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Angiotensin II inhibits growth of cultured embryonic renomedullary interstitial cells through the AT_2 receptor

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Angiotensin II inhibits growth of cultured embryonic renomedullary interstitial cells through the AT_2 receptor. The high abundance of angiotensin II (Ang II) AT_2, relative to the AT_1 receptor subtype in developing kidneys may be related to their potential as mediators of cell growth, although little evidence exists to support this concept. Renomedullary interstitial cells (RMICs) differentiate early in embryonic kidneys and are important in subsequent nephron development. These cells have been shown *in vivo* to possess AT_2 binding sites, although the functional significance of these sites remains unknown. The aim of the current investigation was to examine the actions of Ang II on cultured embryonic renomedullary interstitial cells (ERMICs). ^{125}I-[Sar^1, Ile^8]Ang II specifically bound to AT_1 and AT_2 receptors on ERMICs, and their mRNAs were detected by reverse transcription—polymerase chain reaction (RT-PCR). Angiotensin II (10^{-6} M) increased intracellular IP_3 concentrations at 20 seconds, and decreased intracellular cAMP concentrations after 10 minutes. Angiotensin II (10^{-6} M) induced an increase in [^{3}H]thymidine incorporation, mediated through the AT_1 receptor subtype. Basic fibroblast growth factor (bFGF; 20 ng/ml) also increased ^{3}[H]thymidine incorporation after 24 hours of treatment, an effect that was attenuated by subsequent addition of Ang II (10^{-6} M). This antiproliferative action of Ang II was blocked by PD 123319 (10^{-6} M), an AT_2 receptor antagonist, and was not affected by losartan (10^{-6} M), an AT_1 receptor antagonist. These results indicate a dual role for Ang II in regulating ERMIC mitogenesis: a growth stimulating effect mediated by the AT_1 receptor subtype, and an antiproliferative effect mediated by the AT_2 receptor subtype.

Angiotensin II (Ang II) has well demonstrated roles in the regulation of cardiovascular hemodynamics, secretion of mineralocorticoids and water and electrolyte balance [1, 2]. More recently, Ang II has been reported to have growth promoting properties in a number of cell types [3, 4]. To date, two subtypes of the Ang II receptor have been identified, AT_1 and AT_2. However, most of the known functions of Ang II appear to be mediated through the AT_1 receptor subtype. The high abundance of AT_2 receptors in fetal tissues has lead to the hypothesis that Ang II, acting via the AT_2 receptor, is involved in aspects of organogenesis. *In vitro* studies have suggested the involvement of AT_2 receptors in mediating programmed cell death (apoptosis) [5] and cell differentiation [6, 7].

In a previous study, we reported Ang II promotion of cell proliferation and extracellular matrix (ECM) synthesis in cultured rat RMICs through the AT_{1A} receptor subtype [8]. These findings suggested that the interactions between Ang II and these cells may be important in the progression of renal diseases as well as in normal renal development. Studies on the development of the renal interstitium have suggested that renomedullary interstitial cells (RMICs) differentiate from the nephrogenic mesenchyme early in embryogenesis, and subsequently play an important role in nephron development [9]. At this stage these cells possess both AT_1 and AT_2 receptors. In the current study, embryonic renomedullary interstitial cells (ERMICs) were isolated from embryonic rat kidneys to examine the actions of Ang II on cell proliferation related to their potential role in renal development.

Key words: embryonic renomedullary interstitial cells, angiotensin II, AT_2 receptor, interstitial cells, nephron development.

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METHODS

Cell isolation and culture

Embryos were obtained from time plug mated Sprague-Dawley rats at 15 days of gestation. The age of the embryos was determined from the vaginal plug, the appearance of which was designated as embryonic day 0. The kidneys were dissected and digested with 0.1% collagenase type I (Worthington Biochemical Co., Freehold, NJ, USA) for 30 minutes at 37°C. Digested tissue was sieved through a 75 μm stainless steel mesh and dispersed cells resuspended in a 1:1 mixture of culture medium RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM), conditioned by 3T3 Swiss albino mouse fibroblasts in the log phase of growth. Both culture media were supplemented with 20% fetal calf serum (FCS), 4 mM glutamine, 50,000 U/liter penicillin G and 50 mg/liter streptomycin sulfate. In addition, culture medium RPMI 1640 also contained 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) and 0.22 U/ml insulin (Sigma Chemical Co., St. Louis, MO, USA). Cultures were maintained at 37°C in a 95% O_2/5% CO_2 humidified incubator. Once a homogeneous cell population was obtained, the cells were maintained in media supplemented with 10% FCS only. Sub-confluent cells between passages 3 to 10 were used in all subsequent experiments.

All culture media and supplements, unless otherwise stated, were obtained from CSL Diagnostics (Parkville, VIC, Australia).

All experimental procedures were approved by the Institutional Animal Ethics Committee according to guidelines produced by

the National Health and Medical Research Council of Australia and adopted by State Legislature.

Cell morphology

Sub-confluent cells were fixed in 10% buffered formalin for one hour at room temperature and stained with Oil Red O for demonstration of intracellular neutral lipids. For transmission electron microscopy, cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, 100 mM Na_2HPO_4, 150 mM NaCl, pH 7.35) for one hour at room temperature, postfixed in 2% OsO_4 buffered in 0.1 M PB for one hour at room temperature, dehydrated in graded ethanol solutions and embedded in Epon. Thin (90 to 100 nm) sections were cut, stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop 102 at 60 kV. For scanning electron microscopy, cells were fixed in the same fixative, dehydrated in graded ethanediol solutions and cellosolve, critical point dried, sputter coated with gold and viewed in a Phillips 515 scanning electron microscope at 20 kV. For immunocytochemical localization of α-actin, cells were fixed in Bouin's fluid for one hour at room temperature, washed with phosphate buffered saline (PBS) and incubated with 1 mM proteinase K (Sigma Chemical Co.) for 30 minutes at 37°C. Cells were incubated with 1:10 dilution normal goat serum for 30 minutes at room temperature, and then with a primary antibody (monoclonal, mouse-anti-goat α-actin; Sigma) at a dilution 1:600 in PBS overnight at 4°C. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol, following which the sections were washed and secondary goat-anti-rabbit, biotinylated antiserum applied for one hour at room temperature. The specific immunoreaction was amplified using the avidin-biotin complex (Vectastain ABC kit, Elite, Vector Laboratories, Burlingame, CA, USA) at dilution 1:50 for one hour at room temperature, and the positive immunoreactive staining was identified using 3,-3′-diaminobenzidine tetrahydrochloride dihydrate (DAB; Aldrich Chemical Co. Inc., Milwaukee, WI, USA) at a dilution 1:50 in PBS with 3% H_2O_2. Negative controls were incubated with 1:10 normal goat serum in PBS instead of the primary antiserum. Positive controls in sections of rat aorta were incubated with the primary antiserum.

Reverse transcribed—polymerase chain reaction and Southern blot analysis

RNA was isolated from homogenized embryonic day 15 (E15) rat kidneys and from cultured rat ERMICs. Isolation of RNA was carried out using a method described by Chomczynski and Sacchi [10]. Reverse transcription (RT) reaction of 0.5 μg RNA was performed in 100 mM KCl, 50 mM Tris-HCl (pH 8.4), 6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 500 μM dNTPs (Progen, Darra, QLD, Australia), 12 μg/ml random hexamers (Boehringer Mannheim, Castle Hill, NSW, Australia), 40 units RNasin (Progen, Darra, QLD, Australia), and AMV reverse transcriptase (25 units; Boehringer Mannheim, Castle Hill, NSW, Australia) at 42°C for one hour. The RT reaction was followed by a polymerase chain reaction (PCR). The primers used for the amplification of AT_1 receptor mRNA were: sense 5′ TTGGAAACAGCTTGGTGGTGAT 3′; antisense 5′ CCAGGAAAAGAAGAAGAAAAGCAC 3′ corresponding to regions 131 to 152 and 736 to 759 of the rat AT_{1A} receptor, respectively [11], and are identical in the rat AT_{1B} sequence [12]. The primers used for amplification of AT_{1B} receptor mRNA were: antisense 5′ AGCCTTCTTTAGAGCTTTCCAAATAAG 3′; corresponding to regions 649 to 675 of the rat AT_{1A} receptor, [11], and has a single mismatch with the AT_{1B} receptor mRNA. The sense primer was: 5′ TCCAGCGCCACGCTGT 3′, corresponding to region 20 to 35 of the rat AT_{1B} sequence [12]. The primers used for amplification of the AT_2 receptor mRNA were: sense 5′ GCTGATTTATGATAACTGCTTTAAAC 3′; antisense 5′ AGGTCCAAAGAGCCAGTCATATCTATAAGA 3′; corresponding to regions 74 to 49 and 313 to 342 of the rat AT_2 receptor, respectively [13]. Polymerase chain reaction was performed in a solution containing: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 400 μM dNTPs, *Taq* Polymerase (2.5 units; Bresatec, Adelaide, SA, Australia), 3 μM $MgCl_2$, and each primer at 400 nM. Denaturation, annealing and extension were carried out at 94°C, 60°C and 72°C for one minute each for 30 to 40 cycles, followed by a final extension at 72°C for 10 minutes. The PCR products were analyzed by electrophoresis on a 1.4% agarose gel, then transferred to Hybond N^+ (Amersham, Buckinghamshire, UK) using alkali blotting. The membrane was hybridized with a ^{32}P-labeled oligonucleotide probe in Rapid-Hyb (Amersham, Buckinghamshire, UK) at 42°C for one hour, washed four times in 0.3 M NaCl/0.03 M sodium citrate/0.1% sodium dodecyl sulphate (SDS) pH 7.0 at 50°C, exposed and autoradiographed for 10 minutes. The oligonucleotide probe used for the Southern blot of the AT_{1A} and the AT_{1B} receptor PCR products was to the region 284 to 314 of the rat AT_{1A} receptor [11]. This probe has one mismatch (31nt) between the two subtypes. The oligonucleotide probe used for the Southern blot of the AT_2 receptor PCR product was to the region 72 to 111 of the rat AT_2 receptor mRNA [13].

Receptor binding assay

Sub-confluent cells were made quiescent with serum-free RPMI 1640 medium for 24 hours. Cells were washed twice with PB and harvested into PB containing 10 μm leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM EDTA. Following centrifugation at 3000 rpm for 10 minutes at 4°C, cells were resuspended in PB containing 0.1% bovine serum albumin (BSA), 0.1% bacitracin and 5 mM DTT. Following incubation of cells with 10^{-6} M Ang II amide for non-specific binding (NSB), PB for total binding (TB), 10^{-6} M losartan for AT_2 subtype or 10^{-6} M PD 123319 for AT_1 subtype binding, ^{125}I-[Sar^1, Ile^8]Ang II was added at a dose of 3×10^5 cpm/sample for two hours at rt. The cells were then thoroughly washed and bound radioactivity measured in a Packard gamma counter.

Inositol 1,4,5-triphosphate radioimmunoassay

Sub-confluent RMICs were scraped, resuspended in HEPES buffer (140 mM NaCl, 4.6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.3) and pelleted by centrifugation. This procedure was repeated three times. After the final pelleting, cells were resuspended in HEPES buffer and the protein content of the samples was determined using the Lowry assay [14]. Cells were allowed to equilibrate at 37°C for three minutes, and either vehicle (HEPES buffer) or Ang II (10^{-10} and 10^{-6} M) in the presence or absence of the AT_1 receptor antagonist losartan (10^{-6} M) or the AT_2 receptor antagonist PD 123319 (10^{-6} M), were added for 10 or 15 seconds. The cells were then treated with ice-cold 10% perchloric acid (PCA) for 10 minutes and samples neutralized with a 1:1 mixture of tri-n-octyl amine

and 1,1,2-trichlorotriluoroethane as previously described [15]. Following centrifugation at 10,000 rpm for two minutes, the supernatants were assayed for inositol 1,4,5-triphosphate (IP_3) content using a commercially available kit (Amersham, Buckinghamshire, UK).

cAMP radioimmunoassay

Embryonic renomedullary interstitial cells were grown to subconfluency and prepared for the assay in the manner described for IP_3 measurements. Cell samples were allowed to equilibrate for three minutes at 37°C in the presence or absence of 10^{-3} M 3-isobutyl-1-methylxanthine (IBMX), a non-selective phosphodiesterase inhibitor. Then either vehicle (HEPES buffer) or Ang II (10^{-11} and 10^{-6} M) in the presence or absence of the AT_1 receptor antagonist losartan (10^{-6} M) or the AT_2 receptor antagonist PD 123319 (10^{-6} M) were added. After 1, 2 or 10 minutes, the reaction was stopped by the addition of 10% PCA for 10 minutes at 4°C. Supernatants, obtained after centrifugation at 10,000 rpm for two minutes, were neutralized as described for the IP_3 assay. Neutralized samples were freeze-dried, resuspended in assay buffer (50 mM sodium acetate, 1 mM theophylline, 1 mg/ml BSA, 0.1% sodium azide, pH 5.0) and acetylated with a 1:2 mixture of acetic anhydride and triethylamine. Antibodies to cAMP (1:3600) and ^{125}I-[cAMP] were added to the acetylated samples and left at 4°C for 48 hours to allow binding. Samples were then treated with 5% IgG and 15% polyethylene glycol, incubated for 30 minutes at 4°C, centrifuged at 4000 rpm for 30 minutes at 4°C and the radioactivity of the pellets counted in a Packard gamma counter.

^{3}[H]thymidine incorporation

The ERMICs were plated at a density of 7×10^3 cells/cm^2 in 1:1 RPMI 1640:conditioned DMEM culture medium containing all supplements as indicated above. After 24 hours of plating, the cells were serum-starved for 48 hours. To examine the effect of Ang II on ERMIC proliferation, the cells were treated with serum-free medium (control), or Ang II (10^{-11}-10^{-6} M) with or without 10^{-6} M losartan or 10^{-6} M PD 123319 for 5 to 53 hours. To examine the possible interactions of Ang II and bFGF with ERMIC proliferation, the cells were pre-treated with 20 ng/ml bFGF for 24 hours followed by the addition of 10^{-6} M Ang II with or without 10^{-6} M losartan or 10^{-6} M PD 123319 for additional 24 hours. Throughout the treatment period, the cells examined for evidence of apoptotic figures by phase contrast microscopy. Following treatment, a pulse dose of 1 μCi/ml [^{3}H]thymidine (Amersham) was applied for five hours at 37°C, after which the cells were washed with ice-cold PBS and 5% trichloroacetic acid. The samples were then solubilized with 0.25 M NaOH and 0.1% SDS for 30 minutes at 37°C, and incorporated radioactivity was measured in a Packard liquid scintillation counter.

Statistical analysis

Results are expressed as mean ± SD (unless otherwise stated) and analyzed using two-way analysis of variance and the multiple comparison Tukey test. Statistical significance was assigned to values where $P < 0.05$.

RESULTS

Cell morphology

A homogeneous cell population was reached at passage 3. At this stage, these cells resembled RMICs found *in vivo* [9] as well as cultured RMICs isolated from adult kidneys [8]. The cells were stellate-shaped and projected numerous cytoplasmic processes contacting neighboring ERMICs (Fig. 1B). They showed characteristics of protein synthesizing cells, containing numerous rough endoplasmic reticulum (RER) inclusions with dilated cisternae, a moderate number of mitochondria and condensation of chromatin in the nucleus (Fig. 1A). Characteristic lipid droplets, demonstrated both by light and transmission electron microscopy, were abundant (Fig. 1C). The presence of α-actin filaments was demonstrated by immunocytochemistry (Fig. 1D). Abundant lipid droplets and α-actin filaments are characteristic of both ERMICs *in vivo* [9] and RMICs derived from adult kidneys [8].

Reverse transcription-polymerase chain reaction and Southern blot analysis

A PCR product corresponding to the AT_1 receptor was localized to the homogenized E15 kidney preparation as well as to cultured ERMICs (Fig. 2) [15]. We have previously demonstrated that adult RMICs express only the AT_{1A} subtype whereas whole kidney expresses both AT_{1A} and AT_{1B} receptor mRNA [8]. We examined whether ERMICs express AT_{1B} receptor mRNA using RT-PCR, but could not detect its expression even after 40 cycles and Southern blotting, whereas it was clearly detectable in E15 kidneys. In addition, a band corresponding to the AT_2 receptor subtype was localized to E15 kidneys as well as to cultured ERMICs.

Receptor binding assay

Following a one hour incubation, ERMICs specifically bound ^{125}I-[Sar^1, Ile^8]Ang II (NSB, $0.20 \pm 0.04 \times 10^7$ cpm/mg protein; TB, $1.2 \pm 0.2 \times 10^7$ cpm/mg protein; $N = 5$; $P < 0.001$; Fig. 3). Binding was partially displaced by 10^{-6} M losartan ($0.60 \pm 0.02 \times 10^7$ cpm/mg protein; $N = 2$; $P < 0.01$) and by 10^{-6} M PD 123319 ($0.90 \pm 0.01 \times 10^7$ cpm/mg protein; $N = 2$; $P < 0.01$), indicating the presence of both the AT_1 and AT_2 receptor subtypes.

Effect of angiotensin II on intracellular IP_3

Treatment of ERMICs with Ang II (10^{-6} M) increased intracellular IP_3 concentrations. A marked increase was detected 20 seconds after treatment (control, 1.7 ± 0.7 pmol/mg protein; Ang II, 68.0 ± 2.5 pmol/mg protein; $N = 4$; $P < 0.01$; Fig. 4). This effect was completely abolished in the presence of 10^{-6} M losartan (1.4 ± 0.4 pmol/mg protein; $N = 3$), but not affected in the presence of 10^{-6} M PD 123319 (57.0 ± 2.8 pmol/mg protein; $N = 3$; $P < 0.01$). Angiotensin II (10^{-10} M) had no effect on IP_3 at any time point chosen.

Effect of angiotensin II on intracellular cAMP

No changes in intracellular cAMP concentrations were detected following treatment of ERMICs with Ang II (10^{-6} and 10^{-10} M) for one or two minutes (Fig. 5A), either in the presence or absence of IBMX (10^{-3} M). However, in the presence of IBMX, 10 minutes following treatment with Ang II (10^{-6} M), a decrease in intracellular cAMP concentration was observed (control, 2731 ± 255 fmol/mg protein; Ang II, 1536 ± 194 fmol/mg

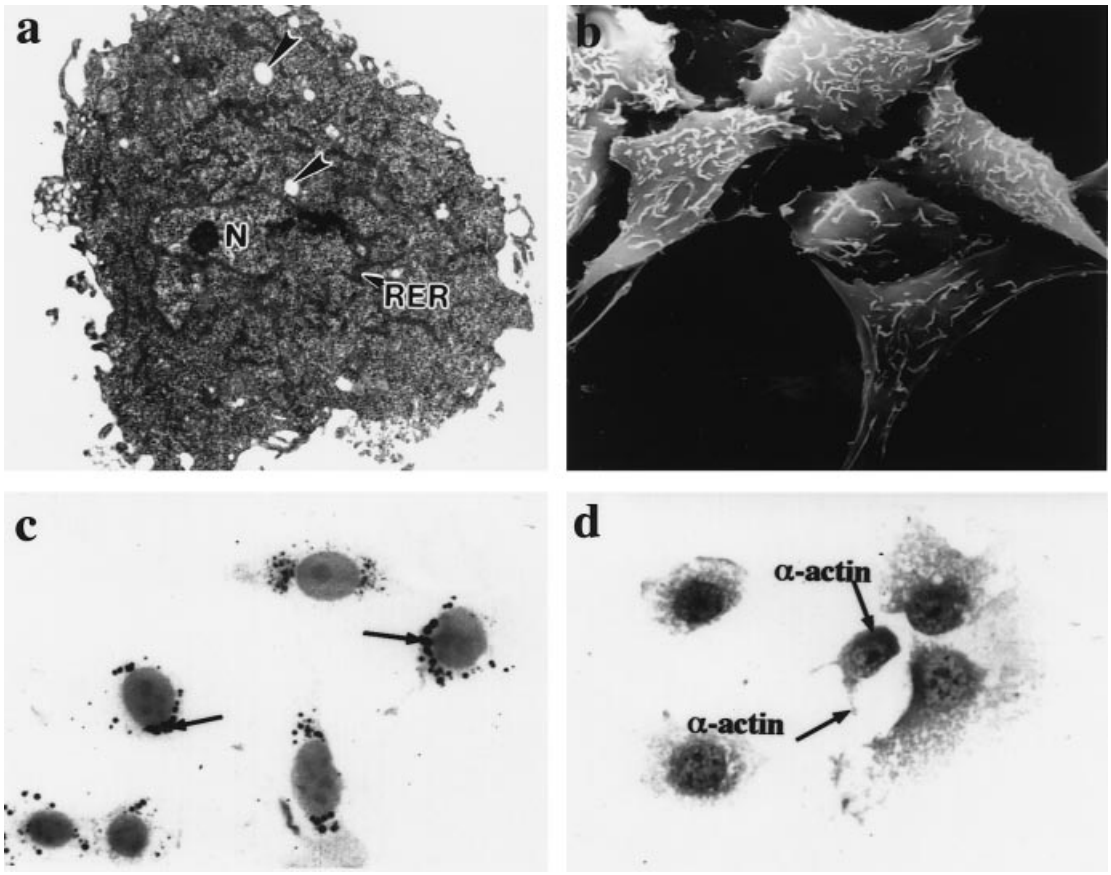

Fig. 1. (*A*) A transmission electron micrograph showing an irregularly shaped embryonic renomedullary interstitial cell (ERMIC) with several cytoplasmic processes, a nucleus (N), abundant rough endoplasmic reticulum (RER) and lipid inclusions (arrowheads) (×3600). (*B*) A scanning electron micrograph of ERMICs demonstrating their irregular shape and the presence of numerous cytoplasmic processes (×3000). (*C*) A wholemount of ERMICs stained with Oil Red O showing numerous lipid droplets (arrows) (×1200). (*D*) α-Actin filaments (α-actin), stained using immunocytochemistry, form dense condensations at peripheral cell contact points (×800).

protein; $N = 4$; $P < 0.05$), (Fig. 5). This effect was completely abolished with 10^{-6} M losartan (1311 ± 119 fmol/mg protein; $N = 3$) but not affected with 10^{-6} M PD 123319 (2289 ± 191 fmol/mg protein; $N = 3$; $P < 0.05$; Fig. 5B).

Effect of angiotensin II on [^{3}H]thymidine incorporation

Treatment of ERMICs with Ang II (10^{-10} to 10^{-6} M) for 5 to 53 hours resulted in time- and dose-dependent increases in [^{3}H]thymidine incorporation. Following treatment for 29 hours with increasing doses of Ang II, significant increases in [^{3}H]thymidine incorporation were observed. The lowest dose of Ang II effective in increasing [^{3}H]thymidine incorporation after 29 hours was 10^{-9} M (control, 1735 ± 110 cpm/well; Ang II, 2112 ± 182 cpm/well; $N = 3$; $P < 0.05$; Fig. 6A). Increases in [^{3}H]thymidine incorporation following treatment with 10^{-6} M were first observed after 11 hours, and increased further after a 29 hours treatment (control, 1735 ± 110 cpm/well; Ang II, 4175 ± 169 cpm/well; $N = 8$; $P < 0.01$; Fig. 6B). The response to Ang II 10^{-6} M was completely abolished in the presence of losartan 10^{-6} M (1284 ± 62 cpm/well), but not affected in the presence of PD 123319 10^{-6} M (3724 ± 79 cpm/well).

Basic fibroblast growth factor (bFGF; 20 ng/ml) also increased [^{3}H]thymidine incorporation after 29 hours of treatment (8799 ±

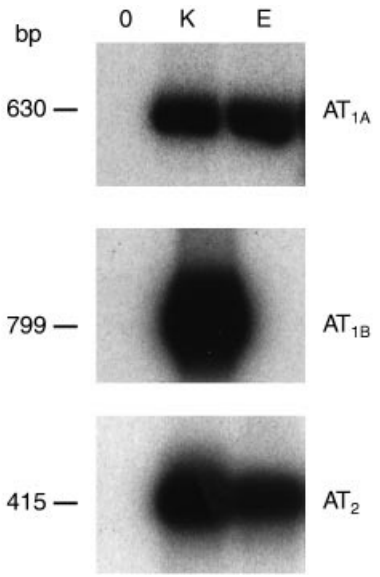

Fig. 2. RT-PCR for AT_{1A} (top panel), AT_{1B} (middle panel) and AT_2 (bottom panel) was performed on RNA from ERMICs (E) and E15 kidney homogenate (K). No DNA was added for negative controls (O). Molecular size markers are given on the left.

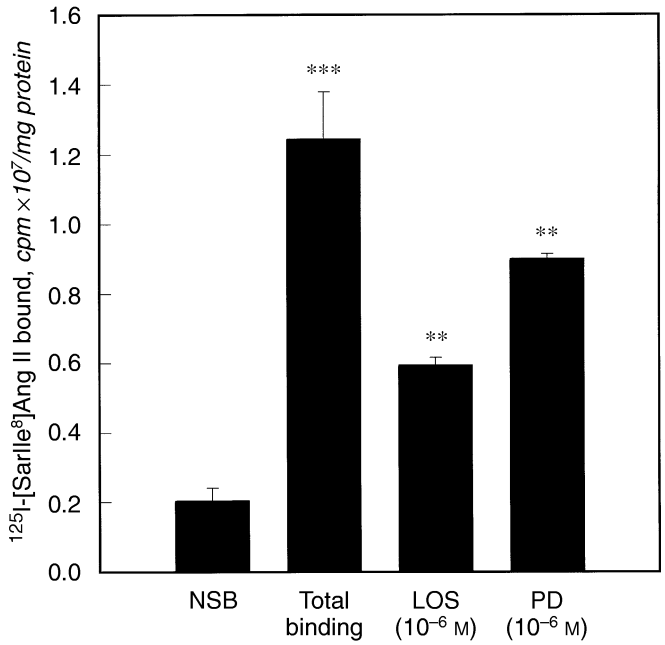

Fig. 3. Binding of angiotensin II (Ang II) to embryonic renomedullary interstitial cells (ERMICs): ^{125}I-[Sar^1, Ile^8] Ang II bound to ERMICs, with low levels of non-specific binding (NSB) also detected. Total binding (TB) of ^{125}I-[Sar^1, Ile^8] Ang II to ERMICs was partially abolished with losartan (10^{-6} M), the AT_1 specific receptor antagonist, and partially with PD 123319 (10^{-6} M), the AT_2 specific receptor antagonist. Values are means ± SD of 2 experiments in duplicate. $^{***}P < 0.001$ vs. NSB.

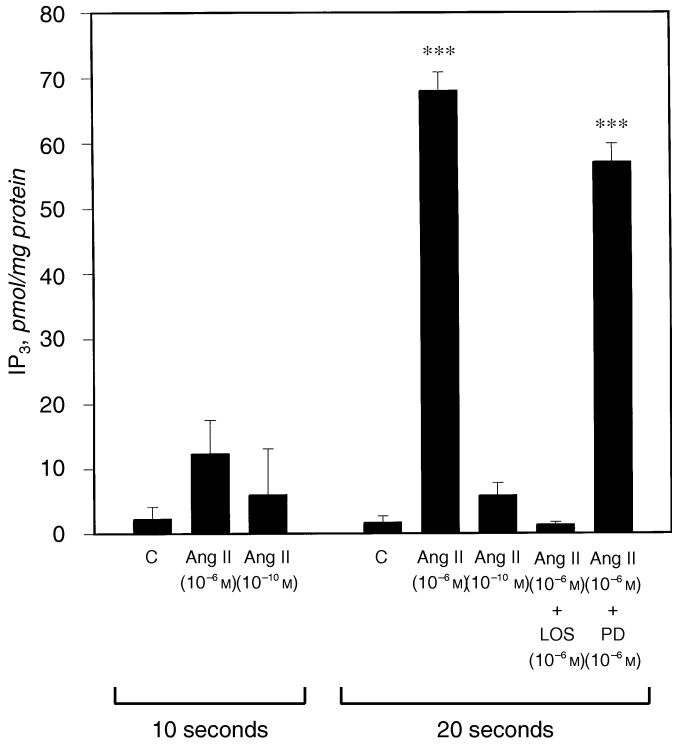

Fig. 4. Effect of angiotensin II (Ang II) on IP_3. No changes in IP_3 concentration were observed 10 seconds following treatment with Ang II. Following treatment of embryonic renomedullary interstitial cells (ERMICs) with Ang II (10^{-6} M) for 20 minutes, IP_3 concentrations were increased. This effect was abolished with losartan (10^{-6} M), but not with PD 123319 (10^{-6} M). Values are means ± SD of 4 experiments in duplicate. $^{***}P < 0.001$ vs. control at the same time point.

966; $N = 5$; $P < 0.001$; Fig. 7). However, in the presence of bFGF, the addition of Ang II (10^{-6} M) during a further 24 hours attenuated the bFGF-induced [^{3}H]thymidine incorporation (3261 ± 284 cpm/well). The ability of Ang II to attenuate cell proliferation induced by bFGF was not altered by losartan (10^{-6} M), but was abolished by PD 123319 (10^{-6} M), indicating that this antiproliferative action of Ang II is mediated through the AT_2 receptor subtype.

During the treatment periods, morphological examination of cells by phase contrast microscopy showed no evidence of apoptosis (nuclear fragmentation or condensation; graphic not shown).

DISCUSSION

The high abundance of AT_2 binding sites in embryonic tissues, including kidney, has suggested their potential importance in the control of cell growth. Although recent studies have reported a role for these receptors in mediating apoptosis [5] and cell differentiation [6, 7], the cells displaying these properties have been derived from either adult or tumor tissues. Thus, the postulated role for the AT_2 receptor in development still remains unclear.

To date, the precise localization of the AT_2 receptors in the developing kidney has not been reported although high levels of mRNA for these receptors have been detected in the interstitium surrounding the collecting ducts [16]. Renomedullary interstitial cells are the predominant cell type in this region and are believed to have an important role in kidney morphogenesis [9]. Thus, characterization of the cellular responses of embryonically derived RMICs to Ang II was undertaken in the present study.

The radiolabeled binding assay and RT-PCR revealed the presence of both AT_1 and AT_2 receptor subtypes and their mRNAs in ERMICs. However, binding of ^{125}I-[Sar^1, Ile^8] Ang II to AT_2 receptors was only observed in the presence of DTT, which has been previously reported to non-selectively increase AT_2 and decrease AT_1 receptor binding [17]. The likely explanation for these effects may be that the cells possess low numbers of AT_2 binding sites.

Increases in intracellular IP_3 concentrations were measured 20 seconds following binding of Ang II to ERMICs. This response had a slower onset than that observed in response to Ang II treatment of RMICs derived from adult kidneys [8], but the reason for this is unclear. Previous studies have indicated that increases in IP_3 and calcium concentration occur following binding of Ang II to AT_1 receptors in a number of cell types [18] and it is likely that a similar pathway is present in ERMICs. The most recent evidence on the signal transduction pathways associated with AT_2 receptor activation indicates an association with Gαi2 and Gαi3 proteins in fetal tissues [19] and inhibition of mitogen-activated protein kinase activity via Gαi and protein phosphatase 2A [20].

We have demonstrated that Ang II acts as a potent mitogen to ERMICs in a time- and dose-dependent manner. This effect was abolished with losartan but not with PD 123319, suggesting that

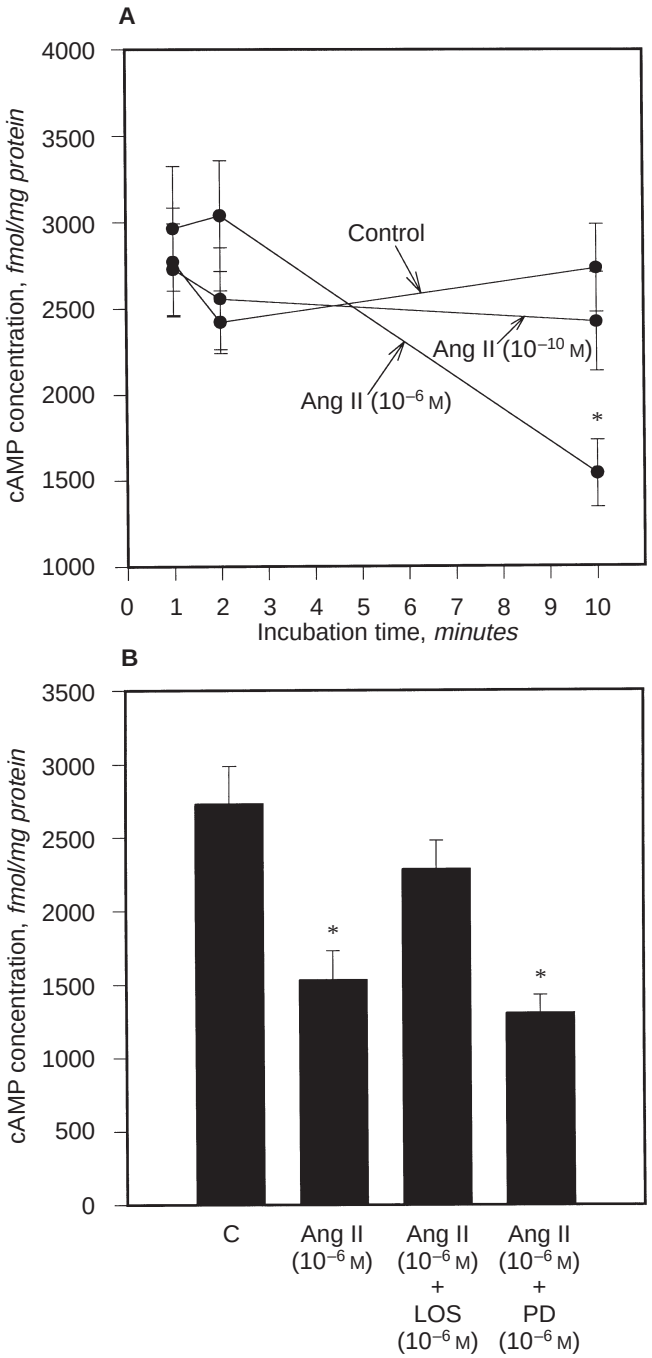

Fig. 5. Effect of angiotensin II (Ang II) on cAMP. (*A*) A decrease in intracellular cAMP concentration was observed 10 minutes following treatment with Ang II (10^{-6} M). Ang II (10^{-10} M) had no effect on cAMP. $^{*}P < 0.05$ vs. control at the same time point. (*B*) Ang II-induced decrease in cAMP concentration was abolished with 10^{-6} M losartan (LOS), but not with 10^{-6} M PD 123319 (PD). Values are means ± SD of 3 experiments in duplicate. $^{*}P < 0.05$ vs. control.

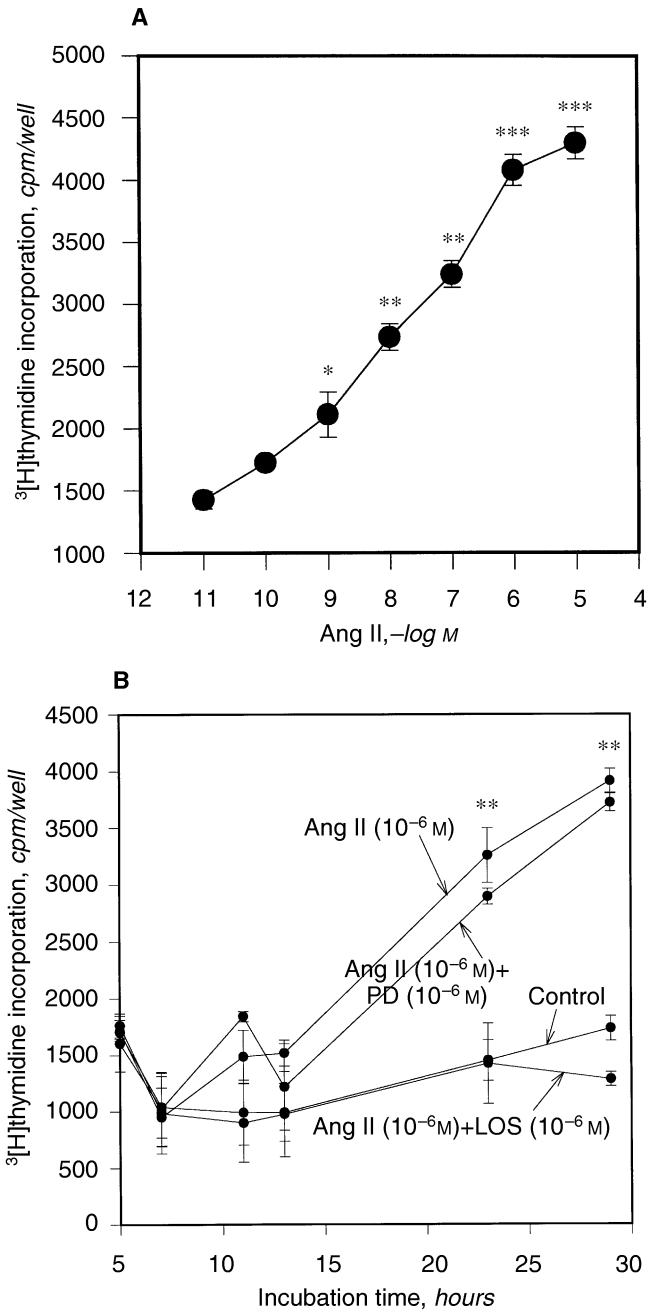

Fig. 6. Effect of angiotensin II (Ang II) on [^{3}H]thymidine incorporation. (*A*) Ang II-induced increase in [^{3}H]thymidine incorporation was dose dependent. The lowest effective dose was 10^{-9} M after 29 hours of treatment. (*B*) Treatment of embryonic renomedullary interstitial cells (ERMICs) with Ang II (10^{-6} M) resulted in an increase in [^{3}H]thymidine incorporation observed after nine hours and increased after 29 hours. This effect was abolished in the presence of losartan (10^{-6} M), but not altered in the presence of PD 123319 (10^{-6} M). Values are means ± SD of 8 experiments in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control at same time point.

the mitogenic effect of Ang II is mediated through the AT_1 receptor. Although the maximal doses inducing cell proliferation and also affecting the second messenger pathways were relatively high (10^{-6} M), studies measuring the interstitial concentrations of Ang II in the kidney have shown greater concentrations of Ang II in the interstitial compartments than in plasma [21]. Thus, the results obtained in the current study following treatment with 10^{-6} M Ang II may reflect the *in vivo* responses of ERMICs to Ang II. The extent of [^{3}H]thymidine incorporation induced by

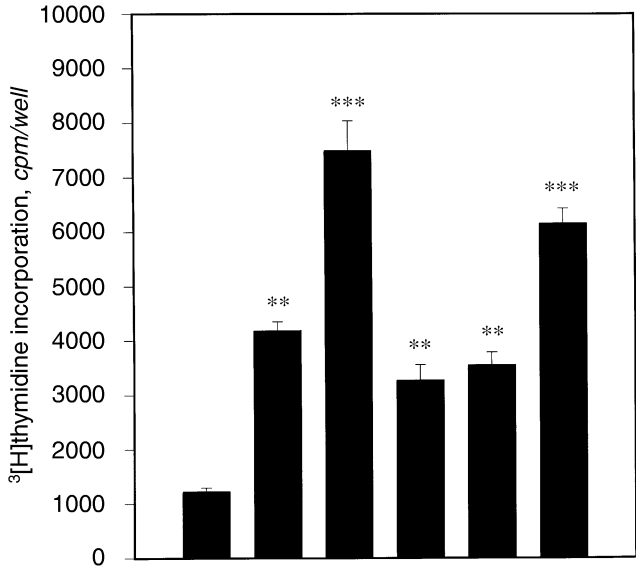

Fig. 7. Effect of basic fibroblast growth factor (bFGF) on [^{3}H]thymidine incorporation. bFGF (20 ng/ml) induced an increase in [^{3}H]thymidine incorporation 29 hours following treatment. Subsequent addition of Ang II (10^{-6} M) for another 24 hours attenuated the bFGF-induced ^{3}[H]thymidine incorporation. This effect was abolished with PD 123319 (10^{-6} M), and not affected with losartan (10^{-6} M). Values are means ± SD of 6 experiments in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control.

Ang II was reduced when compared with that observed in adult RMICs [8]. Considering the embryonic origin of the cells used in the current study, a higher rate of proliferation might have been expected. The contrary finding suggests either that cultured ERMICs posses a lower number of AT_1 binding sites than adult RMICs or that there exist several growth regulating mechanisms in addition to that activated by Ang II.

In addition to Ang II, other vasoactive peptides and/or growth factors also induce mitogenesis in ERMICs (unpublished observations). Among these factors, bFGF was the most potent in inducing cell proliferation. In the present study, ERMICs were treated for 29 hours with bFGF and then with Ang II. After the addition of Ang II, the bFGF-induced increase in [^{3}H]thymidine incorporation was attenuated. This antiproliferative effect of Ang II was not apparent following addition of PD 123319, but was maintained following addition of losartan, suggesting that it was mediated through the AT_2 receptor. The ability of Ang II to inhibit growth induced by bFGF has recently also been reported in coronary endothelial cells [6], where a similar action was also mediated by the AT_2 receptor.

Results obtained in the current study suggest that Ang II, engaging both AT_1 and AT_2 receptors, has a dual role in regulating proliferation of ERMICs. Under conditions when only Ang II is present in the culture medium, it acts as a growth promoting factor via the AT_1 receptor. However, if proliferation had already been promoted by a factor such as bFGF, Ang II activates the AT_2-mediated antiproliferative mechanism. These findings suggest that the antiproliferative action of the AT_2 receptor is not an inherent property, but is only recalled in states of active proliferation.

High levels of AT_2 receptor expression are associated with other developing tissues including adult adrenal gland and ovary [22, 23]. In addition, increased expression of AT_2 receptors has been associated with left ventricular hypertrophy and vascular neointimal proliferation [24, 25]. Thus, the sites of AT_2 expression correspond to sites of active growth and differentiation. This is consistent with the findings of the current study where the function of AT_2 receptors was only apparent under conditions of stimulated growth.

During kidney development, ERMICs differentiate from the nephrogenic mesenchyme to subsequently play a role in nephrogenesis and basement membrane formation [9]. The ability of Ang II to regulate ERMIC proliferation demonstrates an important role for Ang II in kidney morphogenesis. Chronic blockade of the renin-angiotensin system during kidney development results in morphological abnormalities accompanied by impaired renal function [26, 27]. Although such studies have been performed in postnatal animals in which the majority of the receptors are of the AT_1 subtype, these observations highlight the importance of Ang II in regulating developmental processes in the kidney. Blockade of AT_2 receptors from an early stage in embryonic development may provide insight into the physiological relevance of this receptor in renal morphogenesis.

This study has demonstrated the presence of biologically active AT_1 and AT_2 receptors on ERMICs. Both receptors play a role in regulating ERMIC proliferation with opposing effects. Most importantly, this study demonstrates a physiological role for AT_2 receptors on cells that are actively involved in regulating kidney morphogenesis. Thus, through both its receptor subtypes Ang II appears to play an important role in regulating kidney development.

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APPENDIX

Abbreviations used in this article are: Ang II, angiotensin II; RMICs, renomedullary interstitial cells; ERMICs, embryonic renomedullary interstitial cells; AT, angiotensin receptors; RT-PCR, reverse transcription—polymerase chain reaction; bFGF, basic fibroblast growth factor; ECM, extracellular matrix; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HEPES, N-2-hydroxyethlpiperazine-N′-2-ethanesulfonic acid; PB, phosphate buffer; PBS, phosphate buffered saline; DAB, 3,–3′-diaminobenzidine tetrahydrochloride dihydrate; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; NSB, nonspecific binding; TB, total binding; PCA, perchloric acid; IBMX, 3-isobutyl-1-methylxanthine; E15, embryonic day 15.

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