

Inhibition of D₄ Dopamine Receptors on Insulin Receptor Expression and Effect in Renal Proximal Tubule Cells

Ye Zhang, MD, PhD;* Hongmei Ren, BS;* Xi Lu, MS; Duofen He, BS; Yu Han, BS; Hongyong Wang, MD, PhD; Chunyu Zeng, MD, PhD; Weibin Shi, MD, PhD

Background—Ion transport in the renal proximal tubule (RPT), which is increased in essential hypertension, is regulated by numerous hormones and humoral factors, including insulin and dopamine. Activation of dopamine receptor inhibits sodium reabsorption, whereas activation of insulin receptor increases sodium reabsorption in RPTs, and hyperinsulinemic animals and patients have defective renal dopaminergic system. We presume that there is an inhibition of D₄ receptor on insulin receptor expression and effect, and the regulation is lost in spontaneously hypertensive rats (SHRs).

Methods and Results—Insulin receptor expression was determined by immunoblotting, and Na⁺-K⁺-ATPase activity was detected in both Wistar-Kyoto (WKY) and SHR RPT cells. Stimulation of D₄ receptor with PD168077 decreased expression of insulin receptors, which was blocked in the presence of the calcium-channel blocker, nifedipine (10⁻⁶ mol/L per 24 hours), in cell culture medium without calcium or in the presence of inositol 1,4,5-trisphosphate (IP3) receptor blocker (2-aminoethyl diphenylborinate [2-ADB]; 10⁻⁶ mol/L per 24 hours), indicating that extracellular calcium entry and calcium release from the endoplasmic reticulum were involved in the signal pathway. Stimulation of the insulin receptor stimulated Na⁺-K⁺-ATPase activity, whereas pretreatment with PD168077 for 24 hours decreased the inhibitory effects of insulin receptor on Na⁺-K⁺-ATPase activity in WKY cells. However, in SHR cells, inhibition of D₄ receptor on insulin receptor expression and effect were lost.

Conclusions—Activation of D₄ receptor inhibits insulin receptor expression in RPT cells from WKY rats. The aberrant inhibition of D₄ receptor on insulin receptor expression and effect might be involved in the pathogenesis of essential hypertension. (*J Am Heart Assoc.* 2016;5:e002448 doi: 10.1161/JAHA.115.002448)

Key Words: dopamine receptor • hypertension • insulin • renal proximal tubule cells

Essential hypertension affects 25% of the adult population.¹ Ion transport in the renal proximal tubule (RPT) is increased in essential hypertension, which is regulated by numerous hormones and humoral factors, including insulin and dopamine.^{2,3} Activation of dopamine receptor inhibits sodium reabsorption, whereas activation of insulin receptor increases sodium reabsorption in RPTs.^{3,4} Insulin has various effects. Besides its action on glucose metabolism, insulin

affects blood pressure by regulation of vascular smooth muscle cellular proliferation, as well as renal salt and water excretion.⁴⁻⁷ Diabetes mellitus is associated with sodium and water retention.⁸ There are evidences indicating that insulin, through stimulation of Na⁺/H⁺ exchange and Na⁺-K⁺-ATPase activities directly, increases volume absorption in renal proximal tubules.⁶ Dopamine is an endogenous catecholamine that modulates many cellular activities, including blood pressure and transmembrane ion transport.³ Dopamine receptors are classified into the D₁-like and D₂-like subtypes based on their structure and pharmacology. D₁-like receptors, comprised of D₁ and D₅ receptors, stimulate adenylyl cyclase activity, whereas D₂-like receptors, comprised of D₂, D₃, and D₄ receptors, inhibit adenylyl cyclase activity, and regulate/modulate activity of several ion channels.³ Activation of D₁-like or D₂-like receptor induces natriuresis and diuresis.⁹

Increasing pieces of evidence show interactions between insulin and dopamine receptors.¹⁰⁻¹³ Hyperinsulinemic animals and patients have a defective renal dopaminergic system¹⁰; activation of dopamine receptor ameliorates insulin levels in obese humans and improves peripheral insulin sensitivity and renal function in streptozotocin (STZ)-induced type 2 diabetic

From the Department of Cardiology, Daping Hospital, The Third Military Medical University, Chongqing, China (Y.Z., H.R., X.L., D.H., Y.H., H.W., C.Z., W.S.); Chongqing Institute of Cardiology, Chongqing, China (Y.Z., H.R., X.L., D.H., Y.H., H.W., C.Z., W.S.).

*Dr Zhang and Ms Ren are co-first authors.

Correspondence to: Chunyu Zeng, MD, PhD, or Weibin Shi, MD, PhD, Department of Cardiology, Daping Hospital, The Third Military Medical University, Chongqing, China. E-mails: chunyu.zeng01@163.com; stonewb@163.com

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rats.^{11,12} Because both D₄ receptor and insulin receptor exist in the RPTs,¹³ we presume that activation of D₄ receptor inhibits insulin receptor expression and effect, which is aberrant in hypertensive states. In order to test the above hypotheses, we studied D₄ and insulin receptor interaction in immortalized RPT cells from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). These RPT cells behave similarly to freshly obtained RPT cells, at least with regard to dopamine receptors and responses to G-protein stimulation.^{14,15}

Methods

Cell Culture and Sample Preparation

Immortalized RPT cells from microdissected S₁ segments of proximal tubules of 4- to 8-week-old WKY and SHRs were cultured at 37°C in a 95% air/5% CO₂ atmosphere in DMEM/F-12 with transferrin (5 µg/mL), insulin (5 µg/mL), epidermal growth factor (10 ng/mL), dexamethasone (4 µg/mL), and FBS 5% on a 100-mm Petri dish.^{16,17} All animals in this study were handled in accord with the Guide for the Care and Use of Laboratory Animals of the Nation.

Cells were made quiescent by incubation for 2 hours in media without FBS before addition of reagents. Cells (80% confluence) were extracted in ice-cold lysis buffer, sonicated, kept on ice for 1 hour, and centrifuged at 16 000g for 30 minutes. All samples were stored at -70°C until use.

Immunoblotting

Insulin receptor antibody is polyclonal rabbit antihuman antipeptide (Santa Cruz Biotechnology, Santa Cruz, CA).¹⁸ Goat polyclonal p-insulin Rβ antibody (Santa Cruz Biotechnology) was used as the phosphorylated insulin receptor antibody. RPT cells were treated with vehicle (dH₂O), a D₄ receptor agonist (PD168077; Tocris Cookson Ltd, Bristol, UK),¹⁹ or ABT724 (2-[[4-pyridin-2-ylpiperazin-1-yl)methyl]-1H-benzimidazole trihydrochloride; Sigma-Aldrich, St. Louis, MO) or a D₄ receptor antagonist²⁰ (L-745870 [Tocris] or L750667 [Sigma-Aldrich]) at the indicated concentrations and times as described previously.²¹ Transblots were probed with insulin receptor antibody (1:400). The amount of protein transferred onto membranes was determined by immunoblotting for α-actin.

Measurement of Intracellular Calcium Concentration

Twenty-four hours before the experiments, cells were harvested and seeded into 7.5-cm² Petri dishes (Falcon, Franklin Lakes, NJ). Cells were loaded with the calcium indicator, Fura-2AM (5 µmol/L), in HEPES buffered saline. Changes in intracellular calcium ([Ca²⁺]_i) in individual cells were measured

using an Aquacosmos system with band-pass filters for 340 and 380 nm. [Ca²⁺]_i was calculated from the Fura-2 fluorescence ratio (F340/F380) using linear regression between adjacent points on a calibration curve generated by measuring F340/F380 in at least 7 calibration solutions containing [Ca²⁺]_i between 0 and 854 nmol/L. Ca²⁺ concentration in individual groups were measured as previously described.²²

Determination of the Second Messenger(s) Involved in Regulation of D₄ Receptor on Insulin Receptor Expression in WKY Cells

To determine the second messenger(s) involved in the regulation of D₄ on insulin receptor expression in WKY cells, several inhibitors or agonists were used: protein kinase C (PKC) inhibitor (PKC inhibitor 19–31, 10⁻⁶ mol/L)²³; protein kinase A (PKA) inhibitor (PKA inhibitor 14–22, 10⁻⁶ mol/L)²⁴; PKC activator (PMA, 10⁻⁷ mol/L)²⁵; PKA activator (Sp-cAMPs, 10⁻⁷ mol/L)²⁶; calcium-channel blocker (nicardipine, 10⁻⁶ mol/L)²¹; and calcium-channel agonist (BAY-K8644, 10⁻⁶ mol/L).²⁷ Those reagents were added into the cell incubation medium 15 minutes before PD168077 treatment.

PKC inhibitor 19 to 31, PMA, Sp-cAMPs, nicardipine, and BAY-K8644 were purchased from Sigma-Aldrich. PKA inhibitor 14 to 22 was purchased from Calbiochem Company (Darmstadt, Germany).

Real-Time Polymerase Chain Reaction Detecting System of Insulin Receptors

A total of 2 to 3 µg of total RNA extracted from WKY and SHR cells were used to synthesize cDNA, which served as the template for amplification of receptor and β-actin (as a housekeeping gene). For β-actin, the forward primer was 5'-GTGGGTATGGGTCAGAAGGA-3' and the reverse primer was 5'-AGCGCGTAACCCTCATAGAT-3' (GeneBank Accession No.: NM031144). For insulin receptor, the forward primer was 5'-TTCAGGAAGACCTTCGAGGATTACCTGCAC-3' and the reverse primer was 5'-AGGCCAGAGATGACAAGTGACTCCTTGT-3' (GeneBank Accession No.: X57764). Amplification was performed with the following conditions: denaturation at 95°C for 3 minutes, followed by 35 cycles at 95°C for 10 seconds and 60°C for 30 seconds. At the end of each run, a melting curve analysis was performed from 65°C to 95°C to monitor primer dimers or nonspecific product formation. Reactions were performed in triplicate. Insulin receptor mRNA expression was normalized for β-actin mRNA.¹⁸

siRNA

siRNA against D₄ receptor mRNA and its control scrambled RNA were synthesized and purified with reverse-phase

high-performance liquid chromatography as 25-mer phospho-thioate-modified oligodeoxynucleotides (D₄ receptor siRNA sequence 5'-AAGGACCUCAAUGAAUAUGAAGAUAdT-3'; scrambled RNA sequence: 5'-TGACGATAAGAACAATAACdT-3'). Cells were grown in 6-well plates until 60% confluence, and 50 nmol/L of siRNA or control RNA were mixed with 6 μL of Oligofectamine in Opti-MEM medium (Invitrogen Life Technologies, Carlsbad, CA) and incubated for 24 hours, then switched to growth medium and incubated for another 24 hours. Cells were collected and processed for reverse-transcription polymerase chain reaction for D₄ receptor to determine the efficiency of siRNA-induced D₄ receptor gene silencing.

Na⁺-K⁺-ATPase Activity Assay

Na⁺-K⁺-ATPase activity was determined as the rate of inorganic phosphate released in the presence or absence of ouabain.^{28,29} Rat RPT cells were treated with vehicle (dH₂O) and D₄ receptor agonist (PD168077) at the indicated concentrations and durations of incubation. To prepare membranes for Na⁺-K⁺-ATPase activity assay, RPT cells cultured in 21-cm² plastic culture dishes were collected and centrifuged at 3000g for 10 minutes. Cells were then placed on ice and lysed in 2 mL of lysis buffer (1 mmol/L of NaHCO₃, 2 mmol/L of CaCl₂ and 5 mmol/L of MgCl₂). Cellular lysates were centrifuged at 3000g for 2 minutes to remove intact cells, debris, and nuclei. The resulting supernatant was suspended in an equal volume of 1 mol/L of sodium iodide, and the mixture was centrifuged at 48 000g for 25 minutes. The pellet (membrane fraction) was washed 2 times and suspended in 10 mmol/L of Tris containing 1 mmol/L of EDTA (pH 7.4). Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA) and adjusted to 1 mg/mL. Membranes were stored at -70°C until further use.

To measure Na⁺-K⁺-ATPase activity, 100-μL aliquots of membrane fraction were added to an 800-μL reaction mixture (75 mmol/L of NaCl, 5 mmol/L of KCl, 5 mmol/L of MgCl₂, 6 mmol/L of sodium azide, 1 mmol/L of Na₄EGTA, 37.5 mmol/L of imidazole, 75 mmol/L of Tris HCl, and 30 mmol/L of histidine; pH 7.4) with or without 1 mmol/L of ouabain (final volume=1 mL) and preincubated for 5 minutes in a water bath at 37°C. Reactions were initiated by adding Tris-ATP (4 mmol/L) and terminated after 15 minutes of incubation at 37°C by adding 50 μL of 50% trichloroacetate. For determination of ouabain-insensitive ATPase activity, NaCl and KCl were omitted from the reaction mixtures containing ouabain. To quantify the amount of phosphate produced, 1 mL of coloring reagent (10% ammonium molybdate in 10 N of sulfuric acid+ferrous sulfate) was added to the reaction mixture. The mixture was then mixed thoroughly and

centrifuged at 3000g for 10 minutes. Formation of phosphomolybdate was determined spectrophotometrically at 740 nm, against a standard curve prepared from K₂HPO₄. Na⁺-K⁺-ATPase activity was estimated as the difference between total and ouabain-insensitive ATPase activity and expressed as nmol phosphate released per mg protein per minute.

To eliminate the function of proteases and phosphatases, protease inhibitors (1 mmol/L of phenylmethylsulfonyl fluoride, 10 μg/mL of each leupeptin and aprotinin) and a phosphatase inhibitor (50 μmol/L of sodium orthovanadate) were added in all solutions used after drug/vehicle incubations.

Statistical Analysis

Data are expressed as mean±SEM. Comparison within groups was made by repeated-measures ANOVA (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 factorial ANOVA with Holm-Sidak test. A value of *P*<0.05 was considered significant.

Results

Activation of D₄ Receptor Decreases Insulin Receptor Expression in WKY RPT Cells

Stimulation of D₄ receptor with a D₄ agonist, PD168077, decreased insulin receptor expression in a concentration-dependent (10⁻⁹-10⁻⁵ mol/L) and time-dependent (2-30 hours) manner in WKY RPT cells. The inhibitory effect was evident at 10⁻⁸ mol/L, noted as early as 8 hours, and maintained for at least 30 hours (Figure 1A and 1B).

The specificity of PD168077 as a D₄ receptor agonist was also determined by studying the effect of the D₄ receptor antagonist, L745870. Consistent with the study shown in Figure 1A and 1B, PD168077 (10⁻⁷ mol/L per 24 hours), decreased insulin receptor expression. The D₄ receptor antagonist, L745870 (10⁻⁶ mol/L), by itself, had no effect on insulin receptor expression, but reversed the inhibitory effect of PD168077 on insulin receptor expression (Figure 1C).

To further confirm the regulation of D₄ receptor on insulin receptor expression, we used another D₄ receptor agonist (ABT724) and antagonist (L750667). Consistent with Figure 1A through 1C, the inhibitory effect of ABT724 (10⁻⁷ mol/L per 24 hours) was blocked by L750667 (10⁻⁶ mol/L per 24 hours) (Figure 1D and 1E). We also checked the regulation of D₄ receptor on insulin expression in the presence of D₄ receptor siRNA. Transfection with the D₄ receptor siRNA on WKY cells (50 nm, 24 hours) significantly decreased D₄ receptor expression and blocked the inhibitory

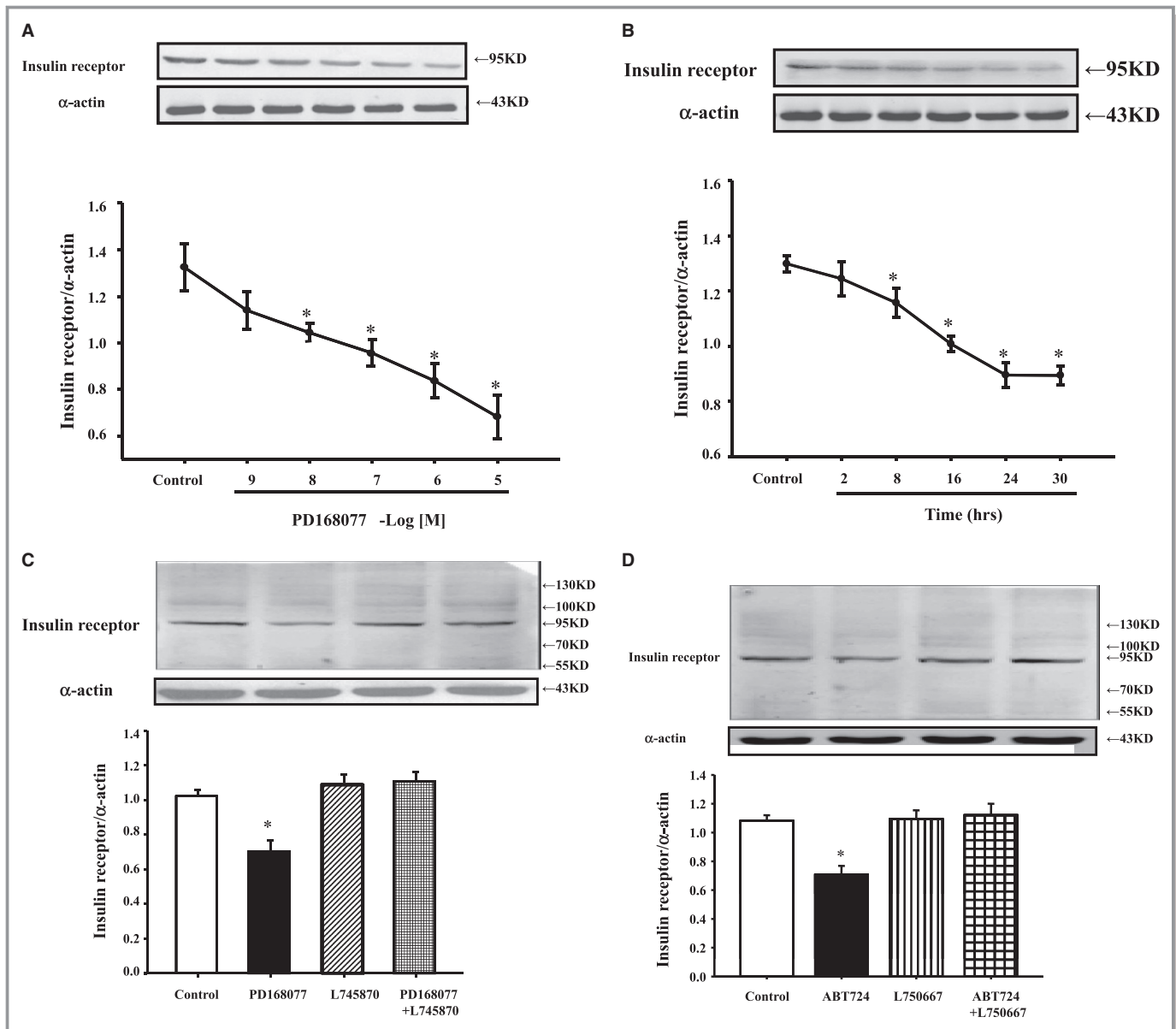


Figure 1. Effect of D₄ receptor on insulin receptor expression in Wistar-Kyoto (WKY) renal proximal tubule (RPT) cells. A, Concentration response of insulin receptor protein expression in WKY cells treated with different concentrations of the D₄ receptor agonist, PD168077, for 24 hours. Results are expressed as the ratio of insulin receptor and α-actin densities (n=6; *P<0.05 vs control). B, Time course of insulin receptor protein expression in WKY cells treated with D₄ receptor agonist, PD168077 (10⁻⁷ mol/L) for different periods. Results are expressed as the ratio of insulin receptor and α-actin densities (n=6; *P<0.05 vs control). C, Effect of a D₄ receptor agonist (PD168077) and a D₄ receptor antagonist (L745870) on insulin receptor expression in WKY cells. Cells were incubated with the indicated reagents (PD168077, 10⁻⁷ mol/L; L745870, 10⁻⁶ mol/L) for 24 hours. Results are expressed as the ratio of insulin receptor and α-actin densities (n=9; *P<0.05 vs others). D and E, Effect of a D₄ receptor agonist (ABT724) and a D₄ receptor antagonist (L750667) on insulin receptor expression in WKY cells. Cells were incubated with the indicated reagents (ABT724, 10⁻⁷ mol/L; L750667, 10⁻⁶ mol/L) for 24 hours. Results are expressed as the ratio of insulin receptor and α-actin densities (n=7; *P<0.05 vs others). F and G, Effect of D₄ receptor siRNA on regulation of D₄ receptor on insulin receptor expression in WKY cells. WKY cells were incubated with D₄ receptor siRNA (50 nm, 24 hours), D₄ receptor expressions were determined by immunoblotting (F, n=5; *P<0.05 vs others). Cells were incubated with the indicated reagents (PD168077, 10⁻⁷ mol/L) for 24 hours with or without the presence of D₄ receptor siRNA. Insulin receptor expressions were determined by immunoblotting. Results are expressed as the ratio of insulin receptor and α-actin densities (G, n=8; *P<0.05 vs others).

effect PD168077 (10⁻⁷ mol/L per 24 hours) on insulin receptor mRNA and protein expressions in WKY cells (Figure 1F and 1G).

It is reported that phosphorylation of insulin receptor will increase its function in RPT cells from both WKY and SHRs; it resulted that PD168077 (10⁻⁷ mol/L per 24 hours) inhibited

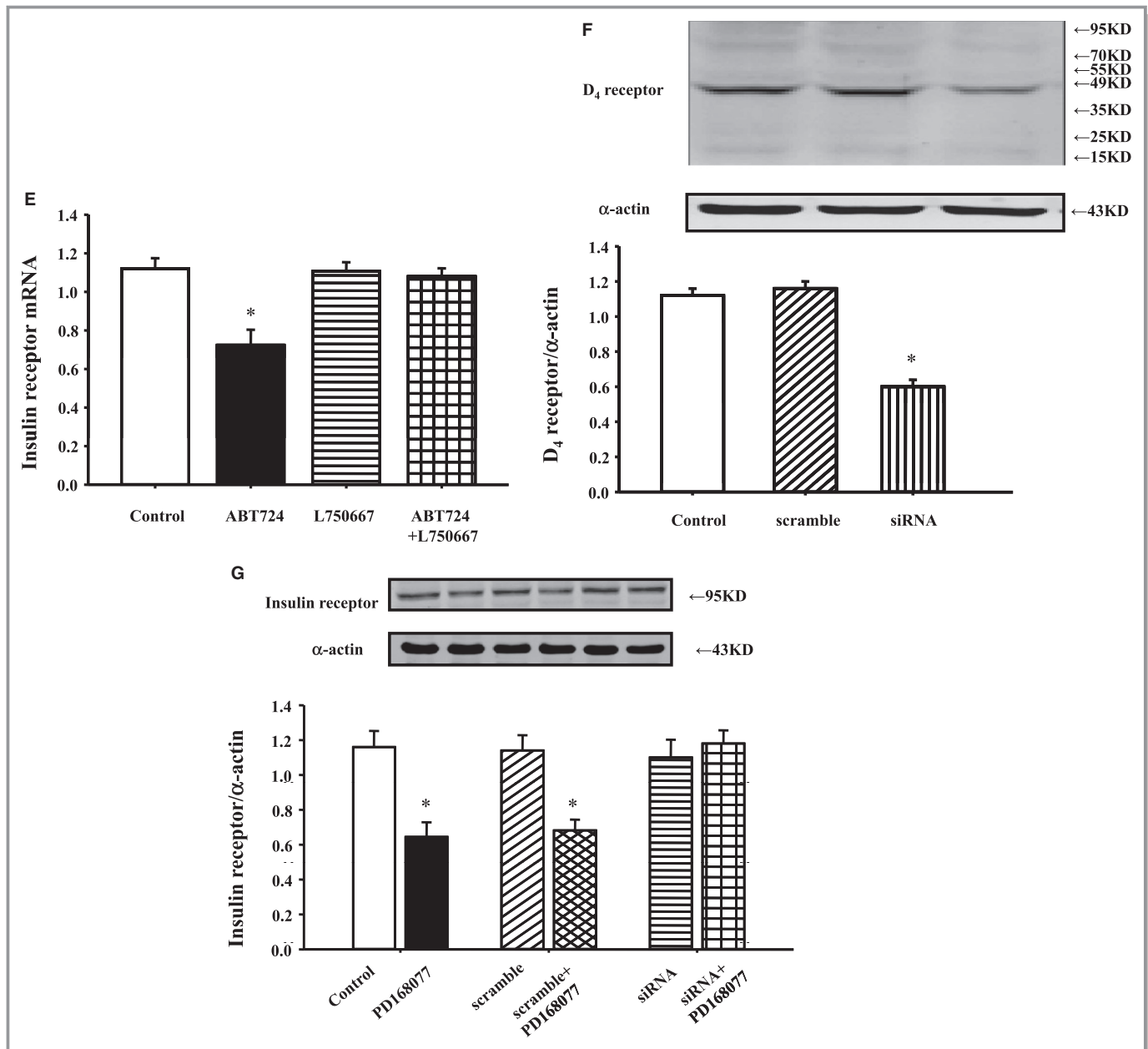


Figure 1. continued.

phosphorylation of insulin receptor in WKY cells, not in SHR cells (Figure 2A and 2B). Moreover, the D₄ receptor antagonist, L745870 (10^{-6} mol/L), by itself, had no effect on insulin receptor phosphorylation, but reversed the inhibition of PD168077 on insulin receptor phosphorylation in WKY cells (Figure 2A).

Extracellular and Intracellular Calcium Mediates the Inhibitory Effect of D₄ Receptor on Insulin Receptor Expression in RPT Cells

To investigate mechanisms of D₄ receptor induced downregulation of insulin receptor, WKY RPT cells were treated with

different agonists or antagonists. Treatment with the calcium-channel blocker, nifedipine (10^{-6} mol/L), or withdrawal of calcium from cell culture medium blocked the inhibitory effect of D₄ receptor on insulin receptor expression (Figure 3A and 3B), indicating that extracellular calcium influx was involved in the signal transduction pathway. To check whether intracellular calcium release from the endoplasmic reticulum was also involved in the mechanisms, we used inositol 1,4,5-trisphosphate (IP₃) receptor blocker (2-aminoethyl diphenyl borate [2-ADB]; 10^{-6} mol/L per 24 hours); the inhibitory effect of D₄ receptor on insulin receptor expression was abolished in the presence of IP₃ blocker (Figure 3C), indicating that both extracellular calcium entry and calcium release from the

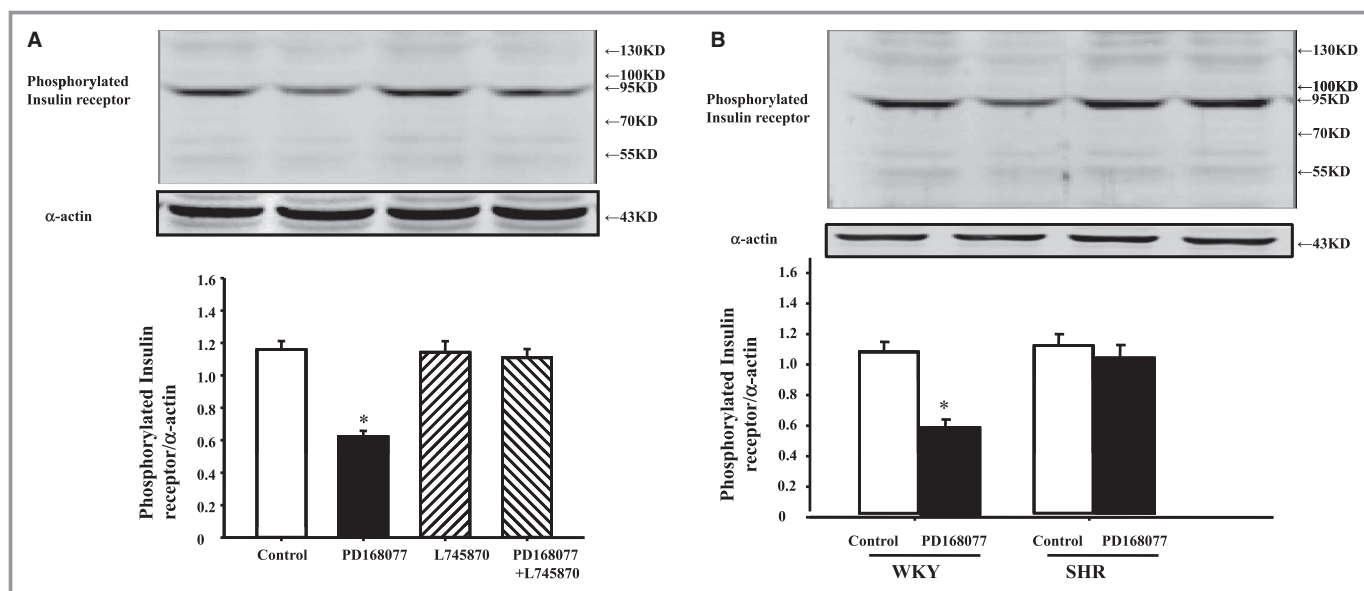


Figure 2. Effect of D₄ receptor on insulin receptor phosphorylation in Wistar-Kyoto (WKY) renal proximal tubule (RPT) cells. A, Effect of a D₄ receptor agonist (PD168077) and a D₄ receptor antagonist (L745870) on insulin receptor phosphorylation in WKY cells. Cells were incubated with the indicated reagents (PD168077, 10⁻⁷ mol/L; L745870, 10⁻⁶ mol/L) for 15 minutes. Results are expressed as the ratio of phosphorylated insulin receptor and α -actin densities (n=9; *P<0.05 vs control). B, Effect of a D₄ receptor agonist (PD168077, 10⁻⁷ mol/L per 15 minutes) on insulin receptor phosphorylation in WKY and SHR RPT cells (n=9; *P<0.05 vs control). SHR indicates spontaneously hypertensive rat.

endoplasmic reticulum were involved in the signal pathway. To further confirm the role of calcium channel and IP₃ receptor, we used calcium-channel activator BAY-K8644 (10⁻⁶ mol/L per 24 hours; Figure 3D-I) or IP₃ (10⁻⁶ mol/L per 24 hours; Figure 3D-II); it resulted that BAY-K8644 or IP₃ decreased insulin receptor expression in WKY RPT cells. The effect of PD168077 (10⁻⁷ mol/L per 15 minutes) stimulation on intracellular calcium concentration in SHR and WKY RPT cells were further studied (Figure 3E and 3F). We found that intracellular calcium concentration between SHR and WKY RPT cells have no significant difference in the basal level. PD168077 (10⁻⁷ mol/L per 15 minutes) increased intracellular calcium in WKY RPT cells, but not in SHRs. This effect was blocked by the presence of nicardipine (10⁻⁶ mol/L per 15 minutes) or 2-ADB (10⁻⁶ mol/L per 15 minutes). We also evaluated the involvement of other key cell-signaling proteins with the use of a PKA inhibitor (PKA inhibitor 14-22, 10⁻⁶ mol/L), PKC activator (PMA, 10⁻⁷ mol/L), and PKA activator (Sp-cAMPs, 10⁻⁷ mol/L). None of these reagents was able to block the inhibitory effect of insulin on D₅ receptor expression (data not shown).

D₄ Receptors Decrease Insulin Receptor Expression in WKY RPT Cells, not in SHR RPT Cells

To investigate whether there is a different regulation between WKY and SHRs, RPT cells from WKY and SHRs

were treated with PD168077 (10⁻⁷ mol/L) for 24 hours; consistent with the results in Figure 1A through 1C, activation of D₄ receptor decreased insulin receptor expression in WKY cells, but not in SHR cells (Figure 4A). We also investigated insulin receptor mRNA levels; consistent with protein results, activation of D₄ receptor with PD168077 (10⁻⁷ mol/L per 24 hours) inhibited insulin receptor mRNA expression in WKY RPT cells, not in SHR cells (Figure 4B). In the presence of the calcium-channel blocker, nicardipine (10⁻⁶ mol/L per 24 hours; Figure 4C-I), or IP₃ receptor blocker (2-; 10⁻⁶ mol/L per 24 hours; Figure 4C-II), dose regulation of D₄ receptor on insulin receptor mRNA expression was lost.

Pretreatment With D₄ Receptor Agonist Decreases the Stimulatory Effect of Insulin Receptor on Na⁺-K⁺-ATPase Activity in WKY RPT Cells, not in SHR RPT Cells

Effects of the D₄ agonist, PD168077 (10⁻⁷ mol/L per 15 minutes), on Na⁺-K⁺-ATPase activity in WKY and SHR RPT cells were explored. Activity of Na⁺-K⁺-ATPase was higher in SHR than WKY RPT cells in the basal level. PD168077 inhibited Na⁺-K⁺-ATPase activity in WKY RPT cells, but not in SHR RPT cells (Figure 5A). The inhibitory effect was disappeared 2 hours after washing with PBS (Figure 5B).

To investigate the physiological significance of D₄ receptor on insulin receptor expression, effects of insulin on Na⁺-K⁺-

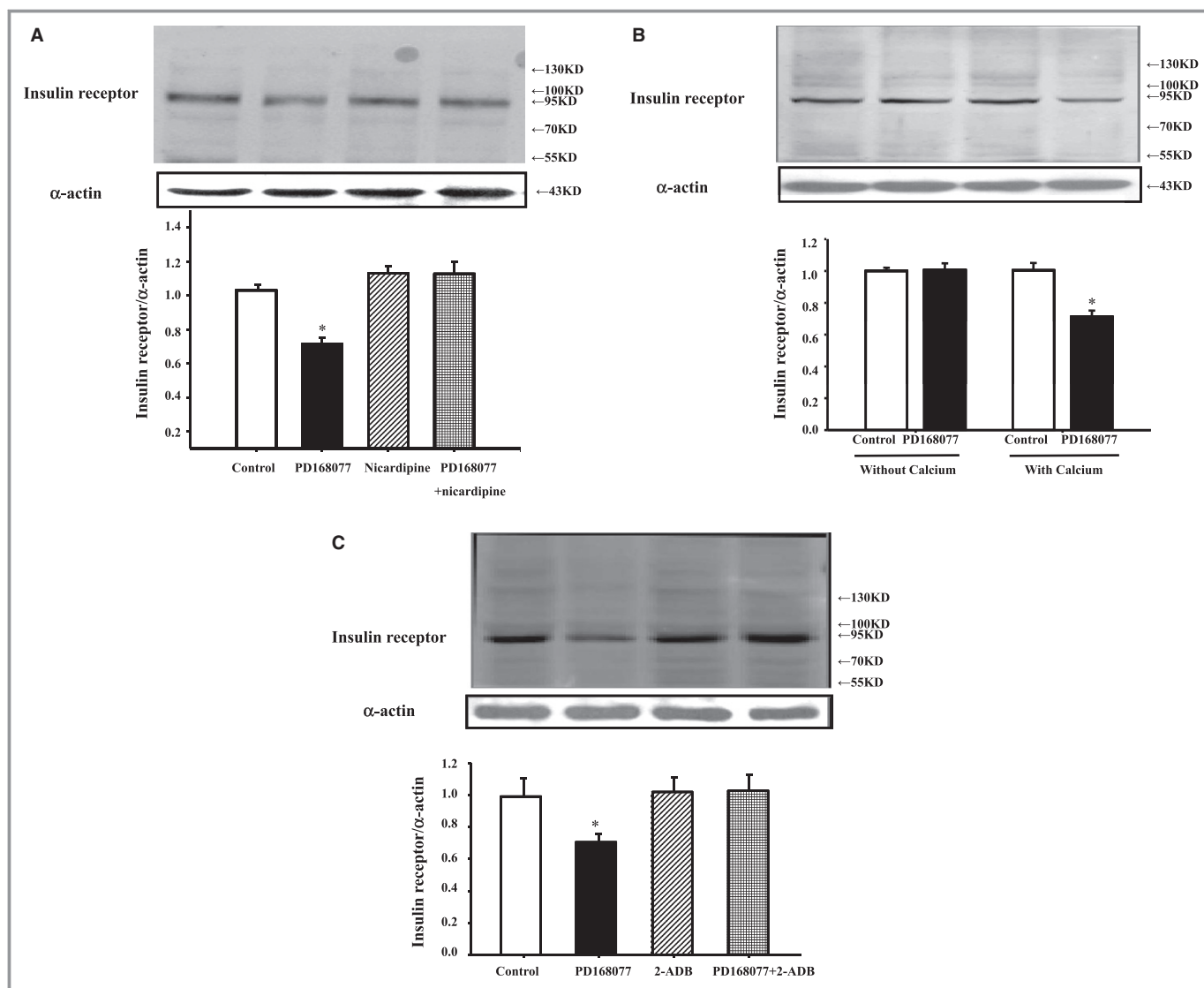


Figure 3. Extracellular and intracellular calcium mediates the inhibitory effect of D₄ receptor on insulin receptor expression in renal proximal tubule (RPT) cells. A, Effect of the D₄ receptor agonist, PD168077 (10⁻⁷ mol/L per 24 hours), and the calcium-channel blocker, nicardipine (10⁻⁶ mol/L per 24 hours), on insulin receptor protein in Wistar-Kyoto (WKY) RPT cells. Cells were incubated with the indicated reagents. Results are expressed as the ratio of insulin receptor and α -actin densities (n=6; *P<0.05 vs others). B, Effect of the D₄ receptor agonist, PD168077 (10⁻⁷ mol/L per 24 hours), on insulin receptor expression in cell culture medium with or without calcium in WKY RPT cells. Results are expressed as the ratio of insulin receptor and α -actin densities (n=6; *P<0.05 vs others). C, Effect of the D₄ receptor agonist, PD168077 (10⁻⁷ mol/L per 24 hours), and inositol 1,4,5-trisphosphate (IP₃) receptor blocker (2-aminoethoxydiphenyl borate [2-ADB]; 10⁻⁶ mol/L per 24 hours) on insulin receptor protein in WKY RPT cells. Cells were incubated with the indicated reagents. Results are expressed as the ratio of insulin receptor and α -actin densities (n=9; *P<0.05 vs others). D, Effect of calcium-channel activator BAY-K8644 (10⁻⁶ mol/L per 24 hours) (I) or IP₃ (10⁻⁶ mol/L per 24 hours) (II) on insulin receptor protein expression in WKY RPT cells. Cells were incubated with the indicated reagents. Results are expressed as the ratio of insulin receptor and α -actin densities (n=9; *P<0.05 vs others). E and F, Effect of the D₄ receptor agonist, PD168077, on intracellular calcium concentration in the presence or absence of pharmacological agents in WKY and spontaneously hypertensive rats (SHRs) cells. Intracellular calcium concentration of RPT cells with or without PD168077 (10⁻⁷ mol/L per 15 minutes) treatment were determined by laser confocal microscopy. The stimulatory effect of PD168077 on intracellular calcium concentration was also tested in the presence of nicardipine (10⁻⁶ mol/L per 15 minutes) or 2-ADB (10⁻⁶ mol/L per 15 minutes). Representative tracings are shown (E) and graphs of the data are shown (F) (Δ [Ca²⁺]_i) shows the difference in calcium concentration between 0 and 60 seconds). (n=6; *P<0.05 vs others).

ATPase activity were determined in RPT cells from WKY and SHR cells. Insulin (10⁻⁷ mol/L per 15 minutes) increased Na⁺-K⁺-ATPase activity, which was reduced by pretreatment

with the D₄ receptor agonist, PD168077 (10⁻⁷ mol/L per 24 hours), in WKY cells. However, in SHR cells, pretreatment with PD168077 had no effect on the insulin-mediated

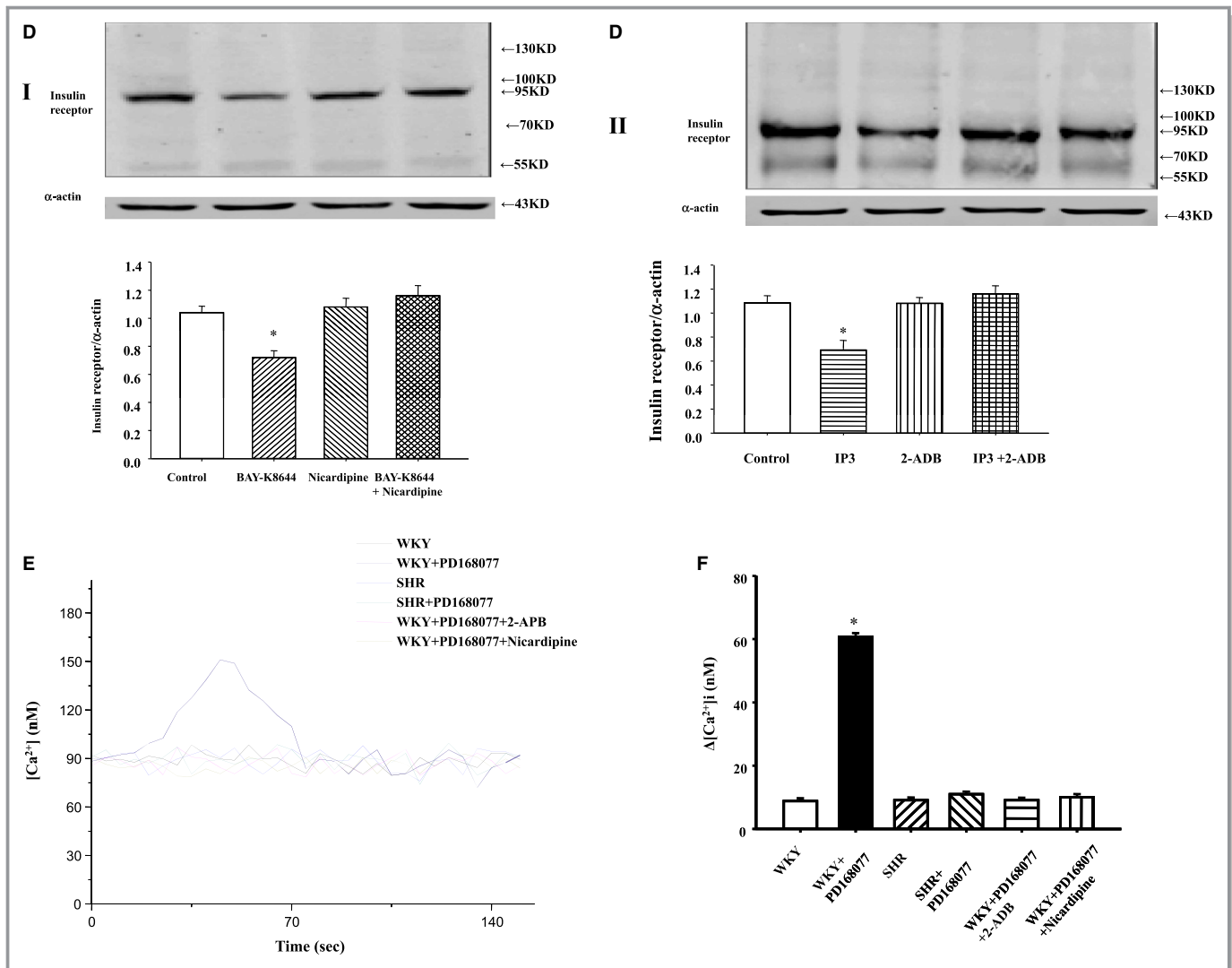


Figure 3. continued.

inhibitory effect on Na⁺-K⁺-ATPase activity in SHR cells (Figure 5C).

Discussion

Insulin receptor exists in RPTs; activation of insulin receptor induces antinatriuresis, which is supported by numerous previous studies. Early studies by Atchley et al. demonstrate that discontinuation of an insulin infusion to diabetic patients results in a brisk natriuresis.³⁰ Miller and Bogdonoff show that infusion of insulin into normal subjects results in a reduction in urinary sodium excretion.³¹ Consistent with those studies, our present study found that insulin increases Na⁺-K⁺-ATPase activity in WKY cells and SHR cells.

Dopamine receptor subtypes are expressed in specific segments of the mammalian kidney. The D₄ receptor has abundant protein expression in the proximal tubule.^{3,13}

Previous studies show interactions between insulin and dopamine receptors. Hyperinsulinemic animals and patients with noninsulin-dependent diabetes have a defective renal dopaminergic system.³² In obese Zucker rats, a model of type 2 diabetes, renal D₁ receptor are downregulated and dopamine fails to produce diuresis and natriuresis; treatment with insulin sensitizer rosiglitazone restores renal D₁ receptor effect.³³ Chronic exposure of cells to insulin causes both reduction in D₁ receptor abundance and its uncoupling from G proteins and diminishes the inhibitory effect of dopamine on Na⁺-K⁺-ATPase activity in hyperinsulinemic rats.³⁴ There are also evidences that activation of dopamine receptor influences insulin effect. Activation of D₂ receptor with bromocriptine simultaneously ameliorates insulin levels in obese humans.¹¹ Fenoldopam treatment improves peripheral insulin sensitivity and renal effect in STZ-induced type 2 diabetic rats.¹² Therefore, we hypothesize that D₄ receptor, along with

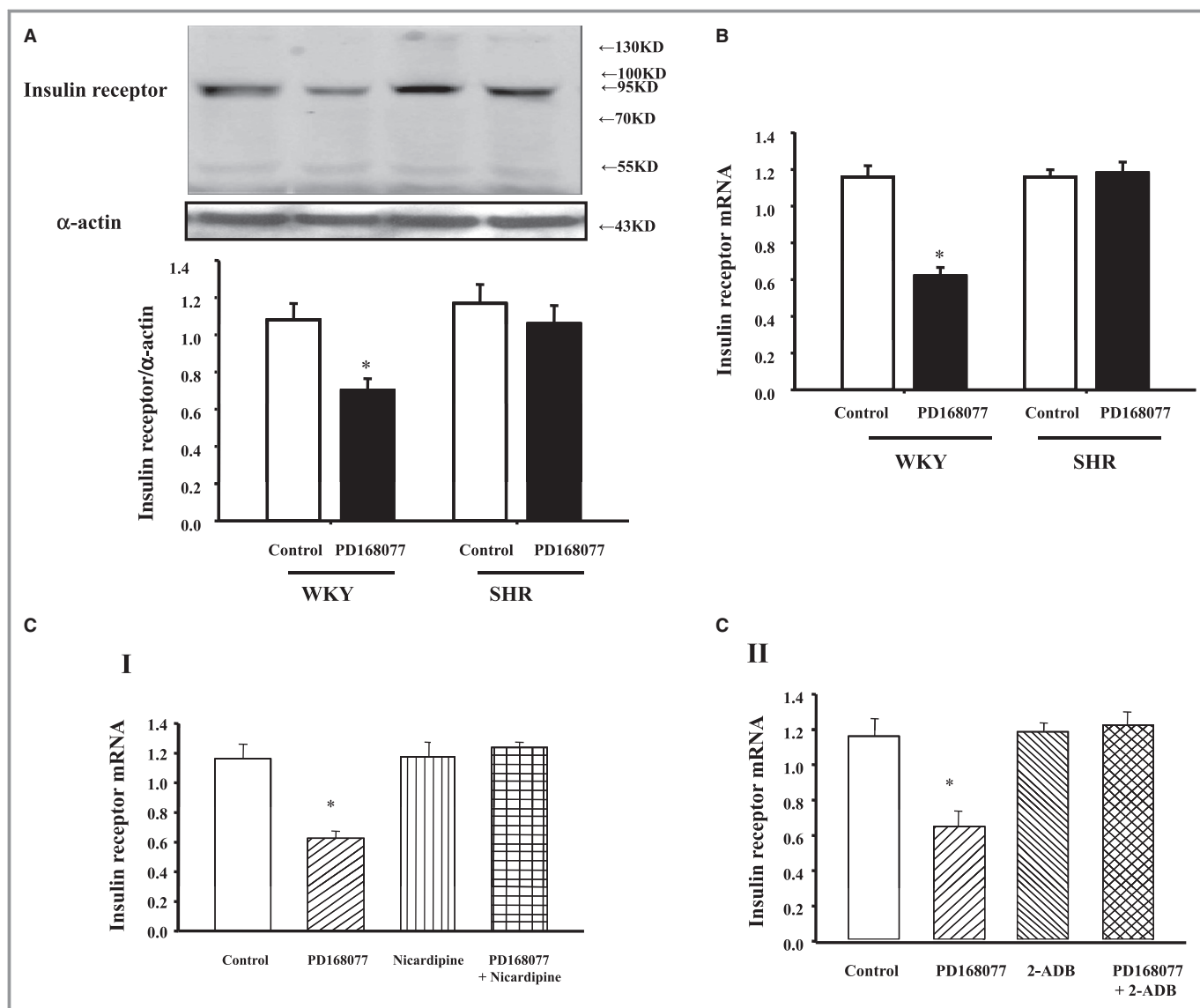


Figure 4. Differential effects of D₄ receptor on insulin receptor expression in renal proximal tubule (RPT) cells from Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs). A, Differential effects of the D₄ receptor agonist, PD168077 (10⁻⁷ mol/L per 24 hours), on insulin receptor protein expression in RPT cells from WKY and SHRs. Cells were incubated at the indicated time points and concentrations. Results are expressed as the ratio of insulin receptor and α-actin densities (n=6; *P<0.05 vs control). B, Differential effects of the D₄ receptor agonist, PD168077 (10⁻⁷ mol/L per 24 hours), on insulin receptor mRNA expression in RPT cells from WKY and SHRs. Cells were incubated at indicated time points and concentrations. Results are expressed as the ratio of insulin receptor and β-actin densities (n=9; *P<0.05 vs control). C, Effect of the D₄ receptor agonist, PD168077 (10⁻⁷ mol/L per 24 hours), in the presence of calcium-channel blocker (nicardipine, 10⁻⁶ mol/L per 24 hours) (I) or inositol 1,4,5-trisphosphate (IP3) receptor blocker (2-ADB; 10⁻⁶ mol/L per 24 hours) (II) on insulin receptor mRNA expression in WKY RPT cells. Cells were incubated with the indicated reagents. Real-time quantitative polymerase chain reaction data were analyzed using the comparative CT method (n=9; *P<0.05 vs others). CT indicates threshold cycle.

insulin receptor in RPTs, might affect insulin receptor expression and effect. This hypothesis is confirmed in this study. Stimulation of D₄ receptor inhibits insulin receptor expression in WKY cells, but not in SHRs. The inhibitory effect of D₄ receptor on insulin receptor expression has physiological significance. Stimulation with insulin increases Na⁺-K⁺-ATPase activities in WKY cells. Pretreatment with D₄ receptor

for 24 hours reduces insulin receptor expression and insulin-mediated Na⁺-K⁺-ATPase activity in WKY cells.

Insulin receptor functions as a tyrosin protein kinase. Phosphorylation of insulin receptor results in a progressive activation of its intrinsic kinase activity and actually plays an important role in regulating the function of the receptor. It is suggested that phosphorylation of the receptor is a major

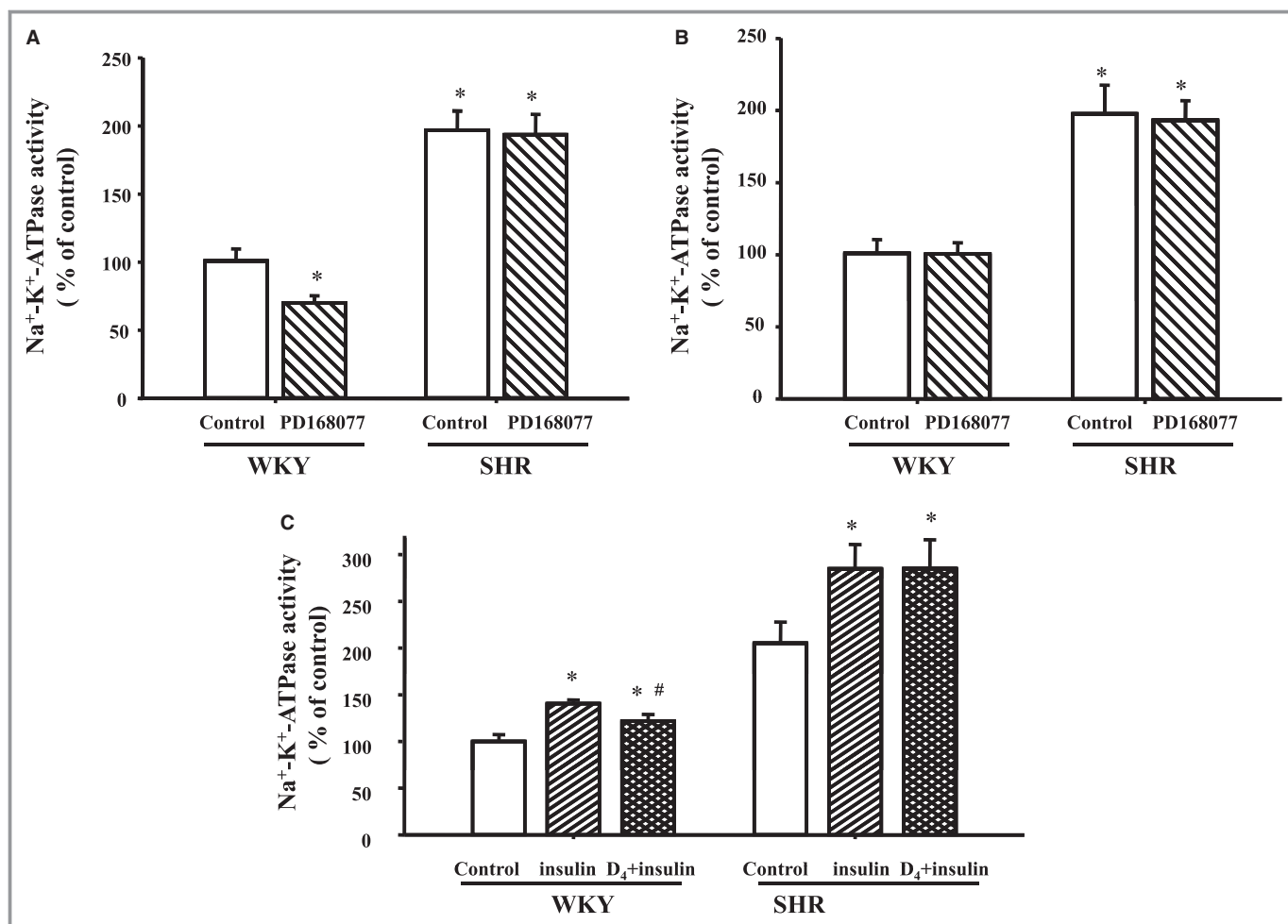


Figure 5. Effect of pretreatment with D₄ receptor on the stimulatory effect of insulin receptor on Na⁺-K⁺-ATPase activity in Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs) cells. A, Renal proximal tubule (RPT) cells from WKY and SHRs were treated with PD168077 (10⁻⁷ mol/L) or vehicle (dH₂O) for 24 hours. Na⁺-K⁺-ATPase activity were investigated in each group. Results are expressed as percent change of control (**P*<0.05 vs control; #*P*<0.05 vs insulin; n=7). B, RPT cells from WKY and SHRs were treated with PD168077 (10⁻⁷ mol/L) or vehicle (dH₂O) for 24 hours. Then, cells were washed 3 times (15 min/times) with serum-free culture medium to remove all the added PD168077 and kept in serum-free culture medium for 2 hours before Na⁺-K⁺-ATPase activity was investigated (**P*<0.05 vs control; #*P*<0.05 vs insulin; n=8). C, Cells were pretreated with PD168077 (10⁻⁷ mol/L) or vehicle (dH₂O) for 24 hours. After PD168077 pretreatment, cells were washed 3 times (15 min/times) with serum-free culture medium to remove all the added PD168077, kept in serum-free culture medium for 2 hours, and then treated with insulin (10⁻⁷ mol/L) for 15 minutes. Results are expressed as percent change of control (**P*<0.05 vs control; #*P*<0.05 vs insulin; n=8).

step in the regulation of its function that is related to kinase activity.^{35–37} We found that stimulation of D₄ receptor inhibits insulin receptor phosphorylation in WKY cells, but not in SHRs. It is in accord with the inhibition of D₄ receptor on effect of insulin receptor.

The mechanism of the decrease in insulin receptors caused by D₄ receptors is not clear. Ca²⁺ is suggested to be critical for expression of insulin receptor. DNA sequence analysis indicated that the promoter region of the gene contains several GGGCGG sequences that may be binding sites for the transcription factor, Sp1 (specificity protein 1).^{38,39} Sp1 could be upregulated by high Ca²⁺ treatment.⁴⁰ Furthermore, Ca²⁺ could stimulate phosphorylation of Sp1 and augment the activity of the gene promoter in an Sp1-binding-dependent

manner.⁴¹ Others also showed that the interactive role of cAMP regulatory element-binding protein and Sp1 is regulated by calcium.⁴² As a D₂-like receptor subtype, activation of D₄ receptor increases intracellular calcium concentration.³ The present study showed that the inhibitory effect of D₄ receptor on insulin receptor expression is blocked by a calcium-channel blocker, withdrawal of calcium in the cell culture medium, or in the presence of IP3 receptor blocker, which blocks the calcium release from endoplasmic reticulum, indicating that calcium mobilization, including extracellular calcium influx and intracellular calcium release from the endoplasmic reticulum, is involved in the signaling pathway. Besides insulin receptor protein expression, our present study found that activation of D₄ receptor decreases insulin receptor mRNA expression in

WKY cells. However, in the presence of a calcium-channel blocker or IP₃ receptor blocker, the stimulatory effect of D₄ receptor on insulin receptor mRNA and protein expression is blocked. It indicates that the regulation may occur at the transcriptional/posttranscriptional level. The detailed mechanisms remain to be elucidated in the future.

The pathogenesis of essential hypertension is complex; both genetic and environmental factors are involved. In hypertensive states, sodium reabsorption in RPTs is increased,³ which is regulated by numerous hormones and humoral factors, including insulin and dopamine. In this study, we found that activation of D₄ receptor reduces insulin receptor expression and insulin-mediated sodium reabsorption by inhibition of Na⁺-K⁺-ATPase activity in the basolateral membranes of RPTs. It might indicate that D₄ receptor is a counterbalance against insulin receptor. However, inhibition of D₄ receptor on insulin receptor expression and effect is lost in SHR, which might be involved in the pathogenesis of essential hypertension.

Conclusions

In summary, we have demonstrated that activation of D₄ receptor, by calcium mobilization from extracellular calcium influx and intracellular calcium release from the endoplasmic reticulum, decreases insulin receptor expression and insulin-mediated stimulatory effect on Na⁺-K⁺-ATPase activity in WKY cells. However, in SHR cells, inhibition of D₄ receptor on insulin receptor expression and effect is lost, suggesting that aberrant inhibition of D₄ receptor on insulin receptor expression and effect might be involved in the pathogenesis of essential hypertension.

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Disclosures

None.

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