Early streptozotocin-diabetes mellitus downregulates rat kidney $\text{AT}_2$ receptors

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Departments of Cellular and Molecular Medicine and Medicine, Division of Nephrology, The Kidney Research Centre, Ottawa Hospital Research Institute and University of Ottawa, Ottawa, Ontario, Canada K1H 8L6; and the Department of Medicine, University of Virginia Health System, Charlottesville, Virginia 22908

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Wehbi, George J., Joseph Zimpelmann, Robert M. Carey, David Z. Levine, and Kevin D. Burns. Early streptozotocin-diabetes mellitus downregulates rat kidney $\text{AT}_2$ receptors. Am J Physiol Renal Physiol 280: F254–F265, 2001.—The interaction of ANG II with intrarenal AT$_1$ receptors has been implicated in the progression of diabetic nephropathy, but the role of intrarenal AT$_2$ receptors is unknown. The present studies determined the effect of early diabetes on components of the glomerular renin-angiotensin system and on expression of kidney AT$_2$ receptors. Three groups of rats were studied after 2 wk: 1) control (C), 2) streptozotocin (STZ)-induced diabetic (D), and 3) STZ-induced diabetic with insulin implant (DI), to maintain normoglycemia. By competitive RT-PCR, early diabetes had no significant effect on glomerular mRNA expression for renin, angiotensinogen, or angiotensin-converting enzyme (ACE). In isolated glomeruli, nonglycosylated (41-kDa) AT$_1$ receptor protein expression ($\text{AT}_{1A}$ and $\text{AT}_{1B}$) was increased in D rats, with no change in glycosylated (53-kDa) AT$_1$ receptor protein or in AT$_1$ receptor mRNA. By contrast, STZ diabetes caused a significant decrease in glomerular AT$_2$ receptor protein expression (47.0 ± 6.5% of C; $P < 0.001; n = 6$), with partial reversal in DI rats. In normal rat kidney, AT$_2$ receptor immunostaining was localized to glomerular endothelial cells and tubular epithelial cells in the cortex, interstitial, and tubular cells in the outer medulla, and inner medullary collecting duct cells. STZ diabetes caused a significant decrease in AT$_2$ receptor immunostaining in all kidney regions, an effect partially reversed in DI rats. In summary, early diabetes has no effect on glomerular mRNA expression for renin, angiotensinogen, or ACE. AT$_2$ receptors are present in glomeruli and are downregulated in early diabetes, as are all kidney AT$_2$ receptors. Our data suggest that alterations in the balance of kidney AT$_1$ and AT$_2$ receptor expression may contribute to ANG II-mediated glomerular injury in progressive diabetic nephropathy.

Ang II is synthesized within the kidney and is a mediator of progressive injury in diabetic nephropathy. Although evidence suggests activation of the intrarenal renin-angiotensin system (RAS) in diabetes (23), the source of intrarenal ANG II formation remains unclear. Anderson et al. (3) demonstrated an increase in kidney renin mRNA and activity in early streptozotocin (STZ)-induced diabetes in rats, and a stimulatory effect of high glucose on proximal tubule angiotensinogen expression was found in cell culture studies (42). In rats with early STZ diabetes, we recently demonstrated increased proximal tubule cell mRNA expression for renin, with reversal by insulin, suggesting that hyperglycemia may selectively augment proximal tubule ANG II synthesis (51).

The glomerulus also has the capacity for synthesis of ANG II (5). Components of the renin-angiotensin system have been detected in cultured rat (8) and human (19) mesangial cells, and glomerular endothelial cells express angiotensin-converting enzyme (ACE) (46). The effect of diabetes on expression of components of the glomerular RAS has not been extensively studied, although glomerular ACE appears to be upregulated in early diabetes in both rats (3) and humans (25).

Once ANG II is formed in the kidney, it appears to exert most of its hemodynamic and nonhemodynamic effects via interaction with plasma membrane AT$_1$ receptors. AT$_1$ receptor stimulation causes enhanced vasoconstriction of the efferent arteriole compared with the afferent arteriole (48), which may contribute to increased glomerular capillary pressure and mechanical stretch-induced glomerular injury (2). In addition, ANG II binds to AT$_1$ receptors present on glomerular mesangial cells, tubular cells, and interstitial cells, resulting in cell hypertrophy, and is associated with stimulation of extracellular matrix production and elaboration of the profibrotic cytokine transforming growth factor-$\beta$ (TGF-$\beta$) (15, 44). A number of studies have demonstrated a downregulation of glomerular and tubular AT$_1$ receptors in early diabetes (7, 9, 11, 16, 49). Nonetheless, the importance of AT$_1$...
receptor activation in mediating progressive diabetic glomerulosclerosis is suggested by studies in experimental diabetes in which AT$_1$ receptor blockade significantly reduced proteinuria and inhibited development of glomerulosclerosis (29, 38), similar to the beneficial effects of ACE inhibitors in diabetic humans (1, 19, 28, 37) and animals (38, 49).

ANG II AT$_2$ receptors have recently been detected in adult rat kidney by immunohistochemistry (24, 27). In contrast to AT$_1$ receptors, AT$_2$ receptors are linked to reduction of blood pressure (14, 35), inhibition of cell growth (22, 26), and apoptosis (47). In glomerular endothelial cells, AT$_2$ receptors stimulate expression of the chemokine regulated on activation of normal T cells expressed and secreted (RANTES), which may contribute to recruitment of monocytes and macrophages in glomerular inflammation (45). The role or regulation of AT$_2$ receptors in diabetic nephropathy, however, is unknown.

In the present studies, we determined the effect of early diabetes in rats on mRNA expression of components of the glomerular RAS, and on glomerular AT$_1$ and AT$_2$ receptor expression. We also performed immunohistochemistry to localize AT$_2$ receptor expression in the normal and diabetic kidney. Our results indicate that diabetes has no significant effect on expression of mRNA for glomerular renin, angiotensinogen, ACE, or AT$_1$ receptors but decreases glomerular AT$_2$ receptors. Furthermore, AT$_2$ receptor expression is decreased in all kidney regions in early diabetes. The data suggest that decreased AT$_2$ receptor expression in the diabetic kidney may be a determinant of the rate of progression of glomerular injury.

METHODS

Animal model. Age-matched male Sprague-Dawley rats, weighing 200–225 g, were used for animal studies, after acclimatization for 5 days. Animals were allowed free access to distilled water and standard rat chow. All studies were approved by the Animal Care Committee of the University of Ottawa.

Rats were rendered diabetic with STZ (65 mg/kg ip; Sigma, St. Louis, MO) dissolved in 0.1 M sodium citrate buffer (pH 4.0). After 24 h, urine was assessed for glucose and ketones with a Keto-Diastix reagent strip (Bayer, Etobicoke, ON), and only those animals with sustained glucosuria were classified as diabetic and were included in further experiments. Rats were divided into three groups: 1) control (C) rats, injected with vehicle (0.1 M sodium citrate buffer, pH 4.0); 2) diabetic (D), rats treated daily with 1–2 units of insulin (Humulin L, Eli Lilly, Indianapolis, IN) subcutaneously to maintain hyperglycemia but prevent ketosis; and 3) diabetic plus insulin implant (D+I) rats, implanted with a sustained-release insulin implant (Linplant, Linshin Canada, Scarborough, ON), to maintain euglycemia. After 2 wk, rats were killed by CO$_2$ narcosis followed by decapitation, and blood was collected for glucose, creatinine, and renin activity analysis. Measurements were performed by the Ottawa Hospital Biochemistry Laboratory (General Campus).

Isolation of glomeruli. Glomeruli were isolated by Percoll gradient centrifugation, modified from a previously described method (39). Renal cortices from both rat kidneys were dissected, and gently minced in a glass petri dish on ice. The tissue was then suspended in a solution containing (in mM) 115 NaCl, 24 Na$_2$CO$_3$, 5 KCl, 1.5 CaCl$_2$, 1.0 MgSO$_4$, 2.0 NaH$_2$PO$_4$, 5.0 glucose, 1.0 alanine, and 10.0 HEPES, pH 7.4, as well as 0.03% collagenase (type IV; Sigma) and 0.01% soybean trypsin inhibitor (Sigma; buffer A). The suspension was gassed with 95% O$_2$-5% CO$_2$ and placed in a 37°C water bath for 45 min. After digestion, the cortical suspension was strained through a 250-μm brass sieve (mesh no. 60, Newark Wire Cloth, ESBE Scientific, Markham, ON) and centrifuged for 1 min at 100 g. The pellet was resuspended in buffer A without collagenase or trypsin inhibitor and centrifuged for 1 min. This was repeated three times. The pellet was resuspended in a 40% Percoll (Sigma) solution of identical ionic composition as buffer A, which had been chilled to 4°C. The Percoll solution was centrifuged at 26,000 g for 30 min at 4°C, and the digested tissue was separated into four distinct bands (F1-F4) after centrifugation, as described (39). The uppermost band of the Percoll gradient (F1 layer) was highly enriched in glomeruli, as described (6). Subsequently, further purification of this band by multiple sieving was performed as described (5). Briefly, the F1 layer was removed and washed with PBS buffer (in mM): 8.5 Na$_2$HPO$_4$, 1.7 NaH$_2$PO$_4$, 145 NaCl, pH 7.4. The tissue was then strained through a 106-μm brass sieve (mesh no. 150) with PBS buffer, and glomeruli were collected on a 75-μm brass sieve (mesh no. 200) immediately below it. The collected tissue consisted of a pure (100%) collection of glomeruli, as determined by light microscopy. Glomerular cells did not undergo significant cell death in this preparation as determined by their ability (>95%) to exclude the vital dye Trypan blue (10 mg/dl) (data not shown).

Competitive RT-PCR. Total RNA was isolated from glomeruli by using a commercial kit (RNAeasy, Qiagen, Chatsworth, CA). RNA quality was assessed by running samples on ethidium bromide-stained 2% agarose-formaldehyde gels and by measuring optical density at 260 and 280 nm. RNA yield varied between 5 and 25 μg per glomerular isolation, and all samples were of high quality as assessed by these standards.

To measure absolute mRNA levels for renin, angiotensinogen, and ACE, a competitive RT-PCR assay was performed on total glomerular RNA by using deletion mutant cRNA for each of these components. Each sample of RNA (62.5 ng) was amplified from rat kidney RNA and ligated into the amplification system for renin, angiotensinogen, and ACE were PCR amplified from rat kidney RNA and ligated into the pCR-Script SK(+) cloning vector (Stratagene, La Jolla, CA). The renin cDNA PCR product was generated with a sense primer, 5'-CTGCCACCTTGGTGTGACTGAG-3', and an antisense primer, 5'-CCAGTATGACAGTGTCATCAG-3', and corresponded to bases 1033–1296 of the rat renin cDNA (264-bp PCR product) (12). The angiotensinogen cDNA PCR product was generated with the sense primer 5'-CCTCGCTCTCTG-3' and the antisense primer 5'-CAGACACTGAGTCTGTTG-3' and corresponded to bases 737–962 of the rat angiotensinogen cDNA (226-bp PCR product) (12). Finally, the ACE cDNA PCR product was generated with the sense primer 5'-GCCACATCCAGTATTTCATGCAGT-3' and the antisense primer 5'-AAGGCTGAATGATGTAAGGTC-3' and corresponded to bases 3013–3454 of the rat ACE
cDNA (442-bp PCR product) (17). All primers were obtained from Oligos Etc. (Wilsonville, OR).

Inverse PCR was performed on the plasmids containing the renin, angiotensinogen, and ACE-partial cDNA sequences, by using sense and antisense primers oriented in a "tail-to-tail" direction, thereby amplifying the cloning vector and a fragment of the cDNA, with a gap between the 5' ends of the PCR product (12). The oligonucleotide primers for inverse PCR of the renin cDNA were sense, 5'-CGACT-GAGCGTTGTGAACCTGACCA-3', corresponding to bases 1166–1183 of the rat renin cDNA, and antisense, 5'-CGACT-GAGATATAGAGTTGACCGTGTG-3', corresponding to bases 1093–1076, and resulting in a 208-bp deletion mutant PCR product (deletion of 56 bp). For angiotensinogen, the sense primer, 5'-AGGCCTGTTCTCAAGGAGGAGG-3', corresponded to bases 847–864 of the rat angiotensinogen cDNA, and the antisense primer was 5'-CAGCTGATCCACCAAAACC-3', corresponding to bases 811–791, resulting in a 169-bp deletion mutant PCR product (deletion of 57 bp). For ACE, the sense primer was 5'-GTCCTGGGCGATGTCATAG-3', corresponding to bases 3337–3358 of the rat ACE cDNA, and the antisense primer was 5'-GAAGGTTGATGTCATGCTC-3', corresponding to bases 3195–3177, resulting in a 301-bp deletion mutant cDNA product (deletion of 141 bp). All inverse PCR reactions were performed for 35 cycles in a PerkinElmer Gene Amp 2400 PCR thermocycler, with hot-start at 96°C for 8 min, followed by denaturation at 96°C for 30 s, annealing at 63°C for 30 s, and elongation at 72°C for 90 s. Self-ligation of the PCR product was performed and plasmids were grown in competent Escherichia coli, followed by plasmid isolation (Qiagen). To confirm correct sequences and orientation, all deletion mutants were sequenced by the University of Ottawa DNA sequencing facility.

Renin, angiotensinogen, and ACE cRNAs were transcribed from deletion mutant plasmids by using T3 or T7 RNA polymerase (Stratagene). The template was then degraded with amplification grade deoxyribonuclease I (DNase I, Life Technologies, Burlington, ON), and the RNA products were purified by phenol/chloroform extraction and ethanol precipitation. RNA was quantified by absorbance at 260 nm.

For competitive RT-PCR reactions, RNA samples were treated with DNase I before reverse transcription, to digest residual genomic DNA. Samples of RNA (total RNA and dilutions of deletion mutant cRNA for either renin, angiotensinogen, or ACE) were then reverse-transcribed by using random hexamers (2.5 μM) and murine leukemia RT (2.5 U/μl) (Gene Amp RNA PCR kit, PerkinElmer, Branchburg, NJ). To control for possible genomic or plasmid DNA contamination, all experiments included a reaction in which RT was omitted from the transcription buffer.

After reverse transcription, the cDNA mixture was amplified by PCR, in a total volume of 100 μl containing 2.5 U AmpliTaq DNA polymerase, 2 mM MgCl2, 1× PCR buffer II (PerkinElmer), and 1 μM each of the sense and antisense oligonucleotide PCR primers for the cDNA of interest. The sense and antisense primers for renin, angiotensinogen, and ACE were identical to those used to generate the nonmutant PCR products described above, of 284-, 226-, and 442-bp sizes, respectively. Preliminary experiments revealed that the yield of PCR products for all three components was linear up to 40 cycles of PCR. Accordingly, PCR was performed for 35 cycles, with a hot-start at 96°C for 3 min, followed by cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 45 s, followed by extension at 72°C for 10 min.

To quantitate PCR products, samples were run on 3% agarose gels stained with Vistra green nucleic acid gel stain (Amersham). To determine the amounts of initial mRNA for renin, angiotensinogen, or ACE, PCR products and their corresponding deletion mutants were quantified by PhosphorImager analysis (Storm 860, Molecular Dynamics, Sunnyvale, CA). The logarithms of the ratio of target to deletion mutant (competitor) species were plotted as a function of the initial amount of deletion mutant cRNA. The initial amount of target mRNA was quantitated by extrapolation. Experiments were eliminated from further analysis if the correlation coefficient (r²) for the generated curve was <0.9.

To establish that the competitive RT-PCR assay could detect predicted changes in mRNA expression, normal rats were placed on diets consisting of either 2.9% NaCl (high salt or HS) or 0% NaCl (zero salt or ZS) for 5 days, because sodium depletion is known to stimulate glomerular renin mRNA expression (36). Competitive RT-PCR for renin mRNA was performed on total RNA isolated from cortex and glomeruli. Figure 1A illustrates a representative experiment in which 62.5 ng of total glomerular RNA were reverse transcribed and coamplified with serial dilutions of mutant renin cRNA, with renin primers. The ZS diet caused an approximately threefold increase in renin mRNA in cortex (Fig. 1B: HS: 925.2 ± 54.7 fg mRNA/62.5 ng total RNA vs. ZS: 2,773.7 ± 348.8 fg mRNA/62.5 ng total RNA; P < 0.001; n = 6), and glomeruli (HS: 2,241.0 ± 582.2 fg mRNA/62.5 ng total RNA vs. ZS: 6,223.8 ± 1,320.5 fg mRNA/62.5 ng total RNA; P < 0.025; n = 5).

Northern analysis for glomerular AT1 receptor mRNA. Northern hybridization for AT1 receptor mRNA in glomeruli was performed by using a 1.2-kb 5' genomic DNA fragment encoding the rat AT1A receptor, essentially as we have previously performed (18). Briefly, total RNA was isolated from glomeruli by using the RNasey kit (Qiagen), and 5-μg samples were run on 1% agarose-2.2 M formaldehyde gels and transferred onto nylon membranes (Schleicher and Schuell, Keene, NH), followed by ultraviolet crosslinking (Bio-Rad UV Linker; Bio-Rad, Montreal, QC). The rat AT1A cDNA probe, which hybridizes to both AT1A and AT1B receptor mRNA, was labeled with [32P]dCTP (3,000 Ci/mmol; Amersham) by the random primer method (Multiprime DNA labeling system, Amersham). Hybridization was performed overnight at 42°C, as described (18), and the membranes were washed at low stringency (2× SSC, 0.1% SDS) where SSC is standard sodium citrate, for 30 min at 23°C, and then at high stringency (0.2× SSC, 0.1% SDS) for 15 min at 65°C. Membranes were then exposed for 48 h at ~70°C to Kodak Biomax MS film, with two Cronex intensifiers (Sigma). After quantitation of the AT1a receptor mRNA signal by densitometry, the membranes were stripped and reprobed with [32P]dCTP-labeled cDNA probe for human β-actin, as we have performed (10).

Immunohistochemistry. Kidneys were removed, cut longitudinally, and immediately placed in Zamboni's fixative (2% paraformaldehyde, 15% picric acid in PBS) for 2 h. The solution was replaced with fresh fixative and incubated at 4°C overnight. The following day the tissue was washed with 10% sucrose in PBS. The sucrose phosphate buffer was replaced daily on each of the following 7 days. Kidneys were then paraffin embedded, and 10-μm sections were cut. Prior to staining, sections were deparaffinized, and endogenous peroxidase activity was blocked by incubating the slides in 0.3% H2O2 in 100% MeOH for 30 min. To block nonspecific binding, sections were incubated in PBS containing 1% milk and 3% goat serum. Sections were then incubated for 48 h at 4°C in a humidified chamber with a rabbit anti-rat polyclonal AT2 receptor antibody (24) diluted 1:100 in PBS containing 1.5% goat serum and 0.5% milk. This antibody has a high

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degree of specificity for the rat AT₂ receptor and has been used to detect AT₂ receptors in the rat adrenal gland, rat brain, and both the rat fetal and adult kidney, with appropriate use of preimmune and preabsorption controls (27).

Furthermore, this AT₂ receptor antibody has been used to demonstrate AT₂ receptor immunostaining in the myocardium and coronary vessels of the neonatal and young rat heart (41). Preabsorption against the peptide antigen blocked any positive immunostaining in these studies, and on immunoblots the AT₂ receptor protein was detected in neonatal cardiac myocytes, but not neonatal cardiac fibroblasts or rat aortic smooth muscle cells (41). After incubation with the AT₂ receptor antibody, the slides were incubated for 30 min at room temperature in a humidified chamber with a biotinylated anti-rabbit IgG, diluted 1:50 in PBS as a secondary antibody. The slides were subsequently placed in 3% H₂O₂ for 10 min prior to incubation with streptavidin-horseradish peroxidase (HRP), diluted 1:50 in PBS for 30 min at room temperature in a humidified chamber. Finally, the slides were incubated with 50 μl of diaminobenzidine (BioGenex, San Ramon, CA) as substrate. The slides were counterstained with hematoxylin (Sigma), dehydrated, fixed with Permount (Fisher Scientific, Ottawa, ON) histological mounting medium, and viewed with a Zeiss Axiophot microscope. To exclude nonspecific binding, all experiments included a control in which the primary antibody was preincubated with a 20-fold excess of immunizing peptide for 1 h at

Fig. 2. Competitive quantitative RT-PCR assay for glomerular renin mRNA from 2-wk diabetic rats. Competitive RT-PCR for renin mRNA from the glomeruli of 2-wk C, D, and D+I rats. Values are means ± SE (n = 6).

**Table 1. Whole animal data**

<table>
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<tr>
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<th>C</th>
<th>D</th>
<th>D+I</th>
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<tbody>
<tr>
<td>Plasma glucose, mM</td>
<td>9.6 ± 0.2</td>
<td>37.8 ± 1.6*</td>
<td>4.9 ± 0.4‡</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>359.3 ± 4.7</td>
<td>267.9 ± 5.6*</td>
<td>350.6 ± 7.6</td>
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<tr>
<td>Kidney weight, g</td>
<td>1.31 ± 0.03</td>
<td>1.49 ± 0.05†</td>
<td>1.25 ± 0.02</td>
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<tr>
<td>Kidney weight as %</td>
<td>0.37 ± 0.01</td>
<td>0.56 ± 0.02*</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>Serum creatinine, μmol/l</td>
<td>52.5 ± 1.1</td>
<td>53.1 ± 1.4</td>
<td>51.7 ± 1.5</td>
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<tr>
<td>Plasma renin activity, ng·l⁻¹·s⁻¹</td>
<td>5.95 ± 1.48</td>
<td>3.40 ± 0.61</td>
<td>4.16 ± 0.62</td>
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Values are means ± SE (n = 12). Measurements were made at the time of death, 2 wk after intervention, except for diabetic rats (D). C, control; D+I, streptozotocin-induced diabetic with insulin implant. *P < 0.001 vs. C and D+I; †P < 0.005 vs. C and D+I; ‡P < 0.05 vs. C.

Fig. 1. Quantitation of glomerular renin mRNA by competitive RT-PCR. A: competitive RT-PCR for renin mRNA from a representative experiment after 2 wk of diabetes. Glomerular total RNA (62.5 ng) was reverse-transcribed and coamplified with serial dilutions of mutant renin cRNA. A representative gel for the PCR products is shown in the inset: Lane 1: a 100-bp DNA ladder; lanes 2, 5, and 8: products with 25 pg of deletion mutant cRNA; lanes 3, 6, and 9: products with 5 pg of deletion mutant cRNA; lanes 4, 7, and 10: products with 0.5 pg of deletion mutant cRNA. The wild-type and mutant cDNA products are observed at 264 and 208 bp, respectively. The unknown amount of mRNA was determined by extrapolation at the point where the log of the ratio of the intensities of the cDNA products is zero. (C), control; (D), diabetic; (D+I), diabetic plus insulin implant. B: validation of competitive RT-PCR for renin mRNA from cortex and glomeruli of rats on a 5-day diet of either high-salt (2.9%), or zero-salt rat chow. Values are means ± SE from cortex (n = 6) and glomeruli (n = 5). *P < 0.001 vs. cortex high salt, and **P < 0.005 vs. glomeruli high salt.
37°C. Slides were quantitatively examined by using the Image-Pro Plus 4.0 software program (Media Cybernetics, Silver Spring, MD). Each slide was analyzed for staining in the cortex, outer medulla, and inner medulla, with three separate fields viewed in each region. Observations were made with the viewer blinded to the origin of the slide.

Western blot analysis of the AT1 and AT2 receptor. Proteins from glomeruli of C, D, and D+I rats were isolated as described (27). Briefly, glomeruli were homogenized in a buffer containing (in mM) 20 Tris·HCl, 100 NaCl, 2 phenylmethylsulfonyl fluoride, 2 EDTA, 2 EGTA, and 10 sodium orthovanadate, as well as 10% glycerol, 10 μg/l leupeptin, and 10 μg/l aprotinin. The homogenate was then centrifuged at 30,000 g for 30 min at 4°C. The supernatants were removed and kept at −80°C until used for further analysis.

Protein concentrations in the supernatant were determined by the Bradford method (Bio-Rad) by using BSA (Sigma) as the standard. Solubilized tissue samples (40 μg) along with prestained standards as molecular weight markers (Bio-Rad) were subjected to SDS-polyacrylamide gel electrophoresis (5% acrylamide stacking gel and 10% running gel). The resolved proteins were transferred onto nitrocellulose membranes (Bio-Rad). The membranes were then blocked overnight at 4°C with 3% skim milk in Tris-buffered saline (TBS), pH 7.6. For AT2 receptor immunodetection, membranes were incubated with a rabbit polyclonal anti-rat AT2 receptor antibody diluted 1:2,000 in TBS with 2% skim milk and 0.01% sodium azide for 2 h at room temperature. Membranes were then washed with TBS containing 0.01% Tween 20 (TBS-T) and incubated with anti-rabbit secondary antibody (1:2,000) conjugated to HRP (Amersham, Oakville, ON). For AT1 receptor immunodetection, membranes were incubated with a rabbit polyclonal anti-rat AT1 receptor antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-T with 2% skim milk for 2 h at room temperature. The AT1 receptor antibody recognizes both AT1A and AT1B receptors in the rodent. Subsequent to washing with TBS-T, mem-

Nonidet P40, and was stirred for 2 h at 4°C. The lysate was then centrifuged at 30,000 g for 30 min at 4°C. The supernatants were removed and kept at −80°C until used for further analysis.

Protein concentrations in the supernatant were determined by the Bradford method (Bio-Rad) by using BSA (Sigma) as the standard. Solubilized tissue samples (40 μg) along with prestained standards as molecular weight markers (Bio-Rad) were subjected to SDS-polyacrylamide gel electrophoresis (5% acrylamide stacking gel and 10% running gel). The resolved proteins were transferred onto nitrocellulose membranes (Bio-Rad). The membranes were then blocked overnight at 4°C with 3% skim milk in Tris-buffered saline (TBS), pH 7.6. For AT2 receptor immunodetection, membranes were incubated with a rabbit polyclonal anti-rat AT2 receptor antibody diluted 1:2,000 in TBS with 2% skim milk and 0.01% sodium azide for 2 h at room temperature. Membranes were then washed with TBS containing 0.01% Tween 20 (TBS-T) and incubated with anti-rabbit secondary antibody (1:2,000) conjugated to HRP (Amersham, Oakville, ON). For AT1 receptor immunodetection, membranes were incubated with a rabbit polyclonal anti-rat AT1 receptor antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-T with 2% skim milk for 2 h at room temperature. The AT1 receptor antibody recognizes both AT1A and AT1B receptors in the rodent. Subsequent to washing with TBS-T, mem-

Fig. 3. Competitive RT-PCR on glomerular angiotensinogen and angiotensin-converting enzyme (ACE) mRNA. A: competitive RT-PCR for angiotensinogen mRNA from the glomeruli of 2-wk C, D, and D+I rats (n = 4). B: competitive RT-PCR for ACE mRNA from the glomeruli of 2-wk C, D, D+I rats (n = 5). Values are means ± SE.

Fig. 4. Effect of early diabetes on glomerular ANG II type 1 (AT1) receptor mRNA expression. Representative Northern blot is shown for glomerular AT1 receptor mRNA (2.3-kb band) in C, D, and D+I rats. Northern blot for β-actin mRNA, indicating equality of RNA loading is depicted below the AT1 mRNA blot. Graph shows densitometric analyses of data, corrected for β-actin signals (n = 4).
branes were incubated with anti-rabbit secondary antibody (1:2,000) conjugated to HRP (Amersham). Proteins were detected by enhanced chemiluminescence (Amersham) on Hy- perfilm (Amersham) according to the instructions provided with the kit.

To ensure equal protein loading, all membranes were stripped and probed with a monoclonal anti-β-actin antibody (mouse ascites fluid; Sigma) that recognizes the β-actin protein at 43 kDa. Signals on Western blots were quantified by densitometry and corrected for the β-actin signal by using the Kodak Digital Science Image Station 440CF and the 1D Image Analysis program.

**Statistical analysis.** Results are expressed as means ± SE. Data were analyzed by one-way ANOVA followed by Bonferroni correction for all pairwise comparisons, or, in the case of the Western blots, by the Mann-Whitney rank-sum test for nonparametric data. A value of $P < 0.05$ was deemed significant.

**RESULTS**

**Whole animal data.** Table 1 illustrates whole animal data for C, D, and D+I rats. Diabetic rats were markedly hyperglycemic and demonstrated glucosuria, similar to previous studies utilizing this model (7, 11). In D+I rats, blood glucose values decreased, and were, indeed, significantly lower than control values (D+I: 4.9 ± 0.4 mM vs. C: 9.6 ± 0.2 mM; $P < 0.05$; $n = 12$). There were significant decreases in body weight, and increases in kidney weights, in the diabetic group. Insulin treatment completely reversed the decreases in body weight, as well the increases in kidney weight. Serum creatinine levels did not change in any of the groups. In the diabetic group, plasma renin activity (PRA) was slightly suppressed, although this did not achieve statistical significance compared with the control group.

**Competitive RT-PCR.** Renin, angiotensinogen, and ACE mRNAs were quantitated from glomeruli by competitive RT-PCR. As shown in Fig. 2, there was an increase in renin mRNA levels in the glomeruli of D rats compared with C rats, although this did not reach statistical significance (C: 2,497.5 ± 405.0 fg mRNA/62.5 ng RNA vs. D: 3,155.5 ± 417.3 fg mRNA/62.5 ng RNA; $P = NS$; $n = 6$). Moreover, there was no significant difference in glomerular renin mRNA between C and D+I rats (D+I: 2,490.0 ± 645.8 fg mRNA/62.5 ng RNA).

With regard to glomerular angiotensinogen and ACE mRNA levels, there were no significant differences...
between rats in C, D, and D+I groups for each of these RAS components (Fig. 3).

Glomerular AT\textsubscript{1} receptor mRNA and protein expression. By Northern analysis, AT\textsubscript{1} receptor mRNA was readily detected in glomeruli from C, D, and D+I rats, as a band at \( \sim 2.3 \) kb. Early diabetes had no significant effect on glomerular AT\textsubscript{1} receptor mRNA expression (Fig. 4).

Western blot analysis was performed on glomerular cell lysates for the AT\textsubscript{1} receptor. A band of the predicted molecular mass of the nonglycosylated form of the AT\textsubscript{1} receptor (41 kDa) (13) was observed on all Western blots, with an additional band at \( \sim 53 \) kDa, likely representing the glycosylated form of the receptor (21). There was no significant change in expression of the 53-kDa glycosylated form of the AT\textsubscript{1} receptor in glomeruli from diabetic rats (5.4 \( \pm \) 2.8% increase; \( P < 0.005 \) vs. C; \( n = 7 \)), although expression of the 41-kDa nonglycosylated receptor protein was significantly increased (Fig. 5: D: 354.4 \( \pm \) 127.4% of C; \( P < 0.005 \) vs. C; \( n = 7 \)), with only partial reversal to C values in the D+I group (D+I: 212.3 \( \pm \) 41.1% of C; \( P < 0.05 \) vs. C; \( n = 7 \)).

Western blot analysis of glomerular AT\textsubscript{2} receptors. By using RT-PCR, mRNA for AT\textsubscript{2} receptors was inconsistently detected in isolated glomeruli (4/7 preparations, data not shown), consistent with other studies indicating a low abundance of this message in adult kidney (30). By Western blot analysis, however, a single band of the predicted molecular mass for the AT\textsubscript{2} receptor (44 kDa) was readily detected in cell lysates from glomeruli, with no other bands observed (27). A significant decrease in AT\textsubscript{2} receptor protein expression was observed in glomerular cell lysates (Fig. 6: D: 47.0 \( \pm \) 6.5% of C; \( P < 0.001 \) vs. C; \( n = 6 \)). The decrease in AT\textsubscript{2} receptor expression was only partially reversed in the insulin-implanted diabetic group (D+I: 66.8 \( \pm \) 8.4% of C; \( P < 0.005 \) vs. C; \( n = 6 \)).

AT\textsubscript{2} receptor immunohistochemistry. In the cortex of C rats, AT\textsubscript{2}-receptor immunostaining was predominantly localized to glomerular endothelial cells, although diffuse staining of cortical tubular segments was also evident (Fig. 7A). In D rats, glomerular staining for the AT\textsubscript{2} receptor was markedly decreased (Fig. 7B), an effect consistently observed in all sections (\( n = 8 \)). Kidney sections from D+I rat sections displayed significant AT\textsubscript{2} receptor staining within glomeruli in all sections (\( n = 8 \); Fig. 7C) to levels not significantly different from control. As a negative control, no glomerular staining was observed in rat kidney sections in which the primary antibody was preincubated with a 20-fold excess of immunizing peptide, demonstrating the specificity of the AT\textsubscript{2} receptor antibody (Fig. 7D).

Figure 8 illustrates representative photomicrographs of the outer medullary region of C, D, and D+I rat kidney sections stained for the AT\textsubscript{2} receptor. All sections depicted some diffuse tubular staining. Intense focal staining was observed in interstitial cells, both in C and D+I rats (Fig. 8, A and C, respectively). In diabetic rats, however, interstitial cell AT\textsubscript{2}-receptor staining was significantly decreased in the outer medullary region (Fig. 8B).

In the inner medulla of control rats, AT\textsubscript{2}-receptor immunostaining was localized to the apical membranes and cytoplasm of inner medullary collecting...
duct (IMCD) cells (Fig. 9A). Both C and D+I rat kidney sections exhibited distinct tubular staining (Fig. 9, A and C), whereas in diabetic rats AT$_2$-receptor immunostaining in IMCD was markedly decreased (Fig. 9B). In both outer and inner medulla, preincubation of AT$_2$ receptor antibody with excess of immunizing peptide completely eliminated AT$_2$ receptor staining (not shown).

Quantitation of AT$_2$ receptor staining by computer-based image analysis revealed a significant decrease in expression of AT$_2$ receptors in all regions of the diabetic kidney compared with control (Table 2). In cortex, insulin implant therapy (D+I) was associated with a complete return of AT$_2$ receptor immunostaining to levels observed in C rats. In the outer and inner medulla, quantitative AT$_2$ receptor immunostaining in D+I rats demonstrated considerable variability, and did not differ significantly from either C or D rats.

**DISCUSSION**

In the present study, early diabetes had no significant effect on glomerular mRNA expression for renin, angiotensinogen, ACE, or AT$_1$ receptors. The major findings are that diabetes was associated with a significant decrease in glomerular AT$_2$ receptor expression, and, indeed, AT$_2$ receptors were decreased throughout the diabetic kidney, as shown by immunohistochemistry. This effect was partially reversed by insulin implants. Our data suggest, therefore, that even if the intraglomerular RAS is not activated in early diabetes, the relative expression of AT$_1$ and AT$_2$ receptors may be important in defining the effects of ANG II in progressive diabetic nephropathy.

In these studies, we used the well-established STZ rat model of diabetes. In all rats, the characteristic increases in plasma glucose, decreases in body weight, and renal hypertrophy were observed after 2 wk (3, 11). In rats with insulin implants, there was a reversal of the effect on body weight, and renal hypertrophy, and plasma glucose levels were decreased compared with C rats, perhaps because of excess insulin administration. Moreover, and importantly, there was no significant change in PRA in the diabetic rats, which is consistent with other reports at this early time point of experimental diabetes (3, 11).

The glomerulus contains all components of the RAS necessary to produce ANG II (8, 19, 46). Indeed, Atiyeh et al. (5) showed that isolated rat glomeruli were capable of ANG II generation, an effect blocked by ACE inhibition in a concentration-dependent fashion. Tank et al. (36) have shown that glomerular renin mRNA is regulated by dietary salt ingestion, an effect reproduced in the present studies. However, no studies have focused on the effects of diabetes on RAS expression in the glomerulus, or on glomerular ANG II production. Accordingly, to determine whether early diabetes altered glomerular mRNA expression for components of the RAS, we used a quantitative competitive RT-PCR method. Preliminary experiments determined that the RT-PCR assay could detect changes in mRNA expression exceeding 20%. Our data suggest that glomerular mRNA expression for RAS components is not signifi-
cantly altered in early diabetes. Alternatively, mRNA levels may be increased to levels below the detectable range for our assay. Indeed, renin mRNA levels were increased, although this did not reach statistical significance. Similarly, we have observed increased expression of proximal tubule renin mRNA after 2 wk in STZ diabetes (51), and increased whole kidney renin mRNA expression has been reported (3). It is also possible that glomerular ANG II levels could be increased in diabetes, independent of mRNA regulation, reflecting changes in mRNA translational efficiency or protein stability. Thus although we did not observe an effect on glomerular ACE mRNA, Anderson et al. (3) have demonstrated an increase in glomerular ACE protein in early STZ diabetes in rats.

Another means of regulation of the intrarenal RAS is at the level of expression of ANG II, AT₁, and AT₂ receptors in target tissue. In the kidney, AT₁ receptors are present in abundance in vascular smooth muscle cells, endothelium, glomerular mesangial cells, podocytes, tubular cells along the entire nephron, and medullary interstitial cells (13, 31, 50). In contrast, AT₂ receptor expression predominates in the fetal kidney and appears to diminish after birth (30). By using a polyclonal anti-AT₂ receptor antibody (identical to the one used in the present studies) Ozono et al. (27) demonstrated AT₂ receptor immunoreactivity in glomeruli, tubules, and interstitial cells from adult rats, with increased expression with sodium depletion. In contrast, immunohistochemical studies by Miyata et al. (24), by using a different antibody, revealed AT₂ receptor expression in all nephron segments in adult rat kidney, except in the medullary thick ascending limb and the glomerulus. Our results indicate that AT₂ receptors are expressed in the adult rat glomerulus, by both Western analysis and immunohistochemistry. Staining was observed in glomerular endothelial cells (Fig. 6), consistent with functional studies demonstrating effects of AT₂ receptor activation in cultured glomerular endothelial cells (45). In addition, we observed AT₂ receptor expression in cortical and outer medullary tubular segments, medullary interstitial cells, and IMCD cells. The reasons for differences in expression pattern compared with the study of Miyata et al. (24) are unclear but could be due to different antibody specificities or tissue preparations. Taken together, however, the data indicate that AT₂ receptors are expressed in the adult kidney, and, furthermore, we demonstrated that preincubation of antibody with the immunizing antigen completely eliminated staining,

Table 2. AT₂ receptor quantitation

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>D+I</th>
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<tbody>
<tr>
<td>Cortex</td>
<td>3,452 ± 1,102</td>
<td>328 ± 84*</td>
<td>4,462 ± 2,630</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>2,760 ± 241</td>
<td>983 ± 365†</td>
<td>4,151 ± 2,175</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>7,995 ± 2,054</td>
<td>1,267 ± 394‡</td>
<td>5,273 ± 1,663</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 4–7). Slides were quantitatively examined by using the Image-Pro Plus 4.0 software program. Each slide was analyzed for staining in the cortex, outer medulla, and inner medulla, with 3 separate fields viewed in each region. Each value represents the number of pixels that exceeded an arbitrary staining threshold per 300 × 300-pixel area. *P < 0.05 vs. C and D+I, †P < 0.05 vs. C.
indicating specificity of the antibody. The AT₂ receptor used in the present studies has also been previously validated by its recognition of the AT₂ receptor in stably transfected COS-7 cells and by its ability to detect AT₂ receptors in a variety of adult and fetal tissues in the rat (27, 41).

Early diabetes caused a significant decrease in AT₂ receptor protein expression, by both Western analysis and immunohistochemistry. In contrast, we observed no change in glomerular AT₁ receptor mRNA, an unexpected increase in expression of the nonglycosylated 41-kDa AT₁ receptor protein (an effect partly reversed by insulin), and no change in expression of the 53-kDa glycosylated receptor. It is noteworthy that decreased glomerular ANG II receptor density has been reported in early diabetes, with a persistent reduction in intrarenal AT₂ receptors by radioligand binding up to 12 wk after STZ diabetes (7, 9, 16, 43). A decrease in AT₁ receptor mRNA has also been demonstrated in whole kidney biopsy samples from humans with type II diabetes (40). In proximal tubule, AT₁ receptor mRNA and protein are both decreased in early STZ diabetes (11, 51). The functional significance of alterations in the relative amounts of glycosylated and nonglycosylated AT₁ receptors within diabetic glomeruli is unclear, although we speculate that this could alter AT₁ receptor binding properties by affecting the numbers of receptors inserted into the plasma membrane.

In contrast to effects on AT₁ receptor expression, we observed a marked decrease in glomerular, and, indeed, intrarenal AT₂ receptor expression in early diabetes. The possible consequences of diminished AT₂ receptor expression merit discussion. In a series of studies, Siragy and Carey (34) have demonstrated that AT₂ receptors are linked to stimulation of intrarenal nitric oxide formation, increases in interstitial cGMP levels (33), and vasodilatation in a kidney-wrap model of hypertension (32). Activation of AT₂ receptors also appears to counteract the hypertensive effects of AT₁ receptor activation, as demonstrated in the AT₂ receptor-knockout mouse (14). Given the present state of knowledge, it is difficult to predict the effects of decreased glomerular AT₂ receptor expression on glomerular function in diabetes. However, decreased total intrarenal expression of AT₂ receptors might promote enhanced tubular sodium reabsorption and increased blood pressure and could also lead to a decrease in AT₂-mediated inhibition of cell growth (22, 26). This could result in an amplification of AT₁-mediated effects on vasoconstriction or cell hypertrophy, contributing to increased nephron injury. In this regard, studies by Miller (23) in humans with early type I diabetes have demonstrated enhanced sensitivity of renal hemodynamic responses to AT₁ receptor antagonism, suggesting a relative augmentation of intrarenal AT₁-mediated receptor activity.

In our studies, insulin-implant therapy did not completely reverse the effects of STZ diabetes on AT₁ and AT₂ receptor expression in all regions. A number of possibilities for this must be considered. First, hyperglycemia may be only one of a number of factors that affect receptor expression in diabetes. Second, it is possible that even though glucose levels were corrected in the insulin implant group (and were indeed lower than controls), transient elevations of glucose may have occurred that could alter receptor expression. Finally, the direct effects of STZ on receptor expression must be considered, because this agent has been described to cause direct tubular nephrotoxicity (4). Notwithstanding these possibilities, the partial reversal with insulin indicates that high glucose at least partly exerts a regulatory effect on AT₁ and AT₂ receptor expression.

In summary, we have shown that early diabetes exerts no significant effect on mRNA expression of glomerular RAS components but significantly decreases AT₂ receptors in glomeruli and other regions of the kidney. The results suggest that altered AT₂ receptor expression in the kidney may be an important determinant of the rate of progression of diabetic nephropathy.

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