Dopamine-Mediated Inhibition of Renal Na,K-ATPase Is Reduced by Insulin

Anees Ahmad Banday, Mohammad Asghar, Tahir Hussain, Mustafa F. Lokhandwala

Abstract—Recently we have reported that rosiglitazone treatment of obese Zucker rats reduced plasma insulin and restored the ability of dopamine to inhibit Na,K-ATPase (NKA) in renal proximal tubules. The present study was performed to test the hypothesis that a chronic increase in levels of insulin causes a decrease in expression of the D1 receptor and its uncoupling from G proteins, which may account for the diminished inhibitory effect of dopamine on NKA in obese Zucker rats. We conducted experiments in primary proximal tubule epithelial cells obtained from Sprague-Dawley rat kidneys. These cells at 80% to 90% confluence were pretreated with insulin (100 nmol/L for 24 hours) in growth factor-/serum-free medium. SKF-38393, a D1 receptor agonist, inhibited NKA activity in untreated cells, but the agonist failed to inhibit enzyme activity in insulin-pretreated cells. Basal NKA activity was similar in untreated and insulin-pretreated cells. Measurement of D1 receptors in the plasma membranes revealed that [3H]SCH-23390 binding, a D1 receptor ligand, as well as D1 receptor protein abundance, was significantly reduced in insulin-pretreated cells compared with untreated cells. SKF-38393 (10 μmol/L) elicited significant stimulation of [35S]GTPγS binding in the membranes from control cells, suggesting that the D1 receptor–G protein coupling was intact. However, the stimulatory effect of SKF-38393 was absent in membranes from insulin-pretreated cells. We suggest that chronic exposure of cells to insulin causes both the reduction in D1 receptor abundance and its uncoupling from G proteins. These phenomena might account for the diminished inhibitory effect of dopamine on NKA activity in hyperinsulinemic rats. (Hypertension. 2003;41:1353-1358.)

Key Words: dopamine ■ insulin ■ Na\(^+\)-K\(^+\)-transporting ATPase ■ receptors, dopamine ■ G proteins

Dopamine (DA) causes inhibition of sodium reabsorption in the renal proximal tubules. This action of DA is mediated by the activation of D1 receptors located on both the brush-border and the basolateral membranes of the proximal tubules and subsequent inhibition of the sodium transporters Na,H-exchanger (NHE) and Na,K-ATPase (NKA). Inhibition of NHE activity results from D1 receptor coupling to the Gs protein and an increase in the levels of intracellular cAMP, which causes activation of protein kinase A (PKA), leading to phosphorylation of the exchanger. D1 receptor coupling with the Gs/11 protein, activation of the phospholipase C/PKC pathway, and subsequent phosphorylation of NKA leads to the inhibition of enzyme activity.

It has been reported that a defect in DA receptor function might contribute to hypertension in animal models as well as in human essential hypertension. For example, DA fails to inhibit NHE and NKA activities in the spontaneously hypertensive rat, and the natriuretic and diuretic response to exogenously administered as well as endogenous DA is also diminished in this model of hypertension. The molecular mechanism for this defect appears to be hyperphosphorylation of the D1 receptor caused by an increase in the constitutive activity of G-protein-coupled receptor kinase (GRK4) in proximal tubular epithelial cells from patients with essential hypertension.

Recently, we have reported that DA was unable to inhibit NHE and NKA activities in obese Zucker rats, which have moderate hypertension and also provide a model for type 2 diabetes. We found that this phenomenon was the result of reduced D1 receptor numbers and uncoupling of the D1 receptor from G proteins in obese Zucker rats. After treatment with rosiglitazone and the lowering of plasma insulin levels, we found that the inhibitory effect of DA on NKA activity was restored in treated obese Zucker rats. Therefore, it appears that hyperinsulinemia, a characteristic of these animals, might have contributed to the D1 receptor dysfunction. To test this hypothesis, we used primary proximal tubular epithelial cells (PTECs) and examined the effect of chronic exposure to insulin on SKF-38393, a D1-like receptor agonist-induced inhibition of NKA, and measured D1 receptors and their coupling with G proteins.

Methods

Materials

Cell culture media were purchased from Gibco-BRL. [3H]SCH-23390, [35S]GTPγS, and 86RbCl were purchased from NEM Life Sciences. Antibodies were purchased from Alpha Diagnostic Inter-
national and Calbiochem-Novabiochem. All other chemicals of the highest purity available were purchased from the Sigma-Aldrich Co.

Cell Culture
Male Sprague-Dawley rats (Harlan, Indianapolis, Ind) weighing 200 to 250 g were used to prepare and purify proximal tubule fragments, as previously described.22,23 The isolated proximal tubules were resuspended in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (DMEM/F12; 1:1, vol/vol) supplemented with 5.73 ng/mL insulin, 5 μg/mL transferrin, 40 ng/mL hydrocortisone, 5 ng/mL selenium, 4 pg/mL 3,3,5-triiodo-L-thyronine, 10 ng/mL epidermal growth factor, 1.2 mg/mL sodium bicarbonate, 0.29 mg/L l-glutamine, 25 U/mL penicillin, 25 μg/mL streptomycin, and 10% fetal calf serum. The biochemical markers Quigley and Gotterer26 with slight modification, as reported earlier.17

Insulin Treatment
Cells (80% to 90% confluent) were incubated without (control) or with insulin (100 nmol/L) for 24 hours in DMEM/F12. After 24 hours, the cells were washed and stabilized for 2 to 3 hours at 37°C in insulin-free DMEM/F12. Subsequently, the cells were used for the NKA assay or membrane preparation. Protein was determined with the use of an assay kit (Pierce).

NKA Assay
We used 2 methods to measure the activity of NKA.17,26 For ATP hydrolysis (method 1), NKA was determined by the method of Quigley and Gotterer26 with slight modification, as reported earlier.17 Cells were incubated without or with SKF-38393 for 10 minutes at 37°C. A cell suspension (0.05 mg protein/mL) was used to assay 1 mmol/L ouabain–sensitive NKA activity by using end-point phosphate hydrolysis of ATP (4 mmol/L). The inorganic phosphate released was determined colorimetrically. For 86Rb uptake (method 2), cells grown in 6-well plates were incubated without or with SKF-38393 for 10 minutes 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 (1.5 mL/well), and radioactivity was measured directly in cell lysate with a gamma counter. NKA activity was determined as the difference between 86Rb uptake in the absence and presence of ouabain.

[3H]SCH Binding
Confluent cells were starved of fetal calf serum for 24 hours in DMEM/F12 with or without insulin (10−7 mol/L). For ligand binding, 25 μg membrane protein was incubated with 4 mmol/L [3H]SCH-23390 in 250 μL (final volume) of binding buffer at 25°C for 1 hour. Nonspecific binding was determined in the presence of 1 μmol/L cold SCH-23390.27

Western Blotting of the D1A Receptor and Gs/11α Proteins
Western blotting of membrane proteins was performed as reported earlier.21,28 In brief, protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transblotted, and incubated with D1A receptor, Gs, or G11α protein-specific, affinity-purified polyclonal antibodies, followed by secondary antibodies conjugated with horseradish peroxidase. The protein bands were visualized with a chemiluminescence reagent kit and quantified by a protein software program (Kodak).

[35S]GTPγS Binding
The GTP binding assay was carried out according to the method of Northup et al.29 as we have reported earlier.16 The final reaction mixture of 90 μL (25 mmol/L HEPES, 15 mmol/L MgCl2, 1 mmol/L dithiothreitol, 100 mmol/L NaCl [pH 8.0], 5 μg protein, and ~100 000 counts per minute of [35S]GTPγS, with or without the D1 receptor agonist SKF-38393) was incubated for 1 hour at 30°C.16

Statistical Analysis
Data are presented as mean±SEM of the 3 to 5 experiments. Statistical analysis was performed with the unpaired Student t test or 1-way ANOVA.

Results
Confluent-monolayer cells showed a typical cobblestone-like appearance and expressed the D1A and PTH receptors and NHE isoform 3 (NHE3 is a distinctive marker for the proximal tubule because it is not expressed in distal tubules), as well as brush-border marker enzymes, alkaline phosphatase and γ-glutamyltranspeptidase. Single-cell monolayers also maintained their transport functions, as determined by sodium-dependent, phlorizin-sensitive glucose uptake. These results suggest that the cultured cells were PTECs and also had functional transport systems.

Effect of SKF-38393 on NKA Activity in Insulin-Treated Cells
At ~80% to 85% confluence, the cells were serum-starved for 24 hours in DMEM/F12 media. Thereafter, we examined the PTECs for a functional response to the D1-like receptor agonist, SKF 38393, and insulin by measuring NKA activity in intact cells. The cells were incubated without (basal) or with SKF-38393 or insulin for 10 minutes, and NKA activity was measured by colorimetric assay and 86Rb uptake. SKF-38393 (1 nmol/L to 1 μmol/L) produced a concentration-dependent inhibition of NKA activity (Figure 1A), and insulin (0.1 nmol/L to 1 μmol/L) produced stimulation of the enzyme activity (Figure 1B), suggesting the presence of functional D1 and insulin receptors in PTECs. Furthermore, the effects of SKF-38393 and insulin on NKA activity were consistent with earlier reports.30,31

Figure 1. Effect of (A) SKF-38393 and (B) insulin on Na,K-ATPase activity in primary proximal tubular epithelial cells. The basal activity of Na+,K+-ATPase was 403.99±20.23 nmol Pi/mg protein per min, or 17.57±0.70 nmol 86Rb/mg protein per min. The values represent mean±SEM of 5 or 6 experiments performed in triplicate.

Nonspecific binding of [35S]GTPγS was determined in the presence of 100 μmol/L cold GTPγS.

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To study the effect of chronic insulin treatment on SKF-38393–mediated inhibition of NKA activity, the cells were incubated with 100 nmol/L insulin for 24 hours in serum-free media. After 24 hours of treatment with insulin, the cells were washed with Krebs-Hanseleit buffer (KHB) and stabilized in insulin-free DMEM/F12 for 3 hours at 37°C, and the effect of SKF-38393 on NKA activity was determined colorimetrically (Figure 2A) or by 86 Rb uptake (Figure 2B). As shown in Figure 2A and 2B, SKF-38393 (100 nmol/L) produced inhibition of NKA activity in control cells but not in insulin-pretreated cells. The basal NKA activities in control (403.9±20.0 nmol Pi/mg protein per minute, or 17.5±0.7 nmol 86Rb/mg protein per minute) and chronically insulin-treated (408.9±21.9 nmol Pi/mg protein per minute, or 17.0±0.9 nmol 86Rb/mg protein per minute) cells were similar. It should be noted that NKA activity in this protocol was measured after 3 hours of stabilization of the cells in insulin-free medium, and we observed no difference in the basal NKA activity between control and insulin-treated cells, whereas in the experiment shown in Figure 1B, the control cells were exposed to insulin for 10 minutes, followed by immediate NKA activity measurement.

**[^3]H]SCH-23390 Binding and D1A Receptor Protein in Insulin-Treated Cells**

The specific binding of [^3]H]SCH-23390, a D1-like receptor ligand, was reduced by 42% in the membranes of insulin-treated cells compared with controls (Figure 3A). Because SCH-23390 is a nonselective ligand for D1-like receptors, we measured D1A receptor protein by Western blotting by using a specific D1A receptor antibody. This antibody labeled a single band with a molecular size of ~50 kDa. Densitometry of the bands revealed that the D1A receptor protein was decreased by 25% in the membranes of insulin-treated compared with control cells (Figure 3B). A similar decrease in D1A receptor protein was observed in whole-cell lysates of insulin-treated cells: control versus insulin-treated cells, 180.2±7.3 versus 138.5±6.1 arbitrary density units (Figure 3B).

**SKF-38393-Induced Stimulation of [^35]S]GTPγS Binding in Membranes of Insulin-Treated Cells**

As shown in Figure 4A, SKF-38393 (10 μmol/L) elicited a 21% stimulation of [^35]S]GTPγS binding in the membranes from control cells. However, the stimulatory effect of SKF-38393 was absent in membranes from insulin-treated cells. There was no significant difference in basal [^35]S]GTPγS binding in membranes from control (120±3.1 fmol/mg protein) and treated (117±5.2 fmol/mg protein) cells.

In addition to the stimulation of GTP proteins, the abundance of Gs and G11α proteins, known to be coupled to D1 receptors, was also measured in the membranes of control and insulin-treated cells. The antibodies for Gs recognized 2 bands (45 and 49 kDa), whereas the antibody for G11α...
Discussion

The present studies demonstrate that chronic exposure of PTECs to insulin abolished the ability of SKF-38393, a D1-like agonist, to inhibit NKA activity. Further experiments revealed that chronic exposure of PTECs to insulin caused a reduction in D1A receptor abundance, which was accompanied by the inability of SKF-38393 to stimulate G proteins, as measured by [35S]GTPγS binding.

DA, through activation of D1 receptors, is known to regulate sodium excretion by inhibiting sodium transport across PTECs.1-4 We have reported that D1 receptor numbers are reduced and that DA is unable to inhibit the sodium transporters NKA and NHE in the proximal tubules of obese Zucker rats.20,21 Furthermore, we have found that if insulin is lowered in these hyperinsulinemic rats, D1 receptor numbers were restored and DA was able to inhibit NKA activity, suggesting a role for insulin in the regulation of D1 receptor function.22 Because treatment of obese Zucker rats with rosiglitazone not only lowers their insulin level but also affects many other blood parameters, such as blood glucose and triglycerides,22 the present study, under more controlled experimental conditions (as described in Methods), shows the effect of insulin alone on the D1 receptor and its function in primary PTECs. Exposure of PTECs to insulin for 24 hours impaired the ability of SKF-38393 to inhibit NKA activity, as measured either colorimetrically or by 86Rb uptake. The reduced inhibition of NKA by SKF-38393 is not due to alteration in the pump activity per se in insulin-treated cells. This was evident from the finding that basal NKA activity, measured either by 86Rb uptake or colorimetrically, was similar in control and chronically insulin-treated cells.

Hyperinsulinemia is a result of insulin resistance in type 2 diabetes and is generally associated with obesity as a metabolic disorder.32 There are numerous studies that suggest hyperinsulinemia as a main cause of elevated renal sodium retention, followed by hypertension, whereas other studies have refuted this notion.33,34 However, insulin has been shown to alter the function of hormones and hormone receptors, which play roles in renal sodium metabolism and the development of hypertension.35,36 A correlation has been observed between hyperinsulinemia and a suppressed renal dopaminergic system, and this suppression of the renal dopaminergic system can contribute to increased sodium reabsorption in type 2 diabetes.37-41 It has also been shown that in healthy humans, an increase in insulin levels leads to a decrease in urinary DA and sodium excretion.40 In addition to reduced/impaired renal DA production, infusion of low-dose DA has been shown to produce a suppressed natriuretic response in type 2 diabetic patients, with or without hypertension, compared with control subjects.41 The reduced natriuretic response to DA in type 2 diabetes was further potentiated when the patients were pretreated with insulin.41 These studies suggest that it is hyperinsulinemia that might be causing impairment in the renal dopaminergic system and the subsequently blunted natriuretic response to exogenous DA in type 2 diabetic patients, irrespective of the state of hypertension.

Evidence to date indicates that insulin therapy causes cerebral edema in patients with diabetic ketoacidosis.42,43 Arief and Kleeman44,45 have reported that insulin enhances the transport of electrolytes into the central nervous system, which may cause addition of osmotically active particles to brain tissue that could cause an uptake of osmotic water. In humans, John et al46 suggested that the insulin-driven Na+/H+ antiport might increase intracellular fluid by promoting Na+ influx into brain cells. Intracranial pressure and fluid shift are controlled by a complex network of membrane pumps and channels, including some that are controlled by insulin.47 Hence, the presence of both sodium transporters in the brain and neuronal swelling in response to in vitro stimulation of the exchanger with insulin has been reported.47,48

In the present studies, we found that insulin treatment caused a significant decrease in the abundance of D1A receptor protein in membranes and in whole-cell homogenates, which might suggest a decrease in the synthesis of receptors and/or increased turnover of receptors. Insulin is known to regulate cell growth and to promote energy storage by interacting with its cell-surface receptors.49 Some insulin
effects occur through altered gene expression. Multiple examples of gene regulation by insulin at both transcriptional and posttranscriptional levels have been described in the last few years. However, the molecular mechanism by which insulin mediates its cytoplasmic and nuclear effects is poorly understood. Insulin responsive elements (IREs) have been recently identified for the c-fos, phosphoenolpyruvate carboxykinase, amylase, and glycerolaldehyde-3-phosphate dehydrogenase genes. In contrast to most sequences that control common regulatory events, these IREs do not share any homology. However, from the present study, it is too early to suggest whether D1 receptor genes do carry IREs. The other possible explanation for decreased D1 receptor protein in whole cells could be the mitogen-activated protein kinase pathway. Zhang et al have reported that insulin blocks the stimulatory effect of high glucose on angiotensinogen gene expression via the mitogen-activated protein kinase signal transduction pathway. Insulin has also been reported to regulate other renal hormone receptors, such as upregulation of angiotensin II AT1 receptors.

The complete loss in the ability of DA to inhibit NKA activity cannot be explained by a mere 25% reduction in the D1 receptor. It is likely that the remaining D1 receptors are unable to couple with G proteins and hence, cannot transduce the cell signaling responsible for inhibiting NKA activity. The GTPyS binding experiment revealed that SKF-38393 was unable to activate G proteins, suggesting defective coupling between the D1 receptor and G proteins. Because the abundance of Gs or Gq/11 and basal GTPyS binding were not reduced, defective coupling of the D1 receptor might not be attributable to these factors. These studies support our earlier findings of defective coupling of the D1 receptor to G proteins in basolateral membranes of obese Zucker rats. Overall, the results obtained in this study under controlled conditions suggest that insulin alone causes down-regulation of D1 receptors and prevents their interaction with G proteins. The mechanism of such a defective coupling/interaction remains to be determined. Nevertheless, our findings do provide a mechanism for insulin-induced impairment in D1 receptor function, which would explain the blunted natriuretic response to DA in type 2 diabetic patients and the reduced ability of DA to inhibit NKA activity in obese Zucker rats.

In conclusion, these results support our hypothesis that chronic exposure of PTECs to insulin causes a reduction in D1 receptor abundance and their uncoupling from G proteins, which might account for the inability of a D1 receptor agonist to inhibit NKA activity. Therefore, it can be suggested that hyperinsulinemia contributes to the defective D1 receptor function in the proximal tubules of obese Zucker rats and that by lowering insulin levels, D1 receptor function can be restored.

Perspective

The present studies have significant clinical relevance, because hyperinsulinemia is a common factor in obesity, diabetes, and hypertension, and our findings demonstrate that insulin alone can cause a significant reduction in D1 receptor numbers, along with receptor–G protein uncoupling. DA, via the D1 receptor, inhibits the sodium transporters in proximal tubules and plays an important role in sodium and water excretion, especially during high sodium intake. The present study shows that chronic exposure of PTECs to insulin could result in the near-complete loss of DA-induced NKA inhibition. Hence, it is likely that in conditions associated with hyperinsulinemia, the activity of endogenously produced renal DA or exogenously administered DA to promote sodium excretion will be compromised. Therefore, use of an alternative pharmacological agent (other than DA) would be required to promote renal sodium excretion in these hyperinsulinemic patients.

Acknowledgments

This study was supported by National Institute of Health grant DK-58743 from the National Institute of Diabetes, Digestive, and Kidney Diseases.

References


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Hypertension. 2003;41:1353-1358; originally published online April 21, 2003;
doi: 10.1161/01.HYP.0000069260.11830.CD
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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