Angiotensin II Increases Pax-2 Expression in Fetal Kidney Cells Via the AT₂ Receptor

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Abstract. Although both the renin angiotensin system (RAS) and the paired homeobox 2 gene (Pax-2) seem critically important in renal organogenesis, whether and how they might interact has not been addressed. The present study asked whether a link between the RAS and Pax-2 exists in fetal renal cells, speculating that such an interaction, if present, might influence renal development. Embryonic kidney explants and embryonic renal cells (mouse late embryonic mesenchymal epithelial cells [MK4] and mouse early embryonic mesenchymal fibroblasts [MK3]) were used. Pax-2 protein and Pax-2 mRNA were detected by immunofluorescence, Western blot, reverse transcription–PCR, and real-time PCR. Angiotensin II (AngII) upregulated Pax-2 protein and Pax-2 mRNA expression via the AngII type 2 (AT₂) receptor in MK4 but not in MK3 cells. The stimulatory effect of AngII on Pax-2 gene expression could be blocked by PD123319 (AT₂ inhibitor), AG 490 (a specific Janus kinase 2 inhibitor), and genistein (a tyrosine kinase inhibitor) but not by losartan (AT₁ inhibitor), SB203580 (specific p38 mitogen-activated protein kinase inhibitor), PD98059 (specific MEK inhibitor), SP600125 (JNK inhibitor), and diphenyleneiodonium chloride (an NADPH oxidase inhibitor). Moreover, embryonic kidney explants in culture confirmed that AngII upregulates Pax-2 gene expression via the AT₂ receptor. These studies demonstrate that the stimulatory effect of AngII on Pax-2 gene expression is mediated, at least in part, via the Janus kinase 2/signal transducers and activators of transcription signaling transduction pathway, suggesting that RAS and Pax-2 interactions may be important in renal development.

Renal morphogenesis involves complex events in which many genes interact. When the normal pattern of nephrogenesis is interrupted, renal abnormalities ensue. More than 40 genes have been reported to participate in renal development, including glial cell line–derived neurotropic factor, RET, Pax-2, Wilms’ tumor suppressor gene, N-Myc, and several components of the renin-angiotensin system (RAS) (1). However, how these putative regulatory factors function and interact to control nephrogenesis is incompletely delineated.

The intrarenal RAS (2–4) seems to play a major role in renal development and repair (5,6). Woods et al. (7,8) showed that the intrarenal RAS is downregulated during the perinatal period in offspring of mothers who are subjected to moderate protein restriction during gestation. Such data suggest that during the perinatal period, angiotensin II (AngII), acting via its receptors, might play an important role in renal development and the long-term control of renal function and arterial pressure (5–8). The importance of the RAS in renal development has also been demonstrated when the RAS is interrupted by inadvertent use of angiotensin-converting enzyme inhibitors in humans (9) or by creating “knockouts” of RAS genes in mice (10).

Paired homeobox genes are important in embryogenesis. There are nine known paired homeobox (Pax) genes, although only Pax-2 and Pax-8 are expressed during fetal renal development. Pax-2 is required during the development of the genitourinary system (11–13), and mutations in Pax-2 lead to renal anomalies. For example, Pax-2 mutations are present in autosomal dominant syndromes such as the renal-coloboma syndrome (14), and 1Neu mice represent a model with similar renal abnormalities (15,16). During renal development, Pax-2 is detected in the caudal mesonephric duct, ureteric bud, and later in mesenchymal condensates induced by the ureteric bud (11–13). Subsequently, Pax-2 is restricted to the distal part of the S-shaped body, and its expression is extinguished as cells differentiate (17). Such studies have indicated that Pax-2 is likely essential for controlling tubular cell proliferation and differentiation (18), mediating mesenchymal-to-epithelial transformation as well as apoptosis (19).

The striking and similar renal anomalies found when either components of the RAS or the Pax-2 gene are mutated led us to hypothesize that there might be a link between the RAS and Pax-2, which, if present, might influence renal development. We used an in vitro approach with cultured fetal renal cells (MK4 and MK3) and fetal renal explants (E14) and found that AngII upregulates Pax-2 gene expression via the AngII receptor (AT₂R). We speculate that the observed upregulation of Pax-2 gene expression by AngII may play an important role in renal organogenesis and perinatal programming.

Materials and Methods

Normal glucose (5 mM) DMEM (catalog #12320) was purchased from Invitrogen (Carlsbad, CA). Oligonucleotides were synthesized by the DNA Synthesis Core Facility at Massachusetts General Hos-
Normal rabbit and mouse IgG and polyclonal anti-AT2R antibody (H-143) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-β-actin monoclonal antibody (clone AC-15) was purchased from Sigma (St. Louis, MO), a polyclonal anti-Pax-2 antibody was purchased from Covance (Richmond, CA), and polyclonal anti-phospho-Janus kinase 2 (JAK2) and anti-JAK2 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). SB203580 (a specific inhibitor of p38 mitogen-activated protein kinase [MAPK]), PD98059 (a specific inhibitor of MEK), SP600125 (an inhibitor of JNK), diphenyleneiodonium chloride (DPI; an inhibitor of NADPH oxidase), AG490 (a specific inhibitor of JAK2), cycloheximide (a protein synthesis inhibitor), and genistein (an inhibitor of tyrosine kinase) all were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). CGP-42112A was purchased from Sigma. The plasmids pcDNA 3.1/Pax-2 and pcDNA 3.1/AT2R were obtained from Dr. Paul Goodyer (McGill University, Montreal Children’s Hospital, Montreal, Quebec, Canada) and Dr. Deng-Fu Guo (CHUM-Hôtel-Dieu, Montreal, Quebec, Canada), respectively.

Depleted FBS (depleted of endogenous steroid and thyroid hormones) was prepared by incubation with 1% activated charcoal and 1% AG 1 × 8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA).

### Table 1. Real-time PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession</th>
<th>Sense (bp)</th>
<th>Antisense (bp)</th>
<th>PCR product size (bp)</th>
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<tr>
<td>Pax 2 (X55781)</td>
<td></td>
<td>5'-AAGCGACAGAACCCGACTATGT-3' (628-649)</td>
<td>5'-ACTCTCTGTCCCTGCCCAT-3' (794-776)</td>
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<td>AT2 R (D16840)</td>
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<td>5'-AGAATTACCCGTGACCAAGT-3' (881-900)</td>
<td>5'-GAAGGGATTAACACAGCTGTT-3' (1087-1067)</td>
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<td>β-actin (NM 007393)</td>
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<td>5'-CGTGCGTGACATCAAAGAGAA-3' (704-724)</td>
<td>5'-GCTCGTGGCAATAGTGATGA-3' (840-820)</td>
<td>116 bp</td>
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### Figure 1. Angiotensin II (AngII) upregulates Pax-2 gene expression as shown by immunofluorescence staining (magnification x400) in MK4 (A) but not in MK3 (B) cells. Cells were incubated in 5 mM d-glucose DMEM containing 1% depleted FBS (dFBS) for 15 min without (left) or with (middle) AngII stimulation at concentration 10⁻⁹ M. As a control, cells were incubated with 5% normal rabbit serum-PBS (right).
CA) for 16 to 24 h at room temperature, as described by Samuels et al. (20).

**Culture of MK3 and MK4 Cells**

MK3 and MK4 cells, previously described, were the gift of Dr. S. Potter (21). The MK3 cell line, which shows fibroblastic morphology, represents early embryonic metanephric mesenchyme before induction by the ureteric bud. MK3 cells express genes characteristic of early mesenchyme, including Hoxa 11, Hoxd 11, collagen I, and vimentin. The MK4 cell line represents later embryonic metanephric mesenchyme undergoing epithelial conversion. MK4 cells are relatively polygonal, or epithelial, in shape and express genes typical of late mesenchyme, including Pax-2, Pax-8, Wnt-4, Cadherin-6, Collagen IV, and LFB3.

MK3 and MK4 cells were cultured in normal glucose (5 mM) DMEM (pH 7.45), supplemented with 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in 95% air and 5% CO₂ at 37°C. Before experiments, cells were synchronized overnight in 5 mM glucose serum-free medium, after which all cells were incubated in DMEM that contained normal glucose plus 1% depleted FBS with or without various concentrations of AngII (10⁻¹¹ M to 10⁻⁷ M) for times indicated.

**Immunofluorescence Studies**

MK3 and MK4 cells were grown in two-chamber slides to 70 to 80% confluence. After exposure to AngII (10⁻⁹ M) for 15 min, cells were washed, fixed with 4% paraformaldehyde/PBS for 20 min, and then washed twice with PBS containing 0.5% BSA. Cells were then permeabilized with PBS containing 0.1% (vol/vol) Triton X-100 and 0.5% BSA for 15 min, washed as before, and then incubated with a primary antibody at 4°C overnight. After washing in PBS, cells were incubated with FITC-labeled goat anti-rabbit IgG for 1 h at room temperature. Immunofluorescence images were recorded with a Wild MPS 46/52 Photoautomat (Wild Leitz, Switzerland). The images are present at x400 magnification. As a negative control, primary antibody was replaced with 5% normal rabbit serum-PBS.

**Western Blot Analysis**

Cells were lysed with 700 µl of lysis buffer (50 mM Tris-HCl [pH 8.0] containing 1% NP-40, 250 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium orthovanadate, and 50 mM NaF). Lysates were sonicated for 30 s, heated at 95°C for 5 min, and centrifuged at 12,000 × g for 10 min at 4°C. Protein concentrations were measured by the Bradford method (Bio-Rad). Small aliquots (20 to 50 µl) of supernatant were subjected to 10% SDS-PAGE and transferred onto a PVDF membrane (Hybond-P; Amersham Pharmacia Biotech). The membrane was first blotted for β-actin and phospho-JAK2 antibody and then reblotted for β-actin and JAK2 antibody. The relative densities of the β-actin and JAK2 bands were measured by computerized laser densitometry. Cells prepared identically were studied in the presence and absence of cycloheximide (10⁻⁶ M) to examine protein stability.

**Reverse Transcription–PCR for Pax-2 mRNA**

Total RNA was prepared using TRIZOL Reagent (Invitrogen). Aliquots of total RNA (2 µg) were used for cDNA synthesis (Super-Script preamplification system; Invitrogen). Subsequently, 2 µl of the cDNA reaction mixture was taken to amplify Pax-2 and β-actin cDNA fragments using the forward primer 5'-TTTGTGAAACGGCGG-GCCCCTTA-3' and the reverse primer 5'-CATTGTCAACAGATGC-CCTCGG-3', corresponding to the nucleotide sequences n + 622 to n + 642 and n + 902 to n + 922 of Pax-2 cDNA (22). Primers specific for rat β-actin (23) (forward and reverse primers 5'-ATGCCATCTCT-GCTCTGGACCTGGC-3' and 5'-AGCATTTCGGTGCAAGTAG-GAGGG-3', corresponding to the nucleotide sequences n + 155 to n + 179 of exon 3 and n + 115 to n + 139 of exon 5 of rat β-actin) were used as internal controls. The amplification cycles were 20 s at 94°C, 20 s at 58°C, and 60 s at 72°C for 35 cycles using Rapid Cycler (Idaho Technology, Salt Lake City, UT). The plasmid pcDNA 3.1/ Pax-2 served as positive control. For identifying Pax-2 and β-actin cDNA fragments, 15 µl of the PCR product was electrophoresed on 1.5% agarose gels and transferred onto a Hybond XL nylon membrane (Amersham Pharmacia Biotech). DIGoxigenin-labeled oligonucleotides (La Roche Biochemicals, Indianapolis, IN) 5'-CCTGGGCGAG-GTACTACGAGACCG-3' and 5'-TCTTTGGCATORCATGAAA-CTACATTTC-3', corresponding to nucleotide n + 747 to n + 760 of Pax-2 cDNA and nucleotide n + 9 to n + 35 of exon 4 of rat β-actin cDNA (23), respectively, were used to hybridize the PCR products on the membrane. After stringent washing, the membrane was detected with a digoxigenin luminescent detection kit (La Roche) and exposed to Kodak BMF film (Amersham Pharmacia Biotech). Pax-2 mRNA levels were normalized by corresponding β-actin mRNA levels.

**Real-Time PCR**

First-strand cDNA was produced from 2 µg of random hexamer primed total RNA using Super-Script preamplification system (Invitrogen). Relative quantification by real-time PCR was carried out using a LightCycler (Roche). Relative quantification by real-time PCR was carried out by the comparative threshold cycle (Ct) method using the relative mRNA expression levels of Pax-2 normalized by corresponding β-actin mRNA levels.

**Figure 2. Dose-dependent AngII upregulation of Pax-2 gene expression.**

Western blot analysis of MK4 cells (A) and MK3 cells (B) is shown. Cells were incubated in 5 mM d-glucose DMEM containing 1% dFBS for 15 min, with or without AngII (10⁻¹¹ M to 10⁻⁷ M). Cells were then lysed and analyzed for Pax-2. The same membrane was reblotted for β-actin. The relative densities of Pax-2 were compared with the β-actin control. The normalized Pax-2 level in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of three independent experiments. *P ≤ 0.05; **P ≤ 0.01.
using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) and DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research), following the protocol described by the supplier. PCR reactions in triplicate underwent 40 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 20 s, and 79°C for 5 s in the thermal cycler (MJ Research). The parameter CT (threshold cycle) value was measured to determine starting copy number of target genes using the standard curve. Lower value of CT indicates higher amount of PCR products. Primers are shown in Table 1.

Embryonic Kidney Culture

Embryonic kidneys were isolated from timed pregnant C57BL/6 mice (E14; Charles River, Wilmington, MA) under sterile conditions and cultured in α-MEM medium without nucleosides (Invitrogen), supplemented with 1 mMol/L β-glycerophosphate, 0.05 mg/ml ascorbic acid, 0.3 mg/ml l-glutamine and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin), and 0.2% BSA. Each kidney explant was cultured in 500 μl of medium in a separate well of a 24-well plate for up to 24 h in the presence or absence of AngII (10⁻⁹ M) with or without PD123319 (10⁻⁶ M).

Statistical Analyses

Three to four separate experiments were performed for each protocol. The data were subjected to t test or ANOVA followed by Bonferroni correction to compare the control and treatment groups in the same experiment. A probability level of P ≤ 0.05 was considered statistically significant.

Results

Pax-2 Is Visualized in MK4 but not MK3 Cells

On the basis of known information (21), we anticipated that Pax-2 would be present in MK4 but not MK3 cells. Immunofluorescence data indicated a low basal level of Pax-2 in MK4 cells and none in MK3 cells (Figure 1). We hypothesized that AngII would increase Pax-2 expression. Indeed, AngII (10⁻⁹ M) upregulated Pax-2 in MK4 cells (Figure 1A) but did not induce Pax-2 in MK3 cells (Figure 1B). Immunostaining seemed intranuclear, consistent with the fact that Pax-2 is a nuclear transcription factor (22,24).

AngII Stimulates Pax-2 Protein Expression in MK4 Cells in a Dose- and Time-Dependent Manner

Western blot results demonstrated that AngII (10⁻¹¹ M to 10⁻⁷ M) stimulates Pax-2 protein expression in a dose-dependent manner in MK4 cells (Figure 2A) but not in MK3 cells (Figure 2B). A maximal response by MK4 cells to AngII
occurred at $10^{-9}$ M (Figure 2C), which was greater in comparison with the increase at either $10^{-7}$M or $10^{-11}$M; thus, the response seemed biphasic. Accordingly, we used AngII ($10^{-9}$ M), at which maximal response occurred, for subsequent experiments.

The stimulatory effect of AngII on Pax-2 expression in MK4 cells (Figure 3A) is time dependent. A stimulatory effect appeared at 5 min after exposure and reached a maximum at 15 min, after which it decreased (Figure 3B). Given this time course, we used 15 min of stimulation for the remainder of the experiments. Moreover, given the rapidity with which AngII stimulates Pax-2 protein expression (minutes), we addressed the possibility of protein stabilization in additional experiments in the absence or presence of cycloheximide (Figure 3C). AngII enhanced and stabilized Pax-2 protein level 15 to 30 min after exposure to AngII in the presence of cycloheximide.

Thirty minutes after AngII exposure, Pax-2 protein level returned to baseline. Thus, Pax-2 protein level declined more slowly in the absence of cycloheximide, suggesting that AngII enhances the stability of Pax-2 protein in MK4 cells.

**AngII Stimulates Pax-2 mRNA Expression in MK4 Cells**

Reverse transcription–PCR (RT-PCR) showed that AngII stimulated Pax-2 expression (expressed as the ratio of Pax-2 mRNA to β-actin mRNA expression) in a dose-dependent manner in MK4 cells (Figure 4A). Again, the maximal effect of AngII occurred at a concentration of $10^{-9}$ M. We also observed that the stimulatory effect of AngII on Pax-2 mRNA expression in MK4 cells started by 15 min, reached a plateau at 30 min, and was sustained to 60 min (Figure 4B). We used 30 min of AngII stimulation for all subsequent experiments.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** (A and B) These data demonstrate the dose- and time-dependent manner of AngII upregulation of Pax-2 mRNA expression as analyzed by reverse transcription–PCR (RT-PCR) in MK4 cells. Cells were incubated in 5 mM D-glucose DMEM containing 1% dFBS for various time period with or without AngII ($10^{-11}$ M to $10^{-7}$ M) and were then harvested and assayed for Pax-2 mRNA levels by RT-PCR. PCR products were hybridized with a digoxigenin-labeled oligonucleotide corresponding to nucleotides n+747 to n+760 of Pax-2 and n+9 to n+35 of exon 4 of rat β-actin, respectively. The relative densities of the PCR band of Pax-2 were normalized using β-actin as a control. The normalized Pax-2 mRNA level in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of three independent experiments. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$. (C) Results of real-time PCR for AngII effect on Pax-2 mRNA in MK4 cells.
Because RT-PCR is only semiquantitative, possibly not reflecting the magnitude of changes, we used quantitative real-time PCR to verify our RT-PCR data. Real-time RT-PCR confirmed quantitatively (Figure 4C) that AngII (10^{-9} M) upregulated Pax-2 gene expression.

**PD123319 Blocks the Stimulatory Effect of AngII on Pax-2 Gene Expression in MK4 Cells**

We next sought to examine which AngII receptor(s) mediated the increase in Pax-2 expression by AngII in MK4 cells. Results of both Western blot and RT-PCR indicated that PD123319 (an AT_2R antagonist [10^{-6} M]) blocks the stimulatory AngII effect (Figure 5), whereas losartan (an AT_1R antagonist [10^{-6} M]) has no effect. There was no additional inhibitory effect when both AT_2R and AT_1R blockers were used, as compared with AT_2R blocker alone. These data suggest that AngII stimulates Pax-2 gene expression via the AT_2 receptor but not via the AT_1 receptor.

To confirm that the effect is via the AT_2 receptor, we also tested the effect of CGP 42112A, an AT_2R agonist, on Pax-2 protein expression; CGP 42112A upregulates Pax-2 protein expression in MK4 cells shown by Western blot (Figure 6, A and B). Furthermore, AT_2R mRNA and protein were detected in both MK3 and MK4 cells by real-time PCR (Figure 6C) and Western blot (Figure 6D), respectively, supporting the observation that AngII signaling via the AT_2R is correct.

Additional inhibitors such as SB203580 (a specific inhibitor of p38 MAPK), PD98059 (a specific inhibitor of MEK), and DPI (an inhibitor of NADPH oxidase) were used in an attempt to begin to understand post-receptor mechanisms. These blockers had no effect at baseline and could not block the AngII effect on Pax-2 expression (Figure 7), indicating that p38 MAPK, p44/42 MAPK, and reactive oxygen species (ROS) generation are likely not involved in AngII upregulation of Pax-2. In additional studies, we observed that AngII stimulates the phosphorylation of JNK in MK4 cells but that SP600125 (JNK inhibitor) could not inhibit the AngII effect on Pax-2 expression (data not shown).

![Figure 5](image_url). Effect of losartan and PD123319 on Pax-2 expression as stimulated by AngII in MK4 analyzed by Western blot (left) and RT-PCR (right). Cells were preincubated in 5 mM d-glucose DMEM containing 1% dFBS for 10 min in the absence or presence of losartan (10^{-6} M) and PD123319 (1 \times 10^{-6} M), then incubated with or without AngII (10^{-9} M) for 15 min or 30 min for Pax-2 expression analyzed by Western blot and RT-PCR, respectively. The same membrane was rebotted for \(\beta\)-actin. The relative densities of the Pax-2 were normalized to the \(\beta\)-actin. The normalized Pax-2 level in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of three independent experiments. \(*P \leq 0.05; ** P \leq 0.01; *** P \leq 0.005.\)
AG490 and Genistein also Block the Stimulatory Effect of AngII on Pax-2 Gene Expression in MK4 Cells

Western blotting demonstrated that AG490 (a specific inhibitor of JAK2) and by genistein (an inhibitor of tyrosine kinase) blocked the stimulatory effect of AngII on Pax-2 gene expression in MK4 cells (Figure 8). As indicated, these compounds had blocked the effect of AngII with a magnitude similar to PD123319. Moreover, these blockers, by themselves, had no effect on Pax-2 expression (data not shown).

AngII Stimulatory Effect on Pax-2 Expression Occurs Via JAK2/STAT Phosphorylation in MK4 Cells

Because the JAK/STAT (signal transducers and activators of transcription) pathway seemed likely to be involved, we performed Western blot analysis, which indicated that AngII stimulates the phosphorylation of JAK2 in MK4 cells in a dose-dependent (Figure 9A) and time-dependent manner (Figure 9B). These results support the notion that AngII upregulates Pax-2 gene expression via the AT2R may be mediated, at least in part, via the JAK2/STAT signal transduction pathway. This stimulatory effect of AngII on JAK2 phosphorylation could be blocked by PD 123319, AG 490, and genistein in MK4 cells (Figure 10). These data indicate that AngII upregulates Pax-2 gene expression via the AT2R. Furthermore, the increase in Pax-2 gene expression may be mediated, at least in part, via the JAK2/STAT signal transduction pathway.

PD123319 Blocks the Stimulatory Effect of AngII on Pax-2 Gene Expression in Kidney Organ Culture

To confirm that the stimulatory effect of AngII on Pax-2 gene expression is not limited to cultured MK4 cells, we examined fetal kidney explants (E14; C57BL/6 strain mice) in culture. Real-time PCR data indicated that PD123319 (10^-6 M) blocks the stimulatory effect of AngII (10^-6 M) on Pax-2 gene expression (Figure 11, ratio of Pax-2 mRNA to β-actin mRNA expression). These ex vivo data in explanted fetal kidneys suggest that AngII stimulates Pax-2 gene expression via the AT2R.

Discussion

PAX genes and the RAS both seem critically important for renal organogenesis (11). The present in vitro and ex vivo
studies support our hypothesis that AngII, a major effector of the RAS, regulates Pax-2 gene expression in MK4 cells (derived from a late embryonic stage in transgenic mice) and mouse embryonic kidney explants, findings that may have substantial implications for nephrogenesis.

Renal morphology in animals that have Pax-2 deficiency or lack RAS genes have provided unique opportunities with which to examine renal development (10). Without a functional Pax-2 gene, renal agenesis occurs (14–16,25,26). Abundant evidence indicates that the RAS is also required during renal development during gestation and in the perinatal period. As demonstrated in vivo and ex vivo studies, all components of the RAS are present in the developing kidney. Local AngII generation contributes in a paracrine manner to the growth and differentiation of the ureteric bud (UB). Iosipiv et al. (27) indicated that UB-derived epithelia express angiotensinogen (ANG) and AT1R during murine metanephrogenesis. However, the expression of AT2R is more than threefold higher as compared with AT1R in undifferentiated mesenchyme of E14 mouse or rat kidney (28,29). By birth, AT1R expression has increased significantly, whereas AT2R rapidly decreases in the first 2 postnatal weeks (28,29). During nephrogenesis, AT1R expression is associated with mesenchymal-epithelial interactions involved in interactions between the UB and the metanephros (29–31), whereas AT2R expression seems to correlate with the differentiation and proliferation of glomerular and tubular cells (32,33). That mutations in RAS genes lead to abnormalities in the development of the renal calyces and pelvis is well established. For example, mice lacking ANG genes (null-mutant) develop severe hypotension and aberrant renal vasculature, temporal delay in glomerulogenesis, and abnormal renal pelvis formation (34–38). Mice lacking angiotensin-converting enzymes and AT1R (null-mutant) have an abnormal phenotype similar to that observed as ANG-deficient mice (10). AT1R null mice exhibit hydronephrosis (31) and do not develop a normal renal pelvis; they also exhibit tubular atrophy and reduction in the size of the renal papilla as compared with wild-type animals (10). In contrast, AT2R null mice display the different phenotype (39,40), one similar to human congenital anomalies of the kidney and urinary tract (renal

Figure 7. The effect of additional inhibitors on Pax-2 stimulation by AngII in MK4 cells as analyzed by Western blot (left) and RT-PCR (right). Cells were preincubated in 5 mM d-glucose DMEM containing 1% dFBS for 10 min in the absence or presence of diphenyleneiodonium chloride (1 × 10⁻⁶ M), SB203580 (1 × 10⁻⁶ M), and PD98059 (1 × 10⁻⁶ M), then incubated with or without AngII (10⁻⁹ M) for 15 min or 30 min for Pax-2 expression analyzed by Western blot and RT-PCR. The same membrane was rebotted for β-actin. The relative densities of Pax-2 were normalized to β-actin. The normalized Pax-2 level in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of three independent experiments. ***P ≤ 0.005.
phenotype including hypoplasia, dysplasia, and ureteral abnormalities), which includes abnormal renal pelvis/ureteric development (41,42).

Like the RAS, Pax-2 is essential for normal nephrogenesis; in fact, Pax-2 mutations are uniquely associated with a special subgroup of congenital anomalies of the kidney and urinary tract (14,43–45). The phenotypic similarities between AT2R null mice and the renal findings with Pax-2 mutation led us to hypothesize that there might be an interaction between AT2R and Pax-2 expression in the development kidney. To our knowledge, whether and how they might interact has not previously been addressed.

We hypothesized that interactions between AngII and Pax-2, if present, might have important implications for further understanding of nephrogenesis. Our results support the concept that AngII can stimulate Pax-2 expression and indicate that nanomolar amounts of AngII (10−9 M) maximally enhance Pax-2 protein level as compared with control (greater than twofold increase). AngII seems to have a biphasic effect; a higher concentration of AngII (10−7 M) also upregulates Pax-2 protein expression, although not to the same extent (Figure 2). A possible explanation for this biphasic effect may be that AngII, at higher concentrations, desensitizes or downregulates AngII receptors. This possibility is supported by studies showing a biphasic response to AngII within the proximal tubule (46–52).

AngII seems to increase Pax-2 protein expression more rapidly than Pax-2 mRNA expression. The peak in protein expression at 15 min is followed by a slow decline. Specifically, AngII augments both Pax-2 protein expression at 15 min and Pax-2 mRNA levels at 15 min and 30 min, respectively, in MK4 cells. One possible explanation for this differential time effect is that AngII might stabilize the half-life of Pax-2 protein and subsequently stimulate the transcription of Pax-2 mRNA. Our studies revealed that cycloheximide attenuated but could not abolish the effect of AngII on Pax-2 protein expression. Such a pattern is similar to that reported by Feliers et al. (53), who recently reported that AngII induced a rapid increase (within 2 min) in vascular epithelial growth factor (VEGF) protein expression that reached a maximum at 15 to 30 min, without changes in VEGF mRNA. The stimulatory effect of AngII on VEGF reported by Feliers et al. seemed to be mediated via both the translocation of VEGF mRNA to polyribosomes and phosphorylation of 4E-BPI and eLF-4E, critical to
the initiation of protein translation. Whether the rapid increase in Pax-2 expression in MK4 cells induced by AngII occurs via similar mechanism(s) remains to be defined.

Our studies show that AngII seems to stimulate Pax-2 expression via the AT2R. The intracellular signaling pathways that might be involved are several. AngII acting as a growth factor within the kidney (48) can directly trigger multiple signal transduction pathways such as p38 MAPK, extracellular signal-regulated kinase, JNK, protein kinase C, ROS generation, and JAK/STAT that are involved in differentiation and/or proliferation (49–51). We performed a number of studies to examine potential signal transduction pathways involved in the effect of AngII on Pax-2 expression.

In addition to intrarenal hemodynamic effects, AngII acts as a growth factor within the kidney (47–54), directly triggering multiple signaling transduction pathways such as p38 MAPK, extracellular signal-regulated kinase, JNK, protein kinase C, ROS generation and JAK/STAT that are involved in differentiation and/or proliferation (55–57). We performed a number of studies to examine potential signal transduction pathways by which AngII might modulate Pax-2 expression. On the basis of our results, we believe that p38 MAPK, p44/42 MAPK, and ROS generation are unlikely to be involved in the AngII upregulation of Pax-2, because SB203580, PD98059, and DPI could not block AngII-induced Pax-2 expression.

The putative involvement of JNK activation would seem possible, on the basis of studies of Cai et al. (58), who reported that activation of JNK by either the upstream kinases MEKK1 or DLK or by expression of Wnt signaling proteins increases Pax-2 phosphorylation and enhances Pax-2 transactivation potential. In additional studies, we observed that AngII stimulates the phosphorylation of JNK in MK4 cells but that SP600125 (JNK inhibitor) could not inhibit the AngII effect on Pax-2 expression. Thus, our present data suggest that the
AngII effect is not likely mediated via the activation of JNK signaling pathway, although discrepant results remain to be delineated.

Recent reports suggest that modulation or activation of JAK/STAT pathway by AngII has pathologic consequences for cardiovascular (56) and renal (59) function. JAK/STAT pathways are activated by multistep phosphorylation cascades after ligand-cell surface receptor binding, which lead directly to target gene promoters in the nucleus, providing mechanisms for transcriptional regulation without second messengers. JAK kinases, once activated, selectively phosphorylate STAT to form homo- and heterodimers, rapidly translocating to the nucleus, and bind to a regulatory element in the promoter of the relevant gene (60). We speculate that the observed interaction of AngII and Pax-2 could lead to AngII–induced phosphorylation of components of the JAK2/STAT pathway to upregulate Pax-2 gene expression. Our data indicate that AG490 and genistein blocked the AngII–mediated upregulation of Pax-2 gene and stimulated the phosphorylation of JAK2 in MK4 cells, suggesting possible mediation via the JAK2/STAT signal transduction pathway.

We hypothesize that existing molecular motifs render this signaling pathway likely. Booz et al. (56) reported that Stat1–Stat2 heterodimers bind to regulatory IFN-stimulated response elements (AGTTTNCNTTTCC); other Stat dimers bind to γ-IFN activated motifs (GAS). The recognized GAS motifs for Stat1, Stat3, Stat4, Stat5a, and Stat5b are TTCN 3 GAA; for Stat6, TTCN 4 GAA. With the exception of Stat2, all Stats can form homodimers. Stat2 functions only as a complex with Stat1. By sequence homology search, we have tentatively identified several putative GAS motifs that are homologous to GAS motifs in human Pax-2 promoter (gb: U45245) at N-651/N-642, N-382/N-374, N-371/N-362, N-382/N-372, and N-152/N-144. We speculate that these sequences are active GAS motifs that might mediate the effect of AT2 R and specific Stats on Pax-2 gene expression.

Pax-2 seems critical for normal renal development, being required for differentiation of renal epithelium from the intermediate mesoderm (62). To date, few genes are known to be regulated directly by Pax-2. In the early metanephric mesenchyme, Pax-2 can activate Wilms’ tumor suppressor gene (63) and glial cell line–derived neurotropic factor (64) genes by direct interactions with cis-acting regulatory sequences. It also has been reported that EGF increased the half-life of Pax-2.
protein in a rat kidney epithelial cell line (NRK-52E) (65), whereas activin A (66) and TGF-β1 (67) downregulated Pax-2 protein in proximal tubule cells. Our present studies indicate that AngII, like EGF (65), enhances Pax-2 protein expression in the presence of cycloheximide (Figure 3C), suggesting that AngII and EGF might have similar action in stabilizing the half-life of Pax-2 protein.

Taken together, the present studies suggest that AngII stimulates Pax-2 gene expression in MK4 cells and E14 explants via the AT1R. It seems that the stimulatory effect of AngII is mediated, at least in part, via the JAK2/STAT signaling transduction pathway. These data indicate that RAS and Pax-2 interact, which may be important in renal development.

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