Rosiglitazone Treatment Restores Renal Dopamine Receptor Function in Obese Zucker Rats

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Abstract—Earlier we have reported a defective dopamine D₁–like receptor function, which was accompanied by a decrease in D1 receptor numbers and the inability of dopamine to inhibit Na,K-ATPase and Na,H-exchanger in proximal tubules of hyperinsulinemic obese Zucker rats. The present study was designed to test the hypothesis that the defect in dopamine receptor function is a result of hyperinsulinemia in obese rats. We designed experiments to study D1 receptor function in obese Zucker rats treated with rosiglitazone, as it lowers plasma insulin by improving insulin sensitivity. A group of untreated lean and obese rats served as controls. Rosiglitazone treatment (10 mg/kg orally, 4 weeks) caused significant decreases in plasma insulin, blood glucose, and blood pressure while causing an increase in renal sodium excretion compared with untreated obese rats. In the isolated proximal tubules obtained from untreated lean rats, dopamine caused concentration-dependent inhibition of the Na,K-ATPase activity, but this inhibitory effect was absent in untreated obese rats. In rosiglitazone-treated obese rats, the inhibitory effect of dopamine on Na,K-ATPase was significantly restored. This was accompanied by a complete restoration of D1 receptor numbers in proximal tubular membranes of treated obese rats. In another set of experiments, treatment of primary proximal tubule epithelial cells in culture medium with insulin caused a significant decrease in the D1 receptor abundance, suggesting a direct role of insulin on D1 receptor regulation. We conclude that hyperinsulinemia causes downregulation of D1 receptor function and lowering of plasma insulin levels leads to restoration of renal D1 receptor function. (Hypertension. 2002;40:667–673.)

Key Words: receptors, dopamine □ hyperinsulinism □ obesity □ rats, Zucker

Within the kidney, dopamine receptors present on proximal tubules play an important role in regulating sodium and water excretion under various physiological conditions.¹ Dopamine receptors exist on both apical and basolateral side of the proximal tubule.² Of the dopamine receptors present on the proximal tubules, D₁ subtype has been implicated in dopamine-mediated natriuresis and diuresis.³ At the level of cellular mechanism of action, the D1 receptors, through coupling with Gs and Gq proteins, are linked to adenyl cyclase and phospholipase C.⁴–⁶ The activation of D1 receptors and subsequent stimulation of second-messenger pathways lead to the inhibition of sodium transporters—Na/H-exchanger (NHE) and Na,K-ATPase (NKA)—and thereby an increase in renal sodium excretion.⁵–⁷⁻⁹

Numerous reports have shown that natriuretic response to the exogenously infused or endogenously produced dopamine is blunted in spontaneously hypertensive rats (SHR).¹⁰,¹¹ owing to a defective D1 receptors in proximal tubules.¹² In vitro studies suggest that dopamine fails to inhibit NKA and NHE in proximal tubules of SHR.¹³,¹⁴ A recent study performed in proximal tubule primary epithelial cells derived from human hypertensive kidneys revealed hyperphosphorylation of D1 receptor caused by hyperactive GRK4 as a mechanism of defective D1 receptors.¹⁵ The defect on D1 receptor is corrected once the GRK4 is downregulated by treating with specific GRK4-antisense.¹⁶

Similarly, we have recently reported that dopamine is unable to inhibit the activity of NKA and NHE in proximal tubules of obese Zucker rats.¹⁶,¹⁷ However, unlike the observations made in SHR, the D1 receptor numbers on both basolateral (BLM) and brush border membrane (BBM) were reduced by 50% in obese compared with littermate lean control rats.¹⁶,¹⁷ The obese Zucker rat is a model of hyperinsulinemia and type 2 diabetes.¹⁸ Insulin has been shown to affect cellular function of various hormones by altering the receptor numbers or receptor sensitivity to their ligands.¹⁹–²¹ It has been reported that natriuretic response to exogenous dopamine was reduced in type 2 diabetic patients.²² On the basis of these studies, we hypothesized that reduction in D1 receptor number and inability of dopamine to inhibit sodium transporters in obese Zucker rats may have been caused by chronic hyperinsulinemia, which if corrected may lead to the restoration of D1 receptor numbers and its function in obese rats. Therefore, in the present study, we treated obese Zucker rats with rosiglitazone, a thiazolidinedione, which has been shown to lower plasma insulin, by improving insulin sensitivity.²³,²⁴ After treatment with rosiglitazone, D1 receptor numbers and the effect of dopamine on NKA activity was

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measured in the proximal tubules of obese rats. Also, additional experiments were performed in the primary epithelial cells to study the effect of insulin on D1 receptor regulation under more controlled experimental conditions.

Methods

Animals and Drug Treatment
Eight- to 9-week-old male obese Zucker rats and age-matched male lean Zucker rats (Harlan Sprague-Dawley Inc) were housed in plastic cages and fed normal rodent chow and tap water ad libitum. The obese rats were separated into two groups: obese control rats and obese rats treated with rosiglitazone. The treatment group received rosiglitazone maleate (10 mg/kg) suspended in 1% carboxy methyl cellulose in distilled water by oral route for 4 weeks. The obese control group received 1% carboxy methyl cellulose. Lean Zucker rats received no treatment. Each group consisted of 6 rats.

General and Biochemical Parameters
Body weights were recorded at the beginning and at the end of the treatment. At the end of treatment, rats were placed in metabolic cages for 24 hours with free access to water. Urine samples were collected and analyzed for urinary sodium and potassium using flame photometry (Corning 480 Flame Photometer). For blood pressure measurement, the rats were anesthetized with sodium pentobarbital (50 mg/kg body wt IP). After a midline incision was made, the aorta was catheterized below the kidneys and blood pressure was measured with a Statham pressure transducer and recorded on a Grass computer.

Isolation and Enrichment of Proximal Tubules
After blood pressure was measured and blood samples were withdrawn, rats were used for the preparation of renal proximal tubules as we have described earlier.\textsuperscript{14} Protein was measured with the use of a kit (Pierce).

Na,K-ATPase Assay
The proximal tubular suspension (1 mg protein/mL) in Krebs buffer was incubated with or without dopamine (1 nmol/L to 1 mmol/L) at 37°C for 20 minutes. After incubation, the tubules were permeabilized by rapid freezing in dry ice/acetone and thawing. Ouabain-sensitive Na,K-ATPase activity was measured\textsuperscript{14} (expressed as nmol Pi/mg protein per minute).

Membrane Preparation
Proximal tubules were homogenized in a buffer containing (mmol/L) 10 Tris-HCl, 250 sucrose, 0.2 phenylmethylsulfonyl fluoride, protease inhibitor cocktail at pH 7.6, and centrifuged at 2500 g for 10 minutes. The supernatant was centrifuged at 24 000 g for 20 minutes. The upper layer of the pellet was resuspended in the same buffer, dounced, and centrifuged again at 24 000 g for 20 minutes. The pellet was washed with washing buffer containing 100 KCl, 100 mannitol, 5 (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]), pH 7.2, and centrifuged at 34 000 g for 30 minutes. Finally, the membrane fraction (containing both BBM and BLM) was resuspended in a small volume of homogenization buffer and stored frozen for further use.

\textsuperscript{3}H SCH 23390 Binding
Binding of \textsuperscript{3}H SCH 23390 to the proximal tubular membranes was performed according to the previously described method.\textsuperscript{4,16} To generate saturation isotherm, the ligand concentration was varied from 1 to 60 nmol/L. Cold SCH 23390 (10 \textmu mol/L) was used for determining the nonspecific binding. The specific binding data were used to determine the B\textsubscript{max} and K\textsubscript{d} values.

Studies in Primary Cell Cultures

Proximal Tubule Epithelial Cell Culture
The primary proximal tubule epithelial cells (PTEC) were prepared and grown in media from proximal tubule fragments isolated from male Sprague-Dawley rats (200 to 250 g), as described earlier.\textsuperscript{23} Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg IP). After a midline incision was made, kidneys were selectively perfused with Krebs-Henseleit buffer with collagenase at a flow rate of 6 mL/min. The kidneys were excised. The cortices were removed, minced, and digested in buffer with collagen for 2 to 3 minutes. The digested suspension was filtered through 80- \mu m mesh and subjected to centrifugation on Ficoll density gradient, as described earlier. In the first set of experiments, the isolated PT fragments were resuspended in DMEM-F12 containing different glucose concentrations (5, 17.5, and 25 mmol/L) supplemented with various growth factors and antibiotics. The proximal tubular suspension was plated in 100-mm diameter dishes and maintained at 37°C, in an atmosphere of 5% CO\textsubscript{2}–95% air. The first media was changed after 72 hours of seeding and then every alternate day. In the second set of experiments, the cells were grown with DMEM-F12 containing 17.5 mmol/L glucose. At 80% to 90% confluency, cells were incubated without (control) and with insulin (100 nmol/L, for 24 hours) in order to investigate the effect of insulin on D\textsubscript{1A} receptor regulation. At ~85% confluency, the cells were starved for 24 hours in their respective antibiotic free mediums without growth supplements. The cells were washed 3 times and used for preparing the plasma membranes as described earlier.\textsuperscript{26}

Western Blotting for D1 Receptor Protein
Equal amounts of membrane proteins (8 \mu g) from control and treated groups of cells were resolved by SDS-PAGE and transferred (transblotted) onto a PVDF membrane (blot). The D1 receptors on the blot were detected by using a D1 receptor affinity-purified polyclonal antibody and anti-rabbit HRP-conjugated antibody. The bands were visualized with an enhanced chemiluminescence reagent kit and quantified by protein software program (Kodak). The specificity of the D1 receptor antibody has been determined earlier.

Statistics
The values are presented as mean±SEM. The results were subjected to 1-way ANOVA to assess significance within a group followed by post hoc Tukey multiple test to assess significance of difference between the groups (lean, obese control, and obese treated with rosiglitazone). The B\textsubscript{max} and K\textsubscript{d} values from the ligand binding data were calculated with the use of Graph Prizm software available on computer.

Chemicals
The insulin-measuring kit was purchased from Linco Research Inc. \textsuperscript{3}H SCH 23390 (75 Ci/mmol) was purchased from NEN Life Sciences. The D1 receptor antibody and Western blotting kit were purchased from Alpha Diagnostic Intl. All other chemicals of the highest purity available were purchased from Sigma Chemical Co or Fisher Scientific Inc.

Results

General Parameters
As shown in the Table, obese rats were significantly heavier than age-matched lean rats. Urine output (24 hours) in both obese control and obese treated rats was similar but significantly higher than in lean rats. Urinary sodium excretion over a period of 24 hours in obese rats was greater than in lean rats. Rosiglitazone treatment of obese rats caused greater urinary sodium excretion than in control obese rats. Urinary potassium excretion in both control and treated obese rats was similar but greater than in lean rats.
Blood Pressure, Blood Glucose, and Plasma Insulin

Both systolic and diastolic blood pressures were significantly higher in obese control compared with lean rats. Rosiglitazone treatment of obese rats caused significant reductions in systolic as well as diastolic blood pressures (Figure 1a). Heart rate was similar in all three groups of rats (Figure 1b).

Fasting blood glucose was significantly higher in obese control rats as compared with lean rats. Similarly, plasma insulin in obese rats was 10-fold higher than in lean rats. Rosiglitazone treatment of obese rats lowered the blood glucose to the level as in lean rats. Plasma insulin levels in obese rats after rosiglitazone treatment were reduced by approximately 50% compared with control obese rats (Figures 1c and 1d).

Effect of Dopamine on Na,K-ATPase Activity in Proximal Tubules

Dopamine produced a concentration-dependent (1 nmol/L to 1 μmol/L) inhibition of the Na,K-ATPase activity in proximal tubules from lean rats. The inhibition of Na,K-ATPase activity by dopamine was absent in obese control rats, but the inhibitory action of dopamine on the enzyme activity was significantly restored in rosiglitazone-treated obese rats (Figure 2). The basal Na,K-ATPase activity (nmol Pi/mg protein per minute) was similar in 3 groups of rats (lean rats, 242.3±15.12; obese control rats, 209.5±46.08; obese rosiglitazone-treated rats, 290.8±30.40).

[3H] SCH 23390 Binding

[3H] SCH 23390 bound in a saturable manner to the proximal tubular membranes from lean, control, and obese rats treated with rosiglitazone. Scatchard analysis of the data revealed a significant decrease in receptor number in obese rat membranes (Bmax: 382.9±23.93 fmol/mg) as compared with lean rats (Bmax: 649.9±90.96 fmol/mg). Treatment with rosiglitazone restored the receptor number (Bmax: 594.0±36.75 fmol/mg) comparable to that in lean rats. The dissociation constant (Kd) values (nmol/L) were similar in lean (61.17±11.30), obese control (41.05±3.98), and obese treated (51.85±2.32) rats (Figures 3a through 3c).

Effect of Glucose and Insulin Treatment on D_1A Receptor Abundance in Primary Epithelial Cells

Using DLA receptor antibody, we observed a single band of ~51 kDa. The specificity of the antibody has been determined earlier. The densitometric analysis of this band revealed that exposure of primary PTEC to varying concentr-
tions (5, 17.5, and 25 mmol/L) of glucose did not affect the expression of D1 receptor protein. In culture cells, 17.5 mmol/L is used as a normal glucose level. Either higher or lower concentrations of glucose did not cause change in the D1 receptor protein expression (Figure 4a). On the other hand, insulin (100 nmol/L) treatment of the primary PTEC caused a significant decrease in the D1 receptor protein expression (Figure 4b).

Discussion

Earlier we have reported that the inhibitory effects of dopamine on NKA and NHE activities in proximal tubules of hyperinsulinemic obese rats were significantly reduced compared with lean rats. We also observed that the reduced inhibition of these transporters was associated with a significant reduction in D1 receptor number in proximal tubular membranes of obese rats. In the present study, we found that if plasma levels of insulin were lowered in obese rats, the ability of dopamine to inhibit the sodium transporters in proximal tubules was restored and membrane D1 receptor numbers were normalized to the level as in lean rats.

Dopamine, endogenously produced or exogenously infused, activates tubular D1 dopamine receptors, leading to the inhibition of sodium transporters, involving multiple second-messenger pathways and subsequently causes an increase in sodium excretion. There have been reports suggesting a negative correlation between renal dopaminergic function and plasma insulin level. In type 2 diabetes, exogenous infusions of dopamine produced attenuated natriuresis and diuresis compared with control subjects. This study suggested that there might be a defect in dopamine receptor and effector coupling resulting in the reduced ability of dopamine to inhibit sodium transporters and causing diminished natriuresis and diuresis. This notion is supported by our studies performed in rat model of hyperinsulinemia/type 2 diabetes, obese Zucker rats. We have found the inhibitory effect dopamine on NKA and NHE activities in proximal tubules of obese Zucker rats was significantly attenuated compared with lean control animals, and these findings are further confirmed in the present study. Reduction in D1 receptor numbers by 50% on both BLM and BBM and uncoupling of the remaining receptors from G proteins could account for the failure of dopamine to activate second messengers and inhibit sodium transporters in proximal tubules of obese Zucker rats.

Similarly, in another model of type 2 diabetes, Wistar fatty rats, dopamine was unable to inhibit the NKA activity in proximal tubules. Insulin influences not only the carbohydrate and lipid metabolism but is also a potent growth hormone that affects cellular transcription. Insulin has been shown to affect the functional response to a number of hormones by altering the receptor number or ligand affinity. It is possible that in type 2 diabetic patients, hyperinsulinemia might have downregulated the D1 receptor and its function, which subsequently led to a diminished natriuretic response to exogenously infused dopamine. In our study, we treated obese rats with rosiglitazone, which lowered plasma insulin by 50% on both BLM and BBM and uncoupling of the remaining receptors from G proteins could account for the failure of dopamine to activate sodium transporters in proximal tubules of obese Zucker rats. Similarly, in another model of type 2 diabetes, Wistar fatty rats, dopamine was unable to inhibit the NKA activity in proximal tubules.
signaling of D1 receptor and its responsiveness to dopamine. Additionally, we also found that D1 receptor numbers on proximal tubular membrane of the treated obese rats were normalized to the level seen in lean rats. Since rosiglitazone treatment caused only partial decrease (∼50%) in plasma insulin, whereas normalizing glucose level similar to the lean rats, it may suggest that it is the hyperglycemia and not hyperinsulinemia responsible for reduction in D1 receptor numbers in obese rats. To address this question, we performed in vitro experiments in primary PTEC under more controlled experimental conditions. We found that either lower or higher glucose concentrations in the media with normal insulin concentration did not cause any change in the D1 receptor abundance. On the other hand, higher levels of insulin with normal glucose in the media caused a significant reduction in D1 receptor abundance in these cells. These data directly support the view that it is the hyperinsulinemia and not hyperglycemia that is responsible for the downregulation of D1 receptors in obese rats. A decrease in D1 receptor number has been reported in various brain regions of rats with diabetes induced by alloxan/streptozotocin.\textsuperscript{30,31} insulin-lowering agents. It is likely that as is the case of changes in D1 receptors, there may exist differences in responsiveness between CNS and peripheral tissues.

In proximal tubules, the Gq-PLC-PKC pathway is involved in D1 receptor–mediated inhibition of NKA.\textsuperscript{32} The complete restoration of D1 receptor number but a partial ability of dopamine to inhibit NKA suggests that the Gq-PLC-PKC pathway may not be fully restored. It may be speculated that further lowering of the plasma insulin levels in obese rats would be required to restore the remaining deficiency in D1 receptor function, as it relates to the Gq-PLC-PKC pathway.

Numerous studies have shown that high blood pressure develops in obese Zucker rats.\textsuperscript{4,33,34} Furthermore, it has been reported that natriuretic response to volume expansion in obese rats is attenuated compared with lean rats,\textsuperscript{35} suggesting impaired mechanisms responsible for natriuresis, including dopaminergic system. In the present study, we found that treating obese Zucker rats with rosiglitazone caused an increase in urinary sodium excretion and a reduction in blood pressure compared with control obese rats. Numerous mechanisms may be responsible for an increased urinary sodium excretion and reduction in blood pressure. For example, since insulin is antinatriuretic,\textsuperscript{36,37} a decrease per se in plasma insulin levels will lead to an increased sodium excretion. Increased activity of angiotensin II, another antinatriuretic hormone, has been reported in proximal tubules of obese compared with lean rats.\textsuperscript{38} It is likely that reduction in plasma insulin levels may have also reduced angiotensin II activity and hence increase in tubular sodium excretion. In vitro studies have shown that insulin upregulates AT1 receptors.\textsuperscript{39} Also, it is likely that a decrease in plasma insulin may have restored the basal natriuretic effect of endogenous dopamine.

**Perspective**

Our findings demonstrating that treatment of obese Zucker rats with the insulin sensitizer rosiglitazone restores dopamine receptor function and lowers blood pressure, have important clinical relevance. Thiazolidenediones such as rosiglitazone are used in the treatment of type 2 diabetes and have been found to be efficacious in some patients in controlling the disease. We have shown that treatment with rosiglitazone, while lowering plasma insulin and blood glucose, is also effective in restoring renal responsiveness to dopamine. Inasmuch as endogenous dopamine plays an important role in maintaining sodium homeostasis during increases in sodium intake, it is likely that patients treated with rosiglitazone will be able to achieve sodium homeostasis through the dopaminergic mechanism. They also will be able to exhibit renal responsiveness to exogenous dopamine. Therefore, these are the added benefits that rosiglitazone therapy will provide to diabetic patients.

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