Diabetic Nephropathy Is Associated with Oxidative Stress and Decreased Renal Nitric Oxide Production

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ABSTRACT

The pathogenesis of diabetic nephropathy remains far from clear, partly due to the lack of a suitable animal model that mimics human renal disease in type 2 diabetes. In this study, the natural history of renal manifestations in ZSF1 rats, a recently developed rodent model of type 2 diabetes, is described. Male ZSF1 rats developed obesity and hyperglycemia by 20 weeks of age on a high-carbohydrate diet. They also developed systolic and diastolic hypertension, hypercholesterolemia, profound hypertriglyceridemia, proteinuria, and renal failure. Renal histology demonstrated changes consistent with early diabetic nephropathy, including arteriolar thickening, tubular dilation and atrophy, glomerular basement membrane thickening, and mesangial expansion. Furthermore, renal nitric oxide production was decreased, and homogenates from renal cortices demonstrated reduced expression of renal endothelial and inducible nitric oxide synthases. These changes were associated with increased urinary levels and renal expression of 8-hydroxydeoxyguanosine, an indicator of mitochondrial oxidative stress, as well as with increased renal peroxynitrite formation. Administration of either insulin or the antioxidant alpha-lipoic acid decreased proteinuria and oxidative stress, but only the former slowed progression of renal failure. We conclude that ZSF1 rats represent the best available rat model to study nephropathy from type 2 diabetes and that the renal lesions are associated with increased oxidative stress and decreased renal nitric oxide availability.


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Despite several recent advances, the pathogenesis of diabetic nephropathy (DN) remains far from clear.¹ The lack of suitable diabetic animal models that develop nephropathy akin to human disease is a major barrier for progress in this field. Notwithstanding the contribution to mechanistic pathways, the in vitro models to study DN have compounded the problem, necessitating more dependable in vivo animal models. Furthermore, the growing epidemic of metabolic syndrome warrants need for animal models that develop hyperlipidemia and obesity in addition to maturity-onset diabetes to mimic complications of human diabetes and metabolic syndrome. We report here a genetically engineered rat that developed features of DN as well as full-blown metabolic syndrome. Furthermore, decreased renal nitric oxide (NO) production was noted in these rats, consistent with our previous observations in mesangial cell cultures in vitro that were exposed to high ambient glucose concentrations. These changes were associated with increased oxidative stress, which partly accounted for decreased NO levels.

Received August 23, 2006. Accepted June 26, 2007.
Published online ahead of print. Publication date available at www.jasn.org.

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RESULTS

The natural course of DN in obese ZSF1 rats was studied for 12 wk from 8 to 20 wk of life. The data showed that the ZSF1 rats significantly increased total body weight (Figure 1), BP, serum creatinine, protein excretion rate, serum cholesterol, triglycerides, and glucose levels by 20 wk (Table 1); however, the lean ZSF1 rats had modest systolic (156 ± 14 mmHg) and diastolic (101 ± 9 mmHg) hypertension but no obesity, hyperglycemia, hyperlipidemia, or proteinuria. All of the obese rats had glycosuria as measured by dipstick and by chemical measurements (254 ± 76 mg/dl). The systolic BP increased from 132 ± 19 to 176 ± 23 mmHg and the diastolic from 88 ± 8 to 103 ± 11 mm/Hg by the 20th week. The rats were clearly hyperglycemic at the 20th week; the plasma glucose levels were 196 ± 22 mg/dl. The most striking feature was the severity of the hyperlipidemias; the total cholesterol was 525 ± 121, and the serum triglyceride levels were 1956 ± 254 mg/dl, rendering the plasma very lipemic. The serum creatinine increased from 0.77 ± 0.19 to 1.47 ± 0.25 mg/dl, and the GFR as measured by endogenous creatinine clearance decreased from 5.72 ± 0.19 to 1.87 ± 0.43 L/kg per d (Table 1).

Table 1. Characteristics of ZSF1 rats at 8 and 20 wk

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>8 Wk (n = 12)</th>
<th>ZSF1 (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>365 ± 35</td>
<td>695 ± 47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>132 ± 19</td>
<td>176 ± 23&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>88 ± 8</td>
<td>103 ± 11&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>126 ± 12</td>
<td>196 ± 22&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.77 ± 0.19</td>
<td>1.47 ± 0.25&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine clearance (L/kg per d)</td>
<td>5.72 ± 0.19</td>
<td>1.87 ± 0.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proteinuria (mg/kg per d)</td>
<td>239 ± 39</td>
<td>534 ± 54&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urinary 8-OHdG (ng/d per kg)</td>
<td>746 ± 110</td>
<td>552 ± 336&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>276 ± 87</td>
<td>525 ± 121&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>369 ± 62</td>
<td>1956 ± 254&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tubular casts (0 to 4)</td>
<td>0</td>
<td>3+&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tubular dilation/atrophy (0 to 4)</td>
<td>0</td>
<td>4+&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glomerular sclerosis (0 to 4)</td>
<td>0</td>
<td>1+</td>
</tr>
<tr>
<td>Arteriolar sclerosis (0 to 4)</td>
<td>0</td>
<td>4+&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.01 versus 8-wk-old ZSF1 rats.
<sup>b</sup> P < 0.001 versus 8-wk-old ZSF1 rats.

The renal histology in ZSF1 rats at 20 wk showed significant tubular atrophy, dilation often with intratubular protein casts, and arteriolar sclerosis (Figure 2) as compared with lean ZSF1 rats and also 8-wk-old obese ZSF1 rats (Figure 3). The renal presence of 8-hydroxydeoxyguanosine (8-OHdG) by immunohistochemistry showed a significant increase compared with 8 wk of age (Figure 4). Urinary excretion of 8-OHdG was increased many fold by the 20th week of life in ZSF1 rats, indicating increased mitochondrial oxidative stress in these rats (3522 ± 336 versus 746 ± 110 ng/d per kg at 8 wk of age; P < 0.01). Expression of endothelial NO synthase (eNOS) and to some extent inducible NO synthase (iNOS) was decreased in the glomeruli and tubules of ZSF1 rats compared with ZSF1 rats at 8 wk of age (Figure 5). In addition, the urinary excretion of NOx was significantly decreased at 20 wk of age compared with 8-wk-old rats.

The urinary NO metabolites (NOx) were significantly decreased in obese ZSF1 rats compared with lean ZSF1 rats at 20 wk of age (5.6 ± 11 versus 22.5 ± 3.9 μM/kg body wt; P < 0.001); however, this was associated with an increase in peroxynitrite formation in renal cortical homogenates (1.376 ± 0.0076 in obese ZSF1 versus 0.515 ± 0.0025 μM/g renal tissue in lean ZSF1 rats). The mesangial expansion and thickening of the glomerular basement membrane (GBM) were more evident on electron microscopy (Figures 6 and 7). The mesangial matrix volume was 1.9 times greater in the obese ZSF1 rats at 20 wk than the obese ZSF1 rats at 8 wk and 1.7 times greater than lean ZSF1 rats at 20 wk (Figure 7). Although tubulointerstitial changes were more obvious than the glomerular changes at 20 wk, the latter in the form of glomerulosclerosis and atrophy were more prominent in older rats at 40 wk (data not shown).

Insulin treatment with insulin minipumps controlled hyperglycemia and glycosuria and decreased proteinuria and slowed renal failure significantly. The blood glucose level decreased from 196 ± 22 to 136 ± 13 mg/dl at the 20th week, and...
the serum creatinine, proteinuria, and 8-OHdG were also significantly decreased (Table 2). The urinary NOx was higher than in obese untreated ZSF1 rats (14.5 ± 3.7 versus 5.6 ± 11 μM/kg; P < 0.05). The glomerular and tubulointerstitial lesions were minimal except for arteriolar thickening representing the hypertensive effects.

To understand the significance of oxidative stress in the renal disease of this rat model and to examine the relation of oxidative stress and renal NO levels, we compared obese ZSF1 rats with a group that were treated with lipoic acid (ALA). The results showed that whereas 8-OHdG was decreased (to 12.1 ± 4.1 versus 5.6 ± 1.1 μM/kg; P < 0.05) in obese ZSF1 rats at 20 wk. The renal lesions and the serum creatinine were not very different from the obese ZSF1 rats, although proteinuria decreased significantly (198 ± 32 versus 534 ± 54 in obese rats; P < 0.01).

Because ZSF1 rats exhibited significant hyperlipidemia, we examined its role in renal disease by feeding another group of rats with niacin mixed in the rat food. At 20 wk, these rats showed marked improvement in triglyceride (35 ± 4 versus 1956 ± 254 in untreated rats; P < 0.001) and cholesterol levels (245 ± 33 versus 525 ± 121 in untreated rats; P < 0.01), but neither the renal function nor the renal histology changed from the untreated ZSF1 rats (see Table 2).

**DISCUSSION**

Although DN has emerged as the leading cause of ESRD, insufficient understanding of pathogenesis accounts for the ineffectiveness of currently available intervention and is significantly affected by the lack of a suitable animal model to study this devastating disease. In this study, we report a new rodent
model of DN that closely mimics human renal disease in type 2 diabetes. The obese ZSF1 rats at 7 wk are normotensive and have normoglycemic and normal renal function; however, the body weights were twice the normal weights for Sprague-Dawley rats of comparable age (Figure 1). A lean counterpart of ZSF1 [(OB) Gmi Crl-fa/H11002/fa/H11002], used as a control, had normal renal structure and function despite modest systemic hypertension.

At 20 wk of age, the obese ZSF1 rats displayed renal failure, severe proteinuria, and hypertension. Together with severe hyperglycemia, the renal disease mimicked nephropathy of type 2 diabetes in humans. Although originally described by Tefovic et al.\(^2\) and additional literature published recently,\(^3\) the characterization of renal disease in ZSF1 rats remains far from complete. The presence of severe hyperlipidemia, especially hypertriglyceridemia, is particularly noteworthy because this degree of hyperlipidemia is not typical of human DN. The presence of moderate to severe hypertension distinguishes ZSF1 rats from Zucker Diabetic Fatty (ZDF) rats and makes the former a more suitable model to study human DN. The lean ZSF1 rats were normal at 20 wk except for modest systemic hypertension.

The renal histology in ZSF1 rats at 20 wk demonstrated thickening of GBM, mesangial expansion, and severe tubular dilation and atrophy. These changes closely resemble human DN, rendering ZSF1 rats a suitable rat model to examine nephropathy of human diabetes. The absence of hydronephrosis in all kidneys examined in this study from ZSF1 rats establishes the superiority of this species over ZDF rats, because the latter often exhibit hydronephrosis and milder hypertension. Furthermore, the thickening of the GBM and mesangial expansion were not documented in the early renal lesions of diabetes in ZDF rats.

Abnormalities of renal NO generation have been linked to pathogenesis of renal disease in diabetes.\(^8,9\) Notwithstanding the complexity and confusions related to the published data in this field, it is clear that whereas in the early phases of DN the NO production and NOS isoform expression in the kidney is upregulated, there is a progressive decline in NO production and specifically NO bioavailability in the kidney with advancing renal failure.\(^10\) Our data from this newer model of DN supports the literature\(^11–13\) in that not only the NOS expression (especially eNOS and iNOS) was downregulated, but also the total renal NO production as reflected by urinary NO\(_x\) levels were sig-
significantly decreased. In this rat model, decrease in NO levels is further contributed by use of NO in the formation of peroxynitrite in renal tissues.

The emergence of 8-OHdG as a measure of DNA oxidation has led to a new way of monitoring oxidative DNA damage that is often observed in diabetes, cardiovascular diseases, and cancer. The nuclear and mitochondrial DNA is a common target of oxidative damage, and guanine is the most nucleic acid base prone to oxidation, which adds a hydroxyl group to the eighth position of the guanine molecule. When such damaged DNA is repaired, 8-OHdG is produced and excreted unchanged in the urine.

Previous studies in experimental diabetes demonstrated decreased renal NO levels as a consequence of increased oxidative stress, primarily as a result of enhanced expression of superoxide dismutase and catalase. In those studies, NOS expression was not studied. Although we did not examine the superoxide dismutase and catalase in our studies and expect some increase in activity, we showed that renal NO production is decreased partly as a result of enhanced oxidative stress and partly as a result of decreased NOS expression. The oxidative stress is evident in enhanced products of mitochondrial oxidative stress and also increased formation of peroxynitrite in the kidney. Although oxidative stress may be an important factor for decreased NO levels, it may not be a major factor for progression of renal disease. This is illustrated by findings of progressive renal failure despite decreased proteinuria in rats that were treated with ALA. Oxidative stress did not play a major role in this rat model of diabetes, specifically in this early phase of nephropathy. Whether there is a greater role in more advanced nephropathy in this model is unknown. These findings are in agreement with most clinical observations that antioxidant therapy fails to retard the progression of renal failure in DN.

One obvious question that stems from characteristics of ZSF1 rats is whether hyperlipidemia contributes to DN in this model. We studied the effects of lipid-lowering therapy on renal disease. We selected niacin, partly because the primary abnormality is severe hypertriglyceridemia and partly because statins may affect renal function through mechanisms other than lipid-lowering effects. Our data demonstrate that despite lowering triglycerides and cholesterol significantly, the renal failure and proteinuria continued with oxidative load and decreased renal NO levels. These data are in conformity with clinical observations in human diabetes.

Treatment of diabetic state with insulin retards the progression and often prevents the development of diabetic nephropathy in human diabetes. In obese ZSF1 rats, administered insulin controlled hyperglycemia and slowed both structural and functional components of renal disease, with amelioration of proteinuria and oxidative stress and increased urinary NOx levels.

Oxidative stress was incriminated as an important mediator in the pathophysiology of DN. Both hyperglycemia and activation of the renin-angiotensin system play a role in the generation of reactive oxygen species (ROS). Observations highlight the relevance of mitochondrial oxidative stress in diabetic vascular complications. Our data from ZSF1 rats clearly demonstrated increased mitochondrial oxidative stress in the kidney as reflected by increased urinary 8-OHdG excretion and by the increased renal presence of 8-OHdG by immunohistochemistry. It is likely that atherosclerosis and hyperglycemia may cause elevation of 8-OHdG; however, the magnitude of changes

Table 2. Characteristics of untreated and treated obese ZSF1 rats at 20 wk of age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Serum Creatinine (mg/d per kg body wt)</th>
<th>Proteinuria (mg/d per kg body wt)</th>
<th>Urine NOx (μM/kg body wt)</th>
<th>8-OHdG (ng/d per kg body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.47 ± 0.25</td>
<td>534 ± 54</td>
<td>5.6 ± 1.1</td>
<td>5322 ± 336</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.94 ± 0.19a</td>
<td>112 ± 28a</td>
<td>14.5 ± 3.7b</td>
<td>2934 ± 978b</td>
</tr>
<tr>
<td>ALA</td>
<td>1.31 ± 0.31</td>
<td>198 ± 32a,b</td>
<td>12.1 ± 4.1b</td>
<td>1827 ± 353b</td>
</tr>
<tr>
<td>Niacin</td>
<td>1.37 ± 0.22</td>
<td>396 ± 36</td>
<td>8.8 ± 2.9</td>
<td>3955 ± 786</td>
</tr>
</tbody>
</table>

aP < 0.01 versus untreated obese ZSF1 rats.
bP < 0.05 versus untreated obese ZSF1 rats.

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(almost eight-fold rise) seen in our study cannot be accounted for by either of those conditions, implicating renal disease as an important contributor to oxidative stress and consequent elevation of 8-OHdG. Aging is associated with oxidative stress (usually a two-fold increase is common)\textsuperscript{26} but not to the extent seen in these studies of ZSF rats with renal failure. Recent studies showed that urinary 8-OHdG not only is a measure of oxidative damage to DNA but also correlates with and is a marker of total body oxidative stress.\textsuperscript{25} Additional studies have shown that urinary 8-OHdG correlated with the severity of DN and retinopathy\textsuperscript{27} and also with future development of DN.\textsuperscript{28} Others\textsuperscript{29} have shown that in streptozotocin-induced diabetic rats, measurement of levels of 8-OHdG from mitochondrial DNA was higher in both renal cortices and papillae and so were the urinary 8-OHdG levels. In contrast, the levels of 8-OHdG in nuclear DNA were not elevated.

The increased ROS in the kidney, especially the superoxide radicals, react with NO to form peroxynitrite, which in turn binds to tyrosine and other protein residues, yielding highly cytotoxic compounds such as nitrotyrosine in the renal and other vascular tissues.\textsuperscript{30} Our findings of increased peroxynitrite in renal cortical homogenates of obese ZSF1 rats strongly support the conclusion that this rat model of DN is associated with severe oxidative stress. This pathway may account partly for the decreased bioavailability of NO in the kidney.

We examined ZSF1 rats, a newer rodent species, for validating as a better model of renal disease in type 2 diabetes and metabolic syndrome. ZSF1 rats developed obesity, hypertension, diabetes, severe hyperlipidemia, and proteinuric renal failure. Renal histology confirmed lesions similar to human diabetic renal disease. Furthermore, the nephropathy was associated with increased mitochondrial oxidative stress and reduced NO bioavailability in the kidney. We conclude that ZSF1 rats constitute the best currently available rodent model to study the nephropathy of type 2 diabetes.

\section*{CONCISE METHODS}

\textbf{ZSF\textsubscript{1} Rats: A Newer Model for DN}

Although several animal models have been used to study diabetes and DN, significant deviations from the human disease limit their usefulness. Specifically, there are no suitable models of nephropathy in type 2 diabetes. The ZDF rat is characterized by obesity, insulin resistance, type 2 diabetes, hyperlipidemia, and proteinuria; however, this model differs from human DN by development of hydronephrosis and the lack of hypertension, which complicates etiopathogenesis of renal disease. A hybrid was developed between a female ZDF and a lean male SHHF (spontaneously hypertensive heart failure) rat, resulting in ZSF1 [(OB) Gmi Crl-fa/fa] rats, which exhibit hypertension (by 12 wk of age) in addition to all of the features of ZDF rats. These rats turn obese shortly after birth and develop diabetes (8 wk), renal disease with proteinuria, and renal failure by 20 wk, with severe renal failure and nephrosis by 46 wk, and die with end-stage renal failure at the age of 12 mo.\textsuperscript{2} Unlike the parental SHHF strain, ZSF1 rats do not develop congestive heart failure\textsuperscript{11}; therefore, the ZSF1 rats (Charles River Laboratories, Indianapolis, IN) constitute the currently best available model to study renal disease in type 2 diabetes. Only male ZSF1 rats were used for this study. A lean counterpart of ZSF1 [(OB) Gmi Crl-fa/−] rats was used as a control.

We examined obese and lean ZSF1 rats obtained at 7 wk of age (200 to 250 g body wt). Studies were initiated at 8 wk, and rats were killed at 20 wk to harvest the kidneys. The rats were fed Purina 5008 chow (Purina Laboratories, St. Louis, MO) to maintain hyperglycemia. A group of 12 rats were used as baseline controls, and after BP readings and 24-h urine samples were obtained, they were killed at 8 wk. Another 12 rats each of obese and lean types were studied from 8 to 20 wk. Body weights, BP, and blood glucose were monitored on a weekly basis. Systolic and diastolic BP was measured noninvasively by tail-cuff plethysmography (Non-Invasive Blood Pressure System; Harvard Apparatus, Holliston, MA). A 24-h urine collection was obtained from all rats at 8 wk and again at 20 wk by housing them in metabolic cages. The urine was examined at the beginning and the end of the study for glycosuria, creatinine clearance, protein excretion rate, 8-OHdG, and NO metabolites. The serum was examined at the start and the end of the study for creatinine, glucose, triglycerides, cholesterol, and NO metabolites.

\section*{General Conditions of Animal Care during the Study Period}

The rats were maintained humanely in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All of the rats were housed in individual metabolic cages and inspected daily by the staff of the animal care facility, who recorded their weekly weights, and research staff recorded BP. The cages have provision to collect the urine and feces separately. Daily food and water intake was monitored. Blood draws were performed the first day of every week and at the end of the study through cannulation into tail vein.

\section*{Assay for Urinary and Renal 8-OHdG}

A relatively simple ELISA procedure with an excellent correlation with HPLC ($r = 0.833$, $P < 0.0001$) was developed.\textsuperscript{32} A commercial ELISA kit is now available (Oxis Research, Portland, OR), and we used this kit to estimate urinary 8-OHdG in Sprague-Dawley rats in a recent study.\textsuperscript{33} The technique and procedural details are simple, and the assay is reliable and reproducible. The presence of 8-OHdG in the renal cortical homogenates was assessed by immunohistochemical evaluation using a specific antibody directed against 8-OHdG (Oxis Research). Tissue levels of 8-OHdG correlate best with the oxidative stress in that organ.

\section*{Peroxynitrite Formation in Renal Tissue}

The presence of peroxynitrite in the renal tissues was determined spectrophotometrically as follows. The standards were prepared by addition of 2.5 $\mu$L of 180 mM solution of peroxynitrite to 1 ml of NaOH solution, and absorbance was read immediately at 302 nm in spectrophotometer. The peroxynitrite in renal cortical homogenates was assessed by measurement absorbance in homogenates (1000 $\mu$L of...
homogenate used) prepared as described previously. The peroxynitrite content was calculated using the standard curve and expressed as \( \mu \text{M/g kidney tissue} \).

**Animal Protocols**

For examination of the effects of insulin, antioxidant, and lipid-lowering treatment on renal function and lesions; urinary \( \text{NO}_2^- \) and oxidative stress, a total of four groups of rats were studied: (1) Control group, (2) ALA therapy, (3) niacin therapy, and (4) insulin therapy. To examine the effect of chronic hyperglycemia on renal disease and function, we treated the rats with insulin by installing subcutaneous osmotic minipumps. Blood glucose levels were maintained at <150 mg/dl by using an osmotic minipump (Model 2004; Alzet, Cupertino, CA), filled with porcine insulin (20 pmol/kg per min) in a glycerine ethanol (1:1 [vol/vol]) mixture, implanted subcutaneously for 4 wk. After 4 and 8 wk, the old minipumps were removed and new ones were inserted. For rats showing persistent hyperglycemia (>250 mg/dl) a second insulin pump was installed, and for blood glucose levels between 150 and 250 mg/dl, subcutaneous insulin was injected to maintain the blood glucose levels <150 mg/dl using a sliding scale (for 200 to 250 mg/dl, one third of a unit per day; for 150 to 200 mg/dl, one fourth of a unit per day).

To examine the role of oxidative stress, we tested the effects of addition of 400 mg/kg ALA to rat chow on one group of ZSF1 obese rats from 8 to 20 wk. The effects of hyperlipidemia on renal structure and function were evaluated by feeding chow mixed with niacin at 400 mg/kg diet to another group of ZSF1 rats from 8 to 20 wk.

**Measurement of GFR and Other Renal Functional Parameters**

Rats were placed in metabolic cages during the period of study when 24-h urine collections were done, and endogenous creatinine clearance was used as a measure of glomerular filtration. Urine and serum creatinine was measured by a creatinine autoanalyzer (Beckman Instruments, Fullerton, CA). Total protein was measured by a spectrophotometric assay as modified by Lowry using bichinchoninic acid reagent (Pierce, Rockford, IL).

**BP Monitoring**

Systemic arterial BP measurements were performed noninvasively by using tail-cuff plethysmography (Model SC1000; Harvard Apparatus). Systolic, diastolic, and mean BP as well as heart rate measurements were performed twice daily in the mechanistic studies and once weekly in the intervention studies. In each setting, the mean of three readings were obtained for each parameter measured. The data were saved automatically on a spreadsheet through a computer interface.

**Measurement of Renal NO Synthesis**

Renal NO synthesis was assayed by measurement of \( \text{NO}_2^- \) and \( \text{NO}_3^- \) content (\( \text{NO}_x \), in the samples using an NO analyzer (Ionics Instruments, Boulder, CO) using the principle of chemiluminescence, which yields more precise and reproducible measurements of NO than other techniques. The details of this technique are described in our previous publication.\(^3\)\(^4\)

**Western Blots to Analyze iNOS and eNOS Expression**

The renal cortical and medullary homogenates from one of the harvested kidneys were used for NOS Western blots. The kidney was harvested in PBS and centrifuged at 1000 rpm for 5 min to sediment cells into a pellet. The complete details of the protocol were described in our previous publication.\(^6\) Immunoblots of the iNOS and eNOS were visualized with enhanced chemiluminescence.

**Renal Histologic Studies and Morphometric Analysis**

At the end of 20 wk, all rats were killed and kidneys were harvested for histopathologic examination by light and electron microscopy. The severity of renal lesions were scored from 0 to 4 by a pathologist who was blinded to the design of the study.

**Measurement of GBM Thickness Measurement**

The electron microscopy photomicrographs of glomeruli were analyzed using the image analysis software associated with AMT (Advanced Microscopy Techniques Corp., Danvers, MA) charged-coupled device system installed on the electron microscopy system. The mean \( \pm \) SEM was calculated from a minimum of six measurements for each datum result.

**Measurement of Glomerular Mesangial Volume**

The electron microscopic photomicrographs were acquired through LSM 510 v3.2, and the mesangial volume was analyzed using the area function analysis in Image J software using LSM Reader 3.2d plug-in (http://rsb.info.nih.gov/ij/). The mean \( \pm \) SEM was calculated from a minimum of six or more measurements for each datum result.

**Statistical Analyses**

Data were analyzed using commercial software (Slide Write for Windows; Advanced Graphics Software, Inc., Encinitas, CA) for statistical significance. All data are expressed as means \( \pm \) SEM. Groups of samples were compared using either independent t test or repeated measures ANOVA as appropriate. In the case of Western blots, quantitative analysis was performed by densitometry, and the most representative of the autoradiographs was used for illustration.

**ACKNOWLEDGMENTS**

The study was funded by a grant from the American Heart Association-Texas Affiliate (grant in aid 0255761Y) and Larry and Jane Woirhaye Memorial Renal Research Foundation.

Parts of this study were presented at the annual meeting of the Southern Society of Clinical Investigations, March 3–5, 2006; Atlanta, GA.

We sincerely acknowledge Tiffany Hough, Mary-Kathryn Hester Department of Pathology, for the help rendered in tissue sections and Dr. Perrett-Gentil, veterinarian in the Department of Laboratory Animal Resources, in the conduct of these experiments.
REFERENCES


DISCLOSURES

None.