuclear extracts were prepared according to the method of Morooka et al. (28). The protein concentration was determined by the method of Lowry et al. (30). The nuclear extracts were stored at −80°C.

Protein electrophoresis and Western blotting. Protein samples were subjected to SDS-PAGE (10% wt/vol) and then transferred to nitrocellulose membranes. Membranes were blocked in 5% powdered milk in Tris-buffered saline with TWEEN (0.2% Tween 20 in 1× Tris-buffered saline) and incubated with 1) anti-SREBP-1, 2) anti–SREBP-2 (1:1,000 dilution; BD Biosciences, San Jose, CA), and 3) lipid transferase-related protein [ADRP] (1:2,000; Roche Biochemicals, Indianapolis, IN) followed by horseradish peroxidase–labeled anti-rabbit IgG (1:15,000 dilution; Molecular Probes, Eugene, OR). Next, samples were washed four times with 1× Tris-buffered saline and then developed using a chemiluminescence detection system (Pierce Biotechnology, Rockford, IL). The signals were quantified in a Phosphor Imager with chemiluminescence detector and densitometry software (Bio-Rad Laboratories, Hercules, CA).

Lipid extraction and analysis. Total lipid was extracted from kidney cortex by the method of Bligh and Dyer (31). Total cholesterol was analyzed using a cholesterol CII kit (Wako Chemicals, Richmond, VA). Triglycerides were determined by the L Type TG H kit (Wako).

Total RNA extraction and real-time PCR. Total RNA was extracted according to Triozol protocol (Invitrogen Life Technologies, Carlsbad, CA). Then, 2 μg of total RNA was subject to DNase digestion, using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories) to make cDNA. qSYBR Green Supermix (Bio-Rad Laboratories) was used for real-time PCR according to manufacturer’s instructions. Primers used are as follows: cyclophilin (sense: TGGAGAGCACAAGACAGACA; antisense: TGGCGGAGTCGCAATAGAT), acyl-CoA carboxylase (ACC; sense: CCCAGCAGAATAGGACTTCTTG; antisense: TCCTTTGTTCCACTAGGAGT), fatty acid synthase (FAS; sense: CCTGAGATGGAGAGCGTCT; antisense: ACCACCATCTCAGGAGGCACA), HMG-CoA reductase (sense: AGCCGAAGCAGCAGCAGAT; antisense: CTGTTGAAATTGCTGTGAATGTT), plasminogen activator inhibitor-1 (PAI-1; sense: GGACACCGCAGCCGACG; antisense: TCTGATGCTGATGGTGCCAGAT), PPAR-α (sense: CTGCAGACAGACCCATCTGGA; antisense: GACCACCGCGCGCAAGG), PPAR-γ (sense: CTGGAGAAGCTATGGACCA; antisense: GGAGTGGGCTGTACCTGCT); TGF-β (sense: TGTAGACTGGGGACGATG; antisense: GTTTACAGGTCGGAGCAGTG), ABCA-1 (sense: GCACTTCGCCTGATGTCT; antisense: CTGATGAGATGGGCTGATC), VEGF (sense: AACGGGAGTAGCTGAGACA; antisense: TGGAGAGTGGAGAGCGTCT); ANP (sense: TGGAGAAGCTATGGACCA; antisense: GGAGTGGGCTGTACCTGCT); LDL receptor (sense: GCATCAGGGGTGGACGAC; antisense: ACGGATACGCTGAGGATTGAG); LDL (sense: TGGAGAAGCTATGGACCA; antisense: GGAGTGGGCTGTACCTGCT); and γ-glutamyltransferase (sense: TGGCCAGACGAGGTTG; antisense: TGGGAGGATGCTGAGGATG). The signals were quantified in a Phosphor Imager with chemiluminescence detector and densitometry software (Bio-Rad Laboratories, Hercules, CA).

Perfusion fixation of mouse kidney. Mice were anesthetized and perfused at a pressure of 180 mmHg through the abdominal aorta, as previously described (4).

Periodic acid Schiff staining, oil red O staining, and immunofluorescence microscopy. Paraffin sections were stained for periodic acid Schiff. Frozen sections were used for oil red O staining to determine the renal accumulation of neutral fats. The stained kidney sections were imaged with an Olympus microscope and scored semiquantitatively in a blinded manner by the renal pathologist (S.L.).

Immunofluorescence microscopy for type IV collagen and fibronectin were
performed as previously described (4). The kidney sections were then imaged with a laser scanning confocal microscope (Zeiss LSM 510).

For adipophilin (ADRP) imaging, paraffin-embedded sections were used, and ADRP was detected by binding to antibodies to ADRP (1:500; Roche Biochemicals) (32,33) in conjunction with Alexa-488–labeled (Molecular Probes) secondary antibodies. Lipid droplets and nuclei were stained with Nile red and 4′,6-diamidino-2-phenylindol, as described previously (32).

Electron microscopy. Perfusion-fixed tissue was immediately postfixed in 1% buffered osmium tetroxide. The sample was dehydrated in a graded series of ethanol and embedded in an epoxy resin. Tissue was surveyed with a series of 1-μm sections for a representative sample. The selected specimens were thin sectioned, viewed, and photographed with an electron microscope (model 201; Phillips Electron Optics, Mahwah, NJ). The sections were read by the renal pathologist (S.L.) for determination of basement membrane thickness and podocyte morphology.

Statistical analysis. SPSS 11.0 for Windows was used for statistical analysis. The results were expressed as the means ± SE. The statistical significance of differences was assessed by one-way ANOVA.

RESULTS

FVB<sup>db/db</sup> mice are obese and hyperlipidemic. As shown in Table 1, at the age of 6 months, FVB<sup>db/db</sup> mice are much heavier than their lean littermates in body, kidney, and liver weight (P < 0.01). Because almost all of the increased mass of the db/db mice is caused by triglyceride accumulation rather than an overall increase in all body compartments, the increased kidney and liver weights represent significant increases in organ weights relative to fat-free mass. Plasma cholesterol and triglyceride levels were much higher in FVB-Lepr<sup>db</sup> mice than control mice.

FVB<sup>db/db</sup> mice develop glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria. Periodic acid Schiff staining clearly reveals mesangial expansion, increased matrix protein accumulation, and tubulointerstitial fibrosis in FVB<sup>db/db</sup> mice (Figs. 1B, C, and D) when compared with age- and sex-matched control mice (Fig. 1A). Immunofluorescence microscopy with anti-fibronectin and anti–type IV collagen (Fig. 2) antibodies indicates increased intensity of immunofluorescence in the glomeruli and tubulointerstitial cells in FVB<sup>db/db</sup> mice, indicating accumulation of extracellular matrix proteins and glomerulosclerosis and tubulointerstitial fibrosis. Furthermore, electron microscopy shows increased glomerular basement membrane thickness and podocyte foot process length in FVB<sup>db/db</sup> mice (Figs. 3A–D). Increased urine-to-albumin ratio usually is an early indication of diabetic nephropathy. This ratio is significantly elevated in FVB<sup>db/db</sup> mice when compared with lean mice (Table 1).

FVB<sup>db/db</sup> mice have increased expression of growth factors. The glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria of FVB<sup>db/db</sup> mice is associated with
significant increases in renal mRNA abundance of TGF-β, PAI-1, and VEGF (Table 2).

**FVB**<sup>db/db</sup> **mice have increased lipid deposits in kidney.**

There is very strong staining of oil red O in the glomeruli of FVB<sup>db/db</sup> (Fig. 4B) and almost no staining in lean mice (Fig. 4A). Adipophilin (ADRP) is a marker of lipid droplets. Immunofluorescence microscopy reveals typical lipid droplets as ring-shaped red dots in the tubules of FVB<sup>db/db</sup> mice (Fig. 4D). In contrast, there are almost no lipid droplets in the lean control mice (Fig. 4C). In addition, Western blot clearly shows a 65% increase of ADRP protein expression in FVB<sup>db/db</sup> mice (Fig. 4E). These data strongly indicate that there are excessive amounts of lipid deposits in the kidneys of FVB<sup>db/db</sup> mice.

**FVB**<sup>db/db</sup> **mice have increased nuclear SREBP-1 and -2 protein abundance.**

In our previous study in animals with type 1 diabetes, we found that SREBPs play a major role in regulating renal lipid metabolism. In this study we
found significant increases in nuclear SREBP-1 and -2 abundance in the kidneys of FVB\textsuperscript{db/db} mice (Figs. 5A and B).

**FVB\textsuperscript{db/db} mice have increased renal expression of genes regulating triglyceride and cholesterol metabolism.**\footnote{Data are the means ± SE, \( n = 8 \) in each group. The relative expression levels were calculated according to the formula \( 2^{-\Delta CT} \), where \( \Delta CT \) is the difference in threshold cycle (CT) values between the target and the internal control.}

\textbf{TABLE 2}

Real-time PCR data for growth factors

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>FVB\textsuperscript{db/db}</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>1.98 ± 1.12</td>
<td>12.88 ± 2.96</td>
<td>0.01</td>
</tr>
<tr>
<td>TGF-β</td>
<td>8.95 ± 4.58</td>
<td>96.42 ± 27.67</td>
<td>0.03</td>
</tr>
<tr>
<td>PAI-1</td>
<td>3.4 ± 0.83</td>
<td>45.02 ± 11.73</td>
<td>0.01</td>
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DISCUSSION

There are well-known changes in diabetes-related renal function and structure, including glomerulosclerosis, proteinuria, and decline in glomerular filtration rate. Several hormonal and metabolic factors, including angiotensin II (34), TGF-β (35), VEGF (36), oxidative stress (37), advanced glycation end products (38), and nitric oxide (39), have been shown to modulate diabetes-related renal disease in rodents. In addition, renal accumulation of lipids has also been proposed to play a role in the pathogenesis of diabetic nephropathy (6–7). Our previous study in streptozotocin-induced type 1 diabetes has shown that altered lipid metabolism plays an important role in the development of diabetic nephropathy. Furthermore, we were able to demonstrate that SREBPs are the key factors linking nephropathy to the dysregulation of lipid metabolism (4).

In **FVB\textsuperscript{db/db}** mice, a type 2 diabetes animal model, we found evidence of diabetic nephropathy, such as increased urine albumin-to-creatinine ratio, increased matrix protein, mesangial expansion, thickening of the glomerular basement membrane, effacement of podocyte foot processes, significant glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria. These renal functional and structural changes are associated with increased mRNA abundance of TGF-β, PAI-1, and VEGF, growth factors that have been shown to play an important role in mediating glomerulosclerosis and proteinuria (35,36,40).

A novel finding of our study is that we found excessive amounts of lipid deposits in the diabetic kidney, as shown by increased oil red O staining, and the presence of lipid bodies, as shown by ADRP immunofluorescence microscopy and ADRP protein levels, using Western blotting. These changes correspond to highly elevated kidney cholesterol and triglyceride content.

We have found that the increases in renal triglyceride and cholesterol content are most likely mediated by
increased nuclear protein levels and transcriptional activities of SREBP-1 and -2. We found that in FVB\textsuperscript{db/db} mice, increased levels of SREBP-1 protein is associated with increased mRNA abundance of ACC and FAS, two key enzymes that mediate increased fatty acid synthesis, which results in increased triglyceride synthesis and accumulation (10,13). Because we found no significant changes in the mRNA abundance of PPAR-\(\alpha\) and its target enzyme, ACO, it is unlikely that decreases in fatty acid oxidation could be responsible for the triglyceride accumulation.

In addition, in FVB\textsuperscript{db/db} mice, increased levels of SREBP-2 protein is associated with increased mRNA abundance of HMG-CoA reductase, a key enzyme that mediates cholesterol synthesis, and LDL receptor, which mediates cholesterol uptake. These alterations in gene expression would result in a significant increase in renal cholesterol content. Because cholesterol accumulation can also be mediated by decreased cholesterol efflux, we also determined the renal abundance of LXR and ABCA-1, important mediators of cholesterol efflux (41,42). There were actu-

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FIG. 4. A and B: Oil red O staining of frozen kidney sections (glomeruli; 40\(\times\) magnification) for control mice (A) and FVB\textsuperscript{db/db} mice (B). C and D: Adipophilin (ADRP) immunofluorescence microscopy (tubules) for control mice (C) and FVB\textsuperscript{db/db} mice (D) (40\(\times\) magnification). Nuclei are blue from 4',6-diamidino-2-phenylindole stain.

FIG. 5. Western blot and densitometric quantification of SREBP-1 (A) and SREBP-2 (B) protein in kidney nuclear extracts. Blot represents eight samples from each group; relative densitometry units are used for comparison. □, control mice; ■, FVB\textsuperscript{db/db} mice. Data are the means ± SE.
ally significant increases in LXR and ABCA-1 mRNA abundance. Thus, the accumulation of renal cholesterol in the FVB<sup>db/db</sup> mouse occurs, despite probable increases in cholesterol efflux.

Previous studies in renal mesangial and tubular cells grown in culture have shown that incubation of these cells with LDL or VLDL induces upregulation of growth factors, including TGF-β (43), PAI-1 (44), and accumulation of extracellular matrix proteins (45), indicating a direct role for lipids in activating the mediators of glomerulosclerosis. Recent studies indicate that VEGF is actively involved in the pathogenesis of diabetic nephropathy (36,46,47). Results from our study do confirm those previous findings and support the theory that altered lipid accumulation is contributing to the development of diabetic nephropathy.

In addition, our study in SREBP-1a transgenic mice suggests that increased renal triglyceride and cholesterol accumulation mediated by increased renal expression of SREBPs do play a critical role in the pathogenesis of glomerulosclerosis and proteinuria. In SREBP-1a transgenic mice, in the absence of any increases in serum glucose, triglyceride, or cholesterol level, we reported that increased renal accumulation of triglycerides results in increased renal abundance of TGF-β, VEGF, type IV collagen, and fibronectin, resulting in glomerulosclerosis and proteinuria (4).

In conclusion, our results indicate that in FVB<sup>db/db</sup> mice that are obese and have type 2 diabetes, glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria are associated with marked alterations in renal lipid metabolism mediated by increased expression of SREBPs, resulting in renal accumulation of lipid bodies, triglycerides, and cholesterol. The increased expression of genes responsible for triglycerides and cholesterol metabolism indicates that increased synthesis rather than decreased oxidation or decreased efflux is the mechanism for triglyceride and cholesterol accumulation. We propose that abnormalities of renal lipid metabolism play an important role in the pathogenesis of diabetic nephropathy.