Insulin causes renal dopamine D1 receptor desensitization via GRK2-mediated receptor phosphorylation involving phosphatidylinositol 3-kinase and protein kinase C

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Banday AA, Fazili FR, Lokhandwala MF. Insulin causes renal dopamine D1 receptor desensitization via GRK2-mediated receptor phosphorylation involving phosphatidylinositol 3-kinase and protein kinase C. Am J Physiol Renal Physiol 293: F877–F884, 2007. First published June 13, 2007; doi:10.1152/ajprenal.00184.2007.—The renal dopamine system plays an important role in sodium homeostasis and a defect in dopamine D1 receptor (D1R) function is present in hypertension, diabetes, and aging. Our previous studies in hyperinsulinemic animals and in renal cell cultures treated with insulin showed decrease in D1R number and defective coupling to G proteins; however, the exact mechanisms remained unknown. Therefore, we investigated insulin-mediated D1R desensitization and underlying molecular mechanism in opossum kidney (OK) cells. Chronic exposure (24 h) of OK cells to 10 nM insulin caused significant decrease in D1R number and agonist affinity. The D1R was hyperserine phosphorylated, uncoupled from G proteins and SKF38393, a D1R agonist, failed to stimulate G proteins and inhibit Na-K-ATPase activity. Insulin increased protein kinase C (PKC) activity and caused G protein-coupled receptor kinase 2 (GRK2) translocation to the membranes. Tyrosine kinase inhibitor genistein and protein kinase C inhibitors and siRNA restored D1R signaling, providing evidence that dopamine D1R-mediated adenylyl cyclase stimulation (1, 12, 15).

Dopamine D1R belongs to the superfamily of heptahelical receptors that modulate the activity of effectors such as adenylyl cyclase by activation of specific heterotrimeric GTP-binding proteins (G proteins) (23). The exposure of G protein-coupled receptors (GPCRs) to agonists often results in a rapid attenuation of receptor responsiveness (9, 11). This process, termed as desensitization, involves phosphorylation of receptors by GPCR kinases (GRKs) and/or by second messenger-dependent [cAMP-dependent protein kinase (PKA) or protein kinase C (PKC)] serine/threonine kinases (9, 11, 19). Homologous desensitization of GPCRs is primarily mediated by GRKs (9, 11, 19, 25). In contrast, second messenger-dependent protein kinases not only phosphorylate agonist-activated GPCRs but also phosphorylate receptors that have not been exposed to agonist (9, 11). Nevertheless, it is now recognized that agonist-independent phosphorylation cannot be ascribed only to second messenger-dependent protein kinases but GRKs are also equally involved (2, 9, 11, 24).

As with most GPCRs, the D1R is known to undergo agonist-mediated desensitization, regulated by its phosphorylated state; this in turn is mediated by GRKs (13, 14, 18, 20, 22, 31). In contrast, a recent study by Rankin et al. (24) showed that GRKs can phosphorylate D1R in absence of agonist activation resulting in constitutive desensitization. Our own studies involving obese Zucker rats and renal proximal tubular cultures exposed to insulin showed that D1R were constitutively phosphorylated and uncoupled from G proteins, and results in the failure of SKF38393 to stimulate G proteins and inhibit Na-K-ATPase activity. D1R-mediated adenylyl cyclase stimulation (1, 12, 15).

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Numerous studies show that renal dopamine via D1 receptor (D1R) activation plays a central role in renal sodium handling especially during sodium-replete state (12, 15). Dopamine produced in the renal proximal tubular cells acts in a paracrine fashion and causes an increase in urinary sodium excretion that is mainly dependent on inhibition of sodium tubular reabsorption (1, 15, 37). This natriuretic effect of dopamine is attributed mainly to the D1R-mediated inhibition of Na-K-ATPase and Na/H exchanger 3 on basolateral and brush-border membranes (1, 6). The signal transduction pathway(s) for D1R-mediated Na-K-ATPase inhibition is complex; it is reported that protein kinase C (PKC) may be involved (1, 15). On the other hand, it has been demonstrated that the inhibition of Na/H exchanger activity in intact cells as well as in brush-border vesicles involves D1R-mediated adenylyl cyclase stimulation (1, 12, 15).

In conclusion, insulin-mediated desensitization involves PI3K, PKC, and GRK2. Insulin causes renal dopamine D1 receptor desensitization via GRK2-mediated receptor phosphorylation involving phosphatidylinositol 3-kinase and protein kinase C. Am J Physiol Renal Physiol 293: F877–F884, 2007. First published June 13, 2007; doi:10.1152/ajprenal.00184.2007.—The renal dopamine system plays an important role in sodium homeostasis and a defect in dopamine D1 receptor (D1R) function is present in hypertension, diabetes, and aging. Our previous studies in hyperinsulinemic animals and in renal cell cultures treated with insulin showed decrease in D1R number and defective coupling to G proteins; however, the exact mechanisms remained unknown. Therefore, we investigated insulin-mediated D1R desensitization and underlying molecular mechanism in opossum kidney (OK) cells. Chronic exposure (24 h) of OK cells to 10 nM insulin caused significant decrease in D1R number and agonist affinity. The D1R was hyperserine phosphorylated, uncoupled from G proteins and SKF38393, a D1R agonist, failed to stimulate G proteins and inhibit Na-K-ATPase activity. Insulin increased protein kinase C (PKC) activity and caused G protein-coupled receptor kinase 2 (GRK2) translocation to the membranes. Tyrosine kinase inhibitor genistein and protein kinase C inhibitors and siRNA restored D1R signaling, providing evidence that dopamine D1R-mediated adenylyl cyclase stimulation (1, 12, 15).

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**MATERIALS AND METHODS**

An OK proximal tubular cell line was obtained from American Type Culture Collection (Manassas, VA). These cells express a variety of detectable and quantifiable functional receptors and show a comparable response to freshly prepared proximal tubules as they relate to dopamine- or insulin-mediated Na-K-ATPase regulation. D1R agonists and R (+)-SKF38393 hydrochloride [an active enantiomer of (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol] were purchased from Sigma (St. Louis, MO). [3H]SCH23390 hydrochloride [R (+)-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol hydrochloride], a D1R antagonist, and [35S]GTPγS [guanosine 5'-[γ-thio]triphosphat-[35S]] were purchased from PerkinElmer (Boston, MA). Antibodies for G proteins, GRKs and D1R, were obtained from Calbiochem (La Jolla, CA), Santa Cruz Biotechnology (Santa Cruz, CA), and Alpha Diagnostic (San Antonio, TX), respectively. PKC assay kit was purchased from Promega (Madison, WI). Inhibitors for PKA (H-89) and PI3K (wortmannin) were obtained from Upstate (Charlottesville, VA) and Invitrogen (Carlsbad, CA), respectively. PKC inhibitor chelerythrine chloride and tyrosine kinase inhibitor genistein were purchased from Sigma. Protease inhibitor (PI) cocktail tablets (complete-11697498) and siRNA for GRK2 were obtained from Roche Diagnostic Gmbh (Indianapolis, IN) and Dharmacon (Lafayette, CO), respectively. All other chemicals of highest purity were purchased from Sigma.

**Cell culture treatment.** OK cells were grown to 80–90% confluence in DMEM:F12 supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% carbon dioxide. Cells were starved overnight in serum-free media and incubated without (vehicle) or with 10 nM insulin for 24 h in DMEM:F12. For membrane preparation, cells were washed in cold PBS and homogenized in 10 Vol of ice-cold homogenization buffer containing 10 mM Tris-HCl, pH 7.6, 50 mM sucrose, PI cocktail, and 0.04 mM phenylmethylsulfonyl fluoride (PMSF). The cell homogenate was centrifuged for 5 min at 750 g (4°C) to separate the membrane (pellet) and cytosol (supernatant) (4).

Transfection of the OK cells with GRK2 siRNA. GeneBank accession number AF087455 (OK-GRK2) was provided to manufacturer (Dharmacon) and to design GRK2 siRNAs (21). GRK2 siRNA and oligofectamine (0.6%) were diluted separately in OptiMem I, mixed, and incubated for 20 min at room temperature. After the cells (40–50% confluent) were washed with PBS, serum-free media was added and cells were incubated with a different concentration of GRK2 siRNA (0.1, 0.2, 0.5, and 2 μM) and 0.6% of oligofectamine for 4 h followed by the addition of the growth medium without antibiotic. The cells were collected after 24–72 h and immunoblotted for GRK2 and actin proteins as described previously (2).

[3H]SCH23390 and [35S]GTPγS binding. For [3H]SCH23390 membrane binding, 50 μg of membrane protein were incubated with 4 nM [3H]SCH23390, a D1R antagonist, in 250 μl (final volume) of binding buffer for 120 min at 25°C (29). Nonspecific binding was determined in the presence of 1 μM unlabeled SCH23390 (4). GTPγS membrane binding assay was performed as described in our previous publications (4, 32). The reaction mixture of 90 μl (final volume) contained 25 mM HEPES, 15 mM MgCl2, 1 mM dithiothreitol, 100 mM NaCl (pH 8.0), 5 μg membrane protein, and ~100,000 CPM of [35S]GTPγS with or without D1R agonist SKF38393. Nonspecific binding of [35S]GTPγS, determined in the presence of 100 μM unlabeled GTPγS, was always less than 2% of total binding.

[35S]GTPγS binding to G proteins. Membranes were resuspended in Krebs-Ringer buffer (KRB) containing 20 mM HEPES (pH 7.4), 154 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgCl2, PI cocktail, and 0.04 mM PMSF (10, 35). The assay mixture (250 μl) containing 200 μg of membrane protein and 2 nm [35S]GTPγS was incubated for 5 min at 25°C, followed by incubation in the absence or presence of D1R agonist SKF38393 for 5 min. In the experiments where D1R antagonist SCH23390 was used, membranes were incubated with the antagonist for 10 min before exposure of [35S]GTPγS (15 min before exposure to the agonist). The reaction was terminated by the addition of 750 μl ice-cold KRB containing 1 mM EDTA and 1 mM EGTA followed by centrifugation at 16,000 g for 5 min at 4°C. The membrane pellets obtained were briefly sonicated in 500 μl of ice-cold 100 mM Tris-HCl immunoprecipitation (IP) buffer, pH 7.5, containing 200 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 0.2% 2-mercaptoethanol, PI cocktail, and 0.04 mM PMSF followed by addition of 1 Vol of IP buffer. The suspension was combined with either specific Gsa or Gix antiseraum at 1:1,000 dilution for 30 min at 25°C. This procedure was followed by the addition of 100 μl protein A-bearing Staphylococcus aureus cells (Pansorbin cells), incubation for 30 min, and centrifugation. The pellet was washed and the immune complex containing the antigen-antibody complex was resuspended in KRB followed by brief sonication and radioactivity was measured by liquid scintillation spectrometry. The radioactivity precipitated by the normal rabbit serum was considered as background and subtracted from all agonist-stimulated values.

[3H]SCH23390 binding to Gsa immunoprecipitated D1Rs. To determine the linkage between D1Rs and G proteins, 200 μg of membrane proteins were solubilized in 1 ml of IP buffer with 0.2% cholate and 0.5% digitonin (10, 35). Solubulized membranes were combined with antisera (1:1,000 dilution) raised against Gsa or Gix proteins for 3 h at 4°C followed by an additional 30-min incubation with 100 μl of Pansorbin cells. The mixture was centrifuged and washed, and the pellet was suspended and incubated for 30 min at 30°C in 500 μl of 50 mM Tris-HCl binding buffer, pH 7.5, which included 5 mM MgCl2 and 1 mM [3H]SCH23390. Nonspecific binding was defined by the addition of 1 μM unlabeled SCH23390. The reaction was terminated by the addition of 9 ml of ice-cold buffer and immediately vacuum filtered over Whatman GF/F filters. The amount of radioactivity on the filter was assessed by liquid scintillation counting, and specific [3H]SCH23390 binding was determined.

Na-K-ATPase assay. Na-K-ATPase activity was determined as described previously (4). Briefly, cells grown in 12-well plates were incubated without or with SKF38393 for 10 min at 37°C. 86Rb+ uptake was initiated by addition of 1 ml DMEM containing 3 μCi/ml 86Rb+. Cells were lysed with 3% sodium dodecyl sulfate and radioactivity as well as protein were measured directly in cell lysate. Na-K-ATPase activity was determined as the difference between 86Rb+ uptake in the absence and presence of ouabain and normalized with protein. We also measured Na-K-ATPase activity by ATP hydrolysis (data not shown). After the SKF38393 treatment, cell suspension (0.1 mg protein/ml) was used to assay 1 mmol/l ouabain-sensitive ATP (4 mM) hydrolysis and inorganic phosphate released was determined colorimetrically (data not shown).

**PKC**. PKC activity was determined by commercially available PKC assay kit as detailed in our previous study (3).

Detection of serine phosphorylation on D1Rs. D1A receptors were immunoprecipitated using the method of Sanada et al. (26) which has been used in our previous studies (5). Briefly, cell membranes (1.5 mg protein/ml) were incubated overnight with 10 μg rabbit dopamine D1A receptor antibody in IP buffer followed by incubation with protein-A/G-agarose beads for 2 h. The ternary complex of D1A receptor-antibody-protein-A/G agarose was washed with IP buffer and then with 50 mM Tris·HCl, pH 8.0. The complex was dissociated in 2X Laemmli buffer and resolved by SDS-PAGE electrophoresis and the proteins were electrotransferred on a PVDF membrane. The membrane was blocked with 4% bovine serum albumin in PBS with 0.1% Tween 20 and immunoblotted with specific phosphoserine antibody or D1A receptor antibody to detect serine phosphorylation on D1Rs and D1R protein, respectively. The densitometric ratio of phosphoserine band and D1A receptor protein band was considered as net D1R phosphorylation.

**REFERENCES**

- **Detection of serine phosphorylation on D1Rs.** D1A receptors were immunoprecipitated using the method of Sanada et al. (26) which has been used in our previous studies (5). Briefly, cell membranes (1.5 mg protein/ml) were incubated overnight with 10 μg rabbit dopamine D1A receptor antibody in IP buffer followed by incubation with protein-A/G-agarose beads for 2 h. The ternary complex of D1A receptor-antibody-protein-A/G agarose was washed with IP buffer and then with 50 mM Tris·HCl, pH 8.0. The complex was dissociated in 2X Laemmli buffer and resolved by SDS-PAGE electrophoresis and the proteins were electrotransferred on a PVDF membrane. The membrane was blocked with 4% bovine serum albumin in PBS with 0.1% Tween 20 and immunoblotted with specific phosphoserine antibody or D1A receptor antibody to detect serine phosphorylation on D1Rs and D1R protein, respectively. The densitometric ratio of phosphoserine band and D1A receptor protein band was considered as net D1R phosphorylation.
Insulin reduced SKF38393-mediated Na-K-ATPase inhibition. Incubation of cells with SKF38393, a D1R agonist, caused concentration-dependent inhibition of Na-K-ATPase activity in vehicle-treated cells but not in insulin-treated cells (Fig. 1A). To elucidate the mechanism of this phenomenon, we used inhibitors of various kinase pathways that are known to be involved in both insulin and D1R signaling. Tyrosine kinase inhibitor genistein (100 nM), PI3K inhibitor wortmannin (100 nM), and PKC inhibitor chelerythrine chloride (1 μM) as well as GRK2 siRNA (2 μM) restored SKF38393 (1 μM)-mediated Na-K-ATPase inhibition in insulin-treated cells, whereas PKA inhibitor H-89 (1 μM) was ineffective (Fig. 1B). We used 1 μM SKF38393 as this concentration showed maximum inhibition (Fig. 1A). Pharmacological inhibitors and GRK2 siRNA did not affect the basal Na-K-ATPase activity (data not shown).

Insulin decreased D1R [3H]SCH23390 binding and affinity. Incubation of OK cells with 10 nM insulin for 24 h caused marked decrease in [3H]SCH23390 binding (Table 1). To determine agonist affinity of D1Rs, [3H]SCH23390, a D1R antagonist, was displaced with SKF38393 in membranes from vehicle- and insulin-treated cells. The analysis of competition binding data revealed that agonist affinity of D1R was reduced by two log units of SKF38393 concentration in insulin-treated cells compared with vehicle (Table 1). Genistein, wortmannin, chelerythrine chloride, and GRK2 siRNA prevented the insulin-induced decrease in D1R number and affinity, whereas PKA inhibitor H-89 (1 μM) failed to influence the effect of insulin on D1R expression or affinity (Table 1).

Insulin reduced D1R G protein coupling. As shown in Fig. 2A, incubation of membranes with SKF38393 followed by immunoprecipitation with Gαs antiserum led to significant increase in [35S]GTPγS binding in vehicle-treated cells but SKF38393 failed to increase [35S]GTPγS binding in insulin-treated cells. Immunoprecipitation with Gαs did not result in SKF38393-induced increase in [35S]GTPγS binding in vehicle-treated cells and the effect of SKF38393 on Gαs was completely blocked by D1R antagonist SCH23390 (Fig. 2A). In assessing basal-specific coupling of D1R to G proteins, we employed direct [3H]SCH23390 binding in immunoprecipitates of Gαs proteins obtained from solubilized cell membranes. The Gαs antiserum-immunoprecipitated [3H]SCH23390 binding sites in insulin-treated cells were significantly reduced compared with vehicle (Fig. 2B), while Gαs antiserum failed to

**Table 1. Effect of I on dopamine D1 receptor ligand binding, affinity, and basal GTPγS binding in OK cells**

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<th>I + G</th>
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<td>[3H] SCH23390 binding, fmol/mg protein</td>
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<td>2.2 × 10^-11</td>
<td>1.51 × 10^-9*</td>
<td>3.2 × 10^-11</td>
<td>1.2 × 10^-11</td>
<td>9.18 × 10^-11</td>
<td>8.9 × 10^-11</td>
<td>2.01 × 10^-9*</td>
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<td><strong>Basal [35S]GTPγS bound, fmol/mg protein</strong></td>
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<td>132.1 ± 6.3</td>
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<td>135.5 ± 6.2</td>
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<td>140.5 ± 8.2</td>
<td>125.1 ± 6.1</td>
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Values are means ± SE. Tyrosine kinase inhibitor genistein (G), phosphatidylinositol 3-kinase inhibitor wortmannin (W), protein kinase C inhibitor chelerythrine chloride (CCI), G protein-coupled receptor kinase 2-specific small interfering RNA (siRNA), and protein kinase A inhibitor (H-89) did not affect the basal [3H]SCH23390 binding, EC50 for [35S]GTPγS binding. Data (n = 6–8, performed in triplicate) were analyzed by ANOVA and post hoc Newman-Keuls multiple comparison test. P < 0.05 was considered statistically significant. *Significantly different from vehicle (V), I. insulin.
coimmunoprecipitate significant D1R binding sites in vehicle- or insulin-treated cells (Fig. 2B). Immunoprecipitates of Gsα antisera were also blotted with a specific D1R and Gsα antibodies. The D1R protein that coimmunoprecipitated with Gsα was significantly reduced in insulin-treated cells compared with vehicle (Fig. 2C). Gsα antisera immunoprecipitated a similar amount of G protein both in vehicle- and insulin-treated cells (Fig. 2D). To elucidate the role of various kinases in insulin-induced D1R G protein uncoupling, GTPγS binding was performed in the presence of various protein kinase inhibitors. In vehicle-treated cells, SKF38393 caused a concentration-dependent increase in [35S]GTPγS membrane binding but failed to produce a similar effect in insulin-treated cells (Fig. 3A). Genistein, wortmannin, chelerythrine chloride, and GRK2 siRNA restored SKF38393-induced [35S]GTPγS membrane binding in insulin-treated cells, whereas H-89 failed to show any effect (Fig. 3B). Basal [35S]GTPγS binding was similar in cells incubated with vehicle, insulin, or kinase inhibitors (Table 1).

**Insulin increased D1R serine phosphorylation.** Because D1R were uncoupled from their cognate G proteins and phosphorylation is known to contribute to receptor desensitization, D1R phosphorylation was evaluated. Following insulin treatment, the D1R demonstrated a significant increase in serine phosphorylation compared with vehicle (Fig. 4). Genistein, wortmannin, chelerythrine chloride, and GRK2 siRNA but not H-89 prevented insulin-mediated D1R hyperphosphorylation (Fig. 4).

**Insulin increased basal PKC activity.** As illustrated in Fig. 5, the basal PKC activity was significantly higher in insulin-treated cells compared with vehicle. Insulin-induced PKC activation was blocked by genistein and wortmannin while GRK2 siRNA and H-89 had no effect on insulin-induced PKC stimulation (Fig. 5).

**Insulin increases GRK2 protein expression and translocation to the membranes.** Since heparin is a nonspecific inhibitor of GRKs and gives conflicting results, we used opossum (Didelphis virginiana)-specific GRK2 siRNA. As shown in Fig. 6A, 48 h after the transfection with 2 μM siRNA, GRK2 protein decreased by ~90% compared with cells incubated with transfection reagent (mock). Incubation of cells with insulin increased GRK2 protein expression in whole cell lysate (WC) as well as membranes (M) of insulin-treated cells (Fig. 6B). However, the extent of increase was much higher in cell membrane fraction compared with cell lysate (Fig. 6B) or cytosol (data not shown) suggesting GRK2 tranlocation from cytosol to membrane. Similar to D1R phosphorylation, insulin failed to increase GRK2 membranous translocation in presence of genistein, wortmannin, chelerythrine chloride, and GRK2 siRNA while H-89 did not prevent the effect of insulin (Fig. 6C).

**DISCUSSION**

In this study, we showed that insulin-mediated dopamine D1R (D1R) heterologous desensitization involved PI3K-PKC-GRK2-D1R serine phosphorylation pathway (Fig. 7). Different types of converging evidence support this conclusion. 1) Insulin reduced D1R expression, affinity and basal coupling to G proteins, and caused D1R serine hyperphosphorylation. SKF38393, a D1R agonist, failed to stimulate G proteins and inhibit Na-K-ATPase activity in insulin-treated cells, indicating D1R desensitization. 2) Tyrosine kinase inhibitor genistein, PI3K inhibitor wortmannin, PKC inhibitor chelerythrine chloride, as well as GRK2 siRNA prevented D1R serine phosphorylation and abrogated D1R desensitization in insulin-treated cells, suggesting that receptor phosphorylation contributes to D1R dysfunction and involves tyrosine kinase, PI3K, PKC,
and GRK2 signaling molecules. 3) Insulin increased basal PKC activity which was sensitive to genistein and wortmannin, whereas GRK2 siRNA failed to block PKC activation, indicating PI3K acts proximal to PKC and GRK2 acts distal to PKC. 4) The increased GRK2 membranous translocation in insulin-treated cells was abolished by genistein, wortmannin, and chelerythrine chloride as well as GRK2 siRNA, suggesting that GRK2 acts at the end of cascade and is responsible for D1R phosphorylation. 5) PKA inhibitor H-89 failed to affect the receptor phosphorylation and/or D1R signaling in insulin-treated cells. Taken together, these data suggest that insulin via PI3K increases PKC activity. PKC increases GRK2 translocation to the membranes, causing D1R serine phosphorylation; this in turn decreases D1R affinity causing uncoupling from G proteins. The D1R downregulation and uncoupling lead to receptor desensitization as evidenced by failure of SKF38393 to stimulate G proteins and inhibit Na-K-ATPase activity (Fig. 7). Finally, the insulin-mediated D1R desensitization does not involve PKA signaling.

In experimental model of diabetes mellitus, various abnormalities of blood pressure regulation and sodium handling have
been demonstrated; however, the precise mechanism involved has not been fully elucidated (8, 29). Several factors are responsible for sodium retention and hypertension in diabetic animals and patients (8, 27, 33). Hyperinsulinemia contributes in part to sodium retention, as insulin can increase sodium reabsorption in proximal tubules (30, 33). On the other hand, there is considerable evidence that hyperinsulinemic animals and patients with noninsulin-dependent diabetes have defective renal dopaminergic system (27, 28, 33). Dopamine is an intrarenal hormone that is mainly produced in proximal tubular cells and inhibits sodium transporters and decreases sodium absorption in autocrine and paracrine fashion. Therefore, an alteration in dopamine signaling caused by hyperinsulinemia may be an important contributing factor for sodium retention under hyperinsulinemic conditions (1, 4, 15, 16, 27, 33).

Previously, we showed that in obese Zucker rats, a model of type 2 diabetes, renal D1R are downregulated and uncoupled from G proteins and dopamine fails to produce diuresis and natriuresis in these animals (5, 32). Treatment of these animals with insulin sensitizer rosiglitazone or antioxidant tempol decreased the plasma insulin levels and restored renal D1R function (5, 32). Since hyperinsulinemia causes changes in D1R function which may contribute to sodium retention and hypertension in diabetes, we undertook the present study to identify the underlying mechanism by which insulin causes desensitization of renal D1R.

We found that exposure of OK cells to SKF38393, a D1R agonist, resulted in a dose-dependent decrease in Na-K-ATPase activity. SKF38393 failed to inhibit Na-K-ATPase activity in cells treated chronically with insulin for 24 h. This effect of insulin was blocked by genistein, a pharmacological inhibitor of tyrosine kinase supporting the involvement of insulin receptor. Furthermore, in insulin-treated cells, the use of PI3K- and PKC-specific inhibitors wortmannin and chelerythrine chloride as well as GRK2-specific siRNA restored the D1R function, whereas PKA inhibitor H-89 was unable to mitigate...
insulin effect. The heterologous D1R desensitization by insulin involved both tyrosine kinase and serine threonine kinases such as PI3K, second messenger-dependent kinase PKC as well as GRK2, but PKA is not involved. These results are in agreement with our previous results in primary that insulin caused a significant decrease in D1R affinity. We compared with vehicles. Furthermore, binding data showed marked decrease in receptor number in insulin-treated cells and restored D1R function (5, 32). Also, the chronic exposure of renal proximal tubular primary cultures from Sprague-Dawley rats to insulin impaired D1R-mediated Na-K-ATPase inhibition (4).

A preponderance of evidence from various in vivo and ex vivo studies suggests that D1R desensitization that results in the waning of receptor response to agonist stimulation is due to a decrease in receptor number as well as uncoupling from G proteins (12, 15, 16). Our D1R ligand binding data revealed a marked decrease in receptor number in insulin-treated cells compared with vehicles. Furthermore, binding data showed that insulin caused a significant decrease in D1R affinity. We further examined the effect of insulin on D1R protein coupling. In agreement with our previous results in primary cultures (4), we observed that D1R agonist SKF38393 failed to increase [35S]GTPγS binding in membranes from insulin-treated cells. Furthermore, the immunoprecipitation results showed decreased basal communoprecipitation of G proteins with D1R ligand binding sites in insulin-treated cells compared with vehicle. These results indicate that D1R are uncoupled from cognate G proteins at basal state and also fail to interact with G proteins after stimulation. Similar to homologous desensitization where GRK2-dependent phosphorylation is mainly responsible for uncoupling from G proteins (20, 31), we observed that insulin-induced D1R G protein uncoupling was abolished by GRK2 siRNA. However, the D1R uncoupling was also sensitive to PI3K and PKC inhibitors. It is noteworthy that insulin failed to decrease the D1R number or coupling in GRK2 siRNA-transfected cells despite increased PKC activity suggesting GRK2 as a terminal kinase responsible for receptor desensitization. Taken together, these results suggest that failure of D1R agonist to inhibit Na-K-ATPase activity in insulin-treated cells may be due to D1R downregulation and uncoupling from G proteins. In addition, the D1R desensitization involved PI3K, PKC, and GRK2 signaling molecules while PKA does not contribute to either loss of affinity or uncoupling.

The observation that desensitized D1R from insulin-treated cells exhibited decrease in affinity and were uncoupled from cognate G proteins at basal state suggests that D1R may have undergone some conformational change. For many GPCRs including D1R, phosphorylation by protein kinases such as PKA or GRKs is an early step in desensitization (9, 11, 14, 20, 24, 31, 34). In this regard, insulin also caused a significant increase in D1R phosphorylation, indicating phosphorylation as an important factor for heterologous desensitization. Unlike the homologous desensitization where PKA has been suggested to play a pivotal role (14, 34), our data show that PKA inhibitor did not influence the effect of insulin on D1R phosphorylation. However, as observed with homologous desensitization, insulin increased D1R phosphorylation via GRK2 pathway. Although PKC inhibitors also blocked the D1R phosphorylation, PKC was not directly involved in this response as insulin failed to increase D1R phosphorylation when cells were transfected with GRK2 siRNA despite a significant increase in PKC activity. However, insulin-mediated GRK2 expression and translocation were PKC dependent, as PKC inhibitors blocked the insulin-dependent increase in GRK2 upregulation whereas H-89, a PKA inhibitor, showed no such effect. Furthermore, we observed that PI3K inhibitor blocked the insulin-mediated PKC activation, GRK2 upregulation, and D1R phosphorylation, suggesting that PKC and GRK2 are activated downstream of PI3K. In support of our data, there are reports that insulin can activate PKC in a PI3K-dependent manner and PKC-induced phosphorylation of GRK2 causes its activation and membranous translocation (7, 17, 36). These results therefore elucidate a signaling cascade for insulin-mediated serine phosphorylation of renal D1R involving PI3K-PKC-GRK2. The hyperphosphorylation of D1R could be responsible for conformational change in receptor binding site leading to decrease in agonist binding, affinity, and uncoupling from G proteins.

The most compelling evidence that emerged from this study is that insulin-mediated heterologous desensitization of D1R involved a novel signal transduction cross talk (Fig. 7). Insulin in a PI3K-dependent manner increased PKC activity causing GRK2 membranous translocation; this in turn increased D1R serine phosphorylation. Hyperphosphorylation of D1R caused conformational changes in receptor binding site leading to loss of affinity and uncoupling from G protein. The functional consequences of these phenomena are reflected in heterologous D1R desensitization as D1R agonist SKF38393 failed to stimulate G proteins and inhibit Na-K-ATPase activity. Therefore, these findings provide an insight of insulin-induced defect in renal D1R signaling which may contribute to sodium retention and hypertension in diabetes.

DISCLOSURES

No conflict of interest exists in this study.

REFERENCES


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