The Genetics of Hypertension Modifies the Renal Cell Replication Response Induced by Experimental Diabetes

Lilia A. Silveira,1 Carlos E. Bacchi,2 Glauce A. Pinto,3 and José B. Lopes de Faria1

To investigate whether the genetics of hypertension modifies renal cell responses in experimental diabetes, we studied the renal cell replication and its regulation by two cyclin-dependent kinase (Cdk) inhibitors, p27kip1 and p21Cip1, in prehypertensive spontaneously hypertensive rats (SHR) and their genetically normotensive counterparts, Wistar Kyoto (WKY) rats, with and without streptozotocin-induced diabetes. In diabetic SHR, the number of proliferating glomerular (0.6 ± 0.3 positive cells/50 glomeruli) and tubulointerstitial (2.8 ± 0.6 positive tubulointerstitial cells/50 grid fields) cells assessed by the bromodeoxyuridine technique was significantly (P = 0.0002) lower than in control SHR (13.2 ± 1.7 and 48.6 ± 9.7, respectively) and control (14.0 ± 1.8 and 63.9 ± 10.6) and diabetic (14.3 ± 3.5 and 66.4 ± 11.5) WKY rats. Proliferating cell nuclear antigen, another marker of cell proliferation, was significantly reduced in replicating glomerular (P = 0.0002) and tubulointerstitial (P < 0.0001) cells in diabetic SHR. In freshly isolated glomeruli, the level of p27kip1 detected by Western blotting was significantly higher in diabetic SHR than in nondiabetic SHR (1.52 ± 0.14 vs. 1.00 ± 0.10% of control, P = 0.014). The expression of p21Cip1 in isolated glomeruli did not differ among the groups of rats. In conclusion, the response of renal cell replication to diabetes differs markedly between prehypertensive SHR and their WKY control rats. The decreased glomerular cell proliferation in prehypertensive diabetic SHR is at least partly mediated by a higher expression of the Cdk inhibitor p27kip1. Diabetes 51:1529–1534, 2002

Susceptibility to diabetic renal disease varies among patients with diabetes (1), and it is not clear why only a subset of these patients develop diabetic nephropathy (2). Although a predisposition to hypertension has been suggested to be an important factor in this susceptibility to diabetic renal disease (3–5), the precise mechanism whereby the genetics of hypertension can increase the risk of nephropathy in individuals with diabetes remains to be established.

In experimental diabetes, there is an early and self-limited increase in mesangial cell replication (6), which is followed by cell-cycle arrest in the G1 phase and concomitant hypertrophy (7–10). Inhibitors of cyclin-dependent kinases (Cdk) such as p27kip1 and p21Cip1 apparently mediate this G1-phase arrest (7,8,11). Abnormalities in cell replication and regulation have been implicated in the glomerular hypertrophy and renal enlargement observed in human and experimental diabetes (8,9,12).

The identification of early abnormalities in the kidneys of diabetic individuals who are destined to become but are not yet hypertensive could provide new insights into the susceptibility to diabetic nephropathy that accompanies hypertension. Because such studies cannot be done readily in humans, we have used spontaneously hypertensive rats (SHR), which exhibit a normotensive period (the first 4 weeks of life) before the development of genetically determined hypertension that subsequently occurs in 100% of the rats (13). In this study, we investigated whether renal cell replication and its regulation by two Cdk inhibitors, p27kip1 and p21Cip1, differ in rats with a predisposition to hypertension (SHR) and their genetically normotensive counterparts, Wistar Kyoto (WKY) rats, made diabetic with streptozotocin (STZ).

**RESEARCH DESIGN AND METHODS**

The protocol for this study complied with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. SHR and WKY rats derived from animals supplied by Taconic (Germantown, NY) and bred in our animal facility were used in this study. Renal cell proliferation and the expression of glomerular p27kip1 and p21Cip1 were done 10 days after the induction of diabetes. Experimental diabetes was induced in 4-week-old prehypertensive male SHR and male WKY rats by injecting STZ (65 mg/kg) (Sigma, St. Louis, MO) dissolved in sodium citrate buffer (pH 4.5) via a tail vein after an overnight fast. Control groups received only vehicle (citrate buffer). Blood glucose levels were measured using an enzymatic colorimetric GOD-PAP assay (Merck, Darmstadt, Germany) 72 h after the injection of STZ or citrate buffer and on the day before killing the rats. Values >15 mmol/l were considered diabetic for these experiments. Systolic blood pressure (three to five determinations per rat) was obtained by tail-cuff plethysmography in

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ABC, avidin-biotin complex; BrdU, 5-bromo-2'-deoxyuridine; Cdk, cyclin-dependent kinase; DAB, diaminobenzidine tetrahydrochloride; PCNA, proliferating cell nuclear antigen; STZ, streptozotocin; TdT, terminal deoxynucleotidyl transferase; TGF, transforming growth factor; TUNEL, TdT-mediated dUTP-biotin nick-end labeling.
unanesthetized rats using an MK III physiograph (Narco Bio-System, Houston, TX). Cell proliferation, assessed by the incorporation of 5-bromo-2'-deoxyuri-
dine (BrDU) (Calbiochem, La Jolla, CA), a thymidine analog that incorporates into DNA in the S phase (14), was examined in seven diabetic SHRs and seven diabetic WKY rats. Numbers of replicating renal cells were also estimated in these same groups of rats by detecting proliferating cell nuclear antigen (PCNA), which is expressed by actively proliferating cells (15). To detect BrDU incorporation into DNA in the S phase, the rats were injected intraperi-
toneally with BrDU (100 mg/kg) dissolved in saline 1 h before killing. An equal number of rats that received saline alone served as the controls. After anesthesia with ether, the kidneys and a portion of the gastrointestinal tract (positive control for BrDU staining) were extracted, fixed in 10% buffered formalin, paraffin-embedded, and cut into 4-μm thick sections for immuno-
staining. The expression of glomerular p27(Kip1) and p21(Cip1) was determined in glomeruli isolated from 29 SHR and 30 WKY rats 10 days after the induction of diabetes.

**Immunohistochemistry.** To detect BrDU, sections were dewaxed, rehy-
drated, and placed in 2N HCl at 51°C for 20 min and in 0.005% trypsin in PBS at 37°C for 2 min for antigen retrieval. After the pretreatment, slides were incubated in 1% nonfat milk in PBS for 1 h to block nonspecific staining. Tissue sections were incubated with a 1:50 dilution of a monoclonal anti-BrdU antibody (Dako, Glostrup, Denmark) for 1.5 h at room temperature. After washing in PBS, a 1:200 dilution of a biotinylated secondary anti-mouse IgG antibody (Vector, Burlingame, CA) was applied for 1 h. Endogenous peroxidase was blocked by incubating the slides in 3% H2O2 for 5 min. To detect PCNA, microwave post-fixation was done using a domestic oven (Panasonic Junior) at 700 W, which was delivered to slides immersed in 0.01 mol/l citrate buffer, pH 6.0, in two 7-min doses separated by a 2-min break, which allowed for buffer replenishment. The slides were allowed to cool to room temperature before being removed from the oven. Sections were then incubated at room temperature for 1 h with primary antibody PC10 (Dako) diluted 1:150. Biotinylated horse anti-mouse (Vector) or mouse anti-human (Dako) antibo-
dies for BrDU and PC10, respectively, were applied for 1 h at room temperature. The slides were then incubated with avidin-biotin complex (ABC) reagent (Vector) for 30 min followed by the addition of diaminobenzi-
dine tetrahydrochloride (DAB) (Sigma) as a substrate-charge solution. After hematoxylin counterstaining and dehydration, the slides were mounted in Entellan (Merck, Darmstadt, Germany). Positive controls for BrDU injection consisted of sections of the gastrointestinal tract of each rat. Negative controls for the reaction consisted of omitting the primary antibody. BrDU and PC10-positive cells were counted in 50 glomeruli; for the tubulointerstitial area, the number of positive cells in 100 grid fields was determined (0.02 mm²).

**Isolation of glomeruli.** Glomeruli were isolated by a differential sieving technique. Briefly, rats were anesthetized, and the kidneys were immediately removed, weighed, and placed in ice-cold Hank’s balanced salt solution (Gibco). The cortices were isolated and finely minced. The pooled material of three to four rats was passed through stainless steel screens (W. Tyler, Mentor, OH) of 60 (pore size 250 μm) and 150 (pore size 106 μm) mesh, and the glomeruli were collected on a 200-mesh screen (pore size 63 μm). The final preparations contained >95% glomeruli as assessed by phase-contrast microscopy. Isolated glomeruli were lysed directly on ice in 300 μl of a buffer containing 2% SDS and 60 mmol Tris-HCl (pH 6.8) supplemented with a cocktail of protease inhibitors (Complete: contains antipain-HCl, chymostatin, leupeptin, bestatin, pepstatin, phosphoramidon, aprotinin, and Eltira; Boehr-
ingser) (18). After centrifugation, the supernatants were transferred to new tubes and the protein concentrations were measured by the Bradford method (19), using BSA as standard.

**Western blotting.** For Western blotting analysis, 30 μg and 60 μg of pooled glomerular protein for p27(Kip1) and p21(Cip1), respectively, in 5% glycater/0.03% bromophenol blue/10 mmol dithiothreitol were loaded into 15% SDS polyacryl-

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**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Control WKY</th>
<th>Diabetic WKY</th>
<th>Control SHR</th>
<th>Diabetic SHR</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>25</td>
<td>19</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>8.1 ± 0.20</td>
<td>24.3 ± 0.88*</td>
<td>7.4 ± 0.27</td>
<td>24.9 ± 0.59†</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>141.5 ± 3.3</td>
<td>118.3 ± 2.5*</td>
<td>109.8 ± 5.3†</td>
<td>79.7 ± 3.2,‡,‡</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>103.9 ± 3.3</td>
<td>112.0 ± 2.7</td>
<td>120.4 ± 0.8*</td>
<td>117.4 ± 3.9*</td>
</tr>
<tr>
<td>Absolute kidney weight (g)</td>
<td>0.62 ± 0.01‡,§</td>
<td>0.60 ± 0.02‡,§</td>
<td>0.48 ± 0.02</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>Kidney to body weight ratio</td>
<td>0.44 ± 0.01</td>
<td>0.51 ± 0.01*,†</td>
<td>0.41 ± 0.01</td>
<td>0.55 ± 0.02*‡,‡</td>
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Data are means ± SE. *P < 0.05 vs. WKY control; †P < 0.05 vs. control SHR; ‡P < 0.05 vs. diabetic WKY; §P < 0.05 vs. diabetic SHR.

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**RESULTS**

As noted by others (20), 4-week-old SHR were smaller than the corresponding WKY rats. Both groups of diabetic rats showed a decrease in body weight 10 days after the injection of STZ compared with their normoglycemic controls (Table 1). Blood glucose levels were similar in the two groups of diabetic rats. Systolic blood pressure was slightly higher in both groups of SHR compared with the WKY control group, but there was no difference between the two diabetic groups. As with body weight, absolute kidney weight was significantly lower in control SHR, and
there was a tendency for it to decrease in both strains of diabetic rats. For this reason, kidney weight was corrected to body weight, as described elsewhere (9,21). Following this correction, the kidney:body weight ratio was similar in nondiabetic WKY rats and SHR. In diabetic rats, this ratio increased significantly in both strains, although that of diabetic SHR was significantly greater than in diabetic WKY rats (0.55 ± 0.02 vs. 0.51 ± 0.01 kidney wt/body wt; P < 0.0001).

To determine whether the greater kidney:body weight ratio in diabetic SHR results from a larger reduction in lean body weight or from kidney hypertrophy, the weights of the heart and spleen were obtained for all experimental groups. If the increase in kidney:body weight ratio was the result of lean body weight loss rather than kidney hypertrophy, it would be expected that the weights of the heart and spleen would also be affected by the correction to body weight; but this was not the case. The heart (WKY, 0.47 ± 0.02 vs. 0.34 ± 0.02 g; SHR, 0.47 ± 0.02 vs. 0.34 ± 0.01 g, control and diabetic rats, respectively) and spleen (WKY, 0.51 ± 0.03 vs. 0.33 ± 0.03 g; SHR, 0.33 ± 0.02 vs. 0.22 ± 0.01 g) weights were significantly lower in diabetic rats of both strains. This relationship was maintained when the ratios of heart (WKY, 0.32 ± 0.02 vs. 0.29 ± 0.01, P = 0.037; SHR, 0.37 ± 0.01 vs. 0.31 ± 0.01, P = 0.0009) and spleen (WKY, 0.35 ± 0.02 vs. 0.27 ± 0.01, P = 0.0001; SHR, 0.26 ± 0.01 vs. 0.20 ± 0.01, P = 0.0065) to body weight were compared. These results were also qualitatively similar when expressed in terms of organ dry weight (the organs were kept at 60°C for 24 h before being weighed). This last observation indicated that the difference in heart and spleen weights between diabetic and control rats could not be attributed to variations in the accumulation of water in these organs.

**Diabetes markedly reduces renal cell replication in SHR.** Figure 1 shows the number of BrdU-positive cells in the glomeruli and tubulointerstitial area of control and diabetic WKY rats and SHR 10 days after the injection of streptozotocin. The number of replicating cells in the glomeruli (13.2 ± 1.7 vs. 14.0 ± 1.8) and tubulointerstitial area (48.6 ± 9.7 vs. 63.9 ± 10.6) of SHR and WKY rats did not differ significantly. In SHR, diabetes significantly decreased the number of proliferating glomerular (13.2 ± 1.7 vs. 0.6 ± 0.3; P = 0.0002) and tubulointerstitial (48.6 ± 9.7 vs. 2.8 ± 0.6; P = 0.0014) cells compared with control SHR (Fig. 1). In contrast, there was no difference in the number of replicating renal cells in glomeruli and the tubulointerstitial area of diabetic and control WKY rats (14.3 ± 3.5 vs. 14.0 ± 1.8 and 66.4 ± 11.5 vs. 63.9 ± 10.6, respectively) (Fig. 1). Qualitatively similar results were obtained for the immunohistochemical localization of PCNA (Figs. 3 and 4). However, the absolute number of proliferating renal cells with PCNA was always higher than observed with BrdU. An increase in the number of cells positive for PCNA compared with BrdU has also been previously described in a variety of conditions (22,23).
TUNEL staining is not modified by diabetes or rat strain. TUNEL staining was a rare event in control rats and was not altered by diabetes. The number of glomerular cells per 100 glomeruli staining positive for TUNEL was 0.71 ± 0.36 in control WKY rats and did not differ from the 1.33 ± 0.33 observed in diabetic WKY rats or the 1.00 ± 0.44 and 0.43 ± 0.20 seen in control and diabetic SHR, respectively. Similarly, the number of tubulointerstitial cells positive for TUNEL did not differ between control (2.86 ± 1.01) and diabetic (4.33 ± 0.71) WKY rats or the control (3.57 ± 0.48) and diabetic (4.71 ± 2.39) SHR. TUNEL staining was readily detected in sections treated with DNase (positive control, Fig. 5).

Increased expression of p27Kip1 in glomeruli isolated from diabetic SHR. To investigate a potential role of cell cycle regulatory proteins in the decreased renal cell replication observed in diabetic SHR, we examined the expression of two cyclin-dependent kinase inhibitors, p27Kip1 and p21Cip1. In freshly isolated glomeruli, the level of p27Kip1 as detected by Western blotting (Fig. 6) was significantly higher in the diabetic SHR (n = 5) than in the nondiabetic SHR (n = 5) (1.52 ± 0.14 vs. 1.00 ± 0.10% of control; P = 0.014). In WKY rats, diabetes did not significantly increase the expression of p27Kip1 (1.33 ± 0.20 vs. 1.00 ± 0.10% of control; P = 0.106). There was no difference in the glomerular expression of p27Kip1 between control rats of the two strains. The glomerular expression of p21Cip1 did not differ between SHR and WKY rats, nor was it significantly modified by diabetes (1.00 ± 0.24 vs. 0.89 ± 0.20 and 1.00 ± 0.22 vs. 1.06 ± 0.03% of control, SHR and WKY and control and diabetic, respectively) (Fig. 7). These results indicate that the decrease in glomerular cell proliferation seen in prehypertensive diabetic SHR may be partly mediated by a higher expression of the cyclin-dependent kinase inhibitor p27Kip1.

DISCUSSION

If hyperglycemia were exclusively responsible for the development of diabetic renal disease, it could be expected that the longer the exposure to diabetes, the greater would be the chance of having diabetic nephropathy. However, this is not entirely true. In patients with type 1 diabetes for >30 years, the risk of developing diabetic nephropathy is relatively low (1,24). Several studies have suggested that susceptibility to diabetic nephropathy may be linked to a predisposition to hypertension (3–5). However, little progress has been made in understanding the mechanisms that associate a predisposition to hypertension with a susceptibility to diabetic renal disease.

In the present study, young diabetic and still-normotensive SHR showed reduced replication of glomerular and tubulointerstitial renal cells, accompanied by a more pronounced kidney enlargement, compared with genetically normotensive WKY rats. An organ can grow in size through an increase in cell number (increased proliferation or decreased apoptosis) or an increase in individual cell size (hypertrophy). An organ can grow in size through an increase in cell number (increased proliferation or decreased apoptosis) or an increase in individual cell size (hypertrophy). It has been suggested that the increased cell size of diabetic glomeruli is due to enhanced protein synthesis (25). The increased cell size, therefore, would represent a response to increased cellularity in the diabetic kidney. TUNEL staining was readily detected in sections treated with DNAse (positive control, Fig. 5).

FIG. 6. Western blots of lysates of isolated glomeruli from control SHR and WKY rats and from rats 10 days after the induction of diabetes with STZ. The membranes were incubated with anti-rat p27Kip1 antibody. Each lane represents a pool of glomeruli from three to four rats.

FIG. 7. Western blots of lysates of isolated glomeruli from control SHR and WKY rats and from rats 10 days after the induction of diabetes with STZ. The membranes were incubated with anti-human p21Cip1 antibody. Each lane represents a pool of glomeruli from three to four rats.
cell size (hypertrophy) (25). In diabetic SHR, the reduction in renal cell replication, the lack of change in the rate of apoptosis, and the presence of larger kidneys suggest that increased renal size is mainly secondary to cell hypertrophy rather than hyperplasia (25). Our results indicate that the increased expression of p27kip1, a Cdk inhibitor, in the glomeruli of diabetic SHR could partly explain the decrease in glomerular cell replication and kidney hypertrophy seen in prehypertensive diabetic SHR. These observations may be relevant to the mechanism underlying the association between a predisposition to hypertension and diabetic nephropathy, and it could lead to identification of early markers of susceptibility to diabetic nephropathy.

Both of the methods used to estimate cell replication showed that there was a marked reduction in renal cell replication in diabetic SHR. However, as previously observed, the number of positive cells stained by PC10 was much higher than that obtained with the BrdU technique (23). This discrepancy has been observed in human and animal tissue stained with these antibodies (23,26). Several reasons have been provided to explain the overexpression of PCNA recognized by PC10 (23). First, PCNA is present in all phases of the cell cycle, with a half-life of ~20 h (27,28). This relatively long half-life leads to the expression of PCNA in cells that have left the cell cycle and are therefore not synthesizing DNA (23). Second, the overexpression of PCNA is particularly prominent in formalin-fixed tissue (23). Third, under certain conditions PCNA expression may be enhanced by growth factors, such as epidermal growth factor (EGF) and transforming growth factor (TGF)-α (28). Immunohistochemistry technique using an antibody to BrdU provides a marker of cell proliferation that is regarded as a "gold standard" (22). Thus, the number of proliferating renal cells observed here with this last technique may reflect the actual number of replicating cells more accurately.

Apoptosis is closely linked to the cell cycle (25), and it has been suggested recently that both p21Cip1 and p27kip1 may protect cells against apoptosis (29,30). As shown here, diabetes did not increase apoptosis in genetically normotensive (WKY) or hypertensive (SHR) rats. These findings agree with data for C57B6 and p21Cip1 knockout mice (23,26). In agreement with the latter study, we have recently observed that the number of replicating renal cells stained with these antibodies (23,26) were slightly lower in control SHR than in the WKY controls (17). In the present study, in which the number of replicating renal cells was similar in control SHR and WKY rats. The rats in the present study were used at ~6 weeks of age, which means that they were ~2 weeks older than those used in previous studies (17).

Our results may be relevant to studies aimed at preventing the development of diabetic renal disease in individuals with a predisposition to hypertension. In this context, the use of an ACE inhibitor in a model of spontaneous diabetes is able to reduce the glomerular expression of Cdk inhibitors such as p16INK4A and p27kip1 (18). In summary, diabetes for 10 days in prehypertensive SHR rats reduces renal cell proliferation and increases the glomerular expression of the Cdk inhibitor, p27kip1, whereas the levels of another Cdk inhibitor, p21Cip1, were negatively regulate cyclin-Cdk complexes by binding to them and inhibiting their kinase activity (37–39). The consequence is G1-phase arrest, and the cells can then undergo hypertrophy (7,8,11). That a high glucose concentration and hyperglycemia can increase the expression of the Cdk inhibitors p27kip1 and p21Cip1 has been demonstrated in cultured mesangial cells (7,10) and in different animal models of diabetes (8,9,11). The Cdk inhibitor that is upregulated in the kidney in animal models of diabetes depends on the experimental conditions (8). In STZ-induced diabetes, the level of glomerular p27kip1 is elevated in BALB/c diabetic mice 48 h after the injection of STZ (11). In another study, the level of p21Cip1 but not p27kip1, was elevated in the glomeruli of female C57B6 mice with STZ-induced diabetes (8). The level of p27kip1 is elevated in the glomeruli of db/db mice, a genetic model of type 2 diabetes (11). As shown here, the levels of p27kip1, but not p21Cip1, a member of the CIP/KIP family of Cdk inhibitors that includes p27kip1, were elevated in diabetic SHR. To our knowledge, no previous study has investigated the expression of Cdk inhibitors in the kidney of SHR. The effects of high glucose on renal hypertrophy, cellular replication, and Cdk inhibitors may be mediated by TGF-β (34,40) and angiotensin II (18,41,42). In addition, gene target experiments have recently demonstrated that the presence of p21Cip1 is a necessary condition for the development of glomerular hypertrophy and proteinuria in diabetic mice (9).
unchanged. Our results provide insight into the cellular mechanism involved in the association between a predisposition to hypertension and the susceptibility to diabetic renal disease.

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