Oxidative stress reduces renal dopamine D1 receptor-Gq/11α
G protein-phospholipase C signaling involving G protein-coupled receptor kinase 2

Anies Ahmad Banday and Mustafa F. Lokhandwala
Heart and Kidney Institute, College of Pharmacy, University of Houston, Houston, Texas

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Banday AA, Lokhandwala MF. Oxidative stress reduces renal dopamine D1 receptor-Gq/11α G protein-phospholipase C signaling involving G protein-coupled receptor kinase 2. Am J Physiol Renal Physiol 293: F306–F315, 2007. First published April 25, 2007; doi:10.1152/ajprenal.00108.2007.—The dopamine D1 receptors (D1R), expressed in renal proximal tubules, participate in the regulation of sodium transport. A defect in the coupling of the D1R to its G protein-effector complex in renal tubules has been reported in various conditions associated with oxidative stress. Because G protein-coupled receptor kinases (GRKs) are known to play an important role in D1R desensitization, we tested the hypothesis that increased oxidative stress in obese Zucker rats may cause GRK2 upregulation and, subsequently, D1R dysfunction. Lean and obese rats were given normal diet or diet supplemented with antioxidant lipoic acid for 2 wk. Compared with lean rats, obese rats exhibited oxidative stress, D1R were uncoupled from Gq/11α at basal level, and SKF-38393 failed to elicit D1R-G protein coupling, stimulate phospholipase C (PLC), and inhibit Na-K-ATPase activity. These animals showed increased basal protein kinase C (PKC) activity and membranous translocation of GRK2 and increased GRK2-Gq/11α interaction and D1R serine phosphorylation. Enzymatic dephosphorylation of D1R restored SKF-38393-induced adenylyl cyclase stimulation but not PLC activation. Treatment of obese rats with lipoic acid restored D1R-G protein coupling and SKF-38393-induced PLC stimulation and Na-K-ATPase inhibition. Lipoic acid treatment also normalized PKC activity, GRK2 sequestration, and SKF-38393-induced adenylyl cyclase stimulation and Na-K-ATPase activity.

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synthesis of inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG) and an increase in intracellular Ca2+ causing PKC activation, which in turn inhibits Na-K-ATPase activity (13, 14, 34, 40, 48). It is also well known that in most hypertensive models, dopamine fails to stimulate D1R signaling and reduce Na-K-ATPase activity (7, 18, 20, 30, 39). However, beyond a basic appreciation, little is known about the signaling molecules PLC, GRK2, and PKC.

**EXPERIMENTAL PROCEDURES**

R(+)-SKF-38393 hydrochloride [an active enantiomer of (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride], a D1R agonist, was purchased from Sigma-Aldrich (St. Louis, MO). Radiolabeled SCH-23390 hydrochloride [R(+)-2,3,4,5-tetrahydro-1-(1H)-3-benzazepin-7-ol hydrochloride], a D1R antagonist, guanosine 5’-(cytidine)triphosphate ([35S]GTPγS), and phosphatidylinositol-4,5-bisphosphate ([3H]PIP2) were purchased from PerkinElmer (Shelton, CT). Antibodies for G proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GRKs, and D1R were obtained from Calbiochem-Novabiochem (La Jolla, CA), Cayman Chemical (Ann Arbor, MI), and Roche Diagnostic International (Indianapolis, IN), respectively. All other chemicals of the highest purity were purchased from Sigma-Aldrich.

**Animals.** Male lean and obese Zucker rats, 8–9 wk old, were purchased from Harlan (Indianapolis, IN), maintained at an ambient temperature with a 12:12-h light-dark cycle, and acclimatized to a lipoic-acid-supplemented diet, an antioxidant-supplemented diet, provided with lipoic acid, an antioxidant-supplemented diet, and, subsequently, receptor functional response. Both lean and obese Zucker rats were provided with lipoic acid, an antioxidant-supplemented diet, for 2 wk. Renal proximal tubules were studied for D1R coupling to G proteins and Na-K-ATPase inhibition as well as the signaling molecules PLC, GRK2, and PKC.

**Preparation of renal proximal tubular suspension.** Renal proximal tubular suspension was prepared as described earlier (10). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip), after midline abdominal incision, the aorta was cannulated below the renal arteries to perfuse the kidneys with an enzyme solution of collagenase and hyaluronidase. Enrichment of proximal tubules was carried out using a 20% Ficoll gradient in Krebs buffer, and protein was determined using the bicinchoninic acid method (Pierce, Rockford-IL) with bovine serum albumin as a standard.

[35S]GTPγS binding to G proteins. Crude membrane preparations were obtained from renal proximal tubules from lean and obese Zucker rats. Proximal tubules were homogenized in 10 volumes of ice-cold homogenization buffer containing 25 mM HEPES, pH 7.4, 100 mM sucrose, 1 mM EGTA, 0.2% 2-mercaptoethanol, PI cocktail, and 0.04 mM phenylmethysulfonyl fluoride (PMSF). The tissue homogenate was centrifuged for 5 min at 750 g (4°C). The resultant supernatant was then centrifuged for 10 min at 48,200 g (4°C). The pellet was resuspended in Krebs-Ringer buffer (KRB) containing 20 mM HEPES, pH 7.4, 154 mM NaCl, 4.8 mM KCl, 1.2 mM KHPo4, 1.2 mM MgCl2, PI cocktail, and 0.04 mM PMSF. Protein values were determined, and 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 (SKF-38393) for 5 min. In the experiments where D1R antagonist (SCH-23390) was used, membranes were incubated with antagonists 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 of 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 buffer, pH 7.5, containing 200 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 0.2% n-mercaptoethanol, PI cocktail, and 0.04 mM PMSF, followed by addition of 1 volume of immunoprecipitation buffer. Normal rabbit serum was added (1:100 final dilution) to each reaction tube and incubated at 25°C with gentle shaking. After 30 min, 100 µl of protein A-bearing Staphylococcus aureus cells (Pansorbin cells) were added, and the incubation continued for another 30 min. The suspension was centrifuged (14,800 g for 3 min), and the supernatant was combined with either specific Gq/11α or Gαs antiserum at 1:1,000 dilution for 30 min at 25°C. This procedure was followed by the addition of 100 µl of 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 using liquid scintillation spectrometry. The radioactivity precipitated by the normal rabbit serum was considered as background and subtracted from all agonist-stimulated values (17).

**Coprecipitation of [3H]SCH-23390 bound receptor with discrete Gα proteins.** To determine linkage between receptor and G proteins, 200 µg of membrane protein were solubilized in 1 ml of immunoprecipitation buffer with 0.2% cholate and 0.5% digitonin. Solubilized membranes were precleared by incubation with normal rabbit serum (1:100 dilution) at 4°C for 60 min, followed by an additional 30 min with 100 µl of Pansorbin cells. The suspension was centrifuged at 4°C, and the supernatant was combined with antisera (1:1,000 dilution) raised against specific peptides of Gq/11α or Gαs 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 nM [3H]SCH-23390. Nonspecific binding was defined by the addition of 1 µM unlabeled SCH-23390. The reaction was terminated by the addition of 9 ml of ice-cold buffer and was immediately vacuum-filtered over Whatman GF/F filters. The amount of radioactivity on the filter was assessed using liquid scintillation counting, and specific [3H]SCH-23390 binding was determined (40).

**Na-K-ATPase assay.** Na-K-ATPase activity was determined as reported previously (10). Briefly, SKF-38393-induced Na-K-ATPase inhibition was determined in renal proximal tubular suspensions (1 mg protein/ml) incubated with or without 1 mM ouabain for 15 min.

**Assay of PLC activity using exogenously added [3H]PIP2 as a substrate.** PLC activity was determined by using an exogenous source of [3H]PIP2 as the primary substrate for PLC and monitoring the release of [3H]IP3 (13, 14). Felder et al. (13, 14) have shown that under similar experimental conditions, incubation of [3H]PIP2 with renal membranes for 10–15 min did not result in the formation of [3H]IP2 ([3H]IP1 (13, 14), [3H]IP2 (0.005 µCi/assay tube) and phosphatidyserine (6 pg/mg protein) were dripped to remove the or
ganic solvents. The phospholipid residue was resuspended in sufficient PLC assay buffer (25 mM Tris-C1, 1.0 mM EGTA, 1.0 mM MgCl$_2$, 0.94 mM CaCl$_2$, and 5 mM LiCl, pH 7.4) to provide 50 μl of emulsion per assay tube. The reaction mixture contained 150 μl of sonicated membranes (suspended in PLC assay buffer, 100–200 μg of protein) and 50 μl of distilled water, with or without SKF-38393. Following a 5-min preincubation of the membranes and SKF-38393 at 37°C, the reaction was started with the addition of 50 μl of phospholipid emulsion. After 10 min, the reaction was terminated by addition of lipid extraction medium [1 ml of methanol-chloroform, 2:1 (vol/vol)], followed by vigorous vortexing. Chloroform (0.5 ml) and distilled water (0.5 ml) were added to the samples and vortexed vigorously. Following a 20-min incubation at room temperature, the samples were centrifuged at 1,200 g for 10 min. One milliliter of the upper aqueous layer was added to 5 ml of scintillation fluid, and the radioactive decay was counted in a liquid scintillation spectrophotometer.

PLC activity was also measured in myo-[H]inositol-labeled membranes (data not shown). The renal proximal tubules were suspended in Dulbecco’s modified Eagle’s medium and incubated with myo-[H]inositol under humidified 95% O$_2$-5% CO$_2$ atmosphere at 37°C for 90 min (13, 14). PLC activity was assayed by determining the release of inositol phosphates following the incubation of 200 μl of membrane suspension in PLC assay buffer with or without SKF-38393 (total volume of 250 μl). The reaction was terminated by the addition of 1 ml of methanol-chloroform [2:1 (vol/vol)], and the products were separated using the procedure of Fielder et al. (13).

**AC assay.** The AC assay was performed using a modification of the method described by Salomon (32). The reaction mixture included 0.5 mM MgCl$_2$, 0.5 mM 3-isobutyl-1-methylxanthine, 0.2 mM EGTA, 0.5 mM dithiothreitol, 1 μM GTP, 0.1 mM ATP, 2 mM phosphocreatine, 5 units of creatine phosphokinase, and 1 μCi of [α-32P]ATP (2.2 × 10$^8$ cpm) in 10 mM imidazole buffer, pH 7.3, with or without SKF-38393. After preincubation at 30°C for 5 min, the reaction was started by the addition of 50 μg of membrane protein. The reaction was terminated 10 min later by the addition of 300 μl of a solution containing 2% SDS, 25 mM ATP, and 1.3 mM cAMP. [32P]cAMP was separated using Dowex and alumina columns.

**Immunoblotting.** Proteins were solubilized in Laemmli buffer, separated by SDS-PAGE, and transferred to nitrocellulose membrane (38). The membranes were blocked and incubated with antisera directed against G proteins, GRK2, GAPDH, or D1R in 0.1% Tris-buffered saline, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunoprecipitation was performed as described previously (38).

**Enzymatic dephosphorylation.** Freshly prepared membranes were resuspended in Tris and EDTA buffer containing 2 mM MgCl$_2$ and exposed to alkaline phosphatase (21, 47). The membranes (80 μg) were incubated with 20 units of alkaline phosphatase in a 100-μl reaction mixture at room temperature for 15–20 min. Okadaic acid (0.1 μM), a serine/threonine phosphatase inhibitor, was used to stop the reaction. The membranes were washed to remove the enzyme; protein was determined and assayed for AC and PLC activities.

**PKC and SOD assay.** Protein kinase C (PKC) was assayed as detailed in our previous publication (5). SOD activity was determined using a commercially available assay kit utilizing a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine.

**Indexes of oxidative stress.** Renal proximal tubular carboxymethyllysine was measured using enzyme-linked immunosorbent assay as described by Koo and Vaziri (24), and malondialdehyde was determined using the method of Mihara and Uchiyama (28). Glutathione was determined colorimetrically as described previously (6).

## RESULTS

Obese animals were significantly heavier than lean rats (lean, 285.0 g; obese, 510 g). Lipoic acid had no effect on food or water intake and weight gain in lean or obese rats compared with respective controls (data not shown). Oxidative stress measured as content of malondialdehyde, a maker of lipid peroxidation, and carboxymethyllysine, a measure of advanced glycation end products, was significantly higher in obese compared with lean animals (Table 1). In addition, compared with lean rats, the levels of glutathione, a pivotal endogenous antioxidant, and SOD activity were markedly reduced in obese animals (Table 1). Supplementation of diet with lipoic acid reduced the malondialdehyde and carboxymethyllysine levels and restored glutathione levels and SOD activity in obese animals (Table 1).

**Lipoic acid restored SKF-38393-induced Na-K-ATPase inhibition in obese rats.** Incubation of proximal tubules with SKF-38393, a D1R agonist, caused concentration-dependent inhibition of Na-K-ATPase activity in lean rats. In obese rats, SKF-38393 failed to cause significant inhibition of Na-K-ATPase activity (Fig. 1A). Lipoic acid treatment restored SKF-38393-induced inhibition of Na-K-ATPase activity in obese animals but had no effect in lean rats. In addition, the D1R-mediated inhibition of Na-K-ATPase activity was PKC sensitive, given that SKF-38393 failed to inhibit renal proximal tubular sodium pump activity from lean rats in the presence of the PKC inhibitor chelerythrine chloride. However, the PKA inhibitor H-89 failed to show any effect (Fig. 1B). The basal PKC activity was higher in renal proximal tubular homogenates from obese compared with lean rats (Table 1). Treatment with lipoic acid normalized the PKC activity in obese rats but had no effect in lean rats (Table 1). The Na-K-ATPase α1-subunit protein content (data not shown) as well as basal activity (nmol·mg protein$^{-1}$·min$^{-1}$) were similar in all four

### Table 1. Effect of lipoic acid on oxidative markers in renal proximal tubules from lean and obese Zucker rats

<table>
<thead>
<tr>
<th></th>
<th>Lean Control</th>
<th>Obese Control</th>
<th>Lean Lipoic Acid</th>
<th>Obese Lipoic Acid</th>
</tr>
</thead>
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<tr>
<td>Body wt, g</td>
<td>290±23.0</td>
<td>500±33.0*</td>
<td>310±21.0</td>
<td>540±40.0*</td>
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<td>Malondialdehyde, nmol/mg protein</td>
<td>0.2±0.04</td>
<td>0.32±0.02*</td>
<td>0.18±0.01</td>
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<td>Carboxymethyllysine, optical density/mg protein</td>
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<td>0.36±0.04*</td>
<td>0.17±0.02</td>
<td>0.24±0.03</td>
</tr>
<tr>
<td>Glutathione, nmol/mg protein</td>
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<td>0.7±0.02*</td>
<td>2.3±0.2</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>Superoxide dismutase, enzyme units/mg protein</td>
<td>2.3±0.2</td>
<td>1.3±0.2*</td>
<td>2.5±0.4</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>Protein kinase C, ng peptide phosphorylated-mg protein$^{-1}$·min$^{-1}$</td>
<td>4.3±0.20</td>
<td>7.6±0.26*</td>
<td>3.8±0.20</td>
<td>4.2±0.30</td>
</tr>
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Data (n = 8 animals) were analyzed using 1-way ANOVA followed by post hoc Newman-Keuls multiple comparison test. *P < 0.05 compared with lean control.
experimental groups (lean control, 257.3 ± 30.2; obese control, 288.2 ± 32.3; lean lipoic acid, 267.3 ± 25.6; and obese lipoic acid, 248.6 ± 35.6).

**Lipoic acid restored D1R-G protein coupling in obese animals.** As shown in Fig. 2A, varying concentrations of SKF-38393 elicited increased $[^{35}S]$GTP$\gamma$S membrane binding in renal proximal tubules from lean rats but not in obese rats. Next, we tried to investigate more specific coupling of D1R to G protein subtype Gq/11. The proximal tubular membranes were incubated with SKF-38393 and $[^{35}S]$GTP$\gamma$S and immunoprecipitated with Gq/11$\alpha$ antibodies. As shown in Fig. 2B, the coupling of D1R to G proteins was reduced in obese rats compared with lean rats. The coupling was specific to D1R and Gq/11$\alpha$ in that the D1R antagonist was able to block it and the Gq$\alpha$ subunit failed to precipitate significant $[^{35}S]$GTP$\gamma$S activity. Similar to agonist-mediated coupling, the basal D1R coupling to Gq/11$\alpha$ was also reduced in obese compared with lean rats (Fig. 2C). The D1R binding sites that coimmunoprecipitated with Gq/11$\alpha$ were significantly reduced in obese compared with lean rats. The reduced D1R-Gq/11$\alpha$ coimmunoprecipitation cannot be attributed to a decrease in protein content of

Fig. 1. Effect of lipoic acid on SKF-38393, a dopamine D1 receptor (D1R) agonist, on Na-K-ATPase inhibition. A: renal proximal tubules from lean control (LC), obese control (OC), lean treated (LT), and obese treated (OT) animals were challenged with the indicated doses of SKF-38393 for 20 min. The data are expressed as a percentage of inhibition produced by the indicated concentration of SKF-38393. A single experiment is shown, representative of 6 individual experiments performed in triplicate. B: renal proximal tubules from lean rats were incubated with 1 μM chelerythrine chloride (CCl) and 1 μM H-89, pharmacological inhibitors of PKC and PKA, respectively, followed by challenge with 1 μM SKF-38393 for 20 min. Bars represent means ± SE of 6 different experiments performed in triplicate. *P < 0.05 compared with respective basal state using Student’s t-test.

Fig. 2. Effect of lipoic acid on SKF-38393, a D1R agonist, on guanosine 5'-[y-thio]triphosphate (GTP$\gamma$S) membrane binding. A: proximal tubular membranes from LC, OC, LT, and OT rats were incubated with varying concentrations of SKF-38393 and $[^{35}S]$GTP$\gamma$S membrane binding over basal by the indicated dose of SKF-38393. A single experiment is shown, representative of 6 individual experiments performed in triplicate, *P < 0.05 compared with respective basal state using 1-way ANOVA followed by Newman-Keuls multiple test.
D1R or G_{q/11}\alpha, since both the proteins are expressed equally in lean and obese rats (data not shown).

Lipoic acid restored SKF-38393-mediated PLC stimulation in obese rats. SKF-38393 caused a concentration dependent increase in PLC activity, as evidenced by hydrolysis of exogenously added \[^{3}H\]PIP2, in renal proximal tubular membranes from lean rats (Fig. 3A). A decreased concentration-response relationship was found in renal membranes from obese rats. Treatment of obese rats with lipoic acid restored D1R-mediated PLC stimulation but had no effect in lean rats (Fig. 3A). Also, lipoic acid treatment had no effect on basal PLC activity in either lean or obese rats (data not shown). Basal as well deoxycholate-stimulated PLC activity was similar in lean and obese rats (Fig. 3B). In addition, preincubation of proximal tubular membranes with D1R antagonist SCH-23390 reduced the SKF-38393-induced PLC stimulation in lean rats (Fig. 3C). Lipoic acid normalized GRK2 sequestration in obese rats. Proximal tubular membranes from obese rats showed a 50% increase in GRK2 protein levels compared with lean rats (Fig. 4A), whereas the cytosolic fraction from obese rats had a comparable decrease in GRK2 protein content (data not shown), indicating translocation of GRK2 to the membranes. The whole cell lysate from lean and obese rats showed similar GRK2 protein expression (Fig. 4B). Supplementation of lipoic acid to obese rats abolished the GRK2 membrane translocation (Fig. 4A). Lipoic acid treatment did not change the GRK2 protein levels in cell homogenates (Fig. 4B).

Lipoic acid reduced GRK2-G_{q/11}\alpha coimmunoprecipitation in obese rats. As shown in Fig. 5A, the immunological experiments using G_{q/11}\alpha antibodies for immunoprecipitation revealed increased coimmunoprecipitation of GRK2 with G_{q/11}\alpha in proximal tubular membranes from obese compared with lean rats. Conversely, the immunoprecipitation with G_{q/11}\alpha antibodies also showed increased interaction between GRK2 and G_{q/11}\alpha in proximal tubular membranes from obese rats (Fig. 5B). Treatment with lipoic acid decreased the coimmunoprecipitation of GRK2 and G_{q/11}\alpha in proximal tubular membranes from obese rats (Fig. 5A).

Alkaline phosphatase treatment restored D1R-mediated AC stimulation but not PLC stimulation in obese rats. A 20-min alkaline phosphatase treatment of renal proximal tubules from lean and obese rats caused a significant decrease in membrane D1R phosphorylation (Fig. 6A). Incubation of proximal tubular membranes with 100 \(\mu\)M SKF-38393 caused significant AC stimulation in lean rats but not in obese rats (Fig. 6B). Pretreatment of membranes from obese rats with alkaline phosphatase restored the D1R-mediated AC stimulation in obese rats but had no effect in lean rats (Fig. 6B). The basal as well forskolin (10 \(\mu\)M)-induced AC activity (pmol·mg protein\(^{-1}\)·min\(^{-1}\)) was similar in lean and obese rats (lean basal, 60.2 ± 12; lean forskolin, 203.3 ± 42.3; obese basal, 55.3 ± 17.6; obese forskolin, 193.3 ± 37.8). SKF-38393 also failed to show significant PLC stimulation in obese compared with lean rats. In addition, pretreatment of membranes with alkaline phosphatase failed to restore the SKF-38393-induced...
PLC stimulation in renal proximal tubular membranes from obese rats (Fig. 6C). As aforementioned, the basal as well deoxycholate-stimulated PLC activities were similar in lean and obese rats (Fig. 3B).

**DISCUSSION**

In the present study we have provided evidence that in renal proximal tubules from obese rats, oxidative stress increases PKC activity and causes membranous translocation of GRK2. Upon translocation, GRK2 interacts with Gq/11 and acts, at least in part, as a regulator of G protein signaling (RGS) leading to the D1R-Gq/11 uncoupling. In agreement with our previous studies, we found that SKF-38393, a D1R agonist, failed to elicit D1R-G protein coupling and inhibit Na-K-ATPase activity in obese rats. SKF-38393 caused a concentration-dependent increase in PLC activity in lean rats but failed to cause similar stimulation in obese rats. In addition, we found increased D1R phosphorylation in obese rats and observed that alkaline phosphatase treatment dephosphorylated the membranes and rescued D1R-AC signaling but failed to restore D1R-PLC pathway. These studies suggest that in addition to phosphorylation, GRK2-Gq/11 interaction also contributes to D1R dysfunction. Therefore, the inability of SKF-38393 to inhibit Na-K-ATPase activity is due to uncoupling of D1R from Gq/11 and failure to activate PLC. Finally, the treatment of obese animals with antioxidant lipoic acid ameliorated oxidative stress, normalized PKC activity and GRK2 seques-

![Image](image-url)
studies, we found that obese animals exhibit oxidative stress as evidenced by increased lipid peroxidation and advanced glycation end products along with decreased levels of glutathione and SOD activity, indicating a compromised antioxidant mechanism (6, 7). Diabetes can induce oxidative stress via several mechanisms (8, 19). These include glucose autoxidation, formation of advanced glycation end products, and activation of polyol pathway (8, 19). Other circulating factors, such as free fatty acid and leptin, also contribute to increased reactive oxygen species generation (8, 19). In agreement with these findings, our group has previously shown that obese Zucker rats are hyperinsulinemic, hyperlipidemic, and hyperglycemic, a hallmark of type 2 diabetes-associated obesity (6, 7). Because the pathogenesis of diabetes as well as its complications involves oxidative stress, the use of antioxidant is an appealing concept (8, 19). Our data show that lipoic acid not only reduces the oxidative stress but restores the normal redox status by normalizing the antioxidant defense mechanism, such as glutathione and SOD.

There is a remarkable parallel in the abnormal dopamine signaling, via D1R, and oxidative stress in hypertension and diabetes (6, 7, 26, 27). White and Sidhu (42) have shown that in spontaneously hypertensive rats (SHR), the renal D1R-G protein coupling defect is contributed by increased oxidative stress. Our own studies in normotensive Fisher 344 rats and streptozotocin-induced type 1 diabetic rats as well as in hypertensive animals provide a convincing correlation between oxidative stress and defective renal D1R function (6, 7, 12, 26, 27). In agreement with our previous findings, incubation of proximal tubules from lean rats with SKF-38393, a D1R agonist, caused a dose-dependent decrease in Na-K-ATPase activity (7). However, no such effect was found in obese animals, whereas treatment of these animals with lipoic acid restored the D1R function, thus providing evidence for the role of oxidative stress in defective D1R signaling. Similar defects have been observed in SHR and Dahl salt-sensitive rats, in which D1R fail to inhibit the NHE3 and Na-K-ATPase activities (18, 20, 30, 35, 42). Although, the exact mechanism for the failure of D1R to inhibit sodium transporters is not known, it is widely believed that defect in coupling of D1R to G proteins and subsequent impaired production of cytoplasmic messengers could be a contributing factor (18, 20, 35, 42).

Evidence to date indicates that stimulated D1R couple to both G_{i3}α and G_{q/11}α and activate AC and PLC, causing inhibition of the sodium transporters NHE3 and Na-K-ATPase (2, 13, 14, 18, 20, 29, 40, 49). The stimulation of AC causes cAMP accumulation and activates PKA, which leads to the inhibition of NHE3 (2, 18, 20). On the other hand, the mechanisms responsible for D1R-mediated Na-K-ATPase inhibition are elusive. In the present study we also found that in lean rats, SKF-38393-induced inhibition of Na-K-ATPase activity was blocked by the PKC-specific inhibitor chelerythrine chloride, whereas the PKA inhibitor H-89 had no effect. Previous studies have shown that in proximal tubules D1R inhibits Na-K-ATPase, involving a PLC-mediated increase in IP_{3} and DAG levels and subsequent PKC activation, whereas forskolin or dibutyryl-cAMP failed to decrease pump activity (2, 13, 14, 18, 20, 34). Although the role of cAMP-dependent kinase in renal D1R-mediated Na-K-ATPase inhibition seems to be controversial, surprisingly, most of studies related to D1R desensitization have been confined to AC stimulation (31, 41, 42).
Therefore, in the present study we investigated the role of oxidative stress-mediated defect in D1R-Gq/11α-PLC signaling. We found that incubation of proximal tubular membranes from obese rats with SKF-38393 failed to increase the hydrolysis of PIP2, suggesting a defect in D1R-mediated PLC stimulation. Treatment of obese animals with lipoic acid restored PLC stimulation in response to SKF-38393. The failure of D1R to activate PLC and increase IP3 and DAG levels may explain the inability of SKF-38393 to inhibit Na-K-ATPase. The defect cannot be attributed to changes in activities of PLC or Na-K-ATPase, because basal activities of these molecules were similar in lean and obese rats. Also, deoxycholate, a direct PLC activator, stimulated PLC to a similar extent in lean and obese rats.

The observation that D1R failed to stimulate PLC and inhibit Na-K-ATPase in obese rats despite similar basal activities indicates that the defect lies proximal to these molecules. Of the many mechanisms, D1R-G protein uncoupling has been shown to contribute to D1R desensitization. Previously, our group has shown in obese Zucker rats that D1R are uncoupled from Gα, and that tempol supplementation restored the coupling, indicating the role of oxidative stress (6, 7). In agreement with these studies we also found that SKF-38393 elicited a concentration-dependent increase in [35S]GTPγS binding in lean rats but not in obese rats. In addition, our results show that SKF-38393 failed to stimulate Gαα in obese rats. Furthermore, immunological experiments showed decreased coimmunoprecipitation of D1R with Gαα in obese compared with lean rats. These results suggest that D1R are uncoupled from Gαα at the basal state and also fail to interact with G proteins after stimulation. Therefore, the inability of stimulated D1R to couple with G proteins may explain the failure of SKF-38393 to activate PLC and inhibit Na-K-ATPase activity in obese animals. Treatment of obese rats with lipoic acid restored the basal as well as agonist-mediated coupling, thus confirming the role of oxidative stress in D1R-G protein uncoupling.

Desensitization of many GPCRs, including D1R, is thought to largely involve their phosphorylation by either second messenger-activated kinases (e.g., PKA or PKC) or GRKs (16, 20, 31, 33, 47). In renal tissues, the serine phosphorylation of the D1R is mainly carried out by GRKs (15, 31, 33, 41). We have previously reported that in obese rats, D1R hyperphosphorylation is mostly contributed by GRK4 and GRK2 (38). Treatment of these rats with the insulin sensitizer rosiglitazone normalized GRKs, reduced D1R phosphorylation, and restored coupling to G proteins (38). In the present study, we also observed an increased translocation of GRK2 to the membranes as well as hyper D1R phosphorylation in obese rats. Treatment of these rats with lipoic acid normalized GRK2 sequestration and restored D1R function, indicating the role of GRK2 in D1R dysfunction. The observation that in obese animals the increased PKC activity was associated with GRK2 translocation and lipoic acid whereas reducing PKC activity also normalized GRK2 sequestration indicates a signal transduction cross talk. In this regard, there are reports suggesting that GRK2 can be phosphorylated by PKC, which leads to its membrane translocation and increased activity (11, 46). Interestingly, numerous reports have suggested that PKC is stimulated by oxidative stress as well as hyperglycemia (reviewed in Ref. 8), both of which are present in obese rats. Oxidative stress can stimulate PKC by direct tyrosine phosphorylation or via activation of phospholipase D, which leads to DAG synthesis (22, 23, 37). On the other hand, hyperglycemia can increase PKC activity via de novo DAG synthesis from glycolytic intermediates (8). In a previous study (3), our group found that exposure of renal proximal tubular cells to H2O2, an oxidant, caused PKC-dependent GRK2 membranous translocation.
phosphorylation or involves additional mechanisms. Recently, it has been shown that at least two members of the GRK family, GRK2 and GRK3, are able to suppress Gαq/11-coupled receptor/PLC signaling even in the absence of kinase activity (9, 43). GRK2-mediated, phosphorylation-independent receptor regulation has now been demonstrated not only in recombinant systems but also for endogenous receptors in cell lines and in primary cultured hippocampal neurons (44, 45). Phosphorylation-independent receptor regulation by GRK2 is mediated through a specific interaction of GTP-loaded Gαq/11 with the RGS homology (RH) domain situated at the NH2 terminus of GRK2 (9, 36). Using immunological experiments, we also found that membranous GRK2 from obese rats showed increased coinmunoprecipitation with Gαq/11. Also, Gαq/11 antisera showed similar increased coprecipitation of these proteins, suggesting that membranous translocation of GRK2 results in its interaction with Gαq/11 and therefore may act as an RGS. Interestingly, treatment of obese rats with lipotic acid reversed the GRK2-Gαq/11 interaction to levels similar to those observed in lean rats. Although these data highlight a novel mechanism by which GRK2 can regulate D1R signaling in renal proximal tubules, they do not exclude the possibility that GRK2-mediated receptor phosphorylation is also an important mechanism in D1R desensitization in obese animals. Interestingly, various reports have shown that human renal D1R phosphorylation is mainly contributed by GRK4 with minor contribution from GRK2 (15, 33, 41). Therefore, it is possible that GRK4-mediated phosphorylation may be responsible for modulating D1R-Gαq/11AC pathway, whereas GRK2, functioning as an RGS, regulates D1R-Gαq/11-PLC signaling.

In conclusion, our data indicate that endogenous GRK2 is a key regulator of renal D1R signaling, utilizing both phosphorylation-dependent and -independent mechanisms to regulate receptor activity. In obese rats the increased oxidative stress causes PKC activation and translocation of GRK 2 to membranes. GRK 2, at least in part, interacts with Gαq/11, acts as an RGS, and decreases D1R coupling to Gαq/11. The D1R-G protein uncoupling diminishes the ability of SKF-38393 to stimulate PLC activity and inhibit Na-K-ATPase activity. Antioxidant lipotic acid, while reducing oxidative stress, restores D1R function, providing a substantial evidence for the role of reactive oxygen species in defective D1R signaling in conditions associated with oxidative stress.

GRANTS

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OXIDATIVE STRESS-MEDIATED RENAL D1 RECEPTOR DYSFUNCTION


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