Angiotensin II Regulation of AT₁ and D₃ Dopamine Receptors in Renal Proximal Tubule Cells of SHR

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Abstract—Dopamine and angiotensin II negatively interact to regulate sodium excretion and blood pressure. D₃ dopamine receptors downregulate angiotensin type 1 (AT₁) receptors in renal proximal tubule cells from normotensive Wistar-Kyoto rats. We determined whether AT₁ receptors regulate D₃ receptors and whether the regulation is different in cultured renal proximal tubule cells from normotensive and spontaneously hypertensive rats. Angiotensin II (10⁻⁸M/24 hours) decreased D₃ receptors in both normotensive (control, 36±3; angiotensin II, 24±3 U) and hypertensive (control, 30±3; angiotensin II, 11±3 U; n=9 per group) rats; effects that were blocked by the AT₁ receptor antagonist, losartan (10⁻⁸M/24 hours). However, the reduction in D₃ expression was greater in hypertensive (60±10%) than in normotensive rats (32±9%). In normotensive rats, angiotensin II (10⁻⁸M/24hr) also decreased AT₁ receptors. In contrast, in cells from hypertensive rats, angiotensin II increased AT₁ receptors. AT₁ and D₃ receptors co-immunoprecipitated in renal proximal tubule cells from both strains. Angiotensin II decreased D₃/AT₁ receptor co-immunoprecipitation similarly in both rat strains, but basal D₃/AT₁ co-immunoprecipitation was 6 times higher in normotensive than in hypertensive rats. Therefore, AT₁ and D₃ receptor interaction is qualitatively and quantitatively different between normotensive and hypertensive rats; angiotensin II decreases AT₁ expression in normotensive but increases it in hypertensive rats. In addition, angiotensin II decreases D₃ expression to a greater extent in hypertensive than in normotensive rats. Aberrant interactions between D₃ and AT₁ receptors may play a role in the pathogenesis of hypertension. (Hypertension. 2003;41[part 2]:724-729.)

Key Words: receptors, dopamine ■ receptors, angiotensin II ■ rats, spontaneously hypertensive ■ kidney

The proximal tubule is a major site of salt and water reabsorption in the mammalian nephron, with up to 60% of the filtrate reabsorbed in this segment. Renal proximal tubule (RPT) function is under hormonal control, with angiotensin II stimulating sodium reabsorption via activation of apical Na⁺/H⁺ exchanger and basolateral [Na⁺][K⁺]ATPase, and with dopamine inhibiting reabsorption by inhibiting these proteins.

The paracrine regulation of sodium reabsorption in the proximal tubule by the renin-angiotensin system occurs via several angiotensin receptor subtypes (AT₁, AT₂, and AT₄). The activation of AT₁ receptors by angiotensin II increases sodium transport, whereas the activation of AT₂ and AT₄ receptors decreases sodium reabsorption in this nephron segment. However, under physiological conditions, the major effect of angiotensin II on sodium transport is stimulatory, via AT₁ receptors.

The dopaminergic system also exerts a paracrine regulatory role on renal sodium transport in the proximal tubule via several receptor subtypes. Dopamine receptors, pharmacologically grouped into D₁-like (D₁ and D₃) and D₂-like (D₂, D₃, and D₄) receptors, as with the angiotensin II receptors, are expressed in brush border and basolateral membranes of RPTs. In contrast to the stimulatory effect of angiotensin II on sodium transport in RPTs, the major consequence of the activation of dopamine receptors is an inhibition of sodium transport via D₁-like and D₃ receptors.

Previous studies have indicated that dopamine and angiotensin II subserve counteracting functions in the proximal tubule. Inhibition of RPT angiotensin II production or blockade of AT₁ receptors increases the natriuretic effect of the D₂-like agonist fenoldopam. D₁-like and D₂-like receptor agonists also antagonize the stimulatory effect of angiotensin II, acting via AT₁ receptors, on RPT luminal sodium transport. The renal vasoconstrictor effect of angiotensin II can also be antagonized by D₁-like receptor agonists. Alterations in the balance of the responsiveness of the proximal tubule to angiotensin II and dopamine have important impli-
cations on net sodium reabsorption. Indeed, an altered interaction between angiotensin II and dopamine has been implicated in genetic hypertension.4,14

In a previous study, we showed that D3 and AT1 receptors co-localized in immortalized RPT cells from normotensive Wistar-Kyoto rats (WKY) and that D3 dopamine receptors decreased AT1 receptor protein expression in these cells.15 We have found that these immortalized RPT cells have characteristics similar to freshly obtained RPT brush border membranes and RPTs, at least with regard to D1 receptors and responses to G-protein stimulation.16–18 The present studies were designed to determine whether angiotensin II regulates AT1 and D3 receptor expression and whether the regulation is different in RPT cells from WKY and spontaneously hypertensive rats (SHR).

Methods

Cell Culture.
All studies have been approved by the Georgetown University Animal Care and Use Committee. Immortalized RPT cells obtained from 4- to 8-week-old WKY and SHR (Charles River Laboratory, Wilmington, Mass) were cultured at 37°C in 95% air/5% CO2 atmosphere in Dulbecco modified Eagle’s medium/F-12 culture media, as previously described.17,19 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 EGTA, 1 mmol/L PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin), sonicated, kept on ice for 1 hour, and centrifuged at 16,000g for 30 minutes. The supernatants were stored at −70°C until use for immunoblotting and/or immunoprecipitation.

Immunoblotting

The antibodies are polyclonal purified anti-peptides. The amino acid sequence for the immunogenic peptide (rabbit anti-human AT1 receptor antibody) is QDCCPKAGRHC. amino acids 15–24 of the AT1 receptor. The specificity of this antibody to the AT1 receptor has been reported.20 The amino acid sequence of the peptide for the rabbit anti-rat D3 receptor antibody corresponds to the third intracellular loop of the D3 receptor.11,21 We have reported the specificity of this D3 receptor antibody.11 Rat RPT cells were treated with vehicle (dH2O), angiotensin II, or an AT1 receptor antagonist (losartan) at the indicated concentrations and times.22 Immunoblotting was performed as previously reported, except that the transblots were probed with the D3 (1:250) or the AT1 receptor antibody (1:400).11,23,24 The amount of protein transferred onto the membranes was determined by Ponceau-S staining and immunoblotting for α-actin.

Immunoprecipitation

RPT cells were incubated with vehicle or angiotensin II (10−8 M) for 24 hours, as described above. The cells were lysed with ice-cold lysis buffer for 1 hour and centrifuged at 16,000g for 30 minutes. Equal amounts of lysates, except as indicated (300 μg protein/mL supernatant for RPT cells from WKY and 900 μg protein/mL supernatant for RPT cells from SHR) were incubated with affinity-purified anti-D3 receptor antibody (1 μg/mL) for 1 hour and G-protein agarose at 4°C for 12 hours. The immunoprecipitates were pelleted and washed 4 times with lysis buffer. The pellets were suspended in sample buffer, boiled for 10 minutes, and subjected to immunoblotting with the AT1 receptor antibody. To determine the specificity of the bands, normal rabbit IgG (negative control) and AT1 receptor antibody (positive control) were used as immunoprecipitants, instead of the D3 receptor antibody. The density of the bands were quantified by densitometry, using Quantiscan as previously reported.17,19,24

Materials

Rabbit anti-rat D3 and anti-human AT1 receptor antibodies were purchased from Alpha Diagnostic International (D3R12A) and Santa Cruz Biotechnology Inc (sc-1173), respectively. Angiotensin II (H-17057002) was purchased from Peninsula Laboratory Inc. Losartan was a gift from Merck & Co (Philadelphia, Penn). Other chemicals for various buffers were of the highest purity available and purchased either from Sigma or GIBCO.

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 2 groups were compared), and comparison among groups (or t test when only 2 groups were compared) was made by ANOVA with the Duncan test. A value of P<0.05 was considered significant.

Results

Specificity of Receptor Antibodies.

The bands for the AT1 receptor (~45 kDa) and D3 receptor (~45 kDa) were found in RPT cells, as previously reported.11,15 Another band of ~40 kDa was noted for the D3 receptor and probably represents a degradation product of the 45-kDa D3 receptor.23 The bands were specific because they were completely blocked when the antibodies were preadsorbed by the immunizing peptide (Figures 1A and 1B, lanes 1 and 2). HEK293 cells, transfected with rat D3 receptor cDNA, expressed bands similar to those noted in RPT cells and were also blocked by immunizing peptide (Figure 1B, lane 1). The pattern of D3 receptor immunoblotting is similar to our previous report.11,15

AT1 Receptors Decrease D3 Receptor Expression in Rat RPT Cells From SHR and WKY

Angiotensin II decreased D3 receptor expression in a concentration- and time-dependent manner in RPT cells from WKY. The inhibitory effect was evident at 10−9M, with a 50% decrease (EC50) in D3 receptor expression at (4.1×10−10M) (Figure 2A). The inhibitory effect of angiotensin II (10−9 M) was noted as early as 8 hours and maintained for at least 30 hours (Figure 2B). In RPT cells from SHR, angiotensin II (10−6 M/24 hours) also decreased D3 expression (SHR: control, 30±3; angiotensin II, 11±3 density units [DU]; WKY: control, 36±3; angiotensin II, 24±3 DU; n=9 per group), but the reduction was greater in SHR (60±10%) than in WKY (32±9%) (Figure 2C). Basal D3 receptor (45 KDa) levels were also lower in SHR compared with WKY, similar to our previous report.11,15

The specificity of angiotensin II as an AT1 receptor agonist was also determined by studying the effect of the AT1 receptor antagonist losartan. Consistent with the study shown in Figures 2A and 2B, angiotensin II (10−6 M/24 hours), decreased D3 receptor expression (control, 25±2 DU; angiotensin II, 19±2 DU; n=8, P<0.05). The AT1 receptor antagonist, losartan (10−5 M), by itself, had no effect on D3 receptor expression (29±3 DU) but reversed the inhibitory effect of angiotensin II on D3 receptor expression (28±2 DU) (Figure 2D).

Angiotensin II Decreases AT1 Receptor Expression in Rat RPT Cells From WKY But Increases It From SHR

To investigate the effect of angiotensin II on its own receptor, RPT cells were incubated with angiotensin II (10−10 M) for 24
hours. Immunoblots showed that angiotensin II decreased its own receptor expression in RPT cells from WKY (control, 26±2 DU; angiotensin II, 17±3 DU; P<0.05, n=5) but increased it in RPT cells from SHR (control, 24±2 DU; angiotensin II, 38±5 DU; n=5 per group) (Figure 3).

**AT₁ Receptor Co-Immunoprecipitates With the D₃ Receptor in Rat RPT Cells**

To determine whether there is a physical interaction between the D₃ and the AT₁ receptor, additional experiments were performed. D₃ receptors were first immunoprecipitated with anti-D₃ receptor antibodies and then probed with anti-AT₁ receptor antibodies. As shown in Figure 4, the band of 45 kDa, representing the co-immunoprecipitated D₃ and AT₁ receptors, was decreased by a 24-hour treatment of angiotensin II (10⁻⁸M) in both RPT cells from WKY and SHR (WKY: control, 45±10 DU; angiotensin II, 16±5 DU; n=7, P<0.05; SHR: control, 7±2 DU; angiotensin II, 3±1 DU; n=7, P<0.05). However, basal co-immunoprecipitation was

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**Figure 1.** Specificity of AT₁ and D₃ antibodies. A, Immunoblot of AT₁ receptors in RPT cells from WKY. Cell lysate protein (100 μg) was subjected to immunoblotting with anti-AT₁ receptor antibody (1:400). The 45-kDa band was completely blocked when the antibody was preadsorbed with immunizing peptide (1:10 w/w). B, Immunoblot of D₃ receptors. Cell lysate protein (100 μg) from HEK293 cells transfected with rat D₃ receptor cDNA (lane 1) and WKY RPT cells (lane 2) had similar bands (45 kDa). Another band of ~40 kDa, probably representing a degradation product of the D₃ receptor, was also noted. The bands were no longer visualized when the antibody was preadsorbed with immunizing peptide (1:10 w/w).

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**Figure 2.** Effect of angiotensin II on D₃ receptor expression in RPT cells from SHR and WKY. A, Concentration-response of D₃ receptor expression in RPT cells from WKY treated with angiotensin II. Immunoreactive D₃ receptor expression was determined after 24-hour incubation with the indicated concentrations of angiotensin II. Results are expressed as relative density units (n=5; *P<0.05 vs control [C], ANOVA, Duncan test). B, Time-course of D₃ receptor expression in RPT cells from WKY treated with angiotensin II. The cells were incubated for the indicated times with 10⁻⁸M angiotensin II. Results are expressed as relative density units (n=7; *P<0.05 vs control [0 time], ANOVA,
greater in RPT cells from WKY than from SHR, similar to our previous report.15

Discussion

There are several novel observations in the present study. First, we show that angiotensin II, via AT1 receptors, decreases D3 receptor expression in rat RPT cells from WKY. This effect is exerted at the AT1 receptor because an AT1 antagonist, losartan, completely blocks the effect of angiotensin II. Second, angiotensin II also decreases D3 receptors in SHR, but the reduction in D3 expression is greater in SHR than in WKY. Third, angiotensin II decreases AT1 receptor expression in RPT cells from WKY but increases it in cells from SHR. Fourth, AT1 receptors co-immunoprecipitate with D3 receptors in rat RPT cells. Angiotensin II stimulation decreases the physical interaction between AT1 and D3 receptors in rat RPT cells from both SHR and WKY to the same degree. However, the basal level of AT1/D3 co-immunoprecipitation is much greater in WKY compared with SHR.

The effect of the D3-like receptor, by itself, on renal sodium transport is controversial; stimulation, no effect, and even inhibition have been reported. The reason for the apparent discrepancies may be related to the different experimental conditions and the specific D3-like receptor involved. However, under certain circumstances, such as during moderate sodium loading, D3 and D2-like receptors can synergistically inhibit sodium transport.11 All of D2-like receptors exist in kidney.3,4 The D3 receptor heterologously expressed in fibroblasts (LTK− cells) increases Na⁺/K⁺ ATPase activity, but the D3 receptors expressed in the kidney are probably in prejunctional nerves because D3 mRNA was not detected in microdissected rat RPTs or rat kidney (M.J. Bek, I. Yamaguchi, Z. Yang, and P.A. Jose, unpublished data, 2000). The D3 receptor is expressed mainly in collecting ducts.4,25 In contrast, D1 receptors, like the D1 and D2 receptors, are expressed in the proximal tubule.11,26 Thus, the D3 receptor may be the major D2-like subtype receptor expressed in RPTs. Quinpirole, a D2-like receptor agonist with a greater selectivity for the D3 and D4 receptor over the D2 receptor, decreases sodium excretion in dogs, whereas 7-OH-DPAT, a D2-like receptor with a greater selectivity for D3 than D2 or D4 receptors, increases both glomerular filtration rate and sodium and water excretion in rats.27,28 PD128907,27 which has a greater selectivity for D3 than for D4 or D5 receptors also increased sodium excretion in salt-loaded but not salt-depleted WKY. These studies suggest that D3 receptors, under certain circumstances, mediate a natriuresis, an effect that seems to be lost in SHR (C. Zeng, L.D. Asico, and P.A. Jose, unpublished data, 2002). Whether the defective effect of D3 receptors on sodium excretion in SHR is primary or secondary to interactions with other receptors is not known. However, preliminary studies from our laboratory indicate that the D3 agonist, PD128907, increases D3 receptor in RPT cells from WKY but has no effect in RPT cells from SHR.15,29

We have reported that mice lacking both D3 receptor alleles developed systemic hypertension and a decreased ability to excrete a systemic sodium load. In addition, renal renin activity and AT1 receptor expression are much higher in the homozygous than in wild-type mice. Z1046, a dopaminergic agonist with a greater selectivity for D3 and D4 receptors over the other dopamine receptors, also increases sodium and water excretion in anesthetized WKY, but this effect is abrogated in SHR.11,30 These data suggest that a D3 receptor antagonist may be a novel treatment for de novo hypertension.

Figure 3. Effect of angiotensin II on AT1 receptor expression in RPT cells from SHR and WKY. The cells were incubated with 10⁻⁸M angiotensin II for 24 hours. Results are expressed as relative densities (n=5; **P<0.05 vs control, ANOVA, Duncan test).

Figure 4. Effect of angiotensin II on the co-immunoprecipitation of D3 and AT1 receptors in rat RPT cells. The cells were incubated with angiotensin II (10⁻⁸M) for 24 hours. Thereafter, the samples were immunoprecipitated with anti-D3 antibodies and immunoblotted with anti-AT1 antibodies (n=7; *P<0.05 vs control; #P<0.05 vs WKY, ANOVA, Duncan test). One immunoblot (45 kDa) is depicted in the inset: (lane 1 indicates positive control; lane 2, negative control; lane 3, vehicle-treated RPT cells of WKY; lane 4, angiotensin II–treated RPT cell of WKY; lane 5, vehicle-treated RPT cells of SHR; and lane 6, angiotensin II–treated RPT cell of SHR). For a positive control, anti-AT1 antibodies (1 μg/mL) were used as the immunoprecipitant; for a negative control, normal rabbit IgG (1 μg/mL) was used as the immunoprecipitant instead of the anti-D3 antibodies and immunoblotted with anti-AT1 antibodies as above.

**Table 1.** Differential effects of angiotensin (10⁻⁸ M/24 hours) on D3 receptor expression in RPT cells from both SHR and WKY. The cells were incubated at the indicated times and concentrations. Results are expressed as relative density units (n=8; *P<0.05 vs others, ANOVA, Duncan test).
receptor defect may play a role in the pathogenesis of some forms of hypertension, most likely via an impaired renal excretory capacity for sodium or via an impaired inhibition/interaction with renin-angiotensin system.

The current study shows that AT1 receptor stimulation decreases D3 receptor expression in RPT cells from both SHR and WKY, but the reduction in D3 expression is greater in SHR than in WKY. The greater reduction in D3 receptor expression in RPT cells caused by angiotensin II in SHR than in WKY may be caused by an increased activity of the AT1 receptor in this rat strain. Indeed young SHR (5 to 7 weeks), the same age group as the rats in which the RPT cells are obtained for immortalization, RPT sodium transport is greater in SHR than in WKY.31–33 Angiotensin II also increases renal proximal tubular sodium reabsorption to a greater extent in SHR than in WKY.33

We also found that angiotensin II decreases AT1 receptor in cells from WKY. There are a few studies on the effect of angiotensin II on AT1 receptor expression in RPT cells. In rabbit RPT cells, a 16-hour incubation with angiotensin II dose-dependently increases AT1 receptor expression, assessed by radioligand binding.34 AT1 receptor mRNAs in rat and rabbit RPT cells are also increased by angiotensin II (10−8M).34,35 However, the systemic administration of angiotensin II that increases systemic blood pressure does not alter total renal AT1 receptor expression, determined by immunoblotting, but decreases AT1 receptor expression (radioligand autoradiography) in glomeruli and inner stripe of the outer medulla. Three days after the infusion of angiotensin II, there is a tendency for a decrease in AT1 expression in the whole kidney and in RPTs, but the changes are small and do not reach statistical significance,36 results that could be taken to support our data. The differences in the results of these studies cannot be explained at this time.

In contrast to the inhibitory effect of angiotensin II on AT1 expression in RPT cells from WKY, angiotensin II produces the opposite effect in RPT cells from SHR. The different effects of AT1 agonist on D3 receptor expression, and its own receptor, could be involved in the pathogenesis of hypertension in SHR. Decreased renal D3 receptor expression and function have been reported in the Dahl salt-sensitive rat.28 However, it is not known whether the D3 receptor dysfunction in these rodent models of genetic hypertension is primary or secondary to a dysfunction of renal D1 receptors.4 An uncoupling of the D3 receptor from its G-protein effector complex in the kidney has been shown to be important in the pathogenesis of genetic hypertension.4,17

The mechanism for the decrease in D3 receptor of the 45-kDa species caused by AT1 receptors was not studied. However, the D3 receptor, a member of the D2-like receptor, has been reported to decrease gene expression by translational and/or posttranslational protein modification.21 It is possible that AT1 receptors regulate D3 receptor expression by similar mechanisms.

We now report that AT1 and D3 receptors can directly interact with each other. In the current studies, the basal level of AT1/D3 co-immunoprecipitation is much lower in RPT cells from SHR than from WKY. AT1 receptor stimulation results in a decreased interaction between AT1 and D3 receptors. The paucity of D3 receptors on the RPT cell membranes could have been responsible for the decreased basal amount of D3 and AT1 receptor co-immunoprecipitation in SHR. Therefore, we tripled the amount of protein used for the immunoprecipitation studies in SHR. In a previous report, we found that doubling the amount of loaded protein from renal cortical membranes from SHR approximate D3 expression in SHR to those observed in WKY.11 Moreover, reversing the antibodies used for immunoprecipitation and immunoblotting, by using the AT1 antibody for immunoprecipitation and the D3 antibody for immunoblotting, gives similar results (data not shown). Therefore, it is unlikely that the low abundance of D3 receptors in SHR could have caused the decreased amounts of D3 and AT1 receptor co-immunoprecipitation in this rat strain. We suggest that the strain differences could be caused by a differential expression in adaptor or interacting proteins. The ability of angiotensin II to decrease the amount of D3/AT1 co-immunoprecipitation to the same degree in WKY and SHR suggests that the putative adaptor or interacting protein normally responds to angiotensin II. Further studies are needed to determine whether the decreased interaction between these 2 receptors is a direct or an indirect mechanism, possibly by the alteration of adaptor proteins. Thus, an adaptor protein for D1 and D3 receptors (protein 4.1N) has been recently identified that is important in their localization in plasma membranes.37 Others have also shown that several G protein–coupled receptors interact directly with each other resulting in homo- or hetero-oligomerization.21,38

In summary, we have demonstrated that AT1 receptors negatively regulate the expression of D3 receptors in rat RPT cells. Furthermore, AT1 and D3 receptors interact in RPT cells, but this interaction is impaired in SHR.

Perspectives
The dopaminergic and renin-angiotensin systems are 2 important systems that regulate sodium excretion and blood pressure.3–5 D1-like and D2-like dopamine receptors synergistically inhibit sodium reabsorption in RPTs.4,11,30,39 The receptor subtypes mediating these synergistic effects are, most probably, D1 and D3 receptors because D1 and D3 receptors are the major dopamine receptors in the RPT cells.4,26 We have preliminary data showing an inhibition of Na+-K+ ATPase activity by the stimulation of both D1 and D3 receptors (data not shown). In SHR, the synergistic interaction between D1-like and D2-like receptors is impaired.11 D1 receptor expression (45 kDa), in renal brush border membranes and RPT cells, is lower in rodent hypertensive models relative to normotensive controls.11,28 Despite similar abundance of D1 receptors in RPTs in human essential and rodent genetic hypertension,4 D1 receptor function is impaired because of increased G protein–coupled receptor kinase (GRK) 4 and decreased phospholipase 2A (PP2A) activities.18,40,41 It is possible that GRK4 and PP2A have a similar effect on D1 receptors, causing the decrease in D1 receptor expression and function. Activation of D1 receptor or D3 receptor decreases AT1 receptor expression in RPT cells from WKY, and those effects are impaired in SHR.2,15 AT1 receptor activity is higher in SHR3–7,33; however, it has not been determined whether the increased AT1 receptor expression or function is primary or secondary to derangements of other systems. We
hypothesize the increased AT₁ receptor activity in SHR is secondary to an impaired dopaminergic system, related to GRK4, D₂, and D₃ receptors. This hypothesis will be tested in future studies.

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References

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