Substantial evidence suggests that the intrarenal renin-angiotensin system (RAS) plays a role in the pathogenesis of diabetic nephropathy. Although the glomerular RAS is activated in the streptozotocin (STZ)-diabetic rat, the status of the glomerular RAS in the Zucker diabetic fatty (ZDF) rat, which is a commonly used genetic model of diabetes, is not known. Angiotensinogen (AGT), angiotensin II (Ang II), angiotensin converting enzyme (ACE), and angiotensin converting enzyme 2 (ACE2) were measured in glomeruli isolated from 4-week-old STZ-diabetic rats and 32-week-old ZDF rats. Glomerular injury was evaluated by histopathologic methods. Both STZ-diabetic and ZDF rats exhibited marked hyperglycemia and renal hypertrophy, but only ZDF rats demonstrated proteinuria and glomerulosclerosis. Glomerular AGT and Ang II levels were increased significantly in STZ-diabetic compared with nondiabetic control rats, accompanied by a reduction in ACE2 activity. In contrast, glomerular AGT, Ang II, and ACE2 were similar in ZDF rats and lean controls. ACE levels were not affected by diabetes in either diabetic model. In conclusion, the glomerular RAS is activated in the STZ diabetic rat but not in the ZDF rat despite a similar degree of hyperglycemia. The mechanism of nephropathy in the ZDF rat may involve factors other than hyperglycemia and RAS activation, such as hypertension and hyperlipidemia. (Translational Research 2008;151:208–216)

Abbreviations: ACE2 = angiotensin converting enzyme 2; AGT = angiotensinogen; Ang = angiotensin; ARB = angiotensin receptor blocker; ELISA = enzyme-linked immunosorbent assay; OZR = obese Zucker rat; PAS = periodic acid-Schiff; PBS = phosphate-buffered saline; RAS = renin-angiotensin system; SE = standard error of the mean; STZ = streptozotocin; TMB = 3,3′,5,5′-tetramethylbenzidine dihydrochloride; ZDF = Zucker diabetic fatty

Diabetic nephropathy is characterized by accumulation of mesangial matrix in the glomerulus that leads to glomerulosclerosis and renal failure. Although the mediators of mesangial matrix expansion in diabetic nephropathy have not been identified fully, a prominent role for the peptide angiotensin (Ang) II has been suggested based on clinical trials in diabetic patients with nephropathy. Treatment with angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) are beneficial in slowing the progression of glomerulosclerosis in patients with both type 1 and type 2 diabetes.\(^1,3,5\) Activation of the intrarenal renin-angiotensin system (RAS) in diabetic patients is suggested by an increased renal vasodilator response to ACE inhibition and angiotensin blockade.\(^5\)

The streptozotocin (STZ)-induced diabetic rat is a model of type 1 diabetes characterized by hyperglycemia and insulin deficiency. Even though proteinuria and glomerulosclerosis develops slowly in this model,
Determination of the role of the intrarenal renin-angiotensin system (RAS) in diabetic nephropathy requires appropriate animal models. Our results suggest that the glomerular RAS is activated in rats administered the islet cell toxin streptozotocin (STZ-diabetic rats) but not in Zucker diabetic fatty (ZDF) rats, a genetic model, despite a similar degree of hyperglycemia.

**Translational Significance**

The glomerular lesions in ZDF rats are those of focal and global glomerulosclerosis and thus are not typical of human diabetic nephropathy. Thus the ZDF rat may not be an appropriate model to investigate the role of the glomerular RAS in glomerular injury in diabetes.

Methods

**STZ-diabetic rat.** Male Sprague-Dawley rats (150–200 g) were made diabetic by a single intravenous injection of STZ (Sigma, St. Louis Mo; 60 mg/kg body weight) into the tail vein. Control rats matched for age and body weight received an equal volume of the vehicle. The diabetic state of the animal was confirmed by the demonstration of nonfasting blood glucose levels > 250 mg/dL 24 h after STZ injection. Food and water intake were given ad libitum. Diabetic rats were assigned to either an untreated group or an insulin-treated group. In the insulin-treated group, rats were implanted with miniosmotic pumps to deliver 3 units of insulin/day. Animals were sacrificed after 4 weeks of diabetes.

**ZDF rat.** Male ZDF rats (fa/fa) and lean control rats (+/?) were purchased from Charles River Laboratories, Inc. (Wilmington, Mass) at 21 weeks of age and maintained on a 5008 Purina diet (Purina, St. Louis, Mo). Hyperglycemia was confirmed by measurement of blood glucose, and no insulin was administered. Animals were sacrificed at 32 weeks of age. Principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed.

**Measurement of physiologic parameters.** The body weights of control and diabetic rats were measured once a week, and blood glucose and urinary protein were measured every 2 weeks. For measurement of blood glucose levels, blood was collected in conscious rats via a needle prick in the tail vein and was analyzed using a glucometer. Twenty-four-hour urine samples were collected from rats housed individually in metabolic cages with free access to water and food. After 24-h urinary volume had been measured, the urine samples were centrifuged and the supernatant was stored at −20°C until assayed for protein using the BIO-RAD method.

**Isolation of glomeruli.** Animals were sacrificed, and kidneys were rapidly removed, weighted, and minced. Glomeruli were isolated by sequentially sieving at 4°C using ice-cold phosphate buffered saline (PBS) buffer. Glomerular suspensions were examined by light microscopy and were found to contain >95% of glomeruli. The suspensions were centrifuged, and the pellet was resuspended in ice-cold PBS, sonicated, and recentrifuged at 13,000 g for 20 min. The resulting supernatant was used for various measurements as outlined below.

**Measurement of angiotensinogen.** Angiotensinogen levels in glomerular extracts were measured by a competitive enzyme-linked immunosorbent assay (ELISA) described in our recent publication. Briefly, a 96-well plate was coated overnight at 4°C with 4 μg/mL of angiotensinogen (1-14) (Sigma). In the wells, samples or standards were mixed with antiangiotensinogen antibody (1:10,000) and incubated at room temperature for 2 h followed by washings and incubation with a peroxidase-conjugated secondary antibody (1:1000) for 1 h. The reaction was developed using 3,3',5,5'-tetramethylbenzidine (TMB) and H₂O₂ stopped with 2N HCl, and read at 450 nm using an ELISA reader (Molecular Devices, Sunnyvale, Calif). Angiotensinogen levels in samples were calculated from a standard curve using angiotensinogen (1-14) as the standard.
Table I. Physical parameters of STZ-diabetic and ZDF rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 6)</th>
<th>STZ (n = 7)</th>
<th>STZ-T (n = 5)</th>
<th>Lean (n = 5)</th>
<th>ZDF (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose, mg/dL</td>
<td>114 ± 6</td>
<td>480 ± 27*</td>
<td>313 ± 92*</td>
<td>115 ± 8</td>
<td>451 ± 18‡</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>330 ± 6</td>
<td>243 ± 17*</td>
<td>269 ± 23*</td>
<td>269 ± 5</td>
<td>470 ± 9</td>
</tr>
<tr>
<td>Kidney wt, (g/kg body wt)</td>
<td>6 ± 0.1</td>
<td>11 ± 0.7*</td>
<td>9 ± 0.5*</td>
<td>5 ± 0.4</td>
<td>12 ± 0.5‡</td>
</tr>
</tbody>
</table>

*P < 0.05 vs control rats.
†P < 0.05 vs lean rats.

After washings, streptavidin-conjugated horseradish peroxidase was added to the wells and was allowed to bind to the immobilized primary antibody and biotinylated Ang II peptide complex in the wells. The final reaction was developed with TMB and H₂O₂ substrate, terminated with 2N HCl, and read at 450 nm using an ELISA reader (Molecular Devices). Because the color intensity in the well depends on the quantity of biotinylated Ang II peptide and immobilized antibody complex, less intensity of the color denotes greater levels of Ang II in the sample. Ang II levels in the samples were calculated from the standard curves run with each assay.

Our previous studies have shown that the Ang II ELISA is specific to Ang II because when endogenous angiotensin peptides were separated first using HPLC and the fractions were tested by ELISA, immunoreactivity to Ang II was detected only in Ang II peptide-containing fractions. The breakdown of the substrate was confirmed using purified ACE2 enzyme (R&D Systems). To measure ACE2 activity, 100 µL of glomerular extract sample was added to 890 µL of assay buffer that contained 75-mmol/L Tris-HCl (pH 7.5), 1-mol/L NaCl, and the reaction was initiated by the addition of 10 µL of the fluorogenic substrate (10-µmol/L final concentration). The reaction mixture was incubated at room temperature, and fluorescence was measured at an excitation wavelength of 320 nm and emission wavelength of 405 nm using a spectrofluorometer (Perkin Elmer) from time 0 to 4 h. Examination of the time curves from the glomerular extracts showed that the formation of the product was linear up to 1 h after which it began to plateau, reaching the maximum at 4 h. Based on these data, enzyme activity was calculated from the 15 min time-point (initial velocity time-point) for all samples. Protein concentration in the sample was determined by the BIO-RAD method, and fluorescence units were normalized to per mg protein concentration. No change in fluorescence release was observed when the substrate was incubated with assay buffer without tissue up to 24 h, which indicates absence of spontaneous substrate breakdown under the assay conditions.

**Dot blot assay for the measurement of ACE and ACE2 protein.** Samples of glomerular extracts from diabetic and nondiabetic rats (10 µL that contain 15, 7.5, and 3.75 µg of protein) were blotted in duplicate onto a nitrocellulose membrane. The nitrocellulose blot was baked at 37°C for 30 min to allow the proteins to bind to the membrane. The blot was treated sequentially as follows: 1) blocked by Tris-buffered saline (TBS, pH 7.6) containing 0.1% Tween-20 and 5% wt/vol nonfat dry milk for 1 h, 2) washed with TBS-1% containing 0.1% Tween-20 for 30 min, 3) reacted with rabbit anti-ACE or rabbit anti-ACE-2 antibody for 1.5 h, 4) washed for 30 min and reacted with biotin labeled anti-rabbit IgG for 1 h, 5) washed for 30 min and reacted with strepavidin-peroxidase for 1 h, 6) washed and developed reaction using diaminobenzidine (Sigma) and hydrogen peroxide as substrate. The intensity of reaction on the blot membrane increased progressively from a greater to a lesser amount of loaded protein. For quantification, the blot reactions with 15-µg loaded protein were scanned in JPEG format and analyzed by image analyses (Image J Software; JAVA imaging software provided by the National Institute of Health and available at [http://rsb.info.nih.gov](http://rsb.info.nih.gov)). Briefly, the images were converted to greyscale, and an average density over the blot reaction area was determined by taking the arithmetic mean.

After incubation, the reaction was terminated with 2N HCl, and absorbance at 450 nm was determined by an ELISA reader (Molecular Devices). The intensity of reaction on the blot membrane was determined by taking the arithmetic mean of each density value.
of densities from 20 random spots that cover the blot reaction area. The mean density of the reaction area was then multiplied by the total area of the blot reaction (in pixels) to yield the magnitude of the overall reaction. The values obtained represented the relative densities of the blot reactions.

**Determination of glomerular injury.** Extent of glomerular injury was assessed by 1 investigator (A.K.S.) in a blinded fashion. Paraffin sections of rat kidneys were stained with periodic acid-Schiff (PAS) reagent to highlight the extracellular matrix and were counterstained with hematoxylin to identify the cells. From each kidney section, 30 randomly selected glomeruli were examined for injury. Each glomerulus was assigned a score for lesions as follows: 0 for normal glomerular architecture; 1 for capillary dilatation and/or mild extracellular matrix expansion; 2 for segmental and/or global sclerosis with capillary collapse. For each rat, the mean glomerular injury score was calculated as the total score of 30 glomeruli divided by 30.

**Statistical analysis.** Values are presented as means ± standard error of the mean (SE), and n denotes the number of rats for each group. Results are compared using 1-way analysis of variance, and Student’s t-test; P < 0.05 is accepted as significant. For figures, data are presented as percentage of control (nondiabetic and lean rats for STZ-diabetic and ZDF rats, respectively).

**RESULTS**

**Blood glucose.** Blood glucose levels in STZ-induced diabetic rats (type 1 diabetes) were significantly greater than those of nondiabetic controls (Table I). The increase in blood glucose was observed as early as 24 h after STZ injection and was maintained throughout the 4-week study period. STZ-diabetic rats treated with insulin (STZ-T) showed a substantial decrease in blood glucose levels compared with STZ-diabetic rats; however, blood glucose levels remained significantly greater in STZ-T rats than in nondiabetic controls (Table I).

ZDF (type 2 diabetes) rats exhibited significantly greater blood glucose levels compared with their respective lean controls (Table I). The marked hyperglycemia observed in the ZDF rat was similar to that present in STZ-induced diabetic rats.

**Body weight.** In STZ-induced diabetic rats, hyperglycemia was found to be associated with significant loss of body weight compared with nondiabetic controls (Table I). The weight loss in STZ-diabetic rats was observed over the 4-week study period, and treatment with insulin partially prevented the weight loss. ZDF rats did not lose weight over the study period and showed comparable body weights with those of lean controls at 32 weeks of age (Table I).

**Kidney weight.** The kidney-weight-to-body-weight ratio increased significantly in STZ-diabetic rats compared with nondiabetic controls (Table I). Insulin treatment in STZ-diabetic rats (STZ-T) resulted in a decrease in the ratio of kidney weight to body weight but failed to normalize it completely compared with nondiabetic controls.

Similarly, ZDF rats showed a significant increase in the ratio of kidney weight to body weight compared with lean control rats (Table I). No hydronephrosis was observed in ZDF rats.

**Urinary protein excretion.** STZ diabetic rats did not exhibit an increase in urinary protein excretion compared with nondiabetic controls, and treatment with insulin did not produce any change in urinary protein excretion (Fig 1).

In contrast, ZDF rats showed a marked and highly significant increase in urinary protein excretion compared with lean control rats (Fig 1). This increase in urinary protein excretion was observed throughout the observation period (at 21 weeks, ZDF: 140 ± 18 mg/24 h vs Lean: 14 ± 3 mg/24 h; at 25 weeks, ZDF: 126 ± 24 mg/24 h vs Lean: 13 ± 3 mg/24 h; at 29 weeks, ZDF: 148 ± 14 mg/24 h vs Lean: 18 ± 2 mg/24 h; and at 32 weeks, ZDF 115 ± 16 mg/24 h vs Lean: 13 ± 1 mg/24 h).

**Kidney morphologic changes.** The glomerular histological findings from PAS-stained kidney sections of STZ-diabetic and ZDF rats are shown in Fig 2, A and B, respectively. In 4-week-old STZ-induced diabetic rats, no remarkable alterations in glomerular morphology were present compared with nondiabetic rats (Fig 2, A). Occasionally, minor changes in glomerular capillaries were observed in STZ-diabetic rats (with or without insulin treatment) as well as in nondiabetic controls. The glomerulosclerosis scores for STZ-diabetic, insulin treated STZ-diabetic, and nondiabetic control rats were similar (Fig 2, A, right panel).
In 32-week-old ZDF rats, glomerular changes were evident in comparison with lean control rats (Fig 2, B). The most common lesions observed were capillary dilation and mesangial expansion accompanied by focal sclerosis and occasionally global sclerosis (Fig 2, B). Although lean rats did not exhibit mesangial expansion or focal sclerosis, mild glomerular capillary dilation was observed in these animals (Fig 2, B). The glomerulosclerosis score was significantly greater in ZDF rats compared with lean controls (Fig 2, B, right panel).

**Glomerular AGT and Ang II.** In STZ-diabetic rats, AGT levels in glomerular extracts were increased significantly (221%) compared with nondiabetic controls (100%) (Fig 3, A). The increase in AGT was accompanied by significantly greater levels of Ang II in STZ-diabetic rats (190 ± 7% for STZ-diabetic rats vs 100 ± 25% for nondiabetic controls) (Fig 3, A). Insulin treatment of STZ-diabetic rats normalized increases in both AGT and Ang II levels (Fig 3, A).

ZDF rats did not show a significant change in glomerular AGT levels compared with lean control rats (Fig 3, B). Also, no difference existed in glomerular Ang II levels between ZDF and lean rats (Fig 3, B). Interestingly, Ang II levels in ZDF rats were substantially less than those in STZ-diabetic rats (ZDF: 26 ± 6 fmol/mg protein vs STZ-diabetic 59 ± 4 fmol/mg protein), indicating that glomerular Ang II levels may be differently regulated in these 2 diabetic models.

**Glomerular ACE and ACE2 activities.** In STZ-diabetic rats, glomerular ACE enzyme activity was similar to that present in nondiabetic controls (Fig 4, A). Insulin treatment in STZ-diabetic rats (STZ-T) increased ACE activity slightly (122%), although not significantly, compared with nondiabetic controls (100%) (Fig 4, A). On the other hand, ACE2 activity was decreased in
STZ-diabetic rats compared with nondiabetic control rats (STZ: 69 ± 17 %; Control: 100 ± 9 %; P = 0.06, n = 5) and was normalized upon insulin treatment (STZ-T: 94 ± 8 vs Control: 100 ± 9%) (Fig 4, A).

Compared with lean rats (100%), ZDF rats showed a slight increase (110%) and decrease (93 %) in ACE and ACE2 activities, respectively (Fig 4, B). These changes were statistically nonsignificant.

Glomerular ACE and ACE2 protein. Figure 5, A shows ACE and ACE2 protein expression in glomerular extracts of STZ-diabetic rats determined by dot blot assay. In STZ-diabetic rats, ACE protein expression did not change compared with nondiabetic controls (Fig 5, A). The density score in STZ-diabetic rats was 106% compared with 100% in nondiabetic control rats (Fig 5, B). Similarly, in insulin-treated STZ-diabetic rats (STZ-T), the density score for ACE protein expression increased slightly and insignificantly (118% vs 100% in controls) (Fig 5, B). ACE2 protein expression in STZ-diabetic rats was somewhat decreased (86%) compared with nondiabetic control rats (100%) (Fig 5, A and B). However, the decrease in ACE2 protein expression of STZ-diabetic rats was not statistically significant. The expression of ACE2 protein in STZ-T was similar to that present in nondiabetic controls (Fig 5, B).

In ZDF rats, glomerular ACE and ACE2 proteins were slightly decreased compared with lean controls, although the differences were statistically insignificant (Fig 5, C and D).

DISCUSSION

The regulation of glomerular Ang II is dependent on the availability of its precursor, AGT, and the activities of ACE and ACE2 that are involved in Ang II formation and degradation, respectively. In confirmation of previous results from our laboratory,10 glomerular Ang
levels were increased in STZ-diabetes, accompanied by increased levels of the substrate AGT. In addition, we found decreased glomerular ACE2 protein levels and activity in STZ-diabetic rats. Because the enzyme ACE2 increases conversion of Ang II to Ang(1-7),16,17 ACE2 downregulation may have contributed to the observed increase in glomerular Ang II levels. However, no changes in any component of the glomerular RAS were observed in the ZDF rat despite similar degrees of hyperglycemia and renal hypertrophy.

Our observations that glomerular ACE2 was decreased in STZ-diabetic glomeruli are consistent with previous reports of reduced ACE2 expression in the STZ-diabetic whole rat kidney.18 Ye et al19 found increased glomerular ACE and decreased ACE2 staining in db/db diabetic mice, and a specific ACE2 inhibitor resulted in increased glomerular fibronectin accumulation. Also, Soler et al20 have demonstrated recently that chronic pharmacologic inhibition of ACE2 activity aggravates glomerular injury in STZ-induced diabetic mice. In another study, deletion of the ACE2 gene in diabetic mice resulted in increased proteinuria and immunostaining for fibronectin compared with diabetic mice with an intact ACE2 gene.21 Thus, it seems that downregulation of glomerular ACE2 may play a role in the increase in glomerular Ang II and matrix protein accumulation observed in diabetes.

Our results seem to suggest an inverse correlation between activation of glomerular Ang II and glomerular injury in the STZ and ZDF rat models of diabetes. STZ-diabetic rats showed increased glomerular Ang II levels but no change in proteinuria and glomerular morphology, whereas ZDF rats exhibited increased proteinuria and glomerular injury but no change in glomerular Ang II. Although STZ-diabetic rats at the time studied (4 weeks post-STZ) did not show evidence of glomerular disease, early changes in glomerular cell physiology are well documented in this model. For instance, previous studies with STZ-diabetic rats have reported increased glomerular mRNA levels of TGF-β122 as well as fibronectin protein and hypertrophy23 within 4 weeks of STZ injection. In cultured glomerular mesangial cells, Ang II increases extracellular matrix synthesis24 and decreases degradation of matrix25; moreover, glucose-induced matrix accumulation can be prevented by Ang II receptor antagonists.25 Taken to-
gether, these findings suggest that activation of glomerular Ang II in the STZ-diabetic rat may trigger initial changes in the glomerular mesangial cell functions related to matrix metabolism.

Even though hyperglycemia in the ZDF rat was of similar magnitude to that observed in the STZ-diabetic rat in our studies, the glomerular RAS was not activated in the ZDF model. Recently, increased whole kidney AGT levels were reported in ZDF rats at 17 weeks of age before histologic changes in the kidneys are evident. However, in these studies, Ang II levels and angiotensin type 1a receptor mRNA levels were not increased significantly compared with values obtained from lean control animals. In the current study with 32-week-old ZDF rats, we did not observe an increase in glomerular AGT and Ang II, although focal and segmental glomerulosclerosis was observed at this time point. Lack of increase in tissue Ang II both before (17 weeks) and after (32 weeks) the development of glomerulosclerosis suggests that the intrarenal RAS is not an etiologic factor in the development of glomerulosclerosis in the ZDF rat. However, the possibility that the RAS is activated at a more intermediate time point cannot be excluded. In this regard, it is noteworthy that increased intrarenal Ang II levels accompanied by increased expression of TGF-β1 and matrix proteins was observed in the obese Zucker rat (the progenitor strain for the ZDF rat) at 21 weeks of age. However, it should be pointed out that the obese Zucker rat (OZR) develops glomerulosclerosis despite the presence of only mild hyperglycemia. This finding suggests a genetic predisposition to glomerulosclerosis in both OZR and ZDF rats that may not be related to blood glucose. Indeed, it has been postulated that hypertension and/or hyperlipidemia rather than hyperglycemia may cause renal changes in the OZR.

Recent studies by Bidani et al. showed mesangial matrix expansion in 36–40-week-old STZ-diabetic rats, although no significant differences in overall glomerulosclerosis score and proteinuria were observed between STZ-diabetic and nondiabetic controls. Thus, the absence of proteinuria and/or histologic changes in 4-week-old STZ-diabetic rats in our study was not unexpected. However, several groups have reported proteinuria during the early phase (1–4 weeks) of diabetes after STZ injection. Some studies have used an albumin ELISA that may detect lower levels of albumin than the standard method for the measurement of total protein used in our studies. Thus, differences in assay techniques may explain these discrepancies. In any event, in comparison with STZ-diabetic rats, it is clear that ZDF rats develop much more marked proteinuria and glomerulosclerosis.

STZ-diabetic rats treated with insulin were still hyperglycemic, which indicates that the dose of insulin used was not sufficient to restore normoglycemia. Despite continued poor glycemic control, this dose of insulin was sufficient to normalize the increased expression of angiotensinogen and Ang II. This finding suggests the possibility that decreased expression of angiotensinogen and Ang II was caused by the effect of insulin alone and not by the decrease in blood glucose. In this regard, insulin per se may modulate the stimulatory effect of hyperglycemia on the RAS. In renal proximal tubular cells, insulin blocks the stimulatory effect of a high glucose level (25 mmol/L) on AGT gene expression. This effect is mediated, at least in part, via the 5′-flanking region of the AGT gene and mitogen-activated protein kinase signal transduction pathway.

In conclusion, despite similar degrees of hyperglycemia, the glomerular RAS is activated in the STZ-diabetic rat but not in the ZDF rat. The reason for these different findings in the 2 models has not been elucidated. However, our findings suggest that the pathogenesis of glomerular injury observed in the ZDF rat is not related to hyperglycemia-induced glomerular RAS activation. Other mechanisms, such as hyperlipidemia, hypertension, genetic susceptibility, or a combination of these factors may be operative.

REFERENCES


