Dopamine-1 Receptor Coupling Defect in Renal Proximal Tubule Cells in Hypertension

Hironobu Sanada, Pedro A. Jose, Debra Hazen-Martin, Pei-Ying Yu, Jing Xu, David E. Bruns, John Phipps, Robert M. Carey, Robin A. Felder

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Dopamine produced by renal proximal tubules (RPT) has been shown to be a paracrine regulator of sodium transport in humans and in animals during sodium-replete conditions. During states of positive sodium balance, endogenous renal dopamine facilitates sodium excretion caused by a decrease in proximal as well as distal ion and water transport. The natriuretic effect of exogenous and endogenous renal dopamine (and dopamine-1 [D₁]-like receptor agonists) is impaired in 2 animal models of hypertension. In the spontaneously hypertensive rat (SHR), the impaired natriuretic effect of dopamine and D₁-like receptor agonists is associated with a decreased ability to inhibit Na⁺-H⁺exchanger and Na⁺,K⁺-ATPase activity in RPT. The decreased ability of dopamine and D₁-like receptor agonists to inhibit these transporters has been related to a defective dopaminergic stimulation of second messenger production by adenylyl cyclase (AC), phospholipase C (PLC), and phospholipase A₂, These phenotypes may be manifestations of a defective gene important in controlling blood pressure, since the defective dopaminergic regulation of RPT transport and sodium excretion cosegregates with hypertension in rats, and disruption of one of the D₁-like receptor genes (D₁A receptor) in mice produces hypertension.

A defective regulation of renal proximal tubule sodium transport by D₁-like receptors is also present in human essential hypertension. We hypothesized that the coupling between a D₁-like receptor and the G protein/effecter enzyme complex may also be defective in some patients with essential hypertension, similar to that seen in animal models of genetic hypertension. Therefore, we compared the effect of dopamine and fenoldopam on AC and PLC activity in human RPT in culture.

Two D₁-like receptors have been cloned in mammals, the D₁ and D₃ receptors, also known as D₁A and D₁B, respectively, in rodents. Since the D₁A receptor seems to

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be important in the regulation of blood pressure in mice. we studied the expression of the D1 receptor in the kidneys and RPT in culture from hypertensive (HT) and normotensive (NT) subjects. We also studied the expression and function of parathyroid hormone–related protein (PTHrP), since there is no impairment of the parathyroid hormone (PTH)/AC cascade in the 2 animal models of genetic hypertension that have an uncoupling of renal D1-like receptors from their effector enzyme complex. Methods

Source of Kidney Tissue
Histologically normal sections of fresh human kidneys from patients (NT subjects: n = 6; mean age, 65 years; 3 men, 3 women; HT subjects: n = 4; mean age, 65 years; 3 men, 1 woman) who had unilateral nephrectomy due to renal carcinoma or trauma were grown in culture. The hospital and outpatient records of the subjects were reviewed and classified into those with either normal blood pressure (n = 5) or essential hypertension (n = 4). All patients signed a consent form agreeing that the tissues taken from them become the property of the Department of Pathology and such tissues can be used for study. All studies were approved by the Institutional Review Board of the University of Virginia Center for the Health Sciences.

Tissue Culture
Human RPT (hRPT) cells were grown in serum-free medium consisting of a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium supplemented with selenium (5 ng/mL), insulin (5 μg/mL), transferrin (5 μg/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and epidermal growth factor (10 ng/mL). hRPT cells (5 x 10⁶ cells per well in 24 well plastic plates coated with 0.075% type I collagen), passage 6 and 7 (except when indicated), were incubated at 37°C in 95% O₂/5% CO₂ for 48 to 72 hours. Electron microscopic studies showed characteristics consistent with RPT origin. The cells express Na⁺/H⁺ exchanger, isoform 3 (NHE-3) and PTH receptors (NHE-3 and PTH receptors are present in proximal tubules; NHE-3 is not present in distal tubules) as well as a brush border marker enyzmes, γ-glutamyltranspeptidase.

Determination of CAMP Accumulation
cAMP accumulation was determined in the presence of 1 mM L-3-isobutyl-1-methylxanthine in Dulbecco’s PBS. The cells were incubated at 37°C for 30 minutes with or without drugs: dopamine and the D1-like receptor agonist fenoldopam, the D1-like receptor antagonist SCH23390 (Research Biochemicals International), PTHrP, amino acids 1 to 34 (Bachem), and forskolin (Sigma Chemical Co).

Determination of PLC
Cytosol and membranes from hRPT were assayed for PLC activity as utilized in our laboratory with [3 H] phosphatidylinositol-4,5-bisphosphate used as substrate. The reaction proceeded for 15 minutes. Any remaining horseradish peroxidase activity was prevented by 1% BSA, 1% nonfat dry milk, and 0.05% Triton X-100. Subsequent incubations were at room temperature. The secondary antibodies (biotinylated horse anti-mouse IgG antibody, 1:200, Vector Laboratories) were also diluted with PBS blocking buffer. Fluorescein-conjugated affinity-purified goat anti-rabbit IgG antibody (Jackson Immunoresearch Laboratories, Inc) was applied at 1:100 for 30 minutes, followed by 0.1 mM L-Tris-HCl, pH 7.5, 0.15 mM L NaCl, and 0.5% Dupont Blocking Reagent for 30 minutes, streptavidin–horseradish peroxidase for 30 minutes, and tetramethylrhodamine tyramide in amplification diluent (1:50) for 10 minutes. Any remaining horseradish peroxidase activity was deactivated with 1% H₂O₂ in PBS (pH 7.0) for 15 minutes. Thereafter, the slides were incubated with anti-fluorescein–horse radish peroxidase (NEN Life Science Products) for 30 minutes and followed by a 10-minute incubation with 1:50 dilution of fluorescein tyramide in ×1 amplification diluent. Immunostaining was detected with the Tyramide Signal Amplification kit (NEN Life Science Products) followed by immunofluorescence visualization.

Immunoblotting and Immunoprecipitation
The tissues or cells were lysed with ice-cold lysis buffer (PBS with 1% NP40, 0.5% Na cholate, 0.1% SDS, 1 mM L-PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM L sodium vanadate). The lysates were incubated with D1 antibody for 1 hour followed with protein-A agarose for 12 hours with rocking at 4°C. The proteins separated by SDS–polyacrylamide gel electrophoresis were electrophoretically transferred onto nitrocellulose membranes. The transblots were washed with buffer and then incubated with peroxidase-conjugated affinity-purified donkey anti-rabbit IgG for 1 hour at room temperature. The immunoblot, visualized with ECL System (Amersham), were quantified by densitometry.

Determination of D1-like Receptor Specificity
We determined the involvement of the D1 receptor using antisense phosphorothioate/proprone-modified oligonucleotides purified by high-performance liquid chromatography (Genosys). Sense (5 mM/L) (5’ ATG AGG ACT CTG AAC ACC 3’) and antisense (5 mM/L) 3’ GGT GTT CAG AGT CCT CAT 5’) oligonucleotides were incubated with cultures of hRPT for 4 to 16 hours, washed, and reincubated for another 4 to 48 hours (total incubation time of 20 to 48 hours). The ability of antisense but not sense oligonucleotides to prevent expression of the D1 receptor was verified by Western blotting.
Sequencing

The sequences of the cDNA from the reverse transcriptase–polymerase chain reaction products of RNA from hRPT in culture were determined by the Sanger dideoxy chain termination method with Sequenase (US Biochemical).

Statistical Analysis

Data are reported as mean±SE. Within-group differences were determined by ANOVA for repeated measures and Scheffe’s test. Differences between HT and NT were determined by t test.

Results

Enzyme Activity Studies

cAMP Accumulation

Basal cAMP accumulation in hRPT was not statistically different between NT (n=6) and HT (n=4) subjects. The D₁-like receptor agonist fenoldopam increased cAMP accumulation in a concentration-dependent manner to a greater degree in NT than in HT subjects. Basal cAMP levels were not statistically different in NT and HT (inset). *P<0.05 NT vs HT, t test; †P<0.05 vs basal activity, ANOVA for repeated measures, Scheffe’s test. Data are mean±SE. n indicates number of experiments; each n represents culture from 1 subject. Error bars are not present if the SE falls within the size of the data point.

PLC Activity

Previous studies in rodents have shown that the D₁-like receptor is linked to both AC and PLC activation.¹,⁷,¹⁰ The ability of D₁-like receptor agonists to stimulate AC and PLC activity is attenuated in rat models of genetic hypertension.¹,⁶–¹⁰ However, in hRPT, fenoldopam (5 μmol/L) stimulated PLC activity to a greater extent in HT than in NT at 1 minute (Figure 4). Since the cells were studied contemparaneously, the preservation of the PLC, forskolin, and PTHrP response but not the cAMP response in HT may be taken to indicate that a selective dedifferentiation of HT cells did not occur with multiple passaging.
Light Microscopic Immunohistochemistry

Kidney Section Studies

Immunohistochemical staining for the D₁ receptor was found in proximal and distal tubules and cortical collecting ducts but was absent in glomeruli, juxtaglomerular apparatuses, small blood vessels, and medulla.¹⁷ No staining was observed in consecutive sections processed with either the antibody preabsorbed against the immunizing peptide or the preimmune serum.¹⁷ As with the D₁ receptor, the PTH/PTHrP receptor was present in both proximal and distal tubules in HT and NT (not shown). No staining was observed in consecutive sections processed with the antibody preabsorbed against the immunizing peptide (not shown).

RPT Cells in Culture

There were no differences in the immunostaining pattern of the D₁ and PTH/PTHrP receptors between hRPT cells from NT and HT (not shown). No staining was observed in consecutive sections processed with either the antibodies preabsorbed against the immunizing peptide or preimmune serum (for the D₁ receptor) (not shown).

Immunofluorescence of D₁ and PTH/PTHrP Receptors in hRPT

The presence of D₁ and PTH/PTHrP receptors in hRPT was corroborated by the immunofluorescence studies; there were also no differences in the immunofluorescence pattern of the D₁ and PTH/PTHrP receptors in hRPT cells from NT and HT (Figures 5). No staining was observed with either the antibodies preabsorbed against the immunizing peptide or preimmune serum (for the D₁ receptor) (not shown).

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Figure 4. Effect of the D₁-like receptor agonist fenoldopam on PLC activity in RPT cells in culture. Fenoldopam (5 μmol/L) increased PLC activity at 1 minute and 30 minutes in HT but only at 30 minutes in NT subjects. Basal PLC levels were not statistically different in NT and HT (not shown). *P<0.05 NT vs HT, t test; †P<0.05 vs basal activity, ANOVA for repeated measures, Scheffé’s test. Data are mean±SE. n indicates number of experiments; each n represents culture from 1 subject.

Figure 5. Immunofluorescence microscopy of D₁ and PTH/PTHrP receptors in RPT cells in culture from NT and HT subjects. Positive immunofluorescent staining was seen for the D₁ receptor (top left, NT; top right, HT) and PTH/PTHrP receptor (bottom left, NT; bottom right, HT) (magnification ×400). No staining was observed with either the preabsorbed antibodies or preimmune antiserum for either PTH/PTHrP or D₁ receptor (not shown).
D1 Receptor Phosphorylation

The uncoupling of the D1-like receptor from its G protein/AC complex is similar but distinct from homologous desensitization that is due to phosphorylation of the receptor after ligand occupation.\textsuperscript{1,10} We hypothesized that the D1 receptor in RPT in hypertension may be already phosphorylated even in the basal state and is therefore essentially desensitized. Since the D1 receptor is phosphorylated at its serine residues,\textsuperscript{19} we compared the serine phosphorylation of the receptor with and without 1 \textmu mol/L fenoldopam. As shown in Figure 7, the quantity of serine-phosphorylated D1 receptor is greater in HT than in NT at basal conditions. Moreover, fenoldopam increased the phosphorylation of the D1 receptor in NT but not in HT subjects. These data are in accord with the hypothesis that the decreased ability of fenoldopam to stimulate cAMP accumulation in HT subjects is associated with increased phosphorylation of the D1 receptor in the basal state.

Discussion

These studies show a selective defect in the D1-like receptor signal transduction pathway involving cAMP in hRPT in culture from human subjects with essential hypertension. The impaired action of D1-like receptors on cAMP accumulation is not due to an aberrant AC enzyme or an epiphenomenon, since forskolin and PTHrP stimulated cAMP accumulation to the same extent in both groups. Because the human proximal tubule cells were studied after several passages, the possible confounding effect of dedifferentiation needs to be considered. However, the preservation of the responses to forskolin and PTHrP makes it unlikely that selective dedifferentiation of the hRPT cells from HT subjects caused the differential signal transduction pathway involving cAMP in hRPT in culture from human subjects with essential hypertension. The decreased ability of D1-like receptors to stimulate cAMP production in hRPT from subjects with essential hypertension is similar to observations in RPT and arterioles in animal models of genetic hypertension.\textsuperscript{1,8,9,22} Because the D1-like receptor-mediated natriuresis is, in part, caused by cAMP, we have suggested that the impaired natriuretic effect of D1-like receptors in the SHR is in part due to decreased D1-mediated stimulation of cytoplasmic second messengers.\textsuperscript{1} The preservation of the natriuretic effect of the D1-like agonist fenoldopam in essential hypertension may be due to an intact D1-like response at more distal sites along the nephron (eg, cortical collecting duct).\textsuperscript{12} The ability of guanine

![Figure 6. Effect of D1 receptor sense and antisense phosphorothioate/propyne-modified oligonucleotides on D1 receptor translation and cAMP accumulation in RPT cells in culture from NT and HT subjects. D1 antisense but not sense oligonucleotides inhibited translation of the D1 receptor (inset, 70 to 90 kDa) and abolished the ability of fenoldopam to stimulate cAMP accumulation in both HT and NT. However, the effect in NT was not complete. The inset shows the immunoblot of D1 receptors in NT and HT (1 = vehicle, NT; 2 = D1 antisense, NT; 3 = D1 sense, NT; 4 = vehicle, HT; 5 = D1 antisense, HT; 6 = D1 sense, HT). Data are mean ± SE. n indicates number of experiments; each n represents culture from 1 subject. *P < 0.01 NT vs HT, t test; #P < 0.03 vs others, ANOVA, Scheffé’s test.](http://hyper.ahajournals.org/)

![Figure 7. Serine-phosphorylated D1 receptor in RPT cells in culture from NT and HT subjects. The D1 receptor was immunoprecipitated with D1 antibody and immunoblotted with phosphoserine antibody as described in Methods. Basal serine phosphorylation was greater in HT than in NT. Fenoldopam increased the phosphorylation of the D1 receptor in NT but not in HT (data corrected for amount of lysate protein). The inset shows the phosphorylated D1 receptor with a duplet band at 70 to 90 kDa (1 = basal, NT; 2 = fenoldopam [5 × 10^{-8} mol/L], NT; 3 = basal, HT; 4 = fenoldopam [5 × 10^{-8} mol/L], HT). Data are mean ± SE. n indicates number of experiments; each n represents culture from 1 subject. *P < 0.05 vs others, ANOVA, Scheffé’s test.](http://hyper.ahajournals.org/)
nucleotides to stimulate AC activity in the RPT is also not impaired in the SHR, suggesting that the defect is probably located proximal to G proteins, presumably at a D1-like receptor.\textsuperscript{1,3} There is organ specificity of the defect since fenoldopam stimulated AC activity in the striatum of the brain to the same extent in SHR and its normotensive control.\textsuperscript{1} There is receptor specificity since PTH stimulated AC activity to the same extent in the RPT of these rat strains, an observation similar to that noted in the present study.\textsuperscript{1,9} There is, however, one appreciable difference. In the SHR, the ability of dopamine and D1-like agonists to stimulate PLC and phospholipase A\textsubscript{2} activity in RPT is also impaired.\textsuperscript{7,10} In the present study, D1-like activation of PLC may be actually greater in HT than in NT subjects. The significance of the apparently greater increase in PLC activity after D1-like receptor stimulation in HT than in NT remains to be determined.

The 2 D\textsubscript{1} receptor subtypes (D\textsubscript{1} and D\textsubscript{5} subtypes) cloned in mammals are expressed in RPT.\textsuperscript{1} There is no difference in the amount or distribution (data not shown) of D\textsubscript{1} or D\textsubscript{5} receptors in the kidney of HT and NT subjects. The apparent uncoupling of a D1-like receptor from AC is also not due to decreased receptor density, similar to studies in genetically hypertensive rats.\textsuperscript{1,9} The ability of antisense D\textsubscript{1} receptor oligonucleotide to completely block the stimulatory effect of fenoldopam on cAMP accumulation in HT, and nearly so in NT, indicates that D\textsubscript{1} receptor function predominates over D\textsubscript{5} receptor function. Moreover, we have reported that disruption of the D\textsubscript{1A} receptor in mice increases blood pressure and produces diastolic hypertension.\textsuperscript{6} Although a defect of the D\textsubscript{1} receptor or its regulation may be involved in hypertension, this dopamine receptor subtype has not been linked to PLC activation.\textsuperscript{1} It is also unlikely that a novel D\textsubscript{1}-like receptor linked to PLC is involved in hypertension because D\textsubscript{1}-like receptor-mediated PLC activation is not impaired in hRPT from HT subjects.

We have not found a mutation of the D1 receptor in our patients with essential hypertension; we have not found a mutation in the D\textsubscript{1A} or D\textsubscript{1B} receptor in genetically hypertensive rats, either.\textsuperscript{22} We have suggested that the uncoupling of a D1-like receptor from its effector complex in RPT is akin to homologous desensitization.\textsuperscript{1} However, the desensitization is ligand independent since it occurs in cultures of RPT that are not exposed to dopamine. (RPT cannot synthesize dopamine in the absence of L-DOPA.)\textsuperscript{1} The present studies show that the renal proximal tubular D\textsubscript{1} receptor is “hyper”-serine-phosphorylated in HT compared with NT and does not respond to fenoldopam stimulation, similar to the cAMP accumulation studies. Since G protein–coupled receptor–related kinases (GRKs) are involved in the phosphorylation of receptors, resulting in their desensitization, it is of interest that GRK activity and GRK2 expression are increased in lymphocytes of patients with essential hypertension.\textsuperscript{24} GRK2 is involved in the phosphorylation of several G protein–coupled receptors, including D\textsubscript{1} receptors.\textsuperscript{23} Increased activity of GRK2 could be responsible for the desensitization of the D\textsubscript{1}-like receptor. However, increased GRK2 activity does not explain the apparent importance of the kidney in the pathogenesis of essential hypertension, since GRK2 is expressed in many organs other than the kidney. Because GRK2 is involved in the desensitization of PTH receptor,\textsuperscript{25} an increase in GRK2 activity should have resulted in the desensitization of the PTH receptor in hypertension. However, renal PTH action is not impaired in rats with genetic hypertension and in humans with essential hypertension.\textsuperscript{1,9} Thus, the desensitization of the D\textsubscript{1} receptor in hypertension may be due to a kinase but not necessarily GRK2.

In summary, we have found that dopamine D\textsubscript{1} and PTH/PTHrP receptors are expressed in RPT of human kidneys. Although the distribution and quantity of D\textsubscript{1} receptor expression are similar in RPT from NT and HT subjects, the ability of dopamine and a D\textsubscript{1}-like receptor agonist to stimulate cAMP production is impaired in HT subjects. The impairment is not due to a defective AC enzyme akin to the ligand-independent desensitization reported in genetically hypertensive rats. The defect is also receptor specific because PTHrP responsiveness is intact. Although the relationship between the increased serine phosphorylation of the D\textsubscript{1} receptor and ligand-independent uncoupling of a D\textsubscript{1}-like receptor remains to be determined, it is suggested that this uncoupling may be involved in the pathogenesis of essential hypertension.

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