Renal Protein Phosphatase 2A Activity and Spontaneous Hypertension in Rats

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Abstract—The impaired renal paracrine function of dopamine in spontaneously hypertensive rats (SHR) is caused by hyperphosphorylation and desensitization of the renal D₁ dopamine receptor. Protein phosphatase 2A (PP₂A) is critical in the regulation of G-protein–coupled receptor function. To determine whether PP₂A expression and activity in the kidney are differentially regulated in genetic hypertension, we examined the effects of a D₁-like agonist, fenoldopam, in renal cortical tubules and immortalized renal proximal tubule cells from normotensive Wistar-Kyoto rats (WKY) and SHR. In cortical tubules and immortalized proximal tubule cells, PP₂A expression and activities were greater in cytosol than in membrane fractions in both WKY and SHR. Although PP₂A expressions were similar in WKY and SHR, basal PP₂A activity was greater in immortalized proximal tubule cells of SHR than WKY. In immortalized proximal tubule cells of WKY, fenoldopam increased membrane PP₂A activity and expression of the regulatory subunit PP₂A-B56α, effects that were blocked by the D₁-like antagonist SCH23390. Fenoldopam had no effect on cytosolic PP₂A activity but decreased PP₂A-B56α expression. In contrast, in immortalized proximal tubule cells of SHR, fenoldopam decreased PP₂A activity in both membranes and cytosol but predominantly in the membrane fraction, without affecting PP₂A-B56α expression; this effect was blocked by the D₁-like antagonist SCH23390. We conclude that renal PP₂A activity and expression are differentially regulated in WKY and SHR by D₁-like receptors. A failure of D₁-like agonists to increase PP₂A activity in proximal tubule membranes may be a cause of the increased phosphorylation of the D₁ receptor in the SHR. (Hypertension. 2000;36:1053-1058.)

Key Words: hypertension, genetic receptors, dopamine dopamine hypertension, essential phosphatase

Essential hypertension is a multifactorial disorder thought to arise from the interactions of environmental factors with multiple genes. Defective regulation of nerve activity, vascular reactivity, endocrine function, and ion transport in genetic hypertension has been linked to abnormalities in signal transduction. Dopamine synthesized by renal proximal tubule cells (RPTC) plays an important paracrine/autocrine role in the regulation of renal function during volume expansion. Dopamine, through different dopamine receptor subtypes, regulates cardiovascular functions by actions on the central and peripheral nervous systems, vascular smooth muscle, and adrenal glands, heart, and kidney. Abnormal regulation of cardiovascular function by various dopamine receptor subtypes has been implicated in the pathogenesis of genetic hypertension. In genetic hypertension, the D₁ dopamine receptor, a G-protein–coupled receptor (GPCR) in renal proximal tubules, is desensitized, serine-hyperphosphorylated, and uncoupled from its G protein effector complex. The functional status of GPCR is determined by the phosphorylation state, a dynamic process controlled by protein kinases and phosphatases. Receptor desensitization occurs after receptor phosphorylation and internalization. In contrast, receptor resensitization requires receptor dephosphorylation, a process mediated by a plasma membrane–associated form of protein phosphatase, a GPCR phosphatase (GRP). GPCR resensitization requires protein phosphatase 2A (PP₂A) activity because okadaic acid, an inhibitor of PP₂A activity, prevents receptor resensitization. Moreover, when phosphorylated receptors translocate from plasma membrane to cytoplasmic vesicles, they become physically associated with PP₂A.

Because PP₂A activity is involved in the dephosphorylation and resensitization of GPCR, it is possible that aberrant D₁ receptor regulation of PP₂A activity may be involved in the desensitization of this GPCR in genetic hypertension. PP₂A is a heterotrimeric protein serine/threonine phosphatase; the holoenzyme consists of a 36-kDa catalytic C subunit, a 65-kDa structural A subunit, and a variable regulatory B subunit. Both the A and C subunits exist as 2 isoforms (α and β), whereas there are 3 families of B subunits: B or PR55,
B’ or B56, and B’’ or PR72. B56 is a new gene family that encodes B56α, β, γ, δ, and ε proteins. The B56β and δ subunits are found predominantly in brain tissue, while the B56α and γ subunits are most abundant in heart and muscle, and the ε subunit is expressed mainly in the testis, lung, and brain. Of the B56 isoforms, B56α appears to be the most abundantly expressed subunit in the kidney.

PP2A activity and protein levels were examined in renal cortical tubules from spontaneously hypertensive rats (SHR) and their normotensive controls, Wistar-Kyoto rats (WKY). To eliminate any confounding influence of dopamine produced by renal proximal tubules from circulating L-dihydroxyphenylalanine (L-DOPA), studies were also performed in immortalized renal proximal tubule cells (RPTC). RPTC cannot synthesize dopamine in the absence of L-DOPA. Here we report that PP2A activity and expression of the PP2A subunit regulatory subunit PP2A-B56α were differentially affected by D1-like receptor stimulation in the kidney of WKY and SHR.

**Methods**

**Materials**

Reagents were obtained from the following: monoclonal anti-human PP2A-B56α (Transduction Labs), polyclonal PP2A A subunits, α, β, and γ (Santa Cruz Biotech Inc); monoclonal anti-human PP2A catalytic subunit (PP2A-C) and Ser/Thr phosphatase assay kit (Upstate Biotech Inc), secondary antibodies for Western blots (Jackson ImmunoResearch Labs Inc), enhanced chemiluminescence Western blotting detection reagents (Amersham Corp), fenoldopam (Smith Kline Beecham Pharmaceuticals), and SCH23990 (RBI Inc).

**Renal Cortical Membranes**

Adult male WKY (body weight 375±43 g, mean arterial pressure [MAP] 98±3 mm Hg, n=4) and SHR (body weight 307±33 g, MAP 161±3 mm Hg, n=4) (Taconic Farms Inc), maintained on standard rat chow until the night before the experiment, were anesthetized with pentobarbital (50 mg/kg body wt IP) and tracheotomized (PE-240). MAP was monitored with Cardiomax II (Columbus Instruments). After a 30-minute equilibration period, saline or fenoldopam in saline (1 μg·kg⁻¹·min⁻¹) was infused (1.2 mL/h) into the right renal artery through the right suprarenal artery for 10 or 30 minutes. The renal cortical cytosol and membrane fractions were prepared in TE buffer (mM): 10 Tris-Cl, pH 7.4, 2 EDTA) and TBST buffer (mM: 10 Tris-Cl, pH 7.4, 150 NaCl, 2 EDTA, 0.5% Triton X-100) as described previously with modification. All buffers contained protease inhibitors (mM): 5 DTT, 2 EDTA, and 1 pofabloc and 10 μg/mL each of leupeptin and aprotinin.

**Preparation of Brush Border Membranes**

Brush border membranes (BBM) (100 μg/sample), prepared by MnCl₂ precipitation and differential centrifugation, were treated with fenoldopam (5 μmol/L) or vehicle (saline) for 30 minutes at room temperature. The BBM were pelleted by centrifugation for 5 minutes, suspended in Laemmli buffer, and boiled for 5 minutes before immunoblotting.

**Immortalized RPTC and Subcellular Fractions**

Immortalized RPTC were maintained in DMEM/F12 supplemented with 5% fetal bovine serum, epidermal growth factor (10 ng/mL), insulin, transferrin, and selenium (5 μg/mL each) at 37°C in humidified 5% CO₂/95% air. The cells were incubated for 1 hour in serum-free DMEM/F12 medium before agonist stimulation with the D₁-like agonist fenoldopam (5 μmol/L). The cells were washed 3 times with TBS and disrupted by Dounce homogenizer or a syringe with a 27-gauge needle in TE buffer. The cell lysates were centrifuged at 2000 rpm for 5 minutes to remove the nuclear fraction. The supernatants were centrifuged at 14 000 rpm for 20 minutes and the resulting supernatant was taken to represent cytosol. The pellets were extracted for 30 minutes on ice with TBST lysis to obtain the membrane fractions. All experiments were carried out at 4°C in the presence of protease inhibitors described above. Protein concentrations were determined by the Bradford method.

**PP2A Activity Assay**

PP2A activity was measured after immunoprecipitation with anti-PP2A catalytic subunit antibody, according to Gupta et al with modifications. The cytosol and membrane preparations were subjected to immunoprecipitation carried out in triplicate (see below). The immune complexes with the protein G beads were initially washed twice with TBS buffer and finally washed with PP2A assay buffer (in mmol/L, 20 MOPS, pH 7.5, 60 2-mercaptoethanol, 100 NaCl, 0.1 mg/mL serum albumin). Immunoprecipitates with mouse IgG served as negative control. The immunoprecipitates were subjected to PP2A assay with a nonradioactive assay kit. In brief, the immune complexes containing PP2A were incubated for 20 minutes at 30°C in 30 μL of assay buffer containing 200 μmol/L of phosphopeptide (KRPTRR). The reactions were terminated by putting the tubes on ice and immediately centrifuging at 8000 rpm for 1 minute. The supernatants (25 μL/well) were pipetted into 96-well microtiter plates containing malachite green solution (100 μL/well). The absorbance was measured at optical density of 630 nm after 15 minutes of development time. PP2A specific activity was expressed as pmol/min per milligram of protein. The lower limit of detection was 20 pmol of Pi (released phosphate from the phosphopeptide).

**Immunoprecipitation and Immunoblotting**

The immunoprecipitation for the PP2A assay was performed by incubation of 50 to 200 μg of protein with 2 μg of PP2A-C monoclonal antibody and protein G beads in TBS buffer with rocking for 2 hours in a cold room. For immunoblotting, 50 to 100 μg of protein was loaded onto polyacrylamide gel. The amount of protein transferred onto the nitrocellulose membrane was verified by Ponceau-S stain. Immunoblotting and quantification of the immunoblots were performed as described previously with Quantiscan. The results were expressed as density units (DU).

**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 Scheffe’s or 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**

D₁-like receptors are expressed to a greater extent in the renal cortex (proximal tubule, distal tubule, cortical collecting) and to a lesser extent in the medulla (medullary thick ascending limb of Henle). Therefore, PP2A expression and activity were studied in the renal cortex.

**In Vivo Studies**

**PP2A Expression**

**Basal Expression**

PP2A, the structural subunit, PP2A-C, the catalytic subunit, and PP2A-B56α, the regulatory subunit, were expressed to a similar extent in renal cortical tubules of WKY and SHR (Figure 1). In agreement with data obtained with the use of tissues other than the kidney, PP2A was more abundant in the cytosol than in the particulate fraction. In WKY rats, PP2A...
subunits predominantly existed in cytosol (PP2A-Aα = 86.9 ± 0.3 DU, PP2A-C = 85.2 ± 1.5 DU, PP2A-B56α = 88.1 ± 4.0, n = 5) and membrane (PP2A-Aα = 13.8 ± 0.4 DU, PP2A-C = 15.2 ± 3.5 DU, PP2A-B56α = 12.2 ± 4.0, n = 5). Similar results were obtained in SHR (data not shown).

Effect of D1-Like Agonist
A 30-minute intrarenal arterial infusion of the D1-like agonist fenoldopam (1 μg/kg body wt per minute) has been shown previously to produce a greater natriuresis in WKY than in SHR.32 In the current experiments, fenoldopam infusion did previously to produce a greater natriuresis in WKY than in SHR. Fenoldopam decreased PP2A-B56α in BBM of SHR (n = 4) and increased it in BBM from WKY (n = 8) (*P<0.05, ANOVA, Scheffe’s test) (for details of methods, see Reference 26). Results are expressed as mean ± SEM. Inset is one immunoblot from 4 independent experiments.

Basal Activity
Basal PP2A activity (pmol/min per milligram of protein) in WKY was 323±97 in the cytosol and 252±45 in the membrane fraction. The corresponding values in SHR were 385±57 and 256±38, respectively.

Effect of D1-Like Agonist
In WKY, the intrarenal arterial infusion of the D1-like agonist fenoldopam, for 10 or 30 minutes, produced no significant change in the cytosol. Fenoldopam also did not change PP2A activity in renal membranes of WKY rats after 10 minutes but did increase it after 30 minutes (12.5±5.0% from control) (P<0.05 n = 4, ANOVA, Duncan’s test). In contrast, in SHR, fenoldopam decreased PP2A activity in both renal cortical tubule cytosol (−17.1±1.7% at 10 minutes, −17.1±4.7% at 30 minutes) (P<0.05 versus vehicle-treated rats) and renal cortical membrane (−14.8±2.8% at 10 minutes, −16.7±3.6% at 30 minutes, n = 4) (P<0.05, ANOVA, Duncan’s test).

In Vitro Studies
We have reported that D1-like receptor function in immortalized RPTC is similar to that in renal proximal tubules freshly obtained from WKY.37,38 The uncoupling of the D1-like receptor in renal proximal tubules in SHR persists in immortalized RPTC from SHR.9,37,38 Thus, D1 receptor protein expression is similar in immortalized RPTC and renal proximal tubules from WKY and SHR.37,38 The D1-like receptor, specifically the D1 receptor subtype, is uncoupled from G protein subunits and effector proteins in immortalized RPTC and renal proximal tubules of WKY and SHR.37,38 Immortalized RPTC from WKY and SHR retain characteristics of renal proximal tubule cells,22 including expression of γ-glutamyl transpeptidase, a renal proximal tubule BBM marker. These cells express the two D1-like receptors, D1 and D2 receptors, G proteins, and Na+/H+ exchanger 3 protein to same extent in WKY and SHR.38

PP2A Expression
Basal Expression
As in the renal cortical tubules, in immortalized RPTC of WKY, PP2A-B56α protein level was greater in cytosol (WKY, 41.5±3.4 DU, n = 4) than in membrane fractions (WKY,
Effect of D<sub>1</sub>-Like Agonist

In membranes from immortalized RPTC of WKY, the D<sub>1</sub>-like agonist fenoldopam (5 μmol/L) increased PP<sub>2A</sub>-B56α protein levels with time (Figure 3A). In cytosol of immortalized RPTC of WKY, fenoldopam decreased PP2A-B56α protein levels in a manner reciprocal with that seen in membranes. The increase in PP<sub>2A</sub>-B56α expression in the membranes caused by 5 μmol/L fenoldopam (80±23% over basal, P<0.05 ANOVA, Duncan’s test) was partially blocked by 5 μmol/L SCH23390 (47±29% over basal, P>0.05 ANOVA, Duncan’s test), which, by itself, had no effect (29±23% over basal, P>0.05 ANOVA, Duncan’s test). In contrast to the WKY rats, fenoldopam produced no change in PP<sub>2A</sub>-B56α expression in either membrane or cytosol in immortalized RPTC of SHR (data not shown).

**PP<sub>2A</sub> Activity**

**Basal Activity**

In the absence of endogenous dopamine, basal PP<sub>2A</sub> activity (pmol/min per milligram of protein) in the membrane was markedly higher in immortalized RPTC of SHR (540±63, n=3) than in immortalized RPTC of WKY (233±21, n=3) (P<0.05, t test). However, PP<sub>2A</sub> activity in cytosol was not different between SHR (496±36, n=3) and WKY (473±95, n=4).

**Effect of D<sub>1</sub>-Like Agonist**

In immortalized RPTC membranes from WKY, fenoldopam (5 μmol/L) increased PP<sub>2A</sub> activity (pmol/min per milligram of protein) with time, peaking at 30 minutes to 278±21 (P<0.05 versus basal activity, n=4); no effect was noted in cytosol (Figure 3B). In contrast, in the immortalized RPTC from SHR, fenoldopam decreased PP<sub>2A</sub> activity in both cytosol and membrane fractions in a time-dependent manner (Figure 3B), in agreement with studies that used renal cortical tubules. The maximum decrease occurred at 30 minutes down to 327±63 in membrane (n=3) and 428±30 in cytosol (n=3) (P<0.05 versus basal activity).

**Effect of D<sub>1</sub>-Like Antagonist SCH23390**

To determine if the effect of the D<sub>1</sub> agonist fenoldopam is mediated by the occupation of the D<sub>1</sub> receptor, studies were performed in the presence of the D<sub>1</sub>-like antagonist SCH23390 (5 μmol/L), a neutral antagonist. Fenoldopam (5 μmol/L, 30 minutes (Figure 3C) increased PP<sub>2A</sub> activity in membrane of WKY (22.6±4.4% from basal) (P<0.05, n=4), which was blocked by SCH23390 (9.3±3.8% from basal, n=4); SCH23390, by itself (3.9±5.7% from basal, n=5), had no effect on PP<sub>2A</sub> activity. Fenoldopam decreased the activity in cytosol of SHR (−18.0±2.8% versus basal, n=4, P<0.05), an effect that was blocked by SCH23390 (−10.4±4.3% from basal, n=4 P>0.05); SCH23390, by itself, had no effect on PP<sub>2A</sub> activity (−8.7±3.6% from basal, n=4 to 5, P>0.05).

**Discussion**

Several studies have shown that GRP plays a role in receptor dephosphorylation that is important in receptor resensitiza-
A high level of receptor phosphatase activity was reported to be associated with sequestered vesicular membranes, capable of dephosphorylating GRK-phosphorylated β-adrenergic receptors. Moreover, the resensitization of GPCR was inhibited by phosphatase inhibitors. GRP has been identified as a member of the family of PPIα.12

PPIIα is generally considered as a cytosolic protein. However, PPIIα is also expressed in cell particulate fractions.12 We also found that PPIIα protein level was much higher in the cytosol than in membranes from renal cortex and immortalized RPTC in both WKY and SHR. However, PPIIα specific activity was not different in cytosol and membrane fractions.

In addition to GPCR resensitization, PPIIα is involved in a broad range of cellular processes, including signal transduction, intermediary metabolism, transcriptional regulation and control of DNA replication, and mitosis. The diversity of PPIIα function is conferred by a variety of targeting/regulatory subunits. Each of the B subunits consists of numerous isoforms and splice variants. The PPIIα regulatory B subunits have been found to have a number of functions, including targeting to distinct intracellular locations, determining substrate and tissue specificity, and acting as receptors of second messengers. Most of the B56 family members are phosphoproteins, and different isoforms have different targeting functions. The function of PPIIα-B56α in hypertension remains to be determined. However, the failure of the D1-like agonist to translocate PPIIα activity from cytosol to membrane in SHR may explain, in part, the "hyper"-serine-phosphorylated D1 receptor in renal membranes in genetic hypertension.5,9 The finding of a difference in PPIIα activity between WKY and SHR in RPTC, where dopamine is present, suggests that dopamine may tonically inhibit the PPIIα activity, especially in the cytosol. Because PPIIα-B56α is important in the targeting of the PPIIα holoenzyme, a defect in B56α function could be involved in the pathogenesis of genetic hypertension. The important finding in our study is the remarkable difference in D1-like receptor-associated PPIIα activity in RPTC membranes between WKY and SHR. The D1-like agonist fenoldopam increased membranes PPIIα activity in WKY but decreased it in SHR. Fenoldopam translocated PPIIα activity from cytosol to the membrane in WKY but not in SHR. We also found that the PPIIα regulatory subunit B56α exists in the BBM, where most of the D1 receptors are located. It is of interest that PPIIα-B56α protein level decreased in the BBM from SHR after fenoldopam treatment. The ability of the D1-like antagonist to block both the increase in PPIIα activity in WKY and the opposite response in SHR support the notion of an abnormality downstream of the D1-like receptor. Because there is no mutation of the D1 receptor in the SHR, this finding suggests that a primary abnormality of PPIIα-B56α may be a cause of the discrepant response of SHR compared with WKY.

Activation of PPIIα necessitates prior phosphorylation of the D1-like receptor. Presumably, activation of a G-protein-coupled receptor kinase (GRK) must have occurred after D1-like agonist stimulation. The phosphorylation of the D1 receptor by GRK by GRK2, GRK3, and GRK5 has been reported to play a role in the desensitization of the D1 receptor. Increased activity and expression of GRK2 has been reported in aortic smooth muscle and lymphocytes from SHR. However, these changes occurred as a consequence of the hypertension. Because the increased phosphorylation of the D1 receptor in renal proximal tubules is ligand independent, the participation of a constitutively activated GRK must have occurred. The existence of this GRK was not determined in the current studies, but GRK6 has been reported to be constitutively activated. We speculate that a constitutively activated GRK (eg, GRK6) causes basal phosphorylation of D1-like receptors in renal proximal tubules in both WKY and SHR. D1-like agonist occupation further increases the phosphorylation of renal D1-like receptors in WKY but not in SHR. PPIIα dephosphorylates and resensitizes renal D1-like receptors in WKY but not in SHR, resulting in a phosphorylated and desensitized D1-like receptor in genetic hypertension.

In conclusion, we have demonstrated that after D1-like agonist activation, PPIIα activity in renal proximal tubules is increased in WKY but decreased in SHR. We speculate that the increased basal levels of serine-phosphorylated D1 receptor in RPTC in hypertension may be a consequence of a defect in dephosphorylation of the phosphorylated D1 receptor. Although we have demonstrated a role of PPIIα in this response, the role of other phosphatases, for example, PPIIβ, in the desensitization of D1-like receptors in hypertension, remains to be determined.

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