A role for angiotensin II AT₁ receptors in ureteric bud cell branching

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Iosipiv, Igor V., and Mercedes Schroeder. A role for angiotensin II AT₁ receptors in ureteric bud cell branching. *Am J Physiol Renal Physiol* 285: F199–F207, 2003. First published March 25, 2003; 10.1152/ajprenal.00401.2002.—Gene-targeting studies have clearly demonstrated that the renin-angiotensin system (RAS) plays an essential role during nephrogenesis (16, 20, 23, 39). The developing kidney expresses all the components of the RAS (7, 22, 41). The activity of the renal RAS is high during fetal and neonatal life and declines during postnatal maturation (7, 41). Renin mRNA and ANG II levels are 20- and 6-fold higher, respectively, in newborn than adult kidneys (7, 43). Interestingly, recent studies in renin knock-in reporter mice demonstrate that juxtaglomerular renin-producing cells originate from the MM and SM at embryo days (E) 11 and 12 before vessel development has occurred (15). During early fetal rat nephrogenesis, immunoreactive angiotensinogen (Ao) is highly expressed in the loose SM (28). To this end, the presence of both Ao and renin in the SM that surrounds the UB branches suggests a potential novel role for local ANG II generation in the paracrine regulation of growth and differentiation of the UB.

AT₁ and AT₂ receptors are abundantly expressed in the nephrogenic zone (22). However, the ontogenic expression of these receptors in the kidney differs: AT₂ receptors are expressed earlier than AT₁ receptors, peak during fetal metanephrogenesis, and rapidly decline postnatally (12). AT₁ receptor expression increases during gestation, peaks perinatally, and declines gradually thereafter (12). The spatial distribution of AT₁ and AT₂ receptor mRNA during ontogeny is also contrasting. AT₁ receptor mRNA is present in mature and maturing glomeruli and in distal and proximal tubules. AT₂ receptor mRNA is present in mesenchymal cells adjacent to the stalk of the UB (12). In addition, AT₁ receptor protein is expressed in the UB and its derivatives of the rat metanephros (28). Thus AT₁ expression appears to correlate with the differentiation and proliferation of glomerular and tubular cells, whereas AT₂ expression is associated with the mesenchymal-epithelial interactions.

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Recent studies by several groups demonstrate that inactivation of the genes that encode components of the RAS in mice causes papillary hypoplasia, hydronephrosis, and urinary-concentrating defect (16, 20, 23). These findings imply that UB-derived epithelial
targets for ANG II actions during renal development. In this work, we tested the hypothesis that ANG II acting via AT1 receptors stimulates UB-branching morphogenesis in vitro. To do so, we characterized the cellular localization of Ao and AT1 receptor proteins during murine nephrogenesis in vivo. Next we determined that cultured murine UB cells express AT1 receptor mRNA and protein. Finally, we investigated the effects of ANG II and its AT1 receptor on branching of UB cells cultured in three-dimensional collagen gels.

METHODS

Immunohistochemistry for AT1 and Ao

Immunolocalization of AT1 receptor protein was performed in C57B/6J mouse embryos from E12 to E16 (n = 4 embryos/age group; Hybrid-Ready Tissue, Novagen). Immunostaining was performed via the immunoperoxidase technique using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA). Sections were deparaffinized in xylene, hydrated in 95–70% alcohol, and washed in PBS (pH 7.2) for 5 min. Endogenous peroxidase activity was quenched by incubation of the sections with 0.3% H2O2 in methanol for 30 min. Sections were first washed in PBS for 20 min and incubated with the blocking antibody (normal rabbit serum) for 20 min and then incubated with the primary antibody diluted in PBS that contained 1% BSA for 90 min. Sections were rinsed in PBS for 10 min and incubated with IgG-biotinylated antibody (anti-rabbit) for 30 min and then washed in biotinylated horseradish peroxidase complex before being exposed to 0.1% diaminobenzidine tetrahydrochloride and 0.2% H2O2 for 3–5 min. Subsequently, the slides were washed in tap water and counterstained with hematoxylin. The primary antibodies used were 1) a polyclonal anti-rat Ao antibody raised in the sheep (1/5,000–1/8,000 concentration; generously provided by Dr. Conrad Sernia; Ref. 4) and 2) a polyclonal rabbit AT1 receptor antibody directed against the NH2-terminal domain of the human receptor (identical sequence in the mouse; Santa Cruz, N-10, sc-1175) at concentrations of 1/100–1/400. AT1 receptor antibody detects both AT1A and AT1B receptor subtypes. The specificity of immunostaining was assessed by preadsorption of the primary antibody with its immunogenic peptide and by the omission of the primary antibody.

Western Blot Analysis

Mouse kidneys and UB cells (generously provided by Dr. Jonathan Barasch, Columbia University) were homogenized in cold lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate) that contained a cocktail of enzyme inhibitors (in mM: 0.02% sodium azide, 1% Nonidet P-40, and 0.5% deoxycholate). The samples were centrifuged for 10 min at 14,000 g at 4°C and the supernatants that contained the proteins were transferred to nitrocellulose membranes. The adequacy of transfer was assessed by Ponceau S staining of the membranes. Nonspecific binding was blocked by incubation of the membranes with PBS that contained 0.2% Tween and 3% BSA overnight at 4°C. The membranes were incubated with the AT1 receptor antibody (1/500 concentration) at room temperature for 1 h. After three washes in PBS-Tween, the nitrocellulose membrane was exposed for 1 h at room temperature to the secondary antibody. Immunoreactive bands were visualized using the enhanced chemiluminescence detection system (ECL, Amersham). The blots were then exposed to Hyperfilm-ECL films.

RT-PCR

RT-PCR was used to determine whether UB cells express ANG II AT1 receptor mRNA. Total kidney and UB cell RNA was extracted using TRIzol reagent (Invitrogen). RNA (0.003 mg) was reverse-transcribed in the presence of 100 ng of random hexamers, 0.001 ml of 10 mM 2-deoxynucleotide 5’-triphosphate (dNTP), 0.002 ml of 10× RT buffer (in mM: 200 Tris·HCl (pH 8.4), 500 KCl, and 15 MgCl2), and 200 U of SuperScript II reverse transcriptase (Invitrogen) as previously described (42). After RNase treatment, cDNA was amplified using the Perkin Elmer Gene Amp PCR System 2400 (tubular structures, Norwalk, CT) from 25% of RT mixture using 20 pmol each of gene-specific primers, 1 U of Tag DNA polymerase, 0.005 ml of 10× PCR buffer, and 0.001 ml of 10 mM dNTP. AT1-specific primers were as follows: sense, 5′-GCATCATCTTTGTGGG-3′; antisense, 5′-GAAGAACGCACATCGCC-3′ (3). Amplification was performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 30 cycles. The AT1 primers amplify both AT1A and AT1B receptor mRNAs.

In Vitro Branching Morphogenesis Assay

UB cells were maintained in MEM media that contained 10% FBS at 37°C in an incubator with 5% CO2. UB cells were initially obtained from microdissected UBs of an E11.5 mouse transgenic for simian virus 40 (SV40) large T antigen (immortomouse, Charles River; Ref. 2). The ureteral epithelial character of UB cells was previously demonstrated by the expression of E-cadherin, cytokeratin, zona occludens-1, c-ret, and lecint (32).

UB cells cultured in gels form processes and branching tubular structures when exposed to various growth factors, which provide a convenient experimental system to analyze mechanisms of epithelial branching morphogenesis (26, 44). Previous studies demonstrate that UB cells grown in collagen gels proceed through defined stages: extension of cellular processes and formation of multicellular cords, which are followed by branching tubular structures (26, 44). A principal advantage of this approach is the ability to examine the direct effect of a specific factor on UB growth that is independent from confounding soluble factors released by the mesenchyme.

In vitro tubulogenesis assay was performed as previously described (34). The cells were used at passages 15–20. The cells were trypsinized and suspended in an 8:1:1 ratio of type I rat-tail collagen (Upstate Biotechnology), 10× DMEM, and 200 mM HEPES (pH 8.0) at 150 × 103 cells/ml. Subsequently, the suspension was dispensed in aliquots into a 96-well culture plate (0.1 ml/well). In pilot experiments, we examined the optimal composition of collagen matrix for UB cell branching. We found that there was no difference in the number of branches formed whether we used collagen alone or a 1:1 mix of type 1 collagen and growth factor-reduced Matrigel for 15,000 cells/ml of gel mix. Therefore, we chose to use collagen alone for the additional experiments. After gelation, 0.1 ml/well of DMEM/F-12 medium with 0.5% FBS or with 0.5% FBS and either 1) ANG II (10−8 to 10−7 M; Sigma), 2) ANG II and AT1 receptor antagonist candesartan (10−6 M; Sigma), or 3) epidermal growth factor (EGF; 40 ng/ml; Upstate Biotechnology) was added to each well on top of the collagen gel. This dose of EGF was previously shown to cause branching of UB cells in collagen gels (32). The growth medium added on top of the gel contained DMEM/F-12 me-
medium as previously described (27). We used 0.5% FBS in the growth medium in an effort to minimize the confounding effects of growth factors present in the serum. Cells were cultured in collagen gels at 37°C with 5% CO2 as described by Qiao et al. (30) for up to 8 days. The media were changed every other day.

The following steps were performed to ensure even well loading with cells. Before suspension of cells in the collagen mix, the cell number was counted using a hemocytometer and Trypan blue dye, and the cells were added to the remainder of the mix to yield a final concentration of 150,000 cells/ml of total collagen mix. To prevent immediate gelation, the mix containing the cells was kept on ice. The cells were dispersed within the mix by gentle vortexing followed by gentle mixing with repeated pipetting of the cells suspended in the mix. In addition, equal loading of the wells with cells was assessed after gelation of the mix. All cells present in a quadrant of selected wells were counted, and then the number was multiplied by 4. The average number of cells per well was 15,000 ± 750 (n = 10 wells), which indicates a coefficient of variability <5%. When branching was determined, all cells and clusters in each well were assessed for the presence of processes/branches. In each well, all cells that had at least one branch were counted. The number of cells with processes or primary branches [an early event in in vitro tubulogenesis (34)] and the number of cells that formed secondary branches were counted directly from the plates in each well using an Olympus IX70 inverted phase-contrast microscope. Each condition was set up in triplicate (n = 4 separate experiments).

Primary branches were defined as processes originating from the cell body. Secondary branches were defined as processes originating from primary branches. A higher number of secondary branches was taken to represent an increased complexity of branching. Images were acquired directly from the plates via an Olympus MagnaFire FW camera and were processed with Adobe PhotoShop 7.0 software.

**Statistical Analysis**

Differences among the treatment groups in cell/process number were analyzed by one-way ANOVA. A P value of <0.05 was considered statistically significant.

**RESULTS**

Localization of Ao and AT1 Receptor Proteins in Mouse Metanephros

**AT1 receptors.** At E12, AT1 immunoreactivity was observed at low levels in a diffuse stippled fashion in the UB and the surrounding MM (Fig. 1A). At E14, AT1 immunoreactivity was highly abundant on both luminal and basolateral aspects of UB branches (Fig. 1B). By E15, AT1 immunoreactivity was present on both luminal and basolateral aspects of UBs and their derivatives (Fig. 1C). Control sections incubated with the omission of the primary antibody showed a marked decrease in staining (Fig. 1D). AT1 was also detected in renal microvessels (data not shown). At E16, AT1 immunostaining was present in proximal tubules (Fig. 2, A and B). In addition, AT1 was weakly expressed in UB branches, SM, and glomeruli. Control sections incubated with the primary antibody preadsorbed with its immunogen showed a marked decrease in staining (Fig. 2C).

**Angiotensinogen.** At E14, Ao immunostaining was present in UB branches and surrounding SM (Fig. 3A). Control sections incubated without the primary antibody showed no specific immunostaining signal (Fig. 3B). By E15, Ao immunoreactivity was observed in UB branches, proximal tubules, and SM (Fig. 3, C and D).

![Fig. 1. Immunolocalization of ANG II type 1 (AT1) receptor protein in the fetal kidney on embryonic day (E)12, E14, and E15. A: AT1 is detectable using an antibody concentration of 1/200. On E12, AT1 immunoreactivity is present at low levels in a diffuse stippled fashion in the ureteric bud (UB) and metanephrogenic mesenchyme (MM). B: on E14, AT1 is expressed on both luminal and basolateral aspects of UB branches. C: AT1 is detectable using an antibody concentration of 1/200. On E15, UB branches are strongly positive for AT1 on both apical and basolateral membranes. D: control section, where the addition of the primary antibody was omitted, demonstrates no staining. G, glomerulus; S, S-shaped glomerulus; CD, collecting duct.](http://ajprenal.physiology.org/DownloadedFrom/10.22032.3747)
Fig. 2. Immunolocalization of AT$_1$ receptor protein in the fetal kidney on E16. AT$_1$ is detectable using an antibody concentration of 1/400. A and B: AT$_1$ is expressed in proximal tubules (PT) but only weakly in UB branches, stromal mesenchyme (SM), and glomeruli. PTs are located in close proximity to the glomeruli and are characterized by thick brush-border epithelia. C: control section incubated with prior preadsorption of the primary antibody with its immunogen shows marked attenuation of staining.

Fig. 3. Immunolocalization of angiotensinogen (Ao) protein in the fetal kidney on E14 and E15. Ao antibody concentration was 1/6,000. A: on E14, Ao immunoreactivity was present in the UB and surrounding SM. B: control section incubated with omission of primary antibody shows absence of specific staining. C: on E15, UB branches and proximal tubules were strongly positive for Ao on both apical and basolateral membranes. D: high-power view shows Ao immunostaining in ampulla of UB branches.
A similar staining pattern was observed on E16 (not shown).

Expression of AT₁ Receptors in UB Cells

To test whether cultured UB cells maintain AT₁ receptor expression, we examined AT₁ receptor gene expression using Western blotting and RT-PCR. As shown in Fig. 3, UB cells expressed abundant AT₁ receptor protein (molecular mass, 41–42 kDa). Control blots in which the primary antibody was preadsorbed with the immunogenic peptide were negative (Fig. 4, A and B). The AT₁ mRNA transcripts were also present in cultured UB cells (Fig. 4C).

ANG II Stimulates UB-Branching Morphogenesis In Vitro

To examine the role of ANG II in UB-branching morphogenesis, we investigated the effects of ANG II on cell process formation in UB cells cultured in three-dimensional type I collagen gels. The number and complexity of processes were quantitated daily via phase-contrast microscopy. Cell process formation and branching morphogenesis depend on multiple factors (including matrix composition, cell density, amount of serum present in culture media, and nature of inducing soluble factor) and are subject to interassay variability.

Under the conditions of our study, the number of cells with processes or branches varied from 60 to 286/well, and the total number of processes varied from 150 to 615/well. Therefore, the branching phenomenon involved 0.4–1.9% of cells originally seeded in a well (15,000). Figure 5 shows phase-contrast photographs that depict the effects of ANG II and EGF (positive control) on cell process formation and branching of UB cells 24 h after cells were plated into collagen gel. In control untreated cells, a few short processes were observed originating from the cell body (Fig. 5A). ANG II caused more extensive changes in cell process formation than control (Fig. 5, B–D). At higher doses such as 10⁻⁵ M, ANG II was capable of causing clearly evident secondary branching as well (Fig. 5D). In the presence of candesartan, cells exposed to ANG II formed shorter and fewer processes as compared to cells exposed to ANG II alone (Fig. 5E). EGF-treated cells, on the other hand, showed long, tube-like formations with secondary and tertiary branches (Fig. 5F).

To further delineate the nature of the branching structures, we visualized the UB cell cultures using immunofluorescence microscopy after double staining for Dolichos biflorus lectin and propidium iodide. Figure 6 shows an example of such an experiment: UB cells form multicellular branching cords when exposed to ANG II.

Quantitative analysis revealed that ANG II tended to increase cell branching at 10⁻⁷ and 10⁻⁶ M concentrations, but statistical significance was not reached until a higher concentration of 10⁻⁵ M was used (Fig. 7). At a 10⁻⁴ M concentration, ANG II increased the number and percent of UB cells with processes per well compared to control cells cultured in the presence of DMEM/F-12 media with 0.5% FBS alone on day 1 (150 ± 13 vs. 100 ± 11%; P < 0.05), day 2 (162 ± 16 vs. 100 ± 9%; P < 0.05), and day 4 (226 ± 24 vs. 100 ± 12%; P < 0.05) following plating (Fig. 7, A and B). ANG II-induced cell process formation was abolished by pretreatment with 10⁻⁶ M candesartan, the AT₁ antagonist [day 1, 150 ± 13 vs. 50 ± 10% (P < 0.01) and day 2, 162 ± 16 vs. 61 ± 13% (P < 0.01); Fig. 8A]. In addition, 10⁻⁵ M ANG II increased the number of cells with secondary branches on day 2 (212 ± 42 vs. 100 ± 22%; P < 0.05), which indicates that ANG II stimulates formation of branching structures with a higher degree of complexity (Fig. 8B). The absolute numbers of cells with secondary branches were as follows: day 1, 23 ± 8.0 vs. 8 ± 2.0 (P = 0.17); day 2, 22 ± 3.5 vs. 8 ± 1.9 (P < 0.05); day 4, 25 ± 7.0 vs. 11 ± 3.1 (P = 0.11; 10⁻⁵ M ANG II vs. control, respectively). Similarly, the number of cells with secondary branches was also decreased in the presence of candesartan: day 1, 205 ± 72 vs. 21 ± 4.1% (P < 0.05); day 2, 212 ± 42 vs. 27 ± 3.2% (P < 0.01); see Fig. 8B. On day 4, the difference did not reach statistical significance at 250 ± 70 vs. 100 ± 23% (P = 0.11).

Studies by Zent et al. (44) demonstrate that branching morphogenesis in UB cells cultured in collagen gels in the presence of 10% FBS can be maintained for up to
12 days. We used a much lower concentration of FBS (0.5%) to minimize confounding influences of multiple growth factors present in the serum on UB cell branching. Cells were monitored for process formation for up to 8 days. The absolute number of cells with processes continued to increase up to day 4 but decreased substantially thereafter: day 1, 118 ± 11 vs. 77 ± 9 (P < 0.05); day 2, 131 ± 13 vs. 80 ± 7 (P < 0.05); day 4, 113 ± 12 vs. 50 ± 6 (P < 0.05); day 6, 63 ± 9 vs. 49 ± 8 (P = 0.22); day 8, 67 ± 12 vs. 50 ± 6 (P = 0.28; 10⁻⁵ M ANG II vs. control, respectively). Although cell viability was not directly assessed, the decline in the number of cells with processes on days 6 and 8 was likely secondary to cell death.

**DISCUSSION**

The present study demonstrates that the UB branches and stroma express AT1 and Ao and that ANG II stimulates branching of UB cells in vitro via activation of the AT1 receptor. To our knowledge, this is the first report to show a direct role for ANG II in UB cell branching.

The kidney is formed by reciprocal inductive interactions between the UB and MM (8). The MM induces UB to elongate and branch repeatedly to form the renal collecting system. In turn, the UB induces the MM to differentiate into the glomeruli and proximal and distal tubules (1, 5). The BF2/FoxD1-positive SM also

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**Fig. 5.** UB cells grown in three-dimensional collagen gels 24 h after plating. A: control (media + 0.5% FBS). B, C, D: ANG II. E: ANG II with candesartan (Cand). F: epidermal growth factor (EGF; 40 ng/ml). 1°, 2°, And 3°: primary, secondary, and tertiary branches, respectively.

**Fig. 6.** Micrographs of UB cells grown for 4 days in collagen gel in the presence of 10⁻⁵ M ANG II. Cells form multicellular branched structures. A: phase-contrast image. B: fluorescence image. Cells were stained with Dolichos biflorus lectin (green) and propidium iodide (red).
plays a key role in renal morphogenesis through release of soluble factors (e.g., retinoic acid, fibroblast growth factor-7, and Wnt2b) that regulate epithelial branching morphogenesis (9, 14, 16, 31). The role of the RAS in UB and collecting-system development has not been defined. However, such a role is likely because 1) the developing kidney expresses all the components of the RAS (7, 22, 41), and ANG II synthesis and AT1/AT2 receptor expression are enhanced during metanephrogenesis (7, 12, 43); and 2) strong evidence derived from genetic studies has shown that inactivation of the Ao, angiotensin-converting enzyme (ACE), or AT1 genes causes abnormalities in the development of the renal medulla (6, 20, 23). Ao-, ACE-, or AT1-mutant mice exhibit thinning of the medulla, atrophy of the papilla, and dilation of the pelvis. These changes are most pronounced postnatally after the formation of the renal papilla. In addition, ACE- and AT1-null animals also have a reduced ability to concentrate urine (16, 23). One possible mechanism may be due to decreased number or altered contractile function of smooth muscle cells along the ureter that lead to defective peristalsis as has been shown in AT1-mutant mice (17).

The role of AT2 in UB branching is unknown. Most recent studies indicate that AT2 counteracts the proliferative actions of AT1 (38). Our preliminary data demonstrate that AT2 is expressed in UB branches during mouse nephrogenesis (11). AT2-mutant mice exhibit ectopic ureteral budding and duplicated collecting systems (24). This suggests that AT2 inhibits aberrant ureteral budding. It is conceivable that unopposed stimulation of AT2 in AT1-mutant mice may hinder UB branching. Therefore, the ultimate effect of ANG II on UB branching may depend on the balance between AT1- and AT2-mediated actions.

In addition, ANG II may influence papillary development by regulating the expression of renal growth factors within or adjacent to papilla. In this regard, downregulation of platelet-derived growth factor mRNA
levels in the renal papilla of Ao-null mice (21) and reduced EGF mRNA levels in AT1-null mice (19) suggest that dysregulation of ANG II-regulated growth factors contributes to abnormal collecting-system formation in these mice. Delineating the effects of the AT1-null mutation on expression of stromal factors necessary for UB branching (BF2, FGF7, Wnt2b, and RAR) will be crucial to our understanding of the mechanism governing the development of the renal medulla.

With regard to the role of ANG II in UB branching per se, studies by Nagata et al. (20) have shown that E12 metanephric kidneys from Ao-null mice grown ex vivo showed favorable branching of UBs that was unaffected by the addition of ANG II. The data were interpreted to suggest that ANG II is not essential for the growth of UBs. However, the absence of ANG II in this model could have been compensated for by other factors derived from the mesenchyme. Therefore, utilization of the UB cell culture model may be more relevant to define the direct role of ANG II in UB-branching morphogenesis. In ongoing work, we are attempting to establish an assay to culture intact UBs isolated from E13 mouse embryos (30). This assay may represent a more physiological system to study the effects of growth factors including ANG II on epithelial branching morphogenesis.

An important finding of this study is that Ao and AT1 are expressed in both UBs and SM. A recent study (15) found that renin is present in the SM at a time when UB branching is just beginning. This raises the intriguing possibility that ANG II can be generated locally in the SM and then act in a paracrine fashion on the adjacent AT1-expressing UBs to induce branching. Similarly, AT1 present in the SM may be important in mediating stromal ANG II signaling to stimulate UB branching. One possible pathway may involve ANG II-induced stimulation of FGF-7 and its coupling with FGF-7 receptors expressed on the UB (29, 31). This possibility is supported by the ability of ANG II to increase basic FGF mRNA levels in luteal cells (37).

Several growth factors have been shown to either stimulate or inhibit UB-branching morphogenesis. The stimulatory factors include EGF and hepatocyte growth factor (32), Gdnf/Ret (40), vitamin A/retinoic acid receptor (16), low-dose bone morphogenetic protein (BMP)-7 (25), pleiotrophin (33), and ANG II. Because ANG II induced process formation in <2% of treated cells, utilization of isolated intact UBs will allow us to better ascertain the relative importance of ANG II in UB-branching morphogenesis. The inhibitory factors include endostatin (13), high-dose BMP-7 (25), BMP-2 (26), BMP-4 (18), and transforming growth factor-β (34). Given the multitude of the signal transduction pathways used by these factors, it is difficult to pinpoint a specific common final pathway that governs UB development. It is more likely that the final common pathway involves cross talk between multiple pathways such as the phosphatidylinositol 3-kinase (PI3-K) and mitogen-activated protein kinase (MAPK) pathways, which are known to commonly be activated by the above factors.

Our present finding that candesartan abrogates ANG II-induced UB cell branching indicates that the AT1 receptor exerts growth-promoting effects on UB cell branching. The signaling events that link AT1 to growth and differentiation of UB cells have not been defined. Several signaling pathways including Ras/Raf/MEK/ERK/activator protein-1 (AP-1) and PI3-K/Akt have been shown to mediate the effects of AT1 on cell proliferation and hypertrophy in vascular smooth muscle cells, fibroblasts, and renal mesangial cells (10, 35, 36). Thus one of the possible mechanisms to lead to increased UB cell proliferation, survival, and morphogenesis may involve AT1-mediated stimulation of the Ras/Raf/MEK/ERK/AP-1 and PI3-K/Akt pathways.

In summary, the present study demonstrates that the AT1 receptor is expressed in the UB and its derivatives during early metanephrogenesis in vivo. In addition, UB-derived cells in culture maintain expression of AT1 receptor mRNA and protein. ANG II, acting via AT1 receptors, stimulates cell process formation and branching in UB cells grown in collagen gels. These data strongly support a direct and independent role for the RAS in the development of the renal collecting system.

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DISCLOSURES
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