

Oxidative Stress Causes Imbalance of Renal Renin Angiotensin System (RAS) Components and Hypertension in Obese Zucker Rats

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Background—Oxidative stress plays an important role in the pathogenesis of hypertension, especially in obesity-related hypertension. The natriuretic and antinatriuretic components of the renal renin angiotensin system (RAS) maintain sodium homeostasis and blood pressure. Here, we test the hypothesis that increased oxidative stress leads to the imbalance of RAS components and hypertension in obese Zucker rats.

Methods and Results—Lean and obese rats received vehicle or tempol, a superoxide dismutase mimetic in the drinking water for 4 weeks. Compared with vehicle-treated lean rats, vehicle-treated obese rats exhibited higher blood pressure and increased renal oxidative stress, accompanied by increased diuretic and natriuretic responses to AT₁R antagonist (Candesartan) and AT₂R agonist (CGP-42112A) and reduced diuretic and natriuretic response to MasR agonist (Ang-[1 to 7]). Moreover, obese rats had higher ACE, AT₁R and AT₂R, lower ACE2 and MasR expressions in the kidney. All of the above-mentioned abnormalities were reversed to some degree by tempol treatment. In primary cultures of renal proximal tubular (RPT) cells from lean and obese rats, tempol treatment also increased AT₂R, ACE2, and MasR expressions but decreased AT₁R and ACE expressions in obese rats.

Conclusions—Taken together, our study indicated that the imbalance of renal RAS components was associated with increased oxidative stress in obese rats. Furthermore, antioxidant treatment with tempol reversed the imbalance of renal RAS components and led to diuresis and natriuresis, which, at least in part, explains the blood pressure-lowering effect of antioxidant supplementation in obesity-related hypertension. (*J Am Heart Assoc.* 2015;4:e001559 doi: 10.1161/JAHA.114.001559)

Key Words: hypertension • obesity • oxidative stress • renin-angiotensin system • tempol

Obesity plays an important role in the pathogenesis of hypertension.^{1,2} Both human and animal models of obesity-related hypertension are characterized by a tendency to retain sodium, which may contribute to the development of hypertension.^{3–5} The causes of the abnormality in sodium homeostasis in obesity-related hypertension remain unclear. However, it is likely that an imbalance between natriuretic and antinatriuretic factors plays an important role.^{6–8} The renin-

angiotensin system (RAS) is a major regulator of sodium and water homeostasis. Among all of the components in RAS, angiotensin II (Ang II) is pre-eminent, by binding to 2 major receptor subtypes, angiotensin II type-1 receptor (AT₁R) and type-2 receptor (AT₂R).⁹ Both receptors are ubiquitously expressed, AT₁R is involved in mediating vasoconstriction and antinatriuresis, which increase in blood pressure,¹⁰ whereas activation of AT₂R is associated with vasodilatation, apoptosis, anti-proliferation, and natriuresis, potentially lowering blood pressure. Therefore, the AT₂R is considered an antagonist of the AT₁R.^{11,12} In addition, the activation of components of the “non-classical pathway,” angiotensin-converting enzyme 2 (ACE2) and other peptidases leads to generation of Ang-(1 to 7) and acting on MasR, also opposes the AT₁R-mediated effects, leading to vasodilatation and natriuresis.¹³ Hyperactivity of the RAS, mainly via enhanced AT₁R function, contributes to the excessive renal sodium reabsorption in obesity-related hypertension.^{14,15} There is evidence that AT₁R activation decreases ACE2 activity and Ang-(1 to 7) production.¹⁶ An imbalance between the antinatriuretic AT₁R and natriuretic agents outside the RAS has also been suggested to be involved in the

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pathogenesis of hypertension in spontaneously hypertensive rats and the hypertension associated with aging.^{17,18}

The precise nature of the imbalance of renal RAS components in human and animal models of hypertension remains to be elucidated. However, there is evidence that obesity, aging, diabetes, and hypertension are associated with increased oxidative stress,^{19–22} in which there is increased activity of the RAS. Oxidative stress can regulate components of the RAS. For example, oxidative stress upregulates renal AT₁R expression; leading to increased stimulation of the activities of Na-K-ATPase, and Na/H exchanger 3 in renal proximal tubules, thus contributing to sodium retention and an increase in blood pressure.²³ Chronic Ang II-infusion, via increased oxidative stress, elevates blood pressure and more importantly causes an imbalance between the hypertensive (ACE, Ang II and AT₁R) and anti-hypertensive components (ACE2, Ang-[1 to 7], Mas and AT₂R) of the RAS in the brain.²⁴ A previous study showed that amelioration of oxidative stress by exercise attenuated the vasoconstrictor axis of the RAS and improved the vasoprotective axis of the RAS in the brain of spontaneously hypertensive rats.²⁵ We hypothesize that oxidative stress would cause the imbalance of the renal RAS components in obese Zucker rats. Tempol, an antioxidant reagent, could reverse the imbalance of renal RAS components in obese Zucker rats, and therefore, lower the obesity-related hypertension, in part, by increased renal sodium excretion. Our current study found that obese Zucker rats fed tempol in drinking water, ameliorated oxidative stress, restored the balance between the natriuretic and antinatriuretic components of the renal RAS, increased sodium excretion, and reduced blood pressure. Thus, therapies that reduce superoxide bioavailability have the potential to ameliorate renal oxidative stress and improve the balance of renal RAS components in obesity-related hypertension.

Materials and Methods

Animal Preparation

The experimental protocols were approved by the Animal Care and Use Committee from The Third Military Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). Ten-week-old male obese (280 to 300 g) and lean (200 to 220 g) Zucker rats (Charles River Laboratory, Wilmington, MA) were maintained in our animal care facility, with a 12-hour light/dark cycle and had free access to standard rat chow and drinking water ad libitum. Lean and obese Zucker rats were randomly assigned to control group and tempol group. Control rats were provided plain tap water for drinking. The tempol group rats were provided with tempol (1.0 mmol/L; Sigma, St. Louis,

MO) dissolved in the drinking water, which was changed twice a day for 4 weeks.

Systolic, diastolic, and mean arterial blood pressures were measured in conscious animals by using a noninvasive tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan) method. Blood pressure was measured every week until the end of the study.

Animal Surgery and Experimental Protocol

The rats were anesthetized with pentobarbital (50 mg/kg body wt, intraperitoneally), and placed on a heated table to maintain rectal temperature between 36 and 37°C. A tracheotomy (PE-240) was performed to facilitate breathing. Anesthesia was maintained by infusing pentobarbital sodium at 0.8 mg/100 g body wt per hour. Catheters (PE-50) were placed into the left external jugular for fluid replacement, and carotid artery for recording systemic arterial pressure and heart rate (Grass Instrument, Quincy, MA). A midline abdominal incision was performed, and both the right and left ureters were catheterized (PE-10). The right suprarenal artery, which originates from the right renal artery, was catheterized (PE-10 heat-stretched to 150 μm) and the vehicle (saline) or reagents were infused at a rate of 40 μL/h.²⁶ The duration of the surgical preparation was about 60 minutes. To maintain a stable urinary output, 5% albumin was used to replace the blood extracted during each period and normal saline solution equal to 1% body weight per hour for insensible fluid loss was maintained. The rats were allowed to stabilize for 120 minutes after surgery prior to the start of the 40-minute urine collections for clearance measurements.

In Vivo Studies on Renal Function

The protocol consisted of 5 consecutive urine collection periods with each period lasting for 40 minutes. The one 40-minute urine collection period when only the vehicle was infused into the right suprarenal artery was considered as baseline period. After the baseline period, the rats received a bolus dose of candesartan (10 μg/kg per minute, i.v.), an AT₁ receptor antagonist,¹⁸ and urine was collected for 3 time periods. Thereafter, the infusate was changed to the vehicle; urine was collected for one period, which was considered as the recovery period. The candesartan data, shown in this study, was the averages of the 3 urine collections during the candesartan infusion. Blood samples (300 μL; replaced with an equal volume of 5% albumin) were collected at the end of each period.

In another set of experiments, similar to the above protocol, after one baseline period, the rats were infused with a specific AT₂R agonist, CGP-42112A (1.0 μg/kg per minute)²⁷ or Ang-(1 to 7) (1.0 μg/kg per minute), a MasR

receptor agonist, for 3 time periods, followed by one recovery period in which only the vehicle was infused.

At the end of the experiment, blood samples were collected and plasma was separated by centrifugation at 1500g for 15 minutes at 4°C. Urine and plasma samples were stored at -20°C until use.

Blood and Urine Analysis

The urine and plasma samples were analyzed for sodium and potassium concentrations using a flame photometer 480 (Ciba Corning Diagnostics, Norwood, MA). Creatinine levels in the plasma and urine were measured using a creatinine analyzer (Beckman, Fullerton, CA). The glomerular filtration rate (GFR) (mL/min) was estimated by creatinine clearance.²⁸ Blood glucose was measured with a glucose analyzer (Roche, Indianapolis, IN), and plasma insulin was measured by rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO). Triglycerides were measured by a triglyceride analyzer (Polymer Technology Systems, Cardiochek, IN). Hematocrit (Hct) was measured by a micro-Hct centrifuge (Haemofuge Heraeus Instr, Germany).

Analysis of Oxidative Stress and Some Peptide Concentration

To measure oxidative stress and angiotensin peptide concentrations in the kidney cortex, the rats were euthanized using CO₂ inhalation. The kidneys were quickly removed and the renal cortices were extracted by methanol. Renal glutathione concentrations were assayed by colorimetric assay kit (21023; OXIS, Foster City, CA). The extent of lipid peroxidation in the renal cortex was determined by using a commercial kit that measured the generation of malondialdehyde (MDA), according to the manufacturer's protocol (Beyotime Institute of Biotechnology, Jiangsu, China). 8-isoprostane in urine was measured by RIA kit (516351; Cayman, Ann Arbor, MI).

Renal renin activity was quantified according to the method described by Giammattei et al²⁹ Ang-(1 to 7) concentrations in the renal cortex were measured by enzyme immunoassay (Bachem, CA).³⁰ Ang II concentrations were measured by using commercially available enzyme immunoassay (EIA) kit (Cayman Chemicals, Ann Arbor, MI) directly after methanol extraction of the renal cortex, as described previously.³⁰ Serum aldosterone concentrations were measured by a commercially available radioimmunoassay kit (DiaSorin, Dietzenbach, Germany), according to the manufacturer's instruction.³¹

Quantitative Real-Time RT-PCR (qRT-PCR) Analysis

Quantitative real-time RT-PCR was used to quantify the renal cortical mRNA expressions of RAS components, ie, ACE, AT₁R, AT₂R, ACE2, and MasR by using specific primers,³² listed in Table 1. Briefly, the rats from these separate groups were euthanized using CO₂ inhalation and the kidneys were quickly removed and immediately frozen on dry ice. Total RNA was isolated from the renal cortices using TRIzol reagent (Invitrogen), following the manufacturer's instructions; cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). Gene expression was measured by the $\Delta\Delta$ CT method and normalized to GAPDH mRNA expression. The data are presented as the fold-change of the gene of interest, relative to that of control rats.

Cell Culture

In vitro experiments utilized primary cultures of RPT cells from lean and obese Zucker rats (14 weeks of age): these RPT cells express all the components of RAS.^{33,34} RPTs were isolated as described previously.²⁸ Briefly, the rats were euthanized using CO₂ inhalation and a midline abdominal incision was made. The aorta was cannulated below the kidneys and kidneys were perfused with collagenase and hyaluronidase. Enrichment of

Table 1. List of Primers Used for Quantitative Real-Time RT-PCR

Gene	Sense	Antisense
ACE	TTGACGTGAGCAACTTCCAG	CAGATCAGGCTCCAGTGACA
AT ₁ R	CAAAGGAGATGGGAGGTCA	TGACAAGCAGTTTGGCTTTG
AT ₂ R	TGCTGTTGTGTTGGCATTCA	ATCCAAGAAGGTCAGAACATGGA
ACE2	ACCCTTCTTACATCAGCCCTACTG	TGTCCAAAACCTACCCACATAT
MasR	CACTGGCCCTCCTGATGAA	GGATGCCAGAATTGAACACAGA
GAPDH	AGACAGCCGCATCTTCTGT	CTTGCCGTGGGTAGAGTCAT

ACE indicates angiotensin-converting enzyme; AT₁R, angiotensin II type-1 receptor; AT₂R, angiotensin II type-2 receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction.

Table 2. General Parameters

Parameter	Lean Control	Lean Tempol	Obese Control	Obese Tempol
Body weight, g	287.8±5.7	289.8±5.2	432.5±6.3*	422.8±7.2*
Kidney weight, g	0.99±0.02	0.98±0.01	1.71±0.02*	1.60±0.01*#
Blood glucose, mmol/L	5.21±0.1	5.17±0.2	8.33±0.2*	6.71±0.1*#
Insulin, nmol/L	0.54±0.02	0.51±0.02	3.99±0.15*	1.38±0.15*#
Triglycerides, mg/dL	63.0±3.8	55.6±2.8	344.5±16.2*	111.9±8.3*#
GFR, mL/g kidney/min	0.92±0.06	0.94±0.05	0.61±0.07*	0.68±0.06*

Effect of tempol on some parameters in lean and obese Zucker rats. Data are shown as mean±SEM (n=8/group). GFR indicates glomerular filtration rate. $P<0.05$ was considered statistically significant. * $P<0.05$ vs lean control; # $P<0.05$ vs obese control.

RPTs was carried out using 20% Ficoll gradient in Krebs buffer. The band at the Ficoll interface was collected and washed by centrifugation at 250g for 5 minutes. Cell viability was determined using the trypan blue exclusion test. RPT cells were cultured using DMEM-F 12 (1:1) supplemented with 10% FBS, epidermal growth factor (EGF) 10 ng/mL, insulin 0.573 ng/mL, and penicillin 25 IU/mL, at 37°C in 95% air and 5% CO₂ in a humidified atmosphere. For experiments, the cells were plated on polystyrene tissue culture dishes at a density of 1.5×10^6 cells/well in 6-well culture plates. At

≈85% confluency, the RPT cells were treated with the SOD mimetic Tempol (0.2 μmol/L, Sigma) for 24 hours. Water was used as a vehicle control. After treatment with the reagent or vehicle, the cells were lysed and immunoblotted for ACE, AT₁R, AT₂R, ACE2, and MasR.

Immunoblotting

The rats from these separate groups were euthanized using CO₂ inhalation and the kidneys were quickly removed. The

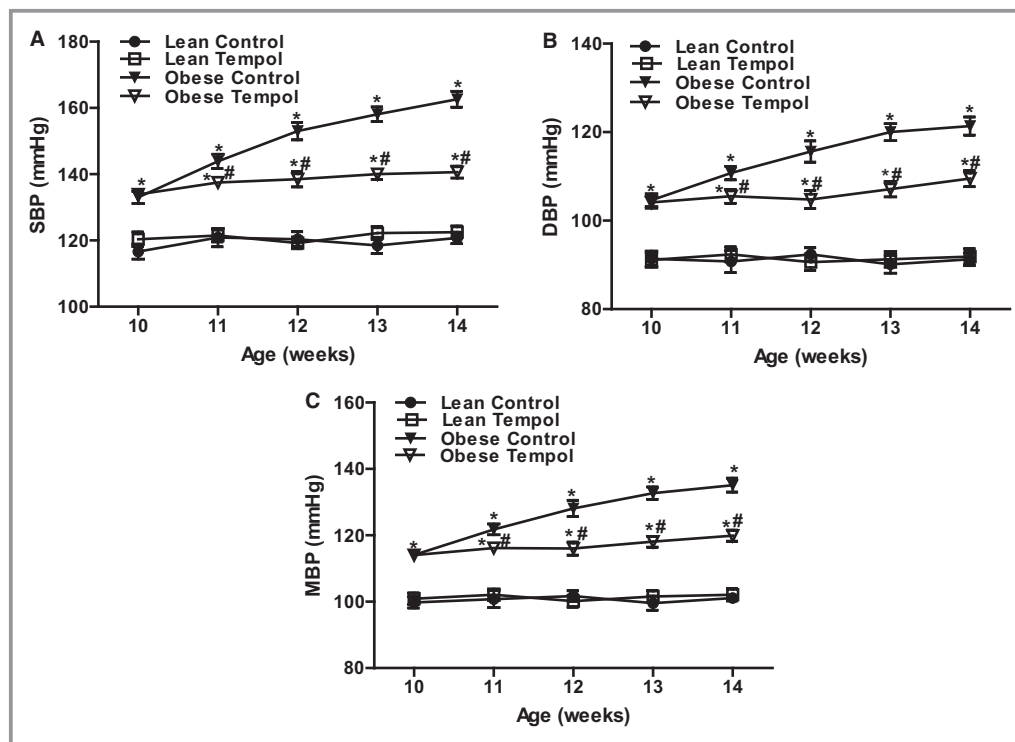


Figure 1. Effect of tempol on blood pressure in lean and obese Zucker rats. Blood pressure, including systolic blood pressure (SBP, A), diastolic blood pressure (DBP, B) and mean blood pressure (MBP, C), was recorded in lean and obese Zucker rats at different ages (10 to 14 weeks). The rats were treated with tempol (1.0 mmol/L) or vehicle for 4 weeks. Data are shown as mean±SEM (n=8/group). * $P<0.05$ vs lean control; # $P<0.05$ vs obese control.

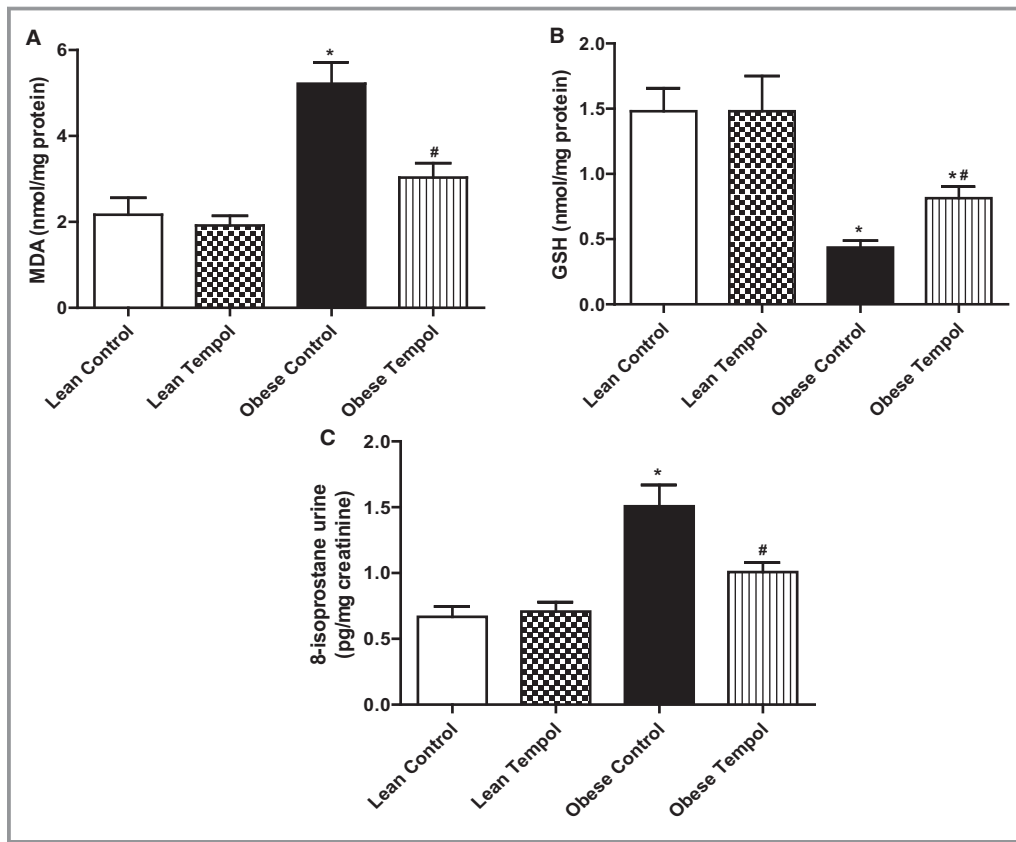


Figure 2. Effect of tempol on oxidative stress in the renal cortex from lean and obese Zucker rats. The indices of oxidative stress included malondialdehyde (MDA, A), Glutathione (GSH, B) and 8-isoprostane (C). The rats were treated with tempol (1.0 mmol/L) or vehicle for 4 weeks. Data are shown as mean±SEM (n=6/group). * $P<0.05$ vs lean control; # $P<0.05$ vs obese control.

renal cortices were homogenized in ice-cold lysis buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF, 10 µg/mL aprotinin, and 10 µg/mL leupeptin). The protein concentrations in the homogenates were quantified by a BCA method using a kit (Pierce, Rockford, IL). After boiling at 95°C for 5 minutes, 50 µg of protein were separated by SDS-PAGE (10% polyacrylamide gel), and then electroblotted onto nitrocellulose membranes (Amersham Life Science, Arlington, TX). The membranes were incubated overnight at 4°C with the primary rabbit polyclonal antibodies against AT₁R, AT₂R, ACE, and ACE2 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA), MasR (1:400; Alomone Labs Ltd, Jerusalem, Israel), and GAPDH (1:500, Santa Cruz Biotechnology), followed by washing and further incubation in horseradish peroxidase-conjugated anti-rabbit/anti-goat secondary antibody (1:10 000 dilution). The bands were visualized by enhanced chemiluminescence kit (Amersham, Arlington, TX), and the band intensities were quantified using Quantity-One software (Bio-Rad, Hercules, CA), and normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression.

Statistical Analysis

The data are expressed as mean±SEM. Comparison within the groups was made by repeated measures ANOVA with Newman-Keuls post hoc test, and comparison among groups was made by 1-way ANOVA with Newman-Keuls post hoc test. Statistical analysis was carried out using a software program (GraphPad Prism version 5; GraphPad Software, San Diego, CA). A value of $P<0.05$ was considered significant.

Results

Animal Characteristics

As shown in Table 2, the parameters, including body and kidney weights and plasma insulin, glucose, and triglyceride levels were higher in obese than lean Zucker rats ($P<0.05$). By contrast, GFR, corrected by kidney weight, was lower in obese than lean Zucker rat (Table 2). Tempol treatment for 4 weeks had no effect on body weight but lowered kidney weight and plasma insulin, glucose, and triglyceride levels in obese

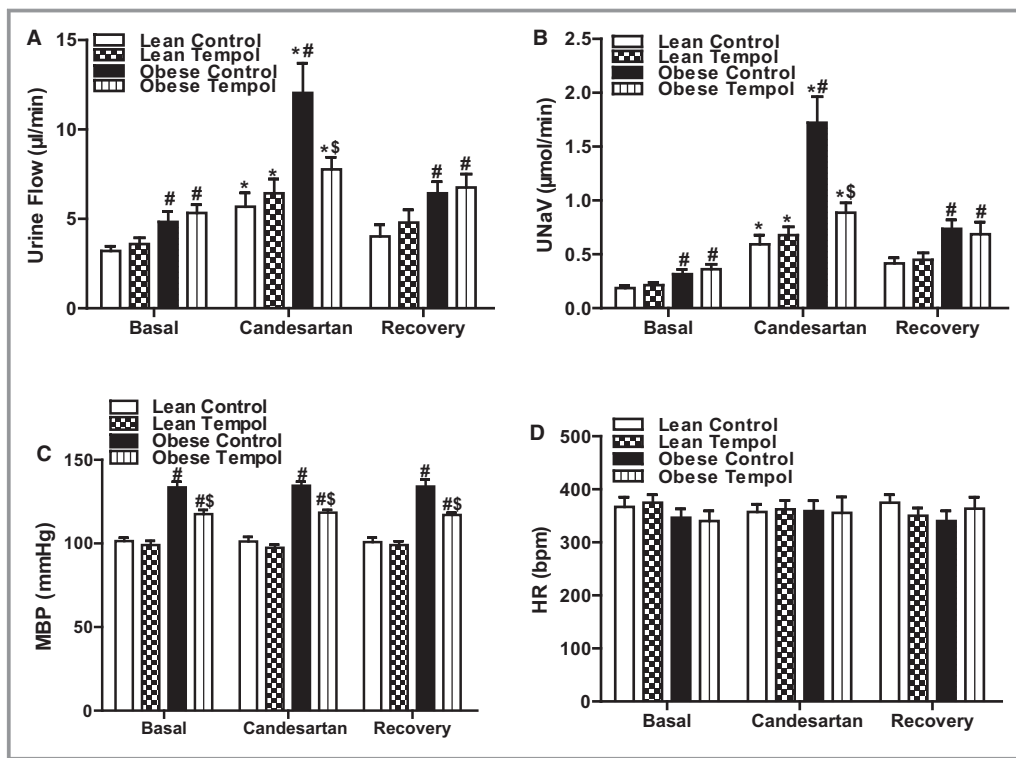


Figure 3. Effect of tempol on AT₁R function in lean and obese Zucker rats. Lean or obese Zucker rats were treated with tempol (1.0 mmol/L) or vehicle for 4 weeks. Urine flow (A), urinary sodium excretion (UNaV) (B), mean blood pressure (MBP) (C), and heart rate (HR) (D) were recorded during the vehicle or candesartan (10 µg/kg body wt per minute) infusion via the right suprarenal artery of anesthetized rats. Values of 3 reagent periods were averaged and shown as 1. Data are shown as mean±SEM (n=6/group). **P*<0.05 vs respective basal using repeated-measures ANOVA followed Newman-Keuls post hoc test; #*P*<0.05 vs lean control within the same treatment and \$*P*<0.05 vs obese control within the same treatment using 1-way ANOVA followed by Newman-Keuls post hoc test. ANOVA indicates analysis of variance; AT₁R, angiotensin II type-1 receptor.

(*P*<0.05), but not in lean Zucker rats. Tempol also did not affect GFR corrected for kidney weight. The basal blood pressures (systolic, diastolic, and mean blood pressures) were higher in obese than lean Zucker rats (Figures 1A through 1C). In the lean Zucker rats, tempol, added to the drinking water for 4 weeks, had no effect on blood pressure, which did not increase with age. However, tempol prevented the increase in blood pressure with age in obese Zucker rats although the blood pressures remained higher than those observed in lean Zucker rats (Figures 1A through 1C).

Effect of Tempol on Oxidative Stress in Obese Zucker Rats

To further determine the role of oxidative stress in lean and obese rats, we measured malondialdehyde (MDA), an index of lipid peroxidation, and glutathione (GSH), a pivotal antioxidant in the kidney. The renal cortical concentration of MDA was higher and GSH level was lower in obese than lean Zucker rats.

Also, urinary 8-isoprostane level was increased in obese relative to lean Zucker rats. Tempol supplementation decreased renal cortical MDA and urinary 8-isoprostane levels and increased renal cortical GSH level in obese Zucker rats but had no effect in lean Zucker rats (Figures 2A through 2C).

Effect of Tempol on Renal RAS Function in obese Zucker Rats

We next determined the effect of tempol on the function of several components of the RAS, including AT₁R, AT₂R, and MasR in lean and obese Zucker rats with or without tempol treatment. The candesartan-mediated diuresis and natriuresis were greater in obese than lean Zucker rats which were reversed by tempol in the obese but not lean Zucker rats (Figures 3A and 3B). Heart rate and blood pressure were not significantly affected by the intrarenal candesartan infusion in all 4 groups of rats (Figures 3C and 3D). CGP-42112A, an AT₂R receptor agonist, also induced a greater diuresis and

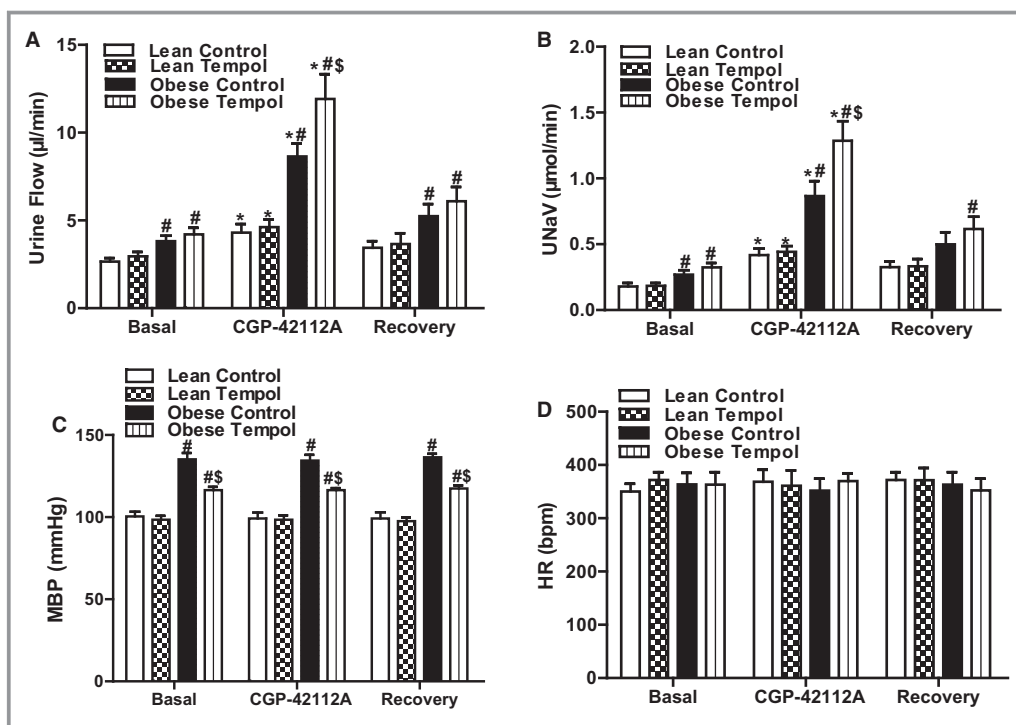


Figure 4. Effect of tempol on AT_2R function in lean and obese Zucker rats. Lean or obese Zucker rats were treated with tempol (1.0 mmol/L) or vehicle for 4 weeks. Urine flow (A), urinary sodium excretion (UNaV) (B), mean blood pressure (MBP) (C), and heart rate (HR) (D) were recorded during the vehicle or CGP-42112A (1.0 µg/kg body wt per minute) infusion via the right suprarenal artery of anesthetized rats. Values of 3 reagent infusion periods were averaged and shown as 1. Data are shown as mean±SEM (n=5/group). * P <0.05 vs respective basal using repeated-measures ANOVA followed Newman-Keuls post hoc test; # P <0.05 vs lean control within the same treatment and \$ P <0.05 vs obese control within the same treatment using 1-way ANOVA followed by Newman-Keuls post hoc test. ANOVA indicates analysis of variance; AT_1R , angiotensin II type-1 receptor.

natriuresis in obese than lean Zucker rats; the AT_2 receptor-mediated diuresis and natriuresis in the obese Zucker rats was augmented by treatment with tempol (Figures 4A and 4B). Heart rate and blood pressure were not significantly different in the 4 groups of rats during CGP-42112A infusion (Figure 4C and 4D).

Ang-(1 to 7) induced a diuresis and natriuresis in both lean and obese Zucker rats. However, the Ang-(1 to 7)-mediated diuresis was similar but the Ang-(1 to 7)-mediated natriuresis was lesser in obese than lean Zucker rats. Tempol treatment enhanced the effect of Ang-(1 to 7) in obese but not in lean Zucker rats (Figures 5A and 5B). Heart rate and blood pressure were not significantly different in all 4 groups of rats during Ang-(1 to 7) infusion (Figures 5C and 5D). There was no difference in Hct among the groups not affected by the protocols (%: basal periods, 31.6 ± 5.8 ; reagent periods, 32.5 ± 4.2 ; recovery periods, 33.2 ± 6.2). To determine if there was any systemic effect of the reagents selectively infused into the right renal artery, urine flow and sodium excretion from the left kidney were also measured. We found that the renal function, including urine flow and sodium excretion, in

the left unperfused kidney was not altered by the vehicle or any of the reagent treatments (data not shown).

Effect of Tempol on Renal RAS Components in Obese Zucker Rats

Because tempol modified the effect AT_1R -, AT_2R -, and MasR-mediated diuresis and natriuresis in obese Zucker rats, we determined if those effects were accompanied by changes in renal cortical mRNA and protein expressions of some components of the RAS, including ACE, ACE2, AT_1R , AT_2R , and MasR. We found that the basal expressions of ACE, AT_1R , and AT_2R were higher, while ACE2 and MasR expressions lower in obese than lean Zucker rats (Figures 6A and 6B), indicating that there was an imbalance between natriuretic and antinatriuretic components of the renal RAS in obese Zucker rats. Tempol had no effect on the RAS components in lean Zucker rats. However, in obese Zucker rats, tempol treatment decreased the renal ACE and AT_1R expressions close to the lean Zucker rat values but increased the renal ACE2 and MasR to lean Zucker rat values and but

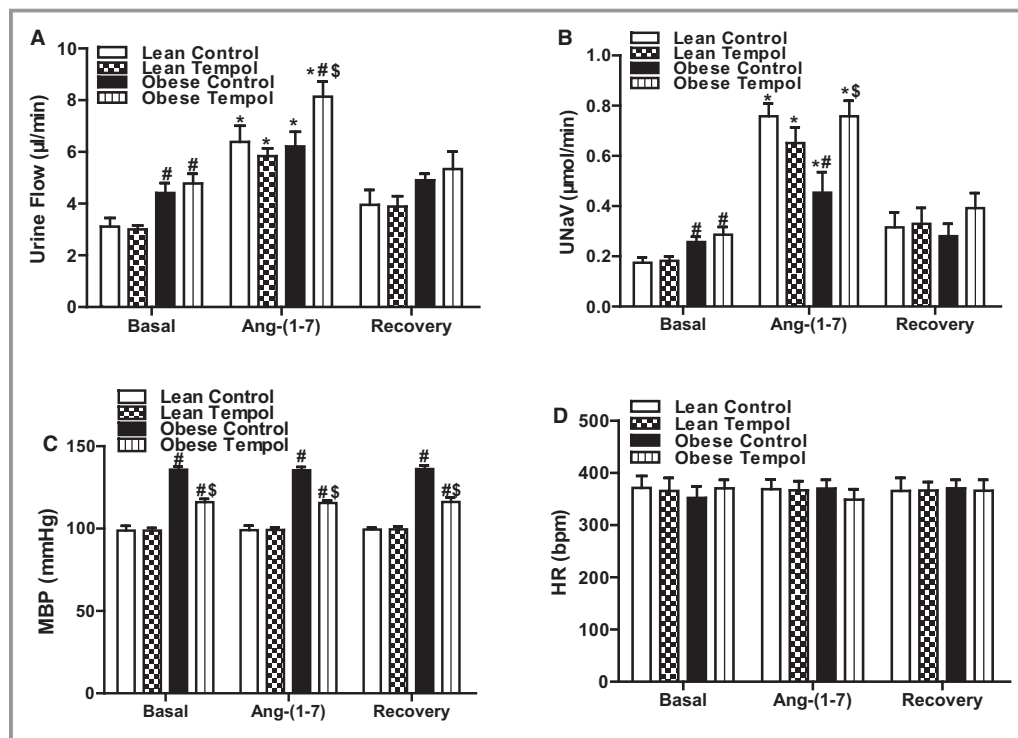


Figure 5. Effect of tempol on MasR function in lean and obese Zucker rats. Lean or obese Zucker rats were treated with tempol (1.0 mmol/L) or vehicle for 4 weeks. Urine flow (A), urinary sodium excretion (UNaV) (B), mean blood pressure (MBP) (C), and heart rate (HR) (D) were recorded during the vehicle or the Ang-(1 to 7) (1.0 µg/kg body wt per minute) infusion via the right suprarenal artery of anesthetized rats. Values of 3 reagent periods were averaged and shown as 1. Data are shown as mean±SEM (n=5/group). * P <0.05 vs respective basal using repeated-measures ANOVA followed Newman-Keuls post hoc test; # P <0.05 vs lean control within the same treatment and $^{\$}$ P <0.05 vs obese control within the same treatment using 1-way ANOVA followed by Newman-Keuls post hoc test. ANOVA indicates analysis of variance; AT₁R, angiotensin II type-1 receptor.

increased further the AT₂R expression in obese Zucker rats (Figures 6A and 6B). We also measured the renal renin (Figure 7A), Ang II (Figure 7B), and Ang-(1 to 7) (Figure 7C), and serum aldosterone concentrations (Figure 7D) in all 4 groups of rats. We found that compared with lean Zucker rats, in obese Zucker rats, renal cortical renin and Ang-(1 to 7) concentrations were lower, while Ang II and aldosterone concentrations were similar. Tempol treatment for 4 weeks had no effect on renal cortical renin, Ang II, Ang-(1 to 7), and serum aldosterone concentrations in lean Zucker rats but decreased renin, Ang II and serum aldosterone concentrations, and normalized Ang-(1 to 7) levels in obese Zucker rats. To study a direct effect of tempol on RAS components, we treated primary cultures of RPT cells from lean and obese Zucker rats (14 weeks) with tempol (0.2 µmol/L). RPT cells from control obese Zucker rats, relative to lean Zucker rats, had increased protein expressions of ACE, AT₁R, and AT₂R, but decreased protein expressions of ACE2 and MasR. Treatment of RPT cells from obese Zucker rats with tempol decreased ACE and AT₁R expression and increased ACE2 and

MasR; AT₂R expression was increased further. There were no effects in RPT cells from lean Zucker rats (Figure 8). All the in vitro data are agreement with the renal cortical values from the in vivo studies.

Discussion

The obese Zucker rat, a model of the metabolic syndrome, exhibits an impaired diuresis and natriuresis that are ascribed to the imbalance between antinatriuretic and natriuretic factors similar to that observed in human essential hypertension and spontaneously hypertensive rats.³⁵ The imbalance is also found in the RAS, ie, increased AT₁R and AT₂R function but decreased MasR function. Although a large body of evidence has accumulated to indicate the presence of increased oxidative stress in hypertension and type II diabetes, it is unclear whether this phenomenon is responsible for the imbalance of renal RAS components and superoxide-lowering therapy with tempol could improve the imbalance in obese Zucker rats. Our current study found that

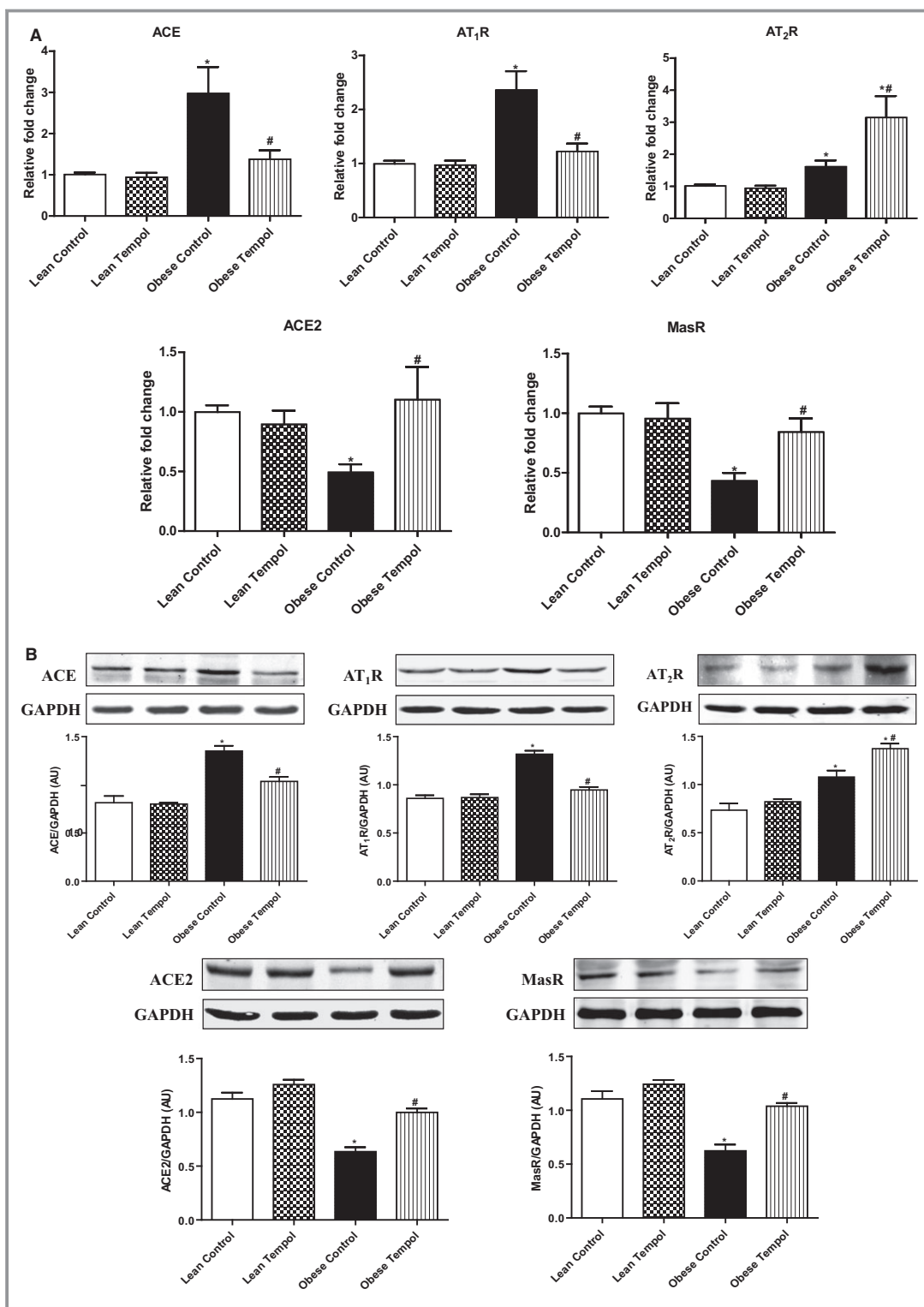


Figure 6. Effect of tempol on the mRNA and protein expressions of RAS components in renal cortex of lean and obese Zucker rats. Those RAS components including ACE, AT₁R, AT₂R, ACE2 and MasR mRNA and proteins were quantified by qRT-PCR and immunoblotting, respectively, and normalized by GAPDH. mRNA expression in renal cortex (A). A representative western blot and densitometric analysis (B). Data are shown as mean±SEM (n=5/group). *P<0.05 vs lean control; #P<0.05 vs obese control. ACE indicates angiotensin-converting enzyme; AT₁R, angiotensin II type-1 receptor; AT₂R, angiotensin II type-2 receptor; qRT-PCR, quantitative real-time polymerase chain reaction; RAS, renin angiotensin system.

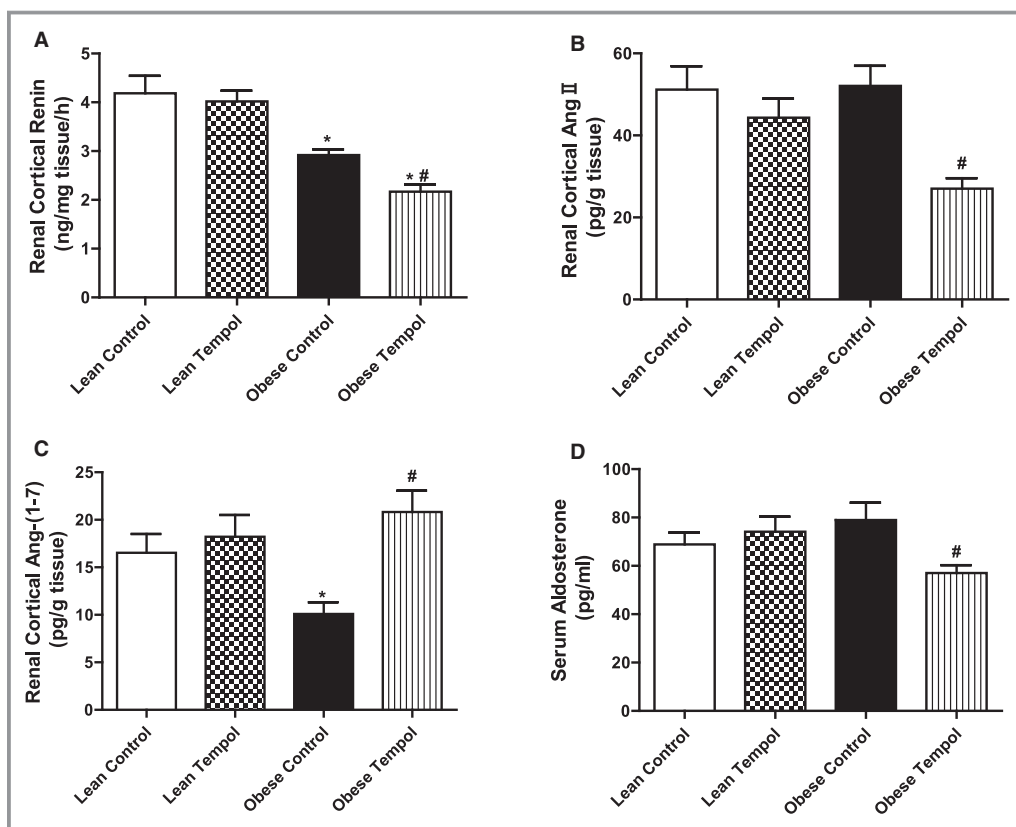


Figure 7. Effect of tempol on renin, angiotensin II, angiotensin (1 to 7), and aldosterone in lean and obese Zucker rats. The concentrations of renin (A), Ang II (B) and Ang-(1 to 7) (C) in renal cortex, and serum aldosterone (D) were quantified using commercially available enzyme immunoassay kits. Data are shown as mean \pm SEM (n=6/group). * P <0.05 vs lean control; # P <0.05 vs obese control.

antioxidant supplementation with tempol not only ameliorated renal oxidative stress and prevented the increase in blood pressure with age in obese Zucker rats. Tempol also restored the balance between the renal natriuretic and antinatriuretic RAS components, which, at least in part, could explain the blood pressure-lowering effect of the antioxidant supplementation in this obesity-related hypertension.

A large amount of accumulated data indicates that oxidative stress is an important contributing factor in hypertension.^{36,37} Hypertensive patients and animal models of hypertension have increased reactive oxygen species production and decreased antioxidant capacity.³⁸ Vaziri et al showed that L-buthionine-sulfoximine, a γ -glutamylcysteine synthetase inhibitor, causes oxidative stress and increases blood pressure in Sprague-Dawley rats. Antioxidant supplementation with tempol attenuates oxidative stress and normalizes blood pressure in these animals.^{39,40} Tempol, a membrane-permeable superoxide dismutase mimetic has been shown to reduce oxidative stress and attenuate hypertension in old FBN hybrid rats, obese Zucker rats, and spontaneously hypertensive rats.^{18,21,41} Our current study also found an imbalance between renal oxidative and

anti-oxidative factors in obese Zucker rats, ie, increased renal lipid peroxidation and nitrosylation and decreased GSH levels. The imbalance in redox status is accompanied by an increase in blood pressure in obese Zucker rats. Tempol treatment restored the balance between superoxide and anti-oxidant factors, and lowered the blood pressure in obese Zucker rats, indicating the important role of oxidative stress in the pathogenesis of obesity-related hypertension. Moreover, our current study excludes the possibility that the lowering of blood pressure is due to weight loss; as we did not observe a change in body weight with tempol treatment in obese Zucker rats.

It is believed that renal RAS plays an important role in the regulation of renal sodium excretion and blood pressure.⁴² Activation of AT₁R decreases sodium excretion while AT₂R and MasR exert the opposite effect; activation of the latter 2 receptors increases sodium excretion.^{10–13} A normal balance among the AT₁R, AT₂R, and MasR keeps sodium excretion and blood pressure in the normal range. However, in hypertensive states, that balance is lost.^{18,23} An enhanced AT₁R function is one of the mechanisms responsible for increased renal sodium reabsorption and blood pressure in obese rats.^{14,15}

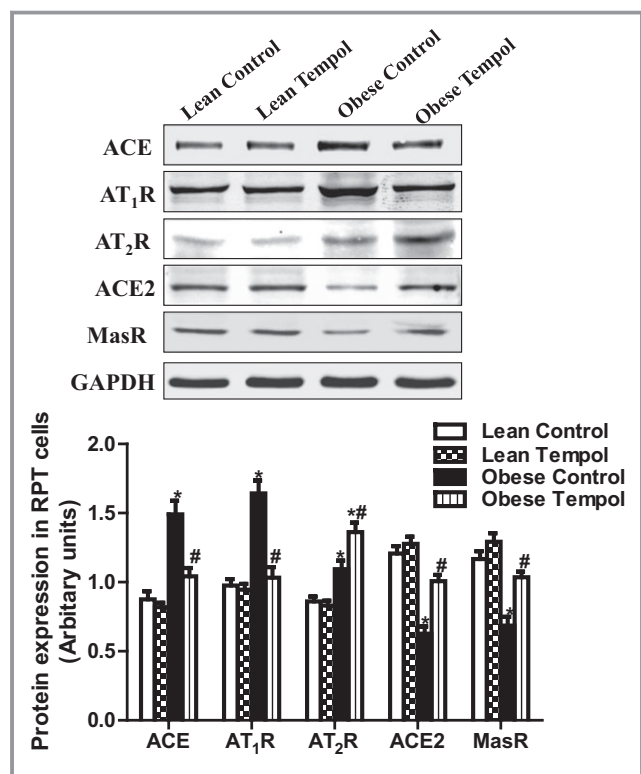


Figure 8. Effect of tempol on the expressions of RAS components in primary cultures of RPT cells from lean and obese Zucker rats (14 weeks). The RPT cells were treated with tempol (0.2 $\mu\text{mol/L}$) for 24 hours. RAS components including ACE, AT₁R, AT₂R, ACE2 and MasR were determined by immunoblotting, and normalized by GAPDH. Data are expressed as mean \pm SEM (n=6 in each group). * $P < 0.05$ vs lean control; # $P < 0.05$ vs obese control. ACE indicates angiotensin-converting enzyme; AT₁R, angiotensin II type-1 receptor; AT₂R, angiotensin II type-2 receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RAS, renin angiotensin system; RPT, renal proximal tubular.

AT₁R activation has been shown to downregulate ACE2 expression and activity and Ang-(1 to 7) production.¹⁶ Our current study also found an imbalance of renal RAS components in obese Zucker rats. Candesartan, an AT₁R blocker, promoted a greater diuresis and natriuresis in obese than lean Zucker rats, while MasR-mediated natriuresis and diuresis were weaker in obese than lean Zucker rats. Consistent with other reports, AT₂R-mediated effect is greater in obese than lean Zucker rats, and is thought to be a compensatory phenomenon against the increased blood pressure of obese Zucker rats.¹² There is strong evidence that an increase in oxidative stress leads to the imbalance of RAS in old rats and rats made hypertensive with angiotensin II.^{18,24} Previous studies have shown that oxidative stress upregulates renal AT₁R and exaggerates Ang II signaling that lead to the increased stimulation of sodium transporters and contribute to the increase in blood pressure.²³ In our current study, we found that tempol treatment reversed the augmented AT₁R

function and restored MasR function. It is also interesting to find that tempol can further increase the compensatory effect of the AT₂R. The increase in AT₂R function and expression may aid in the normalization renal function and blood pressure of obese Zucker rats.

In addition to the AT₁R, AT₂R, and MasR, we also tested for other components of the RAS, including renin, Ang II, Ang-(1 to 7), and aldosterone levels, ACE and ACE2 expression. The AT₂R activation has been reported to negatively regulate renin levels.⁴³ Previous studies have shown that renal renin expression was lower in obese Zucker compared with lean rats.⁴⁴ Thus, it is likely that renin levels could be further reduced by the AT₂R activation with tempol treatment in obese rats. However, our present study found that although renin concentration was lower in obese than lean Zucker rats, Ang II concentration was not different between lean and obese Zucker rats. This could be due to increased activity of ACE that compensates for the decrease in renin, resulting in unchanged Ang II production. Contrary to our results, Sharma et al reported an increase in renal Ang II concentrations in obese compared with lean rats.⁴⁵ The reason for this discrepancy is not clear. However, the different batch and source of rats and different methods used could be reasons of such discrepancy. Consistent with previous reports, there is no difference in aldosterone concentration in lean and obese rats⁴⁶ that may be related to unchanged Ang II production.

Other studies have shown that the balance between ACE and ACE2 is important in determining the development of hypertension.⁴⁷ ACE expression is up-regulated while ACE2 expression is down-regulated in many animal models of hypertension and patients with hypertension.⁴⁸ Consistent with other reports,⁴⁹ our current study also found increased renal ACE expression and decreased renal ACE2 expression in obese Zucker rats. Treatment with tempol normalizes those changes, which might be also involved in its blood pressure effect. A variety of factors, such as hyperglycemia and dyslipidemia, probably contribute to the increased oxidative stress in obese Zucker rats. In accordance with the observations of previous studies,²¹ our current results also showed that tempol treatment reduced plasma lipids and insulin and blood glucose concentrations in obese Zucker rats. Therefore, we speculate the possibility that the decrease in circulating insulin, glucose, and plasma lipids with tempol treatment can further reduce oxidative stress status, and could be also responsible for restoring the balance of RAS components and reducing blood pressure in obese Zucker rats. In our current study, we found that tempol only prevented further increases in blood pressure with age, but did not reduce the blood pressure to the same as lean rats, suggesting that although oxidative stress is an important, but not the only factor involved in the pathogenesis of hypertension.

Based on experimental evidence of the importance of oxidative stress in hypertension, there has been great interest in developing strategies that target reactive oxygen species in the treatment of hypertension. However, antioxidant-based therapies have failed to show therapeutic impact in human patients with hypertension.^{50,51} Although the reasons for these failures are not completely known, it might, at least, be related with the following: (1) antioxidants used; (2) patients included in trials; and (3) the trial design, itself. With respect to antioxidants, it is possible that agents used were ineffective, not appropriate, or that the dosing and duration of therapy were insufficient.⁵² Regarding the individuals included in large trials, most subjects had significant cardiovascular disease that may no longer be reversible.⁵² Another confounding factor is that most of the enrolled subjects were taking aspirin prophylactically. Because aspirin has intrinsic antioxidant properties, additional antioxidant therapy may be ineffective.⁵³ To date, there are no large clinical trials in which patients were recruited based on evidence of elevated reactive oxygen species formation. It is possible that in the absence of oxidative stress, antioxidants are ineffective in the treatment of hypertension.

In conclusion, our current study provides convincing and substantial evidence that oxidative stress causes an imbalance of renal RAS components in obese Zucker rats. Chronic antioxidant supplementation could attenuate oxidative stress, lower blood pressure, and modulate the balance between the natriuretic and antinatriuretic components of the renal RAS in obese Zucker rats.

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Disclosures

None.

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