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Intracellular angiotensin II directly induces in vitro transcription of TGF- β 1, MCP-1 and NHE-3 mRNAs in isolated rat renal cortical nuclei via activation of nuclear AT₁ receptors

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Abstract

The present study tested the hypothesis that intracellular angiotensin II (Ang II) directly induces transcriptional effects by stimulating AT₁ receptors in the nucleus of rat renal cortical cells. Intact nuclei were freshly isolated from the rat renal cortex and transcriptional responses to Ang II were studied using in vitro RNA transcription assays and semi-quantitative RT-PCR. High power phase contrast micrographs showed that isolated nuclei were encircled by an intact nuclear envelop, stained strongly by the DNA marker DAPI, but not by the membrane or endosomal markers. FITC-labeled Ang II and [¹²⁵I]-Val⁵-Ang II binding confirmed the presence of Ang II receptors in the nuclei with a predominance of AT₁ receptors. RT-PCR showed that AT_{1a} mRNA expression was 3-fold greater than AT_{1b} receptor mRNAs in these nuclei. In freshly isolated nuclei, Ang II increased in vitro [α -³²P]CTP incorporation in a concentration manner, and the effect was confirmed by autoradiography and RNA electrophoresis. Ang II markedly increased in vitro transcription of mRNAs for transforming growth factor- β 1 by 143% ($p < 0.01$), macrophage chemoattractant protein-1 by 89% ($p < 0.01$), and the sodium and hydrogen exchanger-3 by 110% ($p < 0.01$). These transcriptional effects of Ang II on the nuclei were completely blocked by the AT₁ receptor antagonist losartan ($p < 0.01$). By contrast, Ang II had no effects on transcription of angiotensinogen and GAPDH mRNAs. Since these transcriptional effects of Ang II in isolated nuclei were induced by Ang II in the absence of cell surface receptor-mediated signaling and completely blocked by losartan, we concluded that Ang II may directly stimulate nuclear AT_{1a} receptors to induce transcriptional responses that are associated with tubular epithelial sodium transport, cellular growth and hypertrophy, and proinflammatory cytokines.

Keywords

Angiotensin II; in vitro transcription; kidney; nucleus; RT-PCR; sodium transport

Introduction

It is now well accepted that endocrine and paracrine angiotensin II (Ang II) activate intracellular signaling pathways and induce diverse biological effects via binding to its cell surface G protein-coupled receptors (9,39,51). Evidence supporting the endocrine and paracrine roles of Ang II in cardiovascular and renal regulation is overwhelming (9,39,51). Indeed, there is little doubt that this mode of Ang II-induced responses plays an essential role in the physiological

regulation of blood pressure, cardiovascular homeostasis, and renal function. In physiological settings, however, binding of Ang II to its cell surface receptors evokes two classical responses; namely rapid activation of intracellular signalling pathways and simultaneous initiation of type 1 (AT₁) receptor-mediated endocytosis (or internalization) of Ang II (9,15,21,49,51). The latter response is thought to desensitize the cellular responses to Ang II by moving extracellular Ang II into the cells for degradation, with internalized receptors being recycled back to the cell surface after dissociation from the agonist (15,21,51). There is evidence that not all internalized Ang II peptides are directed to lysosomes for degradation and some of them may be trafficked to other organelles or the nucleus (3,6,38,41).

Angiotensin II is widely implicated in the development of hypertension and several progressive renal diseases by causing salt and fluid retention and inducing growth and proinflammatory responses (23,42,45,54). For example, Ang II has been shown to induce expression of the epithelial sodium channel (EnaC) (1), the sodium and hydrogen exchanger-3 (NHE-3) (12, 34,40), and proto-oncogenes, growth factors, and hypertrophic marker genes (28,42,50,54). These growth-promoting and proliferative effects of Ang II are thought to be primarily due to activation of cell surface receptors by extracellular Ang II. However, extracellular Ang II is well recognized to internalize with its receptors as a source of intracellular Ang II in vascular smooth muscle cells (VSMCs) (18,46,49) and renal proximal tubule cells after binding to cell surface AT₁ receptors (21,32,34,46,48,51). Moreover, endosomal and/or nuclear Ang II receptors have been identified in the liver and kidney cells (2,36,43,47). This suggests that internalized Ang II may be partly involved in Ang II-induced growth and proinflammatory responses in these tissues by activating cytoplasmic and nuclear Ang II receptors.

In the present study, we used freshly isolated, intact rat renal cortical nuclei to test the hypothesis that internalized or intracellular Ang II directly stimulates AT_{1a} receptors to induce RNA synthesis and in vitro transcription of mRNAs for growth-promoting and proinflammatory cytokines, including angiotensinogen, transforming growth factor- β 1 (TGF- β 1), macrophage chemoattractant protein-1 (MCP-1) and the sodium-hydrogen exchanger-3 (NHE-3). We demonstrated that freshly isolated and intact nuclei bound predominantly AT₁ (AT_{1a}) receptors and that Ang II directly induced transcriptional responses of mRNAs for TGF- β 1, MCP-1 and NHE-3, but not angiotensinogen, via stimulation of the AT₁ receptor in the nucleus. Our results are consistent with an important role of internalized and/or intracellular Ang II and nuclear AT₁ receptors in mediating growth and proinflammatory responses and sodium-retaining effects of Ang II in Ang II-dependent hypertensive renal diseases.

Methods

Materials

Animals—Adult male Sprague-Dawley rats (200–250 g) were purchased from Charles River Laboratories (Wilmington, MA) and maintained in a temperature-controlled room with a 12:12-h light-dark cycle. The animals were fed on a standard rodent chow and had free access to tap water (33,52,55). This study was approved by the Institutional Animal Care and Use Committee of Henry Ford Health System.

Chemicals—Heat-inactivated fetal bovine serum was purchased from ATCC. Val⁵-Ang II and Ang II enzyme immunoassay kits were obtained from Biochem (Peninsula, CA) (32). BCA protein assay kits were bought from Pierce. The AT₁ receptor antagonist losartan was a gift from Merck Pharmaceuticals and the AT₂ receptor antagonist PD 123319 was donated by Pfizer. All fluorescence-labeled peptides or markers, including FITC-labeled Ang II, the fluorescent nuclear marker DAPI, the fluorescent membrane-specific marker Oregon green 488 conjugate-labeled wheat germ agglutinin (WGA) and the fluorescent endosomal marker Alexa Fluor 594-labeled transferrin were obtained from Invitrogen (Molecular Probes).

[α - 32 P]-CTP was purchased from Amersham (specificity: 3000 Ci/mmol). [125 I]-Val⁵-Ang II was radioiodinated and HPLC-purified by Dr. Robert Speth of The University of Mississippi Peptide Radioiodination Service Center (specificity: 2175 Ci/mmol) (31,33). In vitro SP6/T7 RNA transcription system (transcription optimized buffer, DTT, rATP, rGTP, rUTP, rCTP, recombinant RNasin ribonuclease inhibitor, SP6 RNA polymerase, and T7 RNA polymerase) was purchased from Promega. Tri-Reagent was purchased from Molecular Research Center Inc. Forward (or sense) and reverse (or antisense) primers for RT-PCR of mRNAs for AT_{1a} and AT_{1b} receptors (24), angiotensinogen (27), TGF- β 1 (25,50), MCP-1 (5), NHE-3 (29), and the house keeping gene GAPDH (5) were synthesized by Invitrogen.

Isolation of fresh intact cortical nuclei from the rat kidney

Fresh intact nuclei were isolated and purified from the cortex of the rat kidneys using a modification of the method as described by others for preparing the nuclei or nuclear extracts from the rat livers (2,47). Briefly, Sprague Dawley rats were sacrificed by decapitation, and the kidneys were removed quickly. Unless specified elsewhere, all isolation procedures were performed on ice or at 4°C. After the capsule was removed, the kidneys were rinsed with ice-cold the isolation buffer A containing 320 mM sucrose, 3 mM MgCl₂, 20 mM Tris, pH 7.4. The kidneys were cut longitudinally in half with a scalpel blade and the entire medulla (inner and outer) was removed with a fine scissor, so that intact nuclei were isolated only from the renal cortex. The renal cortex of each kidney was then chopped into fine pieces, scooped into a Sorvall centrifuge tube in 15 ml buffer A, and incubated on ice for 15 min. The tissues were then homogenized using a glass homogenizer and motor driven Teflon pestle. The homogenates were filtered through a 50 μ m metal sieve, washed with buffer A if necessary, and centrifuged for 15 min at 1000 X g using a Beckman bench-top centrifuge at 4°C. The supernatants were removed and the pellets resuspended by gentle homogenization with a pipette P5000 in ~ 30 ml of buffer B (2.2 M sucrose, 1 mM MgCl₂, and 10 mM Tris, pH 7.4). The re-suspended homogenates were differentially centrifuged for 60 min at 64,000 X g using a Beckman swing out rotor at 4°C. After centrifugation, the supernatants were removed and cleared before isolated nuclei were suspended and washed twice by centrifugation in 2 ml of buffer A (2,47).

Measurements of rat renal cortical nuclear protein and DNA contents

Protein contents in isolated rat renal cortical nuclei were measured using a BCA protein assay kit (Pierce), as described (13). Nuclear DNA concentrations were determined by a fluorospectrometer (13). Furthermore, isolated nuclei were also stained with the fluorescent nuclear acid marker DAPI (300 nM in PBS), placed on glass cover-slips, and examined using a Nikon Eclipse TE2000-U inverted fluorescence microscope coupled with a Lambda DG4 illumination system (Sutter Instruments) in order to confirm the purity of the nuclei, as described (32,34).

Visualization of the integrity of freshly isolated rat renal cortical nuclei

The integrity of the nuclear structural and functional machinery is important for studying nuclear binding of [125 I]-Val⁵-Ang II or FITC-Ang II and the transcriptional responses to Ang II as described below. Immediately after isolation and purification procedures, the nuclei were visualized without fixation on a phase contrast microscope at low (X 10) and high (X 100) magnifications, as described (47). Special attention was focused on whether the nuclear envelopes or membranes remain intact in isolated nuclei after purification. To exclude the possibility of potential contamination by plasma membranes or endosomal organelles, isolated nuclei were stained with Oregon green 488 conjugate-labeled WGA (100 μ g/ml) or Alexa Fluor 594-labeled transferrin (100 μ g/ml) for 30 min at 22°C. Oregon green 488 conjugate-labeled WGA primarily stains plasma membranes, whereas Alexa Fluor 594-labeled transferrin stains

endosomal organelles (32,55). Unstained fluorescent markers were removed by two washes with PBS, and the nuclei were placed on glass cover-slips and examined using a Nikon Eclipse TE2000-U inverted fluorescence microscope (32,34).

Characterization of AT₁ and AT₂ receptors in isolated rat renal cortical nuclei

Localization of AT₁ and AT₂ receptor binding in isolated nuclei using FITC-labeled Ang II—To co-localize Ang II receptor binding in freshly isolated rat renal cortical nuclei, 10 µg of intact nuclei were placed in each well of 4-well Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL) and incubated in 500 µl of 50 mM Tris buffer containing 1 nM FITC-Ang II (Molecular Probes), 120 mM NaCl, 4 mM KCl, 5 mM MgCl₂, and 1 mM CaCl₂, 10 µg/ml bacitracin and 2 mg/ml D-glucose, pH 7.5, for 60 min at 22°C (32,34). Non-specific binding, AT₁ receptor binding, and AT₂ receptor binding were determined as described below for [¹²⁵I]-Val⁵-Ang II receptor binding (31,32,53). After incubation, the nuclei were gently washed in a fresh volume of the same buffer without FITC-Ang II twice before being counterstained with DAPI (300 nM) for 5 min at 22°C for nuclear localization. The FITC-Ang II-labeled nuclei were examined by a Nikon Eclipse TE 2000-U fluorescence microscope. Fluorescent images of FITC-labeled Ang II (green) and DAPI (blue) in the same nuclei were captured and analyzed using the MetaMorph image system (Universal Image) with filters suitable for FITC (488-nm wavelength) or DAPI (~500 nm) (32,34,56).

Characterization of AT₁ and AT₂ receptor binding in isolated nuclei using [¹²⁵I]-Val⁵-Ang II radioreceptor binding assays—To characterize pharmacological properties of AT₁ and AT₂ receptors in isolated renal cortical nuclei, we used [¹²⁵I]-Val¹-Ang II as the radioligand for Ang II receptor binding assays as described previously (31,32,52,53). Briefly, Ang II receptor binding affinity constant (K_d) and maximal binding sites (B_{max}) were determined from saturation binding curves and Scatchard plot by incubating 100 µg of freshly isolated nuclei in 10 mM sodium phosphate buffer with increasing concentrations of [¹²⁵I]-Val⁵-Ang II alone (0 to 100 nM) (31,32,52,53). K_d and B_{max} were calculated using GraphPad Prism 4.0 (31–33). The specificities of AT₁ and AT₂ receptor binding in freshly isolated nuclei were determined by incubating 100 µg of isolated nuclei with [¹²⁵I]-Val⁵-Ang II (~100 pmol) for 60 min at 22°C in the presence, or absence, of increasing concentrations (0 to 100 µM) of unlabeled competing Ang II, the AT₁ receptor-selective antagonist losartan, or the AT₂ receptor-selective antagonist PD123319, as described (31,32,52,53). Total Ang II receptor binding was calculated as the binding in the absence of competing unlabeled Ang II or its receptor subtype-selective antagonists in the incubation. Non-specific binding was determined as the binding in the presence of 10 µM competing unlabeled Ang II. AT₁ receptor binding was determined in the presence of 10 µM competing unlabeled AT₂ receptor blocker PD 123319, whereas AT₂ receptor binding were calculated as the binding in the presence of 10 µM competing unlabeled AT₁ blocker losartan (31,32,52,53).

RT-PCR of AT_{1a} and AT_{1b} receptor mRNAs—Because the rat kidney expresses two subtypes of AT₁ receptors, AT_{1a} and AT_{1b}, and neither [¹²⁵I]-Val⁵-Ang II nor FITC-labeled Ang II receptor binding assays can distinguish these two receptors, we used semi-quantitative RT-PCR to determine AT_{1a} and AT_{1b} receptor mRNAs in isolated rat renal cortical nuclei, as described (24,37). Briefly, total RNA was extracted from ~200 µg isolated rat renal cortical nuclei using Tri-Reagent RNA isolation protocol (Molecular Research Center Inc., Cincinnati, OH) and the quality of total RNA was verified by spectrophotometry with an OD260/OD280 ratio of 1.9 or above for RT-PCR analyses. First-strand cDNA was synthesized from 1 µg total RNA using SuperScript III-First Strand Synthesis System for RT-PCR kit (Invitrogen) in a final volume of 25 µl containing 25 mM MgCl₂, 10 mM dNTP mixture, 10 µM sense and antisense primer mixers (AT_{1a} or AT_{1b}), 5 U/µl Tag DNA polymerase, and 2 µl cDNA. The sense and antisense primers for AT_{1a} or AT_{1b} receptor mRNA were described by Jöhren et al

(24) (see Table 1). RT-PCR amplification was performed as followed: 35 cycles of denaturation for 2 min at 94°C, annealing for 1 min at 58°C, and extension for 1 min 15 sec at 72°C, respectively. The PCR products were size fractionated on a 2% agarose gel and visualized with ethidium bromide staining and UV transillumination. RT-PCR of GAPDH mRNA was used as a house keeping gene control (24,37).

Effects of Ang II on [α -³²P]CTP incorporation into RNA in isolated rat renal cortical nuclei

The effects of Ang II on total RNA synthesis in isolated rat renal cortical nuclei were studied using a standard in vitro [α -³²P]CTP RNA transcription system (Promega), as described (13, 14). To determine the concentration and response relationship, 100 μ g of freshly isolated nuclei were first stimulated by increasing concentrations of Ang II (0, 1.0, 10 and 100 nM) in the absence of the AT₁ receptor antagonist losartan for 30 min at 37°C. Ang II-pretreated nuclei were then incubated with an in vitro SP6/T7 RNA transcription system consisting of 500 μ M of each ATP, GTP and UTP, 250 μ M CTP, and 2 U/ μ l RNasin at 37°C for 1 hour, with 50 μ Ci [α -³²P]CTP added as an index of in vitro CTP incorporation into RNA during RNA synthesis. To determine the role of AT₁ receptors in mediating [α -³²P]CTP RNA transcription responses to Ang II in isolated nuclei, 100 μ g of nuclei were pre-treated with Ang II (10 nM) in the presence of losartan for 30 min at 37°C, which was followed by incubations with the in vitro SP6/T7 RNA transcription system, as described above. The effect of PD 123319 was not examined because Ang II receptor binding assays detected little AT₂ receptor binding in isolated nuclei. After incubation, RNA was extracted using TRI reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). The extracted RNA was revealed by 2% agarose gel electrophoresis and stained by ethidium bromide. To quantify newly transcribed RNA, RNA in the nuclear extract was precipitated with 10% TCA onto glass fiber filters, washed, and counted in a scintillation counter (Beckman). The newly transcribed RNA on the filters was also exposed to X-ray film for 2 weeks and resultant autoradiographs analysed using a microcomputer imaging device (MCID, Imaging Research Inc. Ontario).

Effects of Ang II on in vitro transcription of mRNAs for angiotensinogen, TGF- β 1, MCP-1, and NHE-3 in freshly isolated rat renal cortical nuclei

To determine whether intracellular Ang II stimulates nuclear Ang II receptors to specifically induce expression of mRNAs for angiotensinogen, TGF- β 1, MCP-1, and NHE-3, the same in vitro SP6/T7 RNA transcription system (Promega) and RNA isolation procedures were followed, with the exception that 500 μ M unlabeled CTP were used in place of [α -³²P]CTP (13,14). We first examined the concentration-dependent responses of TGF- β 1 mRNA expression to Ang II (0, 1.0, 10, and 100 nM) in isolated nuclei to assist determining the optimal Ang II concentration, which was used for studying the responses of other target genes (10 nM). Specifically, first-strand cDNA for each of above target genes was synthesized from 1 μ g total RNA using SuperScript III-First Strand Synthesis System for RT-PCR kit (Invitrogen) in a final volume of 25 μ l containing 25 mM MgCl₂, 10 mM dNTP mixture, 10 μ M of forward (or sense) and reverse (or antisense) primer mixers, 5 U/ μ l Tag DNA polymerase, and 1 μ l cDNA. The sequences of forward (or sense) and reverse (or antisense) primers for angiotensinogen (27), TGF- β 1 (25), MCP-1 (5), and NHE-3 (29), were chosen from the published literature (see Table 1) and synthesized by Invitrogen. RT-PCR amplification was performed as followed: 35 cycles of denaturation for 2 min at 94°C, annealing for 1 min at 58°C, and extension for 1 min 15 sec at 72°C, respectively. The PCR products were size fractionated on a 1.5% agarose gel and visualized with ethidium bromide staining and UV transillumination. mRNA levels were quantified by a micromputerized imaging system (Imaging Research Inc., Ontario) and normalized to the house-keeping GAPDH mRNA (5,13).

Statistical analysis

All data are presented as means $\pm$ SE. Differences in the same parameters between groups of isolated nuclei or between different treatments were compared using one-way ANOVA or unpaired Student's *t*-test. The significance was set at $p < 0.05$.

Results

Characteristics of freshly isolated rat renal cortical nuclei

Although the presence of Ang II receptors in nuclear extracts or nuclear fractions of the rat kidney was reported previously (36,43), the morphological and biochemical properties of freshly isolated rat renal cortical nuclei have not been characterized. Figure 1 shows some unique characteristics of freshly isolated nuclei from the rat renal cortex. At low magnification (10 X), light microscopic imaging shows dense homogeneous round black particles, spreading throughout the microscopic field (Fig. 1A). At high magnification (100 X), phase contrast microscopic imaging reveals the integrity of the nuclei, with a fine layer of nuclear envelope encircling each nucleus (Fig. 1B). Furthermore, these freshly isolated nuclei were readily stained by DAPI, a standard marker of cellular nuclear acids (Fig. 1C; 40 X), but not by the membrane-specific fluorescent marker Oregon green 488 conjugate-labeled WGA (Fig. 1D; 40 X) or the endosomal fluorescent marker Alexa Fluor 594-labeled transferrin (Fig. 1E; 40 X). Finally, high concentrations of DNA were found in these freshly isolated nuclei, as expected (nucleus: 1029 ± 88 μ g/mL DNA vs. cytoplasmic supernatant: 30 ± 5 μ g/mL DNA, $p < 0.001$) (Fig. 1F). Taken together, these data confirm that freshly isolated nuclei bear characteristic morphological and biochemical properties of intact rat renal cortical nuclei.

Localization and characterization of AT₁ and AT₂ receptors in freshly isolated rat renal cortical nuclei

To determine whether AT₁ and AT₂ receptors are present in isolated rat renal cortical nuclei, three different approaches were used. Figure 2 shows in vitro fluorescence imaging of Ang II receptor subtypes in these freshly isolated rat renal cortical nuclei. The nuclei were strongly stained by FITC-labeled Ang II (Fig. 2A; total), which was completely co-localized with the nuclear acid marker, DAPI (Fig. 2B), thus confirming the presence of Ang II receptor binding in isolated rat renal cortical nuclei (Fig. 2C). FITC-labeled Ang II receptor binding was largely displaced by the AT₁ receptor antagonist, losartan (10 μ M; Fig. 2D–2F). In contrast, FITC-Ang II receptor binding was not blocked by the AT₂ receptor antagonist, PD 123319 (10 μ M; Fig. 2G–2I). Further radioreceptor binding assays show that [¹²⁵I]-Val⁵-Ang II bound intact nuclei in a saturable manner (Fig. 3A). Scatchard analysis of [¹²⁵I]-Val⁵-Ang II binding revealed a single class of Ang II receptor binding sites with a B_{max} of 708.5 ± 46.2 fmol/mg nuclear proteins and a K_d of 19.1 ± 3.3 nM (Fig. 3A). Unlabeled Ang II and losartan largely inhibited [¹²⁵I]-Val⁵-Ang II receptor binding, whereas PD 123319 had no significant effect (Fig. 3B). These data suggest that Ang II receptors in freshly isolated rat renal cortical nuclei are predominantly of the AT₁ subtype.

RT-PCR of AT_{1a} and AT_{1b} receptor mRNAs in isolated rat renal cortical nuclei

Figure 4 shows semi-quantitative RT-PCR analyses of AT_{1a} and AT_{1b} receptor mRNA expression in four different nuclear samples, with GAPDH mRNA expression used as a house-keeping control gene. Based on the sense and antisense primers of AT_{1a} and AT_{1b} receptors used, a 300 base pairs (bp) of PCR product was detected for AT_{1a} receptor mRNA, whereas a ~ 800 bp of PCR product was detected for AT_{1b} receptor mRNA. The sizes of AT_{1a} and AT_{1b} PCR products were consistent with what were predicted from the sense and anti-sense primers (24,37). AT_{1a} receptor mRNA expression was about 3-fold higher than that of AT_{1b}

receptor mRNA when identical amplification cycles and exposure conditions were used (Fig. 4).

Effects of Ang II on in vitro [α - 32 P]CTP incorporation into RNA in freshly isolated rat renal cortical nuclei

The concentration-dependent responses of in vitro [α - 32 P]CTP incorporation in isolated nuclei are shown in Fig. 5A. Ang II induced [α - 32 P]CTP incorporation into RNA in a concentration-related manner. At the lower concentration (1.0 nM), Ang II increased [α - 32 P]CTP incorporation by > 30% (control: 20720 ± 1712 cpm/mg nuclear protein vs. 1.0 nM: 28840 ± 2649 cpm/mg protein; n.s.). Higher concentrations of Ang II significantly increased [α - 32 P]CTP incorporation by ~ 150% at 10 nM (51800 ± 10596 cpm/mg protein; $p < 0.05$ vs. control) and ~ 90% at 100 nM (38067 ± 6661 cpm/mg protein; $p < 0.05$ vs. control), respectively. Figure 5B shows representative autoradiographic images of the effects of Ang II (10 nM) on in vitro [α - 32 P]CTP incorporation (middle, $n = 4$), compared with un-stimulated nuclei (left, $n = 4$). The effects of Ang II on [α - 32 P]-CTP incorporation were completely blocked by the AT₁ receptor antagonist, losartan (10 μ M; right; $n = 4$). The quantitative results of these experiments were shown in Fig. 5C. [α - 32 P]-CTP incorporation in non-stimulated nuclei was 32250 ± 2835 cpm/mg nuclear proteins. Ang II stimulation increased [α - 32 P]-CTP incorporation by ~106% (66440 ± 7211 cpm/mg proteins, $p < 0.01$ vs. control nuclei). Blockade of nuclear AT₁ receptors with losartan significantly attenuated Ang II-induced increases in [α - 32 P]-CTP incorporation in the nuclei (39000 ± 3946 cpm/mg proteins; $p < 0.01$ vs. Ang II). Figure 6 further shows the quality of isolated RNA from control (lane 1), Ang II-stimulated (lane 2), and Ang II plus losartan-treated nuclei (Lane 3). As revealed by 2% agarose gel electrophoresis and stained by ethidium bromide, Ang II stimulation increased total RNA synthesis in the nuclei, and the effect was attenuated by losartan (Fig. 6).

Effects of Ang II on TGF- β 1 mRNA expression in freshly isolated rat renal cortical nuclei

Stimulation of the growth factor such as TGF- β 1 mRNA expression by Ang II plays an important role in Ang II-induced cellular growth and hypertrophy, and tissue fibrosis in the kidney (25,50). Yet whether intracellular Ang II induces TGF- β 1 mRNA expression via activation of nuclear AT₁ receptors is not known. Figure 7 shows the concentration-related responses of TGF- β 1 mRNA expression to Ang II in freshly isolated rat renal cortical nuclei. The top panels are representative RT-PCR images of TGF- β 1 mRNA and GAPDH mRNA, respectively, and the bottom panel shows the semi-quantitative results from three experiments. Ang II induced TGF- β 1 mRNA expression in a concentration-dependent manner, with the maximal response observed at 10 nM of Ang II (control: 0.02 ± 0.01 vs. 10 nM Ang II: 0.62 ± 0.08 TGF- β 1 mRNA/GAPDH mRNA ratio; $p < 0.001$). Although Ang II also significantly stimulated TGF- β 1 mRNA expression in isolated nuclei at 1.0 nM (0.22 ± 0.04 TGF- β 1 mRNA/GAPDH mRNA ratio; $p < 0.05$) or 100 nM (0.35 ± 0.04 TGF- β 1 mRNA/GAPDH mRNA ratio; $p < 0.01$), the responses were not as great as those seen at 10 nM (Fig. 7). The role of nuclear AT₁ receptors in mediating Ang II-induced TGF- β 1 mRNA expression in isolated nuclei is shown in Fig. 8. At 10 nM, Ang II markedly increased TGF- β 1 mRNA expression from 0.06 ± 0.01 to 0.56 ± 0.10 TGF- β 1 mRNA/GAPDH mRNA ratio ($p < 0.01$). Losartan significantly blocked Ang II-increased TGF- β 1 mRNA expression in the nuclei (0.18 ± 0.04 TGF- β 1 mRNA/GAPDH mRNA ratio, $p < 0.01$ vs. Ang II) (Fig. 8).

Effects of Ang II on angiotensinogen mRNA expression in freshly isolated rat renal cortical nuclei

Because Ang II has been described to stimulate angiotensinogen mRNA expression in rat hepatic nuclei (13,14), the rat kidney (26,27,42), or in cultured proximal tubule cells (22), the present study determined whether intracellular Ang II induced expression of angiotensinogen

mRNA in freshly isolated rat renal cortical nuclei. Figure 9 shows that at 10 nM, Ang II had no effect on angiotensinogen mRNA expression (control: 0.45 ± 0.06 vs. 0.48 ± 0.06 angiotensinogen mRNA/GAPDH mRNA ratio; *n.s.*). At 10 μ M, losartan also did not affect angiotensinogen mRNA expression (+ losartan: 0.43 ± 0.10 angiotensinogen mRNA/GAPDH mRNA ratio; *n.s.* vs. Ang II or control) (Fig. 9).

Effects of Ang II on MCP-1 mRNA expression in freshly isolated rat renal cortical nuclei

Angiotensin II not only acts as a growth factor but also a pro-inflammatory cytokine by inducing MCP-1 expression (4,5,54). In the present study, incubation of freshly isolated rat renal cortical nuclei with Ang II (10 nM) significantly increased MCP-1 mRNA expression by 89% (control: 0.36 ± 0.07 vs. 0.68 ± 0.10 MCP-1 mRNA/GAPDH mRNA ratio, $p < 0.01$) (Fig. 10). This stimulatory effect of Ang II on MCP-1 mRNA expression was effectively blocked by losartan (0.23 ± 0.06 MCP-1 mRNA/GAPDH mRNA ratio, $p < 0.01$), indicating that the effect was mediated by nuclear AT₁ receptors (Fig. 10).

Effects of Ang II on NHE-3 mRNA expression in freshly isolated rat renal cortical nuclei

Angiotensin II plays a critical role in the regulation of sodium transport in proximal tubules by stimulating NHE-3 expression via actions on apical membrane AT₁ receptors (12,30,34, 40). It is not known whether following receptor-mediated internalization, Ang II may stimulate nuclear AT₁ receptors to induce NHE-3 expression. As shown in Fig. 11, activation of nuclear AT₁ receptors by Ang II induced 110% increases in NHE-3 mRNA expression (control: 0.21 ± 0.03 vs. Ang II: 0.42 ± 0.05 NHE-3 mRNA/GAPDH mRNA ratio, $p < 0.01$). Co-administration of Ang II with losartan significantly reduced NHE-3 mRNA expression to control (0.23 ± 0.05 NHE-3 mRNA/GAPDH mRNA ratio, $P < 0.01$ vs. Ang II).

Discussion

The major goal of the present study was to test the hypothesis that intracellular Ang II, whether synthesized intracellularly or internalized from extracellular fluid, stimulates AT₁ receptors in the nucleus to induce in vitro transcription of the growth factor TGF- β 1, angiotensinogen, the proinflammatory cytokine MCP-1, and the Na⁺/H⁺ exchanger NHE-3. The present study demonstrates three key findings: 1) that freshly isolated nuclei from the rat renal cortex expressed Ang II receptors of primarily the AT_{1a} subtype; 2) intracellular Ang II induced in vitro RNA synthesis by the nucleus by increasing [α -³²P]-CTP incorporation into RNA via activating AT₁ receptors; and 3) Ang II specifically stimulated transcription or expression of TGF- β 1, MCP-1 and NHE-3 mRNAs, but not angiotensinogen mRNA, in the nucleus and these responses were mediated by AT₁ receptors. Because only freshly isolated nuclei were used without significant contaminations by plasma membranes or endosomal organelles, these transcriptional responses to Ang II in the nucleus were independent of cell surface AT₁ receptor- or cytoplasmic AT₁ receptor-mediated signaling cascades. Our results provide evidence that at least in freshly isolated intact nuclei of the rat renal cortex, intracellular Ang II can directly stimulate nuclear AT_{1a} receptors to induce transcription and/or expression of mRNAs for TGF- β 1, MCP-1 and NHE-3.

Whether intracellular Ang II induces biological and/or physiological responses has been debated for decades. The difficulty in arguing for a role of intracellular Ang II is due to the lack of approaches that can definitely separate the responses induced by intracellular Ang II via intracellular or nuclear receptors from those evoked by extracellular Ang II via activation of cell surface receptors. To overcome the technical difficulty, Haller *et al.* microinjected Ang II directly into rat vascular smooth muscle cells (VSMCs) to induce intracellular and nuclear calcium responses to Ang II (19). We recently confirmed these intracellular calcium responses to microinjected Ang II in single rabbit proximal tubule cells (56). De Mello *et al.* dialyzed

Ang II directly into hamster cardiomyocytes and demonstrated that Ang II enhanced the L-type calcium currents (10,11). Alternatively, Cook *et al.* reported that overexpression of a non-secreted form of angiotensinogen or intracellular Ang II (ECFP/AII) and AT₁ receptor fusion proteins (AT₁R/EYFP) in rat hepatoma cells stimulated hepatic cell or VSMC growth and proliferation (6–8). Ang II has also been shown to stimulate RNA synthesis (44) and increase transcription of mRNAs for renin, angiotensinogen, and growth-related factors in rat hepatic nuclei (13,14), but there is no evidence that intracellular Ang II may exert nuclear effects in renal cortical cells.

In the present study, we went to a great length to confirm the identity and quality of our freshly isolated rat renal cortical nuclei and the presence of AT₁ (AT_{1a}) receptors before *in vitro* mRNA transcription studies were performed. The purity and integrity of isolated nuclei were characterized by 1) high power phase contrast images showing intact nuclear envelopes (Fig. 1B); 2) the specific staining with the nuclear acid marker, DAPI (Fig. 1C); 3) the absence of potential contaminations by plasma membranes (Fig. 1D) or endosomal organelles (Fig. 1E); and 4) the presence of high concentrations of DNA contents (Fig. 1F). Because the presence of Ang II receptors in the nucleus is critical for studying the nuclear role of intracellular Ang II, we used three different approaches to confirm whether freshly isolated rat renal cortical nuclei have AT₁ receptors. FITC-labeled Ang II (Fig. 2) and [¹²⁵I]-Val⁵-Ang II binding assays (Fig. 3) demonstrated that the nuclei bound both FITC-labeled Ang II or [¹²⁵I]-Val⁵-Ang II, which were largely displaced by unlabeled competing Ang II (10 μM) and the AT₁ antagonist losartan (10 μM), but not by the AT₂ antagonist PD123319 (10 μM). Thus we conclude that the majority of Ang II receptor binding in isolated rat renal cortical nuclei belongs to the AT₁. Using semi-quantitative RT-PCR, we further demonstrated for the first time that the AT_{1a} receptor dominates the AT_{1b} receptor in a ratio of 3:1 in the nucleus of the rat renal cortex (Fig. 4). These data are consistent with those previously reported in nuclear extracts of the rat renal cortex (36,43), proximal tubule cells (32,34,46,57), and the rat kidney (52,53). However, the B_{max} (~708.5 fmol/mg nuclear proteins) and K_d (~19.1 nM) for Ang II receptors in freshly isolated intact nuclei, as determined by [¹²⁵I]-Sar¹-Ang II binding assays, are somewhat higher than those reported in nuclear extracts of the rat renal cortex (36,43) or rat hepatocytes (2, 47). The differences in AT₁ receptor binding B_{max} and K_d in these studies may reflect the differences in the purity and integrity of nuclear preparations and the conditions of binding assays. For example, Booz *et al.* estimated that when normalized to 5'-nucleotidase activity, the maximal capacity for specific Ang II binding to the liver nuclei reached 2875 fmol. min/μg PO₄, seven-times higher than plasma membranes (2). Pandergrass *et al.* showed that rat renal cortical nuclear fractions had three-time higher B_{max} and two-time higher K_d than plasma membranes (47). Alternatively, a higher K_d value for nuclear Ang II receptor binding in the present study suggests that nuclear Ang II receptors may not be as sensitive to Ang II as cell surface receptors. Higher intracellular Ang II concentrations may be required to stimulate nuclear AT₁ receptors, such as in Ang II-induced hypertension and renal target organ injury (23,42). Indeed, at picomolar concentrations Ang II was shown to physiologically stimulate proximal tubule sodium transport in the rat kidney (20), whereas high picomolar to lower nanomolar Ang II were generally required to induce effects in cultured mesangial cells (17, 31) or proximal tubule cells (34,46,57). In the present study, although Ang II induced RNA transcriptional responses in isolated nuclei in a concentration-dependent manner, a peak response was observed when 10 nM of Ang II was used (Fig. 5A and Fig. 7). Thus Ang II receptor binding characteristics in isolated nuclei is consistent with the concentration and response relationship of Ang II on *in vitro* transcription of RNA and/or target gene mRNA expression.

The effects of intracellular Ang II on the transcription of mRNAs for TGF-β1, MCP-1, and NHE-3 in freshly isolated rat renal cortical nuclei suggest that intracellular Ang II may play an important role in mediating Ang II-induced sodium retention and inflammation and fibrosis

in the kidney. TGF- β 1 is a potent growth factor (25,45,50), whereas MCP-1 is an important proinflammatory cytokine (4,5,54). In vitro, Ang II directly stimulated TGF- β 1 and MCP-1 mRNA expression in mesangial cells and tubular epithelial cells via activation of AT₁ receptors (4,5,25,54). In animal models of Ang II-induced tubulointerstitial inflammation, TGF- β 1 and MCP-1 mRNA expression were substantially increased (42). With respect to sodium retention in hypertensive diseases, Ang II is well recognized to stimulate NHE-3 synthesis and trafficking to apical membranes (12,30,34). Increased NHE-3 expression and function in proximal tubules in the renal cortex by Ang II may contribute to sodium retention and the development of hypertension. Although it is widely thought that these effects may be primarily caused by extracellular Ang II acting on cell surface AT₁ receptors, these mechanisms are probably not involved in Ang II-induced target gene mRNA transcriptional responses observed in isolated nuclei for following reasons. First, cell surface and cytoplasmic Ang II receptors were removed along with plasma membranes and endosomal organelles and only intact nuclei were used in the present study. Second, Ang II-induced nuclear transcriptional responses could be blocked by the AT₁ receptor antagonist losartan. Third, not all target genes in the nucleus were affected by Ang II, since Ang II had no effects on mRNAs for angiotensinogen and GAPDH. Our results therefore support a potential role of intracellular Ang II and nuclear AT_{1a} receptors in mediating these transcriptional responses in the kidney.

However, the signaling mechanisms by which intracellular Ang II activates nuclear AT₁ receptors to induce the expression of TGF- β 1, MCP-1 and NHE-3 mRNAs in isolated nuclei are currently unknown. One of the possibilities may involve nuclear $[Ca^{2+}]_i$, because intracellular Ang II has been shown to increase $[Ca^{2+}]_i$ in the nucleus of VSMCs and proximal tubule cells (19,56) and $[Ca^{2+}]_i$ may play an important role in the regulation of RNA transcription and target gene mRNA expression (28). Cook *et al.* showed that expression of an intracellular Ang II fusion protein in VSMCs induced nuclear accumulation of the AT₁ receptor and activation of cAMP response element-binding protein (CREB), leading to cell proliferation (6,7). Finally, translocation and/or activation of MAP kinases ERK 1/2 or NF- κ in the nucleus is well recognized to play an important role in Ang II-induced cell growth and proliferation (16,17,35,42). Further studies are required to determine whether intracellular Ang II can stimulate AT₁ receptors in the nucleus to activate these transcriptional factors or kinases.

The finding that Ang II did not change transcription of angiotensinogen mRNA in isolated rat renal cortical nuclei is unexpected. Angiotensinogen is the sole substrate for Ang II formation and Ang II is expected to negatively regulate its substrate's production. However, at nanomolar concentrations Ang II stimulated angiotensinogen mRNA transcription in isolated hepatic nuclei, also via AT₁ receptors (13,14). This response may not be surprising given the fact that angiotensinogen is primarily synthesized by hepatic cells (13,14). In the rat kidney, angiotensinogen mRNA expression and protein synthesis were significantly increased in rats chronically infused with Ang II (26,27,42). Ang II has also been shown to stimulate angiotensinogen mRNA expression in proximal tubule cells (22). The reasons underlying these differences between the present and the afore-mentioned studies are not known. It could be due to different approaches or nuclear preparations used, such as cultured cells vs. nuclear extracts, isolated nuclei vs. the entire kidney. For example, Eggena *et al.* found that Ang II had no effects on in vitro transcription of mRNAs for TGF and EGF in isolated hepatic nuclei (14). We reason that extracellular Ang II via cell surface receptor-mediated signaling may mediate the effects of Ang II on angiotensinogen mRNA expression in the kidney.

In summary, we have demonstrate for the first time that in freshly isolated rat renal cortical nuclei, intracellular Ang II bound to nuclear AT₁ receptors and stimulated in vitro RNA synthesis and transcription of mRNAs for TGF- β 1, MCP-1, and NHE-3. By contrast, Ang II did not affect transcription of mRNA for angiotensinogen in these isolated nuclei. These results suggest that intracellular Ang II, either synthesized intracellularly or internalized from

extracellular Ang II, induces novel effects through activation of AT₁ receptors in the nucleus. Thus the effects of intracellular Ang II on in vitro transcription of mRNAs for NHE-3, TGF- β 1, and MCP-1 in the nucleus may play an important role in the development of sodium and fluid retention in Ang II-induced hypertension and renal tubulointerstitial injury in progressive renal diseases.

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Figure 1.

Morphological and biochemical characteristics of freshly isolated rat renal cortical nuclei. A: light microscopic image of the nuclei, 10 X. B: high-power phase contrast image (100 X), showing that the nuclei are encircled by an intact nuclear envelope (arrows). C: DAPI-stained nuclei, 40 X. D: Oregon green 488 conjugate-labeled wheat germ agglutinin (WGA) to stain contaminated plasma membranes, 40 X. E: Alexa Fluor 594-labeled transferrin to stain endosomal organelles, 40 X. F: high DNA concentrations indicate the purity of isolated nuclei. These are representative images from of 3 to 5 different experiments. ** $p < 0.01$ vs. the cytoplasm.

Figure 2.

Localization and subtype specificity of Ang II receptors in freshly isolated rat renal cortical nuclei using FITC-labeled Ang II (see the Methods for details). Panels A, D and G are total FITC-Ang II receptor binding. Panels B, E and H are DAPI-labeled nucleic acids in the same isolated nuclei. Panels C, F, and I are merged images of FITC-Ang II binding and DAPI-labeled nucleic acids in the same nucleus. Panel J shows the levels of AT₁ (> 90%) and AT₂ receptor binding (< 10%) as % of total FITC-Ang II binding. Magnification: 40 X. AU: arbitrary fluorescence intensity unit. ** $p < 0.01$ vs. AT₁ binding. These are representative images from of 3 different experiments.

Figure 3. $[^{125}\text{I}]\text{-Val}^5\text{-Ang II}$ receptor binding characteristics in freshly isolated rat renal cortical nuclei. A: the maximal binding capacity ($B_{\max}$) and binding dissociation constant (K_d) with the insert showing Scatchard analysis. B: Ang II receptor subtype specificity showing the predominance of AT_1 receptor binding in isolated nuclei. ** $p < 0.01$ vs. total binding. These data were determined from 3 different experiments.

Figure 4. Identification of AT_{1a} and AT_{1b} receptor mRNAs in isolated rat renal cortical nuclei using semi-quantitative RT-PCR (see the Methods for details). M indicates the DNA ladder. The levels of AT_{1a} and AT_{1b} receptor mRNAs were normalized by the house-keeping gene GAPDH mRNA (bottom). ** $p < 0.01$ vs. AT_{1a} mRNA. These are representative images from 3 different experiments.

Figure 5.

AT₁ receptors mediated intracellular Ang II-induced in vitro [α -³²P]CTP incorporation into RNA in freshly isolated rat renal cortical nuclei. A: the concentration and effect relationship with a peak response at 10 nM of Ang II ($n = 6$ to 8 samples for each concentration). B: representative autoradiographs with transcriptional responses of four different nuclear samples to Ang II (10 nM) in the absence (middle) or presence of losartan (10 μ M) (right). C: semi-quantitative results on radioactivity from three different groups of nuclear samples ($n = 6-8$) of two repeated experiments. * $p < 0.05$; ** $p < 0.01$ vs. control nuclei and ++ $p < 0.01$ vs. Ang II-treated nuclei.

Figure 6.

AT₁ receptors mediated intracellular Ang II-induced RNA synthesis in freshly isolated rat renal cortical nuclei. A: representative agarose gel electrophoresis from three different experiments shows ethidium bromide-stained RNA extracted from control nuclei (lane 1), Ang II-stimulated nuclei (lane 2), and Ang II plus losartan-treated nuclei (lane 3). Equal amounts of RNA (5 μ g) from each sample or treatment were used. M indicates the RNA ladder of 0.24 to 9.5 kb. B: semi-quantitative results as arbitrary OD units from 3 different experiments. ^{**} $p < 0.01$ vs. control nuclei and ⁺⁺ $p < 0.01$ vs. Ang II-treated nuclei.

Figure 7.

Concentration-dependent transcriptional responses of transforming growth factor-β1 mRNA (TGF-β1) to intracellular Ang II in freshly isolated rat renal cortical nuclei. TGF-β1 mRNA is the expected size, based on the sense and antisense primers used (Table 1). * $p < 0.05$ or ** $p < 0.01$ vs. control nuclei; + $p < 0.05$ vs. previous Ang II concentration(s). N = 3 different experiments.

Figure 8.

AT₁ receptors mediated intracellular Ang II-induced in vitro transcription of TGF-β1 mRNA in freshly isolated rat renal cortical nuclei. Ang II significantly increased transcription of TGF-β1 mRNA in isolated nuclei, and the responses were blocked by losartan. ** $p < 0.01$ vs. control nuclei and ++ $p < 0.01$ vs. Ang II-treated nuclei. N = 3 different experiments.

Figure 9.

Effects of intracellular Ang II on in vitro transcription of angiotensinogen mRNA in freshly isolated rat renal cortical nuclei. Angiotensinogen mRNA is the expected size, as predicted from sense and antisense primers used (Table 1). Ang II and/or losartan had no effects on angiotensinogen mRNA transcription in the nuclei. N = 3 different experiments.

Figure 10.

AT₁ receptors mediated intracellular Ang II-induced in vitro transcription of macrophage chemoattractant protein-1 (MCP-1) in freshly isolated rat renal cortical nuclei. MCP-1 mRNA is the expected size, as predicted from the sense and antisense primers used (Table 1). Ang II significantly increased transcription of MCP-1 mRNA and the responses were blocked by losartan. ** $p < 0.01$ vs. control nuclei and ++ $p < 0.01$ vs. Ang II-treated nuclei. N = 3 different experiments.

Figure 11.

AT₁ receptors mediated intracellular Ang II-induced in vitro transcription of the sodium and hydrogen exchanger-3 (NHE-3) mRNA in freshly isolated rat renal cortical nuclei. NHE-3 mRNA is the expected size, as predicted from the sense and antisense primers used (Table 1). Ang II significantly increased transcription of NHE-3 mRNA and the responses were blocked by losartan. ** $p < 0.01$ vs. control nuclei and ++ $p < 0.01$ vs. Ang II-treated nuclei. N = 3 different experiments.

Table 1

Nucleotide sequences of forward (or sense) and reverse (or antisense) primers for semi-quantitative RT-PCR of target gene mRNAs.

Gene Name	Orientation	Sequence (5' – 3')	Reference
AT _{1a}	Sense	5'-CCAAAGTCACCTGCATCATC-3'	(24,44)
	Antisense	5'-CACAATCGCCATAATTATCCTA-3'	
AT _{1b}	Sense	5'-GTGGAGTGAGAGGGTTCAA -3'	(24,44)
	Antisense	5'-GACATTATTCAGGCAAGCTG-3'	
Angiotensinogen	Sense	5'-TTGTTGAGAGCTTGGGTCCCTTCA -3'	(26,27)
	Antisense	5'-CAGACACTGAGGTGCTGTTGTCCA -3'	
TGF- β 1	Sense	5'-AATACGTCAGACATTTCGGGAA-3'	(25)
	Antisense	5'-GTGGAGTACATTATCTTTGCT-3'	
MCP-1	Forward	5'-TCTACAGAAGTGCTTGAGGTGGTTG-3'	(4,5)
	Reverse	5'-CCTGTTGTTTCACAGTTGCTGCC-3'	
NHE-3	Sense	5'-GGAACAGAGGCGGAGGAGCAT-3'	(29)
	Antisense	5'-GAAGTTGTGTGCCAGATTCT-3'	
GAPDH	Forward	5'-TCTACAGAAGTGCTTGAGGTGGTTG-3'	(5)
	Reverse	5'-CCTGTTGTTTCACAGTTGCTGCC-3'	