Long-Term Antioxidant Administration Attenuates Mineralocorticoid Hypertension and Renal Inflammatory Response

Richard A. Beswick, Hanfang Zhang, Dawnyetta Marable, John D. Catravas, William D. Hill, R. Clinton Webb

Abstract—We previously reported increased monocyte/macrophage infiltration, reactive oxygen species accumulation, and nuclear factor-κB (NF-κB) activation in mineralocorticoid (deoxycorticosterone acetate [DOCA]) hypertensive rats. We tested the hypothesis that prolonged antioxidant administration inhibits superoxide accumulation, lowers blood pressure, and reduces NF-κB activation in DOCA-salt hypertensive rats. DOCA rats exhibited a significant increase in systolic blood pressure compared with sham rats. Aortic rings from DOCA rats exhibited increased superoxide (O$_2^-$) production compared with sham rats. In addition, the treatment of DOCA rats with pyrrolidinedithiocarbamate (PDTC) or 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (Tempol) caused a significant decrease in systolic blood pressure and aortic superoxide accumulation. Monocyte/macrophage infiltration was also significantly decreased in DOCA rats treated with PDTC or Tempol compared with untreated DOCA rats. NF-κB–binding activity was significantly greater in untreated DOCA rats than in either sham rats or PDTC- or Tempol-treated DOCA rats. Also, DOCA rats treated with Tempol exhibited no significant difference in NF-κB–binding activity compared with sham. These results suggest that antioxidants attenuate systolic blood pressure, suppress renal NF-κB–binding activity, and partly alleviate renal monocyte/macrophage infiltration in DOCA-salt hypertension. (Hypertension. 2001;37[part 2]:781-786.)

Key Words: Tempol ■ pyrrolidinedithiocarbamate ■ hypertension, mineralocorticoid ■ nuclear factor-κB ■ monocyte/macrophage

Hypertension imparts an increased risk of myocardial infarction, stroke, renal damage, and blindness. Al though at least 10 genes have been shown to increase blood pressure, the pathogenesis of steroid hypertension has been shown to be primarily linked to mutations that result in ectopic production of the adrenal corticosterone, or aldosterone. Hypertension has also been shown to have proinflammatory actions, which increase the formation of hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$) within tissue and blood. Furthermore, superoxide accumulation has been implicated in the activation of nuclear factor-κB (NF-κB). NF-κB transcriptionally regulates many cellular genes implicated in early immune, acute phase, and inflammatory responses, including interleukin (IL)-1β, tumor necrosis factor-α, IL-2, IL-6, IL-8, inducible NO synthase (iNOS), cyclooxygenase (COX)-2, intracellular adhesion molecules, and many antioxidant systems. Free radicals and other reactive oxygen species (ROS) are generated by all aerobic cells and have been shown to participate in many deleterious reactions, in particular, reduced formation of endothelial NO synthase (eNOS) and increased oxidative stress. Endogenous NO plays an important role in the regulation of blood pressure by maintaining vascular smooth muscle in a partially relaxed state. During hypertension, the endogenous vasodilatory effect of NO is prevented due to interaction with ROS, specifically superoxide, thus resulting in increased vascular resistance and elevation of blood pressure.

Antioxidant treatment has been shown to have beneficial effects on NO metabolism and the pathogenesis observed in angiotensin (Ang) II– and lead–induced hypertension. In vivo and in vitro studies have shown that pyrrolidinedithiocarbamate (PDTC) is a potent antioxidant and NF-κB antagonist. Furthermore, Ang II–receiving rats treated with PDTC exhibit decreased systolic blood pressure and partial amelioration of end-organ damage. In other studies, the antioxidant Tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl) has been shown to normalize blood pressure in spontaneously hypertensive rats (SHR). Tempol is a membrane-stable, membrane-permeable, metal-independent superoxide dismutase (SOD) mimic that has been shown to...
be specific for superoxide. To date, ROS accumulation has been reported in deoxycorticosterone acetate (DOCA)-salt hypertension, SHR and stroke-prone spontaneously hypertensive rats (SHRSP), lead-induced hypertension, and essential hypertension. Previously, we reported that mineralocorticoid hypertensive rats developed increased superoxide formation compounded with increased renal monocyte/macrophage infiltration and NF-κB activation. We therefore tested the hypothesis that prolonged antioxidant administration inhibits superoxide accumulation, lowers systolic blood pressure, and reduces NF-κB activation in mineralocorticoid hypertensive rats.

**Methods**

Silastic was from Dow Corning. DOCA, PMSF, dithiothreitol (DTT), Lucigenin, EDTA, and protease inhibitor cocktail were from Sigma Chemical Co. Pentobarbital was from The Butler Company. Ketamine and xylazine were from Fort Dodge Animal Health. Monocyte/macrophage ED-1 antibody was from Serotec. Tyramide signal amplification (TSA) kit was from NEN Life Science. Mouse HRP and streptavidin-FITC were from Jackson ImmunoResearch.

**DOCA-Salt Rats**

Experiments were conducted on male Sprague-Dawley rats treated with mineralocorticoid for 28 days. All procedures were performed according to institutional guidelines. Male Sprague-Dawley rats were anesthetized with an intramuscular injection of 100 mg/kg ketamine/20 mg/kg xylazine. A midscapular incision was made, and a Silastic sheet containing DOCA (200 mg/kg body wt) was inserted subcutaneously. A right flank incision was made, and a uninephrectomy was performed. Rats treated with DOCA alone received 1% NaCl and 0.1% KCl in drinking water, whereas 10 DOCA-treated rats received drinking water with 1 mmol/L Tempol, 1% NaCl, and 0.1% KCl. Ten DOCA-treated rats also received PDTC (200 mg/kg body wt IP) and were given drinking water that contained 1% NaCl and 0.1% KCl. These concentrations of Tempol and PDTC have been shown to effectively reduce oxidative stress and NF-κB activation in intact rats. Sham-operated rats underwent nephrectomy without the implantation of a Silastic/DOCA pellet. Twenty-eight days after implantation, blood pressure was measured with the tail cuff method (pneumatic transducer), and the rats were anesthetized with a ketamine/xylazine cocktail. The kidney and aorta was carefully removed, cleaned of excess fat, and placed in PSS composed of (mmol/L) NaCl 130, KCl 4.7, KH2PO4 1.18, MgSO4·7H2O 1.17, NaHCO3 14.9, dextrose 5.5, EDTA 0.26, and CaCl2 1.6. The kidney was sectioned into 1- to 2-mm slices, and the aorta was cut into 2- to 3-mm rings.

**Lucigenin Assay**

Lucigenin chemiluminescence was used to measure superoxide production. Details of this assay have been published previously. In recent studies, 5 μmol/L lucigenin has been shown to correlate well with electron spin resonance as a quantitative measure of superoxide production. After preparation, aortic sections were placed in PSS composed of (mmol/L) NaCl 130, KCl 4.7, KH2PO4 1.18, MgSO4·7H2O 1.17, NaHCO3 14.9, dextrose 5.5, EDTA 0.26, and CaCl2 1.6. The kidney was sectioned into 1- to 2-mm slices, and the aorta was cut into 2- to 3-mm rings.
Immunohistochemistry
Frozen kidneys were cryosectioned at 7-μm thickness and air dried as previously described. Sections were fixed with cold acetone, washed with PBS, blocked with blocking buffer (3% calf serum, 0.1% Tween 20, 1 x PBS), and incubated for 60 minutes in a humid chamber at room temperature with primary monoclonal anti-rat monocyte/macrophage ED-1 (1:250 in blocking buffer). After a 60-minute incubation, sections were washed with PBS and incubated with bridging peroxidase-conjugated antibody (HRPsMouse; 1:5000 in blocking buffer) for 30 minutes. Sections were then washed, and TSA was performed for 4 minutes according to a modification of the manufacturer’s protocol. The tyramide was detected with Streptavidin-FITC 1:100 through incubation for 1 hour followed by washing. Immunoreactivity was visualized with a Zeiss Axioplan 2 microscope, and photographs were taken with Zeiss Axiocam. Ten different sections of each kidney (5 kidneys per group) were analyzed. ED-1-labeled monocytes/macrophages from anatomically equivalent sections of each kidney were counted by an outside observer who was blinded as to treatment.

Electrophoretic Mobility Shift Assay
Tissue extractions and electrophoretic mobility shift assay (EMSA) for the transcription factor NF-κB were performed as described previously. Kidneys were frozen and divided into 0.2-g sections. Kidney sections were pulverized in liquid nitrogen with a stainless steel mortar and pestle and resuspended in 1.5 mL hypotonic buffer containing 10 mmol/L HEPES-KOH, pH 7.9, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.5 mmol/L DTT, and protease inhibitor cocktail in a glass homogenizer, followed by incubation for 15 minutes on ice. The tissue was homogenized with 10 strokes in the presence of 1% NP-40. The suspension was centrifuged (13 600g, 4 minutes, 4°C), and the pellet was washed with 1 mL hypotonic buffer. The suspension was recentrifuged (13 600g, 4 minutes, 4°C), and the pellet was resuspended in salt solution (20 mmol/L HEPES-KOH, pH 7.9, 25% glycerol, 1.5 mmol/L MgCl2, 400 mmol/L KCl, 2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, and protease inhibitor cocktail). The salt suspension containing glass beads was rotated on a tube rotator (Sepco Scientific Equipment) for 30 minutes and centrifuged (20 000g, 30 minutes, 4°C). The sample was

Figure 2. Representative immunohistochemical photomicrographs of monocyte/macrophage infiltration in medulla of (A) sham, (B) DOCA, (C) DOCA/PDTC, and (D) DOCA Tempol kidneys (magnification ×20). Semiquantitative scoring analysis of ED-1–positive monocyte/macrophage infiltration showed significantly greater monocyte/macrophage infiltration in DOCA and DOCA/Tempol rats compared with sham rats. Rats treated with DOCA and Tempol exhibited significantly increased monocyte/macrophage infiltration compared with sham rats (E). Results are expressed as mean±SD of 5 rats per group. *P<0.05 vs sham. ΔP<0.05 vs DOCA. Bar represents 5 μm.
immediately frozen in liquid nitrogen. Protein concentration was quantified according to the Bradford method. For EMSA, the NF-κB oligonucleotide was derived from the rat iNOS promoter (−972 to −949) containing the upstream NF-κB–binding site: 5′-TGCCAGGGGATTTTCCCTCT-3′ and 5′-GAGAGAGGGAAAATCCCCCTGG-3′. Each oligomer was labeled with [α-32P]dCTP, and the 3 other nonradiolabeled dNTPs by the Klenow fragment of DNA polymerase I. Renal extract with the same amount of protein (10 to 20 μg) was incubated with 300,000 cpm of [32P]labeled oligonucleotide at 30°C for 30 minutes in the gel shift binding buffer [12 mmol/L HEPES, 10% glycerol, 4 mmol/L Tris-HCl, 60 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 2 μg poly(dL/dC), and 2.5 μg BSA] in a final volume of 25 μL. Subsequently, the free and the oligonucleotide-bound proteins were separated with electrophoresis on a native 5.5% polyacrylamide gel in 0.5× Tris borate-EDTA buffer. After electrophoresis, the gel was dried and exposed to Hyperfilm MP. The intensity of the bands was analyzed with a PhosphorImager (Molecular Dynamics). Competition experiments were conducted by adding excess unlabeled NF-κB oligonucleotide to the binding reaction mixture.

Figure 3. A and B, EMSA for detection of NF-κB shows increased NF-κB binding in DOCA kidney compared with sham kidneys. Treatment with PDTC caused a significant decrease in NF-κB–binding activity compared with sham rats. There was no significant difference in DOCA rats treated with Tempol compared with sham rats. Rats treated with DOCA and Tempol also had a significant decrease in NF-κB–binding activity compared with sham. Supershift assay using p50 and p65 antibodies, which are specific for NF-κB, confirmed NF-κB binding (C). Results are expressed as mean±SD deviation of 4 rats per group. *P<0.05 vs sham. dP<0.05 vs DOCA.
Statistical Analysis

Data are presented as mean±SEM. Statistically significant differences among groups were tested by ANOVA and the Tukey multiple range test or t test as appropriate. A value of P<0.05 was considered statistically significant.

Results

Treatment with the antioxidants PDTC or Tempol reduced systolic blood pressure in DOCA rats (130±5 and 142±5 mm Hg versus 199±3 mm Hg, respectively; 24 DOCA, 12 DOCA/PDTC, and 7 DOCA/Tempol; P<0.05) (Figure 1A). However, the magnitude of the reduction in blood pressure did not reach that observed in sham rats (113±2 mm Hg, P<0.05; 13 sham) (Figure 1A). There was no significant difference in blood pressure between sham rats and sham rats treated with PDTC or Tempol (113±2 mm Hg versus 117±2 and 120±5 mm Hg; 15 sham, 5 sham/PDTC, and 5 sham/Tempol; P<0.05). DOCA rats exhibited increased renal hypertrophy compared with sham rats (0.0080±0.0002 versus 0.0051±0.0001, kidney weight/total body weight; 19 DOCA, 7 DOCA/Tempol, and 15 sham; P<0.05). PDTC or Tempol treatment partially, but significantly, decreased renal hypertrophy compared with untreated DOCA rats (0.0074±0.0002 and 0.0065±0.0004 versus 0.0080±0.0002, kidney weight/total body weight; 19 DOCA and 10 DOCA/PDTC; P<0.05) (Figure 1B).

As a means of measuring oxidative stress within the 4 treatment groups, we measured aortic superoxide production with lucigenin chemiluminescence. DOCA rat aorta had markedly increased superoxide production compared with sham rats (7153 versus 783 versus 3055 cpm/mg; 14 and 12, respectively) (Figure 1C). Treatment of DOCA rats with PDTC or Tempol markedly decreased superoxide production compared with untreated DOCA rats (2498±251 and 2939±469 versus 7153±783, respectively; P<0.05) (Figure 1C). There was no significant difference in PDTC- or Tempol-treated rats compared with sham rats (2498±251 and 2939±469 versus 3055±559 cpm/mg; 8, 8, and 13, respectively; P>0.05) (Figure 1C).

We further investigated monocyte and macrophage infiltration in renal tissue. Monocyte/macrophage infiltration was localized mainly in the renal tubules (Figure 2). DOCA rats exhibited increased monocyte/macrophage infiltration compared with sham rats (42±5 versus 10±2 per field viewed; 5 in all groups, respectively; P<0.05) (Figures 2A, 2B, and 2E). DOCA rats treated with PDTC or Tempol exhibited decreased monocyte/macrophage infiltration compared with untreated DOCA rats (16±3 and 26±4 versus 42±5; respectively, P<0.05) (Figures 2B and 2C through 2E), but the amount of infiltration was higher than for sham rats (Figures 2A and 2C through 2E). There was no significant difference in monocyte/macrophage infiltration in sham/Tempol or sham/PDTC rats compared with untreated sham rats (results not shown).

Using EMSAs, we identified an increase in NF-κB–binding activity in renal tissue from DOCA rats compared with sham rats (Figures 3A and 3C). Treatment with PDTC or Tempol resulted in decreased NF-κB–binding activity compared with untreated DOCA rats (Figures 3A through 3C). Treatment with PDTC also resulted in a significant decrease in NF-κB–binding activity compared with sham rats (0.81±0.12- versus 1.0-fold) (Figures 3A and 3B). There was no significant difference in binding activity between DOCA/Tempol–treated animals and sham animals (Figure 3B). As control, DOCA-salt hypertensive rat renal extracts were incubated with antibodies against NF-κB subunits anti-p50 and anti-p65. Supershift assay confirmed p65 and p50 binding activity (Figure 3C).

Discussion

Several recent studies3,11,14 have provided convincing evidence that hypertension causes increased ROS accumulation. Other studies have also shown that ROS cause activation of NF-κB.5,9 NF-κB plays a critical role in the activation of multiple genes that contribute to the inflammatory response and end-organ damage.13 Here, we report increased NF-κB activation as well as increased ROS accumulation in the mineralocorticoid hypertensive rat. We tested the hypothesis that prolonged antioxidant administration inhibits superoxide accumulation, lowers blood pressure, and reduces NF-κB activation in mineralocorticoid hypertensive rats. We found that antioxidants lower blood pressure, normalize O2·− production, reduce NF-κB activation, and reduce monocyte/macrophage infiltration. Furthermore, although we found that antioxidants reduce NF-κB activation and normalize O2·− production, they only partially correct the elevated systolic blood pressure and monocyte/macrophage infiltration. Thus, it is likely that other mechanisms, not yet fully identified, are participating in the hemodynamics and renal disturbances.

Previous studies have been shown that Ang II-induced hypertension cause renal hypertrophy, NF-κB activation, adhesion molecule upregulation, and monocyte/macrophage infiltration.14 Our findings in this low-renin model of hypertension suggests that NF-κB activation and monocyte/macrophage infiltration may be due to locally produced cellular changes that result in ROS formation. Previously, it has been shown that Ang II, thrombin, platelet-derived growth factor, tumor necrosis factor-α, and lactosylceramide increase NAD(P)H oxidase activity, thus resulting in superoxide accumulation.27–29 Moreover, local cellular changes observed in mineralocorticoid hypertension could also be manifested through the stimulation of NAD(P)H oxidase. Therefore, further studies to better understand the mechanism by which superoxide is generated in the mineralocorticoid hypertensive rat are necessary.

Both superoxide and NO are highly reactive unstable free radicals that react together very rapidly to form peroxynitrate.26 This reaction occurs 3 times faster than the dismutation of superoxide by SOD, thus implying that superoxide generation in vascular tissue may inhibit the physiological function of NO.26 Also, it has been shown that experimental elevation of blood pressure and essential hypertension cause increased superoxide formation and decreased endothelium-dependent relaxation.11,19 Therefore, increased vascular O2·− production observed in the aorta of the mineralocorticoid hypertensive rats may result in alteration in vascular endothelium-dependent relaxation due to decreased NO bioavailability.
Salt retention is characteristic of human hypertension and can be achieved rapidly in the mineralocorticoid hypertensive rat model. We chose to use the mineralocorticoid hypertensive rat model because it shows a markedly depressed renin-angiotensin system and because circulating Ang II has previously been shown to increase monocyte/macrophage infiltration and vasculopathy in the kidney and elsewhere. In other studies, it has also been shown that increased vascular resistance due to arterial hypertension in humans results in increased intrarenal vascular resistance, which causes renal ischemia leading to renal damage. The present study shows that long-term hypertension in the presence of low renin can cause induction in the renal inflammatory response, resulting from ROS accumulation and NF-κB activation. To examine the pathogenesis caused by ROS accumulation, we used 2 potent antioxidants: PDTC and Tempol. From the results obtained in this study, PDTC appears to be a more potent antioxidant than Tempol, and the ability of PDTC to cause complete inhibition of the inflammatory response may be due to more than just its antioxidant effect. Therefore, further studies are necessary to discern some of these effects.

In conclusion, our results indicate that prolonged antioxidant administration normalizes superoxide accumulation, lowers systolic blood pressure, and reduces NF-κB activation in mineralocorticoid hypertensive rats. Also, our results suggest that many of the renal changes that occur during hypertension may be due in part to the induction of signal transduction precursors that initiate the inflammatory response. Therefore, further studies to identify the pathophysiological implication of these precursors on the hypertensive state are necessary.

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