Angiotensin II Upregulates Type-1 Angiotensin II Receptors in Renal Proximal Tubule

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Abstract

Angiotensin II (Ang II) is an important regulator of proximal tubule salt and water reabsorption. Recent studies indicate that rabbit proximal tubule angiotensin II receptors are the type-1 (AT_1R) subtype. We studied the effect of Ang II on proximal tubule receptor expression. Rabbits were treated with either angiotensin converting enzyme inhibitors or a low salt diet to modulate endogenous Ang II levels. In captopril-treated rabbits, liver and glomerular AT_1R mRNA levels increased 242±125 and 141±60%, respectively ($n = 6–7$; $P < 0.05$), as determined by quantitative PCR. In contrast, proximal tubule AT_1R mRNA levels decreased 40±11% ($n = 6$; $P < 0.05$). Binding of ^{125}I Ang II to renal cortical basolateral membranes of captopril-treated rabbits decreased from 2.9±0.55 to 1.4±0.17 fmol/mg protein ($n = 6$; $P < 0.025$). In rabbits fed a sodium chloride–deficient diet for 4 wk, AT_1R mRNA levels decreased 52±11% in liver and 43±7% in glomeruli ($n = 4–5$; $P < 0.05$), whereas they increased 141±85% ($n = 5$; $P < 0.05$) in proximal tubule. In basolateral membranes from rabbits on the sodium chloride–deficient diet, specific binding of ^{125}I Ang II increased from 2.1±0.2 to 4.3±1.1 fmol/mg protein ($n = 7$; $P < 0.05$). To determine whether Ang II directly regulates expression of proximal tubule AT_1 receptors, further studies were performed in cultured proximal tubule cells grown from microdissected S1 segments of rabbit proximal tubules and immortalized by transfection with a replication-defective SV40 vector. Incubation of these cells with Ang II (10^{-11} to 10^{-7} M) led to concentration-dependent increases in both AT_1R mRNA levels and specific ^{125}I Ang II binding. Pretreatment with pertussis toxin inhibited Ang II stimulation of AT_1R mRNA. AT_1R mRNA expression was decreased by either forskolin or a nonhydrolyzable cAMP analogue (dibutryl cAMP). Simultaneous Ang II administration overcame the inhibitory effect of forskolin but not dibutryl cAMP. These results indicate that proximal tubule AT_1R expression is regulated by ambient Ang II levels, and Ang II increases AT_1R mRNA at least in part by decreasing proximal tubule cAMP generation through a pertussis toxin–sensitive mechanism. Upregulation of proximal tubule AT_1R by Ang II may be important in mediating enhanced proximal tubule sodium reabsorption in states of elevated systemic or intrarenal Ang II. (*J. Clin. Invest.* 1995. 95:2012–2019.) Key words: angiotensin II • receptor • kidney • proximal tubule • angiotensin converting enzyme inhibitor

Introduction

The development of nonpeptide angiotensin II (Ang II)[1] receptor antagonists has allowed classification of receptor subtypes. Recent studies have used these compounds to determine the binding properties of renal Ang II receptors. In adult human (1), rat (2), and rabbit kidney (3), binding of Ang II is almost completely inhibited by the type-1 receptor (AT_1R) antagonist, losartan (DuP 753). In the kidney, autoradiography indicates that Ang II receptors are found in highest concentrations on the arterioles, glomerulus, and vasa recta (4). Binding studies of isolated nephron segments have also demonstrated specific binding of Ang II to numerous nephron segments, with the greatest concentration of tubule receptors in the proximal tubule (5).

Proximal tubule Ang II binding sites are present on both apical and basolateral membranes (BLM) (6, 7). Ang II exerts direct effects on proximal tubule transport, independent of alterations in renal or systemic hemodynamics (8, 9). We determined recently that ^{125}I Ang II binding to both basolateral and brush border membranes in rabbit is inhibited completely by losartan and cloned and sequenced cDNA encoding a rabbit kidney cortex type-1 Ang II receptor that is expressed in proximal tubule (10). Genomic Southerns revealed that this rabbit AT_1 receptor cDNA hybridizes to a single DNA band, indicating that a single gene encoding the AT_1 receptor is present in rabbit (10), in contrast to rat (11) and mouse (12), in which at least two genes encode AT_1 receptors (AT_{1a}, AT_{1b}).

The regulation of Ang II receptors is incompletely understood. Binding studies indicate that Ang II receptor density in the glomerulus and vasculature is decreased under conditions of elevated circulating Ang II and is increased when Ang II levels are low (13, 14). Conversely, receptor density in adrenal glomerulosa is increased after salt restriction (15). Recent studies have indicated that Ang II downregulates AT_1R mRNA in rat vasculature and in cultured rat mesangial cells (16–19). However, few studies have investigated regulation of expression of Ang II receptors in proximal tubule. Because of the role that angiotensin II plays in controlling proximal tubule solute and fluid reabsorption and the central role of AT_1 receptors in mediating this process (20–22), the current studies were designed to examine the regulation of rabbit proximal tubule AT_1 recep-

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1. *Abbreviations used in this paper:* ACE, angiotensin converting enzyme; Ang II, angiotensin II; AT_1R, angiotensin II receptor type-1; BLM, basolateral membranes ; RPTC, rabbit proximal tubule cells.

tors in conditions of high and low circulating Ang II. These studies indicate that, unlike AT_1 receptors in glomerulus and in liver, alterations in AT_1 receptor expression in proximal tubule are increased by Ang II.

Methods

Animals. Female New Zealand White rabbits (1.5–2.0 kg) were used for the animal studies. To alter endogenous Ang II levels, a subset of animals were fed a sodium-deficient diet (Teklad Premier Laboratory Diets, Madison, WI) for 4 wk. In preliminary experiments, plasma renin activity increased 3.5-fold after 4 wk of administration of the salt-restricted diet compared with age-matched controls. To inhibit endogenous Ang II production, another subset of animals were given captopril (500 mg/liter) in the drinking water for 8–12 d (23). In preliminary studies to ascertain that angiotensin converting enzyme (ACE) activity was inhibited in these animals, plasma renin activity and Ang I were measured. In rabbits given captopril for 10 d, plasma renin activity increased 7.3-fold and plasma Ang I increased 4.5-fold, while food and water intake were not different between control and captopril-treated animals. In all experiments, age-matched controls were killed at the same time as experimental animals.

Preparation of membranes. Renal cortical BLM were prepared using a modification of the method of Sacktor et al.(24, 25). Protein concentrations were measured using the method of Lowry et al. (26). As we have described previously, BLM are enriched in ouabain-sensitive Na^+-K^+ ATPase activity by 7–10-fold and are not enriched in alkaline phosphatase activity (10).

Separation of glomeruli and proximal tubules. Modification of the methods of Vinay et al. were used (25, 27). Briefly, renal cortices were gently minced and suspended in a solution containing 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1.5 mM $CaCl_2$, 10 mM $MgSO_4$, 2.0 mM NaH_2PO_4, 10 mM Hepes, pH 7.4, (buffer A) with the following additions: 5 mM glucose, 1 mM alanine, 0.03% collagenase (Sigma type I), and 0.01% soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), gassed with 95% O_2/5% CO_2, and maintained at 37°C. The cortical suspension was incubated for 45 min and gently agitated on a rocking platform. The suspension was strained through a large sieve and centrifuged. The pellet was resuspended in oxygenated buffer A and washed and recentrifuged three times. The pellet was then mixed with 100 ml of a 50% Percoll solution with the identical ionic composition as buffer A and which had been previously gassed with 95% O_2/5% CO_2 and chilled to 4°C. The Percoll solution was centrifuged at 15,000 rpm for 30 min at 4°C in a high-speed preparative centrifuge (J 2-21; Beckman Instruments, Inc., Fullerton, CA) using a JA 17 rotor. After centrifugation, the tissue was separated into four distinct bands, as described by Vinay et al. (27). After further washing, the uppermost band was enriched in glomeruli and the lowermost band was enriched in proximal tubule segments.

Development of an immortalized rabbit proximal tubule cell line. A line of SV40 immortalized rabbit proximal tubule cells (RPTC) was developed by transfection of cells grown for 5 d from explants of microdissected early proximal convoluted tubules (S1). Cells were transfected with a vector (p129) containing a replication-defective SV40 (a gift of Katherine Reznikoff of University of Wisconsin, Madison, WI) (28). Immunoblotting with an antibody against the large T antigen (Oncogene Science Inc., Cambridge, MA), indicated the presence of the expected 85-kD band, and immunohistochemistry indicated that all cells express the large T antigen. The cells grew in soft agar, indicating successful transfection and immortalization, and have maintained an epithelioid appearance. Characterization indicated the presence of sodium-coupled phosphate cotransport, stimulation of cAMP accumulation with PTH (threefold increase with 10^{-8} M PTH) but not antidiuretic hormone, and expression of mRNA for phosphoenolpyruvate carboxykinase, consistent with the characteristics of proximal tubule cells in vivo (Robey, R. B., and R. C. Harris, unpublished observations). These cells also increased cytosolic calcium in response to epidermal growth factor and metabolized arachidonic acid to cytochrome P450-mediated regiospecific epoxyeicosatrienoic acids similarly to primary cultures of rabbit proximal tubule cells (Harris, R. C., and J. Capdevila, unpublished observations). Cells were cultured and maintained in DME/F12 supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 95% air/5% CO_2 atmosphere. Before administration of Ang II, cells were made quiescent by incubation for 48 h in media without fetal bovine serum. For studies of AT_1R mRNA expression, agonists were added 4 h before RNA extraction. For binding studies, Ang II or other agonists were added at 16 h and again at 8 h before study. Experiments were performed on confluent cell monolayers of passages 10–25.

^{125}I Angiotensin II binding. Binding assays to BLM were performed using ^{125}I Ang II, essentially as described by Brown and Douglas (6, 7). Briefly, membranes (40–80 μg) were incubated at room temperature in a solution of 100 mM NaCl, 5 mM ethylenediaminetetraacetate, 10 mM Hepes (pH 7.5), 100 mM mannitol, 0.5% bovine serum albumin, 0.1 mM $MgSO_4$, 0.5% trypsin inhibitor, 0.005% aprotinin, 0.1 mM phenylmethylsulfonylfluoride, and 0.1 nM ^{125}I-labeled Ang II. Under these conditions, equilibrium binding occurred within 10–15 min. Binding was routinely terminated after 20 min by addition of 2 ml of ice-cold stop solution (300 mM NaCl, 10 mM Tris [pH 7.5], 100 mM mannitol, 50 mM $MgCl_2$), followed immediately by rapid filtration through presoaked 0.65-μm filters (DAWP; Millipore Corp., Bedford, MA) and 4 washes with 2 ml each of stop solution. Radioactivity bound to the filters was counted in a gamma counter. Binding to the cultured proximal tubule cells was performed using previously described methods (10). For these studies, cells were incubated with ^{125}I Ang II for 4 h at 4°C. Specific binding was determined as total binding minus nonspecific binding in the presence of excess unlabeled Ang II (10^{-6}M). For binding curves, the amount of specific binding (total − nonspecific) was determined in the presence of 0.1 nM ^{125}I-labeled Ang II and increasing concentrations of unlabeled Ang II.

RNA isolation and Northern analysis. Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (29). Samples of RNA were subjected to electrophoresis in denaturing 1% agarose/2.2 M formaldehyde gels transferred overnight by capillary blotting in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate) to Nytran nylon membranes (Schleicher & Schuell, Inc., Keene, NH). Blots were prehybridized for 4 h at 42°C in 30% formamide, 0.1% SDS, 50 mM NaH_2PO_4, pH 7.0, 5 × Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, and 5 × SSC. Blots were hybridized with 2×10^6 cpm/ml of ^{32}P-labeled cDNA overnight at 42°C in a fresh hybridization buffer containing 10% dextran (wt/vol). The cDNA probes were labeled to at least 10^8 cpm/μg by the random priming procedure using a commercially available kit (multiprime labeling kit; Amersham Corp., Arlington Heights, IL). The membranes were washed in 2 × SSC, 0.1% SDS for 15 min at room temperature, followed by two washes in 0.2 × SSC, 0.1% SDS for 15 min at 65°C. The membranes were exposed at −70°C to Kodak X-Omat AR film with an intensifying screen.

Quantitation of AT_1 receptor by PCR. As an internal control for quantitative PCR, an internal MscI/MscI deletion mutant was constructed similar to previously published studies investigating rat AT_1R mRNA regulation (16, 17). Briefly, a deletion mutant was constructed by digesting rabbit AT_1R clone 3 (10) with the restriction enzyme, MscI (which cuts at two sites in this clone, base pair 304 and 593), and religating the deletion fragment. The mutant plasmid was then linearized and cRNA transcribed. For PCR, total RNA (10 μg) and deletion mutant AT_1R cRNA (200 pg) were mixed and reverse transcribed using murine reverse transcriptase (First Strand cDNA synthesis kit; Pharmacia LKB Biotechnology, Piscataway, NJ) and a primer specific for the AT_1R in a final reaction volume of 33 μl. The resultant single strand cDNA mixture was then amplified in a GeneAMP 9600 PCR system using *Taq* polymerase (Perkin Elmer Cetus Corp., Norwalk, CT). The primers used were upstream sense primer (5′-TGGGAATATTTGGGAACAGC-3′) and downstream antisense primer (3′-GTGAATATTTGGTGGGGAAC-5′). PCR was performed at 95°C for 20 s, 55°C for 30 s, and 72°C for 90 s, followed by a 10-min extension at 72°C.

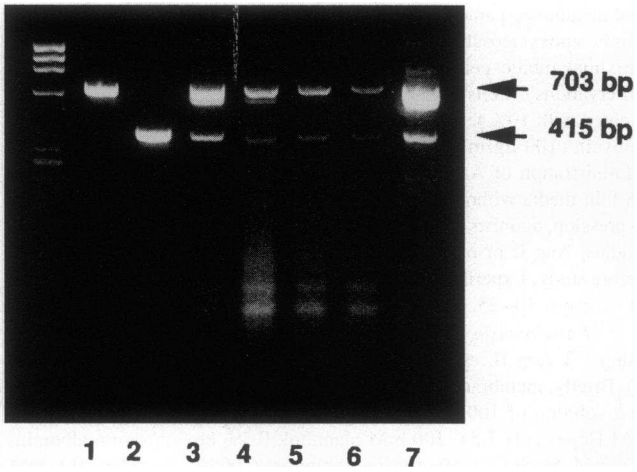

Figure 1. RT-PCR amplification of AT_1R mRNA in rabbit tissues. 200 pg of cRNA of an MscI/MscI deletion fragment of rabbit AT_1R was added to 10 μg of total RNA from rabbit tissues and reversed transcribed. After reverse transcription, PCR was carried out for 35 cycles at 95°C for 20 s, 55°C for 30 s, and 72°C for 90 s using primers as described in Methods. The amplified fragment of intact AT_1R is 703 bp and the MscI/MscI deletion is 415 bp. Lane *1*, liver; lane *2*, deletion fragment; lane *3*, liver + deletion fragment; lane *4*, renal cortex + deletion fragment; lane *5*, glomeruli + deletion fragment; lane *6*, proximal tubule + deletion fragment; lane *7*, adrenal + deletion fragment.

Amplification of intact and mutant AT_1R mRNA by these primers gave 703- and 415-bp fragments, respectively. No amplification occurred in the absence of reverse transcription, indicating that genomic DNA was not being amplified. Preliminary studies indicated linearity of response for at least 40 cycles, and PCR was routinely carried out for 35 cycles. Samples were routinely amplified in the presence of [α-^{32}P]CTP (New England Nuclear, Boston, MA) (3,000 Ci/mmol; 2 μCi/sample). Fig. 1 depicts representative results obtained in tissues from control rabbits, illustrating the 703-bp fragment of native AT_1R mRNA amplified in various tissues, as well as the amplification of the 415-bp deletion mutant fragment. For normalization, parallel samples measured amplification of β-actin, using the primers (5′:AACCGCGAGAAGATGACCCAGATCATGTTT; and 3′:AGCAGCCGTGGCCATCTCTTGCTCGAAGTC) (30). Preliminary studies indicated linearity of β-actin mRNA amplification for > 40 cycles. After gel chromatography on 4% agarose gels, the bands corresponding to the AT_1R, deletion fragment and β-actin were excised and counted by scintillation spectrometry. Results are represented as the ratio of intact and deletion fragment AT_1R mRNA amplified, normalized to the amount of amplified β-actin mRNA. This method provides a relative comparison of the amount of AT_1R mRNA present among the different experimental groups (17, 30).

Statistics. Results are presented as the means±SEM. Results of PCR experiments and ^{125}I Ang II binding to cultured proximal tubule cells were normalized as percentage of control. Statistical comparisons used ANOVA and the Bonferroni modification of Student's t test, with $P < 0.05$ indicating significance. For analysis of ^{125}I Ang II binding as a function of Ang II concentration, results were fit with the ligand binding program, Ultrafit (Biosoft, Cambridge, United Kingdom).

Results

Studies in captopril-treated rabbits. In rabbits treated with the ACE inhibitor captopril for 5–7 d, Northern analysis in six to eight separate experiments indicated that AT_1R mRNA levels were increased in liver, renal cortex, and glomeruli, while expression decreased in the adrenal and proximal tubule (Fig. 2). Because of the varying levels of AT_1R mRNA expression in different tissues, quantitative PCR was performed to quantitate the captopril-induced alterations. As indicated in Fig. 3 *A*, in captopril-treated rabbits, AT_1R mRNA expression increased 242±125% in liver (118±37 vs 274±112 cpm/cpm of mutant AT_1R/cpm of β-actin × 10^6; $n = 6$; $P < 0.05$), 47±12% in renal cortex (177±63 vs 261±102 cpm/cpm of mutant AT_1R/cpm of β-actin × 10^6; $n = 6$; $P < 0.05$), and 141±60% in glomeruli (79±37 vs 158±73 cpm/cpm of mutant AT_1R/cpm of β-actin × 10^6; $n = 7$; $P < 0.05$). In contrast, in proximal tubule suspensions, captopril pretreatment decreased AT_1R mRNA expression by 40±11% (83±33 vs 34±8 cpm/cpm of mutant AT_1R/cpm of β-actin × 10^6; $n = 6$; $P < 0.05$). Adrenal AT_1R mRNA expression was decreased, although not significantly (14±15%; $n = 5$).

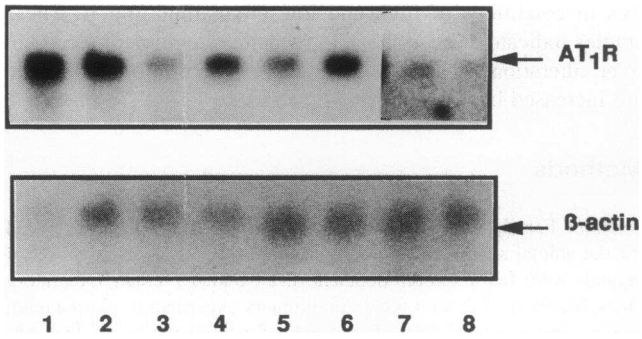

Figure 2. Northern analysis of AT_1R expression in rabbit tissues after captopril treatment. Each lane represents 15 μg of total RNA transferred to nylon membranes and probed with [^{32}P]CTP-labeled rabbit AT_1R cDNA. Lanes *1* and *2*, adrenal; lanes *3* and *4*, renal cortex; lanes *5* and *6*, glomeruli; lanes *7* and *8*, proximal tubules. Control: lanes *1*, *3*, *5*, and *7*; captopril: lanes *2*, *4*, *6*, and *8*. After hybridization, the membrane was exposed to Kodak X-Omat AR film for 24 (lanes *1–6*) or 48 h (lanes *7* and *8*). Blots were then stripped and reprobed with a 2-kb fragment cDNA of human β-actin.

To determine if captopril-induced alterations in AT_1R mRNA levels in proximal tubule were accompanied by alterations in receptor density, ^{125}I Ang II binding studies were performed on rabbit kidney cortical BLM. As indicated in Fig. 3 *B*, specific Ang II binding, determined by incubation of 0.1 nM ^{125}I Ang II in the presence or absence of 10^{-6} M unlabeled Ang II, was decreased 47±8% in BLM from captopril-treated rabbits (2.9±0.6 vs 1.4±0.2 fmol/mg protein; $n = 6$; $P < 0.025$). Scatchard analysis indicated that the altered binding was the result of alterations in available binding sites (B_{max}: 106 vs 67 fmol/mg protein), without alteration in binding affinity (K_d: 4.5 vs 4.9 nM). In both groups, specific ^{125}I Ang II binding was inhibited by the AT_1R-specific inhibitor, losartan (10^{-7} M) (control, 88±10% inhibition; captopril-treated, 89±9% inhibition; $n = 7$).

Studies in NaCl-deficient rabbits. In rabbits fed a sodium chloride–deficient diet for 4 wk, AT_1R mRNA levels were decreased by 52±11% in liver (67±21 vs 30±10 cpm/cpm of mutant AT_1R/cpm of β-actin × 10^6; $n = 4$; $P < 0.05$), by 43±7% in glomeruli (42±19 vs 27±12 cpm/cpm of mutant AT_1R/cpm of β-actin × 10^6; $n = 5$; $P < 0.05$), and by 36±4% in renal cortex (49±11 vs 30±5 cpm/cpm of mutant AT_1R/cpm of β-actin × 10^6; $n = 4$; $P < 0.025$). In contrast, in

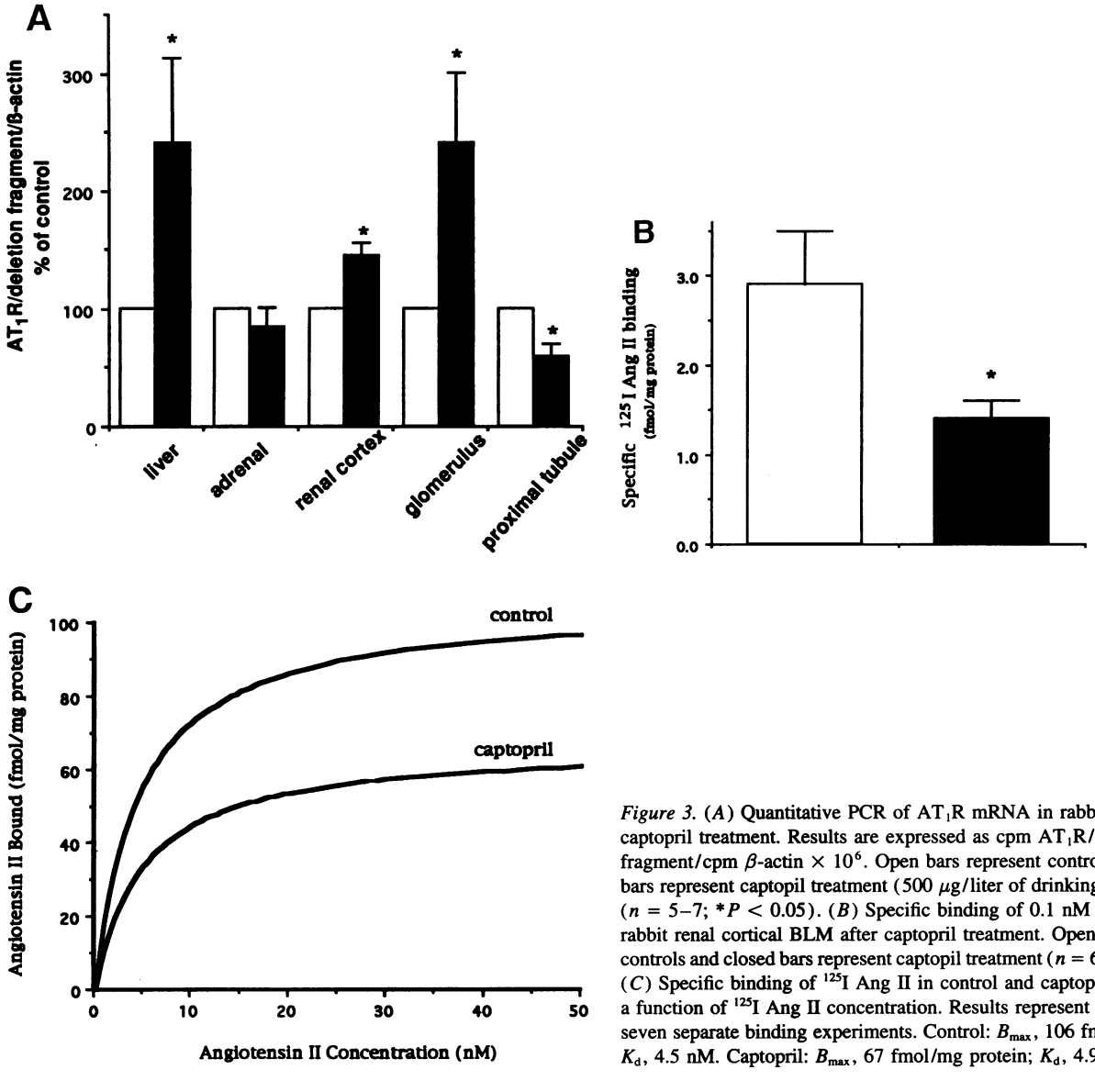

Figure 3. (*A*) Quantitative PCR of AT_1R mRNA in rabbit tissues after captopril treatment. Results are expressed as cpm AT_1R/cpm deletion fragment/cpm β-actin $\times 10^6$. Open bars represent controls and closed bars represent captopil treatment (500 μg/liter of drinking water for 7 d) ($n = 5$–7; $^*P < 0.05$). (*B*) Specific binding of 0.1 nM ^{125}I Ang II to rabbit renal cortical BLM after captopril treatment. Open bars represent controls and closed bars represent captopil treatment ($n = 6$; $^*P < 0.025$). (*C*) Specific binding of ^{125}I Ang II in control and captopril treatment as a function of ^{125}I Ang II concentration. Results represent mean values of seven separate binding experiments. Control: B_{max}, 106 fmol/mg protein; K_d, 4.5 nM. Captopril: B_{max}, 67 fmol/mg protein; K_d, 4.9 nM.

proximal tubule suspensions, AT_1R mRNA levels were increased 141±85% (15±7 vs 32±9; $n = 5$; $P < 0.05$) (Fig. 4 *A*). In kidney basolateral cortical membranes harvested from rabbits on the sodium chloride–deficient diet, specific binding of ^{125}I Ang II increased from 2.1±0.2 to 4.3±1.1 fmol/mg protein ($n = 7$; $P < 0.05$) (Fig. 4 *B*). Losartan (10^{-7} M) inhibited specific binding of ^{125}I Ang II in both groups (control, 98±2% inhibition; low salt, 92±5% inhibition).

Studies in cultured proximal tubule cells. Because the above results suggested that states predisposing to altered levels of circulating and/or local Ang II led to parallel alterations in proximal tubule AT_1R expression, additional studies examined whether Ang II directly regulated expression of proximal tubule AT_1 receptors. For these studies, Ang II receptors in SV40 immortalized RPTC were examined. A concentration response curve with losartan indicated complete inhibition of specific ^{125}I Ang II binding, with K_i of 9×10^{-10} M ($n = 3$) (Fig. 5 *A*). No inhibition of specific binding was noted with the type-2 Ang II receptor inhibitor, CGP 42112 (10^{-7} M) ($n = 2$), indicating that in these cells, all Ang II binding was mediated by AT_1R. Northern analysis indicated increased AT_1R mRNA expression in response to Ang II (Fig. 5 *B*). As shown in Fig. 5 *C*, quantitative PCR in five separate experiments revealed that, when the cells were incubated with Ang II for 4 h, AT_1R mRNA levels increased in a concentration-dependent manner. Ang II at concentrations $> 10^{-11}$ M led to statistically significant increases in expression of AT_1R mRNA. When the cells were incubated with Ang II for 16 h, subsequent specific ^{125}I Ang II binding was significantly increased by concentrations of Ang II $\geq 10^{-11}$ M ($n = 5$) (Fig. 5 *D*). In proximal tubule cells incubated with 3×10^{-10} M Ang II, specific ^{125}I Ang II binding increased by 58±18% (from 4.24±0.19 to 6.65±0.56 fmol/mg protein; $n = 3$; $P < 0.005$). Simultaneous treatment with losartan (10^{-7} M) prevented increases in binding (3.91±0.21 fmol/mg protein; $n = 3$; NS compared with control).

Previous studies (31) have indicated that actions of Ang II in proximal tubule are mediated in part by inhibition of adenylate cyclase via a pertussis toxin–sensitive G protein (G_i). In the cultured proximal tubule cells, Ang II (3×10^{-10} M) increased AT_1R mRNA expression to 192±23% of control ($n = 19$; P

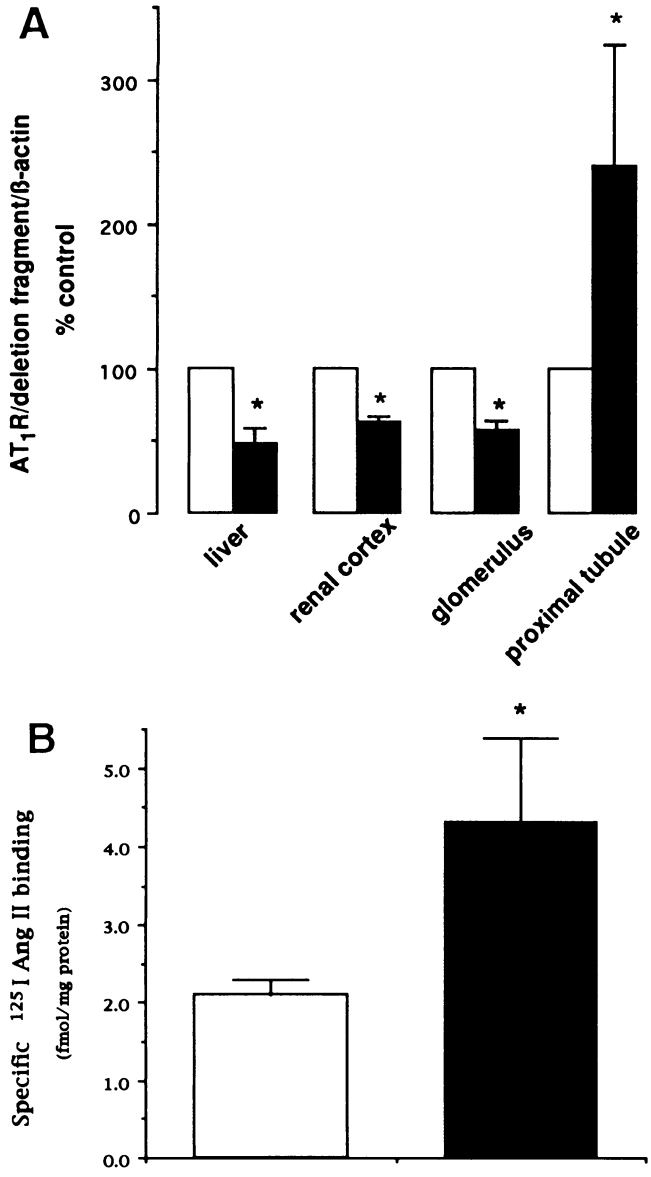

Figure 4. (*A*) Quantitative PCR of AT_1R mRNA in rabbit tissues after chronic salt restriction. Open bars represent control and closed bars represent salt restriction ($n = 4–5$; $*P < 0.05$). (*B*) Specific binding of 0.1 nM ^{125}I Ang II to rabbit renal cortical BLM after chronic salt restriction. Open bars represent control and closed bars represent salt restriction ($n = 7$; $*P < 0.05$).

< 0.005). Preincubation with pertussis toxin (500 ng/ml for 16 h) did not significantly inhibit basal expression of AT_1R mRNA (86±22% of control; $n = 6$) but prevented subsequent Ang II stimulation of AT_1R mRNA expression (113±22% of control; $n = 8$; NS compared with control) (Fig. 6 *A*).

When proximal tubule cells were incubated with either forskolin (10^{-6} M) or the cell permeant nonhydrolyzable cAMP analogue, dibutyryl-cAMP (10^{-4} M), levels of AT_1R mRNA were significantly decreased (forskolin, 52±10% of control; $n = 10$; $P < 0.03$; dibutryl-cAMP,46±3% of control; $n = 11$; $P < 0.02$) (Fig. 6 *B*). After incubation with dibutyryl-cAMP for 16 h, ^{125}I Ang II binding was decreased by 33±4% (2.86±0.23 vs 4.24±0.19 fmol/mg protein; $n = 3$; $P < 0.025$ compared with control).

When proximal tubule cells were incubated simultaneously with forskolin and Ang II (3×10^{-10} M), AT_1R mRNA expression was significantly increased compared with cells incubated with forskolin alone (147±27% of control; $n = 9$; $P < 0.001$ compared with forskolin alone). In contrast, when proximal tubule cells were simultaneously incubated with dibutyryl-cAMP and Ang II, the level of AT_1R mRNA expression was not significantly different than that seen with cAMP alone (62±10% of control; $n = 5$) and was significantly decreased compared with Ang II alone ($P < 0.0001$) (Fig. 6 *B*).

To determine whether protein kinase C–mediated processes were also involved in Ang II stimulation of AT_1R mRNA expression, quiescent proximal tubule cells were incubated for 4 h with phorbol myristate acetate (10^{-7} M). No stimulation of AT_1R mRNA was noted; in fact, expression was inhibited (50±15% of control; $n = 3$).

Discussion

These studies indicate that conditions that alter ambient Ang II levels also modulate AT_1 receptors in rabbit proximal tubule. Proximal tubule receptor density was increased in animals fed a low salt diet and was decreased after treatment with the ACE inhibitor, captopril. Changes in binding parameters were accompanied by alterations in mRNA levels for the receptor. Lewis and Ferguson (23) have previously described that in rats treatment with captopril caused a significant reduction in Ang II binding sites on renal cortical membranes and salt restriction caused a numerical increase in receptor density, although statistical significance was not reached.

Because direct measurements of circulating Ang II levels were not performed in the animal studies, further studies used cultured proximal tubule cells in order to determine whether Ang II directly modulated receptor expression in these cells. Both AT_1mRNA levels and specific ^{125}I Ang II binding were increased in cultured proximal tubule cells in response to incubation with Ang II, indicating that in the proximal tubule, Ang II positively regulated its own receptor. Increases in AT_1R expression occurred with concentrations of Ang II of 10^{-10} to 10^{-7} M, which are considered to be in the physiologic range.

The responses seen in proximal tubule contrast with those reported for vascular and glomerular Ang II receptors, in which receptor density decreased in response to salt depletion or Ang II infusion and increased in response to salt loading or ACE inhibition (13, 14). Similarly, in cultured rat mesangial cells, administration of Ang II led to a decrease of AT_1R mRNA (17). The response of Ang II receptors in the proximal tubule resembled that of the adrenal gland (15). Of interest, in the rat adrenal cortex, $AT_{1a}R$ mRNA increased in response to salt depletion, while $AT_{1b}R$ mRNA decreased (16, 18, 19). Although rabbit adrenal AT_1R mRNA expression was not significantly decreased in the present experiments, in four out of the five experiments, captopril decreased adrenal AT_1R mRNA expression, by an average of 30±8%, suggesting a trend toward inhibition of expression. It is also possible that less profound inhibition with captopril was seen in these studies because RNA was made from total adrenal gland rather than adrenal cortex.

Previous studies in the rat have failed to detect alterations in AT_1R mRNA levels in the kidney in response to alterations in circulating Ang II levels (16). This failure to detect changes in total kidney may be the result of opposing changes in glomerular/vascular and tubular mRNA. In the present studies, al-

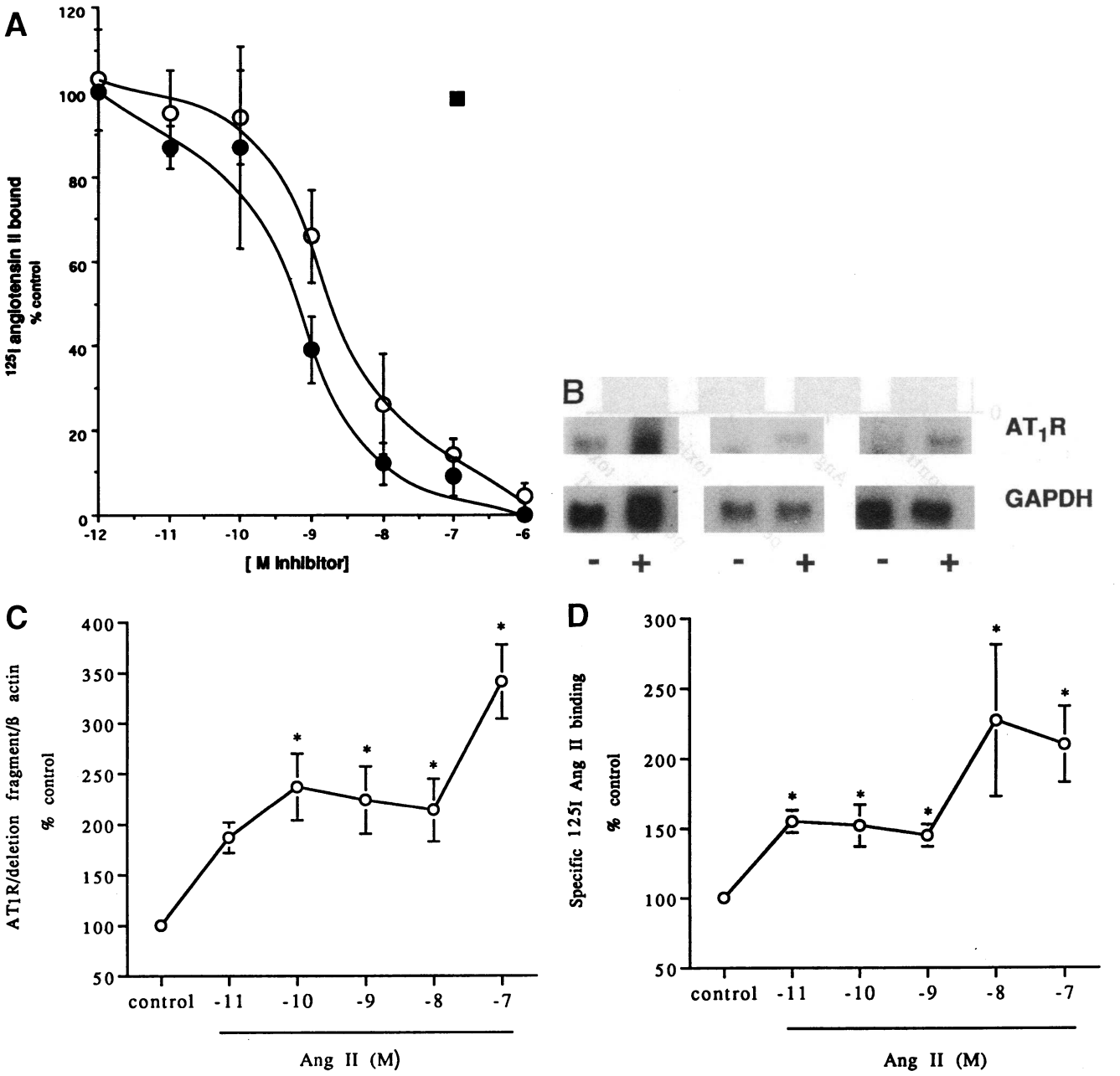

Figure 5. (*A*) Inhibition of specific ^{125}I Ang II binding in SV40 transformed RPTC. Cells were incubated for 20 min at 24°C with 0.1 nM ^{125}I Ang II binding and increasing concentrations of Ang II (*open circles*) or the AT_1R-specific inhibitor, losartan (*closed circles*). For comparison, the lack of inhibition with the AT_2R-specific inhibitor, CGP 42112 (10^{-7} M) (*filled box*), is also indicated. (*B*) Northern analysis of AT_1R mRNA expression in RPTC. 15 μg of total RNA was isolated after 4 h of incubation in the absence (−) or presence (+) of Ang II (10^{-6} M) and probed as described in Fig. 1. Three separate experiments are presented, with expression of the housekeeping gene, GAPDH, also presented for comparison. (*C*) Quantitative PCR of AT_1R mRNA in RPTC after Ang II incubation. RPTC were incubated for 4 h with the indicated concentration of Ang II, RNA was extracted, and RT-PCR was performed as described in Methods ($n = 5$; $^*P < 0.05$). (*D*) Specific ^{125}I Ang II binding in RPTC after Ang II incubation. RPTC were incubated for 16 h with the indicated concentration of Ang II and specific binding of 0.1 nM ^{125}I Ang II was determined ($n = 5$). $^*P < 0.05$ compared with control.

though AT_1R mRNA expression was increased by captopril and decreased by low salt, the magnitude of the change was not as great as that seen in glomeruli. Thus, expression of AT_1R mRNA in renal cortex may represent the averaging of the divergent expression in vascular/glomerular receptors and proximal tubule receptors. Differential responses of $AT_{1a}R$ and $AT_{1b}R$ mRNA in the rat kidney have also not been ruled out. Since the rabbit has a single AT_1 receptor, the differences in expression seen in response to Ang II indicate tissue-specific regulation.

In the cultured proximal tubule cells, elevating intracellular cAMP concentrations decreased AT_1R mRNA expression and specific ^{125}I Ang II binding. Of interest, Makita et al. (17) also found that cAMP decreased AT_1R mRNA expression in

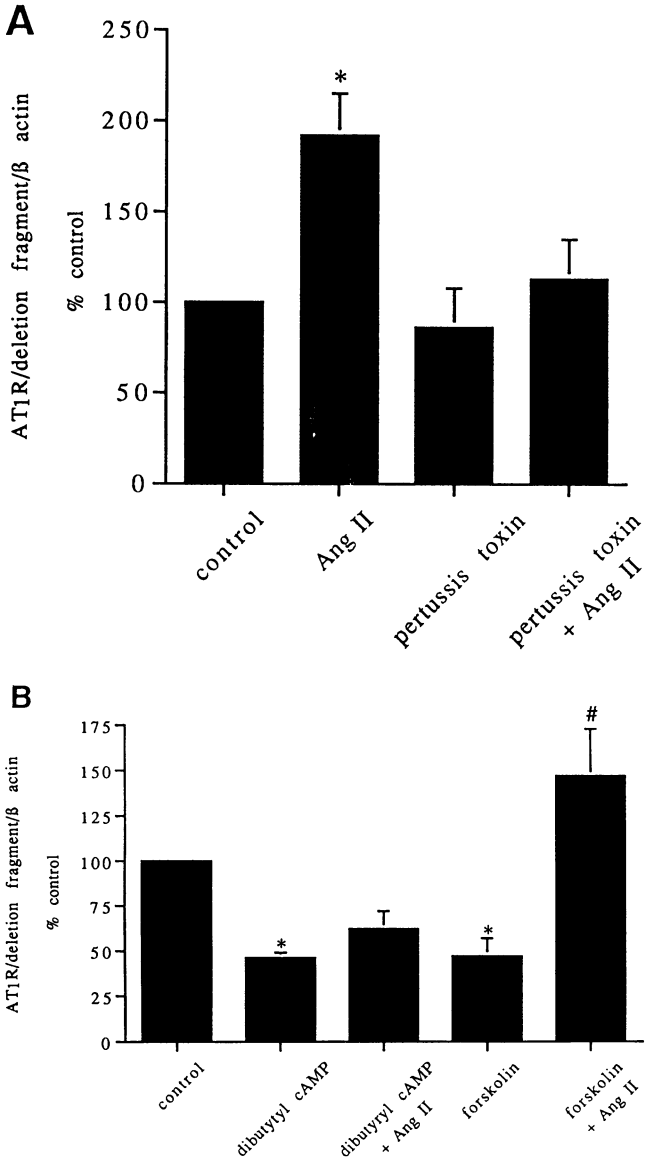

Figure 6. (*A*) Pertussis toxin inhibition of Ang II–mediated AT_1R mRNA expression in RPTC. Cells were preincubated for 16 h with or without 500 ng/ml pertussis toxin and then incubated with or without 3×10^{-10} M Ang II for 4 h before RNA extraction and RT-PCR (n = 6–19). $^*P < 0.05$ compared with control. (*B*) cAMP inhibition of AT_1R mRNA expression in RPTC. Cells were incubated with dibutyryl cAMP (10^{-4} M) or forskolin (10^{-6} M) with or without Ang II (3×10^{-10} M) for 4 h before RNA extraction and RT-PCR (n = 5–11). $^*P < 0.05$ compared with control; $^\#P < 0.05$ compared with forskolin.

cultured rat mesangial cells. In proximal tubule cells, simultaneous addition of Ang II mitigated the forskolin-mediated decrease in AT_1R mRNA expression but did not affect the decrease seen with the nonhydrolyzable cAMP analogue, dibutyryl-cAMP, suggesting that one mechanism of Ang II stimulation of AT_1R mRNA expression in the proximal tubule may involve inhibition of adenylate cyclase activity, with decreases in ambient cAMP levels. In proximal tubule, Ang II is coupled to a pertussis toxin–sensitive G protein (G_i) that mediates inhibition of adenylate cyclase (31, 32). Therefore, the abrogation of Ang II stimulation of AT_1R mRNA expression by pertussis toxin pretreatment is also consistent with mediation of this Ang II response by G_i.

The physiologic significance of the contrasting effects of cAMP and Ang II on AT_1R mRNA expression has not been determined in these experiments. However, acute increases in proximal tubule cAMP levels inhibit apical Na^+/H^+ exchange and proximal tubule reabsorption, and acute Ang II–mediated stimulation of apical Na^+/H^+ exchange and proximal tubule reabsorption is mediated at least in part by inhibition of adenylate cyclase and decreases in ambient cAMP levels (31–33). The present findings suggest that in addition to tonic inhibition of Na^+/H^+ exchange activity, cAMP may also serve to inhibit AT_1R mRNA expression in the proximal tubule tonically, and agents that increase proximal tubule cAMP concentrations may lead to further decreases in Ang II receptor density. Therefore, the balance between Ang II and agents that increase proximal tubule cAMP levels may be important not only in acute modulation of proximal reabsorption but also in regulation of proximal tubule responsiveness to Ang II stimulation.

In the kidney, angiotensinogen mRNA has been localized to the proximal tubule (34). In addition, renin and ACE have also been identified in the proximal tubule (22). The presence of all components of the renin-angiotensin system in proximal tubule suggests that locally produced Ang II could also modulate proximal tubule function. Ang II concentrations in rat proximal tubule lumen were determined by free flow micropuncture and found to be in the range of 10^{-8} M, compared with concentrations in the range of 10^{-10} M in systemic plasma (35, 36). Recent studies by Fox et al. (37) have indicated that in rats institution of a sodium-deficient diet for 7 d induced significant increases in renal Ang I and Ang II levels, and acute administration of an ACE inhibitor decreased plasma and renal Ang II concentrations by 78 and 75%, respectively. The effect of chronic ACE administration was not examined in their study. It is also of interest that a low sodium diet (33) led to an increase in angiotensinogen mRNA in proximal tubule in vivo and Ang II increased angiotensinogen mRNA in cultured mouse proximal tubule cells (38). These data suggest a mechanism whereby dietary salt restriction, by increasing both circulating and local Ang II production and proximal tubule Ang II receptor density, could contribute to the renal conservation of sodium.

In summary, these results demonstrate that, in vivo, ACE inhibition decreases and low salt diet increases proximal tubule AT_1R expression. In cultured proximal tubule cells, administration of Ang II increases AT_1R expression. These studies suggest that in proximal tubule AT_1R expression is regulated by ambient Ang II levels. Upregulation of the proximal tubule AT_1R by Ang II may be important in mediating enhanced proximal tubule sodium reabsorption in states of elevated systemic or intrarenal Ang II.

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