Overexpression of Angiotensinogen Increases Tubular Apoptosis in Diabetes

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ABSTRACT
The intrarenal renin-angiotensin system (RAS) plays an important role in the progression of diabetic nephropathy. We have previously reported that mice overexpressing angiotensinogen in renal proximal tubular cells (RPTC) develop hypertension, albuminuria, and renal injury. Here, we investigated whether activation of the intrarenal RAS contributes to apoptosis of RPTC in diabetes. Induction of diabetes with streptozotocin in these transgenic mice led to significant increases in BP, albuminuria, RPTC apoptosis, and proapoptotic gene expression compared with diabetic nontransgenic littermates. Insulin and/or RAS blockers markedly attenuated these changes. Hydralazine prevented hypertension but not albuminuria, RPTC apoptosis, or proapoptotic gene expression. In vitro, high-glucose medium significantly increased apoptosis and caspase-3 activity in rat immortalized RPTC overexpressing angiotensinogen compared with control cells, and these changes were prevented by insulin and/or RAS blockers. In conclusion, intrarenal RAS activation and high glucose may act in concert to increase tubular apoptosis in diabetes, independent of systemic hypertension.


Diabetic nephropathy (DN) is the leading cause of all ESRD in North America, accounting for 45% to 50% of all cases.1,2 Intensive insulin therapy and chronic treatment with renin-angiotensin system (RAS) blockers are effective in retarding DN progression but do not provide a cure.3–5 The local intrarenal RAS is well accepted.6,7 Renal proximal tubular cells (RPTC) express all components of the RAS.8–10 Angiotensin II (Ang II) levels and RAS genes are elevated in the kidneys of diabetic rats and humans,11–13 implying an important role for the intrarenal RAS in DN progression.

Glomerular changes in DN first consist of hypertrophy and, later, thickening of the glomerular basement membrane, followed by expansion of the mesangial matrix and glomerulosclerosis.14–16 However, the gradual decline of renal function in later stages of DN is invariably associated with tubular atrophy and interstitial fibrosis, hallmarks of ESRD.17,18 In fact, tubular atrophy and interstitial fibrosis are closely associated with loss of renal function and appear to be better predictors of renal disease progression than glomerular pathology.15–18 Tubular atrophy in DN is incompletely understood, although apoptosis is a candidate mechanism. Indeed, apoptosis has been detected in RPTC of diabetic mouse, rat, and human kidneys.19–24

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Our previous study documented that transgenic (Tg) mice overexpressing rat angiotensinogen (rAgt) cDNA in RPTC are prone to develop hypertension, albuminuria, and renal injury. The present study investigated whether intrarenal RAS activation could act in concert with high glucose to enhance RPTC apoptosis independent of systemic hypertension and whether treatment with insulin, RAS blockers, or a combination of both could reverse these events.

RESULTS

Tissue-specific Expression of the rAgt Transgene in Tg Mice

The rAgt-HA transgene was expressed only in the kidneys (Figure 1A) and RPT of adult male Tg mice (Figure 1B) but not in other tissues and RPT of non-Tg mice. Immunostaining confirmed the increased expression of Agt protein in RPTC of Tg mice (Figure 1C). In subsequent experiments, testosterone pellets were not used, because the endogenous testosterone was sufficient to induce rAgt-HA transgene in adult male Tg mice.

Renal Hypertrophy and Albuminuria in Diabetic rAgt-Tg Mice

As expected, STZ administration resulted in elevated blood glucose levels in both non-Tg and Tg mice (Figure 2A) and increased the kidney/body weight and urinary albumin/creatinine ratio (Figure 2, B and C). Insulin normalized these parameters in both diabetic non-Tg and Tg mice. Treatment with RAS blockers also attenuated the kidney/body weight ratio and the urinary albumin/creatinine ratio without affecting blood glucose level. Treatment with insulin plus RAS blockers completely reversed all changes observed in diabetic Tg mice (Figure 2, B and C). Of note, the urinary albumin/creatinine ratio in Tg mice was significantly higher than in non-Tg mice (Figure 2C).

Hypertension in Tg Mice

We detected slight elevations in mean systolic BP in male Tg mice starting at week 11; however, these increases became statistically significant only at week 12 and thereafter (Figure 3A). Hydralazine treatment (commenced on week 14) reduced BP of Tg mice to that of non-Tg mice after 1 wk of treatment (Figure 3A). STZ-induced diabetes did not evoke further increases in BP in non-Tg and Tg mice, and insulin treatment did not reduce BP (Figure 3B). In contrast, treatment with RAS blockers alone or in combination with insulin significantly decreased BP in both diabetic non-Tg and Tg mice (Figure 3B).

Morphologic Studies

As expected, diabetic non-Tg mice developed glomerular and RPTC hypertrophy (Figure 4A, a and b) and exhibited a large number of detached cells and cell debris in the tubular lumen and loss of the brush border in RPTC in diabetic kidneys, indicating RPTC damage. Treatment with insulin (Figure 4A, c) or RAS blockers (Figure 4A, d) attenuated the glomerular hypertrophy and tubular damage in diabetic non-Tg mice. Unlike non-Tg mice, renal structural damage was evident in nondiabetic Tg mice. Histologic findings included vacuole degeneration in RPTC and accumulation of cell debris in the tubular lumen (Figure 4A, e). Kidneys of diabetic Tg mice showed even more severe morphologic changes, including marked tubular luminal dilation, cell debris accumulation inside tubules and loss of brush border in RPTC (Figure 4A, f). Some RPTC even became flattened. Treatment with insulin (Figure 4A, g) or RAS blockers (Figure 4A, h) markedly attenuated, whereas combination of insulin and RAS blockers completely reversed, these abnormalities (Figure 4A, i).

Glomerular volume, RPTC volume, and tubular luminal area were significantly increased in diabetic non-Tg and Tg mice compared with nondiabetic littermates (Figure 4, B, C, and D). Once again, treatment with insulin or RAS blockers...
alone effectively attenuated, whereas the combination treatment fully reversed, these changes. It should be noted that RPTC volume (Figure 4C) but not glomerular volume (Figure 4B) was significantly higher in Tg mice than in non-Tg mice. In contrast, tubular luminal area was significantly lower in Tg mice than in non-Tg mice (Figure 4D). Similar pattern for cell debris quantification was observed in these studies (data not shown).

**Figure 2.** Blood glucose, kidney/body weight ratio, and urinary albumin/creatinine ratio in male non-Tg and Tg mice after 4 wk of STZ-induced diabetes with or without treatment with insulin or RAS blockers. (A) Blood glucose. Blood glucose was measured in the morning without fasting at the end of the study. (B) Kidney-to-body-weight ratio. The kidney-to-body-weight ratio was measured as the weight of two kidneys per body weight. (C) Urinary albumin (µg/ml)/creatinine (mg/dl) ratio. All data are expressed as means ± SD, n = 8 (*P < 0.05, **P < 0.01; ***P < 0.005; NS). Non-Tg (empty bars) and Tg (solid bars) mice.

**rAgt Overexpression Augments Apoptosis in Diabetic RPTC**

Apoptotic cells were detected in RPT and in a few distal tubules of diabetic non-Tg mice (Figure 5, A, a and b). Treatment with insulin (Figure 5A, c) or RAS blockers (Figure 5A, d) significantly attenuated development of apoptosis in RPT and distal tubules in diabetic non-Tg mice. The number of apoptotic RPTC was higher in Tg mice than in non-Tg mice (Figure 5A, a and e) and was even higher in diabetic Tg mice (Figure 5A, f). While treatment with insulin (Figure 5A, g) or RAS blockers alone (Figure 5A, h) effectively attenuated RPTC apoptosis, insulin plus RAS blockers fully prevented RPTC apoptosis (Figure 5A, i). Semiquantitative estimation of the number of apoptotic RPTC confirmed these findings (Figure 5B).

Likewise, immunohistochemistry showed increased staining for active caspase-3 and Bax in RPTC of diabetic non-Tg
Figure 4. Hematoxylin/eosin staining of kidneys of male non-Tg mice and Tg mice after 4 wk of STZ-induced diabetes with or without treatment with insulin, RAS blockers or insulin plus RAS blockers. (A) a, Nondiabetic control; b, STZ-induced diabetes; c, insulin-treated diabetic mouse; d, RAS-blocker-treated diabetic mouse; e, nondiabetic Tg mouse; f, STZ-induced diabetic Tg mice; g, insulin-treated, STZ-induced diabetic Tg mice; h, RAS blockers treated, STZ-induced diabetic Tg mice; i, insulin plus RAS blockers treated, STZ-induced diabetic Tg mice. Magnification, ×600. (B) Mean glomerular volume of male non-Tg and Tg mouse kidneys with or without treatment at week 20. (C) Mean RPTC volume of male non-Tg and Tg mouse kidneys with or without treatment at week 20. (D) Mean tubular lumen area of male non-Tg and Tg mouse kidneys with or without treatment at week 20. Values are expressed as means ± S.E., n = 8 (*P < 0.05; **P < 0.01, ***P < 0.005; NS). Non-Tg (empty bars) and Tg (solid bars) mice.
mice (Figure 6, A, b and Figure 7, A, b) compared with nondiabetic non-Tg mice (Figure 6A, a and Figure 7A, a). Treatment with insulin (Figure 6A, c and Figure 7A, c) or RAS blockers (Figure 6A, d and Figure 7A, d) effectively attenuated these changes. RPTC of Tg mice expressed active caspase-3 and Bax at higher levels (Figure 6A, e and Figure 7A, e) than non-Tg mice (Figure 6A, a and Figure 7A, a). Kidneys of diabetic Tg mice exhibited the highest levels of active caspase-3 and Bax (Figure 6A, f and Figure 7A, f), and these were effectively attenuated by insulin (Figure 6A, g and Figure 7A, g) or RAS blockade (Figure 6A, h and Figure 7A, h). Combination of insulin and RAS blockers resulted in almost complete reversal of these changes (Figure 6A, i and Figure 7A, i). Similar trends were detected by semiquantitation of the number of caspase-3 and Bax positive RPTC (Figure 6B) and by Western blotting of active caspase-3 expression (antibody dilution 1:500) in mouse RPT extracts of male non-Tg and Tg mice.

Figure 5. Apoptosis in male non-Tg and Tg mouse kidneys after 4 wk of STZ-induced diabetes with or without treatment with insulin or RAS blockers, analyzed by TUNEL assay. (A) a, Nondiabetic controls; b, STZ-induced diabetes; c, insulin-treated, diabetic mouse; d, RAS-blocker-treated, diabetic mouse; e, nondiabetic Tg mouse; f, STZ-induced diabetic Tg mouse; g, insulin-treated, STZ-induced diabetic mouse; h, RAS-blocker-treated, diabetic Tg mouse; i, insulin plus RAS-blocker-treated, diabetic Tg mouse. Magnification: ×200. Arrows indicate apoptotic cells in proximal tubule. (B) Bar graph showing semiquantitative analysis of apoptotic RPTC from male non-Tg and Tg mouse kidneys after 4 wk of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. All data are expressed as means ± SD, n = 8 (*P < 0.05, **P < 0.01; ***P < 0.005; NS). Non-Tg (empty bars) and Tg (solid bars) mice.

Figure 6. Immunohistochemical staining of α-active caspase-3 in male non-Tg and Tg mouse kidneys after 4 wk of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers using rabbit anti-α-active caspase-3 antibodies (1:50 dilution). (A) a, Nondiabetic controls; b, STZ-induced diabetes; c, insulin-treated diabetic mouse; d, RAS-blocker-treated diabetic mouse; e, nondiabetic Tg mouse; f, STZ-induced diabetic Tg mouse; g, insulin-treated, STZ-induced diabetic mouse; h, RAS-blocker-treated, diabetic Tg mouse; i, insulin plus RAS-blocker-treated, diabetic Tg mouse. Antibody dilution 1:50. Magnification: ×600. (B) Semiquantitative analysis of caspase-3 staining RPTC from male non-Tg and Tg mouse kidneys after 4 wk of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. All data are expressed as means ± SD, n = 8 (*P < 0.05, **P < 0.01; ***P < 0.005). Non-Tg (empty bars) and Tg (solid bars) mice. (C) Western blot analysis of active caspase-3 expression (antibody dilution 1:500) in mouse RPT extracts of male non-Tg and Tg mice.
Figure 7. Immunohistochemical staining of Bax in male non-Tg and Tg mouse kidneys after 4 wk of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers using rabbit anti-Bax antibodies. (A) a, Nondiabetic control mouse; b, STZ-induced diabetes; c, insulin-treated, diabetic mouse; d, RAS-blocker-treated, diabetic mouse; e, nondiabetic Tg mouse; f, STZ-induced diabetic Tg mouse; g, insulin-treated, STZ-induced diabetic mouse; h, RAS-blocker-treated, diabetic Tg mouse; i, insulin plus RAS-blocker-treated, diabetic Tg mice. Antibody dilution 1:50. Magnification: ×600. (B) Semiquantitative analysis of Bax staining RPTC from male non-Tg and Tg mouse kidneys after 4 wk of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. All data are expressed as means ± SD, n = 8 (*P < 0.05, **P < 0.01, ***P < 0.005). Non-Tg (empty bars) and Tg (solid bars) mice. (C) Western blot analysis of Bax expression (antibody dilution 1:1000) in mouse RPT extracts of male non-Tg and Tg mice.
rAgt Overexpression Enhances Bax and Decreases Bcl-xL mRNA Expression in Mouse RPT

Hyperglycemia alone increased Bax mRNA (Figure 8A) and decreased Bcl-xL mRNA expression (Figure 8B) in both non-Tg and Tg mouse RPT. Combined treatment with insulin and RAS blockers was more effective in reversing these changes than treatment with insulin or RAS blockers alone. These studies demonstrate that hyperglycemia and intrarenal RAS activation act in concert to alter the ratio of Bax and Bcl-xL mRNA expression, consistent with enhanced RPTC apoptosis.

Effect of Hydralazine in Tg Mice

Hydralazine treatment markedly reduced systemic BP in Tg mice without producing significant effects on blood glucose, the kidney/body weight ratio, and albuminuria (Figure 9). Furthermore, hydralazine failed to affect RPTC apoptosis (Figure 10), caspase-3 and Bax immunostaining, and Bax and Bcl-xL mRNA expression (Figure 11). These results indicate that albuminuria and RPTC apoptosis occur independently of systemic hypertension.

Agt Overexpression and High-Glucose-Enhanced Rat Immortalized RPTC Apoptosis In Vitro

Culture of RPTC stably transfected with pRC/RSV or pRSV/rAGT in high-glucose medium led to enhanced apoptosis and caspase-3 activity (Figure 12). Consistent with our in vivo observations, high-glucose-induced RPTC apoptosis and caspase-3 activity were attenuated in the presence of insulin or RAS blockers, with complete inhibition being achieved in the presence of both insulin and RAS blockers (Figure 12). Thus, RAS activation and high glucose can directly induce RPTC apoptosis.

Figure 8. RT-qPCR assays of Bax and Bcl-xL mRNA expression in RPT of non-Tg and Tg mice after 4 wk of STZ-induced diabetes with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. Bax and β-actin or Bcl-xL and β-actin mRNA were run simultaneously in the same RT-qPCR assay. The CT (threshold cycle) value was measured to determine the starting copy number of target genes using the standard curve. Lower CT values indicate higher amounts of PCR products. Bax and Bcl-xL mRNA levels were normalized by corresponding β-actin mRNA levels. Bax and Bcl-xL mRNA levels in nondiabetic non-Tg were considered as 100%. All data are expressed as means ± SD, n = 8 (*P < 0.05, **P < 0.01, ***P < 0.005; NS). Non-Tg (empty bars) and Tg (solid bars) mice.

Figure 9. Blood glucose, kidney/body weight ratio, urinary albumin/creatinine ratio, and mean systolic BP in male non-Tg and Tg mice with or without hydralazine treatment. (A) Blood glucose. Blood glucose was measured in the morning without fasting at the end of the study. (B) Kidney-to-body-weight ratio. The kidney-to-body-weight ratio was measured as the weight of two kidneys per body weight. (C) Urinary albumin (μg/ml)/creatinine (mg/dl) ratio. (D) Mean systolic BP. All data are expressed as means ± SD, n = 8 (*P < 0.05, **P < 0.01; ***P < 0.005; NS). Non-Tg (empty bars) and Tg (solid bars) mice.
DISCUSSION

The present observations indicate that renal rAgt overexpression and high blood glucose act in concert to induce albuminuria and RPTC apoptosis in diabetic Tg mice independent of systemic hypertension.

The transgene expression in our model is kidney-specific because strong rAgt-HA mRNA and Agt protein expression was observed in RPTC of Tg mice. Four weeks post-STZ, the mice exhibited high blood glucose levels, increased kidney to body weight ratio, and albuminuria, characteristics of STZ-induced diabetes. Insulin treatment ameliorated these changes. Treatment with RAS blockers exerted similar beneficial actions, with the exception of blood glucose level, which remained high after RAS blockade. These findings suggest that renal changes can be attributed to the diabetic state per se rather than to a nephrotoxic effect of STZ.

Male Tg mice had significantly higher systolic BP than non-Tg littermates, and STZ-induced diabetes did not lead to significant additional increases in systolic BP. The effectiveness of RAS blockers in reducing BP in Tg mice implicates the involvement of intrarenal RAS activation in the development of hypertension.

Our results highlight an important role for intrarenal Agt gene expression in mediating tubular apoptosis in the diabetic mouse kidney. Indeed, the numbers of TUNEL-positive RPTC were significantly higher in Tg mice than in non-Tg mice and were effectively reduced after treatment with RAS blockers. Losartan reduced the number of apoptotic cells in kidneys of STZ-induced diabetic rats and prevented Ang II-stimulated RPTC apoptosis and glomerular cells in vitro. Consistent with the findings in the TUNEL assay, RPT of diabetic non-Tg and Tg mice exhibited enhanced active

Figure 10. TUNEL assay of apoptotic RPTC in male non-Tg and Tg mouse kidneys with or without hydralazine treatment. (A) Non-Tg control mouse (a and b); Tg mouse (c and d); Tg mouse with hydralazine treatment (e and f). (B) Semiquantitative analysis of apoptotic RPTC. All data are expressed as means ± SD, n = 8 (*P < 0.05, NS). Non-Tg (empty bars) and Tg (solid bars) mice. Note: In a, c and e, magnification: ×200; in b, d and f, magnification: ×600.

Figure 11. Bar graph showing semiquantitative analysis of active caspase-3 and Bax staining in RPTC and RT-qPCR assay of Bax and Bcl-XL mRNA expression in RPT of male non-Tg and Tg mice with or without hydralazine treatment. (A) Active caspase-3 staining RPTC. (B) Bax staining RPTC. (C) Bax mRNA expression. (D) Bcl-XL mRNA expression. Urinary albumin (µg/ml)/creatinine (mg/dl) ratio. (D) Mean systolic BP. The relative densities of Bax (C) and Bcl-xL (D) mRNA were normalized with control β-actin mRNA. Bax and Bcl-xL mRNA levels in nondiabetic non-Tg were considered as 100%. All data are expressed as means ± SD, n = 8 (*P < 0.05, **P < 0.01; ***P < 0.005; NS). Non-Tg (empty bars) and Tg (solid bars) mice.
and perindopril, 10 M glucose) medium plus 0.1% depleted FBS in the absence or presence of insulin (10 M glucose medium). (B) Caspase-3 activity. The graphs represent the mean ± SD of four independent experiments and each treatment group was assayed in duplicate (*P < 0.05, **P < 0.01). pRC/RSV stable transfectants (empty bars) and pRSV/rAgt stable transfectants (solid bars).

Figure 12. Effect of high glucose on RPTC apoptosis and caspase-3 activity in stable transfectants with or without treatment with insulin or RAS blockers or insulin plus RAS blockers. pRC/RSV and pRSV/rAgt stable transfectants were plated at a density of 1 × 10⁴ cells in six-well plates in DMEM supplemented with 5% FBS. When the cells reached 60% confluence, the medium was changed to serum-free DMEM and incubated overnight. Then, the cells were incubated for 24 h in normal glucose (5 mM D-glucose plus 20 mM D-mannitol) or high glucose (25 mM D-glucose) medium plus 0.1% depleted FBS in the absence or presence of insulin (10⁻⁶ M) or RAS blockers (losartan, 10⁻⁶ M; and perindopril, 10⁻⁴ M) or insulin plus RAS blockers and analyzed for apoptosis or caspase-3 activity. (A) TUNEL assay of pRC/RSV and pRSV/rAgt stable transfectants in normal or high-glucose medium. (B) Semiquantitative analysis of apoptotic RPTC. (C) Caspase-3 activity. The graphs represent the mean ± SD of four independent experiments and each treatment group was assayed in duplicate (*P < 0.05, **P < 0.01). pRC/RSV stable transfectants (empty bars) and pRSV/rAgt stable transfectants (solid bars).

caspase-3 and Bax expression. These changes were attenuated by treatment of mice with insulin or RAS blockers alone, with complete inhibition being achieved with insulin plus RAS blockers. Although active caspase-3 and Bax expression was undetectable in wild-type animals by immunostaining and Western blotting, both caspase 3 and Bax were highly expressed in Tg mice. Enhanced Bax expression occurred with concomitant down-regulation of the anti-apoptotic gene Bcl-xL. Treatment with insulin or RAS blockers reversed the Bax/Bcl-xL ratio that was further reduced by insulin plus RAS blockers. An increase in the Bax/Bcl-xL ratio would promote apoptosis and is a likely mechanism of how intrarenal RAS activation and high blood glucose enhanced tubular apoptosis in diabetes.

Next, we addressed whether intrarenal RAS-induced kidney injury was due to elevated systemic BP. Hydralazine treatment of Tg mice decreased hypertension; however, it failed to affect albuminuria, RPTC apoptosis, and proapoptotic gene expression. This study shows for the first time that intrarenal rAgt overexpression does lead to the development of hypertension, and albuminuria and RPTC apoptosis occur independently of systemic hypertension. Our in vitro findings that rAgt overexpression and high glucose can induce RPTC apoptosis further indicate a direct action on the kidney. Taken together, these data provide evidence that intrarenal RAS and high glucose could act in concert to induce tubular apoptosis in diabetes.

The precise molecular mechanism(s) by which intrarenal RAS and high glucose enhanced RPTC apoptosis in our Tg mouse model remain unclear. High glucose and Ang II are known to stimulate ROS, and ROS through activation of p38 MAPK regulates p53 phosphorylation. After translocation to the nucleus, the phosphorylated p53 would enhance Bax gene transcription. Bax translocates to the mitochondria and binds the antiapoptotic proteins Bcl-2 and Bcl-xL and inhibits their protective actions on gate-keeping, ultimately leading to mitochondrial dysfunction and activation of caspase-3. Our data on increased Bax and caspase-3 expression combined with decreased Bcl-xL expression in RPTC lend support to this notion.

The pathophysiological importance of RPTC apoptosis induced by rAgt overexpression remains to be elucidated. Because tubular cells undergo apoptosis in murine and human diabetic kidneys and the present study, we propose that RPTC apoptosis may be an initial mechanism leading to tubular atrophy that has been detected in the kidneys of diabetic patients.

Taken together, our results suggest that activation of intrarenal RAS and high blood glucose act in concert to induce RPTC apoptosis independent of systemic hypertension and most likely lead to tubular atrophy in diabetes. Our data also indicate that treatment with insulin plus RAS blockers could prevent tubular apoptosis and DN progression.

**Concise Methods**

The pKAP2 plasmid containing the kidney androgen-regulated promoter, which is responsive to testosterone stimulation, was obtained from Dr. Curt D. Sigmund (University of Iowa, Iowa City, IA) and has been described elsewhere. A rabbit polyclonal antibody against rAgt was generated in our lab (J.S.D.C.). Anti-Bax and anti-active caspase-3 polyclonal antibody were procured from New England Biolabs Ltd. (Pickering, ON, Canada). Losartan (a nonpeptide Ang II-receptor subtype 1 blocker) and perindopril (an inhibitor of angiotensin-converting enzyme) were obtained from Dupont Merck (Wilmington, DE) and Servier Amérique (Laval, QC, Canada), respectively. Insulin implants (LinBit) (insulin release rate: approximately 0.1 unit/implant/d for >30 d) were purchased from Linshin (Scarborough, ON, Canada). Oligonucleotides were synthesized by InVitrogen, Inc. (Burlington, ON, Canada). Restriction and modifying enzymes were from either InVitrogen, Inc. or La Roche Biochemicals (Dorval, QC, Canada).
Agt Tg Mice
We created Tg mice (C57Bl6 background, line #388) by inserting rAgt cDNA fused with an HA tag (a sequence encoding amino acid residues 98 to 106 (YPYDVPDYA) of human influenza virus hemagglutinin) at the 3′ terminal into a construct containing the kidney androgen-regulated promoter. All animals received standard mouse chow and water ad libitum. The experimental procedures were approved by the Animal Protection Committee of the Centre Hospitalier de l’Université de Montréal (CHUM).

Physiologic Studies
Male Tg mice and non-Tg littermates were divided into four groups (eight mice per group). Group 1 (control group) received vehicle intraperitoneally. Group 2: Streptozotocin (STZ)-induced diabetes: The animals were administered STZ (50 mg/kg in 10 mM sodium citrate buffer, pH 4.5), intraperitoneally, daily for 5 consecutive days. Forty-eight hours after the last STZ administration, blood glucose level was determined with a Side-Kick Glucose Analyzer (Model 1500; Interscience, ON, Canada). Mice with blood glucose >16.6 mM were studied. Group 3: Insulin treatment: On day 3 after the last STZ injection, diabetic mice received a subcutaneous insulin implant (one LinB2 implant per 20 g body weight and a 0.5 LinB2 implant for each additional 5 g body weight) to maintain euglycemia. Blood glucose was monitored twice a week. Group 4: Treatment with RAS blockers: Beginning on day 3 after the last STZ injection, mice received RAS blockers (losartan [30 mg/kg per d] plus perindopril [4 mg/kg per d] in the drinking water). Additionally, STZ-induced diabetic Tg mice treated with RAS blockers plus insulin and Tg mice treated with hydralazine (15 mg/kg per d in drinking water) were also studied.

Systolic BP was monitored with a BP-2000 tail-cuff pressure device (Visitech Systems, Apex, NC) in the morning, at least 3 to 4 times a week for 10 wk after habituating mice to this procedure for at least 30 min per day for 5 d before the first BP reading. Each point represents the mean ± SD of three to four determinations per week per group of animals. Twenty-four hours before euthanasia with CO2, at 4 wk post-STZ administration, the animals were housed individually in metabolic cages to collect urine samples later assayed for glucose and ketone levels (Ketodex, Linco Research, Madrid, Spain). RPTC volume and tubular luminal area were measured from 100 RPTC and 50 RPT per mouse, respectively, with the same software. Outer cortical RPT with similar cross-sectional views and clear nuclear structure were selected. The mean cell volume was estimated by the Nucleator method.

Terminal Transferase-Mediated Deoxyuridine Triphosphate Nick End-labeling (TUNEL) Assay and Immunohistochemical Staining
The percentage of apoptotic RPTC (TUNEL kit; Roche Diagnostics) was estimated semiquantitatively as described by Kumar et al. Immunohistochemical staining was performed by standard avidin-biotin-peroxidase complex method (ABC Staining System; Santa Cruz Biotechnologies, Santa Cruz, CA).

Western Blotting and Real-time Quantitative Polymerase Chain Reaction Assays for Gene Expression
Western blotting was performed as described. Bax and Bcl-xL mRNA expression in mRPTs was quantified by real-time quantitative polymerase chain reaction using forward and reverse primers corresponding to Bax, Bcl-xL, and -actin cDNA (Table 1).

Cell Culture and Caspase-3 Activity Assay
Rat immortalized RPTC stably transfected with the control plasmid pRC/RSV or with a plasmid pRSV/rAgt containing the rAgt cDNA were cultured as described.

Caspase-3 activity was determined fluorimetrically (BD Bioscience Pharmingen, Mississauga, ON, Canada).

Statistics
Data are expressed as mean ± SD. The data are analyzed by one-way ANOVA and the Bonferroni test. P values <0.05 were considered significant.

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DISCLOSURES
None.

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