Mitogen-activated protein kinase upregulation reduces renal D1 receptor affinity and G-protein coupling in obese rats

AA Banday[1], FR Fazili[1], A Marwaha[1] and MF Lokhandwala[1]

[1]*Department of Phamacological and Pharmaceutical Sciences, College of Pharmacy, Heart and Kidney Institute, University of Houston, Houston, Texas, USA*

Reactive oxygen species play a key role in pathophysiology of cardiovascular diseases by modulating G-protein-coupled receptor signaling. We have shown that treatment of animal models of diabetes and aging with tempol decreases oxidative stress and restores renal dopamine D1 receptor (D1R) function. In present study, we determined whether oxidation of D1R and upregulation of mitogen-activated protein kinases (MAPK) were responsible for decreased D1R signaling in obese animals. Male lean and obese Zucker rats were supplemented with antioxidants tempol or lipoic acid for 2 weeks. Compared to lean, obese animals were hyperglycemic and hyperinsulinemic with increased oxidative stress, D1R oxidation and decreased glutathione levels. These animals had decreased renal D1R affinity and basal coupling to G-proteins. SKF-38393, a D1R agonist failed to stimulate G-proteins and adenylyl cyclase. Obese animals showed marked increase in renal MAPK activities. Treatment of obese rats with tempol or lipoic acid decreased blood glucose, reduced oxidative stress, and restored the basal D1R G-protein coupling. Antioxidants also normalized MAPK activities and restored D1R affinity and SKF-38393 induced D1R G-protein coupling and adenylyl cyclase stimulation. These studies show that D1R oxidation and MAPK upregulation contribute to D1R dysfunction in obese animals. Consequently, antioxidants while reducing the oxidative stress normalize the MAPK activities and restore D1R signaling.

Kidney International (2007) **71,** 397–406. doi:10.1038/sj.ki.5002055; published online 27 December 2006

KEYWORDS: dopamine; D1 receptor; G-proteins; lipoic acid; MAP kinase; tempol

Correspondence: *MF Lokhandwala, College of Pharmacy, S & R-2 Bldg, University of Houston, 4800 Calhoun RD, Houston, Texas 77204, USA. E-mail: mlokhandwala@uh.edu*

Received 18 April 2006; revised 31 October 2006; accepted 7 November 2006; published online 27 December 2006

Renal dopamine system is recognized as an important modulator of sodium balance and blood pressure.[1] During increased sodium intake, dopamine causes more than 50% of sodium excretion and participates in maintaining sodium homeostasis.[2] Dopamine exerts its natriuretic and diuretic actions via activation of D1 receptors in the renal proximal tubules.[3,4] In proximal tubules, D1 receptors inhibit activities of Na/H exchanger 3 (NHE3) and Na/phosphate (Pi) co-transporter in luminal membrane and Na/HCO co-transporter and Na/K ATPase (NKA) in basolateral membrane (BLM).[1,4–6] This action of dopamine on NHE3 and Na/HCO co-transporter activities is due to activation of cyclic adenosine mono phosphate (cAMP)/protein kinase A pathway.[1,6–8] Dopamine can inhibit NKA activity by activating G-protein-linked pathway independent of protein kinase A while involving protein kinase C (PKC) and phosphatidylinositol 3 kinase.[7,9,10]

Abnormalities in renal dopamine receptor function are described in human genetic hypertension and abnormal renal D1 receptor function contributes to the development of genetic hypertension and hypertension associated with obesity and diabetes in animals.[1,10–13] The failure of renal dopamine system is not caused by decreased renal dopamine production but by a defective D1 receptor G-protein coupling.[1,10] Although the reduced receptor expression and/or increased receptor phosphorylation have been shown to cause receptor G-protein uncoupling, the exact mechanism is still elusive.[10–13]

Previously, we have shown that in obese Zucker rats, an animal model of obesity and type II diabetes, the defect in renal dopamine D1 receptor was associated with increased oxidative stress.[13] Treatment of these animals with tempol, a superoxide dismutase (SOD) mimetic compound or with insulin sensitizer rosiglitazone decreased oxidative stress and restored D1 receptor G-protein coupling and function.[11,13] Similar observations were made in streptozotocin treated Sprague–Dawley rats and old Fisher 344 rats where antioxidant tempol decreased oxidative stress and restored D1 receptor function, thus indicating that oxidative stress contributes to decreased D1 receptor functioning.[14,15] However, the precise mechanisms

responsible for oxidative stress-associated D1 receptor defect remained to be elucidated.

Reactive oxygen species serve as second messengers in various signal transduction pathways.[16–19] Among many intracellular signaling molecules, reactive oxygen species-induced cellular events have been implicated in part to activation of mitogen-activated protein kinases (MAPK), including the extracellular signal regulated kinase (ERK) 1/ERK2, c-jun NH_2-terminal kinase (JNK), and p38 MAPK.[16–19] Studies have shown that MAPK are activated in cardiovascular diseases such as diabetes and hypertension.[16–19] Furthermore, it is shown that MAPK can modulate dopamine receptor function in various organs including kidneys.[20] The present study was conducted to elucidate the role of MAPK in renal proximal tubular D1 receptor dysfunction of obese Zucker rats.

RESULTS

As shown in Table 1, body weight and blood glucose were significantly higher in obese rats compared to lean rats. Treatment with tempol or lipoic acid showed no effect on the body weight of obese animals but markedly reduced their blood glucose. As shown in Table 2, tempol or lipoic acid showed no effect on body weight and blood glucose of lean rats. Tempol and lipoic acid did not change the water or food intake in lean or obese animals (data not shown).

Antioxidants reduced oxidative stress in obese animals

Compared to lean rats, obese animals had significantly higher plasma and urinary 8-isoprostane levels (Table 1). Renal proximal tubules from obese rats showed significant decrease in reduced glutathione along with increased levels of oxidized glutathione compared to lean rats (Table 1). In addition, the renal nitrotyrosine (Figure 1) and carboxymethyl lysine (Table 1) levels were significantly higher in obese compared to lean rats.

As illustrated in Figure 1b, renal D1 receptors from obese rats have increased carbonyl content compared to lean rats. The Western blotting analysis of various subunits of renal nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase in obese animals did not show any upregulation of this enzyme compared to lean rats (Figure 2a–d). In addition, the low-temperature sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting of endothelial nitric oxide synthase (NOS) showed similar monomer vs dimer expression in lean and obese rats (data not shown). Furthermore, incubation of homogenates with dihydroethidium and NAD(P)H oxidase or NOS substrates caused similar O_2^- production (fluorescence units/min/mg protein) in lean and obese animals, NAD(P)H oxidase – lean: 891.0 ± 121.0, obese: 1049.0 ± 117.0; NOS – lean: 651.0 ± 85, obese: 819.0 ± 113.0. Taken together, these data suggest that NAD(P)H oxidase and NOS are not the predominant source of superoxide production in obese animals. Treatment of obese animals with tempol or lipoic acid significantly decreased the plasma and urinary 8-isoprostane levels, renal nitrotyrosine and carboxymethyllysine levels, and D1 receptor carbonyl content and also restored the renal glutathione levels in obese animals (Table 1 and Figure 1 a and b). In lean rats, antioxidant

Table 1 | Effect of tempol and lipoic acid on body weight, glucose, and oxidative markers in lean and obese Zucker rats

	Lean	Obese	Obese-tempol	Obese-lipoic acid
Body weight, g	280.5 ± 12.0	490.0 ± 16.2^a	540.5 ± 30.2^a	533 ± 20.9^a
Blood glucose, mg/dl	109.1 ± 6.2	209.0 ± 8.2^a	$149.0 \pm 4.3^{a,b}$	$139.3 \pm 3.8^{a,b}$
8-isoprostane, urine (pg/mg creatinine)	593.0 ± 29.2	992.0 ± 32.1^a	625.0 ± 26.3^b	670.0 ± 31.6^b
8-isoprostane, plasma (pg/mg creatinine)	32.1 ± 2.2	49.1 ± 2.4^a	36.1 ± 2.0^b	37.9 ± 2.0^b
CML (optical density/μg protein)	0.38 ± 0.06	1.4 ± 0.1^a	0.54 ± 0.08^b	0.59 ± 0.1^b
GSH nmol/mg protein	0.89 ± 0.05	0.44 ± 0.03^a	1.0 ± 0.1^b	0.99 ± 0.09^b
GSSG pmol/mg protein	28.0 ± 1.1	50.0 ± 3.1^a	32.0 ± 2.9^b	34.0 ± 2.3^b

CML, carboxymethyl lysine; GSH, reduced glutathione; GSSG, oxidized glutathione.
Data (mean $\pm$ s.e.m. of 6–8 different animals) were analyzed by ANOVA and *post hoc* Newman–Keuls multiple comparison test.
$P < 0.05$ was considered statistically significant.
[a]Significantly different from lean rats.
[b]Obese-tempol and obese-lipoic acid significantly different from obese rats.

Table 2 | Effect of tempol and lipoic acid on body weight, glucose, and oxidative markers in lean Zucker rats

	Lean	Lean-tempol	Lean-lipoic acid
Body weight, g	280.5 ± 12.0	310.5 ± 10.0	308.5 ± 11.2
Blood glucose, mg/dl	109.1 ± 6.2	104.1 ± 6.9	100.1 ± 5.3
8-isoprostane, urine (pg/mg creatinine)	593.0 ± 29.2	543.0 ± 22.3	545.0 ± 34.3
8-isoprostane, plasma (pg/mg creatinine)	32.1 ± 2.2	30.1 ± 3.1	29.9 ± 1.6
CML (optical density/μg protein)	0.38 ± 0.06	0.35 ± 0.05	0.34 ± 0.07
GSH nmol/mg protein	0.89 ± 0.05	0.95 ± 0.07	1.0 ± 0.2
GSSG pmol/mg protein	28.0 ± 1.1	22.0 ± 1.9	23.0 ± 1.3

CML, carboxymethyl lysine; GSH, reduced glutathione; GSSG, oxidized glutathione.
Data (mean $\pm$ s.e.m. of 6–8 different animals) were analyzed by ANOVA and *post hoc* Newman–Keuls multiple comparison test.
$P < 0.05$ was considered statistically significant.

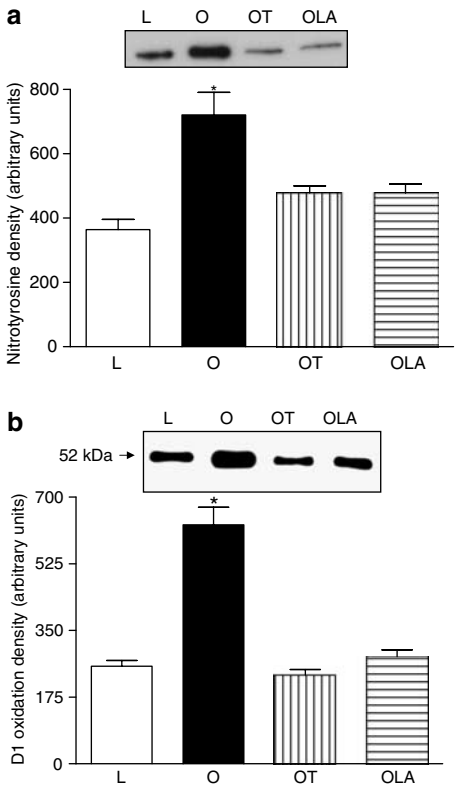

Figure 1 | Effect of antioxidants on oxidative stress. Effect of tempol (T) and lipoic acid (LA) supplementation on (**a**) nitrotyrosine and (**b**) D1 receptor oxidation, in renal proximal tubules from lean (L), obese (O), obese-tempol (OT), and obese-lipoic acid (OLA) rats. Upper panel: representative Western blot. Bars represent density (arbitrary units), mean ± s.e.m. of 5–6 different experiments (animals). Data were analyzed by ANOVA followed by Newman–Keuls multiple test; $P < 0.05$ was considered statistically significant. *Obese (O) significantly different from other groups.

supplementation showed no effect on either oxidative or antioxidative markers (Table 2).

Antioxidants restored renal D1 receptor agonist affinity in obese animals

To determine the affinity of D1 receptors with agonist, we used fenoldopam (FD) to displace [^{3}H]SCH-23390 binding in proximal tubular membranes from lean and obese rats. The agonist affinity for both high- and low-affinity receptors was reduced by a one-log unit of FD concentration in obese compared with lean rats (Figure 3, inset). Treatment with tempol or lipoic acid restored the affinity of renal D1 receptors in obese animals (Figure 3 and inset). Tempol or lipoic acid had no effect on D1 receptor affinity in lean rats (Table 3).

Antioxidants increased co-immunoprecipitation of renal D1 receptors with Gαs in obese animals

In assessing specific coupling of D1 receptors to G-proteins, we employed direct [^{3}H]SCH-23390 binding in immunoprecipitates of Gαs proteins obtained from solubilized membranes of renal proximal tubules. The results shown in Figure 4a demonstrate that Gαs antiserum immunoprecipitated [^{3}H]SCH-23390 binding sites in obese Zucker rats are significantly reduced compared to lean. In obese animals treated with tempol or lipoic acid, the co-immunoprecipitated D1 binding sites with Gαs antiserum were similar to lean rats. Gαi antiserum failed to co-immunoprecipitate significant D1 binding sites. Immunoprecipitates of Gαs antisera were also blotted with a specific D1A and Gαs antibodies. The data presented in Figure 4b shows that the D1A protein that co-immunoprecipitated with Gαs was significantly reduced in obese rats compared to lean rats and treatment with tempol or lipoic acid restored the co-immunoprecipitation of D1α with Gαs proteins. Gαs antisera immunoprecipitated similar amount of Gαs protein both in lean, obese, and antioxidant-supplemented obese rats (Figure 4c). Tempol and lipoic acid showed no effect on basal D1 receptor G-protein coupling in lean rats (data not shown).

Antioxidants restored SKF-38393-induced [^{35}S]GTPγS binding to Gαs proteins in obese animals

Incubation of membranes with SKF-38393 followed by immunoprecipitation with Gαs antiserum showed significant increase in [^{35}S]GTPγS membrane binding in lean rats (Figure 5). However, SKF-38393 failed to increase [^{35}S]GTPγS membrane binding in obese rats. Treatment with tempol or lipoic acid restored the SKF-38393-induced [^{35}S]GTPγS binding in obese rats. In contrast, co-immunoprecipitation with Gαi failed to show the SKF-38393-induced increase in [^{35}S]GTPγS. The activation of G-proteins was D1 receptor specific as the effect of D1 agonist SKF-38393 was completely blocked by D1 antagonist SCH-23390. In lean rats neither tempol or lipoic acid had any effect on SKF-38393-induced GTPγS binding (data not shown).

Antioxidants restored SKF-38393-mediated cAMP production in obese animals

Incubation of proximal tubular membranes with D1 selective agonist SKF-38393 from lean rats resulted in significant elevations in cAMP production. The results summarized in Table 4 indicate that adenylyl cyclase activation in response to SKF-38393 was minimal in membranes from obese rats. Treatment with tempol or lipoic acid restored the SKF-38393-dependent cAMP accumulation in obese rats. Direct enzyme stimulation with forskolin was similar in lean and obese rats (Table 4). Tempol and lipoic acid did not effect the basal or forskolin-induced adenylyl cyclase stimulation (Table 3).

We measured the basal expression and activities of NKA and NHE3, the major sodium transporters of renal proximal tubules in BLM and brush border (apical) membrane (BBM), respectively. No difference was observed in protein abundance (data not shown) or activities of these transporters between lean and obese animals, NKA activity (ouabain-sensitive inorganic phosphate release, nmol/mg protein/min) – lean: 269.0 ± 22.0, obese: 248.0 ± 19.0; NHE3 activity (amiloride-sensitive $^{22}Na^+$ uptake, nmol/mg protein/min) – lean: 2.3 ± 0.2, obese: 2.6 ± 0.3.

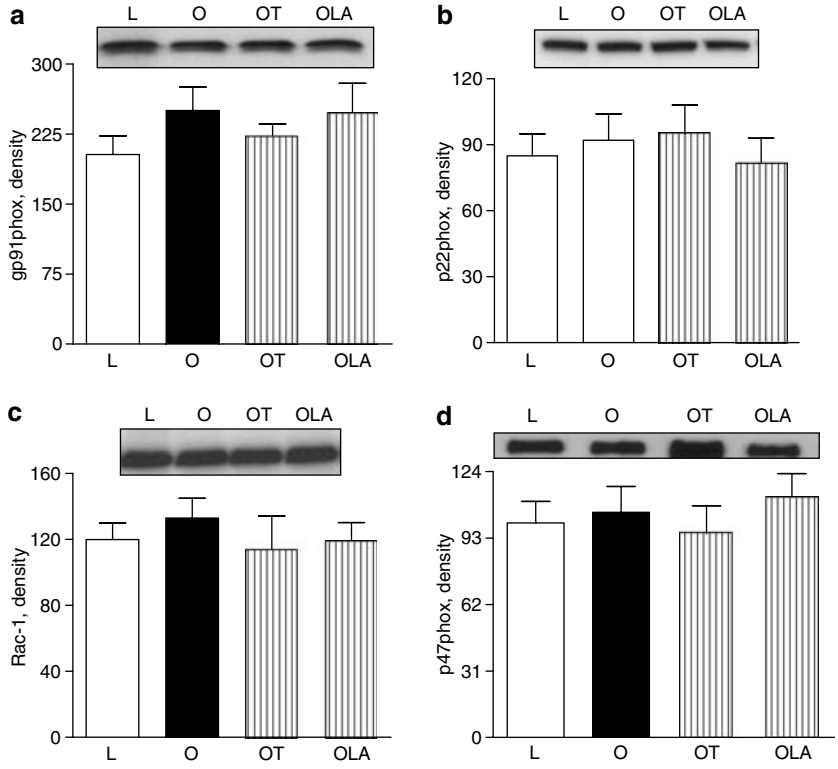

Figure 2 | Effect of antioxidants on NAD(P)H protein expression. Effect of tempol (T) and lipoic acid (LA) supplementation on basal NAD(P)H oxidase protein expression, (**a**) gp91phox, (**b**) p22phox, (**c**) Rac-1, and (**d**) p47phox in renal proximal tubules from lean (L), obese (O), obese-tempol (OT), and obese-lipoic acid (OLA) rats. Upper panels: representative immunoblots. Bars represent density (arbitrary units), mean ± s.e.m. of 5-6 different experiments (animals). Data were analyzed by ANOVA followed by Newman–Keuls multiple test; $P < 0.05$ was considered statistically significant.

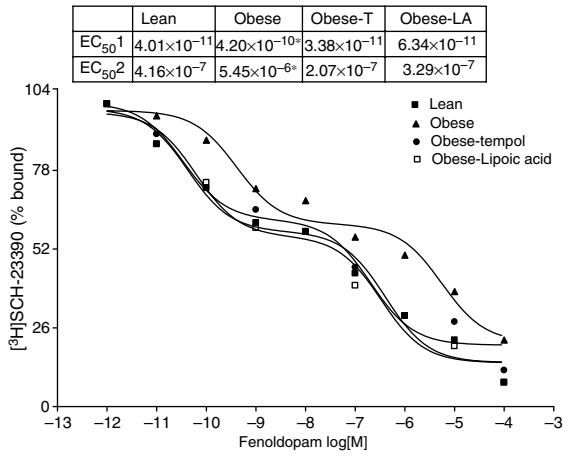

Figure 3 | Effect of tempol (T) and lipoic acid (LA) supplementation on FD displacement of [^{3}H]SCH-23390 in renal proximal tubular membranes from lean, obese, obese-tempol, and obese-lipoic acid rats. Total binding was determined in the absence of any drug, whereas nonspecific binding was determined in the presence of unlabelled SCH-23390 (10 μM). Competition curve was plotted with the percentage of radioligand bound values obtained as means ± s.e. from 4–5 different experiments (animals). Data were analyzed by ANOVA followed by Newman–Keuls multiple test; $P < 0.05$ was considered statistically significant. *Obese significantly different from other groups.

Table 3 | Effect of tempol and lipoic acid on basal D1 receptor affinity, NKA, and NHE3 activity and SKF-38393-induced adenylyl cyclase activation in renal proximal tubular membranes from lean rats

	Lean	Lean-tempol	Lean-lipoic acid
$EC_{50}1$	5.92×10^{-11}	4.58×10^{-11}	3.78×10^{-11}
$EC_{50}2$	4.72×10^{-7}	3.85×10^{-7}	4.22×10^{-7}
NKA activity			
Basal	288.6 ± 20.6	269.2 ± 22.1	255.1 ± 27.9
NHE3			
Basal	2.3 ± 0.2	2.5 ± 0.2	2.1 ± 0.3
Adenylyl cyclase activity			
Basal	55.2 ± 4.4	50.2 ± 3.4	53.2 ± 3.2
SKF-38393 (100 μM)	78.2 ± 4.9[a]	80.0 ± 4.9[a]	74.1 ± 3.2[a]
Forskolin (10 μM)	188.2 ± 12.3[a]	201.2 ± 11.3[a]	195.0 ± 16.4[a]

cAMP, cyclic adenosine mono phosphate; NHE3, Na/H exchanger 3; NKA, Na/K ATPase.
Effective concentration (EC_{50}) is expressed as [M], NKA and NHE3 are expressed as nmol pi/mg protein/min and nmol $^{22}Na^+$/mg protein/min, respectively. Adenylyl cyclase activity was determined as cAMP accumulation (fmol/mg protein). Data (mean ± s.e.m. of 6-8 different animals) were analyzed by ANOVA and *post hoc* Newman-Keuls multiple comparison test. $P < 0.05$ was considered statistically significant.
[a]Significantly different from respective basal.

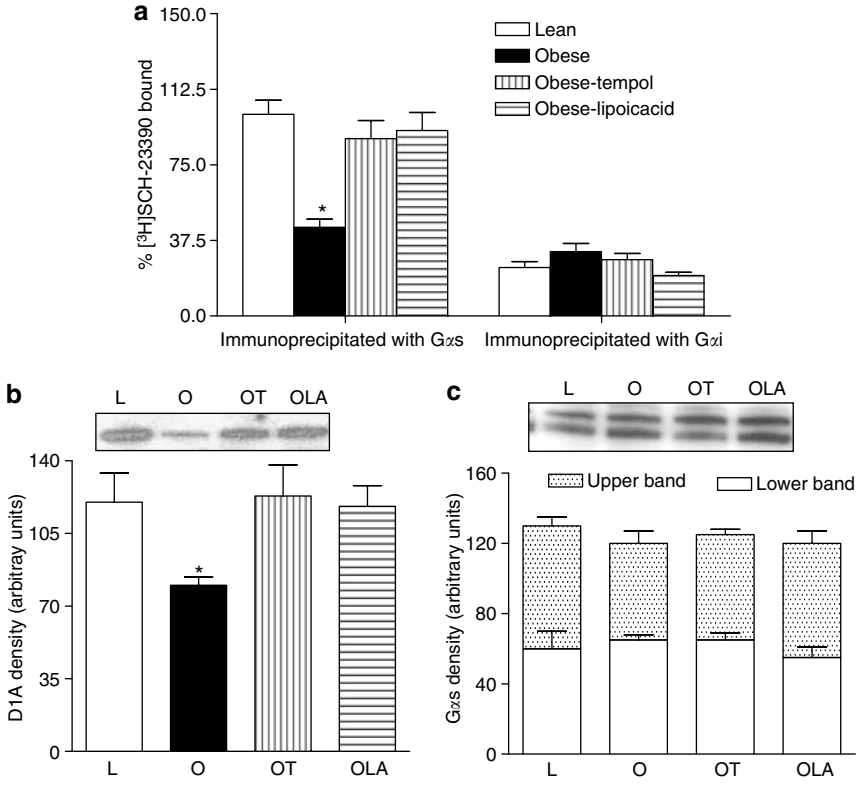

Figure 4 | Effect of tempol (T) and lipoic acid (LA) supplementation on basal D1 receptor G-protein coupling in renal proximal tubular membranes from lean (L), obese (O), obese-tempol (OT), and obese-lipoic acid (OLA) rats. Membrane G-proteins were immunoprecipitated with Gαs or Gαi antibodies and incubated with (**a**) D1 receptor ligand [^{3}H]SCH-23390 or immunoblotted for (**b**) D1α and (**c**) Gαs protein. Upper panel (**b** and **c**): representative immunoblots. Bars represent (**a**) specific [^{3}H]SCH-23390 binding, (**b** and **c**) density arbitrary units, mean ± s.e.m. of 5–6 different experiments (animals). Data were analyzed by ANOVA followed by Newman–Keuls multiple test; $P < 0.05$ was considered statistically significant.

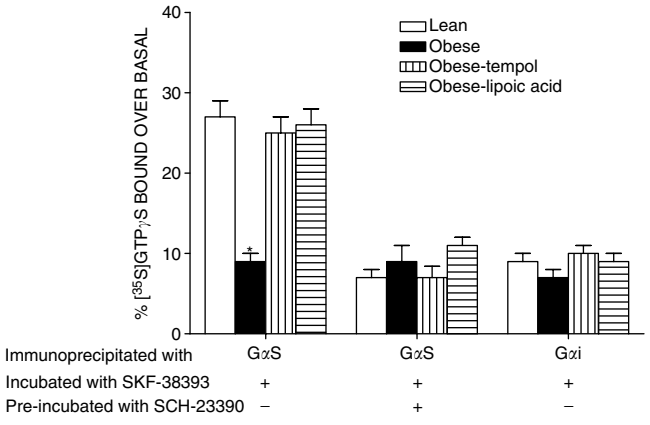

Figure 5 | Effect of tempol and lipoic acid supplementation on SKF-38393-induced D1 receptor G-protein coupling in renal proximal tubular membranes from lean, obese, obese-tempol, and obese-lipoic acid rats. Membranes were incubated with [^{35}S]GTPγS in the presence or absence of SKF-38393 and G-proteins were immunoprecipitated with Gαs or Gαi antibodies. Bars represent specific binding, mean ± s.e.m. of 5–6 different experiments (animals). Data were analyzed by ANOVA followed by Newman–Keuls multiple test; $P < 0.05$ was considered statistically significant.

Antioxidants normalized the basal activities of renal ERK1/2, p38, and JNK in obese animals

As shown in Figure 6a–c, the activities of ERK1/2, p38, and JNK were significantly higher in obese rats compared to lean rats. To investigate the role of oxidative stress on MAPK activation, we studied the effect of tempol and lipoic acid on ERK1/2, p38, and JNK activation. As illustrated in Figure 6a–c, tempol and lipoic acid normalized the ERK1/2, p38, and JNK activities to basal levels in obese animals.

Incubation of proximal tubules from lean and obese animals with 25 μM H_2O_2 for 30 min caused significant increase in malondialdehyde levels; however, the increase was more pronounced in lean proximal tubules compared to obese (Figure 7a). Interestingly, incubation of proximal tubules from lean rats with H_2O_2 caused significant increase in ERK1/2 (Figure 7b) and D1 receptor downregulation and uncoupling from G-proteins (Figure 8a and b). Inhibition of H_2O_2-induced ERK1/2 kinase activation with U-0126 (Figure 7b) attenuated H_2O_2-mediated D1 receptor desensitization (Figure 8a and b). H_2O_2 failed to reduce D1 receptor expression and coupling or upregulate ERK1/2 in proximal tubules from obese animals (Figures 7 and 8). Also, U-0126 failed decrease ERK1/2 activity or restore D1 receptor signaling in proximal tubules of these animals (Figures 7 and 8).

Table 4 | Effect of tempol and lipoic acid on SKF-38393-induced adenylyl cyclase activation in renal proximal tubular membranes from lean and obese animals

	Lean	Obese	Lean-tempol	Obese-lipoic acid
Basal	55.2 ± 4.4	53.2 ± 2.2	50.0 ± 4.2	52.1 ± 3.9
SKF-38393 (100 μM)	78.2 ± 4.9[a]	64.1 ± 3.8	70.0 ± 4.2[a]	73.1 ± 3.6[a]
Forskolin (10 μM)	188.2 ± 12.3[a]	209.0 ± 16.9[a]	216.6 ± 14.3[a]	206.2 ± 17.9[a]

Adenylyl cyclase activity was expressed as cAMP accumulation (fmol/mg protein). Data (mean ± s.e.m. of 6–8 different animals) were analyzed by ANOVA and *post hoc* Newman–Keuls multiple comparison test. $P < 0.05$ was considered statistically significant.
[a]Significantly different from respective basal.

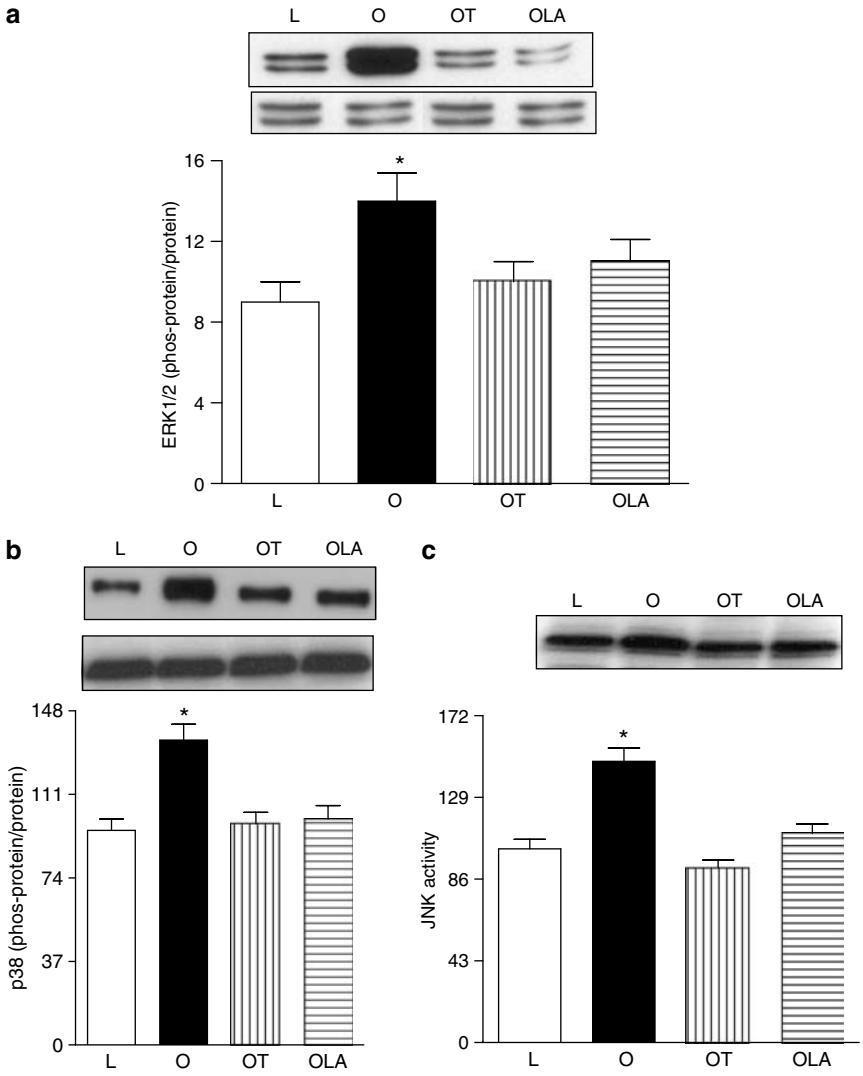

Figure 6 | Effect of antioxidants on MAP kinase activity. Effect of tempol (T) and lipoic acid (LA) supplementation on (**a**) ERK1/2, (**b**) p38, and (**c**) JNK activities in renal proximal tubules from lean (L), obese (O), obese-tempol (OT), and obese-lipoic acid (OLA) rats. (**a** and **b**) Top panel represents ERK1/2 or p38 phosphorylated protein, middle panel represents ERK1/2 or p38 protein abundance, and lower panel (bars) represent the ratio of top and middle panel (phosphorylated protein/protein abundance). (**c**) Upper panel: representative Western blots. Bars represent density (arbitrary units), mean ± s.e.m. of 5–6 different experiments (animals). Data were analyzed by ANOVA followed by Newman–Keuls multiple test; $P < 0.05$ was considered statistically significant. *Obese (O) significantly different from other groups.

DISCUSSION

These results show that defect in D1 receptor function in obese Zucker rats is associated with increased D1 receptor oxidation and upregulation of ERK1/2, JNK, and p38 MAPK activities. Treatment with antioxidants tempol and lipoic acid reduced oxidative stress and normalized ERK1/2, JNK, and p38 MAPK activities. The present study demonstrates that in obese animals the D1 receptors have reduced affinity and are uncoupled from G-proteins in basal state. SKF-38393, a D1 receptor agonist failed to elicit [^{35}S]GTPγS binding and

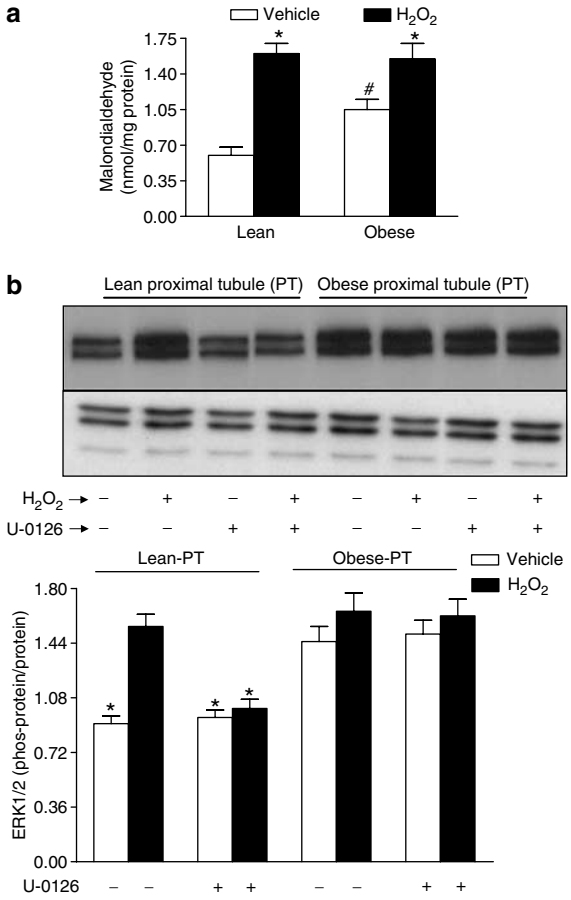

Figure 7 | Effect of H_2O_2 on renal malondialehyde levels and ERK1/2 activity. (**a**) Effect of H_2O_2 on lipid peroxidation in renal proximal tubules from lean and obese Zucker rats. (**b**) Effect of H_2O_2 on ERK1/2 upregulation in renal proximal tubules from lean and obese Zucker rats. Top panel represents ERK1/2 phosphorylated protein, middle panel represents ERK1/2 protein abundance, and lower panel (bars) represents the ratio of top and middle panel (ERK1/2 phosphorylated protein/ ERK1/2 protein abundance). Data were analyzed by ANOVA followed by Newman–Keuls multiple test; $P<0.05$ was considered statistically significant. **(a)** *H_2O_2 significantly different from vehicle, #vehicle-obese significantly different from vehicle-lean. (**b**) *Significantly different from H_2O_2-lean and -obese (vehicle or H_2O_2) groups.

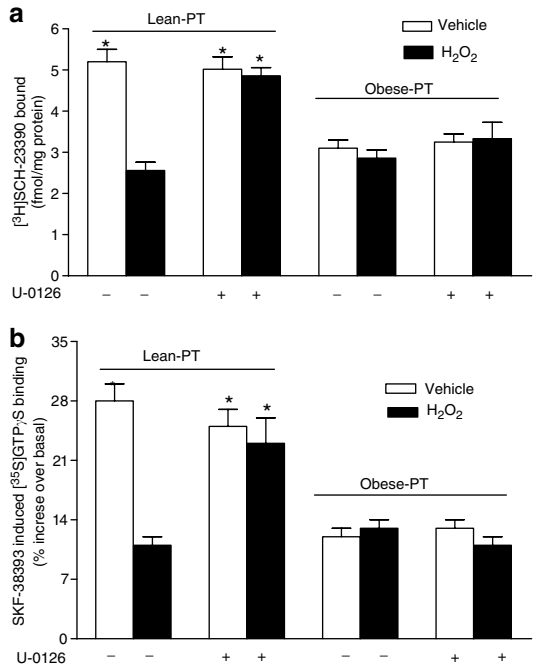

Figure 8 | Effect of H_2O_2 on renal D1 receptor expression and G-protein coupling. Effect of H_2O_2 on (**a**) [^{3}H]SCH-23390 binding and (**b**) SKF-38393-induced [^{35}S]GTPγS binding in proximal tubular membranes from lean and obese animals. Data were analyzed by ANOVA followed by Newman–Keuls multiple test; $P<0.05$ was considered statistically significant. *Significantly different from H_2O_2-lean and -obese (vehicle or H_2O_2) groups.

increase the accumulation of cAMP in renal proximal tubular membranes of obese animals. Furthermore, tempol and lipoic acid restored the D1 receptor affinity and normalized the SKF-38393-induced D1 receptor G-protein coupling and adenylyl cyclase activation. Finally, the direct exposure of proximal tubules to H_2O_2, an oxidant caused upregulation of ERK1/2 and reduced D1 receptor signaling in lean rats. The pre-incubation of proximal tubules with MAPK kinase inhibitor U-0126 inhibited ERK1/2 activation and blocked the oxidative stress-mediated D1 receptor defect.

The novel finding of this study is that in obese animals oxidative stress-mediated upregulation of MAPK is associated with defective D1 receptor signaling. Various studies have shown that diabetes is associated with increased formation of free radicals and decrease in antioxidant potential.[21–25] In present studies, we also observed a significant increase in protein and lipid oxidation, as evidenced by increased nitrotyrosine and 8-isoprostane levels in obese Zucker rats. A variety of different factors such as hyperglycemia and dyslipidemia probably contribute to the increased oxidative stress in obese Zucker rats. Of these factors, glucose autoxidation leads to the production of advanced glycation end products.[22,25] We also observed that obese animals have marked increase in carboxymethyllysine levels, a marker of advanced glycation end product and decrease in reduced glutathione was associated with significant increase in oxidized glutathione, suggesting a failure of the system to restore normal oxidative/antioxidative status. Treatment of obese rats with antioxidants decreased lipid peroxidation, protein oxidation, advanced glycation end product, and decreased urinary excretion of 8-isoprostane. In addition, tempol and lipoic corrected the glutathione levels and restored the redox status, confirming the role of these compounds as useful antioxidants. Contrary to earlier reports, we did not find any change in expression of NAD(P)H subunits from lean or obese rats.[26] The discrepancy may be because most of earlier studies were performed in vascular tissue,[26] whereas in present study we used renal proximal tubules.

At cellular level, the stimulated D1 receptors couple with G-proteins which lead to inhibition of NKA and NHE3 via activation of various kinases such as PKC and protein

kinase A.[1,5–9] Under conditions such as hypertension and diabetes, D1 receptors are uncoupled from G-proteins and thus fail to inhibit sodium transporters which leads to increased sodium retention particularly during modest increases in sodium intake.[1,2,10,13] The mechanism for the uncoupling of D1 receptors from G-proteins is currently unknown, but abnormalities in the desensitization process of D1 receptor might be involved.[1,12,13] Previously, we have shown that in obese Zucker rats, a model of type II diabetes with moderate hypertension, the failure of D1 receptor to couple with G-proteins is not caused by abnormalities in G-proteins.[13] Rather, our previous results suggested that upregulation of G-protein-coupled receptor kinase-2 and subsequent serine hyperphosphorylation of D1 receptors could be responsible for its uncoupling from G-proteins. We also showed that tempol decreased oxidative stress and restored the D1 receptor G-protein coupling, thus implicating the role of oxidative stress.[13]

There are reports indicating that oxidants abolish D1 receptor high-affinity sites and reducing agents preserve and protect such sites from oxidation.[27–29] We observed that in obese Zucker rats there is a significant increase in carbonyl content of D1 receptors, suggesting the specific oxidative modification of the receptor. There are two possible sites on the receptor that can be modulated by free radicals: the ligand binding domain and the G-protein coupling region of the receptor.[29–30] To determine the role of oxidative stress on affinity of D1 receptors, we performed competition binding experiments with the agonist FD, which revealed a significant decrease in the affinity of D1 receptors in obese compared with lean rats. Although a decrease in the agonist affinity toward the D1 receptors may contribute to the reduced D1 receptor activation, it cannot explain the complete functional loss of the D1 receptors. Previously, we have shown that stimulated D1 receptors fail to couple with G-proteins in obese animals.[13] Here, we wanted to determine whether oxidative stress affects the D1 receptor G-protein coupling before stimulation of receptors with agonist as well. The immunoprecipitation results showed decreased basal co-immunoprecipitation of G-proteins with D1 receptor ligand binding sites in obese rats compared to lean animals. Furthermore, D1 receptor ligand SKF-38393 failed to increase the [^{35}S]GTPγS binding in membrane from obese animals. These results indicate that D1 receptors are uncoupled from cognate G-proteins at basal state and also fail to interact with G-proteins after stimulation. Treatment of obese rats with tempol and lipoic acid normalized the carbonyl content of D1 receptors and restored the D1 receptor affinity and coupling to G-proteins.

Incubation of proximal tubular membranes from obese animals with D1 receptor agonist SKF-38393 failed to increase cAMP accumulation suggesting a defect in D1 receptor-mediated adenylyl cyclase activation. Treatment of obese animals with antioxidants restored the adenylyl cyclase activation in response to SKF-38393. The failure of SKF-38393 to activate adenylyl cyclase and increase cAMP levels may explain the inability of D1 receptors to inhibit sodium transporters as was observed in our previous studies.[31] It should be noted that incubation of membranes from both lean and obese rats with forskolin caused similar increases in cAMP levels and immunoblotting experiments showed that the expression of G-proteins was similar in lean and obese rats. Also, the protein abundance and activities of NKA and NHE3 were similar in lean and obese rats, thus indicating the defect in D1 receptor function lies in D1 receptor itself or proximal to G-proteins/effector complex.

The observation that tempol and lipoic acid-induced normalization of MAPK was accompanied by restoration of the D1 receptor affinity and coupling to G-proteins suggests the role of MAPK in oxidative stress-mediated D1 receptor defect. Supplementation of these animals with antioxidants normalized ERK1/2, JNK, and p38 MAPK activities and restored D1 receptor function; thus, implicating the role MAPK in D1 receptor defect. We also observed that incubation of proximal tubules from lean rats with H_2O_2 increased ERK1/2 activity and reduced D1 receptor signaling. In addition, the incubation of proximal tubules with MAPK kinase inhibitor before H_2O_2 exposure blocked both ERK1/2 activation as well as D1 receptor dysfunction, thus reinforcing the role of MAPK in oxidative stress-mediated D1 receptor dysfunction.[32] However, contrary to the observations in lean animals, H_2O_2 failed to upregulate ERK1/2 and reduce D1 receptor signaling in obese animals. This could be because in obese animals ERK1/2 is already upregulated and D1 receptors are also desensitized before H_2O_2 exposure. Although the mechanism for MAPK activation and defective D1 receptor function is not clear, renal D1 receptor stimulation has been shown to activate ERK1/2 and stress-activated JNK and p38 MAPK in time- and concentration-dependent manner.[20] It is possible that MAPK-mediated D1 receptor uncoupling may be a mechanism to limit the overstimulation of D1 receptor pathways via a classical feedback mechanism.[33] It has been reported that renal D1 receptor stimulation increases PKC activity in rats, whereas increased basal activity of PKC in rat renal tubules leads to D1 receptor G-protein uncoupling.[34–36] Normalization of PKC activity by antioxidant supplementation restored D1 receptor function in these animals.[14,15]

In conclusion, we have shown that in obese Zucker rats increased oxidative stress leads to D1 receptor oxidation and ERK1/2, JNK, and p38 MAPK upregulation. This could lead to impaired D1 receptor-mediated inhibition of sodium transporters and natriuresis resulting in sodium retention and subsequently hypertension in obese Zucker rats. Antioxidant supplementation while reducing oxidative stress, prevented D1 receptor oxidation and normalized ERK1/2, JNK, and p38 MAPK activation in obese animals. These phenomena could account for restoration of D1 receptor signaling and ability of dopamine to inhibit sodium transporters and promote sodium excretion.

MATERIALS AND METHODS

Antibodies for G-proteins, MAPK (including c-jun), NAD(P)H, and D1 receptors were obtained from Calbiochem-Novabiochem, Cell Signaling, Transduction Laboratory, and Alpha Diagnostic Intl, respectively. Glutathione colorimetric determination kit, Protein Oxidation Detection Kit, 8-Isoprostane EIA Kit, and protease inhibitor cocktail tablets were purchased from OXIS International Inc. (Foster city, CA, USA), Chemicon International (Temecula, CA, USA), Cayman Chemical (Ann Arbor, MI, USA), and Roche Diagnostic (Indianapolis, IN, USA), respectively. [^{3}H]SCH-23390 and [^{35}S]GTPγS were purchased from NEN Life Sciences. SKF-38393 and all other chemicals of highest purity were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male 7- to 9-week-old lean and obese Zucker rats were purchased from Harlan (Indianapolis, IN, USA) and maintained at an ambient temperature. For treatment purposes, both lean and obese animals were divided into two groups; control groups, where animals were continued on tap water or normal diet and treated groups were animals provided with tempol in tap water or diet supplemented with 0.4% lipoic acid for 2 weeks. Food and water were changed twice a day.

Preparation of renal proximal tubular suspension

Renal proximal tubular suspension was prepared as described earlier.[11,13,14] Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and after a midline incision, the aorta was cannulated below the kidneys and an *in situ* digestion was accomplished by perfusing an enzyme solution of collagenase and hyaluronidase. Enrichment of proximal tubules was carried out using 20% ficoll gradient in Krebs buffer.

[^{35}S]GTPγS binding to G-proteins

Membrane [^{35}S]GTPγS binding to G-proteins was performed according to Friedman *et al.*[37] Briefly, the assay mixture (250 μl) containing 200 μg of membrane protein and 2 nm [^{35}S]GTPγS was incubated for 5 min at 25°C, followed by incubation in the absence or presence of D1 receptor agonist (SKF-38393) for 5 min. The reaction was terminated and G-proteins were immunoprecipitated with Gαs or Gαi antiserum.

Co-immunoprecipitation of [^{3}H]SCH-23390 bound receptor with discrete G_α proteins

Solubilized membranes were combined with antisera (1:1000 dilution) raised against specific peptides of G_α or $G_{\alpha i}$ proteins for 3 h at 4°C followed by an additional 30-min incubation with 100 μl of Pansorbin cells.[38] 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 $MgCl_2$ and 1 nM [^{3}H]SCH-23390. The reaction was terminated by the addition of 9 ml of ice-cold buffer, vacuum filtered, and radioactivity was determined by liquid scintillation counting.

Immunoblotting and MAPK activity

Proteins were solubilized in Laemmli buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane. The membranes were blocked, incubated with antisera directed against G-proteins (G_α, $G_{\alpha i}$), MAPK, and NAD(P)H oxidase in 0.1% Tris-buffered saline followed by incubation with horseradish peroxidase-conjugated secondary antibodies. For ERK1/2 or p38 activation, the blots were incubated for 12 h with antiphosphospecific ERK1/2 and p38 or antibodies that recognize ERK1/2 and p38. JNK activity was measured with a commercially available kit based on phosphorylation of recombinant c-jun.

Adenylyl cyclase assay

The adenylyl cyclase assay was performed as described by Salomon *et al.*[39] The reaction was started by the addition of 50 μg of membrane protein to 1 μCi of [α-^{32}P]ATP ($\sim 2.2 \times 10^6$ c.p.m.) with or without SKF-38393. The reaction was terminated by 2% sodium dodecyl sulfate buffer and [^{32}P]cAMP was separated by Dowex and alumina columns.

Competition radioligand binding assay

FD, a D1 receptor agonist, was used to displace membrane bound radioligand [^{3}H]SCH-23390. The reaction mixture contained 4 nM radioligand, 50 μg membrane proteins, and variable concentration (10^{-10}–10^{-3} M) of FD in a 250-μl assay buffer (final volume) containing (in mM) 50 Tris-HCl, 2 $MgCl_2$, 0.2 sodium metabisulfite, and 0.2 phenylmethylsulfonyl fluoride, pH 7.4. The reaction was carried out for 120 min at 25°C, terminated by filtration, and counted for radioactivity.

NKA and NHE3 assay

Proximal tubular NKA activity was determined by the method of Szczepanska-Konkel *et al.*[40] This method uses plasma membrane preparations containing mainly BLM as determined by marked enrichment of NKA activity, nmol Pi/mg protein/min (lean-homogenate – 25.4 ± 1.8, lean-BLM – 260.2 ± 18.6; obese-homogenate – 28.4 ± 1.7, obese-BLM – 292.2 ± 20.6). NHE3 activity was assayed by measuring 5-(*N*-methyl-*N*-isobutyl)-amiloride-sensitive $^{22}Na^+$ uptake as described in detail by Albrecht *et al.*[41] The enrichment of BBM was confirmed by increased activity of typical BBM enzymes γ-glutamyl-transferase (data not shown) and alkaline phosphatase, nmol Pi/mg protein/min (lean-homogenate – 14.4 ± 1.2, lean-BBM – 148.2 ± 5.6; obese-homogenate – 15.4 ± 1.6, obese-BBM – 162.3 ± 9.7) and decrease in basolateral enzyme activity (data not shown).

Indexes (biomarkers) of oxidative stress

A biooxytech GSH/GSSG-412 kit was used to measure reduced and oxidized glutathione in renal proximal tubules, D1R-DNPH was measured by OxyBlot Protein Oxidation Detection Kit, and 8-ISO $PGF_{2\alpha}$ was measured by radioimmunoassay kit. Carboxymethyllysine was measured by enzyme-linked immunosorbent assay as described by Koo and Vaziri.[42] Endothelial NOS monomer/dimer ratio was measured by low-temperature sodium dodecyl sulfate-polyacrylamide gel electrophoresis low temperature polyacrylamide gel electrophoresis (LT-PAGE).[43] Fluorescence spectrometric assay of O_2^- production was performed as described by Satoh *et al.*[44]

Statistical analysis

Difference between means were evaluated using the unpaired *t*-test or analysis of variance (ANOVA) with Newman–Keuls' multiple test, as appropriate, $P < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS

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

REFERENCES

1. Jose PA, Eisner GM, Felder RA. Renal dopamine receptors in health and hypertension. *Pharmacol Ther* 1998; **80**: 149–182.
2. Hegde SS, Jadhav AL, Lokhandwala MF. Role of kidney dopamine in the natriuretic response to volume expansion in rats. *Hypertension* 1989; **13**: 828–834.
3. Sibley DR. New insights into dopaminergic receptor function using antisense and genetically altered animals. *Annu Rev Pharmacol Toxicol* 1999; **39**: 313–341.
4. Missale C, Nash SR, Robinson SW *et al.* Dopamine receptors: from structure to function. *Physiol Rev* 1998; **78**: 189–225.
5. Aperia AC. Intrarenal dopamine: a key signal in the interactive regulation of sodium metabolism. *Annu Rev Physiol* 2000; **62**: 621–647.
6. Felder CC, Campbell T, Albrecht F *et al.* Dopamine inhibits Na(+)–H+ exchanger activity in renal BBMV by stimulation ofadenylate cyclase. *Am J Physiol* 1990; **259**: F297–F303.
7. Ominato M, Satoh T, Katz AI. Regulation of Na-K-ATPase activity in the proximal tubule: role of the protein kinase C pathway and of eicosanoids. *J Membrane Biol* 1996; **152**: 235–243.
8. Kunimi M, Seki G, Hara C *et al.* Dopamine inhibits renal Na+:HCO_3^- cotransporter in rabbits and normotensive rats but not in spontaneously hypertensive rats. *Kidney Int* 2000; **57**: 534–543.
9. Bertorello AM, Hopfield JF, Aperia A *et al.* Inhibition by dopamine of (Na(+)+K+)ATPase activity in neostriatal neurons through D1 and D2 dopamine receptor synergism. *Nature* 1990; **347**: 386–388.
10. Bek MJ, Eisner GM, Felder RA *et al.* Dopamine receptors in hypertension. *Mt Sinai J Med* 2001; **68**: 362–369.
11. Umrani DN, Banday AA, Hussain T *et al.* Rosiglitazone treatment restores renal dopamine receptor function in obese Zucker rats. *Hypertension* 2002; **40**: 880–885.
12. Sanada H, Jose PA, Hazen-Martin D *et al.* Dopamine-1 receptor coupling defect in renal proximal tubule cells in hypertension. *Hypertension* 1999; **33**: 1036–1042.
13. Banday AA, Marwaha A, Tallam LS *et al.* 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. *Diabetes* 2005; **54**: 2219–2226.
14. Marwaha A, Lokhandwala MF. Tempol reduces oxidative stress and restores renal dopamine D1-like receptor G-protein coupling and function in hyperglycemic rats. *Am J Physiol* 2006; **291**: F58–F66.
15. Asghar M, Lokhandwala MF. Antioxidant supplementation normalizes elevated protein kinase C activity in the proximal tubules of old rats. *Exp Biol Med* 2004; **229**: 270–275.
16. Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 2000; **52**: 11–34.
17. Yoshizumi M, Tsuchiya K, Tamaki T. Signal transduction of reactive oxygen species and mitogen-activated protein kinases in cardiovascular disease. *J Med Invest* 2001; **48**: 11–24.
18. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 2000; **86**: 494–501.
19. Nishiyama A, Yoshizumi M, Hitomi H *et al.* The SOD mimetic tempol ameliorates glomerular injury and reduces mitogen-activated protein kinase activity in Dahl salt-sensitive rats. *J Am Soc Nephrol* 2004; **15**: 306–315.
20. Chen J, Rusnak M, Luedtke RR *et al.* D1 dopamine receptor mediates dopamine-induced cytotoxicity via the ERK signal cascade. *J Biol Chem* 2004; **279**: 39317–39330.
21. Jay D, Hitomi H, Griendling KK. Oxidative stress and diabetic cardiovascular complications. *Free Radical Biol Med* 2006; **40**: 183–192.
22. Rahimi R, Nikfar S, Larijani B *et al.* A review on the role of antioxidants in the management of diabetes and its complications. *Biomed Pharmacother* 2005; **59**: 365–373.
23. Scott JA, King GL. Oxidative stress and antioxidant treatment in diabetes. *Ann N Y Acad Sci* 2004; **1031**: 204–213.
24. Evans JL, Goldfine ID, Maddux BA *et al.* Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr Rev* 2002; **23**: 599–622.
25. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001; **414**: 813–820.
26. Sonta T, Inoguchi T, Tsubouchi H *et al.* Evidence for contribution of vascular NAD(P)H oxidase to increased oxidative stress in animal models of diabetes and obesity. *Free Radical Biol Med* 2004; **37**: 115–123.
27. Sidhu A, Kassis S, Kebabian J *et al.* Sulfhydryl group(s) in the ligand binding site of the D-1 dopamine receptor: specific protection by agonist and antagonist. *Biochemistry* 1986; **25**: 6695–6701.
28. Sidhu A, Kimura K, Vachvanichsanong P. Induction of G protein-independent agonist high-affinity binding sites of D-1 dopamine receptors by beta-mercaptoethanol. *Biochemistry* 1994; **33**: 11246–11253.
29. Kimura K, Sidhu A. Ascorbic acid inhibits 125I-SCH 23982 binding but increases the affinity of dopamine for D1 dopamine receptors. *J Neurochem* 1994; **63**: 2093–2098.
30. Vachvanichsanong P, Kimura K, Sidhu A. Differences in photoaffinity labeling of DA1 receptors in renal proximal tubules from normotensive rat and SHR. *Am J Physiol* 1995; **268**: F1009–F1016.
31. Hussain T, Becker M, Beheray S *et al.* Dopamine fails to inhibit Na,H-exchanger in proximal tubules of obese Zucker rats. *Clin Exp Hypertens* 2001; **23**: 591–601.
32. Koka V, Wang W, Huang XR *et al.* Advanced glycation end products activate a chymase-dependent angiotensin II-generating pathway in diabetic complications. *Circulation* 2006; **113**: 1353–1360.
33. Widmann C, Gibson S, Jarpe MB *et al.* Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* 1999; **79**: 143–180.
34. Hopf FW, Mailliard WS, Gonzalez GF *et al.* Atypical protein kinase C is a novel mediator of dopamine-enhanced firing in nucleus accumbens neurons. *J Neurosci* 2005; **25**: 985–989.
35. Asghar M, Hussain T, Lokhandwala MF. Overexpression of PKC-betal and -delta contributes to higher PKC activity in the proximal tubules of old Fischer 344 rats. *Am J Physiol* 2003; **285**: F1100–F1107.
36. Asghar M, Banday AA, Fardoun RZ *et al.* Hydrogen peroxide causes uncoupling of dopamine D1-like receptors from G proteins via a mechanism involving protein kinase C and G-protein coupled receptor kinase 2. *Free Radical Biol Med* 2006; **40**: 13–20.
37. Friedman E, Butkerait P, Wang HY. Analysis of receptor-stimulated and basal guanine nucleotide binding to membrane G proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal Biochem* 1993; **214**: 171–178.
38. Wang HY, Undie AS, Friedman E. Evidence for the coupling of Gq protein to D1-like dopamine sites in rat striatum: possible role in dopamine-mediated inositol phosphate formation. *Mol Pharmacol* 1995; **48**: 988–994.
39. Salomon Y, Londos C, Rodbell M. A highly sensitive adenylate cyclase assay. *Anal Biochem* 1974; **58**: 541–548.
40. Szczepanska-Konkel M, Yusufi AN, VanScoy M *et al.* Phosphonocarboxylic acids as specific inhibitors of Na+-dependent transport of phosphate across renal brush border membrane. *J Biol Chem* 1986; **261**: 6375–6383.
41. Albrecht FE, Xu J, Moe OW *et al.* Regulation of NHE3 activity by G protein subunits in renal brush-border membranes. *Am J Physiol Regul Integr Comp Physiol* 2000; **278**: R1064–R1073.
42. Koo JR, Vaziri ND. Effects of diabetes, insulin and antioxidants on NO synthase abundance and NO interaction with reactive oxygen species. *Kidney Int* 2003; **63**: 195–201.
43. Kakoki M, Hirata Y, Hayakawa H *et al.* Effects of tetrahydrobiopterin on endothelial dysfunction in rats with ischemic acute renal failure. *J Am Soc Nephrol* 2000; **11**: 301–309.
44. Satoh M, Fujimoto S, Haruna Y *et al.* NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. *Am J Physiol Renal Physiol* 2005; **288**: F1144–F1152.