Renal injury in streptozotocin-diabetic Ren2-transgenic rats is mainly dependent on hypertension, not on diabetes

Andrea Hartner, Nada Cordasic, Bernd Klanke, Michael Wittmann, Roland Veelken, and Karl F. Hilgers

1Children and Youth Hospital, University of Erlangen-Nuremberg, Erlangen; 2Department of Nephrology and Hypertension, University of Erlangen-Nuremberg, Erlangen; and 3Medicine II, Augsburg City Hospital, Augsburg, Germany

Submitted 16 March 2006; accepted in final form 25 September 2006

Hilgers KF. Renal injury in streptozotocin-diabetic Ren2-transgenic rats is mainly dependent on hypertension, not on diabetes. Am J Physiol Renal Physiol 292: F820–F827, 2007. First published October 3, 2006; doi:10.1152/ajprenal.00088.2006.—Induction of streptozotocin (STZ) diabetes in hypertensive rats transgenic for the mouse ren-2 gene (TGR) has been described as a model of progressive diabetic nephropathy. We investigated the long-term course of STZ diabetes in TGR and appropriate Sprague-Dawley control rats (SD) and tested the role of angiotensin-dependent hypertension by treating rats with the angiotensin II type 1 receptor blocker losartan (1 mg·kg⁻¹·day⁻¹) via osmotic minipumps. Five weeks after STZ injection, diabetes developed in TGR and SD. Urinary albumin excretion was increased by diabetes and, to a much higher degree, by hypertension. The effects of hypertension and diabetes were not additive, and only the effects of hypertension were ameliorated by losartan. A similar pattern was observed for cell proliferation and macrophage infiltration in the kidney. In contrast, the effects of hypertension and diabetes on glomerular collagen IV accumulation were additive 5 wk after STZ injection. In a long-term study for 20 wk after STZ, survival was better in STZ-treated TGR than in normoglycemic TGR, whereas all SD survived. Impaired creatinine clearance and increased macrophage infiltration as well as glomerular and interstitial matrix deposition were prominent in TGR compared with SD, regardless of the presence or absence of diabetes. In conclusion, STZ diabetes in TGR may be useful to study glomerular and interstitial matrix deposition early in the course of diabetes. However, the long-term course of this animal model resembles severe hypertensive nephrosclerosis, rather than progressive diabetic nephropathy.

METHODS

Rats were housed in a room maintained at 22 ± 2°C, exposed to a 12:12-h dark-light cycle. The animals were allowed unlimited access to chow (no. 1320; Altromin, Lage, Germany) and tap water. All procedures performed on animals were done in accordance with guidelines of the American Physiological Society and were approved by the local government authorities (Regierung von Mittelfranken). Male rats heterozygous for the mouse ren-2 transgene (TGR) with ANG II-dependent hypertension (28) and age-matched Sprague-Dawley-Hanover (SD) control rats (Mölltegaard, Eijby, Denmark) at an average body weight of 250 g were used for induction of diabetes by intraperitoneal injection of streptozotocin (STZ; 70 mg/kg body wt) (Sigma, Deisenhofen, Germany) dissolved in 0.1 M sodium citrate buffer (pH 4.5) at the age of 12 wk. Two days later, blood was obtained from the tail vein and diabetes was confirmed by measurement of blood glucose with a reflectance meter (Glucometer Elite II; Bayer, Leverkusen, Germany). Only rats with a consistent blood
glucose >250 mg/dl were included. Nineteen TGR-STZ and 17 SD-STZ rats, as well as 16 normoglycemic TGR and 8 normoglycemic SD rats, were followed for 5 wk. Of these, eight SD-STZ, eight TGR, and nine TGR-STZ received a low dose of the AT1 blocker losartan (1 mg·kg body wt·day\(^{-1}\)) for the last 4 wk. The animals were implanted intraperitoneally with osmotic minipumps (Alzet model 2004; Alza Scientific Products, Palo Alto, CA), which delivered 0.25 μl/h for 28 days. In a second set of experiments, 6 SD, 7 SD-STZ, 12 TGR, and 9 TGR-STZ were followed for 20 wk. Blood glucose and systolic blood pressure (measured using tail-cuff plethysmography under light ether anesthesia) were monitored weekly (at 8:00 AM). At the end of the experiment, the rats were killed in metabolic cages for determination of urinary albumin excretion (enzyme immunoassay kit; CellTrend, Luckenwalde, Germany) for 24 h. Subsequently, the rats were equipped with a femoral artery catheter, and arterial blood pressure was measured via transducers (Grass Instruments, Quincy, MA) connected to a polygraph (Hellige, Freiburg, Germany) 4 h after termination of anesthesia. Protein, glucose, urea, and creatinine in serum or urine samples were analyzed with the automatic analyzer Integra 800 (Roche Diagnostics, Mannheim, Germany). Rats were killed, and kidneys were weighed and decapsulated. Renal tissue was fixed in methyl-Carnoy solution (60% methanol, 30% chloroform, and 10% glacial acetic acid) for histology and immunohistochemistry.

Immunohistochemistry. After overnight fixation in methyl-Carnoy solution, tissues were dehydrated by being bathed in increasing concentrations of methanol, followed by 100% isopropanol. After tissues were embedded in paraffin, 3-μm sections were cut with a Leitz SM 2000 R microtome (Leica Instruments, Nussloch, Germany). After deparaffinization, endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 20 min at room temperature. A mouse monoclonal antibody detecting proliferating cells (proliferating cell nuclear antigen, PCNA) was purchased from Santa Cruz Biotechnologies (Heidelberg, Germany) and used at a dilution of 1:50. The mouse monoclonal antibody against the macrophage marker ED-1 was purchased from Serotec (Biozol, Eching, Germany) and used at a dilution of 1:250. Renal cortical collagen I was detected using a rabbit polyclonal antibody (Biogenesis, Poole, UK) at a dilution of 1:1,000. A goat polyclonal antibody to collagen IV (Southern Biotechnology Associates, Birmingham, AL) was used at a dilution of 1:500. Each slide was counterstained with hematoxylin.

Real-time RT-PCR detection of mRNA. Renal cortical tissue extraction and real-time RT-PCR were carried out as described (35). Briefly, first-strand cDNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Darmstadt, Germany) using random hexamers as primers. Final RNA concentration in the reaction mixture was adjusted to 0.5 ng/μl. Reactions without Multiscribe reverse transcriptase were used as negative controls for genomic DNA contamination. PCR was performed with an ABI PRISM 7000 sequence detector system and TaqMan or SYBR green Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany). Primer design was accomplished with PrimerExpress software (Applied Biosystems) for 18S rRNA and tissue inhibitor of metalloproteinase (TIMP)-2. Primer sequences used are as follows: 18S rRNA forward, 5′-TTGATTAAGTCCCTGCCCTTTGT-3′ and reverse, 5′-GCACAATAAAGTCACAGAGGGTAAT-3′; TIMP-2 forward, 5′-GCCTGGAGTTGGAGAAGAAAG-3′, and reverse, 5′-GCACAATAAAGTCACAGAGGGTAAT-3′; and TIMP-2 probe, TCTCCTTCGCCTTCCTGAATTAG. Sequences of primers for transforming growth factor-β1 (TGF-β1), collagen I, collagen III, and TIMP-1 were described previously (14, 19, 33).

Analysis of data. Intraglomerular PCNA- or ED-1-positive cells were counted in all glomeruli of a given kidney section (120–300 glomeruli, no selection) and expressed as cells per glomerular section. Intertstitial PCNA- or ED-1-positive cells were counted in 30 medium-power (magnification ×250) cortical views per section and expressed as cells per square millimeter. Counting was begun in a random cortical field and in consecutive nonoverlapping cortical fields to the right of the previous view without selection; if necessary, counting was continued at the opposite (left) edge of the section. Expansion of interstitial collagen I was measured using Metaview software (Visitron Systems, Puchheim, Germany) in 10 nonoverlapping medium-power cortical views per section, excluding glomeruli, and was expressed as a percentage of stained area per cross section. Glomerular collagen IV staining was measured using Metaview in every third glomerulus per cross section, and the stained area was expressed as a percentage of the total area of the glomerular tuft.

Two-way analysis of variance, followed by the post hoc least significant difference test, was used to compare groups. A P value <0.05 was considered significant. The procedures were carried out using SPSS version 13.0 software (SPSS, Chicago, IL). Values are displayed as means ± SE.

RESULTS

Both SD and TGR developed diabetes mellitus following treatment with STZ (Fig. 1A). In untreated TGR, blood glucose did not differ from that in untreated SD rats (Fig. 1A). Losartan treatment did not significantly alter blood glucose levels (Fig. 1A). Diabetes led to increased water intake in both SD and TGR, which was not altered by losartan (Table 1). Systolic and mean arterial blood pressure were significantly higher in TGR compared with SD rats (Fig. 1B and Table 1). Hypertension in TGR was not significantly affected by STZ treatment (Fig. 1), although intra-arterial measurements showed a trend toward lower mean pressure in TGR-STZ than in TGR (Table 1). Chronic administration of a low dose of losartan did not significantly lower systolic blood pressure (Fig. 1B) but had a modest effect on mean arterial blood pressure in TGR and a marked effect on TGR-STZ (Table 1). Albumin excretion was increased in diabetic animals and even more in hypertensive rats, but a combination of both diseases did not further elevate albuminuria (Fig. 1C). In diabetic and nondiabetic TGR, but not in diabetic SD rats, losartan ameliorated albuminuria (Fig. 1C). Losartan reduced plasma creatinine levels in TGR and TGR-STZ (Table 1).

STZ diabetes led to reduced body weight gain and kidney hypertrophy. Kidney weight-to-body weight ratio was significantly increased in SD-STZ, TGR, and TGR-STZ compared with SD controls. Losartan reduced kidney hypertrophy in TGR and TGR-STZ but not in SD-STZ (Table 1). TGR hypertensive animals had significantly higher heart weight-to-body weight ratios than normotensive SD rats, and this was not affected by STZ diabetes (Table 1). Again, losartan reduced heart weight-to-body weight ratios in TGR and TGR-STZ. Plasma creatinine was significantly elevated only in TGR (Table 1).

Macrophage infiltration was significantly elevated in the kidney interstitium and in the glomerulus only in TGR (Table 2). Treatment with STZ did not further augment interstitial or glomerular macrophage infiltration in TGR (Table 2). Losartan significantly reduced renal interstitial and glomerular macrophage infiltration in all groups (Table 2). Cell proliferation, as counted after immunohistological detection of PCNA, was significantly increased in the kidney interstitium and in the glomerulus only in TGR, with a tendency toward more PCNA-positive cells in SD-STZ and TGR-STZ compared with SD rats.
Evaluation of interstitial fibrosis via the extent of collagen I expansion in the tubulointerstitium revealed significant increases in collagen-positive staining in SD-STZ, TGR, and TGR-STZ compared with SD controls (Fig. 3A). The extent of collagen I expansion in the interstitium of TGR and TGR-STZ was comparable (Fig. 3A). Losartan reduced collagen I expansion in TGR and TGR-STZ but not in SD-STZ (Fig. 3A). The degree of glomerulosclerosis assessed by measuring collagen IV expansion was significantly higher in TGR (Fig. 3B). Diabetes further increased the degree of glomerulosclerosis in TGR (Fig. 3B). This effect was reduced by losartan treatment (Fig. 3B). Matrix expansion was accompanied by a trend toward higher gene expression of TGF-β in TGR and TGR-STZ that reached statistical significance only in TGR (Fig. 4). There also were trends toward higher gene expression of collagens I and III as well as TIMP-1 in TGR (Fig. 4). In histological preparations of the kidneys of both TGR and TGR-STZ, malignant hypertensive lesions were detected, which were not found in SD-STZ (Fig. 5). Treatment with losartan prevented the development of malignant hypertensive lesions in TGR and TGR-STZ (Fig. 5).

The STZ-induced increase of blood glucose levels persisted over 20 wk in SD and TGR (Fig. 6A). Water intake in diabetic rats was still significantly increased after 20 wk (Table 3). Systolic blood pressure was elevated in TGR and diabetic TGR throughout the 20 wk of observation, with a consistent decrease beginning with the 10th wk in diabetic TGR (Fig. 6B). This decrease was even more marked in nondiabetic TGR (Fig. 6B). Arterial blood pressure measurements at the end of the experiment still revealed significantly higher blood pressure in both TGR groups compared with SD groups (Table 3). However, the differences were much smaller after 20 wk than at the early time point of 5 wk (Fig. 6A and Table 1). In the course of the experiment, eight hypertensive rats died. Survival was especially poor in the nondiabetic TGR group, whereas only one rat died in the diabetic TGR-STZ group (Fig. 6C).

Albumin excretion as well as plasma creatinine and plasma urea was significantly elevated only in TGR rats without further increase by concomitant diabetes mellitus of 20-wk duration (Fig. 7A and Table 3). Creatinine clearance was decreased in TGR with and without STZ (Fig. 7B). Cortical and glomerular macrophage infiltration as a marker of inflammatory activity in the kidney was significantly increased only in TGR rats (Table 2). Matrix expansion was detected in diabetic SD rats in the renal cortex (Fig. 8A) but not in

Table 1. Body and organ weights, urine production, water intake, plasma creatinine, and mean arterial blood pressure after 5 wk of diabetes

<table>
<thead>
<tr>
<th>Group</th>
<th>SD</th>
<th>SD + STZ</th>
<th>SD- STZ-Los</th>
<th>TGR</th>
<th>TGR-Los</th>
<th>TGR + STZ</th>
<th>TGR-STZ-Los</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>388.3 ± 9.6</td>
<td>269.1 ± 5.9*</td>
<td>262.0 ± 12.4*</td>
<td>337.9 ± 12.0</td>
<td>377.1 ± 6.3†</td>
<td>265.6 ± 16.1*</td>
<td>272.7 ± 10.7*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Heart weight/body weight ratio, mg/g</td>
<td>3.3 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>5.4 ± 0.3</td>
<td>4.6 ± 0.2†</td>
<td>4.7 ± 0.2*</td>
<td>3.7 ± 0.1†</td>
</tr>
<tr>
<td>Kidney weight/body weight ratio, mg/g</td>
<td>3.2 ± 0.1</td>
<td>5.1 ± 0.1*</td>
<td>5.4 ± 0.2*</td>
<td>4.4 ± 0.1*</td>
<td>3.8 ± 0.1†</td>
<td>5.7 ± 0.2</td>
<td>5.0 ± 0.1†</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>44.7 ± 6.7</td>
<td>167.0 ± 15.0*</td>
<td>136.5 ± 14.3*</td>
<td>64.6 ± 6.8</td>
<td>49.9 ± 8.8</td>
<td>147.4 ± 14.6*</td>
<td>147.2 ± 9.6*</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.31 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.55 ± 0.07*</td>
<td>0.33 ± 0.02†</td>
<td>0.34 ± 0.07</td>
<td>0.24 ± 0.01†</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>120.1 ± 6.5</td>
<td>119.4 ± 3.5</td>
<td>112.7 ± 3.1</td>
<td>212.5 ± 10.3</td>
<td>171.6 ± 10.4†</td>
<td>178.6 ± 17.0*</td>
<td>122.5 ± 5.7†</td>
</tr>
</tbody>
</table>

SD, normotensive normoglycemic Sprague-Dawley control rats; TGR, transgenic hypertensive rats; STZ, streptozotocin treatment; Los, losartan treatment; MAP, mean arterial blood pressure. *P < 0.05 vs. SD, †P < 0.05 vs. respective non-losartan-treated group.
glomeruli (Fig. 8B) compared with control SD rats. In TGR, cortical and glomerular matrix expansion was even more prominent (Fig. 8B), but this was not further aggravated by 20 wk of diabetes (Fig. 8).

**DISCUSSION**

Our data confirm that both STZ diabetes and ren-2-induced hypertension cause some degree of kidney injury. The extent of the hypertension-induced damage was far more pronounced, however. The combination of both models of disease led to additive effects on a few parameters, especially glomerular matrix expansion. These effects were apparent in the early phase of diabetes, 5 wk after STZ injection. Over a prolonged period, the presence or absence of hypertension determined survival and kidney damage, whereas the additional presence or absence of diabetes mellitus had little effect. Thus the model of STZ diabetes in ren-2 transgenic rats may be useful to examine mechanisms of glomerular matrix expansion in early diabetes. However, our data do not support the notion that this model represents human progressive diabetic nephropathy, as suggested by Kelly et al. (16). Rather, severe hypertensive nephrosclerosis determines the outcome of diabetic as well as normoglycemic ren-2 transgenic rats.

Our findings are in agreement with the results of many authors who reported that STZ diabetes does not lead to progressive renal damage in rodents [for review, see O’Donnell et al. (29) for rats and Breyer et al. (4) for mice]. However, our results contrast with those of the Melbourne group reporting a far more pronounced renal damage in TGR-
STZ compared with normoglycemic TGR (16, 17, 27, 37). We cannot fully explain this discrepancy, but several factors may contribute. Kelly, Wilkinson-Berka, Mifsud, and other authors from the Melbourne group used female rats and injected STZ at a younger age (16, 17, 27, 37). Both factors could potentially lead to a less pronounced effect of hypertension on the kidney, because female TGR reportedly exhibit lower blood pressure than male TGR (21). On the other hand, the blood pressure levels reported by Kelly and colleagues (16, 17, 27, 37) for diabetic and normoglycemic TGR are not notably lower than
those measured in our study. Furthermore, Kelly and colleagues (16, 17, 27, 37) may have missed the effects of ren-2-induced hypertension, because these authors did not investigate the respective normotensive controls, neither normoglycemic nor hyperglycemic. Hannover SD rats were included in some studies of the Melbourne group (5, 23), but renal damage was not reported in those articles. Finally, Kelly et al. (16) used a small dose of insulin after STZ treatment, which we did not. The presence or absence of minimal insulin treatment may affect the type and extent of diabetic kidney injury in this model (26).

Our data confirm that both diabetes and ren-2 hypertension induce urinary albumin excretion. However, the effects of hypertension were much more pronounced, and STZ diabetes did not add to the hypertension-induced albuminuria, in contrast to the findings of Kelly et al. (16, 17, 27, 37). A similar pattern was observed for macrophage infiltration as well as cell proliferation in glomeruli and interstitium of the kidney. Losartan ameliorated albuminuria, infiltration, and proliferation in hypertensive rats. The effects of hypertension and diabetes on matrix accumulation were more complex. Interstitial collagen I was increased by diabetes and by hypertension; the effects of both diseases on glomerular collagen IV were additive. The gene expression of TGF-β and collagen I was increased in

Table 3. Body and organ weights, urine production, water intake, plasma creatinine, plasma urea, MAP, and renal macrophage infiltration after 20 wk of diabetes

<table>
<thead>
<tr>
<th>Group</th>
<th>SD</th>
<th>SD-STZ</th>
<th>TGR</th>
<th>TGR-STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>484.7±16.3</td>
<td>352.6±18.4</td>
<td>598.8±24.4</td>
<td>409.8±14.6</td>
</tr>
<tr>
<td>Heart weight/body weight ratio, mg/g</td>
<td>3.6±0.1</td>
<td>4.3±0.2</td>
<td>3.9±0.4</td>
<td>3.8±0.1</td>
</tr>
<tr>
<td>Kidney weight/body weight ratio, mg/g</td>
<td>3.1±0.1</td>
<td>5.2±0.3</td>
<td>3.0±0.3</td>
<td>4.8±0.2</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.35±0.01</td>
<td>0.19±0.10</td>
<td>0.45±0.02</td>
<td>0.31±0.01</td>
</tr>
<tr>
<td>Plasma urea, mg/dl</td>
<td>15.8±1.6</td>
<td>24.6±3.1</td>
<td>58.9±5.6</td>
<td>44.3±0.6</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>18.4±1.2</td>
<td>96.4±28.0*</td>
<td>33.0±9.6</td>
<td>119.3±20.7*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>109.3±2.2</td>
<td>120.4±2.1</td>
<td>128.0±8.6*</td>
<td>135.8±7.1*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. SD.
This observation was somewhat unexpected. In many years of cuff systolic blood pressure had shown little effect of the drug. Almost normalized mean arterial blood pressure, although tail- these animals, as discussed elsewhere (9). TGR. Thus nonhemodynamic effects of ANG II type 1 recep- tors are used (3, 22, 30, 38). On the other hand, we hesitate to interpret our observations in this way, because kidney inflammation and injury are determined by angiotensin-dependent hypertension, irrespective of the presence or absence of STZ diabetes. In other words, this animal model resembles severe hypertensive nephrosclerosis, rather than diabetic nephropathy.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of Rainer Wachtveitl and Miroslava Kupraszewicz-Hutzler.

GRANTS

This study was part B12 of the Interdisziplina¨res Zentrum fu¨r Klinische Forschung at the Hospital of the University of Erlangen-Nuremberg, funded by the Bundesministerium fu¨r Bildung und Forschung (01 KS 0002). In addition, the study was supported by a grant-in-aid (KFO 106, TP2) from the Deutsche Forschungsgemeinschaft. Parts of the data were presented in abstract form at the 31st Annual Meeting of the American Society of Nephrology in Philadelphia, PA, in 1998.
REFERENCES


