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 D<sub>1</sub> dopamine receptor. Protein phosphatase 2A (PP<sub>2A</sub>) is critical in the regulation of G-protein–coupled receptor function. To determine whether PP<sub>2A</sub> expression and activity in the kidney are differentially regulated in genetic hypertension, we examined the effects of a D<sub>1</sub>-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, PP<sub>2A</sub> expression and activities were greater in cytosol than in membrane fractions in both WKY and SHR. Although PP<sub>2A</sub> expressions were similar in WKY and SHR, basal PP<sub>2A</sub> activity was greater in immortalized proximal tubule cells of SHR than WKY. In immortalized proximal tubule cells of WKY, fenoldopam increased membrane PP<sub>2A</sub> activity and expression of the regulatory subunit PP<sub>2A</sub>-B56α, effects that were blocked by the D<sub>1</sub>-like antagonist SCH23390. Fenoldopam had no effect on cytosolic PP<sub>2A</sub> activity but decreased PP<sub>2A</sub>-B56α expression. In contrast, in immortalized proximal tubule cells of SHR, fenoldopam decreased PP<sub>2A</sub> activity in both membranes and cytosol but predominantly in the membrane fraction, without affecting PP<sub>2A</sub>-B56α expression; this effect was blocked by the D<sub>1</sub>-like antagonist SCH23390. We conclude that renal PP<sub>2A</sub> activity and expression are differentially regulated in WKY and SHR by D<sub>1</sub>-like receptors. A failure of D<sub>1</sub>-like agonists to increase PP<sub>2A</sub> activity in proximal tubule membranes may be a cause of the increased phosphorylation of the D<sub>1</sub> 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.¹ ² Defective regulation of nerve activity, vascular reactivity, endocrine function, and ion transport in genetic hypertension has been linked to abnormalities in signal transduction.¹–³ Dopamine synthesized by renal proximal tubule cells (RPTC) plays an important paracrine/autocrine role in the regulation of renal function during volume expansion.³–⁵ 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.³ Abnormal regulation of cardiovascular function by various dopamine receptor subtypes has been implicated in the pathogenesis of genetic hypertension.² ³ ⁶–⁷ In genetic hypertension, the D<sub>1</sub> dopamine receptor, a G-protein–coupled receptor (GPCR) in renal proximal tubules, is desensitized, serine-hyperphosphorylated, and uncoupled from its G protein-effector complex.² ⁸ ⁹ The functional status of GPCR is determined by the phosphorylation state, a dynamic process controlled by protein kinases and phosphatases.¹⁰ ¹¹ Receptor desensitization occurs after receptor phosphorylation and internalization.¹⁰ ¹¹ In contrast, receptor resensitization requires receptor dephosphorylation, a process mediated by a plasma membrane–associated form of protein phosphatase, a GPCR phosphatase (GRP).¹¹ ¹² GPCR resensitization requires protein phosphatase 2A (PP<sub>2A</sub>) activity because okadaic acid, an inhibitor of PP<sub>2A</sub> activity, prevents receptor resensitization.¹³ Moreover, when phosphorylated receptors translocate from plasma membrane to cytoplasmic vesicles, they become physically associated with PP<sub>2A</sub>.¹² ¹⁴ Because PP<sub>2A</sub> activity is involved in the dephosphorylation and resensitization of GPCR, it is possible that aberrant D<sub>1</sub> receptor regulation of PP<sub>2A</sub> activity may be involved in the desensitization of this GPCR in genetic hypertension. PP<sub>2A</sub> 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.¹⁵ Both the A and C subunits exist as 2 isoforms (α and β), whereas there are 3 families of B subunits: B<sub>0</sub> 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, 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).

PP2A regulatory subunit PP2A-B56α were differentially affected by D1-like receptor stimulation in the kidney of WKY and SHR.

Methods

Reagents were obtained from the following: monoclonal anti-human PP2A-C, polyclonal PP2A A subunits, Ax, and Ab (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 (Colin 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 (mmol/L, 10 Tris-Cl, pH 7.4, 2 EDTA) and TBST buffer (mmol/L, 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 (mmol/L): 5 DTT, 2 EDTA, and 1 pefabloc and 10 μg/mL each of leupeptin and aprotinin.

Preparation of Brush Border Membranes

Brush border membranes (BBM) (100 μg/sample), prepared by MNCl 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 F (released phosphate from the phosphopeptide).

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 Scheffé'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.

PP2A Expression

Basal Expression

PP2A Aα, 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 fured 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.

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subunits predominantly existed in cytosol (PP\textsubscript{2A}-A\textalpha=86.9±0.3 DU, PP\textsubscript{2A}-C=85.2±1.5 DU, PP\textsubscript{2A}-B56\alpha=88.1±4.0, n=5) and membrane (PP\textsubscript{2A}-A\textalpha=13.8±0.4 DU, PP\textsubscript{2A}-C=15.2±3.5 DU, PP\textsubscript{2A}-B56\alpha=12.2±4.0, n=5). Similar results were obtained in SHR (data not shown). PP\textsubscript{2A}-B56\alpha expression was greater in BBM relative to PP\textsubscript{2A}-A\textalpha and PP\textsubscript{2A}-C. PP\textsubscript{2A}-B56\alpha was chosen for study because this regulatory protein is ubiquitously expressed and exists as heterotrimer with the PP2A core dimer PP\textsubscript{2A}-A\textalpha and PP\textsubscript{2A}-C.30 In addition, antibodies to PP\textsubscript{2A}-B56 other than PP\textsubscript{2A}-B56\alpha are not commercially available. PP\textsubscript{2A}-B55 was not detected in our samples, in agreement with published reports.31

**Effect of D\textsubscript{1}-Like Agonist**

A 30-minute intrarenal arterial infusion of the D\textsubscript{1}-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 not affect PP\textsubscript{2A}-B56\alpha expression in renal cortical homogenates in either WKY or SHR (data not shown). Fenoldopam increased PP\textsubscript{2A}-B56\alpha expression in BBM in WKY rats (56.7±3.5 DU versus control, 44.0±3.4 DU) (P<0.05, n=8) and decreased it in SHR (41.5±2.6 versus control, 58.7±2.7 DU) (P<0.05, n=4) (Figure 2).

**PP\textsubscript{2A} Activity**

The majority of PP\textsubscript{2A} are heterotrimers that contain a variable regulatory B subunit bound to the A-C dimer.16,33–35 To determine specific PP\textsubscript{2A} activity, we measured PP\textsubscript{2A} activity in immunoprecipitates of the specific monoclonal anti--PP\textsubscript{2A}-C subunit antibody.28,36 The protein phosphatase activity on the immune complex was predominantly PP\textsubscript{2A}, because the A subunit and B56\alpha of PP\textsubscript{2A} were also coimmunoprecipitated with anti-C (data not shown), and the activity was inhibited dose-dependently by a PP\textsubscript{2A} inhibitor, okadaic acid (≈50%, data not shown).

**Basal Activity**

Basal PP\textsubscript{2A} 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 D\textsubscript{1}-Like Agonist**

In WKY, the intrarenal arterial infusion of the D\textsubscript{1}-like agonist fenoldopam, for 10 or 30 minutes, produced no significant change in the cytosol. Fenoldopam also did not change PP\textsubscript{2A} 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 PP\textsubscript{2A} 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 D\textsubscript{1}-like receptor function in immortalized RPTC is similar to that in renal proximal tubules freshly obtained from WKY.37,38 The uncoupling of the D\textsubscript{1}-like receptor in renal proximal tubules in SHR persists in immortalized RPTC from SHR.9,37,38 Thus, D\textsubscript{1} receptor protein expression is similar in immortalized RPTC and renal proximal tubules from WKY and SHR.37,38 The D\textsubscript{1}-like receptor, specifically the D\textsubscript{1} 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 D\textsubscript{1}-like receptors, D\textsubscript{1} and D\textsubscript{5} receptors, G proteins, and Na\textsuperscript{+}/H\textsuperscript{+} exchanger 3 protein to same extent in WKY and SHR.38

**PP\textsubscript{2A} Expression**

As in the renal cortical tubules, in immortalized RPTC of WKY, PP\textsubscript{2A}-B56\alpha protein level was greater in cytosol (WKY, 41.5±3.4 DU, n=4) than in membrane fractions (WKY,
D1-like antagonist SCH23390. Results are expressed as mean±SEM.  

*P<0.05, WKY vs SHR;  

†P<0.05, ANOVA for repeated measures, Scheffé’s test (vs baseline). C, Effect of D1-like agonist fenoldopam (5 μmol/L) in immortalized RPTC from WKY, fenoldopam decreased PP2A activity in both cytosol and membrane fractions in a time-dependent manner; it slightly but significantly increased activity of RPTC from SHR in time-dependent manner. Data for SHR are not included because no changes were noted (n=3). Results are expressed as mean±SEM.  

Figure 3. A, Time course of effect of D1-like agonist fenoldopam on B56α protein expression in immortalized RPTC from WKY. B56α protein expression decreased in cytosol and increased in membrane of RPTC from WKY (n=4) rats in time-dependent manner. Data for SHR are not included because no changes were noted (n=3). Results are expressed as mean±SEM.  

Effect of D1-Like Agonist  

In membranes from immortalized RPTC of WKY, the D1-like agonist fenoldopam (5 μmol/L) increased PP2A-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 PP2A-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 PP2A-B56α expression in either membrane or cytosol in immortalized RPTC of SHR (data not shown).  

PP2A Activity  

Basal Activity  

In the absence of endogenous dopamine, basal PP2A activity (pmol/min per milligram of protein) in the membrane was markedly higher in immortalized RPTC of SHR (540±63 pmol/min/mg protein) than in immortalized RPTC of WKY (233±48 pmol/min/mg protein) (P<0.05, t test). However, PP2A activity in cytosol was not different between SHR (496±36 pmol/min/mg protein, n=3) and WKY (473±36 pmol/min/mg protein, n=4).  

Effect of D1-Like Antagonist SCH23390  

In immortalized RPTC membranes from WKY, fenoldopam (5 μmol/L) increased PP2A activity (pmol/min/mg protein) with time, peaking at 30 minutes to 278±21 pmol/min/mg protein (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 PP2A 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 pmol/min/mg protein (n=3 and 428±50 pmol/min/mg protein (n=3), P<0.05 versus basal activity).  

Effect of D1-Like Antagonist SCH23390  

To determine if the effect of the D1 agonist fenoldopam is mediated by the occupation of the D1 receptor, studies were performed in the presence of the D1-like antagonist SCH23390 (5 μmol/L), a neutral antagonist. Fenoldopam (5 μmol/L, 30 minutes) increased PP2A 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 PP2A 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 PP2A 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 PP2A. PP2A is generally considered as a cytosolic protein. However, PP2A is also expressed in cell particulate fractions. We also found that PP2A protein level was much higher in the cytosol than in membranes from renal cortex and immortalized RPTC in both WKY and SHR. However, PP2A specific activity was not different in cytosol and membrane fractions.

In addition to GPCR resensitization, PP2A 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 PP2A function is conferred by a variety of targeting/regulatory subunits. Each of the B subunits consists of numerous isoforms and splice variants. The function of PP2A-B56a in hypertension remains to be determined. However, the failure of the D1-like agonist to translocate PP2A activity from cytosol to membrane in SHR may explain, in part, the “hyper”-serine-phosphorylated D1 receptor in renal membranes in genetic hypertension. The finding of a difference in PP2A activity between WKY and SHR in RPTC, where dopamine is present, suggests that dopamine may tonically inhibit the PP2A activity, especially in the cytosol. Because PP2A-B56a is important in the targeting of the PP2A holoenzyme, a defect in B56a 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 PP2A activity in RPTC membranes between WKY and SHR. The D1-like agonist fenoldopam increased membranous PP2A activity in WKY but decreased it in SHR. Fenoldopam translocated PP2A activity from cytosol to the membrane in WKY but not in SHR. We also found that the PP2A regulatory subunit B56a exists in the BBM, where most of the D1 receptors are located.

In conclusion, we have demonstrated that after D1-like agonist activation, PP2A 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 PP2A in this response, the role of other phosphatases, for example, PP2B, in the desensitization of D1-like receptors in hypertension, remains to be determined.

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