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Early diabetes mellitus stimulates proximal tubule renin mRNA expression in the rat

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Early diabetes mellitus stimulates proximal tubule renin mRNA expression in the rat.

Background. Enhanced intrarenal angiotensin II (Ang II) activity may contribute to diabetic nephropathy. The proximal tubule is a proposed site of significant intrarenal Ang II production. We determined the effect of early diabetes on mRNA expression of components of the proximal tubule renin-angiotensin system.

Methods. Three groups of male Sprague-Dawley rats were studied after two weeks: (*1*) control (C), (*2*) streptozotocin-induced diabetes (STZ), and (*3*) STZ-induced diabetes, with normoglycemia maintained by insulin implants (STZ-I). Competitive reverse transcription-polymerase chain reaction was used to assay mRNA for renin, angiotensinogen, and angiotensin-converting enzyme in suspensions of proximal tubules; plasma and kidney levels of Ang II were measured by radioimmunoassay, and Western analysis of Ang II subtype 1 (AT_1) receptors was performed.

Results. STZ rats tended to have increased plasma and intrarenal levels of Ang II compared with C and STZ-I rats. In proximal tubules, mRNA for renin was significantly increased in STZ rats, with reversal to control values in STZ-I rats (C, 2432 ± 437 vs. STZ, 5688 ± 890 fg/0.25 μg RNA, $P < 0.05$ vs. C, $N = 9$, vs. STZ-I, 1676 ± 376 fg/0.25 μg RNA, P = NS vs. C). In STZ rats, the AT_1 receptor antagonist losartan caused a further fivefold increase in proximal tubule renin mRNA, associated with proximal tubular renin immunostaining. STZ had no significant effect on mRNA expression for angiotensinogen or angiotensin-converting enzyme in proximal tubules. By Western blot analysis, cortical and proximal tubule AT_1 receptor protein expression was significantly decreased in STZ rats.

Conclusions. These data suggest activation of the proximal tubule renin-angiotensin system in early STZ diabetes, mediated at least partly by enhanced expression of renin mRNA. Increased local production of Ang II could contribute to tubulointerstitial injury in this model.

Key words: angiotensin II, renin-angiotensin system, streptozotocin, tubulointerstitial hypertrophy, diabetic nephropathy.

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Early diabetic nephropathy is characterized by hypertrophy of glomeruli and tubular epithelial cells, thickening of basement membranes, and enhanced renal blood flow and glomerular hyperfiltration [1]. This is accompanied by microalbuminuria and subsequent development of progressive glomerulosclerosis, with accumulation of extracellular matrix proteins in the glomerular mesangium. In addition, tubulointerstitial fibrosis occurs and correlates with the severity of reduction in glomerular filtration rate (GFR) [2, 3].

The renin-angiotensin system (RAS) has been implicated in the pathogenesis of diabetic nephropathy, based in large part on the ability of angiotensin-converting enzyme (ACE) inhibitors or angiotensin II (Ang II) subtype 1 (AT_1) receptor blockers to diminish proteinuria and to retard progressive glomerulosclerosis [4, 5]. In addition to its ability to induce systemic and intraglomerular hypertension [6] and to increase glomerular capillary permeability [7], Ang II is a renal growth factor and causes hypertrophy of mesangial cells and tubular epithelial cells and promotes production of the prosclerotic cytokine transforming growth factor-β (TGF-β) [8, 9]. This suggests that intrarenal Ang II activity may be increased in diabetes. In early diabetes, however, several groups have been unable to demonstrate activation of the intrarenal RAS. For example, Kalinyak et al reported no significant differences in levels of renal renin or angiotensinogen mRNA in rats two weeks after induction of diabetes with streptozotocin (STZ) [10], and Correa-Rotter, Hostetter, and Rosenberg found no significant changes in renal renin mRNA and protein content four weeks after the induction of diabetes [11]. By contrast, in STZ diabetic rats after eight weeks, Anderson, Jung, and Ingelfinger demonstrated increased intrarenal renin mRNA and protein content, as well as enhanced glomerular and renal vascular ACE immunostaining [12].

The lack of a consistent demonstration of activation of the intrarenal RAS in diabetes may be related in part

to differences in time points of study, although the heterogeneous expression of RAS components by cells in the kidney might also contribute. In this regard, although the renal proximal tubule contains all components of the RAS and intraluminal levels of Ang II in the proximal tubule are approximately 1000-fold higher than those circulating in plasma [13], components of the RAS are also expressed in glomeruli and the vasculature [14, 15].

Accordingly, since the proximal tubule is a major site of intrarenal Ang II production, the goal of the present studies was to determine the effect of early STZ diabetes in rats on the expression of components of the proximal tubule RAS. Our data indicate that proximal tubule renin mRNA is significantly up-regulated in early diabetes, with no change in expression of angiotensinogen or ACE mRNA. In diabetic rats, the AT_1 receptor antagonist losartan causes a further significant increase in proximal tubule renin mRNA expression, associated with tubular renin protein immunostaining. STZ diabetic rats have significant renal hypertrophy, elevation of intrarenal Ang II levels compared with insulin-treated rats, and decreased cortical and proximal tubule AT_1 receptor expression.

METHODS

Animal model

Male Sprague-Dawley rats (200 to 300 g) were used for this study and after acclimatization for five days were divided into three groups: (*1*) control (C; vehicle injection), (*2*) diabetic (STZ; administration of STZ, 65 mg/kg in 0.1 mol/L sodium citrate buffer, pH 4.0, intraperitoneally; Sigma, St. Louis, MO, USA), and (*3*) diabetic with insulin implants (STZ + I, STZ 65 mg/kg intraperitoneally and subcutaneous implantation of a sustained release insulin implant; Linplant, Linshin, Scarborough, Ontario, Canada) to maintain euglycemia. The day after administration of STZ, urine was assessed for glucose and ketones using a Keto-Diastix reagent strip (Bayer Inc., Etobicoke, Ontario, Canada), and only those animals with sustained glucosuria were classified as diabetic and were assigned to the STZ or STZ-I groups. Rats in the STZ group received daily injections of one to two units of insulin subcutaneously (Humulin L; Eli Lilly and Co., Indianapolis, IN, USA) to maintain hyperglycemia but prevent ketosis. In some experiments, STZ rats were treated with the AT_1 antagonist losartan, administered by an implanted miniosmotic pump at 25 mg/kg/day (Alza Corp., Palo Alto, CA, USA) beginning 24 hours after STZ. We have previously used this daily dose of losartan, administered subcutaneously, in a rat ischemic kidney model [16]. All rats were allowed free access to distilled water and standard rat chow, and urine was monitored daily for ketones and glucose. After two weeks, rats were sacrificed by rapid CO_2 narcosis and decapitation, and blood was collected for measurement of plasma glucose, plasma renin, serum creatinine (performed by the Ottawa Hospital Biochemistry Laboratory, Ottawa, Ontario, Canada), and plasma Ang II. Kidneys were immediately removed for further analysis. All procedures were approved by the Animal Care Committee of the University of Ottawa.

Measurement of Ang II

Plasma and kidney Ang II levels were measured by a radioimmunoassay using anti-Ang II antibody (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA), monoiodinated ^{125}I-labeled Ang II (Amersham, Arlington Heights, IL, USA), and Ang II standard as previously reported [17]. For these measurements, kidney and plasma samples were collected from anesthetized rats. For assessment of plasma Ang II levels, blood was collected into chilled tubes containing methanol. A blood sample was also taken for hematocrit determination. Blood samples were centrifuged at 4°C for 10 minutes at 100 × *g*, and the supernatant collected and stored at −20°C. One kidney was removed quickly, weighed, immersed in cold methanol (100%) and immediately homogenized with a glass homogenizer.

For the analysis of renal Ang II levels, kidney supernatants were dried overnight in a vacuum centrifuge. The dried residue was reconstituted in 4 mL of 50 mmol/L sodium phosphate buffer, pH 7.4, containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.25 mmol/L thimerosal, and 0.25% heat-inactivated bovine serum albumin (BSA). Plasma and kidney samples were extracted by absorption to and elution with 100% methanol from a phenyl-bonded solid-phase extraction column (Bond-Elut, Varian) for measurement of angiotensin peptides. The eluates were collected and stored at −20°C. Before radioimmunossay, the eluates were evaporated to dryness under vacuum and reconstituted in assay diluent (50 mmol/L sodium phosphate, 1 mmol/L EDTA, 0.25 mmol/L thimerosal, pH 7.4, containing 2.5 mg heat inactivated BSA/mL) and assayed directly. The reconstituted extracts were incubated with Ang II antiserum and ^{125}I-radiolabeled Ang II for 48 hours at 4°C. Bound and free Ang II were separated by dextran-coated charcoal, and the supernatants were counted by a computer-linked γ-counter for five minutes. Results for kidney Ang II levels are reported in femtomoles per gram kidney weight. The sensitivity of the Ang II assay is <5 fmol/g. The percentage of specific binding for Ang II averaged 38%, with a nonspecific binding of 2%.

Angiotensin II [1–8] and angiotensin [2–8] exhibit similar potencies for the Ang II antisera. Angiotensin [3–8] was also detected by the Ang II antisera, but the displacement of ^{125}I-Ang II was not parallel to that elicited by Ang II. Angiotensin [1–10], angiotensin [2–10], and short COOH-terminal fragments of Ang II were only detected at concentrations more than 100-fold higher than those

for Ang II. The Ang II antibody did not display cross-reactivity with angiotensin [1–7] at amounts of up to 10,000 fmol. The recoveries of angiotensin peptides through the extraction and purification procedures were determined by adding ^{125}I-Ang II to some of the samples immediately before application to the solid-phase extraction columns. Ang II levels presented are not normalized based on the extraction efficiency. The recovery of Ang II from plasma was 90 to 95% and from tissues was 80 to 85%.

Isolation of proximal tubule segments

Suspensions of rat proximal tubules were isolated by collagenase digestion of renal cortices, followed by Percoll gradient centrifugation, essentially as described [18]. Briefly, renal cortices were dissected, gently minced, and suspended in a solution containing (in mmol/L) 105 NaCl, 24 $NaHCO_3$, 5 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 2.0 Na_2HPO_4, 5 glucose, 1.0 alanine, 4.0 Na lactate, 10 HEPES (pH 7.4), 0.2% BSA, 0.03% collagenase (type IV; Sigma), and 0.01% soybean trypsin inhibitor (buffer A; Sigma). The suspension was gassed with 95% O_2/5% CO_2 for 30 minutes at 37°C. After digestion, the cortical suspension was strained through a 250 μm brass sieve and then centrifuged for one minute at 100 × *g*. The pellet was resuspended in buffer A without collagenase or trypsin inhibitor and was recentrifuged three times at 100 × *g*. The pellet was then applied to a 40% Percoll solution of identical ionic composition as buffer A, which had been previously chilled to 4°C. The Percoll solution was centrifuged at 26,000 × *g* for 30 minutes at 4°C, and the digested tissue was separated into four distinct bands, as described [18]. The F4 layer, enriched in proximal tubular segments, was removed and utilized immediately for RNA isolation. The procedure yielded a highly purified preparation of proximal tubules (97% by microscopy), and viability of segments was >95%, as determined by exclusion of trypan blue (10 mg/dL).

Competitive reverse transcription-polymerase chain reaction

Total RNA was isolated from proximal tubule segments using a commercial kit (RNeasy; Qiagen, Chatsworth, CA, USA). RNA quality was assessed by running samples on ethidium bromide-stained 1% agarose-formaldehyde gels and by measurement of optical density at 260 and 280 nm. RNA yield was between 10 and 25 μg per proximal tubule preparation, and all samples were of high quality, as assessed by these standards.

To quantitate absolute mRNA levels for renin, angiotensinogen, and ACE, a competitive reverse transcription-polymerase chain reaction (RT-PCR) assay was performed, using deletion mutant cRNA for each of these components. Each sample of RNA (250 ng) was simultaneously reverse transcribed with serial dilutions of deletion mutant cRNA, generated by in vitro transcription from plasmids containing mutant cDNA sequences. To generate deletion mutant cDNA, an inverse PCR method was utilized, essentially as described [19]. Briefly, a partial cDNA sequence for renin, angiotensinogen, or ACE was PCR amplified from rat kidney RNA and ligated into the pCR-Script SK(+) cloning vector (Stratagene, La Jolla, CA, USA). The renin cDNA PCR product was generated with a sense primer, 5′-CTGCCACCTTGTT GTGTGAG-3′, and an antisense primer, 5′-CCAGTAT GCACAGGTCATCG-3′ corresponding to bases 1033 to 1296 of the rat renin cDNA and resulting in a PCR product of 264 bp [19]. The angiotensinogen cDNA PCR product was generated with the sense primer 5′-CCTCG CTCTCTGGACTTATC-3′ and the antisense primer 5′-CAGACACTGAGGTGCTGTTG-3′, corresponding to bases 737 to 962 of the rat angiotensinogen cDNA and resulting in a 226 bp PCR product [19]. Finally, the ACE cDNA PCR product was generated by amplification with the sense primer 5′-GCCACATCCAGTATTT CATGCAGT-3′ and the antisense primer 5′-AACTGG AACTGGATGATGAAGCTGA-3′, corresponding to bases 3013 to 3454 of the rat ACE cDNA and resulting in a 441 bp PCR product [20].

Inverse PCR was performed on the plasmids containing the renin, angiotensinogen, and ACE partial cDNA sequences, 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 [19]. The oligonucleotide primers for inverse PCR of the renin cDNA were sense 5′-CGACTGAGCGTTGTGAACTGTAG CCA-3′, corresponding to bases 1166 to 1183 of the rat renin cDNA, and antisense 5′-CGACTGAGATATAG GATGTGCCAGTG-3′, corresponding to bases 1093 to 1076 and resulting in a 208 bp deletion mutant product (deletion of 56 bp). For angiotensinogen, the sense primer, 5′-AGCGTCGTTCCAAGGGAAGATGAGA GGC-3′, corresponded to bases 847 to 864 of the rat angiotensinogen cDNA, and the antisense primer was 5′-CGACTGAGTGTCACAGCCTGCACAAACC-3′, corresponding to bases 811 to 791, resulting in a 169 bp deletion mutant PCR product (deletion of 57 bp). For ACE, the sense primer was 5′-GTCTCTGCCCTCCAG TGCCTAG-3′, corresponding to bases 3337 to 3358 of the rat ACE cDNA, and the antisense primer was 5′-GAAAGTTGATGTCATGCTC-3′, corresponding to bases 3195 to 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 Perkin-Elmer Gene Amp 2400 PCR thermocycler, with hot-start at 96°C for 8 minutes, followed by denaturation at 96°C for 60 seconds, annealing at 63°C for 60 seconds, and elongation at 72°C for 90 seconds. Self-ligation of the PCR product was performed by adding adenosine

5′-triphosphate (ATP; 1 mmol/L) and 10 U of T4 DNA ligase (Stratagene), followed by an overnight incubation at 17°C. Competent TOP 10F′ *Escherichia coli* bacterial cells (Invitrogen, Carlsbad, CA, USA) were transformed with 4 μL of the ligation reaction, by incubation on ice for 30 minutes, heat-shock at 42°C for 45 seconds, and incubation in 0.45 mL of broth for 60 minutes at 37°C, with shaking at 225 r.p.m. The cells were then plated on Luria-Bertini-agar and grown up for large-scale plasmid preparations, using a Qiagen maxi-plasmid kit. To confirm correct sequences and orientation, all deletion mutants were sequenced in the University of Ottawa DNA sequencing facility.

Renin, angiotensinogen, and ACE RNAs were synthesized from deletion mutant plasmids, using T3 or T7 RNA polymerase (Stratagene). The plasmids were linearized by restriction enzyme digestion, and the transcription was carried out for two hours at 37°C with 1 μg of linearized plasmid DNA; 400 μmol/L each of ATP, cytidine 5′-triphosphate (CTP), guanosine 5′-triphosphate (GTP), and uridine 5′-triphosphate (UTP); and 10 U/μL of T3 or T7 RNA polymerase in 40 mmol/L Tris-HCl (pH 8.0), 50 mmol/L NaCl, 30 mmol/L dithiothreitol, 8 mmol/L $MgCl_2$, and 40 μmol/L EDTA. The template was then degraded with amplification grade DNase I (Life Technologies, Burlington, Ontario, Canada), and the RNA products were purified by phenol/chloroform extraction and ethanol precipitation. RNA was quantitated by absorbance at 260 nm.

For competitive RT-PCR reactions, prior to reverse transcription, all RNA samples were digested with amplification grade DNase I (Life Technologies) for 15 minutes at room temperature to remove any residual genomic DNA. The DNase was inactivated by addition of 2.5 mmol/L EDTA (final concentration) to the reaction mixture. Samples of RNA (total RNA and dilutions of deletion mutant RNA for either renin, angiotensinogen, or ACE) were then reverse transcribed using random hexamers (2.5 μmol/L) and murine leukemia reverse transcriptase (2.5 U/μL; Gene Amp RNA PCR kit; Perkin-Elmer, Branchburg, NJ, USA). To control for possible genomic or plasmid DNA contamination, all experiments included a reaction in which reverse transcriptase was selectively omitted from the transcription buffer.

Following reverse transcription, the cDNA mixture was amplified by PCR, in a 100 μL solution containing 2.5 U Ampli*Taq* DNA polymerase, 2 mmol/L $MgCl_2$, 1 × PCR Buffer II (Perkin-Elmer), and 1 μmol/L 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 previously in this article, of 264, 226, and 441 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 routinely performed for 35 cycles, with a hot-start at 96°C for 3 minutes, followed by cycles at 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 45 seconds, followed by extension at 72°C for 10 minutes.

Following RT-PCR, the PCR products were separated on 3% agarose gels containing Vistra Green nucleic acid gel stain (Amersham). To determine the amounts of initial mRNA for renin, angiotensinogen, or ACE, signals from the PCR products and their corresponding deletion mutants were quantitated by PhosphorImager analysis (Storm 860; Molecular Dynamics, Sunnyvale, CA, USA). The logarithms of the ratio of target to deletion mutant (competitor) species were plotted as a function of the initial amount of deletion mutant RNA in the reverse transcription reaction. The initial amount of target mRNA was quantitated by extrapolating from the point on each curve where the amount of amplified target was equal to the amount of amplified deletion mutant. Experiments were eliminated from analysis if the correlation of fit (r^2) for the generated curve was less than 0.9.

Immunohistochemistry for renin

Rat kidneys were immersed in Zamboni's fixative (2% paraformaldehyde, 15% picric acid in 0.1 mol/L phosphate buffer, pH 7.3) at 4°C overnight. The kidneys were then rinsed with 0.1 mol/L phosphate-buffered saline containing 10% sucrose and incubated overnight at 4°C. Tissue was mounted in paraffin blocks, and 5 μm sections were cut with a microtome and placed on microscope slides. Deparaffinization was performed by heating the slides at 60°C for 5 minutes and then incubating in xylene for 15 minutes at room temperature. The sections were rehydrated by sequential incubation in 100, 95, and 70% ethanol for five minutes each at room temperature. The sections were then washed in distilled water for 2 minutes, and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide treatment for 30 minutes. The sections were then permeabilized with 20 μg/mL proteinase K in Tris-buffered saline (TBS; 0.1 mol/L, pH 7.7) with Tween-20 for 15 minutes at room temperature. The slides were then incubated overnight at 4°C with mouse anti-rat renin monoclonal antibody (Swant, Bellinzona, Switzerland), diluted 1:20 in 0.6% Tris-carrageenan and 0.3% triton-X-100 (TCT) in TBS. The slides were then washed twice with TBS and incubated with anti-mouse biotinylated whole antibody (1:20 in TCT; Amersham) for one hour at room temperature. After washing with TBS, the sections were further incubated with 1:20 dilution of streptavidin-horseradish peroxidase (Amersham) in TCT for one hour at room temperature and then washed twice in TBS and incubated with 3,3′-diaminobenzidine chromogen solution (DAB; Biogenex; ESBE Scientific, St. Laurent, Quebec, Canada) for five minutes. All slides were counterstained in methylene

green for five seconds, washed in water, and dehydrated in 95 and 100% ethanol and xylene. The slides were covered with Permount (Fisher Scientific, Nepean, Ontario, Canada) and a cover slip and were viewed with a Zeiss Axioplan microscope.

Western blot analysis of the AT_1 receptor

Proteins from kidney cortices and proximal tubules of C, STZ, and STZ-I rats were isolated, essentially as described [21]. Briefly, tissue was homogenized in a buffer containing 10% glycerol, 20 mmol/L Tris-HCl, 100 mmol/L NaCl, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L EDTA, 2 mmol/L EGTA, 10 mmol/L sodium orthovanadate, 10 μg/L leupeptin, and 10 μg/L aprotinin. The homogenate was centrifuged at 30,000 × *g* for 30 minutes at 4°C. The resulting pellet was resuspended in the homogenization buffer, which also contained 1% Nonidet P40, and was then stirred for two hours at 4°C. The lysate was then centrifuged at 30,000 × *g* for 30 minutes at 4°C, and the supernatant was removed and utilized for Western analysis.

Protein concentrations in the supernatant were determined by the Bradford method (Bio-Rad, Montreal, Quebec, Canada) using BSA as the standard. Tissue lysates (20 to 40 μg) and prestained molecular weight markers (Bio-Rad) were loaded onto sodium dodecyl sulfate-polyacrylamide electrophoresis gels (SDS-PAGE; 5% acrylamide stacking gel and 10% running gel). The resolved proteins were transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked overnight at 4°C with 3% skimmed milk in TBS, pH 7.6. Membranes were incubated with a rabbit anti-rat polyclonal AT_1 receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000 in TBS containing 0.01% Tween 20 (TBS-T), with 2% skimmed milk for two hours at room temperature. After washing with TBS-T, membranes were incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham, Mississauga, ON, Canada), diluted 1:2000. Proteins were detected by enhanced chemiluminescence (ECL; Amersham) on Hyperfilm (Amersham). Two major protein bands (approximately 41 kD and approximately 53 kD) were detected for the AT_1 receptor on Western blot, likely representing the nonglycosylated and glycosylated forms of the receptor, respectively [22, 23]. To ensure equal loading of proteins, membranes were stripped and reprobed with a monoclonal anti-β-actin antibody (mouse ascites fluid; Sigma), which recognizes the β-actin protein at approximately 45 kD. Signals on Western blots were quantitated by densitometry and corrected for the β-actin signal, using the Kodak Digital Image station 440CF and the ID Image Analysis software program.

Statistics

Results are expressed as means ± SEM. Data were analyzed by one-way analysis of variance followed by Bonferroni correction for all pair-wise comparisons. Significance was assigned at $P < 0.05$.

RESULTS

Whole animal data

As shown in Table 1, at the time of sacrifice, STZ-rats had significant hyperglycemia (plasma glucose: STZ, 25.1 ± 2.2 mmol/L vs. C, 8.14 ± 0.16 mmol/L, $P < 0.001$). In STZ-I rats, plasma glucose levels did not differ significantly from those in control rats. STZ rats experienced significant weight loss over the two-week period, consistent with other studies using this model [24–26]. Kidney weights were significantly increased in STZ-rats (STZ, 1.54 ± 0.03 g vs. C, 1.27 ± 0.04 g, $P < 0.05$), and this was completely reversed in the STZ-I rats (1.24 ± 0.03 g). There were no significant differences in serum creatinine levels among the three groups. Similarly, plasma renin activity was not altered in STZ or STZ-I rats.

Plasma and kidney Ang II levels

In STZ rats, plasma Ang II levels were increased compared with control and STZ-I rats, although this did not reach statistical significance (C 339 ± 64 pmol/L vs. STZ 625 ± 202 pmol/L vs. STZ-I 90 ± 55 pmol/L; Fig. 1). Indeed, plasma Ang II levels were significantly decreased in STZ-I rats, compared with C, by one-way analysis of variance (ANOVA; $P < 0.02$, $N = 3$ to 7). Similarly, although whole kidney Ang II levels in the STZ rat group were higher than kidney levels in C rats, this did not achieve statistical significance (C 244 ± 64 fmol/g vs. STZ 545 ± 124 fmol/g, $P = 0.09$, $N = 6$ to 7). Kidney Ang II levels in STZ rats were significantly higher, however, than those in STZ-I animals (STZ-I 266 ± 53 fmol/g, $P < 0.04$ vs. STZ, $N = 4$ to 7).

Proximal tubule mRNA expression for renin, angiotensinogen, and ACE

Expression of mRNA for renin, angiotensinogen, and ACE was readily detectable in rat proximal tubule suspensions, using RT-PCR. By competitive RT-PCR with deletion mutants, STZ caused a significant increase in mRNA expression for renin in the proximal tubule (C 2432 ± 437 fg/0.25 μg RNA vs. STZ 5688 ± 890 fg/0.25 μg RNA, $P < 0.05$, $N = 9$; Fig. 2), an effect that was reversed in STZ-I rats (1676 ± 376 fg/0.25 μg RNA). In STZ rats, treatment with the AT_1 receptor antagonist losartan (25 mg/kg/day by osmotic minipump) caused a further fivefold increase in proximal tubule renin mRNA expression (STZ 9321 ± 1754 fg/0.25 μg RNA vs. STZ + losartan 52,465 ± 15,003 fg/0.25 μg RNA, $P < 0.025$, $N = 5$; Fig. 3). In contrast, neither STZ or STZ-I had any significant effect on proximal tubule mRNA expression for angiotensinogen (Fig. 4) or ACE (Fig. 5).

Table 1. Whole animal data

Condition	Body weight	Kidney weight	Plasma glucose	Serum creatinine	Plasma renin activity
	g		*mmol/L*	*μmol/L*	*ng/L/second*
C	379.5 ± 9.4	1.27 ± 0.04	8.14 ± 0.16	52.1 ± 1.1	1.85 ± 0.26
STZ	325.2 ± 5.1[a]	1.54 ± 0.03[b]	25.1 ± 2.20[b]	55.4 ± 2.1	2.14 ± 0.32
STZ-I	346.3 ± 4.8	1.24 ± 0.03	7.61 ± 1.39	51.8 ± 1.8	1.75 ± 0.30

Results are means ± SEM for $N = 9$ to 16. Abbreviations are: C, control; STZ, streptozotocin; STZ-I, streptozotocin plus insulin implant.
[a] $P < 0.001$ vs. C
[b] $P < 0.001$ vs. C and STZ-I

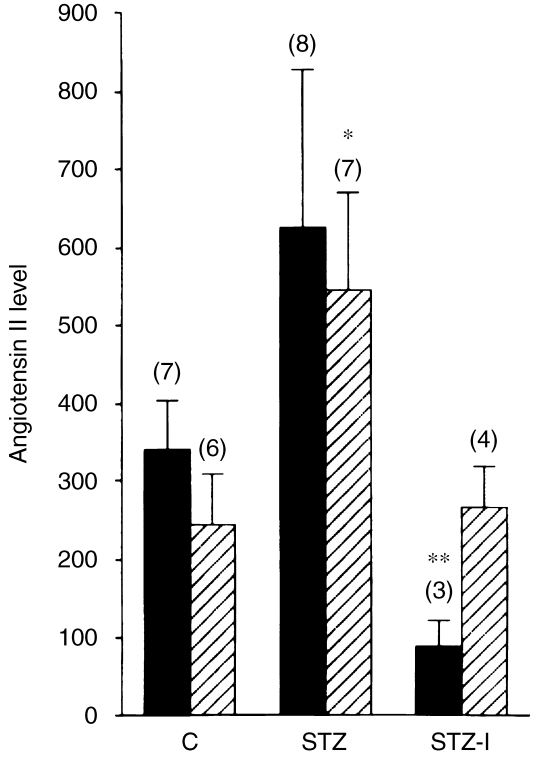

Fig. 1. Plasma and kidney angiotensin II (Ang II) levels. Ang II was measured in plasma (■; in pmol/L) and in whole kidney (▨; in fmol/g), as described in the **Methods** section. Abbreviations are: C, control; STZ, streptozotocin-diabetic rats; STZ-I, streptozotocin-diabetic rats with insulin implants. Results are means ± SEM; the values in parentheses represent numbers of experiments. *$P < 0.04$ vs. STZ-I; **$P < 0.02$ vs. C by one-way ANOVA.

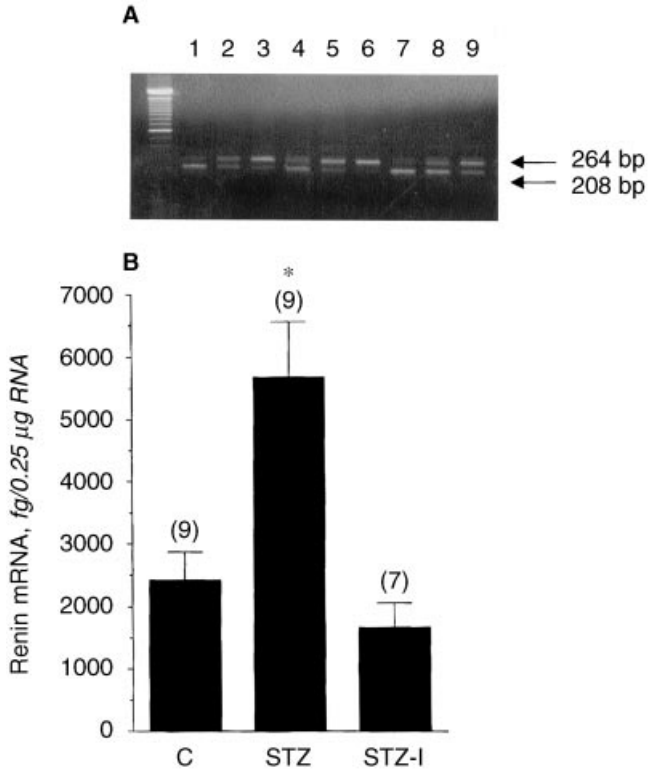

Fig. 2. Increased proximal tubule renin mRNA in streptozotocin (STZ)-diabetes. (*A*) Representative ethidium bromide-stained agarose gel of competitive RT-PCR reaction for renin. Lanes 1–3: control (C) samples, with 10, 2, and 0.5 pg of deletion mutant RNA utilized, respectively. Lanes 4–6: STZ samples, with 10, 2, and 0.5 pg deletion mutant RNA, respectively. Lanes 7–9: STZ rats with insulin implant (STZ-I) samples, with 10, 2, and 0.5 pg deletion mutant RNA, respectively. Bands representing the 264 bp renin PCR product and the 208 bp deletion product are indicated. The first lane at left is a 100 bp DNA ladder. (*B*) Effect of STZ diabetes on renin mRNA. Means ± SEM are shown, and values in parentheses are the numbers of separate experiments. *$P < 0.05$ vs. C or vs. STZ-I.

Renin immunostaining

To determine whether renin protein could be detected in proximal tubules by immunohistochemistry, rat kidney sections were stained with a mouse anti-rat monoclonal antibody against renin. In control and STZ rats, prominent renin staining was observed in juxtaglomerular afferent arterioles and also within glomerular endothelial cells (Fig. 6A). In STZ rats treated with losartan, however, in which a marked increase in proximal tubule renin mRNA was detected, staining for renin was observed in cortical proximal tubules, predominantly along the apical membranes (Fig. 6B, C). As a negative control, no staining was observed in sections in which the primary antibody was omitted (Fig. 6D).

Expression of renal cortical and proximal tubule AT_1 receptors

Previous studies have demonstrated decreased specific binding for Ang II in glomeruli and proximal tubules in early STZ diabetes [27–29]. Since we detected increased

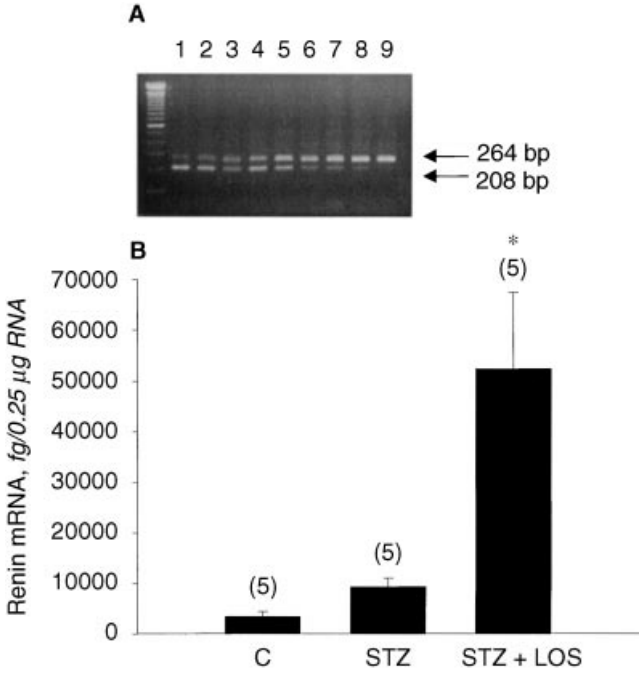

Fig. 3. Effect of losartan on proximal tubule renin mRNA expression. (*A*) Representative agarose gel of RT-PCR samples. Lanes 1–3: C, with 50, 10, and 1 pg deletion mutant RNA, respectively. Lanes 4–6: STZ, with 50, 10, and 1 pg deletion mutant RNA, respectively. Lanes 7–9: STZ + losartan, with 50, 10, and 1 pg deletion mutant RNA, respectively. The first lane at left is a DNA 100 bp ladder. (*B*) STZ rats were treated without (STZ) or with losartan (STZ + LOS), as described in the **Methods** section, and proximal tubule renin mRNA was quantitated by competitive RT-PCR. Control rats (C) received vehicle injection only. Values in parentheses represent numbers of individual experiments. $*P < 0.025$ vs. STZ.

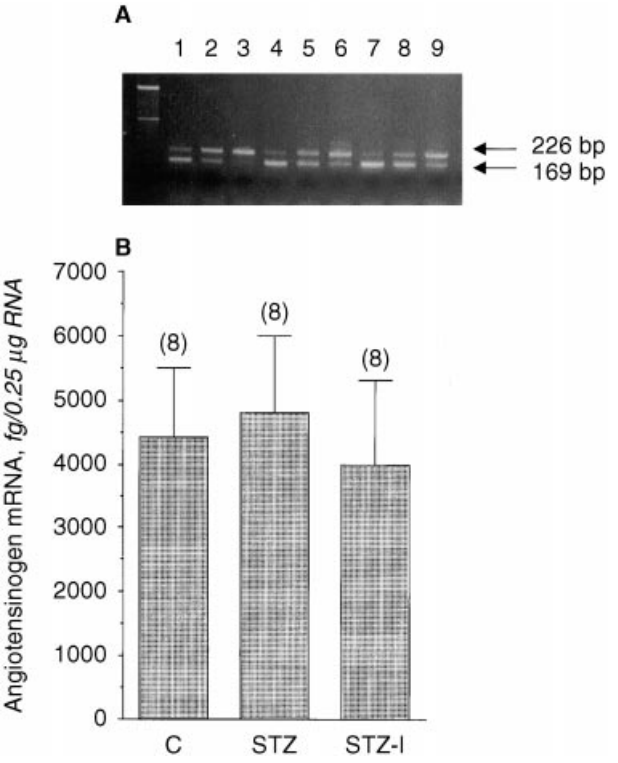

Fig. 4. Effect of STZ-diabetes on proximal tubule angiotensinogen mRNA. (*A*) Representative ethidium bromide-stained agarose gel of competitive RT-PCR reaction for angiotensinogen. Lanes 1–3: C samples, with 10, 2, and 0.2 pg deletion mutant RNA, respectively. Lanes 4–6: STZ samples, with 10, 2, and 0.2 pg deletion mutant RNA, respectively. Lanes 7–9: STZ-I samples, with 10, 2, and 0.2 pg deletion RNA, respectively. Bands depicting the 226 bp angiotensinogen PCR product and the 169 bp deletion product are indicated. The first lane at left is a 100 bp DNA ladder. (*B*) Effect of STZ diabetes on angiotensinogen mRNA. Means ± SEM are shown, and values in parentheses are numbers of separate experiments. P = NS for all comparisons.

renin mRNA in proximal tubules in STZ rats and since kidney Ang II levels tended to increase in this group, we determined the effect of early STZ diabetes on whole cortical and proximal tubule protein expression of AT_1 receptors. On Western blots, two major bands were observed for the AT_1 receptor, one at approximately 41 kD and the other at approximately 53 kD, the latter likely representing the glycosylated form of the receptor, as described (Fig. 7) [22, 23]. Western blot analysis revealed a significant decrease in cortical AT_1 receptor expression in STZ rats (both 41 and 53 kD bands), and this was reversed with insulin implant therapy (C 10.34 ± 0.19 vs. STZ 6.15 ± 0.61 vs. STZ-I 9.07 ± 0.25 arbitrary units; $P < 0.001$, C or STZ-I vs. STZ, $N = 6$). Similarly, proximal tubule AT_1 receptor protein expression was significantly decreased in STZ rats (C 1.92 ± 0.20 vs. STZ 1.33 ± 0.18 arbitrary units, $P < 0.05$, $N = 6$).

DISCUSSION

The major finding of this study is that early diabetes causes significant stimulation of mRNA expression for renin in the proximal tubule, with no effect on mRNA for angiotensinogen or ACE. The AT_1 antagonist losartan, an agent used to prevent the progression of diabetic nephropathy in humans, caused a significant further increase in proximal tubule renin mRNA, associated with tubular immunostaining for renin on kidney sections. We observed increased intrarenal levels of Ang II in rats with diabetes, compared with insulin implant-treated STZ rats. It is also noteworthy that insulin implant therapy to induce normoglycemia prevented the increase in proximal tubule renin mRNA. Finally, there was a significant decrease in expression of cortical and proximal tubule AT_1 receptors in diabetic rats. Taken together, these data suggest that the proximal tubule RAS is activated in early diabetes and may lead to increased local production of Ang II. With AT_1 blockade, there may be a further increase in proximal tubule Ang II synthesis, with this Ang II free to bind to available intrarenal AT_2 receptors. Activation of AT_2 receptors is linked to depression of blood pressure [30] and inhibition

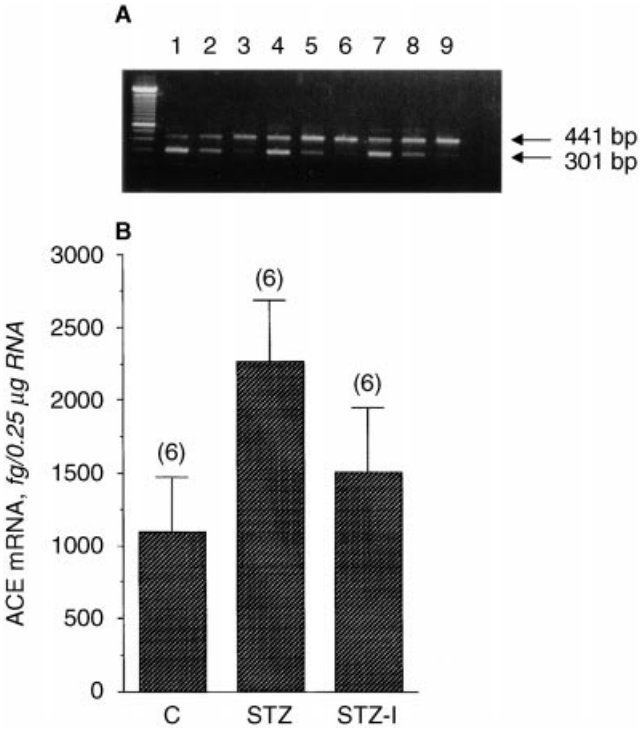

Fig. 5. Effect of STZ-diabetes on proximal tubule angiotensin converting enzyme (ACE) mRNA. (*A*) Representative ethidium bromide-stained agarose gel of competitive RT-PCR reaction for ACE. Lanes 1–3: C samples, with 5, 1, and 0.2 pg deletion mutant RNA, respectively. Lanes 4–6: STZ samples, with 5, 1, and 0.2 pg deletion mutant RNA, respectively. Lanes 7–9: STZ-I samples, with 5, 1, and 0.2 pg deletion mutant RNA, respectively. Bands depicting the 441 bp ACE RT-PCR product and the 301 bp deletion product are indicated. The first lane at left is a 100 bp DNA ladder. (*B*) Effect of STZ-diabetes on ACE mRNA. Means ± SEM are shown, and values in parentheses are numbers of experiments. P = NS for all comparisons.

of renal interstitial fibrosis [31], both desirable effects in the management of diabetic nephropathy.

We utilized the well-described STZ rat model of diabetes, and observed the characteristic elevations of plasma glucose, loss of body weight, and renal hypertrophy after two weeks [24–26]. Plasma renin activity did not significantly change in the diabetic animals, with or without insulin implants, consistent with other reports [10, 11]. STZ diabetes has been associated with tubulotoxicity in the kidney, formation of microfilament aggregates in proximal tubule [32], and impaired tubular protein degradation [33]. The stimulatory effect of STZ diabetes on proximal tubule renin mRNA was unlikely to be due to direct toxicity of STZ, however, since insulin implant treatment reversed this effect. Accordingly, this suggests that hyperglycemia may directly stimulate renin expression in the proximal tubule. Although we were unable to demonstrate significant proximal tubule immunoreactivity for renin in STZ rats, this does not exclude the possibility for enhanced local renin production, since the immunodetection method may not be sensitive enough to reveal small but important changes in renin production. Indeed, the present studies indicate that with a fivefold increase in proximal tubule renin mRNA (with losartan), immunohistochemical staining for tubular renin was readily evident. Furthermore, our localization data are in agreement with studies in mouse kidney, where renin has been reported in the apical portions of proximal tubule cells, at high antiserum concentrations [34].

Competitive RT-PCR was used to quantitate message for renin, angiotensinogen, and ACE in proximal tubule segments. In preliminary experiments, we determined that our assay method reliably produced linear competitive amplification curves, as described [19], and we could detect changes in mRNA expression exceeding 20%. Thus, it is possible that smaller changes in proximal tubule angiotensinogen or ACE mRNA expression in diabetes were not detected in these studies. Aside from the proximal tubule, within the kidney renin mRNA expression predominantly occurs in cells of the afferent arteriole [35]. The purity of our proximal tubule preparation was high (~97%), and it is unlikely that significant, consistent contamination with afferent arterioles occurred that could have influenced the results.

Stimulation of proximal tubule renin mRNA in diabetes is consistent with the studies of Anderson, Jung, and Ingelfinger [12] and Choi et al [36], who demonstrated increased renal cortical renin mRNA by Northern analysis in STZ diabetic rats. However, other investigators have not found increases in total renal renin mRNA expression in experimental diabetes [10, 11]. Because renin mRNA is derived from multiple sources within the kidney, it is possible that these data reflect the limited sensitivity of detection of changes in mRNA expression for renin in whole kidney. Our study is the first to examine RAS expression in proximal tubules in diabetes, a site of high production of Ang II, and a target for diabetes-induced TGF-β production and hypertrophy [9]. Increased proximal tubule renin expression could lead to stimulation of angiotensinogen conversion to the decapeptide Ang I, which may be converted to Ang II either intracellularly or following secretion into the tubular lumen, via membrane-bound ACE. Increased proximal tubule Ang II production in early diabetes might promote tubular cell hypertrophy and interstitial fibrosis, hallmarks of diabetic nephropathy.

Whole-kidney Ang II levels were elevated in STZ rats, although the increase did not reach statistical significance compared with C animals. This may reflect the relative insensitivity of measurements of whole tissue Ang II when assessing proximal tubule Ang II production in the kidney, since other cells (for example, glomeruli, vasculature) may contribute to intrarenal Ang II synthesis [14, 15]. In insulin implant-treated STZ rats, kidney Ang II levels were significantly decreased compared with

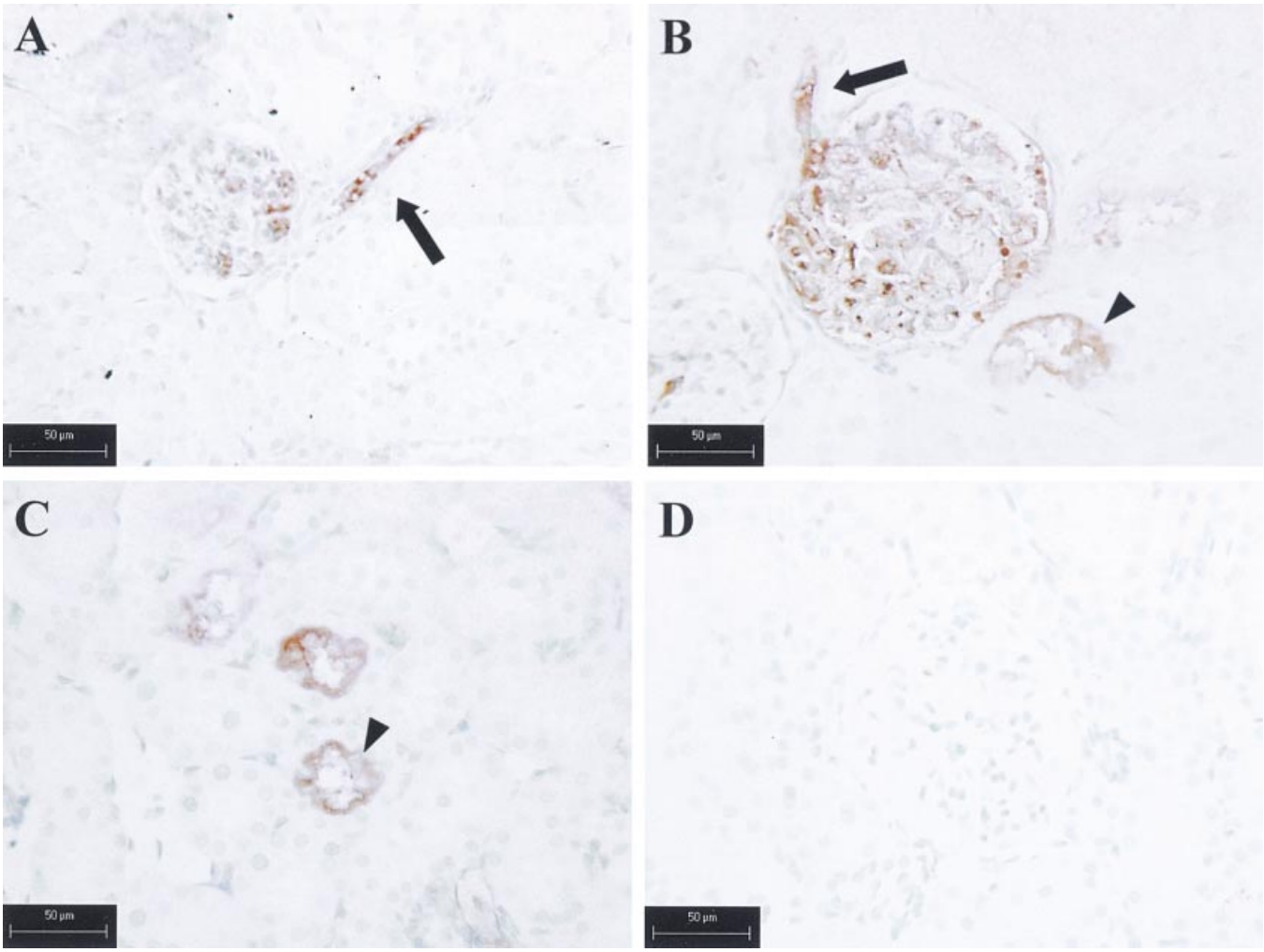

Fig. 6. Effect of losartan on proximal tubule renin protein expression in STZ rats. Immunohistochemistry for renin was performed on rat kidney sections, from STZ (*A*) or STZ + losartan-treated rats (*B* and *C*). In STZ rat kidney, renin immunostaining was observed in afferent arterioles (arrow) and within glomeruli, predominantly in endothelial cells. In losartan-treated STZ rats, renin immunostaining was seen in afferent arterioles (arrow) and in glomeruli and was also observed in cortical proximal tubules, mainly in the apical region of cells (arrowheads, B and C). Photomicrograph (*D*) is a representative section in which staining was performed in the absence of primary antibody, as a negative control. All photomicrographs are representative of sections from three to four separate rats. Magnification ×400.

STZ rats, and plasma Ang II levels were significantly less than levels in C rats. This suggests that insulin may down-regulate expression and activity of the intrarenal RAS, independent of any effect of hyperglycemia. In this regard, Zhang et al have determined that acute exposure (24 hours) to high extracellular glucose stimulates angiotensinogen gene expression in immortalized rat proximal tubular cells in culture, an effect reversed by insulin treatment, although effects on renin mRNA were not addressed in these studies [37, 38].

We observed no significant change in mRNA expression for angiotensinogen in proximal tubule in rats with early diabetes, and although there was an increase in proximal tubule ACE mRNA, this did not reach statistical significance. The lack of effect on angiotensinogen gene expression differs from the cell culture data of Zhang et al noted previously [37, 38]. This may be due to the more prolonged exposure to high glucose, as well as the involvement of other regulatory factors in the in vivo model. Furthermore, proximal tubules were isolated in the presence of normal (5 mmol/L) glucose concentrations in the present studies, which may have caused acute changes in angiotensinogen or ACE mRNA expression.

After eight weeks of STZ diabetes, Anderson, Jung, and Ingelfinger reported a decrease in total renal ACE, with a decrease in immunoreactive tubular ACE and an increase in glomerular ACE [12]. In humans with type II diabetes and nephropathy, a slight decrease in proximal tubular ACE staining has been reported on renal biopsies [39]. In Biobreeding (BB) spontaneously diabetic rats, Everett et al observed an increase in immunoreactive angiotensinogen in proximal tubule after four and

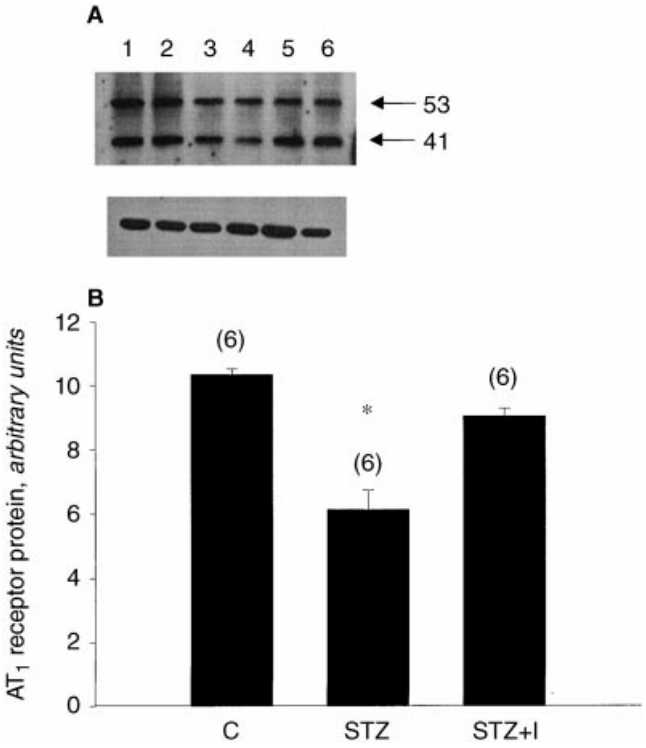

Fig. 7. Effect of STZ diabetes on renal cortical angiotensin II subtype 1 (AT_1) receptor protein. (*A*) Representative Western blot for two experiments showing 41 and 53 kD AT_1 receptor protein bands in samples from C (lanes 1 and 2), STZ (lanes 3 and 4), and STZ-I (lanes 5 and 6) rats. Upper band (~53 kD) likely represents glycosylated form of the AT_1 receptor, which also revealed decreased expression in STZ rats. Below AT_1 receptor Western blot is blot reprobed for the 45 kD β-actin protein, showing equal loading of proteins. (*B*) Effect of STZ diabetes on 41 kD AT_1 receptor expression in kidney cortex. Means ± SEM are shown, and values in parentheses are numbers of experiments. $^*P < 0.001$ vs. C or STZ-I.

eight months [40]. Angiotensinogen mRNA, however, did not change. In summary, our data do not exclude the possibility of time-dependent or strain-specific changes in renal angiotensinogen or ACE mRNA in diabetes, although the evidence clearly points to a role for up-regulation of tubular renin production.

Western blots of cortex proteins revealed a significant decrease in expression of glycosylated and nonglycosylated AT_1 receptors in STZ rats, which reverted to control values with insulin implant therapy. Proximal tubule AT_1 receptor expression was also decreased in STZ rats. These data are consistent with observations of reduced glomerular Ang II binding in early diabetes [27, 28] and the demonstration of reduced AT_1 Ang II receptor binding and mRNA expression in proximal tubules from STZ diabetic rats [29]. Since it appears that proximal tubule AT_1 receptors are up-regulated by Ang II [41], the data suggest that hyperglycemia may induce altered regulation of intrarenal Ang II receptors. The potential role of elevated intrarenal Ang II content in this regulatory process remains to be clarified.

In summary, this study indicates that there is selective up-regulation of renin mRNA in the proximal tubule in early STZ diabetes, and that the process is reversed by insulin therapy. We postulate that this leads to increased local synthesis of Ang II and is associated with a reduction in tubular AT_1 receptor expression. This may lead to enhanced Ang II intracellular signaling, promoting the characteristic tubulointerstitial hypertrophy of early diabetes.

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