Renal Protein Phosphatase 2A Activity and Spontaneous Hypertension in Rats

Peiying Yu, Laureano D. Asico, Gilbert M. Eisner, Ulrich Hopfer, Robin A. Felder, Pedro A. Jose

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 activity and effects were greater in cytosol than in membrane fractions in both WKY and SHR. Although PP₂A expression was 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 receptors, dopamine activity in 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/effecter 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.
B' or B56, and B'' or PR72.16 B56 is a new gene family that encodes B56α, β, γ, δ, and ε proteins.17 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.17–20 Of the B56 isoforms, B56α appears to be the most abundantly expressed subunit in the kidney.17,19,21

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 (RPTC22; RPTC cannot synthesize dopamine in the absence of L-DOPA.23 Here we report that PP2A activity and expression of the PP2A 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, 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). Materials

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.22 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.26 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 al28 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 (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).

In Vivo Studies

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.29 In WKY rats, PP2A

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.3 Therefore, PP2A expression and activity were studied in the renal cortex.

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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 6% laboratory chow and 5% water, were used. 4) (Taconic Farms Inc), maintained on standard

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.

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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). PP2A-B56α expression was greater in BBM relative to PP2A-Aα and PP2A-C. PP2A-B56α was chosen for study because this regulatory protein is ubiquitously expressed and exists as heterotrimer with the PP2A core dimer PP2A-Aα and PP2A-C.30 In addition, antibodies to PP2A-B56 other than PP2A-B56α were not commercially available. PP2A-B55 was not detected in our samples, in agreement with published reports.31

**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 not affect PP2A-B56α expression in renal cortical homogenates in either WKY or SHR (data not shown). Fenoldopam increased PP2A-B56α 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).

**PP2A Activity**

The majority of PP2A are heterotrimers that contain a variable regulatory B subunit bound to the A-C dimer.16,33-35 To determine specific PP2A activity, we measured PP2A activity in immunoprecipitates of the specific monoclonal anti-PP2A-C subunit antibody.28,36 The protein phosphatase activity on the immune complex was predominantly PP2A, because the A subunit and B56α of PP2A were also coimmunoprecipitated with anti-C (data not shown), and the activity was inhibited dose-dependently by a PP2A inhibitor, okadaic acid (∼50%, data not shown).

**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 30 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 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, n=3) than in immortalized RPTC of WKY (233±21, n=3) (\( P<0.05 \), t test). However, PP2A activity in cytosol was not different between SHR (496±36, n=3) and WKY (473±95, n=4).

**Effect of D1-Like Agonist**

In immortalized RPTC membranes from WKY, fenoldopam (5 μmol/L) increased PP2A activity (pmol/min per milligram of protein) with time, peaking at 30 minutes to 278±21 (\( P<0.05 \) versus basal activity) (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 in membrane (n=3) and 428±30 in cytosol (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 (Figure 3C) 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.\textsuperscript{12–14,36,39,40} Moreover, the resensitization of GPCR was inhibited by phosphatase inhibitors.\textsuperscript{12–14,36,39,40} GRP has been identified as a member of the family of PP\textsubscript{2A}.

PP\textsubscript{2A} is generally considered as a cytosolic protein.\textsuperscript{33} However, PP\textsubscript{2A} is also expressed in cell particulate fractions.\textsuperscript{12} We also found that PP\textsubscript{2A} protein level was much higher in the cytosol than in membranes from renal cortex and immortalized RPTC in both WKY and SHR. However, PP\textsubscript{2A} specific activity was not different in cytosol and membrane fractions.

In addition to GPCR resensitization, PP\textsubscript{2A} is involved in a broad range of cellular processes, including signal transduction, intermediary metabolism, transcriptional regulation and control of DNA replication, and mitosis.\textsuperscript{16,33,42} The diversity of PP\textsubscript{2A} function is conferred by a variety of targeting/regulatory subunits. Each of the B subunits consists of numerous isoforms and splice variants.\textsuperscript{16,33,42} The PP\textsubscript{2A} 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.\textsuperscript{17,30} Most of the B56 family members are phosphoproteins, and different isoforms have different targeting functions.\textsuperscript{17,30} The function of PP\textsubscript{2A}-B56α in hypertension remains to be determined. However, the failure of the D\textsubscript{1}-like agonist to translocate PP\textsubscript{2A} activity from cytosol to membrane in SHR may explain, in part, the “hyper-serine-phosphorylated D\textsubscript{1} receptor in renal membranes in genetic hypertension.”\textsuperscript{8,9} The finding of a difference in PP\textsubscript{2A} activity between WKY and SHR in RPTC, where dopamine is absent, but not in renal cortical tubules, where dopamine is present, suggests that dopamine may tonically inhibit the PP\textsubscript{2A} activity, especially in the cytosol. Because PP\textsubscript{2A}-B56α is important in the targeting of the PP\textsubscript{2A} 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 D\textsubscript{1}-like agonist–associated PP\textsubscript{2A} activity in RPTC membranes between WKY and SHR. The D\textsubscript{1}-like agonist fenoldopam increased mem- branous PP\textsubscript{2A} activity in WKY but decreased it in SHR. Fenoldopam translocated PP\textsubscript{2A} activity from cytosol to the membrane in WKY but not in SHR. We also found that the PP\textsubscript{2A} regulatory subunit B56α exists in the BBM, where most of the D\textsubscript{1} receptors are located.\textsuperscript{43,44} It is of interest that PP\textsubscript{2A}-B56α protein level decreased in the BBM from SHR after fenoldopam treatment. The ability of the D\textsubscript{1}-like agonist to block both the increase in PP\textsubscript{2A} activity in WKY and the opposite response in SHR support the notion of an abnormality downstream of the D\textsubscript{1}-like receptor. Because there is no mutation of the D\textsubscript{1} receptor in the SHR, this finding suggests that a primary abnormality of PP\textsubscript{2A}-B56α may be a cause of the discrepant response of SHR compared with WKY.

Activation of PP\textsubscript{2A} necessitates prior phosphorylation of the D\textsubscript{1}-like receptor.\textsuperscript{5–8,41} Presumably, activation of a G-protein–coupled receptor kinase (GRK) must have occurred after D\textsubscript{1}-like agonist stimulation. The phosphorylation of the D\textsubscript{1} receptor by GRK by GRK2, GRK3, and GRK5 has been reported to play a role in the desensitization of the D\textsubscript{1} receptor.\textsuperscript{45} Increased activity and expression of GRK2 has been reported to be constitutively activated in the hypertension.\textsuperscript{46} Because the increased phosphorylation of the D\textsubscript{1} receptor in renal proximal tubules is ligand independent,\textsuperscript{2,3,8,9,38} 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.\textsuperscript{47} We speculate that a constitutively activated GRK (eg, GRK6) causes basal phosphorylation of D\textsubscript{1}-like receptors in renal proximal tubules in both WKY and SHR. D\textsubscript{1}-like agonist occupation further increases the phosphorylation of renal D\textsubscript{1}-like receptors in WKY but not in SHR.\textsuperscript{9} PP\textsubscript{2A} dephosphorylates and resensitizes renal D\textsubscript{1}-like receptors in WKY but not in SHR, resulting in a phosphorylated and desensitized D\textsubscript{1}-like receptor in genetic hypertension.

In conclusion, we have demonstrated that after D\textsubscript{1}-like agonist activation, PP\textsubscript{2A} activity in renal proximal tubules is increased in WKY but decreased in SHR. We speculate that the increased basal levels of serine-phosphorylated D\textsubscript{1} receptor in RPTC in hypertension may be a consequence of a defect in dephosphorylation of the phosphorylated D\textsubscript{1} receptor. Although we have demonstrated a role of PP\textsubscript{2A} in this response, the role of other phosphatases, for example, PP\textsubscript{2B}, in the desensitization of D\textsubscript{1}-like receptors in hypertension, remains to be determined.\textsuperscript{36}


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