Differential D₁ and D₅ Receptor Regulation and Degradation of the Angiotensin Type 1 Receptor

John J. Gildea, Xiaoli Wang, Pedro A. Jose, Robin A. Felder

Abstract—Renal sodium transport is increased by the angiotensin type 1 receptor (AT₁R), which is counterregulated by dopamine via unknown mechanisms involving either the dopamine type 1 (D₁R) or dopamine type 5 receptor (D₅R) that belong to the D₁-like receptor family of dopamine receptors. We hypothesize that the D₁R and D₅R differentially regulate AT₁R protein expression and signaling, which may have important implications in the pathogenesis of essential hypertension. D₁R and D₅R share the same agonists and antagonists; therefore, the selective effects of either D₁R or D₅R stimulation on AT₁R expression in human renal proximal tubule cells were determined using antisense oligonucleotides selective to either D₁R or D₅R. We also determined the role of receptor tyrosine kinase and the proteosome on the D₁R/D₅R-mediated effects on AT₁R expression and internalization. In renal proximal tubule cells, D₅R (not D₁R) decreased AT₁R expression (half-life: 0.47±0.18 hours) and AT₁R-mediated extracellular signal–regulated kinase 1/2 phosphorylation (23±18.9 U with angiotensin II [10⁻⁷ mol/L] versus 81±8.9 U with angiotensin II [10⁻⁷ mol/L] and fenoldopam [D₁R/D₅R agonist; 10⁻⁶ mol/L; P<0.05; n=6]). The fenoldopam-induced decrease in AT₁R expression was reversed by 4-amino-5-(4-chlorophenyl)-7-(t-buty) pyrazolo (3,4-d) pyrimidine (c-Src tyrosine-kinase inhibitor) and clasto-lactacystin ß-lactone (proteasome inhibitor), demonstrating that the fenoldopam-mediated decrease in total cell AT₁R expression is a result of a c-Src- and proteasome-dependent process. D₅R stimulation decreases AT₁R expression and is c-Src and proteasome dependent. The discovery of differential regulation by D₁R and D₅R opens new avenues for the development of agonists selective to either receptor subtype as targeted antihypertensive agents that can decrease AT₁R-mediated antinatriuresis. (Hypertension. 2008;51:360-366.)

Key Words: dopamine 1–like receptor ■ angiotensin type 1 receptor ■ human renal proximal tubular cells ■ D₁ receptor ■ D₅ receptor

The importance of abnormal renal sodium handling in the pathogenesis of hypertension and salt sensitivity has stimulated studies on the interaction between counterregulatory natriuretic pathways. Dopamine and angiotensin II stimulate 2 counterregulatory pathways through specific G protein–coupled receptors in the renal proximal tubule where the natriuretic effect of the dopamine type 1 (D₁R) and dopamine type 5 receptors (D₅R)¹⁻⁶ and the antinatriuretic effect of the angiotensin type 1 receptor (AT₁R)⁷ counterbalance each other to maintain sodium and blood pressure homeostasis. The renal proximal tubule and thick-ascending limb of Henle are the sites of increased sodium reabsorption in human essential hypertension.⁸⁻¹⁰ which is regulated by dopamine¹⁻⁵,⁹⁻¹² and angiotensin II.⁷,⁹,¹⁰,¹³ Angiotensin II is responsible for >50% of sodium reabsorbed by the kidney under basal conditions.⁷,¹³,¹⁴ whereas dopamine is responsible for >50% of sodium excreted under conditions of sodium excess.¹⁻⁵,¹²,¹⁵ Previous studies in normotensive rodents demonstrated that stimulation of both the D₁R and D₅R by D₁-like receptor agonists (because of the lack of availability of pharmacological agents that are selective only to the D₁R or the D₅R) decreased AT₁R protein expression but increased the expression of the D₁R.¹⁶ In the spontaneously hypertensive rat, a rodent model of genetic hypertension and D₁R and D₅R stimulation also decreased AT₁R, but not the D₁R protein, in renal proximal tubular cells (RPTCs).¹⁶ Whether the D₁R and/or the D₅R regulate the AT₁R in human RPTCs or the spontaneously hypertensive rat has not been directly determined.

The natriuretic effect of D₁-like receptor stimulation is enhanced when the biosynthesis of angiotensin II is reduced or when the AT₁R is blocked,¹⁷,¹⁸ suggesting that angiotensin II exerts a tonic control of the dopaminergic pathway. D₁R can regulate AT₁R function by direct receptor/receptor interaction,¹⁶,¹⁹ but a regulatory role of D₅R on AT₁R has not been demonstrated directly. The enhanced antinatriuretic effect of the renin-angiotensin system in genetic hypertension could be further exacerbated by the impaired counteractive natriuretic effect of the renal dopaminergic system.¹⁵ The mechanisms responsible for the counterregulation of the dopaminergic and...
AT1Rs under normal blood pressure conditions are not well understood.

Despite the fact that the D1R and D3R arise from different genes and exhibit 49% amino acid sequence homology, they have similar affinities toward dopaminergic agonists and antagonists. Therefore, to test our hypothesis that there is differential regulation of the AT1R by the D1R and D3R, we selectively silenced the D1R or D3R gene using antisense oligonucleotides selective to either D1R or D3R. In addition, we used human RPTC lines in which the D1Rs are uncoupled from intracellular signaling (D1R-uncoupled RPTC) to determine the selective D1R (in the absence of a functional D3R) effect on AT1R expression in these cells.

Materials and Methods

Human RPTC Cultures

Human kidneys were obtained as fresh surgical specimens from patients who had unilateral nephrectomy because of renal carcinoma or trauma under a university institutional review board–approved protocol that included informed consent that adheres to the Declaration of Helsinki and the most recent version of Title 45, Part 46, US Code of Federal Regulations. Only the visually and histologically normal pole, distal from the affected part of the kidney, was used in our studies. All of the studies were performed in duplicate on 3 established lines of RPTCs. We have reported previously on the characteristics of these cell lines, including cell lines from hypertensive subjects with D1Rs that are uncoupled from G proteins and other effector proteins.

RPTCs were grown at 37°C, 100% humidity, 95% air, and 5% CO2 and fed serum-free medium every 4 days consisting of a mixture of Click’s RPMI 1640 (Quality Biological Inc) supplemented with 5 μg/mL of insulin, 5 μg/mL of transferrin, 5 ng/mL of selenium, 36 ng/mL of hydrocortisone, 4 g/mL of triiodothyronine, 10 ng/mL of epidermal growth factor (Sigma), 100 U/mL of penicillin G, and 100 μg/mL of streptomycin. Before reaching confluence, the cells were subcultured using trypsin-EDTA (0.025%; 0.01%), inactivating the trypsin with 5% serum, spun at 600 g at 4°C, and resuspended in serum-free growth medium for passaging. Cells were used at 70% to 80% confluence in 6-well culture plates between the first and fourth doublings, with experiments performed in duplicate in 3 cell lines.

Drug Treatment

Because highly selective D1R or D3R pharmacological agents do not yet exist, we used the most selective agonists and antagonists to both D1R and D3R (D1-like receptors) available. The cells were incubated for the indicated times with fenoldopam, an agonist selective to both D1R and D3R (Sigma) in the absence or presence of SCH-23390, an antagonist selective to both D1R and D3R (Sigma). When used, SCH-23390 was added 20 minutes before the addition of fenoldopam to block both the D1R and D3R. The drug incubations were terminated with 3 washes of Dulbecco’s PBS. 4-Amino-5-(4-chlorophenyl)-7-(4-butyl) pyrazolo (3,4-d) pyrimidine (FP2), a specific Src nonreceptor tyrosine kinase inhibitor, was used at the 1 μmol/L concentration.

Clasto-lactacystin β-lactone (CLBL), a highly specific cell-permeable proteasome inhibitor, was used at the 10 μmol/L concentration.

Immunoblotting

Cells were homogenized in ice-cold lysis buffer (20 mMol/L of Tris-HCl [pH 7.4], 2 mMol/L of EDTA [pH 8.0], 2 mMol/L of EGTA, 100 mMol/L of NaCl, 10 μg/mL of leupeptin, 10 μg/mL of aprotinin, 2 mMol/L of phenylmethylsulfonyl fluoride, and 1% Nonidet P-40), sonicated, kept on ice for 1 hour, and centrifuged at 26,000g for 30 minutes. The supernatant represented crude cell membranes. Protein concentrations were determined by the BCA protein assay kit (Pierce Biotechnology), using BSA as a standard. The proteins, separated by SDS-polyacrylamide gel electrophoresis, were electrophotographically transferred onto polyvinyliden difluoride membranes (Bio-Rad Laboratories). Equal sample loading and transfer were determined by Ponceau S (Sigma) staining of the polyvinylidene fluoride membrane before immunodetection. Transblots were blocked with 5% nonfat dry milk in Dulbecco’s PBS with 0.05% Tween-20 and incubated with diluted affinity-purified rabbit polyclonal antibody to AT1R (1:400 dilution, sc-1173, Santa Cruz), D1R (1:200 dilution, sc-14001, Santa Cruz), D3R (1:500 dilution, sc-25650, Santa Cruz), phospho-extracellular signal–regulated kinase (ERK) 1 and 2, c-Src, and phosphor tyrosine Y416 c-Src (1:1000, Cell Signaling Inc) for 1.5 hours at room temperature. After 5 washes for 10 minutes each, the membranes were incubated with diluted peroxidase-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology Inc) in 2% milk for 1.5 hours at room temperature and washed as for the primary antibody. The chemiluminescence signal was developed using SuperSignal West Pico substrate (Pierce Biotechnology) for AT1R and phospho- ERK 1 and 2; SuperSignal West Femto (Pierce Biotechnology) for D1R and D3R; and c-Src and was then exposed to x-ray film.

Cell Plasma Membrane Expression of AT1R

Cell-surface membrane sheets were isolated with a detergent-free isolation procedure using sulfo-NHS-SS-biotin. RPTCs, after a 20-minute incubation with the D1R-like receptor agonist fenoldopam (10⁻⁶ mol/L), were washed twice with Hank’s balanced salt solution with magnesium and calcium and then labeled with 1 mmol/L of sulfo-NHS-SS-biotin for 10 minutes. The labeling compound was simultaneously washed and inactivated with 2 washes of Tris-buffered saline. The cells were then scraped off the plate in ice-cold detergent-free lysis buffer (Tris-buffered saline with protease inhibitors), sonicated for 1 second, incubated on ice with streptavidin beads (Ultralink, Pierce) for 20 minutes, washed twice with Tris-buffered saline, and eluted in gel loading buffer (NuPAGE LDS Sample Preparation Buffer with reducing agents, Invitrogen). Immunoblotting for AT1R protein was performed as described above.

Antisense Oligonucleotides

The effects of 50 nM of propyne/phosphorothioate-modified anti-sense oligonucleotides for human (h)D5R (5'-136 nucleotide CATTTCCGGGCTGGAC 153 nucleotide -3'; GenBank accession No. M67439) and hD1R (5'-277 nucleotide GGTGTTCAGAGTC-259 nucleotide -3'; GenBank accession No. X55760) were compared with scrambled sequence controls (hD5R 5'-GTCGCCGAGCTTATGGA-3' and hD1R 5'-GGGACTCTCT ATATCGG-3'). Briefly, cells were grown in 6-well plates until 60% confluence, and 50 nM antisense or scrambled oligonucleotides were mixed with 6 μL of oligofectamine in OptiMEM medium (Invitrogen Life Technologies) and incubated for 24 hours, then switched to growth medium and incubated for another 24 hours. Fenoldopam (10⁻⁶ mol/L) was then added to the medium without growth factors for 2 hours, lysed, and processed for immunoblotting for the D1R, D3R, and AT1R.

Statistical Analysis

The data are expressed as mean±SE. Comparisons within and among groups were made by repeated-measures or factorial ANOVA, respectively, followed by Holm-Sidak or Duncan’s test. A t test was used for 2-group comparisons. A P value of <0.05 was considered significant.

Results

Studies in RPTCs

Effect of D1R/D3R Stimulation on AT1R Expression

We compared AT1R responses to dopaminergic stimulation in human RPTCs with those reported previously in rodent cells using the D1R/D3R agonist fenoldopam. D1R/D3R stimulation decreased the expression of the AT1R in a time-dependent manner, as determined by immunoblotting (Figure 1). The half-life of the AT1R under these conditions (fenoldopam;
Effect of D1R/D5R Stimulation on AT1 Function

To demonstrate that AT1R function was similarly downregulated by D1R/D5R stimulation in our human cell line, we studied a well-established measure of AT1R activity, namely, the angiotensin II–dependent phosphorylation of ERK1/2. Fenoldopam nearly completely inhibited the angiotensin II–dependent increase in phospho-ERK 1/2 (p-ERK 1/2) in human RPTCs (n=3 per group; *P<0.01 vs angiotensin II; ANOVA, Neuman-Keuls test). Selectivity of Fen action on AT1R and an intact p-ERK 1/2 pathway in the Fen-treated cells are confirmed by an increase in p-ERK 1/2 after epidermal growth factor (EGF; 100 ng/ml) stimulation for 10 minutes. One immunoblot is shown in the inset. Veh indicates vehicle control.

The effect of fenoldopam (Fen; 10⁻⁶ mol/L per 4 hours) on angiotensin II (All; 10⁻⁷ mol/L per 10 minutes)–dependent increase in phospho-ERK 1/2 (p-ERK 1/2) in human RPTCs (n=3 per group; *P<0.01 vs angiotensin II; ANOVA, Neuman-Keuls test). Selectivity of Fen action on AT1R and an intact p-ERK 1/2 pathway in the Fen-treated cells are confirmed by an increase in p-ERK 1/2 after epidermal growth factor (EGF; 100 ng/ml) stimulation for 10 minutes. One immunoblot is shown in the inset. Veh indicates vehicle control.

by either D1R scrambled or D5R antisense or scrambled oligonucleotides (D1R antisense: 68.01±4.18% reduction compared with scrambled control; P<0.01; n=3). In contrast, D1R expression was decreased by D1R antisense but not by the D1R scrambled or D5R antisense or scrambled oligonucleotides (D1R antisense: 55.01±6.15% reduction compared with scrambled control; P<0.01; n=3). Similarly treated cells were then split into 2 wells and stimulated with fenoldopam (10⁻⁶ mol/L) or vehicle for 2 hours, lysed, and immunoblotted for the AT1R (Figure 4). In these cells, fenoldopam treatment for 2 hours again decreased AT1R protein expression (Figure 4A), albeit to a lesser extent relative to cells treated with fenoldopam for 4 hours but not with OptiMem (Figure 1). D1R antisense and scrambled oligonucleotides had no significant effect on the fenoldopam-dependent decrease in AT1R protein (Figure 4B). In contrast, D1R antisense but not D1R scrambled oligonucleotides...
blocked the fenoldopam-dependent decrease in AT1R expression by 57.9±13.6% (n=3; P<0.001; Figure 4C). These studies suggest that D5R but not D1R mediates the inhibitory effect of fenoldopam on AT1R protein expression. The increase in AT1R protein in cells not exposed to fenoldopam but treated with D5R antisense oligonucleotides supports the notion that D5R is constitutively active.25

Because the fenoldopam-mediated decrease in AT1R expression occurred too fast to be accounted for by alterations in transcription and translation (half-life: 0.47±0.18 hours), we determined whether the action may have occurred by enhancement of its degradation. In HEK-293 cells heterologously expressing the AT1R and D5R, pulse chase experiments with protein translation inhibited by cycloheximide showed that fenoldopam promoted AT1R degradation via the proteasome.31 Because protein degradation in the proteasome can be triggered by c-Src tyrosine kinase,26 we studied the effect of fenoldopam (10⁻⁶ mol/L) on AT1R expression in cells treated with PP2, a c-Src non-receptor tyrosine kinase inhibitor, or CLBL (a proteasome inhibitor).27 As shown in Figure 5, either PP2 or CLBL completely blocked the fenoldopam (10⁻⁶ mol/L for 4 hours)-mediated decrease in AT1R expression. We selected concentrations of PP2 and CLBL that, by themselves, had no significant effect on AT1R expression; high concentrations of PP2 and CLBL by themselves caused an increase in AT1R expression (data not shown). Presumably, the effect of fenoldopam was exerted via c-Src, because fenoldopam increased c-Src tyrosine phosphorylation at the Y416 site by 2.7±0.4-fold over the control group (vehicle-treated cells), and this increase was completely blocked by the c-Src tyrosine kinase inhibitor PP2 (Figure 6).

Studies in RPTCs With Uncoupled D1Rs
To determine the effect of D1R stimulation on AT1R expression in the absence of functional D1R (D1R uncoupled),23–25 we measured the effect of fenoldopam (10⁻⁶ mol/L) on AT1R expression in both normal human RPTCs and RPTCs with uncoupled D1Rs (uncoupled RPTCs). A 4-hour fenoldopam stimulation produced a similar concentration-dependent decrease in AT1R expression in both RPTCs and uncoupled RPTCs, as determined by immunoblotting (Figure 7). As determined by nonlinear regression analysis, the half-maximal concentrations of fenoldopam were similar in normal RPTCs (24.6 nM) as compared with D1R uncoupled RPTCs (15.8 nM; n=6 per concentration; P>0.05). The congruence of the results (with those described in Figure 4) suggests that the D1R-mediated decrease in AT1R expression is not because of an α-error.

Studies in Surface Labeling of AT1R
AT1R was reduced in our whole-cell preparations. However, we wished to determine whether cell surface expression was reduced, because cell surface receptors are most likely be the

Figure 4. The effect of D1R and D5R antisense (AS) and scrambled (SCR) propyne/phosphothioate oligonucleotides on AT1R protein in human RPTCs. The D1R or D5R antisense and scrambled propyne/phosphothioate oligonucleotides were added to 2 sets of RPTCs in culture using oligofectamine (50 nM for 48 hours; B and C, respectively), and then incubated with fenoldopam (10⁻⁶ mol/L) or vehicle for 2 hours followed by quantitative Western blots using antibodies against the AT1R, according to the procedure described in the Materials and Methods section (n=3 per group; #P<0.001: fenoldopam [Fen] vs Control, in A; &P<0.001 vs AS or SCR, in A, and D1R antisense, in B; oligonucleotides, $P<0.001 vs others and D5R antisense oligonucleotides, C; ANOVA, Newman-Keuls test).

Figure 5. The effect of fenoldopam (10⁻⁶ mol/L per 4 hours), PP2 (10⁻⁶ mol/L, c-Src non-receptor tyrosine kinase inhibitor), and CLBL (10⁻⁵ mol/L, proteasome inhibitor) on AT1R expression in human RPTCs after a 2-hour incubation (n=3 per group; *P<0.01 vs others, ANOVA, Newman-Keuls test). One immunoblot is shown in the inset. The combination of the same concentrations of PP2 and CLBL, in vehicle-treated cells, has no effect on AT1R expression (data not shown).

Figure 6. The effect of a 30-minute incubation of fenoldopam (10⁻⁶ mol/L) and PP2 (10⁻⁶ mol/L, c-Src nonreceptor tyrosine kinase) on c-Src tyrosine (pY416) phosphorylation in human RPTCs (n=3 per group; *P<0.01 vs others, ANOVA, Newman-Keuls test). One immunoblot is shown in the inset. Con indicates control; Veh, vehicle; Fen, fenoldopam.
receptors that are responsive to angiotensin II. Membrane localization of AT1R under identical experimental protocols described for Figure 7 demonstrated that fenoldopam (10^{-9} mol/L per 20 minutes) reduced the control membrane expression from 58,888±1,667 relative fluorescent units to 32,224±4,250 relative fluorescent units (n=6; P<0.05; Figure S1). The magnitude of the decrease in cell surface expression of AT1R induced by a 20-minute incubation with fenoldopam is similar to the decrease in total cellular AT1R expression (Figure 1). These studies indicate that fenoldopam not only can decrease total cellular expression of AT1R but also AT1R expression at the plasma membrane. AT1R degradation induced by fenoldopam starts at the plasma membrane in HEK 293 cells heterologously expressing human AT1R and human D1R but not human D2R,33

**Discussion**

The current study in human RPTCs confirms our previous studies in rodent RPTCs where we demonstrated that simultaneous D1R and D2R stimulation results in a decrease in the expression of the AT1R protein.16 The current studies extend these earlier findings by demonstrating in human RPTCs that the D1R is responsible for AT1R downregulation. We conducted our studies in 6 human RPTC lines, 3 derived from normal subjects (with no significant medical history of hypertension) and 3 that have demonstrated a coupling defect (inhibition of AT1R expression) persists in RPTCs from genetically hypertensive rodents, renal D1R expression is decreased.40 Therefore, the impaired D1-like receptor inhibition of sodium transport in hypertension may be secondary to impairment of both D1R and D2R (function and/or expression), at least in rodents. Indeed, disruption of either the D1R or D2R gene in mice results in hypertension.39,41

The D1R-mediated AT1R downregulation occurs in 30 minutes, too fast for transcriptional-based protein downregulation of AT1R protein expression in the human renal proximal tubule (Dr Pedro A. Jose, unpublished data, 2007). In HEK-293 cells heterologously expressing the AT1R and D1R but not D2R, pulse chase experiments, with protein translation inhibited by cycloheximide, show that fenoldopam also decreases AT1R expression with a half-life of 37.7 minutes, close to the half-life of 28.2 minutes observed in the current report. The process must be related to increased degradation, because the D1R-mediated reduction of AT1R is completely blocked by an inhibitor of proteasome activity. There are examples of protein-linked proteasome- and c-Src tyrosine kinase–mediated protein degradation,26 suggesting that a similar pathway may be active in AT1R downregulation. In fact, we demonstrate that fenoldopam mediates the decrease in total cell AT1R expression as a result of a c-Src- and proteasome-dependent process. c-Src, when tyrosine phosphorylated at Y416 in the activation loop of the enzyme, increases enzymatic activity.42 It has been shown previously that the protein kinase A–dependent phosphorylation of c-Src at serine 17 is followed by Y416, and the addition of PP2 does not prevent the serine 17 phosphorylation but does block the Y416 phosphorylation.43 We now show that c-Src activity is increased by fenoldopam and that blocking c-Src with PP2 prevents the AT1R downregulation. We speculate that the D1R is responsible for the activation of c-Src, because AT1R downregulation by fenoldopam is still operational in D1R uncoupled cells. Our future studies will concentrate on the intermediate pathway connecting the D1R to c-Src activation.

In summary, we have demonstrated that, in RPTCs from human kidneys, there is a transregulatory pathway whereby the D1R receptor downregulates the AT1R. This represents a novel mechanism to explain the dynamic balance between the natriuretic dopaminergic and antinatriuretic angiotensin systems in the kidney.
Perspectives
The human kidney is the primary organ responsible for orchestrating a balance between sodium reabsorption and excretion to maintain fluid and electrolyte balance and, ultimately, blood pressure. Two principal pathways have been identified that act to increase sodium reabsorption (the renin-angiotensin system mainly stimulated by angiotensin II via AT₃,R) or decrease sodium reabsorption (the dopaminergic system working through dopamine produced in the kidney). It has been of interest to determine which cell-surface receptors are responsible for transducing these counterbalancing signals to enable the development of targeted pharmaceuticals. In this article we have delineated how these 2 systems work to regulate each other. More importantly, we have identified a novel AT₃,R regulatory pathway that relies on the independent action of the D₃,R that is mechanistically distinct from the D₃,R.

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Disclosures
None.

References
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