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Posttranscriptional mechanisms contribute to osmotic regulation of ANG type 1 receptors in cultured rat renomedullary interstitial cells

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Lee, Sunghou, Zheng Wu, Kathryn Sandberg, S-E. Yoo, and Christine Maric. Posttranscriptional mechanisms contribute to osmotic regulation of ANG type 1 receptors in cultured rat renomedullary interstitial cells. Am J Physiol Regul Integr Comp Physiol 290: R44–R49, 2006.—Previously, we showed that ANG II receptors in cultured rat renomedullary interstitial cells (RMICs) are osmatically regulated (19). The current study examined the mechanisms underlying this osmotic regulation in RMICs cultured in isosmotic (300 mosmol/kgH2O) and hyperosmotic (600 mosmol/kgH2O) conditions. Radioligand competition analysis coupled with RNase protection assays (RPA) and ligand-mediated receptor internalization studies revealed that RMICs primarily express the type 1a angiotensin receptor (AT1aR). When cultured under hyperosmotic conditions, the density (Bmax) of AT1R in RMIC membranes decreased by 31% [Bmax (pmol/mg protein): 300 mosmol/kgH2O, 6.44 ± 0.46 vs. 600 mosmol/kgH2O, 4.42 ± 0.37, n = 8, P < 0.01], under conditions in which no detectable changes in AT1aR mRNA expression or in the kinetics of ligand-mediated AT1R internalization were observed. RNA electromobility shift assays showed that RNA protein complex (RPC) formation between RMIC cytosolic RNA binding proteins and the 5′ leader sequence (5′LS) of the AT1aR was increased 1.5-fold under hyperosmotic conditions [5′LS RPC (arbitrary units): 300 mosmol/kgH2O, 0.79 ± 0.08 vs. 600 mosmol/kgH2O, 1.17 ± 0.07, n = 4, P < 0.01]. These results suggest that the downregulation of AT1aR expression in RMICs cultured under hyperosmotic conditions is regulated at the posttranscriptional level by RNA binding proteins that interact within the 5′LS of the AT1aR mRNA. The downregulation of AT1aR expression under hyperosmotic conditions may be an important mechanism by which the activity of ANG II is regulated in the hyperosmotic renal medulla.

angiotensin II; angiotensin receptors; osmolality; posttranscriptional regulation; RNA binding proteins

THE RENIN-ANGIOTENSIN SYSTEM (RAS) plays an important role in regulating systemic blood pressure and fluid and electrolyte balance (4, 21). Although more than one peptide has thus far been reported to mediate the physiological action of the RAS in the kidney (1), still to date, the main active peptide of the RAS is ANG II. ANG II mediates its actions through a number of receptor subtypes; however, the majority of its actions are mediated through the AT1 receptor subtype (AT1R). In the kidney, AT1Rs are widely distributed: they are localized to cortical mesangial cells and proximal tubules (11) and renomedullary interstitial cells (RMICs) (35). Given the localization of AT1Rs in both the renal cortex and medulla, we do not commonly think that the mechanisms that regulate their expression and activity may be different. As a result of functional urine-concentrating mechanisms, renal medullary cells are normally exposed to very high extracellular concentrations of NaCl (6). It is thus conceivable that these constant fluctuations in extracellular osmolality will affect the activity of ANG II and will contribute to the regulation of its cell surface receptor. To date, very little is known about the osmotic regulation of AT1Rs in the renal medulla.

Because of the technical difficulties in examining the direct physiological role of cells residing in the renal inner medulla, including RMICs, our knowledge in this regard is rather limited. Thus far, RMICs have been proposed to play an important role in regulating renal medullary structure and function under both physiological and pathophysiological conditions (17, 18, 23). RMICs express receptors for a number of vasoactive peptides, including ANG II (AT1) (36), atrial natriuretic peptide (ANP: NPRA and NPRB) (8), endothelin (ET-1: ETA and ETB) (32), and bradykinin (5). The fact that RMICs express these receptors suggests that their role in the renal medulla is not merely to provide structural support to medullary nephron segments and collecting ducts but may well play an active physiological role in regulating renal medullary function.

We have previously reported that AT1Rs in cultured RMICs are sensitive to changes in extracellular osmolality (19). When cultured under hyperosmotic conditions, binding of ANG II to AT1Rs is reduced compared with isoosmotic conditions. However, the underlying mechanisms for the osmotic sensitivity and regulation of AT1Rs are not as yet understood. The current study investigated the molecular mechanisms underlying the osmotic regulation of AT1Rs in cultured RMICs.

METHODS

Materials. 125I-labeled [Sar1,Ile8]ANG II (2,176 Ci/mmol) was purchased from Peptide Radioiodination Center (Pullman, WA). ANG II (human) and [Sar1,Val5,Ala8]ANG II (saralasin) were purchased from Sigma (St. Louis, MO), and PD123319 was purchased from Research Biochemical International (Natick, MA). The nonpeptide AT1R antagonist SK1080 was obtained from the Korea Research Institute of Chemical Technology, Taejon, South Korea.

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Culture of RMICs. RMICs were isolated from the kidneys of Sprague-Dawley rats and characterized according to the method described previously (18). The cells were maintained at 37°C in a humidified atmosphere of 95% air-5% CO2 in isosmotic (300 mosmol/kg H2O) culture medium RPMI 1640 (Biofluids, Rockville, MD) containing 25 mM HEPES and 0.27 g/5 ml insulin. Cells between passages 10 and 25 were used in all experiments. Cell viability was assessed by determining levels of lactate dehydrogenase released into the media (Roche Molecular Biochemicals, Indianapolis, IN).

Manipulation of culture media osmolality. Cells were plated at a density of 1 × 10⁵ cells/cm² in culture medium RPMI 1640 (300 mosmol/kg H2O) and allowed to attach for 24 h. The osmolality of the media was then either kept at 300 mosmol/kg H2O or increased to 600 mosmol/kg H2O by addition of NaCl (80 mM) and mannitol (100 mM). Our preliminary studies determined that using either NaCl or mannitol alone as an osmotic stimulus resulted in similar cell responses (e.g., ANG II binding to AT1 receptors) compared with the combination of NaCl and mannitol (unpublished observations). We chose to use the combined NaCl and mannitol because cell viability was better preserved using this combination stimulus. The osmolality of the culture media was verified by the freezing point depression method using a Micro-Osmometer (Advanced Instruments, Norwood, MA). After changes in media osmolality, the cells were grown for 24 h; then they were harvested or further analysis was performed, as described below.

Preparation of membrane fractions for radiolabeled ligand binding. The cells were first rinsed with phosphate-buffered saline (PBS) at room temperature. All further steps were performed on ice at 4°C. The cells were scraped, washed with phosphate-buffered saline, pelleted, and homogenized in membrane homogenization buffer [10 mM Tris (pH 7.2), 3 mM MgCl2, and 100 mM NaCl] (29). The homogenate was centrifuged at 100,000 × g for 1 h, and the resulting supernatant was further centrifuged at 44,000 × g for 60 min. After the final pellet was resuspended in membrane binding buffer (10 mM Tris (pH 7.2) containing 3 mM MgCl2), the protein content was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Radiolabeled ligand binding. For the equilibrium binding analysis, membrane fractions (5 μg) were incubated with increasing concentrations of [125I]-labeled [Sar1, Ile8]ANG II (0.05–4 nM) in membrane binding buffer containing 0.2% BSA for 1 h at room temperature. Specific binding of [125I]-labeled [Sar1, Ile8]ANG II was defined as the total binding minus nonspecific binding. Nonspecific binding was determined experimentally in the presence of 1 μM unlabeled saralasin. In competition experiments, membrane fractions were incubated with increasing concentrations of specific receptor antagonists in membrane binding buffer containing 0.2% BSA and 0.3–0.5 nM [125I]-labeled [Sar1, Ile8]ANG II.

Binding reactions were terminated by the addition of ice-cold membrane binding buffer and rapid filtration through glass fiber filters (GF/C Whatman, presoaked with assay buffer) using a Brandel cell harvester (Brandel M-24R, Rockville, MD). The filters were washed with ice-cold membrane binding buffer, and membrane-bound radioactivity that was trapped on the filters was measured in a Packard Cobra II γ-counter. The dissociation constant (Kd) and the maximum number of receptor-specific binding sites (Bmax) were calculated from nonlinear regression analysis using a software program PRISM (GraphPad Software, San Diego, CA). The inhibitory constants (Ki) for angiotensin receptor subtype-specific antagonists were determined by calculating the half-maximum inhibitory dose derived from nonlinear curve fitting.

Ribonuclease protection assay. The cells were washed with PBS at room temperature, and total RNA was isolated using Tri reagent (Molecular Research Center, Cincinnati, OH). AT1R mRNA in RMICs was measured by ribonuclease protection assay (RPA) using RPA III (Ambion, Austin, TX) according to the manufacturer’s protocol. The specific AT1R cRNA probe (20,000 cpm), which produces a protected fragment of 95 bases, was hybridized with 10 μg RMIC total RNA at 42°C for 16 h, as described previously. The pTRI-actin (mouse antisense control template; Ambion, Austin, TX) was used to make a 304-base cRNA probe, which produces a protected fragment of 245 bases with T7 RNA polymerase and γ-[32P]CTP by in vitro transcription. RNase digestion with RNase T1 was carried out at 37°C for 30 min. After the precipitation of protected fragments, the samples were separated by electrophoresis on a denaturing 5% polyacrylamide gel. After electrophoresis, the gel was transferred to filter paper, dried, exposed to Phosphor imager screen, and the total abundance of AT1R mRNA was quantitated by Image-Quan software (IQMac V1.2). The expression level of AT1R mRNA normalized to β-actin levels in the same sample.

Receptor-ligand internalization assay. Subconfluent RMICs were incubated in isosmotic (300 mosmol/kg H2O) or hyperosmotic (600 mosmol/kg H2O) media for 24 h. The cells were then collected and plated at 2 × 10⁵ cells/well in a 24-well plate and allowed to attach for 3 h. The receptor-ligand internalization assay was then carried out as previously described (7). After the cells were washed twice with serum-containing RPMI 1640, the cells were incubated in cell binding buffer [50 mM Tris (pH 7.8), 120 mM NaCl, 4 mM KCl, 5 mM MgCl2, 2 mg/ml bovine serum albumin (BSA) and 0.5 μM of [125I]-labeled [Sar1, Ile8]ANG II for 5–40 min at 37°C followed by rapid chilling on ice. At the indicated times, the cells were washed four times with cell binding buffer and incubated for 4 min with or without acetic acid solution [5 mM ice cold acetic acid in 150 mM NaCl, pH 2.5]. The cells were then solubilized with 1% SDS, 1% Triton X-100 in 10 mM Tris-HCl, pH 8.0, and radioactivity was counted in a Packard Cobra II γ-counter. After subtraction of nonspecific binding, results were expressed as the ratio [AT1]i/[AT1]s, based on [AT1]i= [AT1]s × [AT1]ki, or [AT1]i/[AT1]s = kki/kki, where [AT1]i and [AT1]s are internal and surface [125I]-[Sar1, Ile8]ANG II, respectively, and kki and kki are the internalization and exocytic rate constants. In parallel experiments, the protein concentration of each well was measured using the Bio-Rad DC protein assay.

Preparation of labeled AT1R 5′ leader sequence cRNA. The 5′ leader sequence (5′/LS) (271 nt) of the rat AT1R cDNA was subcloned into the pcRI vector (InVitrogen, San Diego, CA) by TA cloning. The 5′LS plasmid was linearized using the restriction enzyme XhoI. Radiolabeled 5′LS cRNA was prepared using T7 RNA polymerase and γ-[32P]GTP by in vitro transcription (Promega, Madison, WI) for the synthesis of a single-stranded RNA probe (385 bases) with high specific activity. After electrophoresis in an 8 M urea-5% polyacrylamide gel, the RNA probe was eluted from the gel in elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.1% sodium dodecylsulfate) for 1–2 h at 60°C. The radiolabeled cRNA was precipitated with 7.5 M ammonium acetate and ethanol and resuspended in diethyl pyrocarbodi-treated water.

Preparation of cytosolic extracts for RNA electromobility shift assay. The cells were scraped and homogenized in cell homogenization buffer (25 mM Tris buffer, pH 7.4, containing 0.1 mM EDTA, 40 mM KCl, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.2 unit/ml aprotinin and 10 μg/ml antipain). The homogenates were centrifuged at 300,000 × g for 10 min at 4°C, and the supernatant was then centrifuged at 20,000 × g for 20 min at 4°C. The supernatant was layered on top of a 30% sucrose cushion solution containing 10 mM Tris (pH 7.6), 1 mM potassium acetate, 1.5 mM magnesium acetate and 2 mM diithiothreitol (DTT) and centrifuged at 230,000 × g for 3 h at 4°C. The supernatant was collected, concentrated 2-fold with Microcon YM-10 (Millipore, Bedford, MA), and the protein content determined using the Bio-Rad Protein Assay.

RNA electromobility shift assay. Cytosolic extracts (10–30 μg), were incubated with 10⁶ cpm of 32P-labeled 5′LS cRNA probe, 10 mM DTT, 40 units RNase inhibitor, 10× binding buffer [100 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES, pH 7.6), 400 mM KCl, 30 mM MgCl2, and 50% glycerol] for 20 min at 30°C. T1 RNase (4 U) and heparin sulfate (100 μg) were added to the
reaction and incubated for 15 min. The samples were then electrophoresed at 200 volts in a 4% polyacrylamide gel, the gel was exposed to a Phosphor imager screen, and RNA binding protein was quantitated by ImageQuant software (IQMac V1.2).

Statistical analysis. Results are expressed as means ± SE. Statistical significance was evaluated by unpaired Student’s t-test except for the internalization studies, where one-way-ANOVA with Student-Neuman-Keuls multiple comparison test was used. P < 0.05 was considered statistically significant.

RESULTS

AT₁R binding in RMIC membranes. Equilibrium radioligand binding studies using the dual AT₁R and AT₂R peptide ligand ¹²⁵I-labeled [Sar¹, Ile⁸]ANG II were performed on membranes prepared from RMICs that were cultured under isoosmotic conditions (300 mosmol/kgH₂O). Specific binding of ¹²⁵I-labeled [Sar¹, Ile⁸]ANG II increased in a linear manner between 1 and 30 µg membrane protein and reached saturation by 1 h at room temperature (data not shown). All subsequent binding experiments were performed on 5 µg membrane protein with a 1-h incubation period at room temperature. A representative equilibrium binding curve is shown in Fig. 1A along with Scatchard transformation of the data (Fig. 1B). Scatchard analysis revealed that the ligand interacts with a single population of receptor sites with a Kₐ of 0.48 ± 0.04 nM and a Bₘₐₓ of 6.44 ± 0.46 pmol/mg protein.

Radioligand competition experiments were also performed. Membranes prepared from RMICs cultured under isoosmotic conditions, were incubated with ¹²⁵I-labeled [Sar¹, Ile⁸]ANG II and the AT₁R specific nonpeptide antagonist, SK1080 or the AT₂R-specific nonpeptide antagonist, PD123319 (Fig. 1C). PD123319 was a poor competitor of ¹²⁵I-labeled [Sar¹, Ile⁸]ANG II binding (Kᵢ > 10 µM), whereas SK1080 completely and potently abolished the ¹²⁵I-labeled [Sar¹, Ile⁸]ANG II binding (Kᵢ = 2.39 ± 0.13 nM). These data suggest that the vast majority of ANG II receptors expressed on RMIC membranes cultured under isoosmotic conditions are of high affinity and of the AT₁R subtype.

Effect of osmolality on AT₁R binding in RMIC membranes. To examine the effects of an osmotic challenge on AT₁R receptor binding, RMICs were cultured in a hyperosmotic media at 600 mosmol/kgH₂O. Scatchard transformation (Fig. 1B) of saturation binding curve data (Fig. 1A) revealed that the density of AT₁R binding sites was decreased by 33% in RMICs cultured under hyperosmotic conditions compared with isoosmotic conditions (Bₘₐₓ = 4.42 ± 0.37 pmol/mg protein, P < 0.01). No changes in ligand binding affinity (Kₐ = 0.56 ± 0.03 nM) were observed under hyperosmotic conditions.

To determine whether changes in media osmolality altered ANG II receptor subtype expression, radioligand competition studies were performed. PD123319 remained a poor competitor of ¹²⁵I-labeled [Sar¹, Ile⁸]ANG II binding (Kᵢ > 10 µM), whereas SK1080 potently abolished ¹²⁵I-labeled [Sar¹, Ile⁸] ANG II binding at inhibitory concentrations (Kᵢ = 3.70 ± 1.2 nM) that were indistinguishable from concentrations observed in membranes prepared from RMICs cultured under isoosmotic conditions (Fig. 1C), suggesting that even under hyperosmotic conditions, the predominant receptor subtype expressed in RMICs is the AT₁R.

Fig. 1. Angiotensin receptor binding in rat renomedullary interstitial cells (RMICs) cultured under isoosmotic and hyperosmotic conditions. A: ¹²⁵I-labeled [Sar¹, Ile⁸]ANG II saturation binding curves on membranes prepared from RMICs grown under isoosmotic (300 mosmol/kgH₂O) (●) and hyperosmotic (600 mosmol/kgH₂O) (○) conditions. B: linear Scatchard transformation of the saturation binding curves. C: competition binding analysis in the presence of SK1080 (AT₁R antagonist) (●, ○) or PD123319 (AT₂R antagonist) (●, ◆) on membranes prepared from RMICs grown under isoosmotic (●, ●) and hyperosmotic conditions (○, ◆). Data are expressed as the means ± SE from three experiments each performed in duplicate.

No significant differences in the percentage of nonviable cells were observed between isoosmotic and hyperosmotic conditions (data not shown).

Effect of osmolality on AT₁R mRNA expression in RMICs. The levels of AT₁R mRNA expression in RMICs were determined by an RPA that we previously developed, which differentiates between the AT₁A and AT₁B receptor mRNA subtypes (33). To test the dose response of extracted total RNA, the specific AT₁A cRNA probe (20,000 cpm) was hybridized with total RNA (2–30 µg) from RMICs with β-actin as an internal control. The ratio of the signal in AT₁A cRNA to β-actin was unchanged up to 10 µg under these experimental conditions (data not shown). Therefore, 10 µg of total RNA was used to compare the steady-state level of AT₁R mRNA in RMICs cultured under isoosmotic and hyperosmotic conditions. The
levels of AT1aR mRNA expression when normalized to β-actin were not significantly different in RMICs cultured under isosmotic and hyperosmotic conditions [AT1aR/β-actin (arbitrary units): 300 mosmol/kgH2O, 0.17 ± 0.01 vs. 600 mosmol/kgH2O, 0.13 ± 0.01, n = 4] (Fig. 2). No AT1bR mRNA was detected in cultured RMICs by RPA (data not shown).

Effect of osmolality on AT1R internalization in RMICs. To determine whether osmolality alters the degree of ligand-mediated AT1R endocytosis, receptor-ligand internalization studies were performed in RMICs cultured under isosmotic and hyperosmotic conditions (Fig. 3). Total specific bound radioligand typically accounted for 85–95% of total surface bound radioligand (acid-sensitive fraction), indicating that the majority of AT1R were internalized by 40 min. The sum of specific membrane-bound or internalized radioligand did not exceed 10% of the total radioligand added per well, so the amount of available radioligand was not limiting. Under these experimental conditions, there were no significant differences in the degree of receptor internalization in RMICs cultured under isosmotic or hyperosmotic conditions [endocytosis rate constant (min−1): 300 mosmol/kgH2O, 0.076 ± 0.008 vs. 600 mosmol/kgH2O, 0.043 ± 0.012].

Effect of osmolality on AT1R RNA-protein complex formation in RMICs. We have previously shown that alterations in the levels of cytosolic proteins, which bind to the 5′LS of the AT1aR are associated with inverse changes in AT1aR protein expression (15, 16, 22). To determine whether osmolality alters the levels of RNA-protein complex (RPC) formation in the 5′LS in RMICs, we performed RNA EMSAs with radiolabeled 5′LS on cytosolic extracts prepared from RMICs cultured under isosmotic and hyperosmotic conditions (Fig. 4A). The degree of 5′LS RPC formation was increased by 1.5-fold in RMICs cultured under hyperosmotic conditions compared with cells cultured under isosmotic conditions, [5′LS RPC (AU): 300 mosmol/kgH2O, 0.79 ± 0.08 vs. 600 mosmol/kgH2O, 1.17 ± 0.07; n = 4, P < 0.05].

DISCUSSION

The present study demonstrates that under hyperosmotic conditions, the density of AT1Rs on cell membranes of cultured RMICs is decreased, under conditions in which AT1aR mRNA expression and agonist-mediated AT1R internalization are not detectably altered. Additionally, we provide evidence suggesting that high osmolality regulates AT1aR expression, in part, by a posttranscriptional mechanism involving cytosolic protein binding within the 5′LS of the AT1aR mRNA.

Fig. 2. AT1aR mRNA levels in RMICs cultured under isosmotic and hyperosmotic conditions. AT1aR mRNA levels were determined by RNase protection assay (RPA). A: electrophoresis profile of the RPA. Lane 1, negative controls (no RNA); lane 2, AT1aR and β-actin RNA probes without hybridization to RMIC RNA; lane 3, AT1aR and β-actin RNA probes hybridized with mRNA isolated from RMICs grown under isosmotic conditions (300 mosmol/kgH2O); lane 4, AT1aR and β-actin RNA probes hybridized with mRNA isolated from RMICs grown under hyperosmotic conditions (600 mosmol/kgH2O); and lane 5 RNA size markers. B: AT1aR mRNA levels in RMICs grown under isosmotic and hyperosmotic conditions determined by RPA and expressed in arbitrary units normalized to the β-actin. The data are expressed as the means ± SE and represent four experiments performed in duplicate.

Fig. 3. 125I-labeled [Sar1,Ile8]ANG II-AT1R internalization kinetics in RMICs cultured under isosmotic and hyperosmotic conditions. A: time course of 125I-labeled [Sar1,Ile8]ANG II-AT1R internalization kinetics in RMICs cultured under isosmotic (300 mosmol/kgH2O) and hyperosmotic (600 mosmol/kgH2O) conditions expressed as the amount of 125I-labeled [Sar1,Ile8]ANG II-AT1R binding as a function of time. B: ratio of internalized radioligand to cell surface 125I-labeled [Sar1,Ile8]ANG II-AT1R binding as a function of time in RMICs grown under isosmotic (○) and hyperosmotic (●) conditions. Values shown are means ± SE obtained from three independent experiments performed in triplicate.
gests that osmolality regulates AT1R density independently of receptor desensitization, oligomerization, and receptor cycling. However, these findings do not rule out the possibility that posttranslational mechanisms such as phosphorylation is followed by recycling of AT1Rs to the cell surface, leading to interactions with many proteins in-...
by which the activity of ANG II is fine-tuned depending on the underlying hydration and salt intake.

In conclusion, the present data suggest that AT1R membrane density in RMICs is reduced under hyperosmotic conditions through a posttranscriptional regulatory mechanism that involves proteins that bind within the 5'UTR of the AT1R mRNA. Therefore, posttranscriptional regulation of AT1R expression by RNA binding proteins in RMICs may contribute to regulation of renal function under both physiological and pathophysiological conditions.

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