Tempol Reduces Oxidative Stress, Improves Insulin Sensitivity, Decreases Renal Dopamine D1 Receptor Hyperphosphorylation, and Restores D1 Receptor–G-Protein Coupling and Function in Obese Zucker Rats

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Oxidative stress plays a pathogenic role in hypertension, particularly the one associated with diabetes and obesity. Here, we test the hypothesis that renal dopamine D1 receptor dysfunction in obese Zucker rats is caused by oxidative stress. One group each from lean and obese Zucker rats received tempol, a superoxide dismutase mimetic in drinking water for 2 weeks. Obese animals were hypertensive, hyperglycemic, and hyperinsulinemic, exhibited renal oxidative stress, and increased protein kinase C activity. Also, there was hyperphosphorylation of D1 receptor, defective receptor–G-protein coupling, blunted dopamine-induced Na^+-K^+-ATPase inhibition, and diminished natriuretic response to D1 receptor agonist, SKF-38393. However, obese animals had elevated levels of plasma nitric oxide and urinary cGMP. In addition, L-*N*-nitroarginine and sodium nitroprusside showed similar effect on blood pressure in lean and obese rats. In obese animals, tempol reduced blood pressure, blood glucose, insulin, renal oxidative stress, and protein kinase C activity. Tempol also decreased D1 receptor phosphorylation and restored receptor G-protein coupling. Dopamine inhibited Na^+-K^+-ATPase activity, and SKF-38393 elicited a natriuretic response in tempol-treated obese rats. Thus in obese Zucker rats, tempol ameliorates oxidative stress and improves insulin sensitivity. Consequently, hyperphosphorylation of D1 receptor is reduced, leading to restoration of receptor–G-protein coupling and the natriuretic response to SKF-38393. *Diabetes* 54: 2219–2226, 2005

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CML, carboxymethyllysine; DAG, 1,2-diacylglycerol; FE_{Na}, fractional excretion of sodium; GFR, glomerular filtration rate; GRK, G-protein–coupled receptor kinase; L-NNA, L-*N*-nitroarginine; MDA, malondialdehyde; PKC, protein kinase C; SNP, sodium nitroprusside; SOD, superoxide dismutase; [^{35}S]GTPγS, guanosine 5′-(γ-thio)triphosphate[^{35}S].

Diabetes and hypertension are two of the most common diseases, and the frequency of both diseases increases with concomitant obesity. Type 2 diabetes, which accounts for 80–90% of all diabetes, is characterized by a tendency to retain sodium (1–3). The origin of these changes in sodium homeostasis remains unclear. In experimental models of diabetes, several abnormalities of blood pressure regulation and sodium handling have been reported (1–4). It is likely that an overactivity of antinatriuretic factors and a dysfunction in natriuretic factors contribute to sodium retention (5–7). Dopamine promotes sodium excretion via activation of renal D1 receptors (8). Dopamine inhibits sodium reabsorption via inhibition of Na^+-K^+-ATPase and the Na^+-H^+ exchanger in renal proximal tubules (9,10). In many hypertensive states, the ability of the kidney to excrete sodium is diminished (11). Because there is a close relationship between renal D1 receptor function and urinary sodium excretion, it is speculated that a defect in renal dopamine receptor function may contribute to impaired sodium homeostasis and hypertension in diabetic patients (2,4).

Although the precise nature of renal D1 receptor dysfunction in human and animal models of hypertension and in obese Zucker rats remains to be elucidated, there is increasing evidence that the reduced renal effects of dopamine are due to defects in the D1 receptor itself, in both ligand binding and the receptor–G-protein coupling (7,11–13). We have shown that renal D1 receptors in obese Zucker rats are unable to bind ligands and couple to G-protein despite normal G-protein expression (7). We have also shown that dopamine failed to recruit D1 receptors from cytosol to cell membranes in proximal tubules from these animals (7).

Despite the coexistence of insulin resistance, abnormal glucose tolerance, hypertension, abdominal obesity, and dyslipidemia, the mechanism of the interaction remains unclear and controversial. Nevertheless, there is increasing evidence in the literature that recognizes oxidative stress as being associated with clustering of diabetes, obesity, and hypertension (14–20). In hypertension, oxidative stress is increased, and antioxidant defenses are

diminished (16). Antioxidant agents such as α-tocopharol, ascorbic acid, lipoic acid, and tempol are reported to lower blood pressure in hypertensive models (17–19). Tempol is a membrane-permeable and metal-independent superoxide dismutase (SOD) mimetic and has been used for the removal of intracellular and extracellular O_2^-. Although tempol does not scavenge H_2O_2, it prevents H_2O_2-mediated injury by reducing the intracellular concentrations of Fe^{2+} and hence the formation of hydroxyl radicals (20,21).

Previously, we have reported that the insulin sensitizer rosiglitazone improved insulin sensitivity and normalized blood glucose in obese Zucker rats (7). Recently, in a follow-up study, we were able to show that D1 receptor dysfunction in these animals is not intrinsic but contributed by factors associated with obesity and type 2 diabetes (22). Of the many factors that coexist with both diabetes and obesity is oxidative stress (14,23,24). Because obese Zucker rats are known to have high levels of oxidative stress (23,24), we investigated the possibility that antioxidant supplementation would reduce oxidative stress, improve insulin sensitivity, and restore renal D1 receptor function. We examined various markers of oxidative stress and D1 receptor function in control and tempol-supplemented lean and obese Zucker rats.

RESEARCH DESIGN AND METHODS

4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol); (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (SKF-38393), a D1 receptor agonist; L-*N*-nitroarginine (L-NNA); and sodium nitroprusside (SNP) were purchased from Sigma (Fluka/RBI). *R*-(+)-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol hydrochloride ([^{3}H]SCH-23390 hydrochloride), a D1 receptor antagonist, and guanosine 5′-(γ-thio)triphosphate[^{35}S] ([^{35}S]GTPγS) were purchased from NEM Life Sciences. Antibodies were purchased from Alpha Diagnostic International and Calbiochem-Novabiochem. The protein kinase C (PKC) assay kit was purchased from Promega. All other chemicals of highest purity available were purchased from Sigma-Aldrich.

Animal treatment. Nine-week-old male obese and lean Zucker rats (Harlan, Indianapolis, IN) were housed in plastic cages with free access to normal rodent diet. Animals (both lean and obese) were divided in two groups: one group received 1 mmol/l tempol in drinking water (n = 10), and the other group, which was kept on tap water (n = 10), served as control. Tempol-supplemented water was changed three times a day for 15 days.

Surgical procedures for renal function studies. Surgical procedures for blood pressure measurement and renal function studies were performed essentially as described in our previous publication (25). Rats were anesthetized with Inactin (100–150 mg/kg i.p.) and prepared for the measurement of blood pressure, heart rate, and intravenous drug administration. A stabilization period of 40 min was allowed before the administration of vasoactive agents L-NNA (10 mg/kg body wt; a nitric oxide synthase inhibitor) and SNP (a nitrovasodilator; 4–6 μg · kg body wt^{-1} · min^{-1}).

Experimental protocol for renal function studies. The effect of SKF-38393 on sodium and water excretion was determined in all four groups. The protocol consisted of a 45-min stabilization period after the surgery followed by five consecutive 30-min collection periods: C1, C2, D, R1, and R2. During C1 and C2, saline alone was infused; during D, SKF-38393 (3 μg · kg^{-1} · min^{-1} in saline) was infused; and during R1 and R2 (recovery), only saline was infused. Urine samples were collected throughout the 30-min periods, and blood samples (500 μl) were collected at the end of each period. Plasma was separated by centrifuging blood samples at 1,500*g* for 15 min at 4°C. Urine and plasma samples were stored at −20°C until analyzed for creatinine, sodium, and potassium (25).

Urine and plasma analysis. Sodium and potassium concentrations in the urine and plasma were measured using a flame photometer 480 (Ciba Corning Diagnostics, Norwood, MA). Plasma and urine creatinine levels were measured with a creatinine analyzer (model 2, Beckman, Fullerton, CA). Blood glucose and triglycerides were measured with a glucose analyzer (Roche Diagnostic) and triglyceride analyzer (Polymer Technology Systems). Plasma insulin was measured by radioimmunoassay using a rat insulin kit (RI-13k; Linco Research, St. Charles, MO). Plasma NO (nitrate + nitrite) levels were estimated by a colorimetric assay kit (Calbiochem, San Diego, CA). Urinary cGMP was measured with a cGMP ELISA kit (R & D Systems, Minneapolis, MN). Homeostasis model assessment, which incorporates measures of both fasting plasma concentrations of glucose and insulin, was used to calculate an index of insulin resistance as insulin (microunits per milliliter) × glucose (millimoles per liter)/22.5 (26). To evaluate renal function, urine volume was measured gravimetrically, and urine flow (milliliters per minute) was calculated. The fractional excretion of sodium (FE_{Na}) (percentage) was calculated based on clearance of sodium and creatinine. The glomerular filtration rate (GFR) (milliliters per minute) was calculated from creatinine clearance (25).

Preparation of renal proximal tubular suspension. Renal proximal tubular suspension was prepared as described previously (25). Briefly, enrichment of proximal tubules was carried out using 20% Ficoll gradient in Krebs buffer. The band at Ficoll interface was collected and washed by centrifugation at 250*g* for 5 min. Proximal tubular viability was checked using the Trypan blue exclusion test. Proximal tubules from a group of animals were treated with tempol (1 μmol/l–1 mmol/l) or SNP (1 μmol/l–1 mmol/l) for 40 min. Cell lysate, cytosol (35,000*g* supernatant), and membrane fractions (35,000*g* pellet) were isolated from proximal tubules by differential centrifugation, a standard method routinely used in our laboratory. Protein was determined by bicinchoninic acid method (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard.

Indexes (biomarkers) of oxidative stress. Proximal tubules were homogenized in buffer containing 10 mmol/l HEPES, 320 mmol/l sucrose, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, and protease inhibitor cocktail and centrifuged at 12,000*g* for 10 min. Carboxymethyllysine (CML) was measured by enzyme-linked immunosorbent assay as described by Koo and Vaziri (27), and malondialdehyde (MDA) was determined by the method of Urchiyama and Mihara (28).

Western blotting, immunoprecipitation, and serine-phosphorylation of D1A receptors. Membrane proteins were resolved on SDS-PAGE and transblotted as described previously (22,25). The specific antibodies were used to detect the D1A receptor proteins. The D1A receptor was immunoprecipitated as described by Jose et al. (13).

Measurement of [^{35}S]GTPγS binding. To determine the D1 receptor–G-protein coupling, membrane [^{35}S]GTPγS binding was stimulated by 1 μmol/l SKF-38393. Nonspecific [^{35}S]GTPγS binding was determined in the presence of 100 μmol/l unlabeled GTPγS. Specific binding was calculated as the difference between total and nonspecific binding (22,25).

Effect of SKF-38393 on Na^+-K^+-ATPase activity. Na^+-K^+-ATPase activity was determined as reported previously (9,25). Briefly, SKF-38393–induced Na^+-K^+-ATPase inhibition was determined in proximal tubular suspensions (1 mg protein/ml) incubated with or without 1 mmol/l ouabain at 37°C for 15 min.

[^{3}H]SCH binding. Fifty micrograms of membrane protein was incubated with 50 nmol/l [^{3}H]SCH-23390, a D1 receptor antagonist, in 250 μl (final volume) of binding buffer for 120 min at 25°C. Nonspecific binding was determined in the presence of 1 μmol/l unlabeled SCH-23390 (21,25).

PKC activity. PKC activity was determined by a commercially available PKC assay kit as detailed in our previous study (29).

Statistical analysis. Differences between means were evaluated using the unpaired t test or analysis of variance with Newman-Keuls multiple test, as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

As shown in Table 1, body weights of 11- to 12-week-old obese rats were markedly higher than lean rats. Plasma insulin levels of obese rats were nearly eight times higher than lean rats, whereas plasma glucose was elevated ~50%. In addition, the blood triglyceride levels were about ninefold higher in obese compared with lean rats. Together, these results suggest that obese Zucker rats present a typical model of type 2 diabetes with concomitant obesity. Tempol treatment normalized the blood glucose levels, because no significant difference was observed in glucose levels of obese treated animals compared with lean rats. Tempol treatment of obese rats caused 70 and 64% decreases in plasma insulin levels and blood triglyceride levels, respectively. However, the levels of plasma insulin and blood triglycerides of tempol-treated obese rats remained significantly higher than lean control/treated rats. Homeostasis model assessment showed that

TABLE 1
Effect of tempol on basal parameters

Parameter	LC	OC	LT	OT
Body wt (g)	261.0 ± 10.0	462.0 ± 14.0*	253.0 ± 8.0	448.0 ± 16.0*
Blood glucose (mmol/l)	5.27 ± 0.3	8.83 ± 0.7*	5.14 ± 0.4	5.80 ± 0.4†
Insulin (nmol/l)	0.57 ± 0.04	4.65 ± 0.40*	0.49 ± 0.05	1.4 ± 0.10*†
Triglycerides (mg/dl)	60.0 ± 4.0	430.0 ± 20.0*	52.0 ± 5.0	155.0 ± 10.0*†
MDA (nmol/mg protein)	0.55 ± 0.05	0.91 ± 0.08*	0.51 ± 0.07	0.59 ± 0.08†
CML (optical density/μg protein)	0.62 ± 0.07	1.21 ± 0.10*	0.55 ± 0.04	0.67 ± 0.08†
HOMA	0.26 ± 0.01	2.20 ± 0.10*	0.26 ± 0.01	0.73 ± 0.10*†
Mean blood pressure (mmHg)	89.0 ± 3.0	109.5 ± 3.0*	88.9 ± 5.0	99.6 ± 3.1†
GFR (ml/min)	0.95 ± 0.12	1.20 ± 0.32	0.77 ± 0.08	1.53 ± 0.3

Data ($n = 8$ animals) were analyzed by ANOVA followed by Newman-Keuls test multiple test. $P < 0.05$ was considered statistically significant. *Significantly different from LC and LT. †Significantly different from OC. HOMA, homeostasis model assessment; LC, lean control; OC, obese control; LT, lean treated; OT, obese treated.

tempol treatment significantly improved insulin sensitivity in obese animals but did not alter insulin sensitivity in lean rats. Plasma NO (nitrate + nitrite) levels were markedly increased in obese compared with lean rats (lean vs. obese 12.5 ± 0.6 vs. 26.0 ± 1.0.1 μmol/l). Urinary cGMP (nanomoles per milliliter of urine) was fourfold higher in obese animals compared with lean animals (lean versus obese 2.8 ± 0.6 vs. 9.11 ± 1.5 μmol/l). Furthermore, mean blood pressure was significantly elevated in obese control rats compared with lean control rats; tempol supplementation significantly reduced the blood pressure in obese rats compared with obese controls, but did not produce any change in mean blood pressure of lean rats. Although, tempol increased the GFR in obese animals, due to large variations, GFR in all the groups remained statistically nonsignificant (Table 1). Vasoactive agents L-NNA and SNP caused similar changes in blood pressure in lean and obese animals (L-NNA, mmHg increase over basal [lean, 7.8%; obese, 12.3%]; SNP, mmHg decrease over basal [lean, 44.1%; obese, 40.2%], $P < 0.05$).

Effect of tempol on oxidative stress. To determine oxidative stress in lean and obese rats, we measured MDA, a measure of lipid peroxidation, and CML, an index of advanced glycation. As shown in Table 1, the levels of renal MDA and CML were, respectively, 40 and 48% higher in obese animals compared with lean rats. Tempol treatment eliminated the increased oxidative stress in obese animals by normalizing the levels of both MDA and CML (Table 1).

Effect of tempol on D1 receptor expression. The specific binding of [^{3}H]SCH-23390, a D1 receptor antagonist was significantly reduced in proximal tubular membranes from obese rats compared with lean rats. Tempol treatment normalized the [^{3}H]SCH-23390 membrane binding in obese rats, while having no effect on ligand binding in lean rats (Fig. 1*A*). The Western blot analysis of D1A receptor revealed no significant difference in protein content of receptor in whole-cell lysate, cytosol, or membranes. Also, tempol did not affect the protein expression of D1A receptor in lean or obese rats (Fig. 1*B*). Treatment of proximal tubules from lean or obese animals with 1 mmol/l SNP or 1 mmol/l tempol had no effect on D1 receptor ligand binding (Table 2).

Effect of tempol on SKF-38393-induced D1 receptor–G-protein coupling. SKF-38393, a D1 receptor agonist, elicited a 35% increase in [^{35}S]GTPγS membrane binding in proximal tubules from lean rats but failed to stimulate [^{35}S]GTPγS membrane binding in obese rats (Fig. 2). Treatment with tempol restored the SKF-38393-induced [^{35}S]GTPγS membrane binding in proximal tubules from obese rats. There was no significant difference in basal [^{35}S]GTPγS membrane binding in proximal tubules from control or treated lean and obese animals. Tempol also showed no significant effect on [^{35}S]GTPγS membrane binding in lean rats. Incubation of proximal tubules with 1 mmol/l SNP or 1 mmol/l tempol did not cause any change in basal or SKF-38393–induced [^{35}S]GTPγS membrane binding from lean or obese rats (Table 2).

Effect of tempol on D1 receptor serine-phosphorylation. The uncoupling of the D1 receptor–G-protein could be due to receptor desensitization. We hypothesized that the D1 receptor in obese rats may already be phosphorylated in the basal state and therefore essentially desensitized. As shown in Fig. 3, the D1A receptors in proximal tubular membranes from obese animals were hyperphosphorylated at serine residues compared with lean rats. Treatment of obese rats with tempol normalized this D1A receptor serine phosphorylation.

Effect of tempol on PKC activity. As shown in Fig. 4, the basal PKC activity in renal proximal tubular homogenates was significantly higher in obese animals compared with lean rats. Tempol treatment decreased the elevated level of PKC activity in obese rats, but it did not affect PKC activity of lean rats.

Effect of tempol on dopamine-induced Na^+-K^+-ATPase inhibition. The incubation of renal proximal tubules from lean rats with dopamine (1 nmol/l–1 μmol/l) caused a concentration-dependent inhibition of Na^+-K^+-ATPase activity (Fig. 5). Incubation of proximal tubules from obese rats with similar concentrations of dopamine failed to induce significant inhibition of Na^+-K^+-ATPase activity. However, dopamine produced concentration-dependent inhibition of Na^+-K^+-ATPase in proximal tubules from tempol-treated obese animals. Tempol had no significant effect on tubular viability as well as on dopamine-induced Na^+-K^+-ATPase inhibition in lean rats. The basal Na^+-K^+-ATPase activity was similar in all four experimental groups. When proximal tubules from lean or obese animals were treated with SNP (1 μmol/l–1 mmol/l), a significant decrease in Na^+-K^+-ATPase activity was observed at concentrations >0.5 mmol/l in both lean and obese rats (Table 2).

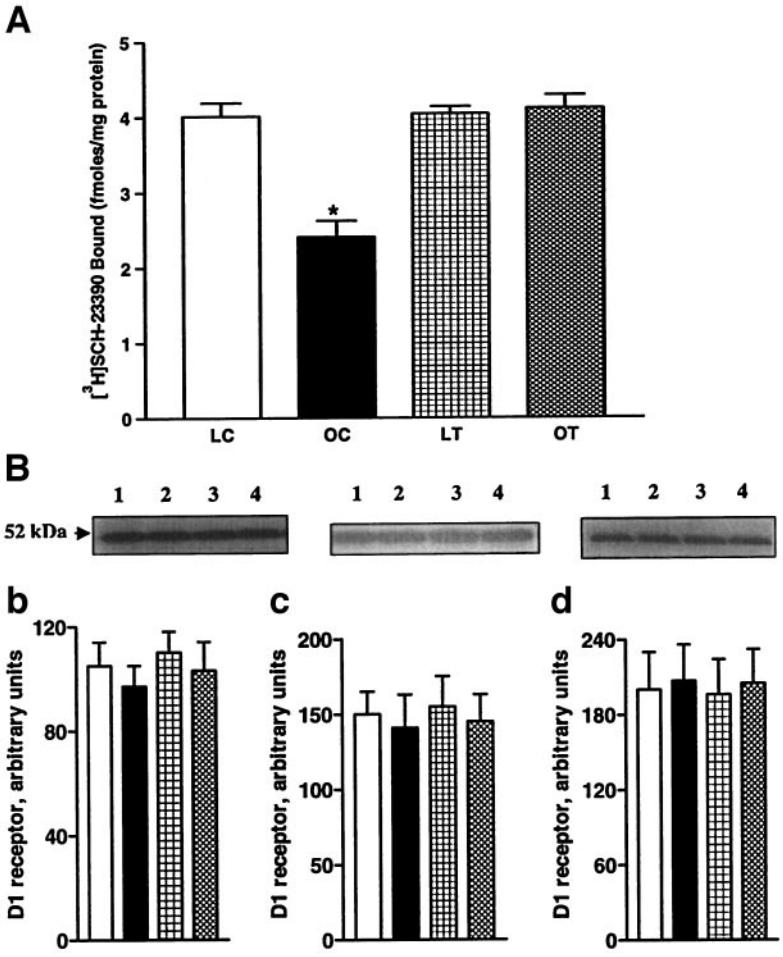

FIG. 1. ***A*****: Effect of tempol supplementation on [^{3}H]SCH-23390 binding in renal proximal tubular membranes of lean and obese Zucker rats. *Significantly different from other groups.** ***B*****: Effect of tempol supplementation on dopamine D_{1A} receptor protein expression in renal proximal tubules.** ***Top panel*****: Representative Western blots.** ***Bottom panel*****: Bars represent density (arbitrary units); means ± SEM of five to six different experiments (animals).** ***a*****: Whole-cell lysate, 8–10 μg of protein;** ***b*****: membrane, 2–4 μg of protein; and** ***c*****: cytosol, 15–18 μg of protein, from lean control (□), obese control (■), lean treated (⊞), and obese treated (▨). Data were analyzed by ANOVA followed by Newman-Keuls multiple test; $P < 0.05$ was considered statistically significant.**

Effect of tempol on D1 receptor–mediated sodium excretion. Intravenous administration of SKF-38393 (3 μg · kg · body wt^{-1} · min^{-1}) failed to increase urine flow and FE_{Na} in obese control rats (Fig. 6). However, in obese treated rats, SKF-38393 significantly increased urine flow and FE_{Na}. Tempol treatment did not alter the response to SKF-38393 in lean rats. Tempol treatment increased basal (C) fractional sodium excretion in lean as well as obese rats. No changes in mean blood pressure and heart rate were produced by SKF-38393 in any of the groups (data not shown).

DISCUSSION

The obese Zucker rat, a model of type 2 diabetes, also exhibits a moderate degree of hypertension (7,30). Moreover, these animals have a defect in dopamine D1 receptor function similar to the one observed in human essential hypertension and spontaneously hypertensive rats (11,12). Although a large body of data has accumulated to indicate increased oxidative stress in hypertension and type 2 diabetes (14–20), it is unclear whether this phenomenon is responsible for decreased insulin sensitivity, impaired D1

TABLE 2
Effect of 1 mmol/l SNP and 1 mmol/l tempol on [^{3}H]SCH-23390 and [35S]GTPγS binding and Na^+-K^+-ATPase activity in proximal tubules from lean and obese animals

	Vehicle		SNP		Tempol	
[^{3}H]SCH-23390 bound (fmol/mg protein)						
Lean	3.9 ± 0.2		4.1 ± 0.3		4.3 ± 0.4	
Obese	2.5 ± 0.2*		2.6 ± 0.2*		2.8 ± 0.3*	
[^{35}S]GTPγS bound (pmol/mg protein)	Control	SKF-38393	Control	SKF-38393	Control	SKF-38393
Lean	3.0 ± 0.2	4.5 ± 0.3†	3.3 ± 0.3	4.8 ± 0.3†	3.2 ± 0.4	4.6 ± 0.3†
Obese	3.3 ± 0.2	3.7 ± 0.3	3.5 ± 0.2	3.9 ± 0.3	2.9 ± 0.3	3.3 ± 0.3
Na^+-K^+-ATPase (nmol Pi · mg protein^{-1} · min^{-1})						
Lean	312.1 ± 11.2		243.4 ± 10.9‡§		320.3 ± 15.2	
Obese	329.5 ± 14.4		259.9 ± 9.8‡§		318.6 ± 16.5	

Data ($n = 6$ animals) were analyzed by ANOVA followed by Newman-Keuls test multiple test. $P < 0.05$ was considered statistically significant. *Significantly different from lean. †Significantly different from control. ‡Significantly different from vehicle. §Significantly different from tempol.

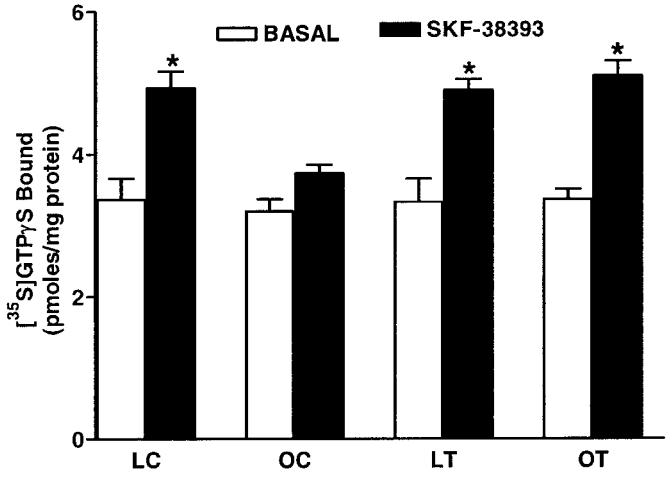

FIG. 2. Effect of tempol supplementation on SKF-38393–induced [^{35}S]GTPγS binding in renal proximal tubular membranes of lean control (LC), obese control (OC), lean treated (LT), and obese treated (OT) Zucker rats. *Significantly different from basal. Bars represent means ± SE of five to six different experiments (animals) performed in triplicate. Data were analyzed by ANOVA followed by Newman-Keuls multiple test; $P < 0.05$ was considered statistically significant.

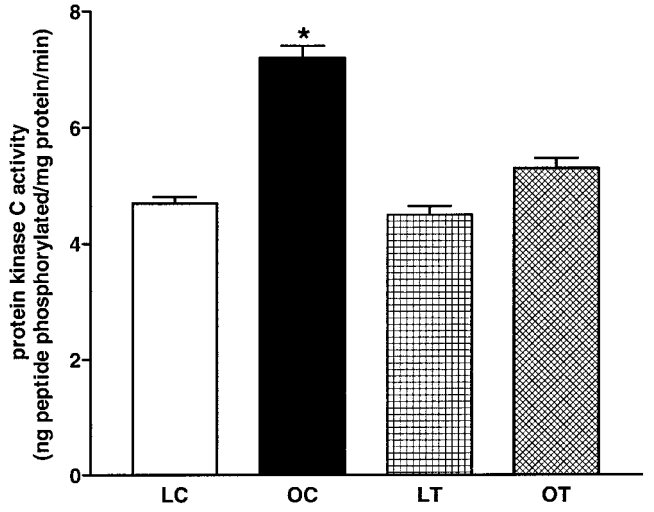

FIG. 4. Effect of tempol supplementation on PKC activity in renal proximal tubular homogenate of lean control (LC), obese control (OC), lean treated (LT), and obese treated (OT) Zucker rats. *Significantly different from other groups. Bars represent means ± SE of five to six different experiments (animals) performed in triplicate. Data were analyzed by ANOVA followed by Newman-Keuls multiple test; $P < 0.05$ was considered statistically significant.

receptor function, and increased blood pressure observed in obese Zucker rats. The results presented here provide evidence for the participation of oxidative stress in D1 receptor dysfunction and other abnormalities observed in obese Zucker rats, because the membrane-permeable free radical scavenger tempol can ameliorate oxidative stress, improve insulin sensitivity, decrease blood pressure, and restore D1 receptor function. Our data show that in obese Zucker rats, tempol treatment reduces renal oxidative stress, blood glucose, and blood pressure. It also normalized renal PKC activity and markedly lowered blood triglycerides and plasma insulin levels. Furthermore, tempol normalized the D1 receptor ligand binding and serine phosphorylation and restored D1 receptor–G-protein coupling and dopamine-induced Na^+-K^+-ATPase inhibition. The functional consequence of these changes was reflected in the restoration in the ability of D1 receptor agonist to promote sodium excretion.

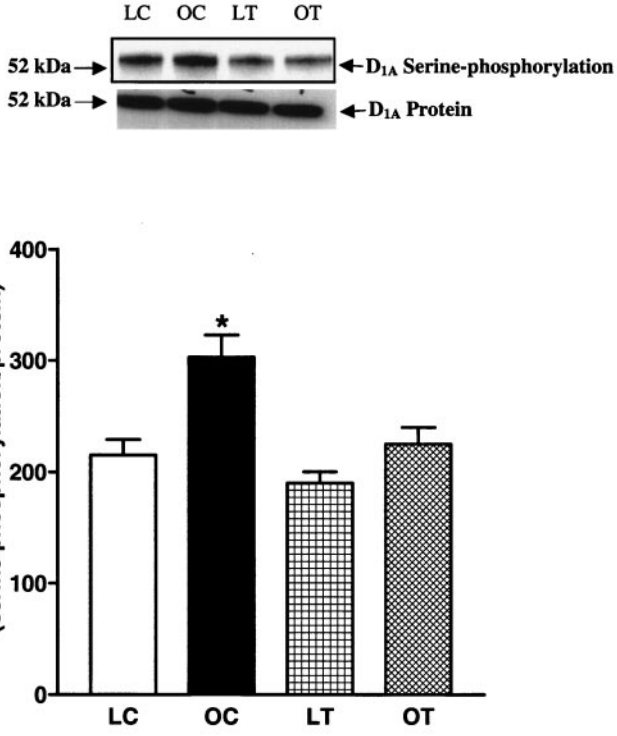

FIG. 3. Effect of tempol supplementation on serine–dopamine D_{1A} receptor phosphorylation in renal proximal tubular membranes of lean control (LC), obese control (OC), lean treated (LT), and obese treated (OT) Zucker rats. *Top panel*: Representative Western blots. *Bottom panel*: Bars represent densitometric analysis of serine-phosphorylation of D_{1A} receptor normalized to protein density. *Significantly different from other groups. Data (n = 5–6 animals) were analyzed by ANOVA followed by Newman-Keuls multiple test; $P < 0.05$ was considered statistically significant.

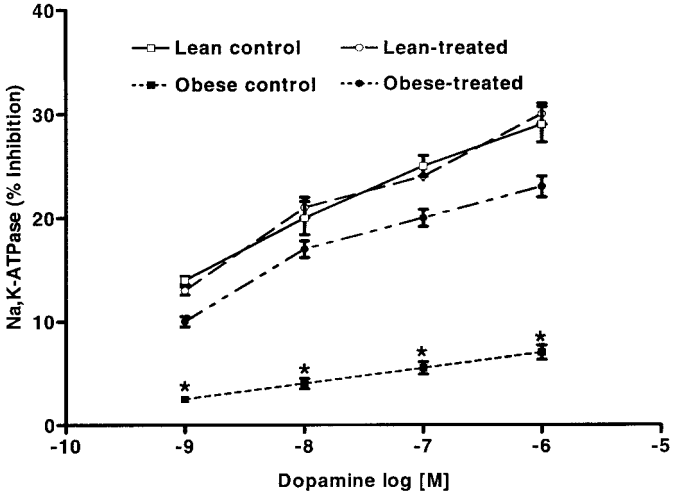

FIG. 5. Effect of tempol supplementation on dopamine-induced inhibition of Na^+-K^+-ATPase activity in renal proximal tubules of lean and obese Zucker rats. Basal Na^+-K^+-ATPase activity (nanomoles of P_i per milligram of protein per minute) was similar in all the experimental groups: lean control (LC), 307 ± 9; obese control (OC), 333 ± 16; lean treated (LT), 321 ± 12; obese treated (OT), 327 ± 14. *Significantly different from other groups at respective concentrations. Lines represent means ± SE of five to six different experiments (animals) performed in triplicate. Data were analyzed by ANOVA followed by Newman-Keuls multiple test; $P < 0.05$ was considered statistically significant.

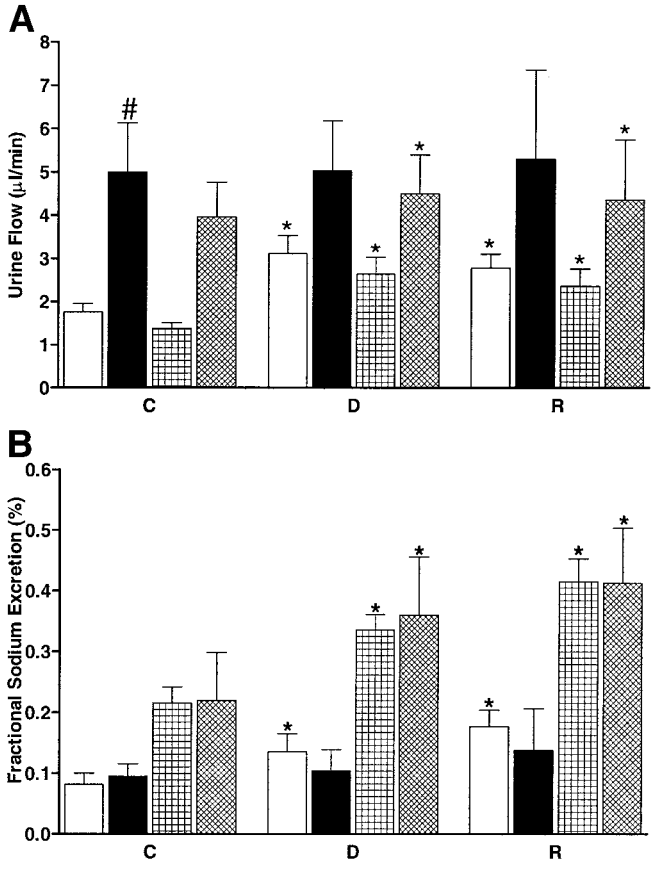

FIG. 6. Urine flow (*A*) and FE_{Na} (*B*) before, during, and after 3 μg · kg^{-1} · min^{-1} SKF-38393 in lean (□), obese (■), lean treated (⊞), and obese treated (▨) rats. C, basal values before drug administration; D, values during drug administration; R, values after drug infusion was terminated. All the time intervals (C, D, and R) were 30 min. Two control (C1 and C2) and two recovery (R and R2) collections were averaged and shown in figure. Bars represent means ± SE, respectively, $n = 4–6$; *$P < 0.05$ from control values of the same group using ANOVA and Neuman-Keuls test; $^{\#}P < 0.05$ from control lean rats using Student's unpaired *t* test.

There is compelling evidence that oxidative stress is an important contributing factor for a variety of cardiovascular disorders, and obese Zucker rats are known to have increased advanced glycation and lipooxidation end product, elevated plasma 8-epi-prostaglandin F_2, and increased NADH oxidase activity in retina (15,23,24). Our results also indicate that obese Zucker rats are under increased oxidative stress as evidenced by increased renal CML, a marker of advanced glycation end product, and MDA, an index of lipid peroxidation. One of the novel observations of our study is that 2-week treatment with tempol not only lowered the oxidative stress but also improved the insulin sensitivity and decreased blood pressure. Administration of antioxidant enzymes such as SOD and catalase has been shown to prevent or treat hypertension (31,32). However, the potential benefits of the systemic administration of SOD are limited, because SOD does not permeate biological membranes and is, therefore, unable to remove O_2^- produced intracellularly. Tempol is a membrane-permeable SOD mimetic and has been shown to attenuate hypertension (20). During hypertension, the endogenous vasodilatory effect of NO is prevented due to its interaction with ROS, specifically superoxide, which transforms NO to peroxynitrite and decreases its bioavailability, resulting in increased vascular resistance (33). Via its SOD mimetic action, tempol increases the bioavailability of NO to maintain regulation of normal blood pressure (33). Therefore, it can be speculated that because obese animals are known to have high oxidative stress, their NO levels and subsequent signaling could be compromised, and tempol may be exerting its beneficial effects by restoring NO levels. However, we found that NO and its second messenger levels are elevated in obese animals. Also, vasoactive agents such as L-NNA and SNP showed similar effects on blood pressure in lean and obese animals. In addition, our in vitro studies showed that a similar concentration of SNP was required to inhibit NKA activity in lean and obese proximal tubules. Although the scavenging of superoxide radicals remains the central mechanism for its blood pressure–lowering mechanism, the effect of tempol on direct sympathetic nerve activity inhibition and heme-oxygenase system is also well documented (34,35). Recently, Yang et al. (35) reported that tempol inhibits O_2^--induced degradation of hypoxia-inducible factor-1α. Hypoxia-inducible factor-1α mediates the transcriptional activation of many oxygen-sensitive genes like heme-oxygenase (*HO-1*). *HO-1* is expressed in kidney and metabolizes heme molecules to produce biliverdin and CO (36). CO plays an important role in the regulation of a variety of cell functions, including an increase in the production of cGMP by stimulating guanylate cyclase and activation of Ca^{2+}-dependent large-conductance K^+ channels, which may be responsible for CO-induced vasodilation of renal arterial vessels (36–38). It is suggested that tempol modulates the activity of redox sensitive *HO-1* and thus play a vital role in sustaining a close relationship between heme metabolism, kidney function, and blood pressure regulation (35,38).

A variety of different factors probably contribute to the defect in D1 receptor function in obese Zucker rats. We have observed D1 receptor dysfunction in streptozotocin-induced hyperglycemic rats and in renal proximal tubular cultures exposed to insulin or fatty acids (21,25). Obese Zucker rats exhibit hyperinsulinemia, hyperglycemia, dyslipidemia, and increased oxidative stress, and, thus, all of these factors can contribute to the impairment in D1 receptor function (7,23,24). Interestingly, in obese animals, treatment with tempol improved insulin sensitivity, decreased plasma insulin and blood triglycerides, and normalized blood glucose. Based on our observations, we suggest that the effect of tempol is due to its ability to improve insulin sensitivity leading to normalization of blood glucose and a marked decrease in triglycerides. The decrease in circulating insulin, glucose, and triglycerides can further decrease the oxidative stress and thus have a cumulative effect in restoring the D1 receptor response.

Various factors have been suggested to alter renal function in obesity including hyperinsulinemia and insulin resistance (39). The impaired pressure natriuresis in obese rats could be caused by either reduced GFR or increased sodium reabsorption. However, it is reported that in obese animals, both GFR and renal blood flow are significantly increased rather than decreased (40,41). Therefore, sodium retention and altered pressure natriuresis in these animals appear to be due to increased tubular sodium reabsorption. The causes of increased tubular sodium

reabsorption in obese Zucker have not been fully elucidated. Recent studies from our laboratory suggest that impaired D1 receptor function may be important in causing sodium retention in these animals (6,7). As observed in the present and previous studies (7), the natriuretic response to D1 receptor agonist is impaired in obese Zucker rats. In further examining the molecular mechanism contributing to D1 receptor dysfunction, we found that obese Zucker rats have elevated renal PKC activity, and D1A receptor hyper-serine phosphorylation and tempol supplementation normalized both PKC activity as well as serine phosphorylation.

The failure of dopamine to inhibit Na^+-K^+-ATPase cannot be explained by a mere loss of 40% receptor numbers/ligand binding. Theoretically, the remaining 60% receptors should enable the ligand to activate secondary enzyme complex and thus perform the downstream task. As revealed by [^{35}S]GTPγS binding experiments, SKF-38393 was unable to stimulate G-proteins, suggesting D1 receptor–G-protein uncoupling. Thus in obese animals, the defect in D1 receptor function could be attributed primarily to receptor–G-protein uncoupling, resulting in loss of functional response. It should be noted that in these animals, there is no reduction in G-proteins, effector enzyme activity per se, renal Na/H-exchanger, or Na^+-K^+-ATPase activity (6,7).

One of the mechanisms responsible for uncoupling may be hyperserine phosphorylation of D1 receptor in obese Zucker rats. Like most G-protein–coupled receptors, D1 receptor undergoes both a second messenger–dependent kinase and G-protein–coupled receptor kinase (GRK)-mediated phosphorylation reaction that leads to its desensitization (42,43). Although in the present study, we found increased PKC activity, it is reported that PKC cannot the phosphorylate D1A receptor because the receptor lacks the phosphorylation sites for PKC (44). Because GRKs are involved in the phosphorylation of D1 receptors, resulting in their desensitization, it is of interest that GRK-2 and -4 activities and expression are increased in patients with essential hypertension (13,45). Increased activity of GRK-2 could be responsible for the hyper-serine phosphorylation and desensitization of the D1 receptors because this kinase is activated by PKC, and we observed a significant increase in basal PKC activity (46). It is worth noting that we also observed increased expression of GRK-4 and translocation of GRK-2 from cytosol to membranes in proximal tubules of obese Zucker rats (47).

The observation that in obese rats tempol-normalized PKC activity along with D1A receptor serine phosphorylation provides a link between oxidative stress, PKC stimulation, and receptor phosphorylation. There is overwhelming evidence that PKC is stimulated by oxidative stress as well as hyperglycemia, both of which are present in obese rats (48–50). PKC is activated by 1,2-diacylglycerol (DAG) produced from receptor-mediated hydrolysis of inositol phospholipids. Both oxidative stress and hyperglycemia increase DAG synthesis. Oxidative stress increases DAG via hydrolysis of phosphatidylcholine by activating phospholipase D (48). Konish et al. (49) have shown that almost all PKC isoforms (namely α, β, and γ of cPKC; δ and ε of nPKC; and ξ of aPKC) are tyrosine phosphorylated and catalytically activated by H_2O_2. On the other hand, hyperglycemia increases de novo DAG synthesis from the glycolytic intermediate dihydroxyacetone phosphate through reduction of the latter to glycerol-3-phosphate and stepwise acylation. Increased de novo synthesis of DAG activates PKC both in cultured vascular cells and in retina and glomeruli of diabetic animals (50).

In conclusion, our studies provide substantial evidence that tempol, an SOD mimetic, can mitigate oxidative stress, improve insulin sensitivity, and restore D1 receptor–G-protein coupling and function in obese Zucker rats. At the cellular level, tempol decreased PKC activity, which could at least in part be responsible for normalization of D1 receptor serine phosphorylation and subsequent D1 receptor–G-protein coupling. These phenomena could account for restoration of dopamine-induced inhibition of Na^+-K^+-ATPase activity and the ability of dopamine to promote sodium excretion.

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