Published in final edited form as:
J Hypertens. 2012 June ; 30(6): 1176–1184. doi:10.1097/HJH.0b013e3283532099.

Angiotensin II AT_2 receptor decreases AT_1 receptor expression and function via nitric oxide/cGMP/Sp1 in renal proximal tubule cells from Wistar–Kyoto rats

Jian Yang[a,b,*], **Caiyu Chen**[a,b,*], **Hongmei Ren**[a,b], **Yu Han**[a,b], **Duofen He**[a,b], **Lin Zhou**[a,b], **Ulrich Hopfer**[c], **Pedro A. Jose**[d], and **Chunyu Zeng**[a,b]

[a]Department of Cardiology, Daping Hospital, The Third Military Medical University, Chongqing

[b]Chongqing Institute of Cardiology, Chongqing, P.R. China

[c]Department of Physiology and Biophysics, Case Western Reserve School of Medicine, Cleveland, Ohio

[d]Division of Nephrology, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland, USA

Abstract

Background—The renin–angiotensin (Ang) system controls blood pressure, in part, by regulating renal tubular sodium transport. In the kidney, activation of the angiotensin II type 1 (AT_1) receptor increases renal sodium reabsorption, whereas the angiotensin II type 2 (AT_2) receptor produces the opposite effect. We hypothesized that the AT_2 receptor regulates AT_1 receptor expression and function in the kidney.

Methods and results—In immortalized renal proximal tubule (RPT) cells from Wistar–Kyoto rats, CGP42112, an AT_2 receptor agonist, decreased AT_1 receptor mRNA and protein expression ($P < 0.05$), as assessed by reverse transcriptase-polymerase chain reaction and immunoblotting. The inhibitory effect of the AT_2 receptor on AT_1 receptor expression was blocked by the AT_2 receptor antagonist, PD123319 (10^{-6} mol/l), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (10^{-4} mol/l), or the nitric oxide-dependent soluble guanylate cyclase inhibitor 1H-[1,2,4] oxadiazolo-[4,3-a] quinoxalin-1-one (10^{-5} mol/l), indicating that both nitric oxide and cyclic guanosine monophosphate (cGMP) were involved in the signaling pathway. Furthermore, CGP42112 decreased Sp1 serine phosphorylation and reduced the binding of Sp1 to AT_1 receptor DNA. Stimulation with Ang II (10^{-11} mol/l per 30 min) enhanced Na^+-K^+-ATPase activity in RPT cells, which was prevented by pretreatment with CGP42112 (10^{-7} mol/l per 24 h) ($P < 0.05$). The above-mentioned results were confirmed in RPT cells from AT_2 receptor knockout mice; AT_1 receptor expression and Ang II-stimulated Na^+-K^+-ATPase activity were greater in these cells than in RPT cells from wild-type mice ($P < 0.05$). AT_1/AT_2 receptors co-localized and co-immunoprecipitated in RPT cells; short-term CGP42112 (10^{-7} mol/l per 30 min) treatment increased AT_1/AT_2 receptor co-immunoprecipitation ($P < 0.05$).

Conclusions—These results indicate that the renal AT_2 receptor, via nitric oxide/cGMP/Sp1 pathway, regulates AT_1 receptor expression and function, which may be important in the regulation of sodium excretion and blood pressure.

Correspondence to Chunyu Zeng, MD, PhD, Department of Cardiology, Daping Hospital, The Third Military Medical University, Chongqing City, 400042, P.R. China. Tel: +86 23 68757808; fax: +86 23 68757808; chunyuzeng01@163.com.
*Jian Yang and Caiyu Chen contributed equally to this work.

Conflicts of interest There are no conflicts of interest (financial or otherwise).

Keywords

AT_1 receptor; AT_2 receptor; hypertension; renal proximal tubule cells

INTRODUCTION

Essential hypertension is a major risk factor for stroke, myocardial infarction, heart failure, and kidney failure. The kidney plays a major role in the long-term control of blood pressure by regulation of sodium excretion [1]. Patients with polygenic essential hypertension have increased sodium transport in the renal proximal tubule and medullary thick ascending limb, which are regulated by numerous hormones and humoral factors, including angiotensin (Ang) II [2]. Ang II regulates sodium excretion via several Ang receptor subtypes [angiotensin II type 1 (AT_1), type 2 (AT_2), and type 4 (AT_4)] [3]; stimulation of AT_1 receptor increases sodium reabsorption, whereas AT_2 receptor produces the opposite effect [2,4].

The AT_2 receptor is ubiquitously expressed in fetal tissues but is also expressed in adult tissues [5,6], including kidney and artery. Apart from an AT_2 receptor-mediated natriuretic effect, the AT_2 receptor also promotes vasodilation and apoptosis of vascular smooth muscle cells (VSMCs) [7,8]. Activation of the AT_2 receptor decreases AT_1 receptor expression in VSMCs [9]. Aortic AT_1 receptor expression and vascular response to Ang II are greater in AT_2 receptor knockout than wild-type mice [10]. Therefore, these effects of AT_2 receptor may counter the AT_1 receptor-mediated effect on blood pressure. However, whether or not the AT_2 receptor regulates AT_1 receptor expression and function in kidney is not known. We hypothesized that activation of the AT_2 receptor may negatively regulate AT_1 receptor expression and function in renal proximal tubule (RPT) cells. Therefore, in the present study, we studied the regulation of AT_1 receptor expression by the AT_2 receptor in RPT cells from Wistar–Kyoto rats and AT_2 receptor knockout mice.

MATERIALS AND METHODS

Cell culture

Immortalized RPT cells from 4–8-week-old Wistar–Kyoto rats were cultured at 37°C in 95% air and 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium/F-12 culture media, as previously described [11,12]. The cells (80% confluence) were extracted in ice-cold lysis buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/l EDTA, 1 mmol/l ethylene glycol tetraacetic acid (EGTA), 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin], sonicated, kept on ice for 1 h, and centrifuged at 16 000*g* for 30 min. All samples were stored at −70°C until use.

Immunoblotting

The antibodies are polyclonal purified antipeptides. The amino acid sequence of the immunogenic peptide (rabbit antihuman AT_1 receptor antibody) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) is QDDCPKAGRHC, amino acids 15–24 of the AT_1 receptor. The goat AT_2 receptor antibody (Santa Cruz Biotechnology Inc.) corresponds to the C-terminal cytoplasmic domain of the AT_2 receptor. Rat RPT cells were treated with vehicle (dH_2O) or the AT_2 receptor agonist, CGP42112 (Sigma, St Louis, Missouri, USA), at the indicated concentrations and time points. Immunoblotting was performed as previously reported [13,14], except that the transblots were probed with the AT_1 receptor antibody (1 : 400) and the AT_2 receptor antibody (1 : 400). The amount of protein transferred onto the membranes

was determined by immunoblotting for α-actin (Santa Cruz Biotechnology Inc.). The receptor densities were normalized by α-actin.

Reverse transcriptase-polymerase chain reaction of AT_1 receptors

A total of 2 μg of total RNA extracted from RPT cells was used to synthesize cDNA and served as a template for amplification of AT_1 or AT_2 receptor and βactin which served as the house-keeping gene control. For β-actin, the forward primer was 5′-GTGGGTATGGGTCAGAAGGA-3′ and the reverse primer was 5′-AGCGCGTAACCCTCATAGAT-3′ (GenBank accession no. BC 063166). For the AT_1 receptor, the forward primer was 5′-CCAAAGTCACCTGCATCATC-3′ and the reverse primer was 5′-CACAATCGCCATAATTATCCTA-3′ (GenBank accession no. NM-030985). The amplifications were both performed with the following conditions: 35 cycles of denaturation at 94°C for 2 min, annealing for 30 s at 52.5°C, and extension for 45 s at 72°C. For AT_2 receptor, the forward primer was 5′-CT TCAATCTGGCTGTGGC-3′ and the reverse primer was 5′-GCATATTTCTCAGGTGGG-3′ (GenBank accession no. NM-012494). The amplification was performed with the following conditions: 35 cycles of denaturation at 95°C for 2 min, annealing for 30 s at 57°C, and extension for 45 s at 72°C. The AT_1 receptor and AT_2 receptor mRNA expressions were normalized by β-actin mRNA [15].

Immunoprecipitation

Renal proximal tubule cells were incubated with vehicle or CGP42112 (10^{-7} mol/l) for 30 min, as described above. The cells were lyzed with ice-cold lysis buffer for 1 h and centrifuged at 16 000*g* for 30 min. Equal amounts of lysates were incubated with affinity-purified antibodies (1.0 μg/ml) for 1 h and G-protein agarose at 4°C for 12 h. The immunoprecipitates were pelleted and washed four times with lysis buffer. The pellets were suspended in sample buffer, boiled for 10 min, and subjected to immunoblotting with antibodies.

In one series of experiments, the sample was immunoprecipitated with the AT_2 receptor antibody and then immunoblotted with the AT_1 receptor antibody. In another series of experiments, the sample was immunoprecipitated with Sp1 antibody (Millipore Co., Bedford, Massachusetts, USA) or rabbit antihuman NHE3 antibody (Santa Cruz Biotechnology Inc.) and then immunoblotted with antiphosphoserine antibody (Zymed Laboratories, San Francisco, California, USA). The bands were quantified by densitometry, using Quantiscan as previously reported [16].

Na^+-K^+-ATPase activity assay

Na^+-K^+-ATPase activity was determined as the amount of inorganic phosphate released in the presence or absence of ouabain, as described previously [13,17]. To prepare membranes for assay of Na^+-K^+-ATPase activity, RPT cells, cultured in 21-cm^2 plastic culture dishes, were washed twice with 5 ml chilled phosphate-free buffer (2.36 mmol/l NaCl, 0.54 mmol/l $NaHCO_3$, 0.4 mmol/l KCl, and 0.12 mmol/l $MgCl_2$), and centrifuged at 3000*g* for 10 min. The cells were then placed on ice and lyzed in 2 ml of lysis buffer (1 mmol/l $NaHCO_3$, 2 mmol/l $CaCl_2$ and 5 mmol/l $MgCl_2$). Cell lysates were centrifuged at 3000*g* for 2 min to remove insoluble debris. The supernatant was suspended in an equal volume of 1 mol/l NaI, and the mixture was centrifuged at 48 000*g* for 25 min. The pellet (membrane fraction) was washed twice and re-suspended in 10 mmol/l Tris with 1.0 mmol/l EDTA (pH 7.4). Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, California, USA) and adjusted to 1 mg/ml. The membranes were stored at −70°C until further use.

To measure Na^+-K^+-ATPase activity, 100 μl aliquots of the membrane fraction were added to 800 μl reaction mixture (75 mmol/l NaCl, 5 mmol/l KCl, 5 mmol/l $MgCl_2$, 6 mmol/l sodium azide, 1 mmol/l Na_4EGTA, 37.5 mmol/l imidazole, 75 mmol/l Tris HCl, and 30 mmol/l histidine; pH 7.4), with or without 1 mmol/l ouabain (final volume 1 ml), and preincubated for 5 min in a water bath at 37°C. Reactions were initiated by adding Tris-ATP (4 mmol/l) and terminated after 15 min of incubation at 37 °C by adding 50 μl of 50% trichloracetate. For the determination of ouabain-insensitive ATPase activity, NaCl and KCl were omitted from the reaction mixtures containing ouabain. The amount of phosphate produced was quantified by adding to the reaction mixture to 1 ml of coloring reagent (10% ammonium molybdate in 10N sulfuric acid+ferrous sulfate). After thorough admixture and centrifugation at 3000*g* for 10 min, the amount of phosphomolybdate in the supernatant was quantified spectrophotometrically at 740 nm against a standard curve prepared from K_2HPO_4. Na^+-K^+-ATPase activity was estimated as the difference between total and ouabain-insensitive ATPase activity and expressed as mmol phosphate released per μg protein per min.

Cell surface Na^+-K^+-ATPase protein expression

Cultured rat RPT cells were starved in serum-free medium for 2 h and then treated with the AT_2 receptor agonist CGP42112 (10^{-7} mol/l) for 24 h or Ang II (10^{-11} mol/l) for 30 min. Cell impermeable, noncleavable sulfosuccinimidyl-6-(biotinamido) hexanoate (final concentration 250 μg/ml) was added into the medium 20 min before the end of the drug treatment [18]. The cells were washed three times (ice-cold PBS), lyzed with a lysis buffer, sonicated, and placed on ice for 1 h. The supernatants from the cell lysates were immunoprecipitated with the anti-Na^+-K^+ ATPase α1 subunit (Santa Cruz Biotechnology Inc.) or rabbit antihuman NHE3 and then subjected to immunoblotting. The membrane sheets were blocked with 10% milk in wash buffer for 1 h, washed with wash buffer three times, and incubated with peroxidase-conjugated streptavidin (1 : 5000 dilution, 30 min; Jackson Immuno-Research Laboratory). The biotinylated protein bands were visualized by enhanced chemiluminescence. The intensity of the band represents Na^+-K^+-ATPase α1 subunit expression or NHE3 expression at the cell surface membrane.

Confocal microscopy of double-stained renal proximal tubule cells

Renal proximal tubule cells, grown on coverslips, were fixed and permeabilized with 100% methanol (30 min). Reactions with antibodies were performed as described previously [18]. The AT_2 receptor (1 : 300) was visualized using an IgG affinity-purified polyclonal goat antihuman AT_2 receptor antibody followed by Alexa Fluor 568-rabbit antigoat IgG antibody (red; Molecular Probes; Eugene, Oregon, USA). The AT_1 receptor was visualized using an IgG affinity-purified polyclonal rabbit antihuman AT_1 receptor antibody (1 : 300), followed by fluorescein isothiocyanate-conjugated goat antirabbit IgG antibody (green; Molecular Probes). Immunofluorescence images were acquired (Olympus AX70 laser confocal microscopy) at an excitation wavelength of 480 and 560 nm; emission was detected at 535 and 645 nm. Cells that were treated with only Alex 488-goat antirabbit IgG or Alexa Fluor 568-rabbit antigoat IgG antibodies revealed no immunofluorescence, and omission of the anti-AT_1 receptor antibody showed no red or yellow color after merging the images (data not shown).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed with the Light-shift Chemiluminescent EMSA Kit (Pierce Chemical Co., Rockford, Illinois, USA) according to manufacturer's recommendations [19]. The system, using a nonisotopic method to detect DNA–protein interactions, includes an enhanced luminol substrate for horseradish peroxidase with optimized blocking and washing buffers that together produce sensitivity

equivalent to radioactive (^{32}P) systems. A synthetic DNA double-stranded oligonucleotide probe containing the sequence of the rat AT_1 receptor gene promoter between nucleotides −40 and −6 bp (5′-GGAACCTGCAGAGCAGCGACGCCCCCTAGGCTATA-3′, 3′-CCTTGGACGTCTCGT CGCTGCGGGGGATCCGATAT-5′, containing Sp1 site) was labeled with biotin and incubated with the nuclear extracts. After the reaction, the DNA–protein complexes were subjected to a 6% native polyacrylamide gel electrophoresis and transferred onto a nylon membrane (Millipore Corp.). After transfer, the membrane was immediately cross-linked for 15 min on a UV transilluminator. A chemiluminescent detection method using a luminal/enhancer solution and stable peroxide solution (Pierce Chemical Co.) was used, as described by the manufacturer, and membranes were exposed to X-ray films for 30 s to 5 min before development.

Statistical analysis

The data are expressed as mean±SEM. Comparison within groups was made by ANOVA for repeated measures (or paired *t*-test when only two groups were compared), and comparison among groups (or *t*-test when only two groups were compared) was made by ANOVA with Duncan's test. A value of *P* less than 0.05 was considered significant.

RESULTS

Expression of AT_2 receptors in renal proximal tubule cells

Due to the abundant expression of the AT_2 receptor in rat kidney during embryonic period, we used the samples from embryonic rat kidney (16 days) as positive controls. Immunoblotting showed the specific AT_2 receptor protein (≈41 kDa) bands in RPT cells (Fig. 1a), which were no longer visible when antibodies were preadsorbed by the immunizing peptide (Fig. 1a). AT_2 receptor mRNA expression in RPT cells and embryonic rat kidney is shown in Fig. 1b.

AT_2 receptor stimulation decreases AT_1 receptor expression in renal proximal tubule cells

Activation of AT_2 receptors with the AT_2 receptor agonist, CGP42112, decreased AT_1 receptor protein expression in a concentration and time-dependent manner. The inhibitory effect was evident at 10^{-9} mol/l, as early as 8 h, and maintained for at least 30 h (data not shown). CGP42112 also decreased AT_1 receptor mRNA expression in a concentration-dependent (Fig. 2a) and time-dependent manner (data not shown), indicating that regulation of AT_2 receptor on AT_1 receptor expression occurred, at least, at the transcriptional level.

The specificity of CGP42112 as an AT_2 receptor agonist was also determined by studying the effect of AT_2 receptor antagonist PD123319. Stimulation with CGP42112 (10^{-7} mol/l per 24 h) decreased AT_1 receptor expression in RPT cells. The AT_2 receptor antagonist, PD123319 (10^{-6} mol/l), by itself, had no effect, but reversed the inhibitory effect of CGP42112 on AT_1 receptor expression (Fig. 2b).

Pretreatment with CGP42112 decreases the stimulatory effect of Ang II on Na^+-K^+-ATPase activity in renal proximal tubule cells

Consistent with a previous study [20], Ang II, incubated for 30 min, produced a biphasic effect on Na^+-K^+-ATPase activity in a concentration (10^{-12}–10^{-8} mol/l)-dependent manner; the maximum stimulatory effect occurred at 10^{-11} mol/l (data not shown). The stimulatory effect of Ang II (10^{-11} mol/l) on Na^+-K^+-ATPase activity was also time (5–30 min)-dependent (data not shown).

To confirm the effect of AT_2 receptor on AT_1 receptor function, we studied the effect of Ang II on Na^+-K^+-ATPase activity in RPT cells with or without pretreatment with the AT_2

receptor agonist CGP42112. Fig. 3 shows that pretreatment with CGP42112 (10^{-9}–10^{-7} mol/l per 24 h) decreased the Ang II-mediated stimulatory effect on Na^+-K^+-ATPase activity in a concentration-dependent manner. The cells pretreated with CGP42112 for 24 h were washed three times (15 min/wash) with serum-free culture medium to remove all the added CGP42112, kept in serum-free culture medium for 2 h, and then treated with vehicle or Ang II for 30 min.

The effect of the AT_2 receptor agonist, CGP42112, was exerted at the AT_2 receptor because pretreatment with the AT_2 receptor antagonist PD123319 (10^{-6} mol/l per 24 h) reversed the inhibitory effect of CGP42112 (10^{-7} mol/l) on Ang II-mediated stimulation of Na^+-K^+-ATPase activity (Fig. 3).

To investigate whether or not the above-described effect of Ang II or CGP42112 involved changes in Na^+-K^+-ATPase protein expression, membrane Na^+-K^+-ATPase α1 subunit protein expression was also measured. We found that neither Ang II (30 min) nor CGP42112 (24 h) altered membrane protein expression of Na^+-K^+-ATPase α1 subunit in RPT cells (data not shown). CGP42112 (10^{-9}–10^{-6} mol/l per 24 h) also had no effect on the protein and mRNA expressions of AT_2 receptors in RPT cells (data not shown). These results suggest that the ability of the AT_2 receptor to impair the AT_1 receptor-mediated stimulation of Na^+-K^+-ATPase activity was not related to alterations in expression of Na^+-K^+-ATPase and AT_2 receptor *per se*.

In addition to studying the effect of AT_2 receptor stimulation on Ang II-stimulated Na^+-K^+-ATPase activity, we also studied the effect of AT_2 receptor stimulation on Ang II-mediated phosphorylation and cell surface receptor expression of NHE3, a protein that is responsible for the majority of sodium transport in the luminal membrane of RPT cells. We found that Ang II (10^{-11} mol/l) increased cell surface NHE3 expression and decreased NHE3 phosphorylation in RPT cells. The pretreatment of renal proximal tubule cells with the AT_2 receptor agonist CGP42112 (10^{-7} mol/l per 24 h) impaired these effects of Ang II (data not shown).

Role of nitric oxide/cyclic guanosine monophosphate in the inhibitory effect of AT_2 receptors on AT_1 receptor expression in renal proximal tubule cells

Due to the involvement of nitric oxide and cyclic guanosine monophosphate (cGMP) in AT_2 receptor signaling [21,22], and the inhibitory effect of AT_2 receptor on AT_1 receptor-mediated phospholipase D activation in kidney [23], we investigated a nitric oxide/cGMP mechanism for the AT_2 receptor-mediated down-regulation of AT_1 receptor expression. RPT cells were treated for 24 h with the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} mol/l) and the soluble guanylyl cyclase inhibitor 1H-[1,2,4] oxadiazolo-[4,3-a] quinoxalin-1-one (ODQ, 10^{-5} mol/l). Whereas L-NAME or ODQ, by them selves, had no effect on AT_1 receptor expression, inhibition of nitric oxide production or blockade of guanylyl cyclase activity blocked the inhibitory effect of AT_2 receptor on AT_1 receptor expression (Fig. 4a and b).

To further confirm the role of nitric oxide and cGMP on the AT_2 receptor-mediated inhibition of AT_1 receptor expression, RPT cells were treated for 24 h with the nitric oxide donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP, 50–300 μmol/l), and cGMP analog, 8-bromo-cGMP (8Br-cGMP, 50–300 μmol/l). We found that either SNAP or 8Br-cGMP decreased AT_1 receptor expression in a concentration-dependent manner (data not shown).

Role of Sp1 in the inhibitory effect of AT_2 receptors on AT_1 receptor expression in renal proximal tubule cells

The ubiquitous transcription factor Sp1 is important in the regulation of AT_1 receptor expression [24,25]. To determine whether or not the regulation of the AT_1 receptor by the AT_2 receptor is mediated by Sp1, RPT cells were treated with the potent and specific Sp1 blocker, mithramycin A. Pretreatment with mithramycin A (1.0 μmol/l per 24 h), which by itself had no effect on AT_1 receptor expression, blocked the inhibitory effect of CGP42112 on AT_1 receptor expression (Fig. 5a). A further experiment found that stimulation with CGP42112 (10^{-7} mol/l per 30 min) decreased Sp1 phosphorylation (control=58.0±4.0 DU; CGP42112=42.0±4.2 DU; n=5; P<0.05) but had no effect on the Sp1 expression (control=1.0±0.1 DU; CGP42112 1.0±0.08 DU; n=8; P=NS). Using EMSA, we also found that AT_2 receptor stimulation with CGP42112 (10^{-7} mol/l per 1.0 h) decreased the amount of Sp1 bound to AT_1 receptor DNA (Fig. 5b); addition of 50 times molar excess of unlabeled probe eliminated this band, confirming the specificity of the binding.

AT_2 receptor co-localizes and directly interacts with the AT_1 receptor in renal proximal tubule cells

To determine whether or not AT_2 and AT_1 receptors can directly interact, we used immunofluorescence laser confocal microscopy and co-immunoprecipitaion studies. Both AT_2 and AT_1 receptors co-localized at the plasma membrane and cytoplasm of RPT cells (Fig. 6a). There was direct interaction because the AT_2 and AT_1 receptors co-immunoprecipitated in the basal state, which was increased following stimulation of the AT_2 receptor with CGP42112 (10^{-7} mol/l per 30 min) (Fig. 6b).

AT_1 receptor expression in renal proximal tubule cells from AT_2 receptor knockout mice

To further confirm the regulation of AT_1 receptor expression by the AT_2 receptor, we studied the expression of AT_1 receptor in RPT cells from AT_2 receptor knockout mice [26]. Consistent with the pharmacological data, AT_1 receptor expression was found to be increased in RPT cells from AT_2 receptor knockout mice (Fig. 7a). Furthermore, the stimulatory effect of Ang II (10^{-11} mol/l per 30 min) on Na^+-K^+-ATPase activity was also increased in this cell line (Fig. 7b).

DISCUSSION

The AT_2 receptor is highly expressed in fetal kidney. Its expression gradually decreases after birth but continues to be expressed in the adult kidney, albeit at low levels [5,27]. Indeed, stimulation of AT_2 receptors inhibits the activity of Na^+-K^+-ATPase in the proximal tubules isolated from adult rats, mice, and rabbits [21,28–31].

There is increasing evidence for interactions between AT_1 and AT_2 receptors. AT_1 receptor expression in VSMCs is decreased with AT_2 receptor over-expression and increased in some organs, such as the lung and adrenal gland, of AT_2 receptor knockout mice [9,10,32,33]. However, the mechanism by which the AT_2 receptor opposes the effect of the AT_1 receptor on sodium transport is not clear. Therefore, we studied a rat RPT cell line that has been shown to faithfully mimic the effects of G-protein-coupled receptors *in vivo*, including dopamine receptors and Ang II receptors [12,15,34]. Our current study shows that the AT_2 receptor agonist CGP42112 decreases AT_1 receptor mRNA and protein expressions in RPT cells. This regulation is functionally relevant; pretreatment with the AT_2 receptor agonist, CGP42112, for 24 h attenuates the stimulatory effect of Ang II on Na^+-K^+-ATPase activity. Because CGP42112 does not affect Na^+-K^+-ATPase protein expression, we suggest that in the long term (24 h) decrease in AT_1 receptor expression, not the Na^+-K^+-ATPase transporter *per se*, is responsible for the attenuated Ang II-mediated stimulation of Na^+-K^+-

ATPase in CGP42112-treated cells. Therefore, the inhibition of AT_1 receptor expression produced by the stimulation of AT_2 receptors could be an important mechanism to oppose AT_1 receptor-mediated sodium retention.

The AT_2 receptor can also antagonize the stimulatory effect of Ang II on sodium transport by direct protein–protein interaction. We found that AT_1 and AT_2 receptors co-localize and co-immunoprecipitate in RPT cells; stimulation of AT_2 receptors with CGP42112 for 30 min increases the amount of AT_2/AT_1 receptor co-immunoprecipitation. It is possible that the increase in their co-immunoprecipitation would lead to fewer AT_1 receptors that are available to increase sodium reabsorption, leading to increased basal sodium excretion. However, the counter-regulatory effect of the AT_2 receptor on AT_1 receptor function may also involve an indirect interaction. Many studies have demonstrated that nitric oxide and cGMP are involved in the physiological effects of AT_2 receptors. AT_2 receptor activation increases the production of nitric oxide and cGMP through bradykinin-dependent or independent pathways [22,35,36]. In the vasculature, AT_2 receptors down-regulate AT_1 receptor expression and induce vasodilation by activation of bradykinin, nitric oxide, and the cGMP cascade [9,22,37,38]. In the kidney, activation of the AT_2 receptor, via a nitric oxide/cGMP-dependent pathway, inhibits Na^+-K^+-ATPase activity, renin production, and AT_1 receptor-mediated phospholipase D activation [21,23,39]. Our present study is in agreement with these reports; both nitric oxide synthase inhibitor L-NAME and guanylyl cyclase inhibitor ODQ abrogate the down-regulation of AT_1 receptor expression caused by CGP42112. To further investigate the mechanism by which long-term AT_2 receptor stimulation decreases AT_1 receptor expression, we studied the effect of AT_2 receptor on Sp1, a regulator of AT_1 receptor promoter activity [24,40–42]. We found that stimulation of AT_2 receptors decreases the phosphorylation of Sp1, and decreases the binding of nuclear protein to AT_1 receptor DNA. Moreover, blockade of Sp1 with mithramycin A abolishes the ability of an AT_2 receptor agonist to inhibit AT_1 receptor expression, indicating that the AT_2 receptor inhibits AT_1 receptor expression, via Sp1.

To ensure that the results of the pharmacological studies are specific and not due to nonspecific pharmacological effects, we studied the consequences of deletion of the AT_2 receptor gene. We used an RPT cell line derived from AT_2 receptor knockout mice. These cells have been shown to possess typical transporters of the renal proximal tubule making them suitable to study the consequences of the lack of the AT_2 receptor on AT_1 receptor expression and function [26]. In the present study, the protein expression of AT_1 receptors in RPT cells from AT_2 receptor knockout mice is increased; the stimulatory effect of Ang II on Na^+-K^+-ATPase activity is also increased. These results are consistent with the ability of AT_2 receptors to inhibit AT_1 receptor expression and function in immortalized rat RPT cells.

In summary, we have demonstrated that the AT_2 receptor regulates the expression and function of the AT_1 receptor *in vitro*. Activation of AT_2 receptors decreases AT_1 receptor expression at the transcription level that involves Sp1 and attenuates the ability of Ang II to increase Na^+-K^+-ATPase activity in rat RPT cells, effects that are corroborated in studies of RPT cells from AT_2 receptor knockout mice. AT_2 and AT_1 receptors directly interact which is increased by stimulation of AT_2 receptors. Apart from the regulation of transcription and direct protein–protein interaction, the AT_2 receptor may also regulate the AT_1 receptor via nitric oxide/cGMP pathway. The multifaceted regulation of the AT_1 receptor by the AT_2 receptor may play an important role in the renal physiological and pathological mechanisms involving increased activity of the renin–angiotensin system.

Acknowledgments

These studies were supported in part by grants from Natural Science Foundation Project of CQ CSTC (CSTC, 2009BA5044), grants from the National Natural Science Foundation of China (30925018, 31130029, 81070559, 81100500), and grant from the National Institutes of Health (R01HL092196).

Abbreviations

AT_1 receptor	angiotensin II type 1 receptor
AT_2 receptor	angiotensin II type 2 receptor
cGMP	cyclic guanosine monophosphate
EMSA	electrophoretic mobility shift assay
RPT	renal proximal tubule
RT-PCR	reverse transcriptase-polymerase chain reaction
VSMC	vascular smooth muscle cell

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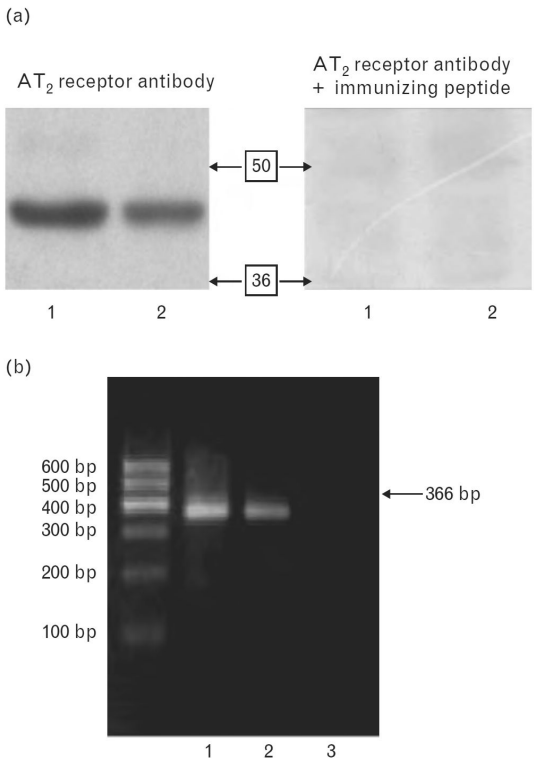

FIGURE 1.

AT_2 receptor protein and mRNA expression in rat RPT cells. (a) AT_2 receptor protein expression in RPT cells. Cell lysate proteins (100 μg) from kidneys of embryonic rats (lane1) and RPT cells (lane 2) were subjected to immunoblotting with anti-AT_2 receptor antibody (1 : 400). In RPT cells and rat embryonic kidney, the 41 kDa bands were no longer visible when the antibody was preadsorbed with the immunizing peptide (1 : 10 w/w incubation for 12 h). The numbers between the autoradiographs indicate the molecular sizes. (b) AT_2 receptor mRNA expression in RPT cells. AT_2 receptor RT-PCR products were analyzed in 10% polyacrylamide gel stained with ethidium bromide. An amplification product of the predicted size (366 bp) is seen in RT-PCR reaction using RNA (4–6 μg) from kidneys of embryonic rats (lane1) and RPT cells (lane 2). No amplification is seen in the absence of RPT cell RNA (lane 3). RPT, renal proximal tubule.

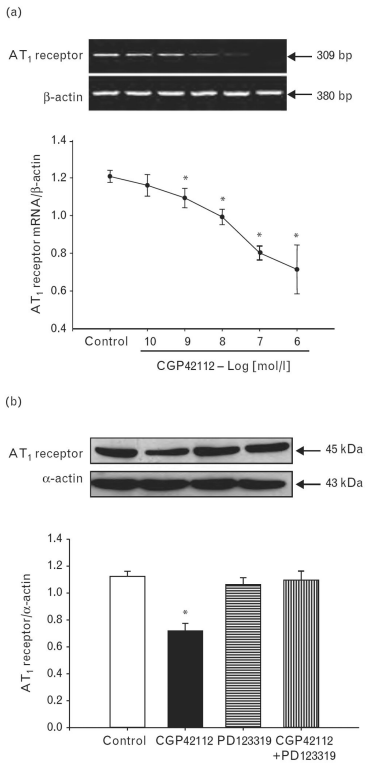

FIGURE 2.

Effect of the AT_2 receptor agonist, CGP42112, on AT_1 receptor expression in rat RPT cells. (a) Concentration–response of AT_1 receptor mRNA expression in RPT cells treated with CGP42112. AT_1 receptor mRNA expression was determined after 24 h incubation with the indicated concentrations of CGP42112. Results are expressed as the ratio of AT_1 receptor to β-actin mRNA densities (n=5, $^*P < 0.05$ vs. control, ANOVA, Duncan's test). (b) Effect of the AT_2 receptor agonist, CGP42112, and antagonist, PD123319, on AT_1 receptor protein expression in RPT cells. The cells were incubated with the indicated reagents (CGP42112, 10^{-7} mol/l; PD123319, 10^{-6} mol/l) for 24 h. Results are expressed as the ratio of AT_1 receptor to α-actin densities (n=5, $^*P < 0.05$ vs. others, ANOVA, Duncan's test). RPT, renal proximal tubule.

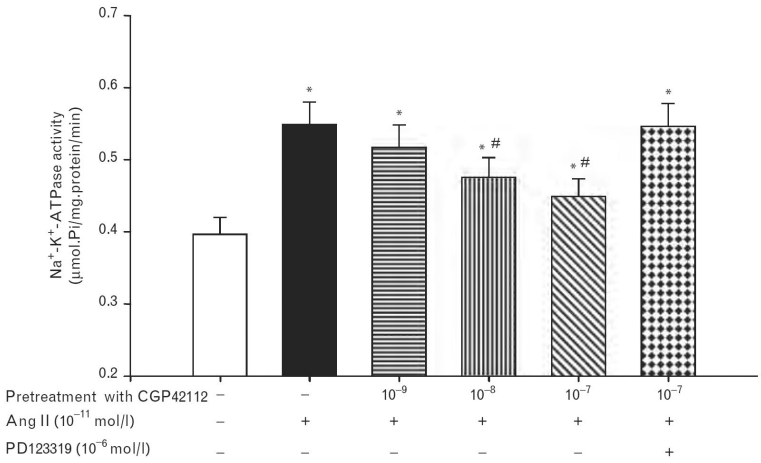

FIGURE 3.

Effect of pretreatment with an AT_2 receptor agonist, CGP42112, on the stimulatory effect of Ang II on Na^+-K^+-ATPase activity in RPT cells. The cells were pretreated with CGP42112 (10^{-7} mol/l) or vehicle (dH_2O) for 24 h. After CGP42112 pretreatment, the cells were washed three times (15 min/wash) with serum-free culture medium to remove all the added CGP42112, kept in serum-free culture medium for 2 h, and then treated with Ang II (10^{-11} mol/l) for 30 min. For the PD123319 blockade study, the cells were simultaneously treated with PD123319 and CGP42112. Results are expressed as μmol phosphate released per mg protein per min (*$P < 0.05$ vs. control, #$P < 0.05$ vs. Ang II alone, n=4–6, ANOVA, Duncan's test). RPT, renal proximal tubule.

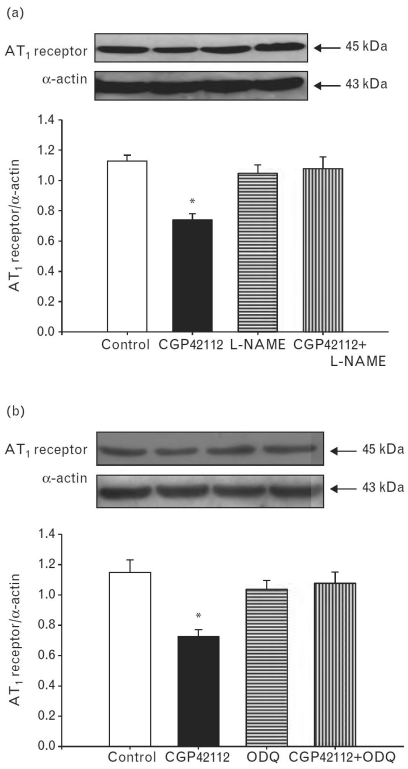

FIGURE 4.

Role of nitric oxide and cGMP in the inhibition of AT_1 receptor expression by AT_2 receptor stimulation in RPT cells. The RPT cells were incubated with the indicated reagents [CGP42112, 10^{-7} mol/l; L-NAME, 10^{-4} mol/l (a); ODQ, 10^{-5} mol/l (b)] for 24 h. Results are expressed as the ratio of AT_1 receptor to α-actin densities (n=4–6, *$P < 0.05$ vs. others, ANOVA, Duncan's test). RPT, renal proximal tubule.

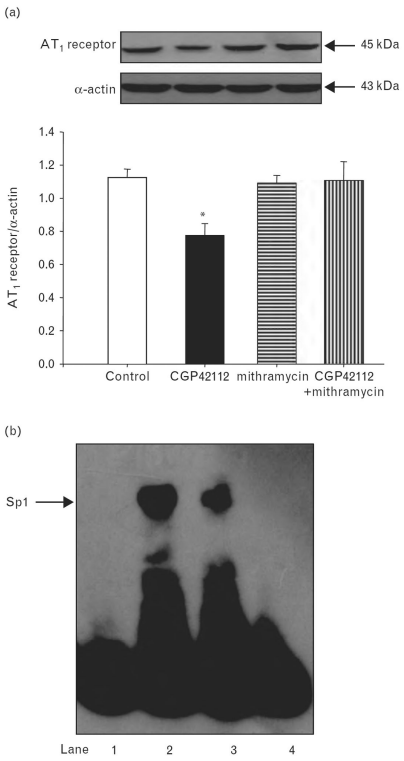

FIGURE 5.

Role of Sp1 in the inhibition of AT_1 receptor expression by AT_2 receptor stimulation in rat RPT cells. (a) Effect of Sp1 blocker in the inhibition of AT_1 receptor expression by AT_2 receptor stimulation in RPT cells. The RPT cells were incubated with the indicated reagents [CGP42112, 10^{-7} mol/l; Sp1 blocker, mithramycin A (1.0 μmol/l)] for 24 h. Results are expressed as the ratio of AT_1 receptor to α-actin densities (n=5, $^*P < 0.05$ vs. others, ANOVA, Duncan's test). (b) EMSA of nuclear protein from RPT cells. Binding activity of AT_1 receptor gene promoter (−40 to −6 bp), containing Sp1 site, was examined in the nuclear protein from vehicle-stimulated (lane 2) or CGP42112 (10^{-7} mol/l per 1.0 h)-stimulated (lane 3) RPT cells by EMSA. No nuclear extracts or 50 times of unlabeled probe were added to the reaction mixture as controls (lane 1 and 4, respectively). CGP42112 decreased the amount of binding (lane 3). EMSA, electrophoretic mobility shift assay; RPT, renal proximal tubule.

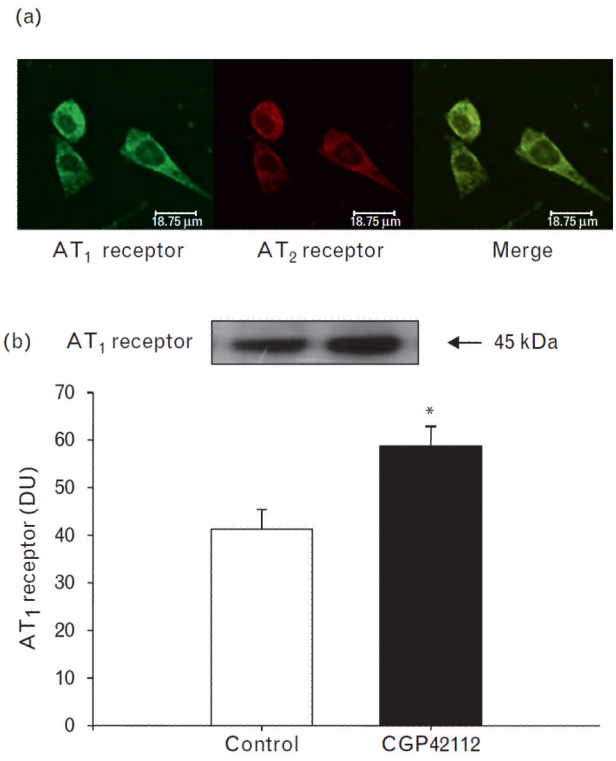

FIGURE 6.

The linkage of AT_2 and AT_1 receptor in rat RPT cells. (a) AT_2 and AT_1 receptor co-localization in rat RPT cells. The cells grown on coverslips were washed, then fixed and double-immunostained for AT_2 and AT_1 receptors, as described in the Methods section. Co-localization appears as yellow after merging the images of Alexa 488-tagged AT_1 receptor (green) and Alexa 568-tagged AT_2 receptor (red). (b) Effect of the AT_2 receptor agonist, CGP42112, on AT_1/AT_2 receptor co-immunoprecipitation in RPT cells. The cells were incubated with CGP42112 (10^{-7} mol/l) for 30 min. Thereafter, the samples were immunoprecipitated with anti-AT_2 receptor antibodies and immunoblotted with anti-AT_1 receptor antibodies. Results are expressed as relative density units (DU) ($n = 5$, $^*P < 0.05$ vs. control, *t*-test). RPT, renal proximal tubule.

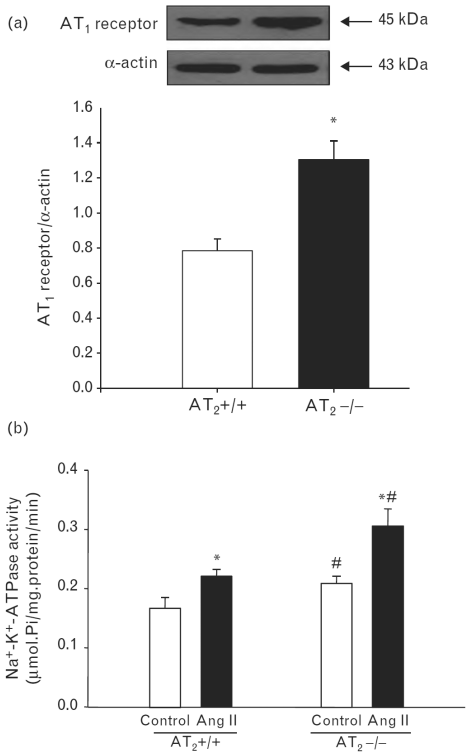

FIGURE 7.

Expression and function of AT_1 receptor in RPT cells from AT_2 receptor knockout mice. (a) Immunoreactive AT_1 receptor protein expression in RPT cells from AT_2 receptor knockout mice ($AT_2^{-/-}$) and their wild-type controls ($AT_2^{+/+}$). Results are expressed as the ratio of AT_1 receptor of α-actin densities (*$P < 0.05$ vs. wild-type control, $n = 4$, t-test). (b) Effect of Ang II on Na^+-K^+-ATPase activity in RPT cells from AT_2 receptor knockout mice ($AT_2^{-/-}$) their wild-type controls ($AT_2^{+/+}$). Results are expressed as μmol phosphate released per mg protein per min (*$P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. $AT_2^{+/+}$, $n = 6$–7, ANOVA, Duncan's test). RPT, renal proximal tubule.