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Antioxidant treatment normalizes nitric oxide production, renal sodium handling and blood pressure in experimental hyperleptinemia

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Abstract

Recent studies suggest that adipose tissue hormone, leptin, is involved in the pathogenesis of arterial hypertension. However, the mechanism of hypertensive effect of leptin is incompletely understood. We investigated whether antioxidant treatment could prevent leptin-induced hypertension. Hyperleptinemia was induced in male Wistar rats by administration of exogenous leptin (0.25 mg/kg twice daily s.c. for 7 days) and separate groups were simultaneously treated with superoxide scavenger, tempol, or NAD(P)H oxidase inhibitor, apocynin (2 mM in the drinking water). After 7 days, systolic blood pressure was 20.6% higher in leptin-treated than in control animals. Both tempol and apocynin prevented leptin-induced increase in blood pressure. Plasma concentration and urinary excretion of 8-isoprostanes increased in leptin-treated rats by 66.9% and 67.7%, respectively. The level of lipid peroxidation products, malonyldialdehyde + 4-hydroxyalkenals (MDA+4-HNE), was 60.3% higher in the renal cortex and 48.1% higher in the renal medulla of leptin-treated animals. Aconitase activity decreased in these regions of the kidney following leptin administration by 44.8% and 45.1%, respectively. Leptin increased nitrotyrosine concentration in plasma and renal tissue. Urinary excretion of nitric oxide metabolites (NO_x) was 57.4% lower and cyclic GMP excretion was 32.0% lower in leptin-treated than in control group. Leptin decreased absolute and fractional sodium excretion by 44.5% and 44.7%, respectively. Co-treatment with either tempol or apocynin normalized 8-isoprostanes, MDA+4-HNE, aconitase activity, nitrotyrosine, as well as urinary excretion of NO_x, cGMP and sodium in rats receiving leptin. These results

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indicate that oxidative stress-induced NO deficiency is involved in the pathogenesis of leptin-induced hypertension.

Keywords: Leptin; Arterial hypertension; Oxidative stress; Obesity; Nitric oxide

Introduction

The prevalence of overweight and obesity has increased dramatically in recent years. Among chronic adverse consequences of obesity, cardiovascular complications including arterial hypertension are one of the most important. However, the pathogenesis of obesity-associated hypertension is incompletely understood. Recent studies point to the role of leptin, a peptide hormone secreted by adipose tissue, in cardiovascular complications of obesity (Correia and Haynes 2004). Plasma leptin concentration is markedly increased in obese individuals (Considine et al., 1996). Chronic leptin administration increases blood pressure (Shek et al., 1998) and some studies suggest that hypertension develops in obese animals only if they are hyperleptinemic (Mark et al., 1999; Aizawa-Abe et al., 2000). In addition, increase in plasma leptin has been reported in some non-obese humans with essential hypertension (Agata et al., 1997). Taken together, these data suggest that hyperleptinemia contributes to the pathogenesis of obesity-associated hypertension. Although hypertensive effect of leptin is generally attributed to the stimulation of sympathetic nervous system (Shek et al., 1998), other mechanisms such as increase in vasoconstrictor endothelin-1 (Quehenberger et al., 2002) may also be important. Indeed, some studies indicate that sympathoexcitation cannot solely explain leptin-induced blood pressure elevation (Bernal-Mizrachi et al., 2002).

Recent studies indicate that oxidative stress contributes to the development of hypertension in various animal models including obesity-associated hypertension (Dobrian et al., 2001a; Manning et al., 2003; Vaziri 2004). Reactive oxygen species (ROS), especially superoxide anion radical (O_2^-), scavenge nitric oxide (NO) by binding it to form peroxynitrite ($ONOO^-$) and thus limit its vasodilatory effect on vascular smooth muscle cells. Apart from regulating vascular tone, ROS may increase blood pressure by modulating renal sodium handling. NO increases natriuresis by causing renal vasodilation and inhibiting tubular sodium reabsorption (Ortiz and Garvin, 2002a). Excessive intrarenal O_2^- production induces Na^+ retention and blood pressure elevation by compromising local NO availability (Zou et al., 2001; Makino et al., 2002).

Leptin increases ROS production in vitro (Bouloumie et al., 1999; Yamagishi et al., 2001). Recently, we have demonstrated that 7-day leptin administration induces systemic and intrarenal oxidative stress and NO deficiency, the latter evidenced by reduced urinary excretion of NO metabolites and of its second messenger, cyclic GMP (Beltowski et al., 2004). In addition, hyperleptinemia was associated with decreased natriuresis and fractional excretion of sodium. These data suggest that ROS-mediated inactivation of NO contributes to leptin-induced hypertension by causing vasoconstriction and/or renal sodium retention. However, because hypertension per se can increase ROS production (Ungvari et al., 2003), it is unclear whether oxidative stress is involved in the pathogenesis of leptin-induced hypertension or is simply the consequence of increased blood pressure. Therefore, in the present study we investigated whether antioxidant treatment could decrease blood pressure and normalize NO production as well as Na^+ excretion in hyperleptinemic animals.

Materials and methods

Experimental protocol

The study was performed on adult male Wistar rats weighing 208 ± 8 g (mean ± S.E.M.) before the experiment. The study protocol was approved by the Bioethics Committee of the Lublin Medical University. The animals were kept at a temperature of 20 ± 2 °C with 12-h light/dark cycle (lights on at 7:00 am). After two weeks of acclimation, they were randomized into 7 groups (n = 8 each): 1) control group, fed standard rat chow ad libitum; 2) leptin-treated group, which received leptin injections (0.25 mg/kg twice daily s.c. for 7 days); 3) pair-fed group, in which the amount of food served was adjusted to food intake in the leptin-treated group; 4) ad libitum-fed group receiving superoxide dismutase mimetic, 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (tempol) in the drinking water at a concentration of 2 mM; 5) ad libitum-fed group receiving NAD(P)H oxidase inhibitor, apocynin (2 mM in the drinking water); 6) leptin-treated group receiving tempol; and 7) leptin-treated group receiving apocynin. Animals not treated with leptin received s.c. injections of 0.25 mg/kg bovine serum albumin (BSA). Leptin and BSA injections were made every 12 h, between 7:00 and 8:00 am and between 7:00 and 8:00 pm. The approximate doses of both tempol and apocynin calculated from water intake were 200 μmol/kg/day. Before starting the respective treatments, a 24-h urine collection was made in all animals kept in individual metabolic cages. The second urine collection was performed during the last 24 h of treatment. Urine was collected into sterile containers with 10 μl of 2 mM EDTA and 10 μl of 0.01% butylated hydroxytoluene (BHT) to prevent formation of isoprostanes in vitro.

Systolic blood pressure (SBP) was measured with a tail-cuff method in conscious restrained animals prewarmed with a light lamp for 5 min at 37 °C. Three consecutive measurements were taken and the average value was recorded. SBP measurements were started 5 days before the beginning of treatment to train the animals to the procedure and were performed every day throughout the experiment between 1:00 and 2:00 pm, i.e. about 6 h after preceding injection. After completion of the second urine collection and the last SBP measurement, the animals were anesthetized with pentobarbital (50 mg/kg). The abdominal cavity was opened and blood was withdrawn from the abdominal aorta into EDTA-containing tubes. The kidneys were removed and frozen in liquid nitrogen. The kidneys, plasma, and urine samples were stored at −80 °C until analysis. For the assay of isoprostanes, plasma and urine samples were stored in the presence of 0.05% BHT. For the measurement of cyclic GMP, 3-isobuthyl-1-methylxanthine (IBMX) was added to the samples (30 μl of 10 mM IBMX per 0.5 ml of sample) to prevent breakdown of cGMP by phosphodiesterases.

Indices of oxidative stress

Plasma and urinary 8-isoprostanes, the products of ROS-mediated peroxidation of arachidonic acid, were measured by enzyme immunoassay (EIA) using 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI, USA), after extraction on C-18 SPE cartridges (Waters Corporation, Milford, MA, USA). The procedure of extraction and assay was described in detail previously (Beltowski et al., 2004). The limit of the sensitivity of the assay was 5 pg/ml whereas the intra- and interassay CV were 5% and 8%, respectively. The recovery of 8-isoprostane standard added to the samples averaged 79%.

The level of intrarenal oxidative stress was assessed by measuring malonyldialdehyde + 4-hydroxyalkenals (MDA+4-HNE) in the renal cortex and medulla using Bioxytech LPO-586 assay kit (Oxis International, Portland, OR, USA). In addition, aconitase activity was measured in renal tissue. Aconitase is inactivated by superoxide and peroxynitrite and is used as an index of intracellular O_2^- production (Takemoto et al., 2001). The enzyme activity was expressed as nanomole of isocitrate converted to *cis*-aconitate per milligram of protein during 1 min.

Nitrotyrosine assay

Peroxynitrite nitrates protein tyrosine residues form nitrotyrosine (NT). Nitrotyrosine level in plasma and tissues is used as an indirect index of $ONOO^-$generation. NT was measured by enzyme immunoassay (EIA) using BIOXYTECH Nitrotyrosine-EIA kit (Oxis International). Unlike commonly used Western blotting and HPLC methods which detect only protein-bound and free nitrotyrosine, respectively, EIA detects total NT pool. The assay is specific since the antibodies used do not cross-react with either non-nitrated tyrosine or other nitrated amino acids. However, the disadvantage is that this method is semiquantitative because anti-nitrotyrosine antibodies demonstrate different affinity toward various nitrated proteins (Inoue et al., 2002).

Samples of renal cortex and medulla were homogenized in 10 volumes of 100 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA and were centrifuged at 4500 $\times g$ for 15 min. Plasma or tissue homogenate (100 μl) was added to the wells coated with monoclonal mouse anti-nitrotyrosine antibody raised against nitrated keyhole limpet hemocyanin and was incubated for 1 h at room temperature. The wells were washed and filled with 100 μl of biotin-labeled polyclonal goat anti-nitrotyrosine antibodies. After 1-h incubation and washing the wells, 100 μl of streptavidin peroxidase solution was added. The plate was incubated for 1 h, washed, and 100 μl of tetramethylbenzidine was added to the wells. The reaction was stopped after 30 min by adding 100 μl of 2.0 M citric acid and the absorbance was read at 450 nm in a microplate reader. Standard curve was constructed using known concentrations (0–1500 nM) of nitrotyrosine. The detection limit of the assay was 2 nM and the intra- and inter-assay CV were 2.3% and 8.7%, respectively.

Other assays

Nitric oxide metabolites (nitrates + nitrites, NO_x) were assayed in plasma and urine by the colorimetric method of Griess after enzymatic conversion of nitrates to nitrites by nitrate reductase, using Total Nitric Oxide Assay Kit (R and D Systems Ltd, Abingdon, Oxon, United Kingdom). Cyclic GMP was measured by competitive EIA using Cyclic GMP Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI, USA). Plasma and urinary creatinine was assayed by the colorimetric method. Glomerular filtration rate (GFR) was calculated as creatinine clearance. Plasma and urinary Na^+ was measured by flame photometry. Fractional excretions of Na^+ and NO_x were calculated as the ratios between urinary excretion and the amount filtered (GFR × plasma concentration). Renal generation of cGMP (nephrogenous cGMP in urine) was calculated as the difference between urinary excretion and filtered load of the nucleotide (Pham et al., 1997). Plasma leptin was measured using Leptin Enzyme Immunoassay Kit (Cayman Chemical). Protein was assayed in tissue homogenates by the method of Lowry et al. (1951).

Reagents

Recombinant human leptin was purchased from R and D Systems. Other reagents were obtained from Sigma-Aldrich.

Statistics

Data are reported as mean ± S.E.M. from eight animals in each group. Baseline and post-treatment urinary excretion of isoprostanes, NO_x, cGMP and Na^+ in a given group was compared by repeated-measures ANOVA. Comparisons between different groups were performed by ANOVA and Newmann–Keul's post-hoc test. $P<0.05$ was considered significant.

Results

Food intake, water intake, body weight, plasma leptin, and blood pressure

There were no significant differences in food intake, water intake, body weight and blood pressure between groups before treatment and these variables did not change in the control group during the experiment. Food intake was significantly lower in leptin-treated animals only on the 6th and 7th day of treatment, however, final body weight was similar in all groups. Neither tempol nor apocynin had any effect on food intake in ad libitum-fed and in leptin-treated rats (Table 1). Water intake did not differ between groups. Plasma leptin was more than three-fold higher in leptin-treated than in control rats. Pair-feeding caused 40.5% decrease in plasma leptin. Tempol and apocynin had no effect on plasma leptin either in ad libitum-fed or in leptin-treated rats (Table 1). After 7 days of treatment, systolic blood pressure was by 20.6% higher in leptin-treated than in control animals, whereas pair-feeding did not change blood pressure. Tempol and apocynin had no effect on SBP in rats which did not receive leptin, however, each of these drugs normalized blood pressure in leptin-treated animals (Table 1).

Table 1
Food intake, water intake, body weight, plasma leptin, and blood pressure

	Control	Leptin-treated	Pair-fed	Ad libitum-fed + tempol	Ad libitum-fed + apocynin	Leptin-treated + tempol	Leptin-treated + apocynin
Food intake (g/day)[a]	25 ± 3	19 ± 3*	19 ± 2*	24 ± 3	26 ± 2	18 ± 2*	19 ± 3*
Water intake (ml/day)[a]	24 ± 2	21 ± 3	25 ± 3	26 ± 2	22 ± 3	25 ± 3	21 ± 2
Body weight (g)[b]	229 ± 2	227 ± 6	225 ± 4	227 ± 7	228 ± 6	236 ± 8	232 ± 7
Plasma leptin (ng/ml)[b]	4.12 ± 0.20	13.97 ± 0.96***	2.04 ± 0.15*	3.96 ± 0.17	4.01 ± 0.21	13.78 ± 1.10***	14.01 ± 0.97***
Systolic blood pressure (mm Hg)[b]	126 ± 5	152 ± 7*	121 ± 5	124 ± 6	121 ± 3	128 ± 3	132 ± 4

[a]During 7th day of treatment, [b]after 7days of treatment, *$P<0.05$, and ***$P<0.001$ vs. control group.

Indices of oxidative stress

Plasma concentration and urinary excretion of isoprostanes increased in leptin-treated rats by 66.9% and 67.7%, respectively. Pair-feeding had no effect on plasma and urinary isoprostanes. Tempol and apocynin restored plasma and urinary isoprostanes to normal level in leptin-treated animals but had no effect in ad-libitum-fed rats (Fig. 1).

In the animals receiving leptin, the concentration of MDA+4-HNE was 60.3% higher in the renal cortex and 48.1% higher in the renal medulla in comparison to the control group (Fig. 2, top). Aconitase activity decreased following leptin treatment in the renal cortex and medulla by 44.8% and 45.1%, respectively (Fig. 2, bottom). Pair-feeding had no effect on either MDA+4-HNE or aconitase activity. Tempol and apocynin did not change MDA+4-HNE and aconitase activity in ad-libitum-fed rats but normalized their level in leptin-treated rats (Fig. 2).

Nitrotyrosine in plasma and renal tissue

Increased ROS generation leads to enhanced formation of peroxynitrite, which reacts with lipids and nucleic acids as well as nitrates protein tyrosine residues. Consequently, NT accumulates in plasma and tissues while urinary NO_x excretion decreases because less NO is directly oxidized to nitrites and nitrates (Vaziri et al., 2001; Zhan et al., 2004). Plasma nitrotyrosine concentration was higher in leptin-treated than in control animals by 57.9%. Co-administration of apocynin completely normalized plasma nitrotyrosine in leptin-treated rats. Also tempol tended to reduce plasma nitrotyrosine in animals treated with leptin but its effect was not significant (Fig. 3). Leptin treatment increased NT concentration in the renal cortex and medulla by 119.6% and 73.8%, respectively. In both parts of the kidney, apocynin prevented increase in NT induced by leptin. Tempol completely normalized NT in the renal medulla but only partially corrected it in the renal cortex (Fig. 3). Interestingly, whereas neither of antioxidants had any effect on NT concentration in the renal cortex and plasma of ad libitum-fed animals, both tempol and apocynin markedly decreased NT in the renal medulla of these rats (Fig. 3), suggesting that significant

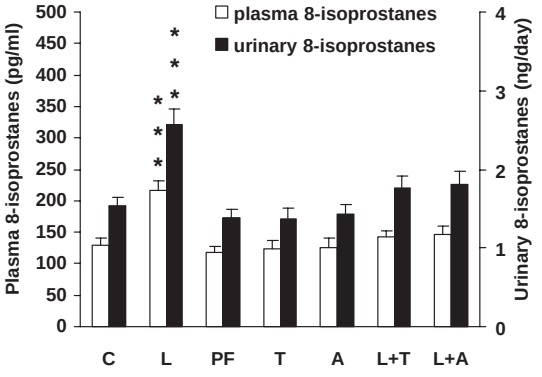

Fig. 1. The effect of leptin treatment (0.25 mg/kg s.c., twice daily for 7 days) on plasma concentration (white bars, left scale) and urinary excretion (black bars, right scale) of 8-isoprostanes. C—control group, L—leptin-treated group, PF—pair-fed group, T—ad libitum-fed group receiving tempol, A—ad libitum-fed group receiving apocynin, L+T—group receiving leptin and tempol, and L+A—group receiving leptin and apocynin. $^{***}P<0.05$ vs. control group.

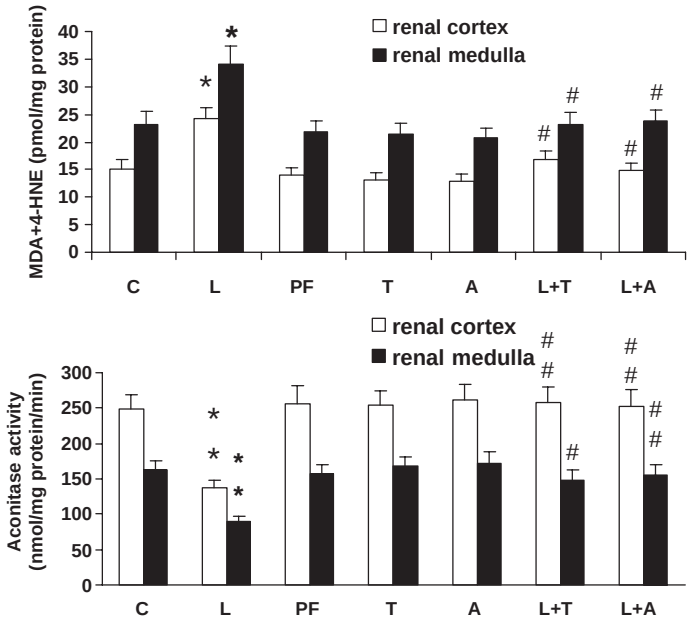

Fig. 2. The effect of leptin and antioxidant treatment on lipid peroxidation products (malonyldialdehyde + 4-hydroxyalkenals, MDA+4-HNE, top panel) and aconitase activity (bottom panel) in the kidney. $^{*}P<0.05$, $^{**}P<0.01$ vs. control group, $^{\#}P<0.05$, and $^{\#\#}P<0.01$ vs. leptin-treated group.

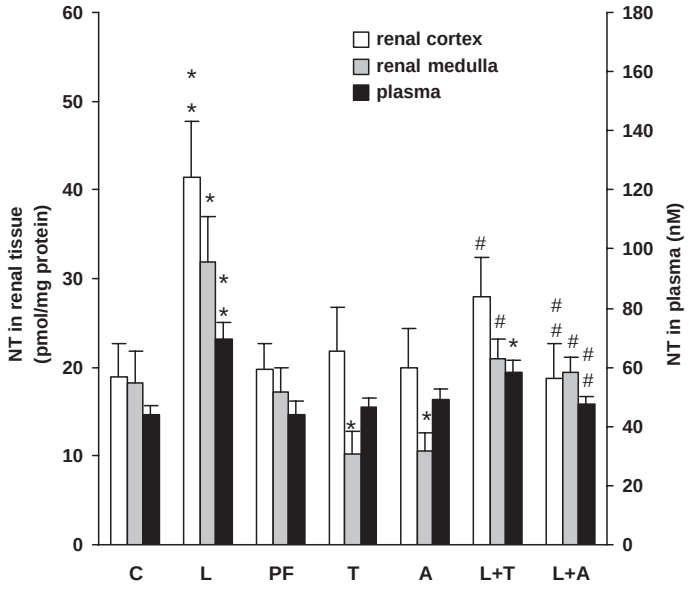

Fig. 3. Nitrotyrosine (NT) concentration in renal tissue (left axis) and plasma (right axis). $^{*}P<0.05$, $^{**}P<0.01$ vs. control group, $^{\#}P<0.05$, and $^{\#\#}P<0.01$ vs. leptin-treated group.

amounts of nitrotyrosine are formed in this part of the kidney under baseline conditions. Pair-feeding had no effect on NT level either in plasma or in the renal tissue.

Nitric oxide metabolites and cyclic GMP

Plasma concentration of NO_x did not differ between groups. Urinary excretion of NO metabolites ($U_{NO_x}V$) was 57.4% lower in leptin-treated than in control animals. In pair-fed group, urinary NO_x excretion tended to be lower than in control group but the difference did not reach the level of significance ($P=0.06$). As previously discussed (Beltowski et al., 2004), this may be accounted for by reduced nitrate intake due to food restriction. However, urinary NO_x excretion was significantly lower (−46.8%, $P<0.01$) in leptin-treated than in pair-fed rats despite identical food intake. Fractional excretion of NO metabolites ($FENO_x$) was 55.7% lower in leptin-treated animals than in the control group. Pair-feeding had no effect on fractional NO_x excretion. Tempol and apocynin did not change either absolute or fractional NO_x excretion in ad libitum-fed animals, however, each of these antioxidants normalized $U_{NO_x}V$ and $FENO_x$ in leptin-treated rats (Fig. 4, top).

Urinary cGMP excretion ($U_{cGMP}V$) followed the pattern observed for $U_{NO_x}V$, i.e. it was 32.0% lower in leptin-treated than in control rats but was not changed in pair-fed group. Tempol and apocynin normalized urinary cGMP excretion in leptin-treated animals, whereas neither of these drugs had any effect on $U_{cGMP}V$ in rats not treated with leptin (Fig. 4, bottom). Plasma cGMP did not differ between

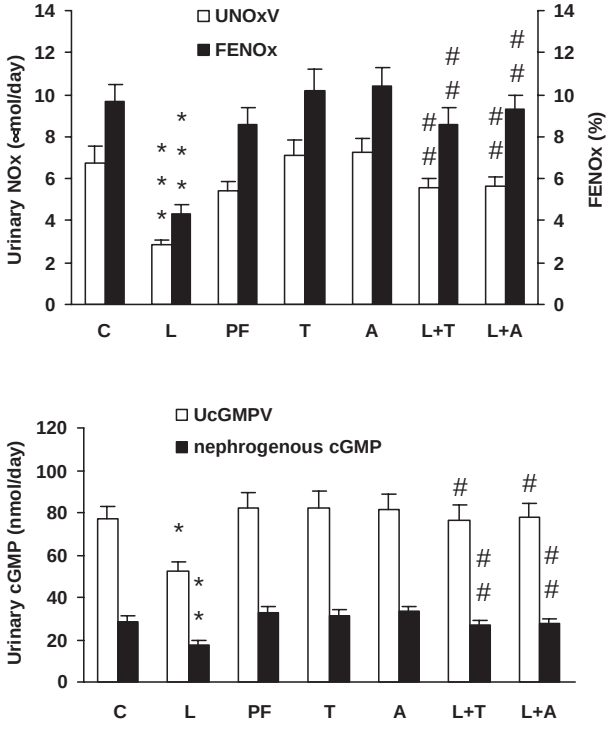

Fig. 4. Top panel: absolute (UNO_xV) and fractional ($FENO_x$) excretion of nitric oxide metabolites, nitrites + nitrates. Bottom panel: absolute urinary excretion of cGMP (UcGMPV) and excretion of nephrogenous cGMP. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. control group, $^{\#}P<0.05$, and $^{\#\#}P<0.01$ vs. leptin-treated group.

Table 2
Renal function and electrolyte balance on 7th day of treatment

	Control	Leptin-treated	Pair-fed	Ad libitum-fed + tempol	Ad libitum-fed + apocynin	Leptin-treated + tempol	Leptin-treated + apocynin
Urine output (ml/day)	23 ± 4	19 ± 4	24 ± 4	25 ± 4	23 ± 2	21 ± 3	23 ± 3
Creatinine clearance (ml/min)	2.33 ± 0.25	2.59 ± 0.28	2.18 ± 0.20	2.14 ± 0.19	2.37 ± 0.21	2.15 ± 0.16	2.45 ± 0.19
UNaV (mmol/day)	2.27 ± 0.22	1.26 ± 0.15*	1.78 ± 0.18	2.23 ± 0.19	2.39 ± 0.20	2.16 ± 0.21	2.23 ± 0.26
$FENa^+$ (%)	0.47 ± 0.04	0.26 ± 0.02**	0.40 ± 0.03	0.51 ± 0.04	0.48 ± 0.05	0.50 ± 0.04	0.45 ± 0.04

UNaV—absolute urinary excretion of sodium and $FENa^+$—fractional excretion of sodium.
*$P<0.05$ and **$P<0.01$ vs. control group.

groups (not shown). The amount of nephrogenous cGMP was 38% lower in leptin-treated than in control group. Leptin-induced decrease in nephrogenous cGMP was prevented by co-treatment with either tempol or apocynin (Fig. 4, bottom).

Renal function and sodium balance

There were no differences in urine output, creatinine clearance and sodium excretion between groups before treatment and these variables did not change in the control group during the experiment (not shown). Urine output and creatinine clearance were similar in all groups at the end of the experiment (Table 2). Plasma concentrations of sodium did not differ between groups. Absolute and fractional urinary Na^+ excretion was lower in leptin-treated than in control animals by 44.5% and 44.7%, respectively. Pair-feeding had no effect on natriuresis. Tempol and apocynin increased absolute and fractional Na^+ excretion in leptin-treated rats to the level observed in the control group (Table 2).

Discussion

Tempol is a low-molecular-weight membrane permeable antioxidant which catalytically scavenges superoxide both in vitro and in vivo. Previous studies have documented that chronically administered tempol normalizes blood pressure in various animal models of hypertension such as spontaneously hypertensive rat (Schnackenberg and Wilcox, 1999), Dahl salt-sensitive rat (Zicha et al., 2001), hypertension induced by angiotensin II (Nishiyama et al., 2001), deoxycorticosterone acetate and high-salt diet (Beswick et al., 2001a), endothelin (Sedeek et al., 2003), dexamethasone (Zhang et al., 2004), high-fructose diet (Onuma and Nakanishi, 2004), lead intoxication (Vaziri et al., 2001), and one-kidney one-clip hypertension (Dobrian et al., 2001b). This is accompanied by reduction of oxidative stress, increase in systemic NO production and decrease in nitrotyrosine level (Vaziri et al., 2001, 2003). Other antioxidants such as vitamin E, ascorbic acid and α-lipoic acid also lower blood pressure in hypertensive animals and humans (Vaziri, 2004). The results of this study suggest that leptin-induced hypertension may be added to the growing list of experimental models in which antioxidant treatment is effective.

Antihypertensive effect of tempol is mainly attributed to the improved availability of vascular NO (Nishiyama et al., 2001). However, tempol may lower blood pressure also by regulating renal Na^+ handling. Several lines of evidence support this possibility. First, tempol abolished leptin-induced intrarenal oxidative stress as evidenced by normalization of MDA+4-HNE, renal nitrotyrosine concentration and aconitase activity. Second, tempol increased fractional excretion of NO metabolites, which is affected by intrarenal NO production (Beltowski et al., 2004). Third, tempol increased the amount of nephrogenous cGMP. Finally, antioxidant treatment normalized fractional sodium excretion, suggesting that tempol prevented increase in tubular Na^+ reabsorption induced by hyperleptinemia. Taken together, these data indicate that treatment with tempol results in preservation of intrarenal NO by protecting it from ROS-mediated inactivation and thus improves its natriuretic activity.

Although tempol increased NO production in leptin-treated rats, it is unclear whether its hypotensive and natriuretic effects should be solely attributed to NO. In some studies acute hypotensive effect of tempol was NO-independent (Xu et al., 2001). ROS may induce vasoconstriction not only by scavenging NO but also by other mechanisms. For example, peroxynitrite nitrates inactivates prostacyclin synthase (Zou and Ullrich, 1996) and isoprostanes may constrict blood vessels directly by activating thromboxane receptors (Audoly et al., 2000). Tempol may also improve the NO- and prostacyclin-independent component of endothelium-dependent vasorelaxation mediated by endothelium-derived hyperpolarizing factor (Adeagbo et al., 2003). In addition, natriuretic effect of tempol infused into the renal medullary interstitium was not abolished by NO synthase blockade (Zou et al., 2001). In vitro, superoxide stimulates whereas tempol inhibits chloride reabsorption in the medullary thick ascending limb in NO-independent manner (Ortiz and Garvin, 2002b). Tempol decreases the activity of the sympathetic nervous system (SNS) including renal sympathetic nerves (Shokoji et al., 2004). Leptin stimulates SNS (Dunbar et al., 1997) and norepinephrine released by renal sympathetic endings enhances Na^+-transport especially in the proximal tubule (DiBona and Kopp, 1997). Thus, hypotensive and natriuretic effects of tempol in leptin-treated rats might be in part mediated via the SNS. In addition, intrarenally generated ROS accelerate the breakdown of 20-hydroxyeicosatetraenoic acid (20-HETE), a cytochrome P450-dependent arachidonate metabolite which inhibits Na^+ reabsorption in the medullary thick ascending limb, whereas tempol prevents this effect (Hoagland et al., 2003). It remains to be established whether one of these or the other, yet unidentified, NO-independent mechanism contributes to antihypertensive and natriuretic effect of tempol in hyperleptinemic rats.

NAD(P)H oxidase is a multimeric O_2^- generating enzyme consisting of membrane-bound subunits, $gp91^{phox}$ and $p22^{phox}$, as well as of cytosolic components, $p47^{phox}$, $p67^{phox}$ and small GTP-binding protein, Rac1. First identified in phagocytes, NAD(P)H oxidase is widely expressed in the cardiovascular system and the kidney and is the principal source of superoxide in these organs (Geiszt et al., 2000; Zou et al., 2001; Lassegue and Clempus, 2003). Vascular and renal expression and/or activity of NAD(P)H oxidase is up-regulated in various models of hypertension including hypertension induced by experimental obesity (Zalba et al., 2001; Sonta et al., 2004, Shinozaki et al., 2004). Apocynin inhibits NAD(P)H oxidase by blocking the assembly of $p47^{phox}$ and $p67^{phox}$ both in vitro and in vivo and decreases blood pressure in mineralocorticoid-induced hypertension (Beswick et al., 2001b) and in angiotensin II-induced hypertension (Virdis et al., 2004). We observed that apocynin mimicked the effect of tempol on isoprostanes, nitrotyrosine, NO production, cGMP excretion, natriuresis and blood pressure, suggesting that NAD(P)H oxidase is a dominant source of ROS also in leptin-induced hypertension. There are several potential mechanisms through which leptin could stimulate NAD(P)H

oxidase. Leptin augments endothelin-1 production by endothelial cells (Quehenberger et al., 2002) and endothelin-1 stimulates vascular NAD(P)H oxidase (Duerrschmidt et al., 2000). Leptin may also activate protein kinase C (Maingrette and Renier, 2003), which is a well-known regulator of vascular and renal NAD(P)H oxidase (Sonta et al., 2004). NAD(P)H oxidase is activated by angiotensin II (Lassegue and Clempus, 2003). The effect of leptin on plasma angiotensin level has not been studied, however, Bornstein and Torpy (1998) have demonstrated that 7-day i.p. administration of leptin at a dose of 0.12 mg/kg increases plasma renin activity in the rat. Finally, leptin stimulates production of proinflammatory cytokines such as tumor necrosis factor-α (Loffreda et al., 1998), which activates NAD(P)H oxidase not only in phagocytes but also in the vasculature (Li et al., 2002).

The major limitation of this study is that we did not examine NAD(P)H oxidase expression and/or activity in the kidney and vasculature of leptin-treated rats. This will be done in ongoing study, together with examining the expression of main antioxidant enzymes. Nevertheless, up-regulation of NAD(P)H oxidase is not the definite evidence of its causative role in a given model of hypertension for several reasons. First, increased blood pressure per se stimulates vascular NAD(P)H oxidase, as recently demonstrated in experimental aortic coarctation (Sindhu et al., 2005). Second, dietary antioxidants partially normalize $p91^{phox}$ and $p22^{phox}$ expression in the kidney of spontaneously hypertensive rat, suggesting that oxidative stress may secondarily up-regulate NAD(P)H oxidase (Zhan et al., 2004). Finally, our model of hyperleptinemia is associated with positive sodium balance which also stimulates renal NAD(P)H oxidase (Kitiyakara et al., 2003). Thus, the effect of apocynin may be more reliable evidence that increased ROS production is primarily accounted for by NAD(P)H oxidase than increased expression of this enzyme.

Conclusions

We have demonstrated that administration of superoxide scavenger, tempol, attenuates systemic and intrarenal oxidative stress, normalizes NO production and renal sodium excretion as well as reduces blood pressure in rats with experimentally induced hyperleptinemia. The effects of tempol are reproduced by apocynin, suggesting that NAD(P)H oxidase is a main source of reactive oxygen species in leptin-induced hypertension. These data indicate that oxidative stress-induced NO deficiency is involved in the pathogenesis of hypertension associated with hyperleptinemia and suggest that antioxidant treatment may be useful in controlling blood pressure in hyperleptinemic obese individuals.

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