Activation of D$_3$ Dopamine Receptor Decreases Angiotensin II Type 1 Receptor Expression in Rat Renal Proximal Tubule Cells


Abstract—The dopaminergic and renin angiotensin systems interact to regulate blood pressure. Disruption of the D$_3$ dopamine receptor gene in mice produces renin-dependent hypertension. In rats, D$_2$-like receptors reduce angiotensin II binding sites in renal proximal tubules (RPTs). Because the major D$_3$-like receptor in RPTs is the D$_3$ receptor, we examined whether D$_3$ receptors regulate angiotensin II type 1 (AT$_1$) receptors in rat RPT cells. The effect of D$_3$ receptors on AT$_1$ receptors was studied in vitro and in vivo. The D$_3$ receptor agonist PD128907 decreased AT$_1$ receptor protein and mRNA in WKY RPT cells and increased it in SHR cells. PD128907 increased D$_3$ receptors in WKY cells but had no effect in SHR cells. D$_3$/AT$_1$ receptors colocalized in RPT cells; D$_3$ receptor stimulation decreased the percent amount of D$_3$ receptors that coimmunoprecipitated with AT$_1$ receptors to a greater extent in WKY than in SHR cells. However, D$_3$ receptor stimulation did not change the percent amount of AT$_1$ receptors that coimmunoprecipitated with D$_3$ receptors in WKY cells and markedly decreased the coimmunoprecipitation in SHR cells. The D$_3$ receptor also regulated the AT$_1$ receptor in vivo because AT$_1$ receptor expression was increased in kidneys of D$_3$ receptor–null mice compared with wild type littermates. D$_3$ receptors may regulate AT$_1$ receptor function by direct interaction with and regulation of AT$_1$ receptor expression. One mechanism of hypertension may be related to increased renal expression of AT$_1$ receptors due decreased D$_3$ receptor regulation. (Circ Res. 2006;99:494-500.)

Key Words: AT$_1$ receptor ■ D$_3$ receptor ■ renal proximal tubule cells ■ hypertension
characteristics similar to freshly obtained RPTs and renal brush border membranes, at least, with regard to the D3 receptor.9,10,21 In this study, we determined whether D3 receptors interacted with AT1 receptors. We also examined AT1 receptor expression in kidneys of D3 null mice. We now report that D3 and AT1 receptors colocalize in rat RPT cells. Furthermore, D3 receptor occupancy decreases expression of AT1 receptors and their communoprecipitation with D3 receptors. The interaction between D3 and AT1 receptors also occurs in vivo because AT1 receptor expression is increased in the kidneys of D3 receptor–null mice.

**Materials and Methods**

**D3 Dopamine Receptor–Null Mice**

Mice homozygous for the D3 null receptor were bred from heterozygous mice in C57BL/6 background (The Jackson Laboratory, Bar Harbor, Me); nontransgenic mice served as controls.18 The mice were anesthetized with pentobarbital (50 mg/kg IP), tracheotomized, and blood pressure determined from the femoral artery. The renal cortices were prepared for immunoblotting as described.9,10,19,21 All experiments were approved by the Georgetown University Animal Use and Care Committee.

**Cell Culture**

Immortalized RPT cells from Wistar–Kyoto and spontaneously hypertensive rats (SHRs)9,10,20,21 were cultured at 37°C in 95% air/5% CO2 atmosphere in DMEM/F-12. Embryonic thoracic aortic smooth muscle cells22,23 (passage 10 to 20) from normotensive Berlin–Druckrey IX (A10; CRL 1476, American Type Culture Collection, Manassas, Va) were cultured at 37°C in 95% air/5% CO2 atmosphere in DMEM. The cells (80% confluency) were extracted in ice-cold lysis buffer, sonicated, kept on ice for 1 hour, and centrifuged at 16,000g for 30 minutes. All samples were stored at −70°C until use.

**Immunoblotting**

Polyclonal rabbit anti-human AT1 receptor antibodies (QDDCPK-AGRHC, 15 to 24 position on AT1 receptor) (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) and rabbit anti-rat D3 receptor antibodies (CHVSPELYR, 405 to 413 position on the D3 receptor) (Alpha Diagnostic International, San Antonio, Tex) were used. The specificity of these antibodies has been reported.9,10,24–26 Rat RPT cells were treated with vehicle, D3 receptor agonist (PD128907) (Sigma Co, St Louis, Mo)16 or the D3 receptor antagonist (U99194A) (Research Biochemicals International, Natick, Me); nontransgenic mice served as controls.18 The mice were anesthetized with pentobarbital (50 mg/kg IP), tracheotomized, and blood pressure determined from the femoral artery. The renal cortices were prepared for immunoblotting as described.9,10,19,21 All experiments were approved by the Georgetown University Animal Use and Care Committee.

**RT-PCR of AT1 Receptors**

A total of 2 to 3 μg of total RNA extracted from both WKY and SHR cells was used to synthesize cDNA and served as a template for amplification of AT1, and for β-actin sequences as an endogenous standard.16 For β-actin, the forward primer was 5′-GTGGGTATGGGCTAGACCGTT3′- and the reverse primer was 5′-AGGGCGTAACCCCTCATGAT-3′ (GenBank accession no. BC 063166). The amplification was performed with the following conditions: 35 cycles of denaturation at 94°C for 2 minutes, annealing for 30 seconds at 52.5°C, and extension for 45 seconds at 72°C. For AT1, the forward primer was 5′-CACAAGTCACCTGCTCATC-3′ and the reverse primer was 5′-CACAACTGGCATATTATCTA-3′ (GenBank accession no. NM-030985). The amplification was performed with the following conditions: 35 cycles of denaturation at 94°C for 2 minutes, annealing for 30 seconds at 52.5°C, and extension for 45 seconds at 72°C.30 The AT1 receptor mRNA expression was normalized by β-actin mRNA.

**Immunoprecipitation**

RPT cells were incubated with vehicle, or a D3 receptor agonist, PD128907 (10−7 mol/L), for 24 hours as described above. PD128907 has a 10- to 20-fold greater affinity for the D3 than the D2 receptor.16 Cell lysates (800 μg protein/mL supernatant) were incubated with D3 receptor or AT1 receptor antibody (1 μg/mL) for 1 hour and protein-G agarose at 4°C for 12 hours. The immunoprecipitates were suspended in Laemmli buffer, boiled for 10 minutes, divided into 2 aliquots, electrohoresed, transferred, and subjected to immunoblotting with the AT1 and D3 receptor antibodies.

In 1 series of experiments, the sample immunoprecipitated with the D3 receptor antibody was divided into 2 aliquots, 1 immunoblotted with the AT1 receptor antibody and the other immunoblotted with the D3 receptor antibody. The data are shown as the ratio of D3 receptor immunoprecipitate/AT1 receptor immunoblot and D3 receptor immunoprecipitate/D3 receptor immunoblot. To determine the specificity of the bands, normal rabbit IgG (negative control) and AT1 receptor antibody (positive control) were used as the immunoprecipitates instead of the D3 receptor antibody.21

In another series of experiments, we reversed the antibodies used for immunoprecipitation and immunoblotting. The samples were immunoprecipitated with the AT1 receptor antibody and divided into 2 aliquots. One was immunoblotted with the D3 receptor antibody and the other was immunoblotted with the AT1 receptor antibody. The data are shown as the ratio of AT1 receptor immunoprecipitate/D3 receptor immunoblot and AT1 receptor immunoprecipitate/AT1 receptor immunoblot. To determine the specificity of the bands, normal rabbit IgG (negative control) and D3 receptor antibody (positive control) were used as the immunoprecipitates instead of the AT1 receptor antibody.21

**Immunofluorescence Microscopy**

RPT cells, treated with vehicle or PD128907 (10−7 mol/L) for 24 hours at 37°C, as above, were fixed and permeabilized as reported.31 The D3 receptor was visualized using an IgG affinity-purified polyclonal rabbit anti–rat D3 receptor antibody followed by Alexa 488–goat anti–rabbit IgG antibody (Molecular Probes, Eugene, Ore). The AT1 receptor was visualized using a mouse anti–AT1 receptor monoclonal antibody (Abcam Limited, Cambridgeshire, UK), followed by an Alexa Fluor 568–goat anti–mouse IgG antibody (Molecular Probes). Immunofluorescence images were acquired (Olympus AX70 laser confocal microscopy) at an excitation wavelength of 480 nm and 560 nm; emission was detected at 535 and 645 nm. Cells that were treated with only Alexa 488–goat anti–rabbit IgG or Alexa Fluor 568–goat anti–mouse IgG antibodies revealed no immunofluorescence, and omission of the anti-AT1 antibody showed no red or yellow color after merging (data not shown).

**Statistical Analysis**

The data were 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 Duncan’s test. Corresponding periods between 2 different groups were analyzed by independent t test. A value of P<0.05 was considered significant.
Results

D3 Receptors Decrease AT1 Receptor Expression in WKY RPT Cells and Increase It in SHR RPT Cells

The D3 receptor agonist PD128907 decreased AT1 receptor expression in a concentration- and time-dependent manner. The inhibitory effect was evident at 10^{-7} mol/L with a 50% decrease (EC50) in AT1 receptor expression at 7.3 \times 10^{-10} mol/L (Figure 1A). The inhibitory effect of PD128907 (10^{-7} mol/L) was noted as early as 2 hours and maintained for at least 24 hours; a 50% decrease (t1/2) in AT1 receptor expression occurred at 7.1 hours (Figure 1B). In contrast, in RPT cells from SHRs, PD128907 (10^{-7} mol/L per 24 hours) increased AT1 receptor expression (WKY: control = 1.0 \pm 0.1 DU; PD128907 = 0.5 \pm 0.1 DU; SHR: control = 0.9 \pm 0.1 DU; PD128907 = 1.6 \pm 0.2 DU; n = 8) (Figure 1C).

The specificity of PD128907 as a D3 receptor agonist was also determined by studying the effect of the D3 receptor antagonist U99194. Consistent with the study shown in Figure 1A and 1B, PD128907 (10^{-7} mol/L per 24 hours) decreased AT1 receptor expression (control = 1.2 \pm 0.1 DU, PD128907 = 0.6 \pm 0.1 DU, n = 5; P < 0.05). The D3 receptor antagonist, U99194 (10^{-7} mol/L), by itself, had no effect on AT1 receptor expression (U99194 = 1.2 \pm 0.1 DU) but reversed the inhibitory effect of PD128907 on AT1 receptor expression (PD128907 + U99194 = 1.1 \pm 0.1 DU, n = 5) (Figure 1D).

To investigate a mechanism for D3 receptor downregulation of AT1 receptor, the RPT cells from WKY and SHRs were treated with PD128907 for 24 hours. Activation of the
D3 receptor decreased AT1 receptor mRNA expression in WKY cells, but increased it in SHR cells (WKY: control/ H11005 1.1/ H11006 0.1 DU, PD128907/ H11005 0.8/ H11006 0.03 DU; SHR: control/ H11005 0.9/ H11006 0.1 DU, PD128907/ H11005 1.3/ H11006 0.1 DU; P<0.05, n=6) (Figure 1E).

D3 Receptor Agonist Increases D3 Receptor Expression in WKY RPT Cells, Not in SHR Cells

To investigate the effect of a D3 receptor agonist on its own receptor, RPT cells from SHR and WKY rats were incubated with the D3 receptor agonist, PD128907 (10⁻⁷ mol/L), for 24 hours. Immunoblots showed that the D3 receptor agonist increased its own receptor expression in WKY RPT cells, not in SHR cells (WKY: control=1.1±0.1 DU, PD128907=0.8±0.03 DU; SHR: control=0.9±0.1 DU, PD128907=1.3±0.1 DU; P<0.05, n=6) (Figure 2).

D3 Receptor Colocalizes With the AT1 Receptor in Rat RPT Cells

To determine whether there is a potential for a direct or an indirect interaction between D3 and AT1 receptors, we studied the colocalization of D3 and AT1 receptors in rat RPT cells using immunofluorescence laser confocal microscopy. Both D3 receptor and AT1 receptor were found throughout the whole cell. It was also evident that the D3 receptor and AT1 receptor colocalized in these cells, especially in the cellular membrane (Figure 3).

To determine whether there is a physical interaction between the D3 and the AT1 receptors, additional experiments were performed. When D3 receptors were immunoprecipitated with the D3 receptor antibody and immunoblotted with the AT1 receptor antibody, and the data were normalized by D3 receptor immunoprecipitated and immunoblotted with D3 receptor antibody (Figure 4A), basal coimmunoprecipitation levels were higher in WKY than in SHR cells. However, D3 receptor decreased AT1 receptor mRNA expression in WKY cells, but increased it in SHR cells (WKY: control/ H11005 1.1/ H11006 0.1 DU, PD128907/ H11005 0.8/ H11006 0.03 DU; SHR: control/ H11005 0.9/ H11006 0.1 DU, PD128907/ H11005 1.3/ H11006 0.1 DU; P<0.05, n=6) (Figure 1E).

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AT₁ Receptor Expression Is Increased in the Kidneys of D₃ Receptor–Null Mice

To determine whether the apparent D₃ regulation of AT₁ receptors in vitro occurred in vivo, we determined AT₁ receptor expression in D₃ receptor–null mice. We have reported that arterial blood pressure and renin release were increased in D₃ receptor–null mice in mixed B129 and C57BL/6 background. The ability of the homozygous D₃ receptor–null mice to excrete an acute sodium load was also impaired.

In the current study, we found that pentobarbital-anesthetized homozygous D₃ receptor–null mice, in C57BL/6 background, had higher systolic (SBP) and diastolic (DBP) blood pressures (SBP = 117 ± 5 and DBP = 85 ± 2 mm Hg; n = 5) than wild-type C57BL/6 mice (SBP = 94 ± 2 and DBP = 69 ± 3 mm Hg; n = 5; P < 0.05, t test). Immunoreactive AT₁ receptors were also greater in the kidneys of D₃ receptor–null mice than in wild-type C57BL/6 mice (null mice, 1.4 ± 0.1 DU; wild-type mice, 0.7 ± 0.1 DU; n = 5) (Figure 5). To determine whether there was any effect of the D₃ receptor on vascular AT₁ receptor expression, we treated A10 cells with different concentrations of the D₃ receptor agonist PD128907 (10⁻⁴ mol/L to 10⁻⁷ mol/L). We found that stimulation of the D₃ receptor had no effect on AT₁ receptor expression in A10 cells (Figure 6).

Discussion

There are several novel observations in our study. First, we showed that a D₃ receptor agonist PD128907 decreased AT₁ receptor protein and mRNA expression in rat WKY RPT cells; in contrast, the D₃ receptor agonist increased AT₁ receptor expression in SHR RPT cells. This effect was clearly exerted at the D₃ receptor because a D₁ receptor antagonist, U99194, blocked the effect of the D₃ receptor agonist. Second, the D₃ receptor agonist increased D₃ receptor protein expression in WKY cells but had no effect in SHR cells. Third, AT₁ receptor expression was increased in the kidneys of D₃ receptor–null mice. Fourth, we demonstrated that D₃ receptors colocalized and coimmunoprecipitated with AT₁ receptors in rat RPT cells. D₃ receptor stimulation with PD128907 decreased the percent amount of D₃ receptors that coimmunoprecipitated with AT₁ receptors to a greater extent in WKY than in SHR cells. However, D₃ receptor stimulation did not change the percent amount of AT₁ receptors that coimmunoprecipitated with D₃ receptors in WKY cells because PD128907 decreased AT₁ receptor protein in WKY cells (Figure 4B) (WKY: control = 1.1 ± 0.2 DU, PD128907 = 1.3 ± 0.1 DU; SHR: control = 0.4 ± 0.05 DU, PD128907 = 0.15 ± 0.04 DU; P < 0.05, n = 5). In the SHR, D₃ receptor stimulation with PD128907 markedly decreased the coimmunoprecipitation because PD128907 increased AT₁ receptor protein in SHR cells.

Several studies have shown that the dopaminergic/angiotensin systems interact to regulate renal function. As stated in the introduction, sodium transport in the kidney, especially in the luminal membrane of the proximal tubule, is reciprocally regulated by these systems. For example, dopamine, via D₁-like receptors, antagonizes the stimulatory effects of angiotensin II on Na⁺ uptake in rat RPTs and brush border membrane vesicles. Also, angiotensin-converting enzyme inhibition or an AT₁ receptor blockade enhances the natriuretic effect of a D₁-like agonist. The renal vasoconstrictor effect of angiotensin II can also be antagonized by D₁-like receptor agonists.

The effect of the D₁-like receptor, by itself, on renal sodium transport is controversial: stimulation, no effect, and
even inhibition have been reported.8 However, under certain circumstances, such as during moderate sodium loading, D1- and D2-like receptors can synergistically inhibit sodium transport.25 The inhibitory effect of dopamine on sodium transport can be antagonized by both D1-like and D2-like receptor blockers.13,25,33 A D2-like agonist, bromocriptine, has also been reported to antagonize the stimulatory effect of angiotensin II on Na+/K+-ATPase activity in RPTs.12

The mechanism for the antagonistic effect of D2-like receptors on angiotensin II action is not well understood. Aminochrome, a metabolite of the dopamine oxidative pathway, has been reported to decrease AT1 expression in a dopaminergic neuronal cell line RCSN3 of rat substantia nigra.34 Bromocriptine, a D2-like agonist, has also been reported to decrease AT1 receptor expression in rat RPTs.12 However, because bromocriptine has equal affinity to D2 and D3 receptors,16 it is not known which D2-like receptor mediates the inhibitory effect of bromocriptine on AT1 receptor expression in rat RPTs.

The current studies show that the D3 receptor participates in the regulation of AT1 receptor expression in RPT cells. A role for D2 receptors cannot be excluded because D2 receptors are also expressed in rat renal cortex.8 However, the D2 receptors expressed in rat RPTs are probably prejunctional because D2 receptor mRNA is not detected in the immortalized rat RPT cells (unpublished observations). It is also not clear whether the inhibitory effect of D2 receptors on AT1 receptor expression is direct or indirect. The D3 receptor, but probably not the D2 receptor, is the D2-like receptor that inhibits renin release.14 Thus, plasma renin levels are elevated in D3 receptor–null mice but not in D2 receptor–null mice.15,19 Because RPT cells can also generate angiotensin II,25,35 it is possible that a D3 receptor–mediated decrease in angiotensin II formation could have led to the decrease in AT1 receptor expression because angiotensin II has been reported to increase AT1 receptor mRNA in RPTs.36 However, long-term infusion of angiotensin II in vivo in rats has no effect on AT1 receptor expression in RPTs.37 We also have preliminary observations indicating that AT1 receptor expression in immortalized RPT cells is not increased by incubation with angiotensin II.26 We, therefore, suggest that D3 receptors, independent of angiotensin II, can regulate AT1 receptor expression.

The mechanism for the decrease in AT1 receptors caused by D3 receptors was also investigated in this study. Stimulation of the D3 receptor for 24 hours decreases AT1 receptor mRNA expression in WKY cells but increases it in SHR cells. This result is consistent with AT1 receptor protein result and indicates that the regulation of D3 receptors on AT1 receptor expression may occur at the transcriptional/posttranscriptional level. The decrease in AT1 receptor protein expression at 2 hours, however, is probably not attributable to alterations at the transcriptional/posttranscriptional level. In the SHRs, renal G protein–coupled receptor kinase 4 (GRK4) expression and activity are increased18 and may be responsible for the impaired D3 receptor function in the kidney and hypertension. The GRK4 variant, A142V, but not GRK4 wild-type transgenic mice have increased AT1 receptor protein.39 We have preliminary data indicating that the D3 dopamine receptor increases the degradation of the AT1 receptor.40 Therefore, the possibility that the D3 receptor directly or indirectly increases the degradation of the AT1 receptor cannot be ruled out. In SHRs, the decreased expression of D3 receptor, even if constitutively active, may not be sufficient to impair the degradation of AT1 receptor and, thus, the absence of a difference in total cell AT1 receptor expression between WKY and SHRs. Stimulation of D3 receptors would assist in the degradation of AT1 receptors in WKY rats, but this does not occur in SHRs, hence the increase in AT1 receptor protein with D3 receptor stimulation in the latter rat strain. In addition, in the SHRs, stimulation of an altered D3 receptor (secondary to increased GRK4 activity) may be responsible for the increase in AT1 receptor mRNA.

We found by both morphological (confocal microscopy) and biochemical (immunoprecipitation) methods that D3 and AT1 receptors can directly interact with each other. When D3/AT1 receptor coimmunoprecipitation is normalized by D3 receptor or AT1 receptor, the basal levels of D3/AT1 receptor coimmunoprecipitation are higher in WKY cells as compared with SHR cells. However, when data of D3/AT1 receptor coimmunoprecipitation are normalized by D3 or AT1 receptor, the ratios are different. Normalization by D3 receptor shows that stimulation with PD128907 decreases the percent amount of D3 receptors that coimmunoprecipitate with AT1 receptors to a greater extent in WKY than in SHR cells. This occurs because PD128907 increases D3 receptor expression in WKY but not in SHR cells.32 Normalization of the data with AT1 receptor shows that PD128907 does not change the D3/AT1 receptor coimmunoprecipitation in WKY cells but markedly decreases it in SHR cells.

Taking into account that PD128907 increases D3 receptor expression but decreases AT1 receptor expression in WKY cells, we suggest that in WKY cells, PD128907 decreases the amount of D3 receptors bound to AT1 receptors while keeping the same amount of AT1 receptors bound to the D3 receptor. Therefore, more D3 receptors can exert their effects while keeping AT1 receptors in check; D3 receptors are available to inhibit sodium reabsorption, and fewer AT1 receptors are available to increase sodium reabsorption in RPTs in WKY rats.

In SHR cells, PD128907 increases AT1 receptor expression but does not affect D3 receptor expression. The data in Figure 4A and 4B suggest that D3 receptor stimulation with PD128907 makes more AT1 receptors than D3 receptors available to exert their effects, more sodium reabsorption is 1 consequence.

D3 receptor–null mice in a C57BL/6 background have higher blood pressures than wild type C57BL/6 mice, confirming our previous study of D3 receptor–deficient mice with mixed B129 and C57BL/6 background. The D3 receptor–deficient mice in the previous study have increased renal renin activity, and their blood pressures are normalized by AT1 receptor blockade with losartan.18 It was not determined in the previous study whether the hypertension in the D3 knockout mice is caused by an increase in angiotensin II levels and/or an increase in AT1 receptors. Consistent with the ability of D3 receptors to inhibit AT1 receptor expression in immortalized RPT cells, AT1 receptor expression is increased in the kidneys of D3 receptor–null mice. It is, therefore, likely that the hypertension in D3 receptor–null mice is also related to an increase in AT1 receptor expression.
In summary, we have demonstrated that the D₃ receptor regulates AT₁ receptor expression in vivo and in vitro. It increases its own expression but decreases AT₁ receptor expression in RPT cells from normotensive WKY rats. In contrast, in RPT cells from SHR, the D₃ receptor no longer regulates its own expression and actually increases AT₁ receptor expression. D₃ and AT₁ receptors colocalize and coimmunoprecipitate in RPT cells. Basal D₃ and AT₁ receptor coimmunoprecipitation is greater in WKY than SHR cells, but PD128907 had different effects on the percent amount of D₃ or AT₁ receptor that coimmunoprecipitated with the other receptor. It is possible that an impaired interaction between D₃ and AT₁ receptors play a role in the pathogenesis of genetic hypertension.

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**Disclosures**

None.

**References**


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