Angiotensin II AT$_2$ receptor regulates ureteric bud morphogenesis

Renfang Song, Melissa Spera, Colleen Garrett, Samir S. El-Dahr, and Ihor V. Yosypiv

Section of Pediatric Nephrology, Department of Pediatrics, Hypertension and Renal Center of Excellence, Tulane University Health Sciences Center, New Orleans, Louisiana

Submitted 11 March 2009; accepted in final form 18 December 2009

IN THE CLASSIC RENIN-ANGIOTENSIN system (RAS), renin cleaves angiotensinogen (AGT) to generate angiotensin (ANG) I, which is then converted to ANG II by the angiotensin-convert- ing enzyme (ACE). ANG II is the principal effector peptide growth factor of the RAS which acts via two major G protein-coupled receptors: AT$_1$R and AT$_2$R (4). Convincing evidence indicates that the RAS is critical for proper kidney development. Use of ACE inhibitors or AT$_1$R antagonists during fetal life, as well as mutations in the genes encoding AGT, renin, ACE, or AT$_1$R in humans, are associated with renal tubular dysgenesis (18). Genetic inactivation of AGT, renin, ACE, or AT$_1$R in mice causes pelvic dilatation (hydrenephrosis) and hypoplastic papilla (15, 34, 36, 46, 47). Mutations in the AT$_2$R gene in mice are associated with increased incidence of double ureters and vesicoureteral reflux (35). Collectively, these findings indicate that the ureteric bud (UB) and its derivatives, the collecting ducts, are principal targets for the RAS.

UB outgrowth from the nephric duct is followed by repetitive branching to form the renal collecting system (ureters, pelvis, calyces, and collecting ducts) (1, 14, 19, 43). Concurrently, emerging UB tips induce surrounding mesenchymal cells to undergo mesenchymal-to-epithelial transition (MET) and form proximal epithelial elements of the nephrons (from the glomerulus to the distal tubule). Even subtle decreases in the efficiency of UB branching result in a profound decrease in nephron endowment (42). Together, aberrant UB branching and nephrogenesis cause renal hypodysplasia and lead to eventual progression to chronic renal failure (6, 28).

We have recently reported that ANG II induces the expression of glial-derived neurotrophic factor (GDNF)/c-Ret/Wnt11 signaling pathway genes, promotes cell proliferation preferentially in UB tip cells and stimulates UB branching (52). Here, we demonstrate an essential role for ANG II AT$_2$R in UB morphogenesis. AT$_2$R is expressed in the UB epithelia during metanephric development. Pharmacological antagonism of the AT$_2$R during early stages of UB development or genetic inactivation of the AT$_2$R in mice result in impaired UB branching. Finally, inhibition of AT$_2$R signaling downregulates GDNF/Ret/Wnt11 pathway gene expression, decreases proliferation, and induces apoptosis of the UB cells.

MATERIALS AND METHODS

Immunohistochemistry for AT$_2$R. Immunolocalization of AT$_2$R protein was performed in C57BL/6J mouse embryos (E) from day E11.5 to E16 (n = 4/lage group; Hybrid-Ready Tissue, Novagen). Immunostaining was performed by the immunoperoxidase technique using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA). A polyclonal rabbit AT$_2$R antibody directed against the NH$_2$-terminal domain of the AT$_2$R (N-19, sc-7421, Santa Cruz) was utilized at concentrations of 1/100 to 1/400. Specificity of immunostaining was documented by the elimination of immunoreactivity after preabsorption of the primary antibody with its immunogen or omission of the primary antibody.

Western blot analysis. Mouse kidneys and UB cells (generously provided by Dr. Jonathan Barash, Columbia University) were homogenized in cold lysis buffer containing a cocktail of enzyme inhibitors. The samples were centrifuged, and the supernatants were homogenized in cold lysis buffer containing a cocktail of enzyme inhibitors. The samples were Western blotted to antibodies specific to AT$_2$R, GDNF, c-Ret, Wnt11, and Spry1. Protein bands were imaged using ImageJ and band intensities determined.

RT-PCR. Semi-quantitative RT-PCR was utilized to determine whether cultured UB cells and mesenchymal (MK4) cells express AT$_2$R mRNA. The MK4 cell line represents embryonic metanephric mesenchyme undergoing epithelial conversion. MK4 cells are epithelial in shape and express genes typical of late mesenchyme, including paired box 2 (Pax-2), Pax-8, Wnt-4, and cadherin-6 (51). UB, MK4
cells, and total kidney RNA was extracted using the TRIzol reagent (Invitrogen). RNA (3 μg) was reverse-transcribed. cDNA was amplified using the PerkinElmer Gene Amplification system 2400 (Cetus Instruments, Norwalk, CT) from 25% of the RT mixture using gene-specific primers: sense 5'-ATTCCTGTTCCTCCTAC-3'; antisense 5'-GTAACACGGTGTGCAAA-3'.

In vitro tubulogenesis assay. The in vitro tubulogenesis assay was performed as previously described (17). UB cells were initially obtained from microdissected UB of an E11.5 mouse transgenic for simian virus 40 (SV40) large T antigen (Innnotro-mouse, Charles River Laboratories, New York, NY) (2). UB cells were used at passage numbers 10–15. The cells were trypsinized, and 150 × 10^5 cells/well were suspended in type I rat-tail collagen (Upstate Biotechnology), 10 × DMEM, 200 mM HEPES (pH 8.0) in an 8:1:1 ratio at 150 × 10^5 cells/ml. After gelation, DMEM/F12 medium with 0.5% FBS or medium with 0.5% FBS containing ANG II (10^{-5} M, Sigma, St. Louis, MO) alone or ANG II and AT_{2R} antagonist PD123319 (10^{-5} M, Sigma) was added to each well on top of the collagen gel. After a 48-h incubation period at 37°C and 5% CO_{2}, individual cells/well were scored for the presence of cell processes directly from the plates utilizing an Olympus IX70 inverted phase-contrast microscope, and the average number of processes per cell was calculated. Each condition was set up in triplicate (n = 4 separate experiments). Images were acquired directly from the plates via an Olympus MagnaFire FW camera and processed with Adobe Photoshop 7.0.

Metanephric organ culture. Wild-type CD1 mice embryos (Charles River) were dissected aseptically from the surrounding tissues on E12.5. Embryos from AT_{2R} mutant mice were dissected on E14.5. The day when the vaginal plug was observed was considered to be E0.5. CD1 mice metanephi were grown on an air-fluid interface on polycarbonate Transwell filters (0.5 μm, Corning Costar) inserted into six-well plates containing DMEM/F12 medium (GIBCO BRL) alone, in the presence of ANG II or AT_{2R} antagonist PD123319 (10^{-6} M, Sigma) for 48 h at 37°C and 5% CO_{2}, as previously described (51) and then processed for the whole mount immunohistochemistry or in situ hybridization. AT_{2R} mutant mice in C57/BL6 background, originally generated by Dr. Tadashi Inagami (Vanderbilt University, Nashville, TN) (24), were obtained from Dr. Alvin Schmaier (Case Western Reserve University). AT_{2R} mutant metanephi were immediately processed for the whole mount immunofluorescence using an anti-cytokeratin antibody (Sigma). The effect of drug treatment was studied in paired kidneys obtained from the same fetus (i.e., the left kidney was incubated with media and the right kidney with PD123319, or the left kidney with media and the right kidney with CGP42112).

In situ hybridization. AT_{2R} mRNA expression and the effect of PD123319 (10^{-6} M) on the GDNF/c-Ret/Wnt11 pathway and Sprouty (Spry) 1 gene expression was examined by whole mount in situ hybridization. Mouse full-length AT_{2R} cDNA was from Open Biosystems (Rockford, IL). c-Ret, GDNF, Wnt11, and Spry1 cDNAs were kind gifts from Drs. F. Costantini, A. McMahon, and J. Licht. Preparation of RNA probes and whole mount in situ hybridization were performed according to protocols (http://www.hhmi.ucla.edu/derobertis) established in the De Robertis laboratory. Five embryonic kidneys per treatment group per probe were examined. All experiments were done at least twice. The metanephi were photographed using an Olympus model SC35 camera mounted on an Olympus model BH-2 microscope, and digital images were captured using Adobe Photoshop software.

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR was utilized to determine whether AT_{2R} antagonist PD123319 alters GDNF, c-Ret, Wnt11, and Spry1 mRNA expression in the whole metanephi grown ex vivo. E12.5 CD1 mice metanephi were grown on an air-fluid interface in the presence of media (control) or AT_{2R} antagonist PD123319 (10^{-6} M, Sigma) for 24 h at 37°C and 5% CO_{2}. In addition, we examined the effect of genetic inactivation of AT_{1R} in mice on GDNF, c-Ret, Wnt11, Spry1, and bone morphogenetic protein 4 (BMP4) mRNA levels in the kidney on E13.5. SYBR Green quantitative real-time RT-PCR was conducted using Mx3000P equipment (Stratagene, La Jolla, CA) using MxPro QPCR software (Stratagene) as described previously (16). The quantity of each target mRNA expression was normalized by that of GAPDH mRNA expression. Three RNA samples per treatment group were analyzed in triplicate in each run. PCR was performed three times.

Cell proliferation and apoptosis assays. To investigate the role of endogenous ANG II and AT_{2R} in UB cell proliferation and survival, E12.5 CD1 mice metanephi were grown on filters in the presence of DMEM/F12 medium alone (n = 10) or with AT_{2R} antagonist PD123319 (10^{-6} M, n = 10) for 24 h at 37°C. In addition, cell proliferation and apoptosis were examined in E13.5 metanephi from AT_{2R}^{-/-} and AT_{2R}^+/+ mice (n = 4/genotype). The kidneys were fixed in 10% neutral buffered formalin overnight at 4°C, processed for paraffin embedding, and 4-μm-thick sections were cut. Cell proliferation was examined by incubating the sections in PBS+3% BSA with anti-phospho-histone H3 (ph3) antibody (1:50, Cell Signaling, Danvers, MA) overnight at 4°C. Following washings in PBS/Tween and blocking with IgG, the sections were incubated with anti-cytokeratin antibody (1:200, Sigma). For the negative controls, the primary antibody was replaced with PBS/3% BSA solution. After the final wash in PBS, 4'-6-diamidino-2-phenylindole (DAPI; Invitrogen, Eugene, OR) was added to the mounting media to mark the nuclei. The number of ph3-positive (red) and cytokeratin-positive (green) cells was determined in a blinded fashion in four randomly selected UBs of each kidney section by fluorescent microscopy. A cell proliferation index (percentage of ph3-positive cells) was calculated from the ratio of ph3-positive to total nuclei. Apoptosis was assessed by terminal uridine triphosphate (UTP) nick-end labeling (TUNEL; TACS TdT Kit, R&D Systems, Minneapolis, MN) as previously described (52) or with an anti-cleaved caspase 3 antibody (Cell Signaling). The number of TUNEL-positive cells per UB tip or stalk was determined in each kidney section (n = 10 kidneys/group; 3 sections/kidney), and the mean number of TUNEL-positive cells per UB tip or stalk was calculated. In addition, the number of ph3- and caspase 3-positive cells per UB or mesenchyme was determined in three sections/kidney in AT_{2R}^{-/-} and AT_{2R}^+/+ mice (n = 4 kidneys/genotype). All experiments involving mice were approved by Tulane Institutional Animal Care and Use Committee.

Statistical analysis. Data are presented as means ± SE. Differences among the treatment groups in mRNA levels, the number of UB tips, and the number of TUNEL-positive cells in media vs. PD123319 were analyzed by Student's t-test. Differences among the treatment groups for cell proliferation index were analyzed by the Mann-Whitney rank sum test. A P value of <0.05 was considered statistically significant.

RESULTS

Expression of the AT_{2R} protein in developing metanephos in vivo. We previously demonstrated that AT_{2R} protein is expressed in the UB as early as E12 of murine development (26). In this study, we examined the expression of the AT_{2R} protein in mouse kidneys on E11.5–E16. On E11.5 (T-stage UB), AT_{2R} immunostaining was localized on both luminal and basolateral aspects of the UB followed by the induced mesenchyme (Fig. 1). On E12, distinct but modest AT_{2R} immunostaining was present in the medullary stroma, vesicles, and UB tips (Fig. 1). On E13, strong expression was observed in UB branches and tips, followed...
by the stroma and maturing glomeruli, which expressed \( \text{AT}_2\text{R} \) in the tuft but not in podocytes. On \( E14 \), \( \text{AT}_2\text{R} \) remained prominent on both luminal and basolateral membranes of UB branches followed by nephron progenitors and medullary stroma, whereas the cortical stroma was mostly \( \text{AT}_2\text{R} \) negative (Fig. 2). On \( E16 \), \( \text{AT}_2 \) was expressed diffusely in inner cortical tubules, which resembled morphologically proximal tubules (Fig. 2).

**Effect of ANG II and \( \text{AT}_2\text{R} \) antagonist PD123319 on UB cell morphogenesis in vitro**. UB cells cultured in gels form processes and branching tubular structures when exposed to various growth factors, providing a convenient experimental system for analyzing mechanisms of epithelial branching morphogenesis (25). A principal advantage of this approach is the ability to examine the direct effect of a specific factor on UB cell growth that is independent from confounding soluble factors released by the mesenchyme. Importantly, cultured UB cells maintain expression of \( \text{AT}_2\text{R} \) mRNA and protein (Fig. 3, A and B). ANG II increased the number of UB cells with processes per well compared with control cells cultured in the presence of DMEM/F12 media with 0.5% FBS alone (16.9 ± 0.9 vs. 9.1 ± 2.6, \( P < 0.05; n = 4 \) experiments). The effect of ANG II on UB cell branching was abrogated by pretreatment with the \( \text{AT}_2\text{R} \) antagonist PD123319 (10\(^{-6}\) M, 6.9 ± 1.6 vs. 9.1 ± 2.6, \( P < 0.01; n = 4 \) experiments) (Fig. 3, C–G). The results demonstrate that \( \text{AT}_2\text{R} \) is expressed during UB cell morphogenesis and is capable of stimulating branching of UB cells in vitro. This observation is consistent with the ability of \( \text{AT}_2\text{R} \) to induce neurite outgrowth and elongation (17).

**Effect of \( \text{AT}_2\text{R} \) antagonist PD123319 or genetic inactivation of \( \text{AT}_2\text{R} \) in mice on UB morphogenesis in whole metanephric kidney**. To examine the role of endogenous ANG II and its \( \text{AT}_2\text{R} \) in UB branching in the intact metanephros, we utilized the \( \text{AT}_2\text{R} \) antagonist PD123319. Treatment of \( E12.5 \) metanephiroi grown ex vivo with PD123319 (10\(^{-6}\) M) decreased the number of UB tips compared with control after 48 h (34 ± 1.0 vs. 43 ± 0.8, \( P < 0.01; n = 5/treatment \) group) (Fig. 4, A, B, and E). In contrast, treatment of metanephiroi with the \( \text{AT}_2\text{R} \) agonist CGP42112 (10\(^{-9}\) M) increased the number of UB tips compared with control (48 ± 1.8 vs. 39 ± 12.3, \( P < 0.05; n = 5/treatment \) group) (Fig. 4, C–E). Importantly, at 10\(^{-9}\) M concentration, CGP41221 acts as a selective agonist of the \( \text{AT}_2\text{R} \) (5). These findings are consistent with the results obtained in UB cells demonstrating that ANG II-induced effects on UB cell branching are mediated in part via \( \text{AT}_2\text{R} \). Moreover, these findings indicate that the inhibitory effect of PD123319 on branching of UB cells cultured in three-dimensional gels is a physiologically relevant event since it can be recapitulated in the intact organ culture.

To address the concerns of the specificity of the \( \text{AT}_2\text{R} \) antagonist in UB development, we examined UB branching in \( \text{AT}_2\text{R} \) null mice on \( E13.5 \). The number of UB tips was decreased in metanephiroi from \( \text{AT}_2\text{R}\text{ null} \) compared with \( \text{AT}_2\text{R}\text{ wildtype} \) littermates (36 ± 1.8 vs. 48 ± 1, \( P < 0.01; n = 6/group \) (Fig. 4, F–H). In summary, our results indicate that \( \text{AT}_2\text{R} \) signaling stimulates early UB branching and are consistent with previous reports which implicate \( \text{AT}_2\text{R} \) in renal branching morphogenesis (8).
**AT2R gene is expressed in mesenchyme and UB in vivo.** To determine the spatial expression of the AT2R gene, we mapped localization of AT2R mRNA in the metanephros by in situ hybridization on E11.5–E13.5. On E11.5, AT2R mRNA is expressed predominantly in the ureter and mesenchyme adjacent to the UB tips (Fig. 5A). On E12.5, AT2R mRNA is expressed throughout branching UB and mesenchyme (Fig. 5B). On E13.5, AT2R mRNA is present in the UB, mesenchyme, and maturing glomeruli (Fig. 5C). These data are in concert with our findings demonstrating that cultured UB and MK4 cells express AT2R mRNA (Fig. 3A). Localization of AT2R expression in the kidney mesenchyme is consistent with earlier studies in rodents using in situ hybridization (26). Given that AT2R null mice demonstrate a decreased rate of apoptosis of mesenchymal cells around the UB (35), it is conceivable that an absence of timely apoptosis of mesenchymal cells in AT2R-deficient mice may hinder mesenchyme-UB cross talk to affect ure-
Fig. 4. Effect of media, PD123319, CGP42112, or genetic inactivation of the AT2R on UB branching in mouse metanephroi. A and B: after 48 h in culture, kidney explants were costained with anti-pancytokeratin antibody to label the UB (red) and Lotus tetragonolobus agglutinin (LTA) to label proximal tubule epithelia (green). C and D: UB is visualized with anti-pancytokeratin antibody (green). The number of UB tips was compared between the treatment groups. A–D: representative images of branching UBs in paired metanephroi. A: left kidney, media; B: right kidney, PD123319 (PD). C: left kidney, media. D: right kidney, CGP42112 (10^{-6} M; CGP). E: bar graph showing the effect of media, PD123319, or CGP42112 on the number of UB tips. F–H: effect of genetic inactivation of the AT2R on UB branching on E13.5. Metanephroi were costained with anti-pancytokeratin antibody to label the UB (green) and anti-WT1 antibody to label the mesenchyme and podocytes (red). F: AT2R^{+/−} kidney. G: AT2R^{−/−} kidney. H: bar graph showing the effect of AT2R genotype on the number of UB tips. Numbers on A–D and F and G indicate the number of UB tips.

Figures

This possibility is supported by the findings that activation of AT2R induces Pax-2 gene expression in renal mesenchymal cells (54). Together, it is conceivable that both UB and mesenchymal AT2R stimulate UB branching.

Effect of AT2R antagonist PD123319 or genetic inactivation of AT2R in mice on GDNF, c-Ret, Wnt11, Spry1, and BMP4 gene expression in metanephric kidney. The GDNF/c-Ret/Wnt11 signaling pathway is a major positive regulator of the UB branching morphogenesis program (10, 33, 44). In a previous study, we demonstrated that ANG II upregulates GDNF/c-Ret/Wnt11 gene expression in embryonic metanephric kidneys grown ex vivo (52). In the present study, we examined the effect of endogenous ANG II and its AT2R on GDNF, c-Ret, Wnt11, and Spry1 gene expression during early stages of UB morphogenesis. Whole mount in situ hybridization showed a decrease in Spry1, GDNF, c-Ret, and Wnt11 gene expression in metanephroi treated with PD123319 (10^{-6} M) for 24 h (Fig. 6A). To allow a more quantitative analysis of changes in GDNF, c-Ret, Wnt11, and Spry1 gene expression, we examined the effect of PD123319 on GDNF, c-Ret, Wnt11, and Spry1 mRNA levels in cultured metanephroi by quantitative real-time RT-PCR. Treatment with PD123319 resulted in a significant decrease in GDNF (0.59 ± 0.06 vs. 1.0 ± 0, P < 0.01), c-Ret (0.57 ± 0.03 vs. 1.0 ± 0, P < 0.01), Wnt11 (0.62 ± 0.11 vs. 1.0 ± 0, P < 0.05), and Spry1 (0.52 ± 0.04 vs. 1.0 ± 0, P < 0.01) mRNA levels compared with control (Fig. 6B). In accordance with these findings, GDNF (0.73 ± 0.05 vs. 1.0 ± 0, P < 0.01), c-Ret (0.76 ± 0.05 vs. 1.0 ± 0, P < 0.01), Wnt11 (0.53 ± 0.05 vs. 1.0 ± 0, P < 0.01), and Spry1 (0.74 ± 0.06 vs. 1.0 ± 0, P < 0.01) mRNA levels were lower in E13.5 metanephroi of AT2R^{−/−} compared with AT2R^{+/−} mice (Fig. 6B). Therefore, the stimulatory effects of endogenous ANG II on GDNF/c-Ret/Wnt11 gene expression are mediated, in part, via the AT2R. Since genetic deficiency or pharmacological antagonism of AT2R decreases UB branching, the AT2R-mediated effect on GDNF/c-Ret/Wnt11 is physiologically important. An observed decrease in Spry1 mRNA levels in PD123319-treated and AT2R^{−/−} metanephroi suggests that AT2R-dependent stimulation of GDNF/c-Ret/Wnt11 gene expression cannot be attributed to downregulation of Spry1, an antagonist of c-Ret signaling.

To test the hypothesis that a decrease in UB branching observed in AT2R^{−/−} mice is mediated by BMP4, a known inhibitor of UB branching (35), we examined the effect of genetic inactivation of AT2R in mice on BMP4 gene expression in E13.5 metanephroi by real-time qPCR. BMP4 mRNA levels were higher in AT2R^{−/−} compared with AT2R^{+/−} mice (1.26 ±
0.05 vs. 1.0 ± 0.0, P < 0.01). These findings indicate that the stimulatory effects of endogenous AT2R on UB branching are mediated, in part, via downregulation of BMP4. Alternatively or additionally, since AT2R is expressed in the mesenchyme and GDNF mRNA levels are lower in AT2R−/− metanephroi, the observed decrease in UB branching may be secondary to a direct effect on GDNF.

Effect of AT2R antagonist PD123319 or genetic inactivation of AT2R in mice on cell proliferation in embryonic kidney. To investigate the cellular mechanisms leading to stimulation of UB branching by endogenous ANG II and its AT2R, we examined the effect of AT2R antagonism or genetic inactivation of the AT2R on proliferation of the UB epithelium, utilizing an antibody to pH3. Treatment of intact metanephroi with PD123319 (10−6 M) for 48 h in vitro decreased cell proliferation index in the UB tip (5.5 ± 0.7 vs. 20.2 ± 2.9; P < 0.01) and stalk (5.9 ± 1.9 vs. 15.3 ± 2.8; P < 0.05) cells compared with control (Fig. 7, F–J). The number of proliferating cells was lower in the UB of AT2R−/− than AT2R+/+ mice (49 ± 8 vs. 86 ± 8; P < 0.05) (Fig. 8, F–J). In contrast, the number of pH3-positive cells in the mesenchyme did not differ (374 ± 41 vs. 383 ± 75; P = 0.9) (Fig. 8, F–J). These results demonstrate a direct stimulatory effect of ANG II AT2R on UB cell proliferation.

AT2R antagonism promotes apoptosis of UB cells. Aberrant apoptosis is a cardinal feature of renal dysplasia and hypoplasia (49). In this regard, attenuated apoptosis of mesenchymal cells during fetal metanephrogenesis has been reported in AT2R-deficient mice (35). In this study, we examined the effect of AT2R antagonism or genetic inactivation of the AT2R on cell apoptosis in intact metanephroi. Treatment of E12.5 metanephroi with PD123319 (10−6 M) for 24 h significantly increased the number of TUNEL-positive cells in the UB tips and stalks (tips: 0.76 ± 0.2 vs. 0.19 ± 0.1, P < 0.05; stalks: 0.88 ± 0.2 vs. 2.1 ± 0.4; P < 0.01) (Fig. 7, A–E). Interestingly, apoptotic cells were also detected in the nephrogenic zone and stroma (Fig. 7, A–C). Given that AT2R protein is expressed in stromal cells (Fig. 2, A and C), it is conceivable that stromal AT2R signaling is important in nephrogenesis and UB morphogenesis. The number of caspase 3-positive cells was increased in the UB of AT2R−/− compared with AT2R+/+ mice (7.8 ± 0.9 vs. 1.5 ± 0.5; P < 0.05) (Fig. 8, A–E). In contrast, the number of caspase 3-positive cells in the mesenchyme did not differ (50 ± 13 vs. 63 ± 14; P = 0.5) (Fig. 8, A–E). These findings indicate an inhibitory role of endogenous ANG II and its AT2R on apoptosis of UB cells, suggesting a role for the AT2R in epithelial cell survival during UB branching.
DISCUSSION

The present study demonstrates that ANG II AT₂R is expressed in the UB epithelia, metanephric mesenchyme, and stroma and plays an essential role during early stages of UB morphogenesis. Inhibition of AT₂R signaling impairs UB branching, downregulates GDNF, c-Ret, Wnt11, and Spry1 gene expression, decreases proliferation, and induces apoptosis of the UB cells.

The ANG II AT₂R is a developmentally regulated G protein-coupled receptor that is highly expressed in the fetal kidney (16, 24, 26, 35). AT₂R mRNA is present in undifferentiated mesenchyme at the time of UB outgrowth from the nephric duct and is detected in the UB on E13.5 in the mouse (35). Temporally, AT₂R expression peaks during early fetal metanephrogenesis and rapidly declines postnatally (16). The fetal kidney expresses all the other components of the classic RAS. Angiotensinogen (AGT) and AT₁R are expressed in both the UB and adjacent stromal mesenchyme as early as on E12 in the mouse (25). Renin-expressing cells are present in the stroma on E12 (29). ACE is detected in the embryonic kidney in rodents and humans (45). Collectively, the fetal metanephros has the capacity to both generate ANG II and to transmit its actions. It is intriguing that ANG II levels are higher in the fetal and newborn than adult kidney (31, 50). To this end, high ANG II levels in the embryonic kidney, coupled with upregulated AT₂R expression, suggest an important role for the AT₂R in metanephric development during fetal life.

Studies conducted in humans demonstrate that AT₂R mutations may be linked to congenital abnormalities of the kidney and urinary tract (CAKUT). In this regard, a single polymorphism in the AT₂R, the A1332G transition, is associated with ureteropelvic junction stenosis, vesicoureteral reflux, megau- reter, hypoplastic, and multicystic dysplastic kidneys (20, 35, 41). Deletion of the AT₂R gene in mice causes a duplicated collecting system and hydronephrosis (37). These forms of CAKUT are observed in 3.1% of AT₂R null newborns. Intrigu- ingly, aberrant UB budding is seen in up to 60% of AT₂R-deficient embryos on E11, suggesting that the majority of these malformations undergo self-correction in utero. It is conceivable that initial redundant UB outgrowth in these mice is subsequently suppressed by inhibitors of UB branching such as Spry1, BMP2, or BMP4 (7, 32). Interestingly, AT₂R is expressed between the nephric duct and the mesenchyme just cranial to the normal UB branching site in wild-type embryos on E11 (26). AT₂R null mice demonstrate a decreased rate of apoptosis of mesenchymal cells around the developing UB (35). AT₂R may mark mesenchymal cells destined to undergo apoptosis. Hence, the absence of timely apoptosis of mesenchymal cells in AT₂R null mice may create a physical barrier which separates UB from the mesenchyme and hinders recip-
rocal interactions of genes required for proper ureteric branching.

One mechanism by which aberrant AT2R function may disrupt UB-mesenchyme cross talk is inhibition of the interactions between GDNF secreted by metanephric mesenchymal cells and its tyrosine kinase receptor c-Ret expressed in the nephric duct and subsequently in the UB tip cells (38). The GDNF/c-Ret/Wnt11 pathway is a major positive regulator of UB branching in the metanephros (3). Like c-Ret, Wnt11 is expressed in the UB tip cells and interacts genetically with GDNF/c-Ret to induce UB branching (3). Recent work from our laboratory demonstrated that ANG II-induced UB branching is accompanied by activation of the GDNF/c-Ret/Wnt11 pathway (52). Our present findings that AT2R deficiency or antagonism downregulates GDNF, c-Ret, and Wnt11 expression indicate that activation of this pathway by AT2R is critical in ANG II-induced signaling to stimulate UB morphogenesis. Recent work from our laboratory demonstrated that ANG II-induced UB branching is accompanied by activation of the GDNF/c-Ret/Wnt11 pathway (52). Our present findings that AT2R deficiency or antagonism downregulates GDNF, c-Ret, and Wnt11 expression indicate that activation of this pathway by AT2R is critical in ANG II-induced signaling to stimulate UB morphogenesis.

Interestingly, ANG II, acting via AT2R, induces the expression of Pax-2, a transcription factor present in both the UB and metanephric mesenchyme (12). Genetic inactivation of Pax-2, GDNF, c-Ret, or Wnt11 in mice causes significant impairment of UB morphogenesis (12, 33, 44). Given that GDNF or c-Ret expression is activated by Pax-2 (9, 33), it is conceivable that a decrease in GDNF/c-Ret/Wnt11 gene expression observed in the present study is due to inhibition of Pax-2-stimulating effects on GDNF and c-Ret. Thus AT2R signaling controls a hierarchy of gene expression critical for UB morphogenesis.

Colocalization of AGT, renin, AT2R, and wing helix transcription factor Foxd1 expression in the stroma (21, 24, 25, 27) suggests potential interactions. In this regard, genetic inactivation of the Foxd1 gene in mice reduces UB branching and alters c-Ret expression (21, 27). Furthermore, AGT and renin promoters contain a putative binding site for Foxd1 (53). Whether Foxd1 acts upstream of the RAS to regulate UB branching remains to be determined.

An important finding of the present study is that AT2R protein is expressed on both luminal and basolateral aspects of the UB branches and in adjacent stromal mesenchyme. Since AGT and renin are expressed in the stroma, it is conceivable that ANG II can be generated in the stroma and act in a paracrine fashion on the adjacent AT2R-expressing UBs to stimulate branching. This model is supported by our present findings that AT2R antagonism abrogates ANG II-induced branching in UB cells grown in the absence of the mesenchyme. In addition, AT2R present in the stroma may be important in mediating stromal ANG II signaling to stimulate UB branching.

Fig. 7. Effect of AT2R antagonist PD123319 on UB cell apoptosis and proliferation. A–D: apoptotic cells were identified by terminal uridine triphosphate (UTP) nick-end labeling (TUNEL; brown staining). A: media. B and C: PD123319 (10^-6 M). D: kidney treated with TACS-nuclease to generate DNA breaks in every cell (positive control). E: bar graph shows the effect of media or PD123319 on the number of TUNEL-positive cells in the UB tip and stalk cells. F–I: proliferating cells are identified by anti-phospho-histone H3 (pH3) antibody (red staining). UB epithelia are visualized with anti-cytokeratin antibody (green). J and I: media. H and I: PD123319 (10^-6 M). PD123319-treated metanephroi have less pH3 staining in UB (arrows) compared with control (media; marked by rectangles in G and I). J: bar graph shows the effect of media or PD123319 on cell proliferation index in the UB tip and stalk cells.
The balance of cell proliferation and apoptosis plays a critical role in UB branching and nephron endowment (13, 30, 39). The present study demonstrates that inhibition of endogenous AT2R signaling induces apoptosis and inhibits proliferation in UB tip and stalk cells. We speculate that AT2R plays an important role in the expansion of the ampulla, subsequent branching, and directional bud elongation. Given that BMP2 and BMP4 inhibit UB branching (32, 40), decreased UB branching in AT2R-null mice may be mediated, in part, by enhanced BMP2/BMP4 signaling. Our present findings of increased BMP4 gene expression in AT2R-null metanephroi suggest that the stimulatory effects of endogenous AT2R on UB branching are mediated, in part, via downregulation of BMP4. Notably, AT2R-deficient mice have smaller mean glomerular tuft volume compared with wild-type mice (8). Whether AT2R deficiency results in decreased nephron endowment remains to be determined. AT2R-dependent apoptosis may result from activation of MAP kinase phosphatase 1 or Src homology 2 domain-containing protein-tyrosine phosphatase 1, leading to inactivation of ERK1/2 (11, 22). AT2R-dependent activation of Pax-2 in whole metanephroi and mesenchymal cells is mediated by the JAK2/STAT pathway (54).

In summary, the present study demonstrates that ANG II AT2R is expressed in the UB and mesenchyme during nephric development. Aberrant AT2R signaling downregulates GDNF, c-Ret, and Wnt11 gene expression, decreases proliferation and induces apoptosis of the UB cells, and impairs UB branching. These results support the hypothesis that abnormal collecting system development in AT2R-deficient mice is at least partly due to dysregulation of the UB branching morphogenesis program as well as aberrant UB cell proliferation and apoptosis.

ACKNOWLEDGMENTS

We thank Mercedes Schroeder (Tulane University) for assistance with the in vitro tubulogenesis assay, Drs. Frank Costantini (Columbia University Medical Center), Andrew P. McMahon (Harvard University), and Jonathan D. Licht (Northwestern University) for providing the probes for in situ hybridization, and Dr. Alvin H. Schmaier (Case Western Reserve University) for providing AT2R mutant mice originally generated by Dr. Tadashi Inagami (Vanderbilt University, Nashville, TN).

GRANTS

This work was supported by National Institutes of Health (NIH) Grants P20 RR17659 and DK-71699 (I. Yosypiv). S. El-Dahr is supported by NIH Grants DK-56264 and DK-62250.

DISCLOSURES

No conflicts of interest are declared by the authors.
REFERENCES


