Antioxidant Enzymes and Effects of Tempol on the Development of Hypertension Induced by Nitric Oxide Inhibition

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Background: This study analyzed whether hypertension induced by N\textsuperscript{\textsubscript{w}}-nitro-L-arginine methyl ester (L-NAME) is associated with dysregulation of the main antioxidant enzymes (superoxide dismutase [SOD], catalase, glutathione peroxidase [GPX], and glutathione reductase [GR]) and whether chronic administration of tempol ameliorates this hypertension.

Methods: Four groups of male Wistar rats were used: 1) control rats; 2) rats treated with L-NAME (35 mg/100 mL in drinking fluid); 3) rats treated with tempol (18 mg/100 mL in drinking fluid); and 4) rats treated with L-NAME plus tempol. All treatments were maintained for 6 weeks. Body weight, systolic blood pressure (BP) determined by the tail-cuff method, and heart rate were measured once per week. At the end of the experimental period, direct BP and morphologic, metabolic, plasma, and renal variables were measured. Enzymatic activities were measured in the kidney (cortex and medulla) and heart (right and left ventricles).

Results: Rats with L-NAME–induced hypertension showed increased copper–zinc (Cu-Zn) SOD activity in the renal cortex and medulla and the left and right ventricles, which was reduced by tempol administration. The manganese (Mn) SOD activity was increased by L-NAME and reduced by tempol in the renal cortex but was unchanged in other tissues. Catalase activity was not affected by L-NAME or tempol treatments in any tissue. Both GPX and GR activities were increased by L-NAME and reduced by tempol in the renal cortex and medulla but were not affected in the ventricles. Tempol reduced BP and total urinary excretion of 8-hydroxy-2\textsuperscript{-}deoxyguanosine in L-NAME–treated animals but did not affect either variable in controls.

Conclusions: We conclude that L-NAME–induced hypertension is associated with an upregulation of antioxidant SOD, GPX, and GR activities. Moreover, the results indicate that tempol attenuates hypertension on nitric oxide–deficient rats and that oxidative stress participates in the established phase of this type of hypertension. Am J Hypertens 2005;18:871–877 © 2005 American Journal of Hypertension, Ltd.

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Clinical and experimental studies indicate that oxidative stress contributes to the development of hypertension in human beings\textsuperscript{1,2} and animals.\textsuperscript{3–11} The induction of oxidative stress by glutathione depletion causes severe hypertension in rats.\textsuperscript{3} Several studies have demonstrated that oxidative stress is involved in the pathogenesis of arterial hypertension in genetic animal models\textsuperscript{4–6} and in secondary forms of arterial hypertension.\textsuperscript{7–10} Some of these experimental models showed increased tissue levels of superoxide or hydrogen peroxide or both, which have many prohypertensive actions, including direct vasoconstriction,\textsuperscript{11,12} antinatriuresis,\textsuperscript{13} and sympathetic stimulation.\textsuperscript{14} On the other hand, several investigators have proposed that oxidative stress contributes to the generation or maintenance of hypertension via inactivation of nitric oxide (NO),\textsuperscript{4,7,8} inhibiting its vasodilatory and natriuretic actions, and via nonenzymatic generation of vasoconstrictor isoprostanes from arachidonic acid peroxidation,\textsuperscript{5,15} with direct vasopressor\textsuperscript{16} and antinatriuretic\textsuperscript{15} effects.

Hypertension produced by NO synthesis inhibition is associated with increased oxidative stress\textsuperscript{17,18} and chronic oral administration of quercetin, a flavonoid with antiox-
idant properties, had protective effects on rats with hypertension induced by \(N^\text{3-} \text{nitro-L-arginine methyl ester (L-NAME)} \). \(^{18}\)

Tempol (4-hydroxy-2,2,6, 6-tetramethyl piperidinoxyl) is a stable, low–molecular-weight superoxide dismutase (SOD) mimetic that is metal independent and cell membrane permeable. \(^{19}\) Tempol has been used to evaluate the contribution of oxidative stress to the pathogenesis of different models of arterial hypertension. Thus, tempol treatment of hypertensive rats reduced blood pressure (BP) and superoxide anion production in genetic \(^{4–6,20}\) and secondary forms of hypertension. \(^{7,8}\) Several investigators \(^{4,5,9,10}\) have proposed that a major component of the antihypertensive effect of tempol is related to improved NO availability.

This study aimed to determine whether hypertension induced by NO inhibition, produced by oral administration of L-NAME, is associated with dysregulation of the main antioxidant enzymes, ie, SOD, catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GR), in the kidney (cortex and medulla) and heart (left and right ventricles), organs primarily related to BP control. A further aim was to test whether chronic administration of tempol ameliorates L-NAME–induced hypertension.

**Methods**

**Animals**

A total of 40 male, age-matched Wistar rats born and raised at the animal center of the University of Granada were used. The experiments were performed according to European Union guidelines for ethical care of animals. Rats that initially weighed 250 to 275 g were randomly assigned to different experimental groups. All animals had free access to standard rat diet with 0.5% sodium content (rodent toxicology diet, B&K, Barcelona, Spain) and tap water ad libitum, except where stated. The rats were randomly divided into four groups: 1) control group (designated CONTROL); 2) L-NAME–treated group (designated L-NAME); 3) tempol-treated group (TEMPOL); and L-NAME–plus tempol-treated group (L-NAME+TEMPOL) \( (n = 10 \text{ per group}) \). The L-NAME was given by gavage \( (35 \text{ mg/kg/day}) \), and tempol was given in the drinking water \( (180 \text{ mg/L}, \sim 18 \text{ mg/kg/day}) \). The concentration of tempol was adjusted every 2 days according to the animals’ fluid intake to ensure the administration of a similar dose to L-NAME–treated and control groups. All treatments were started simultaneously and maintained for 6 weeks.

**Experimental Protocol**

Body weight and tail systolic BP (SBP) were determined weekly throughout the experiment. The SBP was measured by tail-cuff plethysmography in unanesthetized rats \( (LE \ 5001-\text{Pressure Meter, Letica SA, Barcelona, Spain}) \). At least seven determinations were made at every session, and the mean of the lowest three values within a range of 5 mm Hg was used to obtain the SBP level.

After the time course study, all animals were housed in metabolic cages with free access to food and respective drinking fluids. After 2 days of adaptation, food and water intake and urine values were gathered during 2 consecutive days. The values obtained each experimental day were averaged for statistical purposes. The urinary variables measured were diuresis, natriuresis, kaliuresis, creatinine, proteinuria, and total excretion of 8-hydroxy-2-deoxyguanosine \( (8 \text{ OHdG}) \), an index of oxidative stress.

After the metabolic study, the femoral artery was cannulated and exteriorized at dorsum of neck. After a 24-h recovery period, direct BP and heart rate (HR) were recorded continuously for 60 min. The values obtained during each of the last 30 min were averaged to obtain the mean BP value. Blood samples from femoral catheter were used to determine plasma urea, creatinine, and electrolytes. Finally, the rats were killed by exsanguination. After the treatment period, kidneys and heart were weighed and the heart was divided into right ventricle and left ventricle plus septum to study morphologic variables. Samples from renal cortex and medulla and both ventricles were immediately harvested, cleaned, snap-frozen in liquid nitrogen, and stored at \( –70^\circ \text{C} \) until their processing for measurement of enzymatic activities.

**Enzymatic Determinations**

**Preparation of Tissue Homogenate** Homogenates \( (25\% \text{ wt/vol}) \) of kidney (cortex and medulla) and heart (left and right ventricle) of each animal were prepared in a solution containing 50 mmol/L potassium phosphate buffer \( (\text{pH} \ 7.4) \), 1 mmol/L ethylenediaminetetraacetate, and 1 mmol/L dithiothreitol using a Polytron homogenizer \( (\text{Omni International, Warrenton, VA}) \). Tissue homogenates were centrifuged at 3000 rpm for 10 min at 4°C to discard cellular debris. The supernatant was precipitated by ketone precipitation method. A portion of supernatant was used to determine protein concentration by the method of Lowry et al. \(^{21}\)

**Superoxide Dismutase Activity** The SOD activity was measured spectrophotometrically by the method of McCord \(^{22}\) with slight modifications. The assay is performed in 3 mL of 50 mmol/L potassium phosphate buffer at \( \text{pH} \ 7.8 \) containing 1 mmol/L cytochrome C, 1 mmol/L xanthine, and sufficient xanthine oxidase to produce a cytochrome C reduction rate at 550 nm of 0.025 absorbance units per min. Parallel measurements were also performed in presence of 1 mmol/L potassium cyanide \( (\text{KCN}) \), selective inhibitor of copper–zinc \( (\text{Cu–Zn}) \) SOD, to differentiate Cu-Zn SOD and Mn SOD isoenzymes.

**Catalase Activity**

The CAT activity was determined by the procedure of Aebi. \(^{23}\) Decrease in absorbance at 240 nm was monitored.
for 1 min. The activity was calculated using H₂O₂ extinction coefficient of 0.041 mmol⁻¹cm⁻¹.

Glutathione Peroxidase Activity

The GPX activity was measured spectrophotometrically. The assay comprised 50 mmol/L potassium phosphate buffer (pH 7.6), 2 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L reduced glutathione (GSH), 1 mmol/L NaN₃, 0.2 mmol/L NADPH, and 1 U/mL glutathione reductase (GR). The activity was calculated using a molar extinction coefficient for NADPH of 6.22 mmol⁻¹cm⁻¹ at 340 nm.

Glutathione Reductase Activity

The GR activity was determined by the procedure of Carlberg and Mannervik with minor modifications. The assay solution contained 50 mmol/L potassium phosphate buffer (pH 7.6), 2 mmol/L NADPH, and 20 mmol/L oxidized glutathione. The reaction was initiated by addition of H₂O₂ and absorbance at 340 nm was recorded. The activity was calculated using molar coefficient for NADPH of 6.22 mmol⁻¹cm⁻¹ and expressed in units per milligram of protein.

Analytical Procedures

Plasma and urinary electrolytes and creatinine were measured in an autoanalyzer (Beckman CX4, Brea, CA). The urine protein concentration was measured by the Bradford method. Urinary excretion of 8OHdG was determined using a commercial competitive enzyme-linked immunoassay kit, as described by the supplier (Genox Corp., Baltimore, MD).

Statistical Analyses

Results are expressed as means ± SEM. Evolution of tail SBP with time was compared using a nested design. When the overall difference was significant, the Bonferroni method with an appropriate error was used. Other variables measured at the end of the experimental period were compared with one-way analysis of variance, and subsequent pairwise comparisons were performed with the Newman-Keuls test.

Results

Blood Pressure and Heart Rate

Figure 1 summarizes BP data. Figure 1A depicts the evolution of tail SBP measured by plethysmography, and Fig. 1B shows final MAP measured by direct recording in conscious rats. Treatment with L-NAME induced a time-dependent rise in tail SBP, which was significantly attenuated by coadministration of tempol. Tail SBP was lower in L-NAME+TEMPOL rats throughout the last 4 weeks of the study versus values in rats not treated with L-NAME. Administration of tempol to normal rats did not significantly modify BP. The BP measurements from femoral catheter in conscious rats at the end of the experiment confirmed values obtained by indirect method. The HR measured by plethysmography was similar in all groups throughout the time course (data not shown); this observation was confirmed at the end of the study by direct HR recording. The HR values in experimental groups at the

FIG. 1. Time course of systolic blood pressure (SBP) measured by the tail-cuff method (A), and final mean arterial pressure (MAP) measured by direct recording (femoral artery) in conscious rats (B). CONTROL = control rats; L-NAME = rats treated with N^ω-nitro-L-arginine methyl ester (L-NAME); L-NAME+TEMPOL, rats treated with L-NAME plus tempol; TEMPOL = rats treated with tempol. *P < .001 versus CONTROL, +P < .01 versus L-NAME.

FIG. 2. Activity of copper–zinc superoxide dismutase (Cu-Zn SOD) in tissues of experimental groups. *P < .05 versus CONTROL; +P < .05 versus L-NAME. Abbreviations as in Fig. 1.
end of the study were as follows: CONTROL, 363.7 ± 11; TEMPOL, 361.5 ± 14; L-NAME, 336.5 ± 10; and L-NAME+TEMPOL, 341 ± 12 (mm Hg).

Antioxidant Enzyme Activity

The L-NAME hypertensive rats showed significant increased Cu-Zn SOD activity in the renal cortex and medulla and in the left and right ventricles (Fig. 2). This variable was reduced to normal values by tempol administration in all tissues of L-NAME treated rats. The Mn SOD activity was increased by L-NAME and reduced by tempol in the renal cortex but was not significantly changed in other tissues (Fig. 3). We observed that the CAT activity was not affected by L-NAME or tempol treatments in any tissue, although there was a tendency to an increase in the renal medulla of L-NAME and L-NAME+TEMPOL groups (Fig. 4). The CAT activity was 10-fold higher in the renal cortex and medulla versus left and right ventricles. Both GPX and GR activities were increased by L-NAME and reduced to normal values by tempol in the renal cortex and medulla, but these treatments did not affect these activities in the ventricles (Figs. 5 and 6).

Morphologic, Plasma, and Urinary Variables

No significant differences in weekly body weight increases (data not shown) or final body weight (Table 1) were observed among the experimental groups. Absolute and relative kidney weights were not significantly affected by any treatment. The L-NAME group showed absolute (left/ right ventricle weight) and relative (left ventricle/body weight) left ventricular hypertrophy, which was not affected by tempol. There were no significant differences in plasma sodium, potassium, urea, or creatinine levels among experimental groups (data not shown). No significant differences were observed in food or fluid intake among any groups. There were no significant differences in diuresis, natriuresis, kaliuresis, proteinuria, or creatinine clearance between the control and experimental groups, except that proteinuria was significantly higher in L-NAME–treated groups (P < .05) versus their respective controls (L-NAME, 7.04 ± 0.94; L-NAME+TEMPOL, 6.75 ± 0.32; CONTROL, 3.72 ± 0.48; TEMPOL, 3.50 ± 0.25 mg/100 g/24h). Urinary excretion of 8-OHdG was significantly increased (P < .01 v Control) in L-NAME group versus controls. Tempol reduced urinary excretion of 8-OHdG in L-NAME rats (P < .05 v L-NAME) but did not significantly affect this variable in control rats. Total urinary excretion of 8-OHdG in the experimental groups was (ng/100 g/24 h): L-NAME, 135 ± 9.8; L-NAME+TEMPOL, 72 ± 7.3; CONTROL, 66 ± 8.0; TEMPOL, 60 ± 6.2.

Discussion

In this study, we found that the L-NAME group had a significant increase in urinary 8-OHdG, a sensitive and stable biomarker of oxidative stress in vivo. This observation is consistent with several reports showing that hypertension from chronic NO deficiency is associated with increased oxidative stress. The present study was designed to determine the effect of chronic NO deficiency, which causes hypertension and oxidative stress, on the activity of SOD, CAT, and GPX enzymes, principal components of the antioxidant defense system. The L-NAME
hypertensive rats showed significantly increased Cu-Zn SOD activity in the renal cortex and medulla and in the left and right ventricles. However, Mn SOD activity was increased by L-NAME and reduced by tempol only in the renal cortex. These findings rule out a quantitative deficiency of intracellular SOD isoforms in this model. Furthermore, increased SOD activity in tissues of L-NAME hypertensive rats may be a compensatory response to NO deficiency. In this context, it has been hypothesized that NO may act as a scavenger of superoxide. Thus, it is known that $O_2^-$ reacts with NO to form peroxynitrite, and the rate constant of the reaction between NO and $O_2^-$ to form peroxynitrite is threefold that between superoxide dismutase (SOD) and $O_2^-$ to form hydrogen peroxide. In support of this hypothesis, tempol-treated, NO-deficient rats showed a reduction to normal SOD activity values, probably due to the SOD mimetic activity of tempol.

The CAT activity in the renal cortex and medulla and left and right ventricles of the present L-NAME hypertensive rats was similar to that in controls. Therefore, CAT activity was not affected in tissues of chronic NO-deficient hypertensive rats. Both GPX and GR activities were increased by L-NAME and were reduced by tempol in the renal cortex and medulla, but these treatments did not affect these enzymatic activities in the ventricles. These findings suggest that oxidative stress in L-NAME–induced hypertension may not be due to a primary downregulation of CAT, GPX, or GR, at least during the established phase when measurements were performed. In fact, GPX and GR were elevated. Although the reason for these increases is not clear, they may be related to reduced $O_2^-$ inactivation caused by inhibition of NO synthesis, as indicated by the return to normal levels after tempol administration.

The present results indicate that long-term tempol administration attenuates development of hypertension in L-NAME hypertensive rats without affecting ventricular hypertrophy or proteinuria, as reported in spontaneously hypertensive rats. However, normal control animals exhibited no discernible response to tempol administration, as previously reported. This observation, together with the fact that tempol reduced 8-OHdG in L-NAME rats, argues against a nonspecific effect of tempol on BP and demonstrates that, in the absence of oxidative stress, antioxidant therapy has no effect on BP. Interestingly, tempol reduced BP in L-NAME rats but not to normal values, indicating the participation of factors other than oxidative stress in the pathogenesis of L-NAME–induced hypertension.

It has been proposed that NO inactivation is an important mechanism by which oxidative stress contributes to hypertension. Administration of tempol or other antioxidants improves NO availability and ameliorates hypertension in rats, and its antihypertensive action is blocked after NOS inhibition. Therefore, the antihypertensive actions of long-term tempol treatment were attributed to increased NO availability and reduced oxidative stress. However, the tempol-induced BP reduction in NO-deficient hypertensive rats clearly indicates that the antihypertensive effects of tempol can also be produced by NO-independent pathways. Indeed, it was recently demonstrated that prevention of hypertension development by tempol is independent of NO availability improvements in Dahl salt-sensitive rats and rats with ACTH-induced hypertension.

The mechanisms by which tempol reduces BP in L-NAME hypertensive rats are unclear, although a leftward shift in the renal pressure–natriuresis relationship may offer an explanation, as these rats had the same
sodium excretion as L-NAME control rats but with decreased BP. It has been reported that the hypertensive effect of tempol is accompanied by an increase in medullary blood flow, which persists with NO synthase blockade. Given the important role of medullary blood flow in long-term BP control, tempol might reduce BP in L-NAME hypertensive rats by increasing medullary blood flow and facilitating sodium excretion.

Alternatively, the antihypertensive effect of tempol may derive from an improvement in endothelial dysfunction. Several investigators observed that the BP reduction produced by chronic tempol treatment of hypertensive rats is accompanied by increased endothelium-dependent vasodilation. This antihypertensive mechanism may participate in L-NAME-induced hypertension despite NO blockade, because tempol alleviates hypertension and improves endothelium-derived hyperpolarizing factor–mediated vasodilation induced by ACh in DOCA-salt rats. Therefore, tempol treatment of L-NAME hypertensive rats may increase the activity of endothelium-derived hyperpolarizing factor, responsible for endothelium-dependent vasodilation in these animals.

Recent studies showed that tempol decreases blood pressure, renal sympathetic nerve activity, and HR in SHR and DOCA-salt rats to a greater degree than in control rats, and that these responses are not altered by L-NAME pretreatments. It was proposed that augmented superoxide production contributes to hypertension development via activation of the sympathetic nervous system and that tempol reduces BP, inhibiting the sympathetic activity. However, the absence of any HR changes in tempol-treated rats suggests that the effect on sympathetic function did not play a major role in the antihypertensive effect of this drug in our study. On the other hand, the study design does not allow other actions of tempol on the sympathetic nervous system to be ruled out.

In conclusion, these results indicate that oxidative stress participates in the established phase of L-NAME–induced hypertension and that tempol attenuates this hypertension by mechanisms that do not appear to be related to sympathetic inhibition. Furthermore, this type of hypertension was associated with a compensatory upregulation of antioxidant SOD, GPX, and GR activities that was reversed by tempol administration.

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**References**


