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ORIGINAL ARTICLE

Activation of angiotensin II type 1 receptors increases D_4 dopamine receptor expression in rat renal proximal tubule cells

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Both the dopaminergic and renin–angiotensin systems play important roles in the regulation of blood pressure. Our previous study showed that the stimulation of dopaminergic D_4 receptors reduced angiotensin II type 1 (AT_1) receptor expression in renal proximal tubule (RPT) cells. In this study, we tested whether AT_1 receptors, in return, would regulate D_4 receptor expression and function in RPT cells. Expression of the D_4 receptor from Wistar-Kyoto (WKY) or spontaneously hypertensive rats (SHRs) RPT cells and renal cortex tissues were determined by western blot, and Na^+–K^+ ATPase activity was determined using an enzyme assay. Urine volume and urine sodium of WKY rats and SHRs treated with or without D_4 receptor stimulation were measured. Thus, activation of AT_1 receptors with angiotensin II (Ang II) increased D_4 receptor protein expression in RPT cells, and this increase was blocked by nicardipine, a calcium influx blocker. The D_4 receptor agonist PD168077 inhibited Na^+–K^+ ATPase activity in WKY RPT cells but not in SHR RPT cells. Ang II pre-treatment promoted D_4 receptor-mediated inhibition of Na^+–K^+ ATPase in RPT cells in WKY rats but not in SHRs. Meanwhile, Ang II pre-treatment augmented the natriuretic effect of PD168077 in WKY rats but not in SHRs. In conclusion, AT_1 stimulation can regulate the expression and natriuretic function of dopaminergic D_4 receptors in RPT cells and might be involved in the pathogenesis of essential hypertension.
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Keywords: angiotensin II type I receptor; dopaminergic receptor; hypertension; renal proximal tubule cell; sodium excretion

INTRODUCTION

The kidney plays a major role in the long-term regulation of blood pressure.[1] Natriuresis and diuresis are regulated by numerous hormones and humoral factors, including angiotensin II (Ang II) and dopamine. Dopamine and Ang II are two important regulators of sodium and water transport in the kidney, but they serve counteracting functions.[1,2] The activation of AT_1 receptors by low concentrations of Ang II causes an increase in renal sodium reabsorption.[3] By contrast, the activation of D_1-like (D_1 and D_5 subtypes) and D_2-like dopaminergic receptors (D_2, D_3, and D_4 subtypes) causes a decrease in renal sodium reabsorption.[4–6]

Na^+–K^+ ATPase, which provides the driving force for sodium across the apical membrane in the proximal tubule of the kidney, plays an important role in sodium reabsorption. Na^+–K^+ ATPase is a heterodimer consisting primarily of α and β subunits. The α-subunit is the catalytic unit that binds sodium, potassium and ATP. The β-subunit is essential for targeting the α-subunit to the cell membrane and stabilizing the Na^+–K^+ ATPase.[7–9] The dopaminergic D_1 and D_2-like receptors can inhibit Na^+–K^+ ATPase activity, thereby blocking tubule sodium reabsorption and inducing natriuresis.[10–13] Thus, D_1 and D_2-like receptors regulate water–sodium retention and balance the blood pressure through a Na^+–K^+ ATPase-dependent mechanism.

Dopaminergic and AT_1 receptors are both expressed in the brush border and basolateral membranes of the renal proximal tubule (RPT).[14–16] The interaction between dopaminergic receptors and Ang II receptors has been found in previously published papers.[17–19] The activation of D_3 dopaminergic receptors decreases AT_1 receptor expression in RPT cells from Wistar-Kyoto (WKY) rats.[18] Conversely, the stimulation of AT_1 receptors decreases D_3 receptor expression in WKY RPT cells.[19] The D_4 receptor is an important dopaminergic receptor for the function of natriuresis and diuresis in human and rodent renal proximal tubules. It antagonizes vasopressin- and aldosterone-dependent sodium reabsorption in the cortical collecting duct, and D_4 dopaminergic receptor-deficient mice develop hypertension.[20] However, whether stimulation of AT_1 receptors affects the expression and function

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of D_4 receptors is unknown. Our previous study proved that D_4 and AT_1 receptors colocalized in RPT cells, and stimulation of D_4 receptors decreases AT_1 receptor expression in RPT cells from WKY rats[21] as well as in vascular smooth muscle cells.[22]

In this study, we aimed to identify the effects of AT_1 receptors on D_4 receptor expression, Na^+–K^+ ATPase activity and natriuresis and to investigate the underlying mechanism regulating this process. The AT_1 and D_4 receptor interaction in immortalized RPT cells from WKY rats and spontaneously hypertensive rats (SHRs) was detected. These immortalized RPT cells were used because they behave similarly to freshly isolated RPT cells in response to the stimulators and inhibitors of dopaminergic and AT_1 receptors and of other G protein-coupled receptors (GPCRs).[23]

METHODS

Cell culture

Immortalized RPT cells (Taconic, Germantown, NY, USA) from WKY rats and SHRs were cultured at 37 °C in 95% air and 5% CO_2 in DMEM/F-12 (HyClone, Logan, UT, USA) with transferrin (5 μg ml^{-1}), insulin (5 μg ml^{-1}), epidermal growth factor (10 ng ml^{-1}), dexamethasone (4 μg ml^{-1}) and fetal bovine serum 10% in a 100-mm Petri dish.[18] Cells were incubated in media without fetal bovine serum for 2 h before the addition of reagents.

Preparation of kidney and RPT cells

The WKY rats and SHRs (SLRC Laboratory Animals, Shanghai, China) were anesthetized with pentobarbital (50 mg kg^{-1}), after which the kidneys were removed and the rats were killed (pentobarbital, 100 mg kg^{-1}). The renal cortices or cultured RPT cells were homogenized in an ice-cold lysis buffer (phosphate-buffered saline with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} PMSF, 10 μg ml^{-1} aprotinin and 10 μg ml^{-1} leupeptin), sonicated, kept on ice for 1 h and centrifuged at 16 000*g* for 30 min. The supernatants were stored at − 80 °C until use.[24] All experiments were approved by the Third Military Medical University Animal Use and Care Committee.

Western blot analysis

Rats and RPT cells were treated with vehicle (dH_2O), an AT_1 receptor agonist (Ang II; Sigma-Aldrich, St Louis, MO, USA), or an AT_1 receptor antagonist (losartan; Merck, Darmstadt, Germany). Cells were serum-starved 2 h before cell lysis for immunoblotting. Immunoblotting was performed as previously reported.[25] The blots were probed with polyclonal goat anti-D_4 receptor antibodies (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-actin (Santa Cruz Biotechnology) was used for the normalization.[18]

Determination of the signaling pathways involved in the regulation of D_4 receptor expression by AT_1 receptors in WKY cells

To determine the regulatory pathways involved in the AT_1 receptor-mediated regulation of D_4 receptor expression in RPT cells, several inhibitors or agonists, including a protein kinase C inhibitor (19–31, 10^{-6} mol l^{-1}; Sigma-Aldrich), a protein kinase A inhibitor (14–22, 10^{-6} mol l^{-1}; Calbiochem, Darmstadt, Germany) and a calcium channel blocker (nicardipine, 10^{-7} mol l^{-1}; Sigma-Aldrich) were used. These reagents were added into the incubation medium 15 min before the addition of Ang II.

Na^+–K^+ ATPase activity assay

Na^+–K^+ ATPase activity was determined as the rate of inorganic phosphate release in the presence or absence of ouabain.[26] Rat RPT cells were pre-treated with Ang II or vehicle (dH_2O) for 24 h. After washing for 15 min, the cells were treated with a D_4 receptor agonist (PD168077; Tocris Cookson, Bristol, UK) or vehicle (dH_2O) for 15 min. The cellular lysates of RPT cells were collected and centrifuged at 48 000*g* for 25 min. The pellet (membrane fraction) was washed two times and suspended in 10 mmol l^{-1} Tris containing 1 mmol l^{-1} EDTA (pH 7.4). Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) and adjusted to 1 mg ml^{-1}. Na^+–K^+ ATPase activity was measured by adding 100 μl of membrane fraction to an 800 μl reaction mixture, which was pre-incubated for 5 min in a water bath at 37 °C. The amount of phosphate produced was quantified by the addition of 1 ml of coloring reagent to the reaction mixture. The mixture was then mixed thoroughly and centrifuged at 3000 *g* for 10 min. The resulting phosphomolybdate was quantified spectrophotometrically at 740 nm using a standard curve prepared from K_2HPO_4. The difference between total and ouabain-insensitive ATPase activity was taken as Na^+–K^+ ATPase activity and expressed as μmol phosphate released per mg protein per minute. Protease inhibitors (1 mmol l^{-1} phenylmethylsulfonyl fluoride, 10 μg ml^{-1} each leupeptin and aprotinin) and a phosphatase inhibitor (50 μmol l^{-1} sodium orthovanadate) were used to eliminate the effect of proteases and phosphatases.[27]

Reverse transcriptase-polymerase chain reaction of D_4 receptors

The total RNA was extracted from RPT cells. The cDNA was synthesized by using the extracted RNA that served as a template to amplify D_4 receptors and β-actin. For D_4 receptors, the forward primer was 5′-GAT GTG TTG GAC GCC TTT CT-3′ and the reverse primer was 5′-TCG GCA TTC AAG ATG GTG TA-3′. The amplification conditions of D_4 receptors were as follows: 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 β-actin, the forward primer was 5′-GTG GGT ATG GGT CAG AAG GA-3′ and the reverse primer was 5′-AGC GCG TAA CCC TCA TAG AT-3′. The amplification conditions of β-actin were the same as the D_4 receptor.[28] The D_4 receptor was corrected based on the density of β-actin.

Immunoprecipitation

Equal amounts of cell lysates (1000 μg protein ml^{-1} supernatant) were incubated with polyclonal anti-phosphoserine antibody (Abcam, Cambridge, MA, USA; D_4 receptor phosphorylation; 2 μg ml^{-1}) overnight at 4 °C and then with protein-G agarose for 2 h at room temperature. The immunoprecipitate was subjected to immunoblotting with the anti-D_4 receptor antibody.

Determination of the effect of PD168077 on urine volume and urine sodium of WKY rats and SHRs

The rats were pre-treated with Ang II (10 ng kg^{-1} per day, for 1 week) or vehicle (dH_2O). Urine and sodium excretions were measured in WKY rats and SHRs kept in metabolic cages for 24 h after the injection of PD168077 (0.3 mg kg^{-1}) via tail vein. Urine sodium was measured by an electrolyte analyzer.

Statistical analysis

The data are expressed as the means ± s.e.m. Comparison within groups was made by repeated measures ANOVA (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 factorial ANOVA using the Holm–Sidak test. A value of $P<0.05$ was considered significant.

RESULTS

AT_1 receptors increase D_4 receptor expression in RPT cells

Immortalized RPT cells from WKY rats were treated with varying concentrations and durations of the AT_1 receptor agonist Ang II for 24 h. We found that Ang II increased D_4 receptor expression in a concentration- (10^{-10}–10^{-6} mol l^{-1}) and time- (2–30 h) dependent manner. The stimulatory effect was evident at 10^{-9} mol l^{-1} (Figure 1a) and the stimulatory effect of Ang II (10^{-7} mol l^{-1}) was noted as early as 2 h and was maintained for at least 30 h (Figure 1b). To determine whether the increase in D_4 receptor expression was induced by AT_1 receptor activation in RPT cells, we pre-treated WKY RPT cells with the AT_1 receptor antagonist losartan (10^{-7} mol l^{-1}) for 30 min. The results showed that the Ang II-induced increase of D_4 receptor protein expression was blocked by the pre-treatment of losartan. Losartan alone did not affect the D_4 receptor expression in

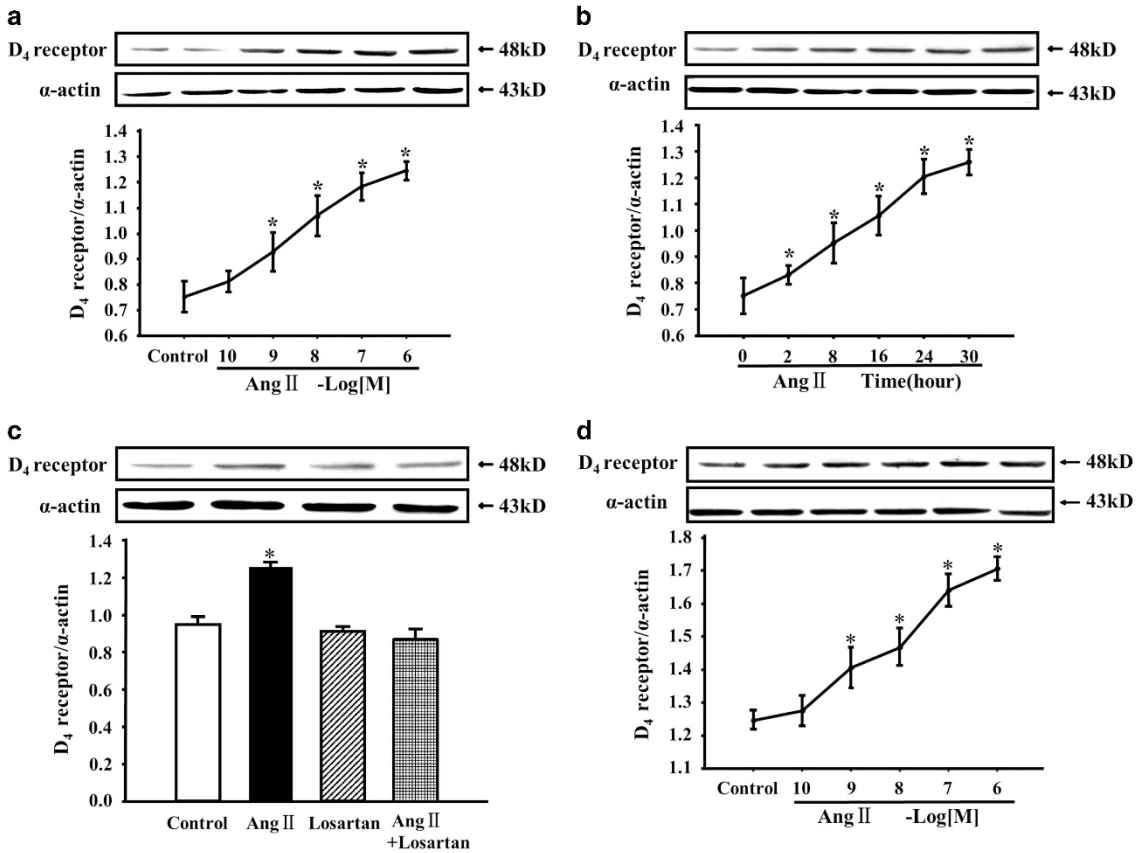

Figure 1 Effect of Ang II on D_4 receptor protein expression in RPT cells. (**a**) Concentration response of D_4 receptor protein expression in WKY RPT cells treated with varying concentrations of the AT_1 receptor agonist (Ang II) for 24 h. The results are expressed as the ratio of D_4 receptor and α-actin densities ($n=7$; $^*P<0.05$ *vs.* control). (**b**) Time course of D_4 receptor protein expression in WKY RPT cells treated with Ang II (10^{-7} mol l^{-1}) for varying durations of incubation. The results are expressed as the ratio of D_4 receptor and α-actin densities ($n=7$; $^*P<0.05$ *vs.* control). (**c**) Effect of Ang II and an AT_1 receptor antagonist (losartan) on D_4 receptor expression in RPT cells from WKY rats. The cells were incubated with the indicated reagents (Ang II, 10^{-7} mol l^{-1}; losartan, 10^{-7} mol l^{-1}) for 24 h. The results are expressed as the ratio of the D_4 receptor and α-actin densities ($n=4$; $^*P<0.05$ *vs.* others). (**d**) Concentration response of D_4 receptor protein expression in SHR RPT cells treated with varying concentrations of Ang II for 24 h. The results are expressed as the ratio of D_4 receptor and α-actin densities ($n=5$; $^*P<0.05$ *vs.* control).

RPT cells (Figure 1c). Meanwhile, we treated immortalized RPT cells from SHRs with varying concentrations of Ang II for 24 h. The results showed that Ang II also increased D_4 receptor expression in a dosage-dependent manner in SHR RPT cells. Like that in WKY RPT cells, the stimulatory effect was significant at 10^{-9} mol l^{-1} in SHR RPT cells (Figure 1d).

Calcium mediates the stimulatory effect of AT_1 receptors on D_4 receptor expression in RPT cells

To investigate the mechanism of AT_1 receptor downregulating D_4 receptor expression, RPT cells from WKY rats were treated with several agonists or antagonists. As protein kinase A, protein kinase C and Ca^{2+} were key cell signaling pathways, the protein kinase A inhibitor (14–22, 10^{-6} mol l^{-1}), the protein kinase C inhibitor (19–31, 10^{-6} mol l^{-1}) and the L-type Ca^{2+} channel blocker (nicardipine, 10^{-7} mol l^{-1}) were added into the incubation medium 15 min before the addition of Ang II. D_4 receptor expressions were detected by immunoblotting. The results showed that nicardipine blocked the stimulatory effect of Ang II on D_4 receptor expression (Figure 2) while the protein kinase A inhibitor and the protein kinase C inhibitor did not (data not shown). Nicardipine alone had no effect on D_4 receptor expression (Figure 2).

The comparison of the regulation of AT_1 receptors on D_4 receptor expression in WKY rats and SHRs

We next studied whether there was different regulation of AT_1 receptors on D_4 receptor expression in RPT cells from WKY rats and SHRs. We compared D_4 receptor expression in WKY RPT cells and SHR RPT cells after Ang II (10^{-7} mol l^{-1}, 24 h) treatment by using a reverse transcriptase-PCR (RT-PCR) analysis and immunoblotting. It was noticed that basal D_4 receptor mRNA and protein expression were higher in RPT cells from SHRs than that from WKY rats (Figures 3a and b). The results also showed that Ang II increased mRNA and protein expressions of D_4 receptors in RPT cells from both WKY rats and SHRs (Figures 3a and b). This result was also confirmed *in vivo*. After treatment with Ang II (10 ng kg^{-1} per day) for 1 week, the renal cortices were collected and processed for immunoblotting of D_4 receptors. The results showed that Ang II increased the protein expression of D_4 receptors in WKY rats and SHRs (Figure 3c).

Ang II augmented D_4 receptor-medicated inhibition of Na^+–K^+ ATPase in WKY RPT cells but not in SHR RPT cells

To investigate the influence of stimulation of the AT_1 receptor on D_4 receptor function, we evaluated the activity of the D_4 receptor downstream molecule Na^+–K^+ ATPase. In RPT cells from both WKY rats and SHRs, the cells were pre-treated with Ang II (10^{-7} mol l^{-1})

for 24 h. After washing for 15 min, the cells were treated with a D_4 receptor agonist (PD168077, 10^{-11} mol l^{-1}) for 15 min. The results showed that the activity of Na^+–K^+ ATPase was higher in SHR than WKY RPT cells at the basal level. PD168077 decreased Na^+–K^+ ATPase activities in WKY RPT cells, but not in SHR RPT cells. Pre-treatment with Ang II (10^{-7} mol l^{-1}) for 24 h increased the inhibitory effect of PD168077 on Na^+–K^+ ATPase activity in WKY RPT cells. However, in SHR RPT cells, pre-treatment with Ang II had no effect on the D_4-mediated inhibitory effect of Na^+–K^+ ATPase activity in SHR RPT cells (Figure 4a). Further, PD168077 significantly increased urine volume and sodium excretion in Ang II-pre-treated WKY rats, but not in SHRs (Figures 4b and c). Additionally, we used immunoprecipitation to investigate the phosphorylation of D_4 receptors in WKY and SHR RPT cells. The results showed that the phosphorylation of D_4 receptors was higher in SHR RPT cells than WKY RPT cells (Figure 4d).

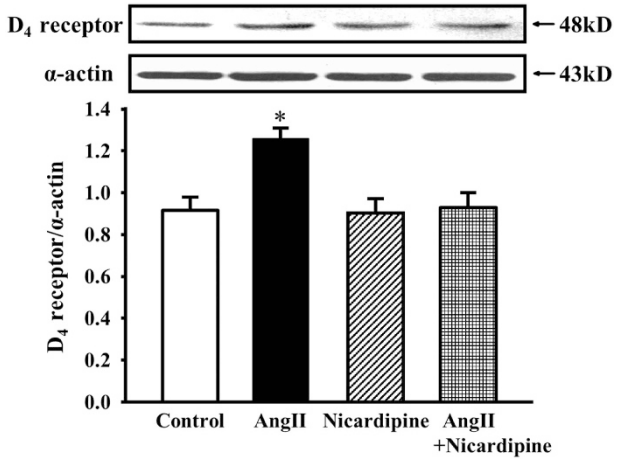

Figure 2 Role of calcium in the stimulatory effect of AT_1 receptors on D_4 receptor expression in WKY RPT cells. Ang II (10^{-7} mol l^{-1}) and the calcium channel blocker, nicardipine (10^{-7} mol l^{-1}), were used to treat WKY RPT cells for 24 h. The results are expressed as the ratio of the D_4 receptor and α-actin densities ($n=6$; $^*P<0.05$ *vs.* others).

DISCUSSION

In a physiological state, the activation of renal D_4 receptors decreases Na^+–K^+ ATPase activity, an important sodium transporter, in basolateral membranes in RPT cells, which thereby increases sodium and water excretion and lowers blood pressure.[29] In this study, we found that stimulation of AT_1 receptors can increase D_4 receptor expression in rat RPT cells, which may be of physiological significance since it enhances the effect of D_4 receptors on sodium excretion to balance the blood pressure. The regulation of AT_1 stimulation on D_4 receptor expression is associated with an AT_1-mediated calcium influx. The D_4 receptor protein expression is higher in SHR than in

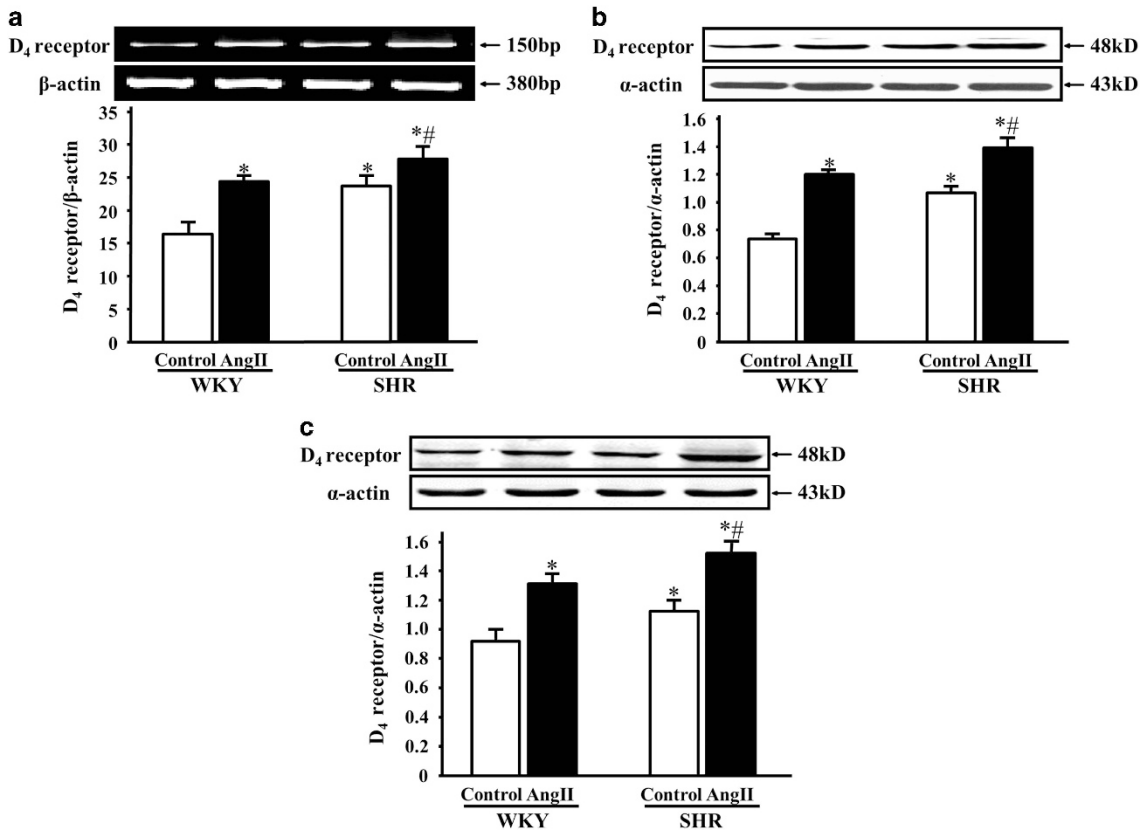

Figure 3 Effect of Ang II on D_4 receptor expression in RPT cells from both WKY rats and SHRs. (**a**, **b**) Effect of Ang II on D_4 receptor expression in RPT cells from both WKY rats and SHRs. After treatment with the AT_1 agonist, Ang II (10^{-7} mol l^{-1}), for 24 h, the cells were collected and processed for reverse transcriptase-PCR (**a**) or immunoblotting (**b**) for the D_4 receptor. The results are expressed as the ratio of D_4 receptor and α-actin densities ($n=5$–6; $^*P<0.05$ *vs.* WKY control; $^{\#}P<0.05$ *vs.* SHR control). (**c**) Effect of Ang II on D_4 receptor expression in the kidneys of both WKY rats and SHRs. After treatment with the AT_1 agonist (Ang II), the renal cortices were collected for immunoblotting for D_4 receptor. The results are expressed as the ratio of the D_4 receptor and α-actin densities ($n=4$, $^*P<0.05$ *vs.* control).

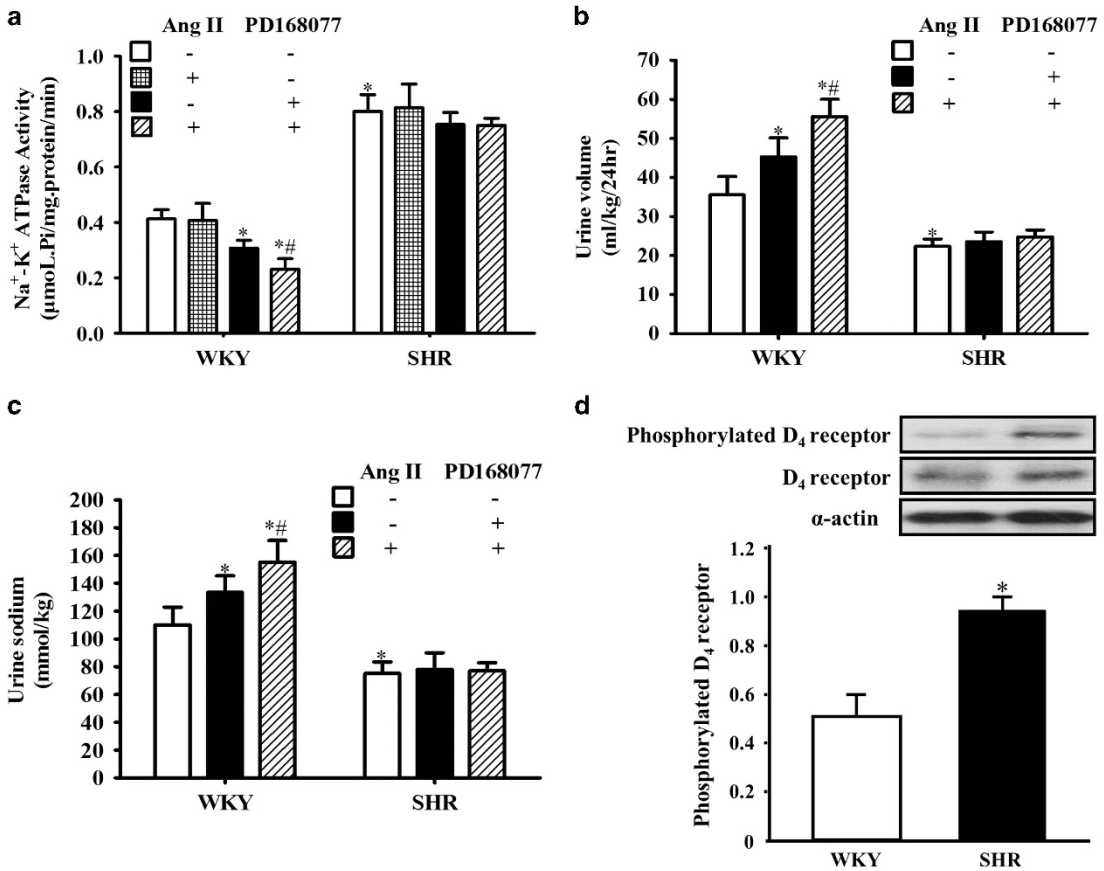

Figure 4 Effect of AT_1 receptor agonists on the function of D_4 receptors. (**a**) Effect of pre-treatment with Ang II on the inhibitory effect of D_4 receptors on Na^+–K^+ ATPase activity in WKY and SHR RPT cells. The cells were pre-treated with Ang II (10^{-7} mol l^{-1}) or vehicle (dH_2O) for 24 h. After washing for 15 min, the cells were treated with PD168077 (10^{-11} mol l^{-1}) for 15 min. The results are expressed as μmol phosphate released per mg protein per minute ($n=5$; $^*P<0.05$ *vs.* control; $^{\#}P<0.05$ *vs.* PD168077 alone). (**b**) Effect of pre-treatment with Ang II on the inhibitory effect of D_4 receptors on urine volume in WKY rats and SHRs. The rats were pre-treated with Ang II, or vehicle (dH_2O) for 1 week. Urine volume (normalized by body weight) was measured for 24 h in rats after PD168077 or vehicle injection ($n=5$; $^*P<0.05$ *vs.* control; $^{\#}P<0.05$ *vs.* PD168077 alone). (**c**) Effect of pre-treatment with Ang II on the inhibitory effect of D_4 receptors on urine sodium in WKY rats and SHRs. Urine sodium excretion was measured in PD168077-treated or vehicle-treated rats ($n=5$; $^*P<0.05$ *vs.* control; $^{\#}P<0.05$ *vs.* PD168077 alone). (**d**) D_4 receptor phosphorylation in WKY and SHR RPT cells. The RPT cell lysate protein was immunoprecipitated with an anti-phosphoserine antibody and immunoblotted using an anti-D_4 antibody ($n=3$; $^*P<0.05$ *vs.* WKY).

WKY RPT cells. However, the D_4 receptor agonist could not decrease Na^+–K^+ ATPase activity in SHR RPT cells, indicating that there were D_4 receptor dysfunctions in a hypertensive state. Thus, the increasing D_4 receptor expression by Ang II would augment the inhibitory effect of the D_4 receptor on Na^+–K^+ ATPase activity in WKY RPT cells, but this effect is absent in SHR RPT cells.

The dopaminergic and renin–angiotensin systems play an important role in regulating the blood pressure. High NaCl intake increases the renal synthesis of dopamine and dopaminergic receptor activity, which decreases epithelial sodium transport, whereas a sodium deficit activates the renin–angiotensin-aldosterone system, which increases epithelial sodium transport.[30] Several studies have shown that the dopaminergic and renin–angiotensin systems interact to regulate renal function.[18,19,21,31,32] For example, D_1-like receptor agonists antagonize the stimulatory effects of Ang II on NaCl uptake in rat RPT cells and brush border membrane vesicles.[32] Additionally, an AT_1 receptor blockade enhances the natriuretic effect of D_1-like agonists.[31] Moreover, activation of D_3 dopaminergic receptors decreases AT_1 receptor expression in WKY RPT cells but increases AT_1 receptor expression in SHR RPT cells.[18] In return, AT_1 receptors negatively regulate the expression of D_3 receptors in rat RPT cells.[19] The interaction between the renin–angiotensin and dopaminergic systems has also been determined in our studies. Our previous study proved that stimulation of D_4 receptors influences AT_1 receptor expression in RPT cells.[21] In this study, we demonstrated that stimulation of AT_1 receptors, in return, increases D_4 receptor expression in both WKY and SHR RPT cells. These findings indicate that the interaction between renin–angiotensin and dopaminergic systems is also involved in maintaining sodium balance.

The mechanism for the increase in D_4 receptor expression caused by the AT_1 receptor in WKY rats was also investigated in this study. We found that stimulation of AT_1 receptors increases D_4 receptor mRNA expression in both WKY and SHR RPT cells. This result is consistent with changes at the protein level and indicates that the regulation occurs at the transcriptional level. We also found that calcium mediates the stimulatory effect of the AT_1 receptor on D_4 receptor expression in RPT cells. It is known that the response of intracellular calcium concentrations to Ang II is mediated by AT_1 receptors in the podocytes of the intact glomerulus and is partly due to Ca^{2+} entry from the extracellular space.[33] L-type Ca^{2+} channels are major Ca^{2+} channels that mediate Ca^{2+} influx in RPT cells.[34] Ca^{2+} influx through L-type Ca^{2+} channels triggers Ca^{2+} dynamics and various Ca^{2+}-dependent signaling pathways. For example, Ca^{2+} is needed for the opening of the inositol 1,4,5-trisphosphate receptor

($InsP_3R$), which activates a cascade of Ca^{2+} release from the endoplasmic reticulum store.[35] Thus, we speculate that the AT_1 receptor-mediated regulation of D_4 receptor expression is dependent on the L-type Ca^{2+} channel-mediated Ca^{2+} influx in RPT cells and affects D_4 receptor expression at the transcriptional level. Further studies are needed to investigate how calcium signals stimulate D_4 receptor expression.

Our data suggest that D_4 receptor function is aberrant in SHRs. It is known that phosphorylation plays an important role in regulating the function of the dopaminergic receptors, which are G protein-coupled receptors.[36] Phosphorylation makes dopaminergic receptors high-affinity binding partners for arrestin proteins, which stop further G protein activation and promote receptor internalization, recycling or degradation.[37] Therefore, our results indicate that the difference in the phosphorylation level of D_4 receptor proteins might be associated with the aberrant function of D_4 receptors in SHRs.

In conclusion, we have demonstrated that AT_1 receptors upregulate D_4 receptor expression in RPT cells via the activation of calcium channels. The inhibitory effect of D_4 receptors on Na^+–K^+ ATPase activity was enhanced by AT_1 receptor stimulation in WKY RPT cells but not in SHR RPT cells. These results were also confirmed *in vivo*. This effect might be involved in the pathogenesis of essential hypertension.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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1 Chugh G, Pokkunuri I, Asghar M. Renal dopamine and angiotensin II receptor signaling in age-related hypertension. *Am J Physiol Renal Physiol* 2013; **304**: F1–F7.
2 Liu X, Wang W, Chen W, Jiang X, Zhang Y, Wang Z, Yang J, Jones JE, Jose PA, Yang Z. Regulation of blood pressure, oxidative stress and AT_1R by high salt diet in mutant human dopamine D_5 receptor transgenic mice. *Hypertens Res* 2015; **38**: 394–399.
3 Zeng C, Wang Z, Hopfer U, Asico LD, Eisner GM, Felder RA, Jose PA. Rat strain effects of AT_1 receptor activation on D_1 dopamine receptors in immortalized renal proximal tubule cells. *Hypertension* 2005; **46**: 799–805.
4 Jiang X, Chen W, Liu X, Wang Z, Liu Y, Felder RA, Gildea JJ, Jose PA, Qin C, Yang Z. The synergistic roles of Cholecystokinin B and dopamine D_5 receptors on the regulation of renal sodium excretion. *PLoS ONE* 2016; **11**: e0146641.
5 Gildea JJ, Shah IT, Van Sciver RE, Israel JA, Enzensperger C, McGrath HE, Jose PA, Felder RA. The cooperative roles of the dopamine receptors, D_1R and D_5R, on the regulation of renal sodium transport. *Kidney Int* 2014; **86**: 118–126.
6 Chen Y, Asico LD, Zheng S, Villar VA, He D, Zhou L, Zeng C, Gastrin Jose PA. Gastrin and D_1 dopamine receptor interact to induce natriuresis and diuresis. *Hypertension* 2013; **62**: 927–933.
7 Aperia A. 2011 Homer Smith Award: to serve and protect: classic and novel roles for Na^+-K^+-adenosine triphosphatase. *J Am Soc Nephrol* 2012; **23**: 1283–1290.
8 Feraille E, Doucet A. Sodium-potassium-adenosine triphosphatase-dependent sodium transport in the kidney: hormonal control. *Physiol Rev* 2001; **81**: 345–418.
9 Gattineni J, Baum M. Developmental changes in renal tubular transport-an overview. *Pediatr Nephrol* 2015; **30**: 2085–2098.
10 Carey RM. Theodore Cooper Lecture: renal dopamine system: paracrine regulator of sodium homeostasis and blood pressure. *Hypertension* 2001; **38**: 297–302.
11 Aperia A, Bertorello A, Seri I. Dopamine causes inhibition of Na^+-K^+ ATPase activity in rat proximal convoluted tubule segments. *Am J Physiol* 1987; **252**: F39–F45.
12 Bertorello A, Hokfelt T, Goldstein M, Aperia A. Proximal tubule Na^+-K^+ ATPase activity is inhibited during high-salt diet: evidence for DA-mediated effect. *Am J Physiol* 1988; **254**: F795–F801.
13 Aperia AC. Intrarenal dopamine: a key signal in the interactive regulation of sodium metabolism. *Annu Rev Physiol* 2000; **62**: 621–647.
14 Asghar M, Tayebati SK, Lokhandwala MF, Hussain T. Potential dopamine-1 receptor stimulation in hypertension management. *Curr Hypertens Rep* 2011; **13**: 294–302.
15 Choi MR, Kouyoumdzian NM, Rukavina Mikusic NL, Kravetz MC, Roson MI, Rodriguez Fermepin M, Fernandez BE. Renal dopaminergic system: pathophysiological implications and clinical perspectives. *World J Nephrol* 2015; **4**: 196–212.
16 Cuevas S, Villar VA, Jose PA, Armando I. Renal dopamine receptors, oxidative stress, and hypertension. *Int J Mol Sci* 2013; **14**: 17553–17572.
17 Ortiz PA, Garvin JL. Intrarenal transport and vasoactive substances in hypertension. *Hypertension* 2001; **38**: 621–624.
18 Zeng C, Liu Y, Wang Z, He D, Huang L, Yu P, Zheng S, Jones JE, Asico LD, Hopfer U, Eisner GM, Felder RA, Jose PA. Activation of D_3 dopamine receptor decreases angiotensin II type 1 receptor expression in rat renal proximal tubule cells. *Circ Res* 2006; **99**: 494–500.
19 Zeng C, Asico LD, Wang X, Hopfer U, Eisner GM, Felder RA, Jose PA. Angiotensin II regulation of AT_1 and D_3 dopamine receptors in renal proximal tubule cells of SHR. *Hypertension* 2003; **41**: 724–729.
20 Bek MJ, Wang X, Asico LD, Jones JE, Zheng S, Li X, Eisner GM, Grandy DK, Carey RM, Soares-da-Silva P, Jose PA. Angiotensin-II type 1 receptor-mediated hypertension in D_4 dopamine receptor-deficient mice. *Hypertension* 2006; **47**: 288–295.
21 Chen K, Deng K, Wang X, Wang Z, Zheng S, Ren H, He D, Han Y, Asico LD, Jose PA, Zeng C. Activation of D_4 dopamine receptor decreases angiotensin II type 1 receptor expression in rat renal proximal tubule cells. *Hypertension* 2015; **65**: 153–160.
22 Yu C, Chen J, Guan W, Han Y, Wang WE, Wang X, Wang H, Jose PA, Zeng C. Activation of the D_4 dopamine receptor attenuates proliferation and migration of vascular smooth muscle cells through downregulation of AT1a receptor expression. *Hypertens Res* 2015; **38**: 588–596.
23 Zeng C, Armando I, Luo Y, Eisner GM, Felder RA, Jose PA. Dysregulation of dopamine-dependent mechanisms as a determinant of hypertension: studies in dopamine receptor knockout mice. *Am J Physiol Heart Circ Physiol* 2008; **294**: H551–H569.
24 Gildea JJ, Wang X, Jose PA, Felder RA. Differential D_1 and D_5 receptor regulation and degradation of the angiotensin type 1 receptor. *Hypertension* 2008; **51**: 360–366.
25 Luo H, Wang X, Wang J, Chen C, Wang N, Xu Z, Chen S, Zeng C. Chronic NF-κB blockade improves renal angiotensin II type 1 receptor functions and reduces blood pressure in Zucker diabetic rats. *Cardiovasc Diabetol* 2015; **14**: 76.
26 Kotlo K, Shukla S, Tawar U, Skidgel RA, Danziger RS. Aminopeptidase N reduces basolateral Na^+-K^+ ATPase in proximal tubule cells. *Am J Physiol Renal Physiol* 2007; **293**: F1047–F1053.
27 Sorbel JD, Brooks DM, Lurie DI. SHP-1 expression in avian mixed neural/glial cultures. *J Neurosci Res* 2002; **68**: 703–715.
28 Yang J, Chen C, Ren H, Han Y, He D, Zhou L, Hopfer U, Jose PA, Zeng C. 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. *J Hypertens* 2012; **30**: 1176–1184.
29 Gomes P, Soares-Da-Silva P. D_2-like receptor-mediated inhibition of Na^+-K^+ ATPase activity is dependent on the opening of K^+ channels. *Am J Physiol Renal Physiol* 2002; **283**: F114–F123.
30 Natarajan AR, Eisner GM, Armando I, Browning S, Pezzullo JC, Rhee L, Dajani M, Carey RM, Jose PA. The renin-angiotensin and renal dopaminergic systems interact in normotensive humans. *J Am Soc Nephrol* 2016; **27**: 265–279.
31 Chen C, Lokhandwala MF. Potentiation by enalaprilat of fenoldopam-evoked natriuresis is due to blockade of intrarenal production of angiotensin-II in rats. *Naunyn Schmiedebergs Arch Pharmacol* 1995; **352**: 194–200.
32 Sheikh-Hamad D, Wang YP, Jo OD, Yanagawa N. Dopamine antagonizes the actions of angiotensin II in renal brush-border membrane. *Am J Physiol* 1993; **264**: F737–F743.
33 Nitschke R, Henger A, Ricken S, Gloy J, Muller V, Greger R, Pavenstadt H. Angiotensin II increases the intracellular calcium activity in podocytes of the intact glomerulus. *Kidney Int* 2000; **57**: 41–49.
34 Bozic M, Valdivielso JM. Calcium signaling in renal tubular cells. *Adv Exp Med Biol* 2012; **740**: 933–944.
35 Yatabe MS, Yatabe J, Takano K, Murakami Y, Sakuta R, Abe S, Sanada H, Kimura J, Watanabe T. Effects of a high-sodium diet on renal tubule Ca^{2+} transporter and claudin expression in Wistar-Kyoto rats. *BMC Nephrol* 2012; **13**: 160.
36 Chen K, Fu C, Chen C, Liu L, Ren H, Han Y, Yang J, He D, Zhou L, Yang Z, Zhang L, Jose PA, Zeng C. Role of GRK4 in the regulation of arterial AT_1 receptor in hypertension. *Hypertension* 2014; **63**: 289–296.
37 Gurevich EV, Gainetdinov RR, Gurevich VV. G protein-coupled receptor kinases as regulators of dopamine receptor functions. *Pharmacol Res* 2016; **111**: 1–16.