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# Renal and metabolic effects of tempol in obese $ZSF_1$ rats—distinct role for superoxide and hydrogen peroxide in diabetic renal injury

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**Abstract**

Oxidative stress, that is, overproduction of reactive oxygen species and reduced antioxidant system activity, is implicated in the pathogenesis of diabetic complications; and therefore, superoxide dismutase (SOD) mimetic tempol should be protective in diabetic kidney. However, the effects of tempol in metabolic syndrome–associated renal injury have not been thoroughly examined. In this study, we examined the effects of 9 weeks of treatment with tempol on metabolic status, renal oxidative stress, and kidney function and structure in obese, diabetic, hypertensive $ZSF_1$ rats and their nondiabetic, hypertensive, lean littermates. The obese rats had significantly reduced total SOD and catalase activity, increased peroxidase activity and lipid peroxidation, and higher level of protein oxidation in renal cortical tissue compared with their lean littermates. These changes were accompanied by renal injury (proteinuria; reduced excretory function; and markedly increased glomerular and interstitial inflammation, proliferation, and collagen IV synthesis). Tempol treatment slightly increased total SOD activity, significantly reduced lipid peroxidation and peroxidase activity, but had no effect on catalase and protein oxidation. Tempol had no effects on blood pressure, renal hemodynamics and excretory function, and proteinuria in obese rats, yet improved insulin sensitivity and reduced renal inflammatory, proliferative, and fibrotic changes. Because tempol possesses no catalase activity and, in diabetes, not only SOD but also catalase is inhibited, it is possible that the toxicity of hydrogen peroxide ($H_2O_2$) remains unaltered under tempol treatment. This study suggests that superoxide and $H_2O_2$ may have distinct roles in the pathogenesis of diabetic renal injury, with superoxide mainly being involved in inflammatory, proliferative, and fibrotic changes, and $H_2O_2$ in glomerular hemodynamics and proteinuria.


## 1. Introduction

There is a growing body of evidence indicating that oxidative stress is increased in both diabetes and metabolic syndrome and that it may contribute to the development of obesity-related diabetes, hypertension, and nephropathy. The balance between the production of reactive oxygen species (ROS) and the antioxidant defense system activity determines the level of oxidative stress. In this regard, overproduction of superoxide ($O_2^{\cdot-}$) in diabetes is well documented [1]. Hyperglycemia (via autooxidation of glucose, polyol pathway, and advanced glycation), increased free fatty acids, and oxidized low-density lipoproteins all lead to overproduction of mitochondrial nicotinamide adenine dinucleotide. In turn, the excess of nicotinamide adenine dinucleotide (NAD) produces an increase in mitochondrial proton gradient; so the single electrons are transferred to oxygen, leading to the formation of $O_2^{\cdot-}$ [2]. The diabetic environment also increases NAD phosphate (NADPH) oxidase activity; and nonphagocytic form of the enzyme is considered a key molecular source for hyperproduction of $O_2^{\cdot-}$ in cardiorenal cell compartments, including vascular smooth muscle cells, endothelial cells, fibroblasts, and mesangial cells [3-5]. Diabetes is also characterized by inhibition of the 2 main antioxidant enzymes: superoxide dismutase (SOD), which converts $O_2^{\cdot-}$ to hydrogen peroxide

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($H_2O_2$), and catalase (CAT), which provides the subsequent degradation of $H_2O_2$ to water and molecular oxygen [1,3]. The abundant ROS production, together with insufficient activity of the antioxidant defense system in diabetes, suggests potentially beneficial effects of antioxidant therapy in metabolic syndrome–associated renal disease. However, the systemic administration of antioxidant enzymes such as SOD or CAT is limited (*a*) because of the large structure of these enzymes that does not allow them to permeate biological membranes and to attenuate the detrimental effects of ROS, produced intracellularly, and (*b*) because of partial glomerular filtration and, therefore, reduced renal bioavailability. To overcome these limitations, ROS scavengers with low molecular weight have been developed.

Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl) is a stable, metal-independent, low–molecular weight SOD mimetic with an excellent cell permeability. Tempol has been shown to be renoprotective and to attenuate oxidative stress–mediated renal dysfunction and injury caused by renal ischemia, hemorrhagic shock, or endotoxin [6-8]. Furthermore, tempol reduces blood pressure and renal oxidative stress in spontaneously hypertensive rats (SHR) [9], decreases elevated blood pressure and renal inflammatory response in mineralocorticoid-induced hypertension [10], and attenuates hypertensive renal damage in the Dahl salt-sensitive rats [11]. In rats with angiotensin II–induced hypertension, tempol does not alter blood pressure [12,13], yet increases glomerular filtration rate (GFR), urine flow, and sodium excretion; and these changes are associated with reduced 8-isoprostane but not $H_2O_2$ renal excretion [12]. In 2-kidney 1-clip hypertensive rats, tempol reduces blood pressure and renal vascular resistance (RVR) and increases GFR [14].

However, the effects of tempol in metabolic syndrome and associated renal injury have not been thoroughly examined; and the available data are inconclusive. Thus, in normotensive, insulin-resistant, obese Zucker rats, tempol reduces blood pressure and improves insulin sensitivity [15]; and in normotensive streptozotocin (STZ) diabetic rats, tempol reduces blood pressure and renal oxidative stress, but fails to reduce proteinuria [16].

The goal of this study was to investigate the effects of long-term treatment with tempol on metabolic status, blood pressure, renal oxidative stress, and kidney function and structure in obese (Ob; fa/fa$^{cp}$), diabetic, and hypertensive $ZSF_1$ rats and their lean (Ln; fa/+; +/+), nondiabetic, hypertensive littermates. The obese $ZSF_1$ rat model was developed by crossing lean normotensive female Zucker diabetic fatty rats with lean male spontaneously hypertensive heart failure rats. Obese (fa/fa$^{cp}$) $ZSF_1$ rats are hypertensive, hyperlipidemic, and diabetic, and develop marked renal dysfunction [17-19]. The use of lean controls permitted the renal effects of tempol to be studied in genetically identical (except for *fa* gene) hypertensive animals in the presence (Ob) or absence (Ln) of metabolic syndrome and renal disease.

## 2. Materials and methods

### 2.1. Animals

Sixteen 11-week–old, obese (body weight, 427 ± 5 g), male $ZSF_1$ rats and 14 lean (357 ± 5 g) littermates were obtained from Genetic Models (Indianapolis, IN). Animals were housed in the University of Pittsburgh Medical Center animal care facility (temperature, 22°C; light cycle, 12 hours; relative humidity, 55%) and allowed to acclimatize for 10 days before taking baseline measurements. Rats were given ad libitum a Pro Lab RMH 3000 rodent diet (PMI Nutrition, St Louis, MO). Institutional guidelines for animal welfare were followed, and the Institutional Animal Care and Use Committee approved the experimental protocols.

### 2.2. Metabolism cage studies

Before initiating the treatment, rats were placed in metabolic cages; and after a 48-hour acclimatization period, 24-hour urine volume and food and water intake were measured. Rats were removed from the metabolic cages and weighed, and blood samples were drawn from the tail vein and used for determination of plasma lipids. Metabolic cage studies were repeated after 2, 5, and 9 weeks of treatment. Total protein concentration was measured in the urine samples by a spectrophotometric assay using bicinchoninic acid reagent (Pierce, Rockford, IL) and a modification of the method of Lowry et al [20]. Urine glucose concentrations were measured by a spectrophotometric assay using the Infinity Glucose Reagent (Sigma Diagnostics, St Louis, MO), and urine samples were analyzed for creatinine by Creatinine Analyzer (Model 2; Beckman Instrument, Fullerton, CA). Plasma samples were analyzed in duplicates for triglycerides and cholesterol levels (Sigma Diagnostics). Prandial plasma levels of insulin and leptin were measured after 9 weeks of treatment using the Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem, Chicago, IL) and Mouse Leptin ELISA Kit (Crystal Chem), respectively. After baseline metabolism cage studies (week 0) were completed, animals were randomly assigned to drink tap water (Ln- and Ob-$ZSF_1$ groups) or 1 mmol/L solution of tempol (Sigma Diagnostics; Ln- and Ob-tempol groups). The selected dose of tempol (1 mmol/L) was shown to reduce blood pressure and renal oxidative stress and vasoconstriction in SHR [9] and to decrease elevated blood pressure and renal inflammatory response in mineralocorticoid-induced hypertension [10].

### 2.3. Acute measurements of renal hemodynamics

At 20 weeks of age, after 9 weeks of treatment, animals were fasted overnight; and next morning, blood samples were taken from tail vein for measurements of blood glucose. Precision QID Blood Glucose Test Strips kits (Medisence, Bedford, MA) were used to measure blood glucose levels. Next, each rat was anesthetized with pentobarbital (45 mg/kg intraperitoneally) and instrumented for measurements of blood pressure and renal hemodynamics. Briefly, a PE-240

polyethylene catheter was inserted into the trachea to facilitate breathing. A PE-50 catheter was inserted into the left carotid artery and connected to a digital blood pressure analyzer (BPA; Micro-Med, Louisville, KY) for continuous measurement of systolic, diastolic, and mean arterial blood pressure and heart rate. Two PE-50 cannulas were placed in the left jugular vein for infusion of $^{14}$C-inulin and supplemental anesthetic, respectively. Next, through midline abdominal incision, the left kidney was exposed; and a PE-10 catheter was inserted into the left ureter to facilitate collection of urine. A flow probe (Model 1RB; Transonic Systems, Ithaca, NY) was placed on the left renal artery for determination of renal blood flow (RBF), and intravenous infusion of $^{14}$C-inulin (0.035 $\mu$Ci/50 $\mu$L saline per minute) was initiated. A 60-minute stabilization period was permitted before two 30-minute clearance periods were conducted. Mean arterial blood pressure and RBF were recorded at \5-minute intervals and averaged during a 30-minute urine collection. A midpoint blood sample (100 $\mu$L) for measurement of radioactivity and hematocrit was collected. Urine volume was determined gravimetrically, and plasma and urine $^{14}$C-inulin radioactivity was measured (liquid scintillation analyzer, Model 2500TR; Packard Instrument, Downers Grove, IL). Inulin clearance (an estimate of GFR) and RVR were calculated.

### 2.4. Renal histopathology and immunohistochemical studies

After conducting acute measurements of renal function, animals were euthanized by anesthetic overdose; and kidneys were removed and weighed. The right kidney was fixed in 10% formalin buffer for subsequent light microscopy and immunohistochemistry. The kidney tissue sample was sectioned and processed into paraffin blocks for light microscopy. Five-micrometer tissue sections from formalin-fixed, paraffin-embedded renal cortices were dewaxed and stained with periodic acid Schiff stain for histologic assessment. Kidney slices were examined by light microscopy and were scored in a blinded fashion by one of the investigators (ES). Histopathologic features were assessed semiquantitatively on 10 high-power fields (×400) and included focal segmental and focal global glomerulosclerosis, tubular atrophy (0-3$^+$), interstitial inflammation (0-3$^+$), tubular dilation (0-4$^+$), arterial medial hypertrophy, and arteriolar sclerosis (0-3$^+$). For labeling of collagen IV, renal cortical segments (5 $\mu$m) were incubated overnight at 4°C with rabbit anti–mouse collagen IV antibody (dilution 1/500) obtained from Chemicon International (Temecula, CA). For labeling of proliferating cell nuclear antigen (PCNA), a primary monoclonal mouse antibody (1/200 dilution; Dako, Carpenteria, CA) was used. A rat ED1 antibody (Serotec, Raleigh, NC) specific for a monocyte/macrophage cytoplasmatic antigen was used to label glomerular and interstitial macrophages. To assess the nonspecific staining, the primary antibody was replaced with phosphate-buffered saline (PBS). Sections were washed and further developed according to the directions of the manufacturer (Dako) using the LSAB2 kit that contained a second antibody linked to avidin and peroxidase-conjugated biotin. A SAMBA 4000 image analyzer (Image Products International, Chantilly, VA) with specialized computer software (Immuno-Analysis, version 4.1; Microsoft, Richmond, WA) and a color video camera were used to quantitatively assess the immunochemical staining for collagen IV and PCNA. Software designed for immunostaining analysis enabled the operator to set density threshold values by averaging several fields on the negative control tissues in which the primary antibody was replaced with PBS. Background subtraction was then performed automatically on every tissue. Ten high-power fields (×400) were assessed for staining density or positively marked cells for ED1. The results are reported as the labeling index, which represents the percentage of the total examined area that stained positively. Staining intensity of positive areas was also assessed (mean optical density), and a mean quick score was then calculated (mean optical density × labeling index).

### 2.5. Measurement of SOD, CAT, and peroxidase activities

Renal cortical tissue samples were homogenized by lysis buffer (0.1% sodium dodecyl sulfate in PBS) in the presence of protease inhibitors. The tissue suspension was centrifuged at 15 000*g* for 30 minutes at 4°C. The resulting supernatant was used for determination of protein concentration and measurement of enzymatic activities. Total SOD activity was measured by using cytochrome *c*–based spectrophotometric assay [21]. The samples were mixed in 1/10 mixture of 50 $\mu$mol/L xanthine in 0.001 mol/L NaOH and 10 $\mu$mol/L cytochrome *c* in 0.1 mmol/L EDTA and 50 mmol/L phosphate buffer followed by the addition of 0.18 mg of xanthine oxidase in 0.1 mmol/L EDTA and 50 mmol/L phosphate buffer. The absorbance was recorded at 550 nm for 3 minutes. *One unit of SOD activity* was defined as the amount of the enzyme required to inhibit the rate of cytochrome *c* oxidation by 50%. Catalase activity was assayed by the method of Aebi [22] that observes the substrate ($H_2O_2$) conversion rate. The enzyme assay contained 1.0 mL of freshly prepared $H_2O_2$ solution (15 mmol/L in phosphate buffer) and 20 $\mu$L of tissue sample. The decrease in absorbance was read in a spectrophotometer at 240 nm against a blank for 60 seconds. Enzyme activity was expressed as CAT activity units per milligram of protein. The amount of units was calculated from calibration curve that was plotted using the dilutions of standard bovine liver CAT (Sigma Diagnostics). Total peroxidase activity was determined using Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, Oregon). A working solution containing 100 $\mu$mol/L Amplex Red and 2 mmol/L $H_2O_2$ was prepared immediately before use from stock solutions. Reactions were initiated by the addition of 20 $\mu$L of the Amplex Red/$H_2O_2$ working solution to 80 $\mu$L of sample (8 times diluted by

PBS). Fluorescent measurements of reaction product were made with excitation wave of 571 nm and emission of 585 nm. The kinetics were measured every 5 seconds for a total of 5 minutes. A series of dilutions of standard horseradish peroxidase solution was used for calibration.

### 2.6. Measurement of oxidative markers

The level of oxidative stress was controlled by measuring protein dityrosine cross-links formation as previously described [23]. For this purpose, the supernatant from tissue homogenates was subjected to spectrofluorimetric analysis using excitation at 315 nm and emission at 410 nm. The same samples were used for analysis of the level of total protein nitration. Sample aliquots (15 mg of protein in 10 $\mu$L) were transferred to nitrocellulose membrane (BioRad, Hercules, CA) using the BioRad dot blot system. The membrane was blocked with 5% milk for 1 hour and incubated at 4°C overnight with the first anti–3-nitrotyrosine antibody (Calbiochem, San Diego, CA). Membranes were washed 3 times in PBS containing 0.5% Tween 20 solution and then incubated at room temperature for 1 hour with horseradish peroxidase–conjugated mouse secondary antibody (Pierce) at 1:5000 dilution. The detection of horseradish peroxidase in dots was performed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Chemiluminescence was measured by Epi Chemi II bioimaging darkroom (Biovision, Mountain View, CA). Densitometric analysis was performed by using ImageJ National Institutes of Health software, and dot densities were normalized on protein concentration. The lipid peroxidation in plasma was analyzed using Lipid Hydroperoxide Assay Kit (Cayman Chemicals, Ann Arbor, MI).

### 2.7. Statistical analysis

Statistical analyses were performed using the Number Cruncher Statistical software program (Kaysville, UT). Group comparison for data from metabolic studies (repeated measurements) was performed by a 1F and 2F hierarchical analysis of variance (ANOVA), followed by a Fisher least significant difference test for post hoc comparisons. Comparison of data from acute experiments (single-point data) was performed by Student *t* test. The probability value of *P* less than .05 was considered statistically significant. All data are presented as mean ± SEM.

## 3. Results

### 3.1. Effects on the metabolic parameters and renal hemodynamics and excretory function

The measurements of metabolic parameters are presented in Table 1. Compared with their lean littermates, at 11 weeks of age, obese $ZSF_1$ rats had fully developed metabolic

Table 1
Metabolic parameters in Ln and Ob $ZSF_1$ rats and animals treated with tempol for 9 weeks

| Treatment | n | Body weight, g | Food intake, g/(kg 24 h) | Fluid intake, mL/(kg 24 h) | Urine volume, mL/(kg 24 h) | Urine glucose, g/(kg 24 h) | Plasma triglycerides, mg/dL | Plasma cholesterol, mg/dL |
|---|---|---|---|---|---|---|---|---|
| *Wk 2* | | | | | | | | |
| Ln-$ZSF_1$ | 7 | 390 ± 7 | 65 ± 2 | 81 ± 7 | 45 ± 1 | 0.01 ± 0.01 | 27 ± 11 | 93 ± 4 |
| Ln-tempol | 8 | 404 ± 4 | 65 ± 2 | 83 ± 5 | 38 ± 2 | 0.01 ± 0.01 | 194 ± 16 | 89 ± 2 |
| Ob-$ZSF_1$ | 8 | 530 ± 7* | 81 ± 2* | 170 ± 10* | 129 ± 10* | 10.7 ± 0.75* | 1757 ± 201* | 196 ± 8* |
| Ob-tempol | 8 | 507 ± 9* | 90 ± 2* | 209 ± 15* | 172 ± 13* | 17.2 ± 2.23* | 1787 ± 141* | 199 ± 8* |
| *Wk 5* | | | | | | | | |
| Ln-$ZSF_1$ | 7 | 438 ± 9 | 50 ± 2 | 82 ± 8 | 39 ± 1 | 0.02 ± 0.01 | 249 ± 43 | 85 ± 4 |
| Ln-tempol | 8 | 431 ± 6 | 49 ± 1 | 72 ± 8 | 38 ± 2* | 0.01 ± 0.01 | 250 ± 15 | 86 ± 3 |
| Ob-$ZSF_1$ | 8 | 565 ± 5* | 83 ± 5* | 201 ± 23* | 157 ± 10* | 18.9 ± 1.75* | 3149 ± 108* | 230 ± 7* |
| Ob-tempol | 8 | 536 ± 10* | 86 ± 2* | 255 ± 17* | 222 ± 15*,† | 25.5 ± 3.43* | 3664 ± 266* | 259 ± 11* |
| *Wk 9* | | | | | | | | |
| Ln-$ZSF_1$ | 7 | 466 ± 10 | 41 ± 6 | 77 ± 9 | 40 ± 7 | 0.02 ± 0.01 | 287 ± 17 | 81 ± 6 |
| Ln-tempol | 8 | 477 ± 7 | 53 ± 5 | 75 ± 5 | 37 ± .7 | 0.02 ± 0.01 | 293 ± 6 | 86 ± 4 |
| Ob-$ZSF_1$ | 8 | 606 ± 16* | 77 ± 2* | 211 ± 19* | 161 ± 11* | 16.5 ± 3.33* | 3791 ± 446* | 286 ± 8 |
| Ob-tempol | 8 | 567 ± 9*,† | 71 ± 8* | 250 ± 32* | 192 ± 19* | 20.7 ± 3.93* | 2843 ± 204*,† | 252 ± 12*,† |
| 2F ANOVA | | | | | | | | |
| Treatment (A) | $P \leq$ | .043 | .250 | .127 | .043 | .407 | .776 | .456 |
| Phenotype (B) | $P \leq$ | .001 | .001 | .001 | .001 | .013 | .001 | .001 |
| Time (C) | $P \leq$ | .001 | .001 | .066 | .003 | .001 | .001 | .308 |
| A × B interaction | $P \leq$ | .007 | .426 | .053 | .009 | .009 | .060 | .441 |
| A × C | $P \leq$ | .205 | .566 | .633 | .107 | .192 | .051 | .618 |
| B × C | $P \leq$ | .548 | .001 | .003 | .001 | .053 | .001 | .264 |
| ABC | $P \leq$ | .211 | .414 | .069 | .589 | .168 | .042 | .594 |

Fisher least significant difference test:
* $P < .05$ vs Ln-$ZSF_1$.
† $P < .05$ vs Ob-$ZSF_1$.

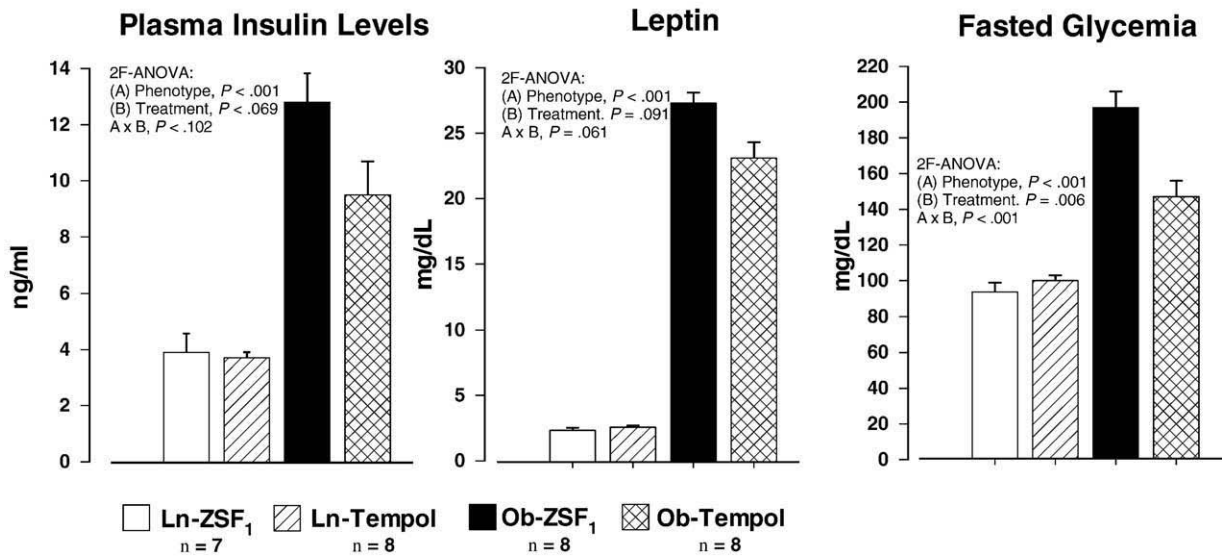


Fig. 1. The effects of 9 weeks of treatment with tempol (1 mmol/L in drinking water) on glucose, insulin, and leptin levels in Ln and Ob $ZSF_1$ rats.

syndrome; that is, animals were already overtly obese and had elevated lipids, polyphagia, polydipsia, polyuria, and glycosuria. Obese animals also had elevated blood glucose, insulin, and leptin levels (Ob control, 20 weeks of age; Fig. 1). However, before initiating the treatments, as well as 2 weeks into treatments, urinary protein excretion was similar in obese controls compared with their lean littermates (Fig. 2), suggesting that the metabolic syndrome preceded the development of renal injury. In control obese animals, body weight, urinary protein and glucose excretion, and plasma triglycerides and cholesterol levels continued to rise over the study period.

After 9 weeks of treatment, in obese animals, tempol mildly reduced body weight (by ~5%), produced a slight but significant decrease in plasma triglycerides and cholesterol levels (Table 1), and significantly reduced fasted blood glucose levels (Fig. 1). Tempol also reduced elevated insulin and leptin levels in Ob rats by 25% and 15%, respectively ($P < .05$, paired $t$ test; Fig. 1); had no effect on time-dependent increase in proteinuria (Fig. 2); increased urinary volume; and tended to increase glycosuria in obese animals (treatment × phenotype interaction, $P < .009$; Table 1). Tempol had no effect on metabolic parameters in lean animals.

Both lean and obese animals were hypertensive, and Ob animals had slightly higher blood pressure (phenotype, $P < .001$; Table 2). Tempol had divergent effect on the blood pressure in obese and lean animals, that is, tended to reduce and increase blood pressure in Ln and Ob rats, respectively (phenotype × treatment interaction, $P < .0l$). At 20 weeks of age, obese controls had significant renal hypertrophy, increased RVR, and markedly reduced renal plasma flow (RPF) and GFR compared with their lean littermates (Table 2). Tempol had no effect on renal hemodynamics and excretory function in obese animals, yet significantly reduced RVR and increased RPF and GFR in lean rats (Table 2).

### 3.2. Effects on renal tissue inflammation, proliferation, and fibrosis

Renal histopathologic analysis revealed significant glomerular and interstitial changes in obese controls. Adult, 20-week–old, obese $ZSF_1$ rats had significant tubular dilation/atrophy, pronounced interstitial inflammation, and initial/

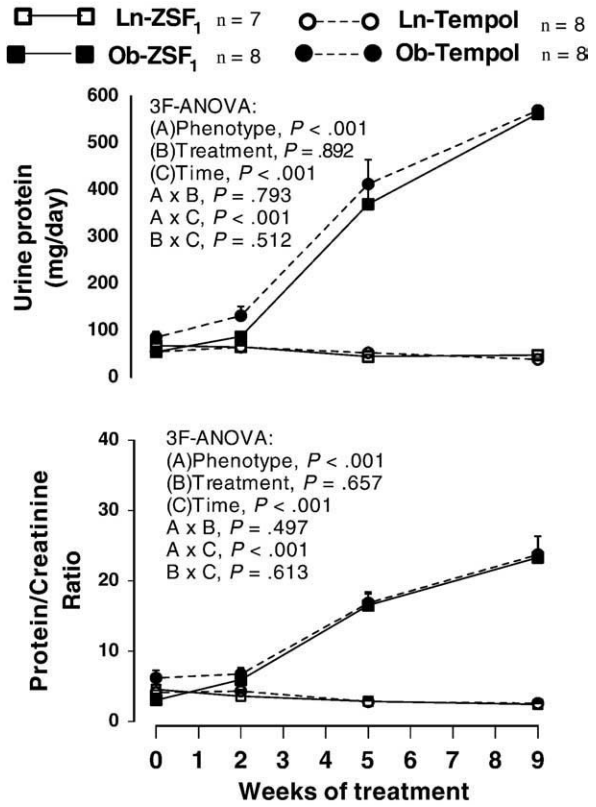


Fig. 2. Urinary protein and creatinine excretion in control Ln and Ob $ZSF_1$ rats and in the animals treated with tempol (1 mmol/L in drinking water).

Table 2
Renal hemodynamic and excretory function in lean and obese $ZSF_1$ rats after 9 weeks of treatment with tempol

| Parameters | Lean control | Lean tempol | Obese control | Obese tempol | 2F ANOVA: $P \leq$ | | |
|---|---|---|---|---|---|---|---|
| | | | | | Phenotype (A) | Treatment (B) | A × B interaction |
| | | | | | Ln vs Ob | Temp vs cont | |
| Body weight, g | 462 ± 9 | 464 ± 8 | 634 ± 10 | 591 ± 10* | .001 | .046 | .013 |
| Kidney weight, g | 2.97 ± 0.1 | 2.83 ± 0.1 | 4.44 ± 0.24 | 4.76 ± 0.23 | .001 | .475 | .057 |
| Kidney–body weight ratio, g/kg | 6.42 ± 0.31 | 6.03 ± 0.13 | 7.03 ± 0.23 | 7.91 ± 0.47 | .033 | .526 | .106 |
| Mean blood pressure, mm Hg | 151 ± 3 | 144 ± 6 | 155 ± 2 | 164 ± 3† | .001 | .605 | .007 |
| Systolic blood pressure, mm Hg | 172 ± 5 | 166 ± 3 | 174 ± 4 | 186 ± 5† | .015 | .339 | .002 |
| Diastolic blood pressure, mm Hg | 131 ± 2 | 126 ± 2 | 137 ± 3 | 143 ± 2† | .001 | .750 | .003 |
| Heart rate, beats/min | 400 ± 5 | 394 ± 3 | 365 ± 6 | 374 ± 4 | .001 | .750 | .200 |
| RBF, mL/(min g kidney) | 5.78 ± 0.3 | 7.92 ± 0.3§ | 2.78 ± 0.3† | 2.94 ± 0.2† | .001 | .007 | .004 |
| Hematocrit, % | 48 ± 1 | 46 ± 1 | 44 ± 1 | 42 ± 1 | .002 | .037 | .787 |
| RPF, mL/(min g kidney) | 3.04 ± 0.18 | 4.28 ± 0.19§ | 1.56 ± 0.14† | 1.68 ± 0.19† | .001 | .001 | .005 |
| RVR, (mm Hg mL)/(min g kidney) | 30.4 ± 2.8 | 19.5 ± 2.6§ | 61.9 ± 3.8† | 61.1 ± 3.3† | .001 | .161 | .103 |
| Urine volume, μL/(30 min g kidney) | 189 ± 49 | 216 ± 49 | 420 ± 45‡ | 600 ± 51‡ | .001 | .036 | .121 |
| GFR, mL/(min g kidney) | 1.54 ± 0.1 | 1.85 ± 0.07§ | 0.85 ± 0.01† | 0.83 ± 0.02† | .001 | .119 | .063 |

Fisher post hoc LDS test:
* $P < .05$ vs Ob-control.
† $P < .05$ vs Ln groups.
‡ $P < .05$ vs all other groups.
§ $P < .05$ vs Ln-control.

mild (~3% to 4%) glomerulosclerosis. Quantitative immunohistochemical analysis of renal cortical sections stained for markers of inflammation (ED1+ cells), proliferation (PCNA), and early fibrosis (collagen IV) is presented in Fig. 3; and representative examples of glomerular staining for collagen IV are shown in Fig. 4. As compared with lean littermates, control obese animals had increased staining for glomerular and interstitial ED1+ cells (Fig. 3A), glomerular and tubular PCNA (Fig. 3B), and glomerular collagen IV (Fig. 3C). Tempol significantly decreased the number of ED1+ cells in lean animals and in the interstitium, but not in glomeruli of obese animals. The number of cells positive for PCNA staining (labeling index, Fig. 3B) was markedly increased in glomeruli and tubules from obese animals compared with lean littermates; and tempol reduced the number of tubular cells positive for PCNA in both lean and obese animals, but had no effects on the elevated PCNA expression in glomeruli from obese animals. Finally, area positive for collagen IV was markedly extended in obese controls; and tempol reduced collagen IV accumulation in both lean (Figs. 3C and 4A, B) and obese (Figs. 3C and 4C, D) rats. Tempol had no effect on the mild glomerulosclerosis seen in obese animals (data not shown).

### 3.3. Effects on oxidative stress and antioxidant system

The activities of the 2 main antioxidant enzymes—SOD and CAT—and the total activity of cellular peroxidases in renal cortical tissue are shown in Fig. 5. Activity of SOD was reduced in obese animals (Fig. 5A), and tempol treatment produced a mild increase in SOD activity in Ob rats. Because tempol does not accumulate in tissues and readily goes out during the sample preparation, it is unlikely that augmented SOD activity reflects the activity of tempol. Besides, the rate of dismutase reaction catalyzed by tempol is much slower than the one induced by natural enzyme [24]; therefore, the actual impact of tempol on the measured total SOD activity (rate of cytochrome *c* oxidation) should be insignificant. In contrast, lean animals treated with tempol had reduced SOD activity. This could display a natural mechanism of cell adaptation to excessive intracellular SOD activity. It is plausible that, as the own SOD activity in lean animals remains high, the additional increase in SOD activity created by tempol stimulates inhibition of endogenous SOD.

Catalase activity was found to be markedly reduced in obese animals (Fig. 5B), and tempol had no effects on the enzyme activity in both lean and obese rats. Total peroxidase activity in obese animals was increased by 40%, but this increase did not reach statistical significance. Importantly, tempol caused a significant reduction of total peroxidase activity in both obese and lean animals (Fig. 5C) and restored the enzyme activity in obese animals even below the initial levels in lean controls.

The levels of protein dityrosine, protein nitration, and plasma lipid peroxidation as the markers of oxidative stress are presented in Fig. 6. The level of protein dityrosine in renal cortical tissue (Fig. 6A) was increased in obese rats, and tempol treatment had no effect on the amount of renal dityrosine. In contrast, protein nitration (Fig. 6B) and lipid peroxidation levels (Fig. 6C), initially elevated in animals with metabolic syndrome, were reduced after 9 weeks of treatment with tempol.

## 4. Discussion

In the present study, tempol was effective in correcting some of the pathologic changes in obese diabetic animal,

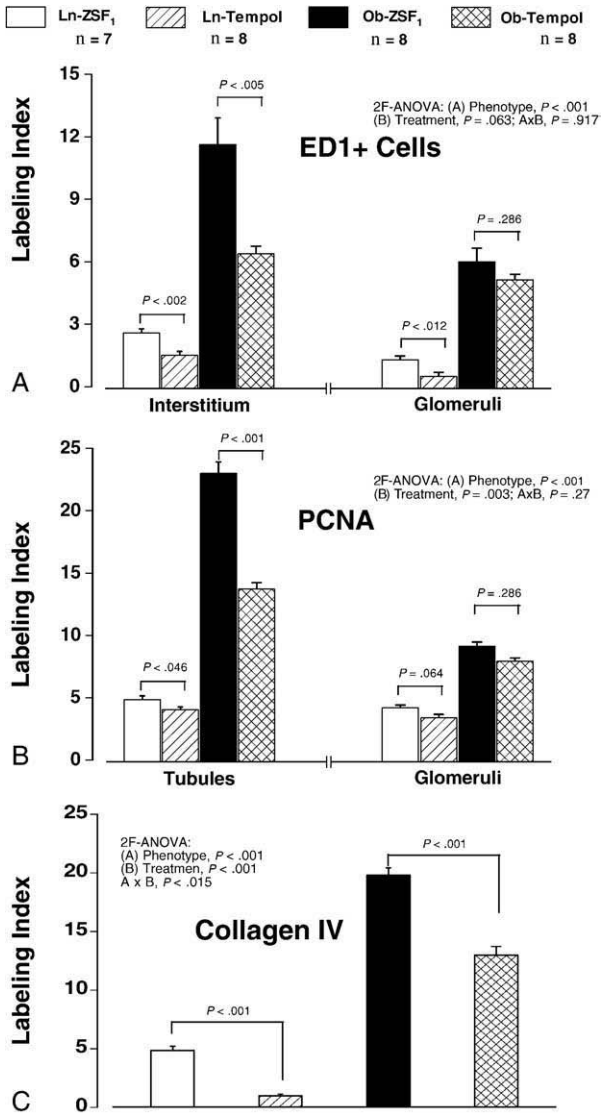


Fig. 3. Assessment of immunohistochemical staining for (A) inflammatory ($ED1^+$ cells), (B) proliferative (PCNA), and (C) fibrotic (collagen IV) changes in the kidney of control Ln and Ob $ZSF_1$ rats and in animals treated with tempol (1 mmol/L). Staining was assessed by quantitative image analysis. Results represent mean ± SEM of the labeling index.

while failing to mitigate the others. Thus, tempol produced mild but significant reduction in body weight, decreased the elevated plasma triglycerides levels, and improved insulin sensitivity in obese diabetic rats. Tempol also attenuated tubulointerstitial inflammatory and proliferative responses, and glomerular and interstitial fibrotic changes in diabetic kidney. In aggregate, these effects would be expected to result with reduced renal injury. However, tempol had no effect on increased RVR, reduced GFR and proteinuria, and even tended to exacerbate hypertension in obese animals. In contrast, in lean, nondiabetic, but hypertensive $ZSF_1$ rats, tempol significantly reduced RVR; increased GFR; tended to decrease blood pressure; and even reduced the mild proliferative, inflammatory, and fibrotic changes present in nondiabetic kidney in lean animals.

The lack of antihypertensive effect of tempol in obese $ZSF_1$ rats is somewhat surprising and contradicts the early reports of antihypertensive effects in several models of hypertension, [9-11,13]. However, tempol has no effects on blood pressure in angiotensin II–induced hypertension and in nitric oxide (NO)–deficient SHR [12,13,25]. Hypertension in $ZSF_1$ rats is associated with severe endothelial dysfunction, that is, vascular NO deficiency [26], which corroborates, but do not explain, previous studies in SHR. It seems that the effect of tempol on blood pressure is complex and may not only be related to its SOD mimetic activity [27], but under exaggerated oxidative stress may depend on the levels of $H_2O_2$ (*infra vide*).

In addition to hypertension, tempol had no effect on another independent risk factor for renal disease: proteinuria. This is also surprising given the fact that after 9 weeks of treatment, tempol improved insulin sensitivity and reduced plasma lipid levels. However, tempol was reported to have no effect on proteinuria in STZ diabetic normotensive rats [16] and in rats with angiotensin I–-induced hypertension [13]. Furthermore, in rats with hyperthyroidism, tempol decreases elevated blood pressure, plasma lipid peroxidation, and renal 8-isoprostane excretion (ie, reduces systemic and renal oxidative stress), yet has no effect on proteinuria [28]. The absence of the effects on proteinuria in obese $ZSF_1$ rats raises the question regarding the overall benefits of SOD mimetics in nephropathy associated with metabolic syndrome. However, the measurements of the renal activity of antioxidant enzymes and markers of oxidative damage may shed more light on and provide explanation for the partially protective effects of tempol in diabetic kidney.

Long-term tempol treatment produced a slight increase in total SOD levels and reduced total peroxidase activity, protein nitration, and lipid peroxidation in obese animals. However, the significantly reduced activity of CAT seen in obese animals was not altered by tempol; and tempol was unable to reduce the levels of dityrosine, a marker of protein oxidative damage.

The oxidative stress (ie, increased $O_2^{\cdot-}$ levels) contributes to the development of insulin resistance [29], whereas SOD mimetics or therapeutic agents that enhance the activity of endogenous SOD improve insulin sensitivity [30,31]. This may explain the observed beneficial effects of tempol on metabolic status in obese diabetic animals.

Down-regulation of SOD and the subsequent excessive release of $O_2^{\cdot-}$ contribute to the inflammatory, proliferative, and fibrotic renal injury [32], whereas overexpression of SOD and SOD mimetics reduces inflammatory, proliferative, and fibrotic injury in both nondiabetic and diabetic kidney [10,33-36]. Therefore, it is not surprising that tempol had antiproliferative, anti-inflammatory, and antifibrotic effects in diabetic kidney (Fig. 3). The adverse renal effects of the excessive $O_2^{\cdot-}$ production may also be due in part to its interaction with NO. This interaction has 2 important biological consequences: reduced NO bioavailability and production of peroxynitrite. By reducing NO bioavailability,

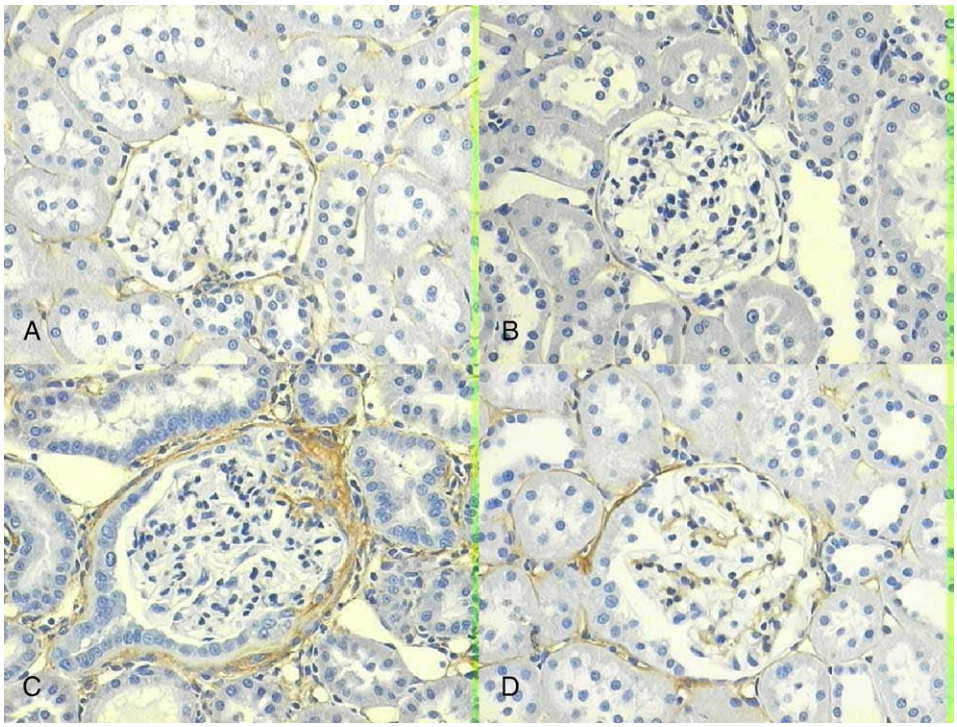


Fig. 4. Representative collagen IV immunochemistry of cortical sections from control lean (A) and obese (C) $ZSF_1$ rats and lean (B) and obese (D) animals treated with tempol. Magnification, ×400.

$O_2^{\cdot-}$ diminishes the anti-inflammatory, antiproliferative, and vasodilatory properties of NO; and reduced NO availability is believed to play an important role in the development of diabetic complications [37]. The interaction between $O_2^{\cdot-}$ and NO also results in the formation of peroxynitrite, a potent oxidative and nitrative agent that has a pathologic role in cardiovascular disease, diabetes, and diabetic complications [38]. The notion that in the present study tempol significantly reduced the formation of protein nitrotyrosine (a specific marker of peroxynitrite generation) suggests that, in addition to superoxide scavenging, the beneficial effects of tempol in obese animals may be due, at least in part, to the reduced $ONOO^-$ formation (Fig. 7).

Superoxide anion and peroxynitrite could be the sources of hydroxyl radical, one of the most reactive and deleterious ROS capable to induce DNA, protein, and lipid oxidation. However, as tempol increases the rate of superoxide utilization, it is expected to decrease the amount of $^{\cdot}OH$ formation and extent of $^{\cdot}OH$-mediated reactions. Moreover, tempol induces a rapid DNA-$Fe^{2+}$ oxidation and thus may interrupt the Fenton reaction and subsequent $^{\cdot}OH$ radical formation from $H_2O_2$ [39]. Therefore, it is plausible that $^{\cdot}OH$-mediated processes could also be decreased after tempol treatment (Fig. 7). Indeed, we found a significant reduction of plasma lipid peroxidation in both lean and obese tempol-treated animals.

Similar to previous studies in diabetic subjects and animals [40,41], in the present study, reduced activity of CAT in the renal cortex was detected in Ob rats compared with their lean nondiabetic littermates. Catalase is responsible for $H_2O_2$ neutralization (ie, its conversion to $H_2O$); and therefore, increased renal cortical $H_2O_2$ levels should be expected. Tempol had no effects on the reduced CAT activity. Importantly, under condition of reduced CAT activity, tempol may even be expected via dismutase reaction to further increase $H_2O_2$ levels [16,42].

In the present study, tempol significantly reduced total peroxidase activity in both lean and obese rats. Under conditions of reduced CAT activity and stressful levels of $H_2O_2$, peroxidase reduces $H_2O_2$ to water and simultaneously oxidizes second reductants such as tyrosine. Importantly, the renal levels of dityrosine were increased in both tempol-treated and control Ob rats, suggesting that the enhanced protein oxidation in obese animals is due first and foremost to the uncontrolled production of $H_2O_2$ (Fig. 7). However, the decreased tissue peroxidases did not prevent protein oxidation in the obese group, suggesting that under the excess of substrate ($H_2O_2$) even a reduced activity of cell peroxidases is sufficient to mediate severe protein damage. Myeloperoxidase, one of the most vigorous peroxidases, is abundantly present in monocytes [43]. Tempol inhibited the renal macrophages infiltration, and this could explain the reduction in total peroxidase activity. However, the activities of peroxidases in other cell compartments may remain the same and, under excessive $H_2O_2$ production, may produce significant protein oxidation.

Multiple lines of evidence indicate that overproduction and/or reduced inactivation of $H_2O_2$ may induce proteinuria.

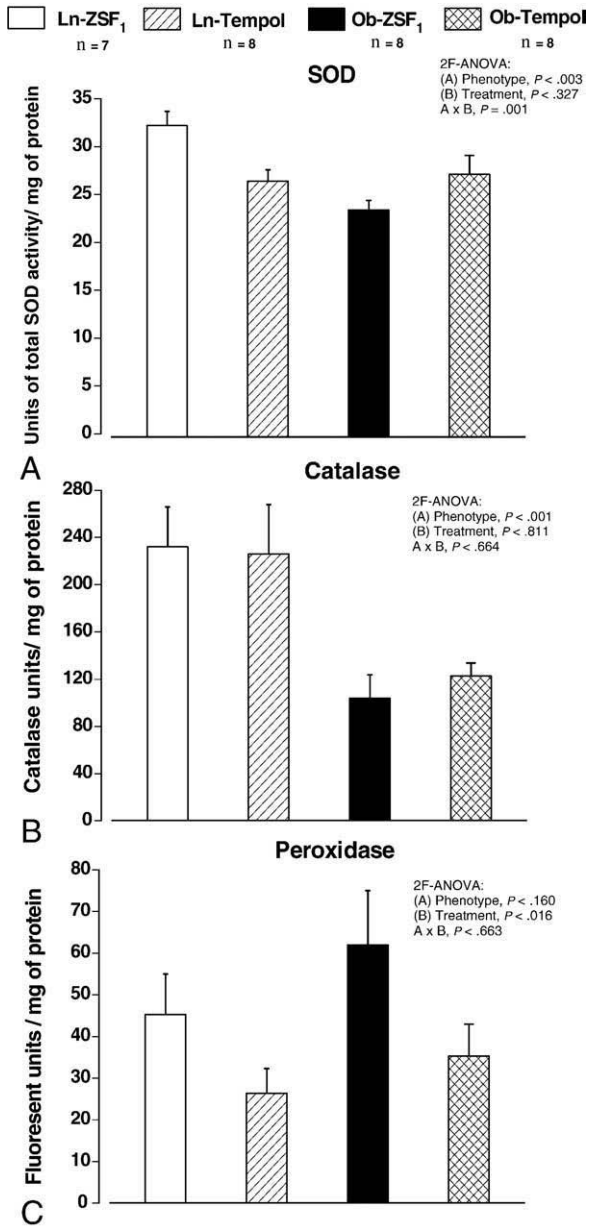


Fig. 5. The SOD (A), CAT (B), and peroxidase (C) activity in renal cortical tissue homogenates from control Ob and Ln ZSF1 rats and Ob and Ln animals treated with tempol for 9 weeks.

Thus, $H_2O_2$ infused into the renal artery causes massive transient proteinuria [44,45]; and CAT, but not SOD, markedly reduces proteinuria [46,47]. Therefore, it is plausible that in diabetic rats proteinuria is largely due to the reduced CAT activity and excessive $H_2O_2$ production.

In the present study, tempol significantly reduced RVR and tended to decrease blood pressure in lean animals; and this is in accordance with previous studies in hypertensive animals [10-13]. Yet, tempol had no effect on markedly increased RVR and even tended to increase blood pressure in obese animals. The excessive production of $H_2O_2$ may explain the differences in blood pressure and RVR response to tempol between obese and lean animals.

Hydrogen peroxide is a potent renal vasoconstrictor in diabetic mice and rats [48,49], and increased renal medullary $H_2O_2$ leads to hypertension [50]. It seems that increased renal formation of $H_2O_2$ by tempol counteracts its renal vasodilatory and antihypertensive effects. In this regard, renal interstitial infusion of tempol increases renal medullary $H_2O_2$; and coinfusion of CAT completely abolishes this increase and potentiates renal vasodilatory response to tempol [51]. Furthermore, although tempol completely blocks SOD inhibitor-induced accumulation of superoxide, it fails to attenuate SOD inhibitor-induced hypertension, unless CAT is coinfused [51]. Therefore, under oxidative stress and sufficient CAT activity, tempol reduces RVR and

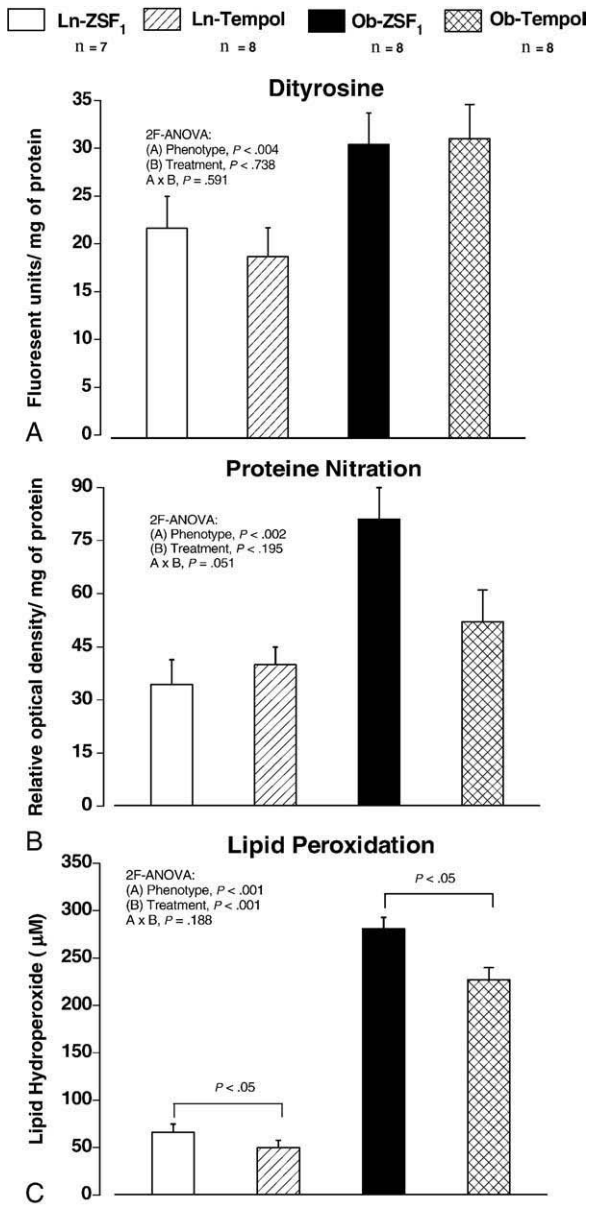


Fig. 6. Dityrosine (A) and protein nitration (B) in renal cortical tissue homogenate and lipid peroxidation (C) in plasma from Ob and Ln ZSF1 rats and Ob and Ln animals treated with tempol for 9 weeks.

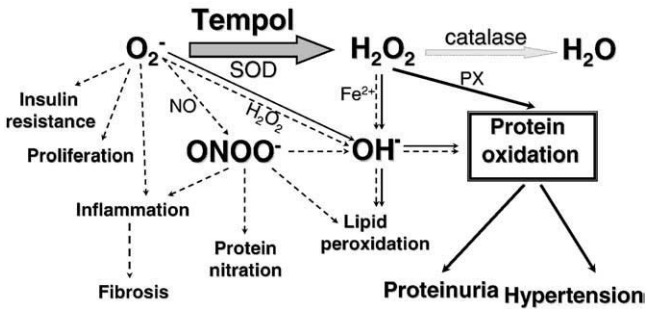


Fig. 7. The schematic summary of tempol action in diabetic kidney. The dashed arrows show the processes that are reduced by tempol, dashed + solid arrows represent those that could be diminished, and the solid arrows represent those that remain unchanged. Tempol, as an active SOD mimetic, catalyzes the dismutase reaction of superoxide conversion into $H_2O_2$. Thus, tissue concentrations of superoxide and, therefore, the extent of $O_2^{\cdot-}$-dependent pathologic processes are expected to languish. However, as the activity of CAT is diminished, the level of $H_2O_2$ increases and $H_2O_2$-mediated pathologic processes are not altered.

blood pressure in lean ZSF1, but not in obese animals that have reduced CAT activity and excessive oxidative stress and $H_2O_2$ production. Furthermore, because of the significantly higher fluid intake, diabetic rats consumed ~50 mg/(kg d) of tempol vs ~15 mg/(kg d) of tempol ingested by their nondiabetic lean littermates. The increased tempol consumption (ie, augmented dismutase reaction and subsequent $H_2O_2$ production in the presence of reduced CAT activity and $H_2O_2$ removal) may explain the tendency of tempol to exacerbate hypertension in obese animals, but to reduce blood pressure in lean $ZSF_1$ rats.

In *db/db* or streptozotocin diabetic mice, overexpression of endogenous cytosolic $Cu^{2+}/Zn^{2+}$ SOD was reported to prevent proteinuria [33,34]. This would suggest that SOD mimetics such as tempol should attenuate diabetic proteinuric disease. However, in diabetic mice with overexpressed SOD, CAT activity is normal; and activity of glutathione peroxidase, another enzyme that reduces $H_2O_2$ to water, is even increased. This is in contrast to diabetic patients and to diabetic STZ and ZSF1 rats, in which CAT activity is reduced. Furthermore, apocynin, a specific inhibitor of NADPH oxidase (a main enzyme for $O_2$ anion production in cardiovascular cells), but not tempol, attenuates angiotensin II–induced (NADPH-mediated) proteinuria [13]. This suggests that inhibition of $O_2^{\cdot-}$ production (and subsequent reduced $H_2O_2$ levels) rather than potentiation of SOD reaction by tempol (and subsequent increase in $H_2O_2$) attenuates proteinuria. Altogether, the above discussed studies emphasize the significances of proper function of CAT when increased superoxide production through dismutase reaction generates excessive amount of $H_2O_2$ and suggest that, under severe oxidative stress, the overall effect of tempol is determined by the level of CAT activity.

In conclusion, this study suggest that $O_2^{\cdot-}$ and $H_2O_2$ may have distinct roles in the pathogenesis of diabetic renal injury, with $O_2^{\cdot-}$ mainly being involved in inflammatory, proliferative, and fibrotic changes, and $H_2O_2$ in glomerular hemodynamics, proteinuria, and hypertension. In diabetic kidney, dismutase reaction catalyzed by tempol does not completely eliminate the toxicity of ROS. In diseases like metabolic syndrome that are characterized by inhibition of both SOD and CAT activities, restoration of SOD activity will only result with reduced severity of the pathologic processes mediated by $O_2^{\cdot-}$, whereas the changes induced by $H_2O_2$ will not be altered. This study highlights the importance of balanced antioxidant therapy in the treatment of diabetic complications (ie, use of combined SOD/CAT mimetics [52]) and underscores the need for detailed studies of the role of distinct ROS in the pathobiology of cardiovascular and renal disease in diabetes.

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