

Antioxidant Effects of Vitamins C and E Are Associated With Altered Activation of Vascular NADPH Oxidase and Superoxide Dismutase in Stroke-Prone SHR

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Abstract—Ascorbic acid (vitamin C) and α -tocopherol (vitamin E) have antioxidant properties that could improve redox-sensitive vascular changes associated with hypertension. We determined whether vitamins C and E influence vascular function and structure in hypertension by modulating activity of NADPH oxidase and superoxide dismutase (SOD). Adult stroke-prone spontaneously hypertensive rats (SHRSP) were divided into 3 groups: control (C; n=6), vitamin C-treated (vit C, 1000 mg/day; n=7), and vitamin E-treated (vit E, 1000 IU/day; n=8). All rats were fed 4% NaCl. Blood pressure was measured weekly. After 6 weeks of treatment, the rats were killed, and mesenteric arteries were mounted as pressurized preparations. Vascular O_2^- generation and NADPH oxidase activity were measured by chemiluminescence. Vascular SOD activity and plasma total antioxidant status (TAS) were determined spectrophotometrically. Blood pressure increased from 212 ± 7 to 265 ± 6 mm Hg in controls. Treatment prevented progression of hypertension (vit C, 222 ± 6 to 234 ± 14 mm Hg; vit E, 220 ± 9 to 227 ± 10 mm Hg). Acetylcholine-induced vasodilation was improved ($P < 0.05$), and media-to-lumen ratio was reduced ($P < 0.05$) in the treated rats. O_2^- was lower in vitamin-treated groups compared with controls (vit C, 10 ± 4 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ dry tissue weight; vit E, 9.6 ± 3.5 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ dry tissue weight; C, 21 ± 9 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ dry tissue weight; $P < 0.05$). Both vitamin-treated groups showed significant improvement ($P < 0.01$) in TAS. These effects were associated with decreased activation of vascular NADPH oxidase (vit C, 46 ± 10 ; vit E, 50 ± 9 ; C, 70 ± 16 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ dry tissue weight, $P < 0.05$) and increased activation of SOD (vit C, 12 ± 2 ; vit E, 8 ± 1 ; C, 4.6 ± 1 U/mg; $P < 0.05$). Our results demonstrate that vitamins C and E reduce oxidative stress, improve vascular function and structure, and prevent progression of hypertension in SHRSP. These effects may be mediated via modulation of enzyme systems that generate free radicals. (*Hypertension*. 2001; 38[part 2]:606-611.)

Key Words: antioxidants ■ resistance ■ remodeling ■ hypertension, malignant

Oxidative damage induced by reactive oxygen species is caused by increased production of superoxide anion (O_2^-) and its metabolites and/or by reduced bioavailability of antioxidant defenses. This imbalance between pro-oxidants and antioxidants gives rise to cellular oxidative stress, which plays an important role in the pathogenesis of hypertension.¹⁻³ Reactive oxygen species may act through several mechanisms to mediate vascular change in hypertension: (1) direct actions on endothelial cells and vascular smooth muscle cells (VSMCs), resulting in structural and functional damage; (2) scavenging of the important vasodilator NO; (3) production of peroxynitrite, a potent constrictor and lipid-oxidizing radical; (4) effects on endothelial cell eicosanoid metabolism; and (5) oxidative modification of LDL.^{4,5}

Many studies support a role for altered redox status in hypertension. At the cellular level, concentrations of O_2^- and H_2O_2 are increased, and activity of NADPH oxidase, the

major O_2^- -generating enzyme in vascular cells, is increased.⁶⁻⁸ Endothelium-dependent vasodilation is impaired in hypertension, probably because of increased quenching of NO by O_2^- .⁹ Treatment with antioxidants such as allopurinol¹⁰ and hydrosoluble coenzyme Q10¹¹ improve endothelial function and lower blood pressure in hypertensives, who have been shown to have decreased concentrations of plasma antioxidants.¹² Emerging evidence demonstrates that vitamins with antioxidant properties, such as ascorbic acid (vitamin C) and α -tocopherol (vitamin E), also have blood pressure-lowering effects.

A large epidemiological study recently reported that dietary intake of ascorbic acid correlates inversely with hypertension and its clinical sequelae.¹³ In mild to moderate hypertensive patients, treatment with ascorbic acid (500 mg/day) significantly improved systolic and diastolic blood pressure¹⁴ and increased plasma HDL cholesterol in female

Received March 28, 2001; first decision April 26, 2001; revision accepted May 22, 2001.

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hypertensives.¹⁵ Vitamin C normalized vascular hyperresponsiveness to norepinephrine, as measured by forearm blood flow, in hypertensive patients.¹⁶ Furthermore, impaired endothelium-dependent vasodilation in peripheral and epicardial arteries in hypertensive subjects was improved by intra-arterial infusions of ascorbic acid.^{17,18} In experimental models of hypertension, vitamin C, alone or in combination with vitamin E, accelerates degradation of *S*-nitrosoglutathione, increases synthesis of NO, and reduces blood pressure.^{19,20} Data relating to antihypertensive effects of vitamin E are conflicting. Most clinical trials failed to demonstrate beneficial effects of vitamin E supplementation in hypertensive patients.^{21,22} However, in experimental models of hypertension, vitamin E reduces blood pressure. In spontaneously hypertensive rats (SHR), dietary supplementation of α -tocopherol for 3 months prevented development of increased blood pressure, reduced lipid peroxides in plasma and vessels, and enhanced the total antioxidant status.^{23,24} These effects were attributed to increased activation of vascular NO synthase (NOS) by α -tocopherol.^{24–26} In Dahl salt-sensitive rats, vitamin E administration ameliorated renal and vascular injury but did not significantly reduce blood pressure.²⁷

Mechanisms underlying putative blood pressure-lowering effects of antioxidant vitamins have not been fully elucidated. Both vitamins C and E, which are potent scavengers of free radicals, stimulate activation of NOS activity and increase NO synthesis in endothelial cells.^{19,24–26} These effects could contribute to improved endothelium-dependent vasodilation in hypertension. Vitamin E inhibits expression of adhesion molecules,²⁸ which could influence cell-cell interactions and, consequently, vascular structural changes associated with hypertension. Furthermore, γ -tocotrienol has been shown to improve superoxide dismutase activity in vessels from SHR,²⁹ suggesting that antioxidant vitamins alter activity of enzyme systems that generate reactive species.

The aim of the present study was to investigate whether vitamins C and E influence progression of blood pressure elevation in stroke-prone SHR (SHRSP) by modulating the vascular redox state through changes in activation of NADPH oxidase and superoxide dismutase. Furthermore, we tested the hypothesis that blood pressure effects of antioxidant vitamins ameliorate vascular functional and structural changes associated with hypertension.

Methods

Animal Experiments

At 16 weeks of age, SHRSP were divided into 3 groups: control ($n=6$), vitamin C-treated (ascorbic acid, 1000 mg/d; $n=7$) and vitamin E-treated (α -tocopherol, 1000 IU/d; $n=8$). Ascorbic acid was added to drinking water, and α -tocopherol was mixed in sesame oil and added to the chow. All rats were placed on a high-salt diet by adding 4% NaCl to the food to accelerate the progression and severity of hypertension. Rats were studied for 6 weeks. Systolic blood pressure (SBP) was measured weekly by the tail-cuff method and recorded on a model 7 polygraph fitted with a 7-P8 preamplifier and PCPB photoelectric pulse sensor (Grass Instruments Co). The average of 3 pressure readings was obtained. Rats were killed by decapitation at 22 weeks of age.

Study of Small Arteries

Superior mesenteric arteries were taken from the part of the mesenteric vascular bed that feeds the jejunum, 8 to 10 cm distal to the pylorus, and placed in cold physiological salt solution. A third-order branch of the mesenteric arterial tree (2 mm long) was dissected and mounted in a pressure myograph chamber as we have previously described.³⁰ Intraluminal pressure was set to 45 mm Hg with a servocontrolled pump. Endothelium-dependent relaxation was assessed by measuring the dilatory response of small arteries precontracted with norepinephrine (5×10^{-5} M) to cumulative doses of acetylcholine (ACh) (10^{-7} to 10^{-5} M). Previous studies in salt-loaded SHRSP demonstrated that inhibition of NO generation with NOS blockers abolishes ACh-stimulated responses, indicating that ACh-mediated vasodilation depends on endothelium-derived NO in this model.³¹ Endothelium-independent relaxation was assessed in norepinephrine-precontracted vessels exposed to sodium nitroprusside (10^{-7} to 10^{-4} M). Lumen and media dimensions were measured with the intraluminal pressure maintained at 45 mm Hg. Media cross-sectional area was calculated as $[(\pi/4) \times (D_e^2 - D_i^2)]$, where D_e and D_i are the external and lumen diameters, respectively.

Detection of Vascular O_2^- by Lucigenin Chemiluminescence

The thoracic aorta was cleaned of adherent adipose tissue, and 5-mm-long rings were cut and incubated in HEPES buffer. Rings were maintained at 37°C for 30 minutes; rinsed; then gently transferred to test tubes containing warmed HEPES buffer and Lucigenin (5 μ mol/L), an acridylum dinitrate; and allowed to equilibrate in the dark for 10 minutes at 37°C. Lucigenin chemiluminescence was then recorded every 1.8 seconds for 3 minutes with a luminometer (AutoLumat LB953, EG&G Berthold). Chemiluminescence was expressed as counts/second. Luminescence was also measured in tubes containing buffer and Lucigenin without vascular rings, and these blank values were subtracted from the chemiluminescence signals obtained from the aortic rings. O_2^- generation was quantified against a standard curve of O_2^- generation by xanthine/xanthine oxidase as previously described.^{6,8} Tissue O_2^- formation was expressed as $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ dry tissue weight dry tissue weight.

Measurement of Total Antioxidant Status

Blood was collected from tail arteries in EDTA-containing tubes. Plasma was obtained by centrifuging blood at 1000g for 10 minutes. Plasma total antioxidant status (TAS) was measured using the Calbiochem total antioxidant status assay kit (Calbiochem-Novobiochem Corp) according to the manufacturer's instructions. The assay relies on the ability of antioxidants in the plasma to inhibit oxidation of 2,2' azino-bis-[3-ethylbenz-thiazoline-6-sulfonic acid] (ABTS) to $ABTS^+$ by metmyoglobin. The amount of $ABTS^+$ produced is monitored by reading the absorbance at 600 nm. Under these reaction conditions, the antioxidants in the plasma cause suppression of the absorbance at 600 nm to a degree that is proportional to their concentration. The final plasma antioxidant concentration was obtained using the following formula: antioxidant concentration (mmol/L) = [factor \times (absorbance of blank - absorbance of sample)]; factor = [concentration of standard / (absorbance of blank - absorbance of standard)].

Measurement of NADPH Oxidase Activity

Aortic segments were prepared as described above for measurement of O_2^- . NADPH oxidase was measured as described previously.³² Activity of NADPH oxidase was measured in a luminescence assay with 5 μ mol/L Lucigenin as the electron acceptor and 100 μ mol/L NADPH as the substrate. The reaction was started by the addition of 100 μ L of sample. Luminescence was measured every 1.8 seconds for 5 minutes in a luminometer (AutoLumat LB 953, Berthold). A buffer blank was subtracted from each reading. The amount of O_2^- generated was calculated by comparison with a standard curve using xanthine/xanthine oxidase.⁸ Activity was expressed as $\text{nmol } O_2^- \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ dry tissue weight protein.

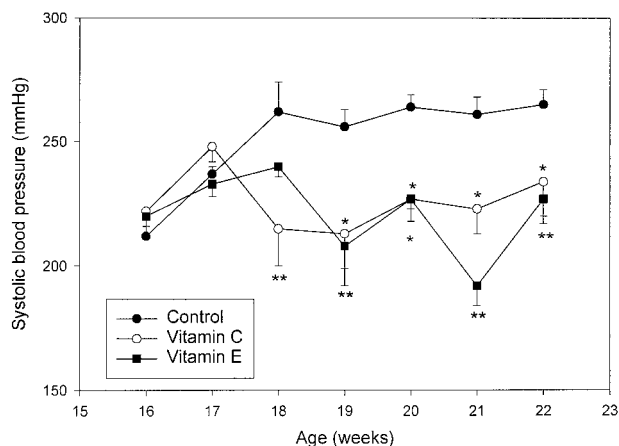


Figure 1. Line graphs demonstrate SBP in control and vitamin-treated groups. From 16 weeks of age, SHRSP were fed a high-salt diet (4% NaCl) * $P < 0.05$, ** $P < 0.01$ vs control counterpart.

Measurement of Superoxide Dismutase Activity

Aortic segments were washed with 0.9% NaCl containing 0.16 mg/mL heparin to remove erythrocytes, which interferes with the assay. The tissue was homogenized, centrifuged, and exposed to 400 μ L extraction reagent (ethanol/chloroform, 62.5/37.5 v/v). The sample was then vortexed and centrifuged at 3000g for 5 minutes. Activity of superoxide dismutase was measured using a kit from Calbiochem (Calbiochem-Novobiochem Co). The assay kit utilizes 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo(c)fluorine reagent. This reagent undergoes alkaline autoxidation, which is accelerated by superoxide dismutase. Autoxidation of the reagent yields a chromophore, which absorbs maximally at 525 nm. The assay was performed according to manufacturer's instructions. Activity of superoxide dismutase was expressed as U/mg protein.

Data Analysis

Data are presented as mean \pm SEM. Statistical analysis was performed using ANOVA for repeated measures or Student's *t* test. $P < 0.05$ was considered significant.

Results

Blood Pressure

SBP increased progressively for the first 2 weeks following salt loading and then plateaued at ≈ 263 mm Hg in the control group (Figure 1). Vitamin C and vitamin E prevented progression of blood pressure elevation. Six weeks after salt loading, SBP was significantly lower in the vitamin C- (234 ± 14 mm Hg) and vitamin E (227 ± 10 mm Hg)-treated groups compared with untreated controls (265 ± 6). Body weight did not differ significantly between groups throughout the duration of the experiment (data not shown).

Effects of Vitamins C and E on Vascular Structure and Function

The media-to-lumen ratio was significantly reduced in the vitamin C ($9.1 \pm 0.9\%$, $P < 0.05$) and vitamin E groups ($6.3 \pm 0.8\%$, $P < 0.01$) compared with controls ($13.0 \pm 1.0\%$). Media thickness was also significantly reduced ($P < 0.05$) in the vitamin C (19.9 ± 1.2 μ m) and vitamin E (17.8 ± 0.8 μ m)-treated groups versus controls (23.6 ± 1.0 μ m). Media cross sectional area was not influenced by either vitamin treatment (data not shown).

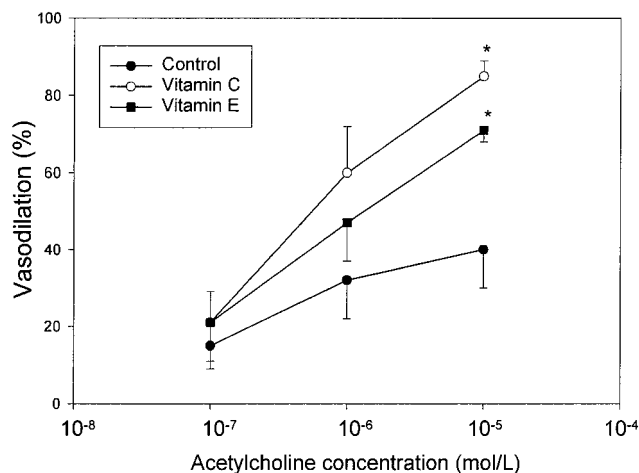


Figure 2. Line graphs demonstrate vascular relaxations to acetylcholine in control and vitamin-treated salt-loaded SHRSP. Segments of small mesenteric arteries were mounted as pressurized systems as described in the Methods section. Relaxations were studied after the arteries had been precontracted with norepinephrine (5×10^{-5} M). Each data point is expressed as the mean \pm SEM. * $P < 0.05$ vs control group.

As shown in Figure 2, vitamin C and vitamin E significantly increased ($P < 0.05$) maximal Ach-induced relaxation compared with that of controls. These results indicate improved endothelium-dependent function. Endothelium-independent vasorelaxation in response to maximal sodium nitroprusside concentration was similar in all groups (control, $70 \pm 14\%$; vitamin C, $75 \pm 11\%$; vitamin E, $68 \pm 16\%$).

Effect of Vitamins on Vascular O_2^- Generation and Plasma Antioxidant Status

Superoxide anion concentration, as measured by Lucigenin chemiluminescence, was significantly lower in the vitamin-treated groups compared with the control group (Figure 3). These findings were associated with significantly increased plasma total antioxidant concentrations in the vitamin C- (1.2 ± 0.1 nmol/L, $P < 0.05$) and vitamin E (1.4 ± 0.1 nmol/L, $P < 0.01$)-treated groups compared with the control group (0.85 ± 0.06 nmol/L).

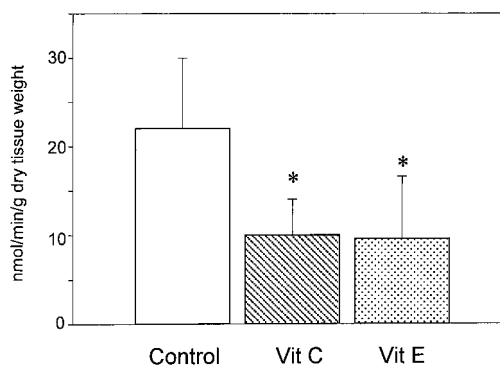


Figure 3. Vascular O_2^- production assessed by Lucigenin chemiluminescence (5 μ mol/L). Aortic segments from untreated and vitamin-treated rats were studied. Results are mean \pm SEM. * $P < 0.05$ vs control.

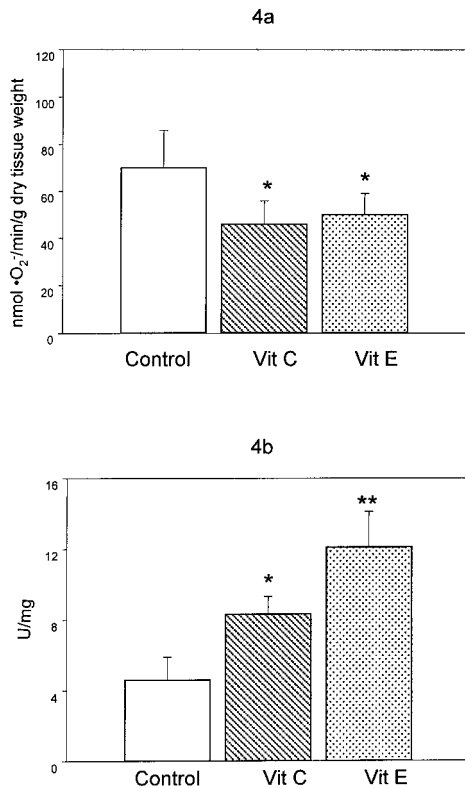


Figure 4. Bar graphs demonstrate activity of NADPH oxidase (Figure 4a) and superoxide dismutase (Figure 4b) in aortic segments from untreated and vitamin-treated salt-loaded SHRSP. Results are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs control group.

Effect of Vitamins on Activation of NADPH Oxidase and Superoxide Dismutase

Activity of NADPH oxidase, a major source of O_2^- in the vasculature, was significantly lower in salt-loaded SHRSP following 6 weeks of treatment with vitamin C or vitamin E compared with untreated rats (Figure 4a). These findings were associated with a significant rise in superoxide dismutase activity in the vitamin-supplemented rats compared with the control group (Figure 4b).

Discussion

The major findings of the present study are that (1) blood pressure-lowering effects of vitamins C and E are associated with improved endothelium-dependent vasodilation and amelioration of vascular structural changes, (2) vitamin supplementation decreases vascular oxidative stress and improves total antioxidant status, and (3) antioxidant properties of vitamins C and E are associated with decreased activation of NADPH oxidase and increased activity of superoxide dismutase. These data suggest that in addition to the known free radical scavenging properties of antioxidant vitamins, they may influence the vascular redox state in severely hypertensive rats by modulating O_2^- -generating enzyme systems.

Vitamins C and E prevented the progression of hypertension in salt-loaded SHRSP. Previous studies investigating effects of these vitamins in experimental models of hypertension have shown, for the most part, significant antihyperten-

sive effects.^{19,23–25} Clinical studies reported blood pressure-reducing actions of vitamin C, particularly in elderly patients.^{13–18} However, clinical data relating to vitamin E have been disappointing. Results from the Heart Outcomes Prevention Evaluation (HOPE) trial²¹ and the Collaborative Group of the Primary Prevention Project,²² in which hypertensive patients were treated with vitamin E (400 IU/d or 300 mg/d, respectively) did not demonstrate any clinically relevant effects on blood pressure. Reasons for these conflicting data may relate, in part, to the fact that in experimental studies, vitamin E is supplemented at higher doses (800 to 1000 IU/d) than those used in clinical trials (300 to 500 IU/d).

In the model studied here, the blood pressure-lowering actions of vitamins C and E were accompanied by improved endothelium-dependent vasodilation and decreased vascular hypertrophy, suggesting that these processes are redox-sensitive. Our findings are in keeping with others that salt-induced hypertension, as well as SHRSP, are hypertensive models of oxidative stress.^{3,9} Mechanisms by which antioxidant vitamins influence vascular function could be through free radical scavenging, which decreases NO quenching by O_2^- , thereby increasing bioavailability of the potent vasodilator NO. Vitamins C and E have also been shown to directly stimulate activity of NOS by increasing intracellular tetrahydrobiopterin, which would further increase NO synthesis.^{17,24,29} Although we did not assess the NO system in our model, previous studies demonstrated that antioxidant vitamins increase NOS activity and NO generation in arteries from SHR.^{23,24} Free radicals cause extensive cellular damage, facilitate lipid peroxidation, increase intracellular free Ca^{2+} concentrations, and stimulate inflammatory- and growth-signaling events in VSMCs.^{33–35} These processes could contribute to vascular structural changes associated with hypertension, especially because O_2^- and H_2O_2 stimulate hypertrophy and hyperplasia.³⁵ In both vitamin-treated groups, media thickness was less than that in untreated control rats. These results suggest that antioxidants improve integrity of vascular structure, possibly by preventing the cellular damage induced by oxygen free radicals.

Supplementation with vitamins C and E decreased vascular O_2^- and increased total plasma antioxidant status. These findings confirm the antioxidant properties of vitamins C and E and indicate that treatment influences generation of oxygen free radicals and improves antioxidant defenses in our model. Processes contributing to these effects are probably related to the direct scavenging actions of vitamins C and E. However, it is possible that these vitamins may also influence the vascular redox state by modulating activity of enzyme systems that generate reactive oxygen species. We demonstrate the novel findings that in the vitamin-treated rats, activation of NADPH oxidase is decreased and activity of superoxide dismutase is increased. Because NADPH oxidase is the major source of superoxide anion in vascular cells, decreased activation of the enzyme would result in reduced generation of the oxygen free radical. On the other hand, enhanced activation of superoxide dismutase would lead to increased dismutation of O_2^- , which further decreases O_2^- concentration. From our study, we cannot elucidate which of these systems is more important, but together they could contribute

to overall reduction in generation of reactive oxygen species and improved oxidative status, as we observed in the treated rats. Mechanisms whereby antioxidant vitamins influence NADPH oxidase and superoxide dismutase are ill defined, but they may play an important role in the regulation of protein expression of the enzymes at the transcriptional or posttranslational levels.³⁶ It is also possible that vitamins C and E directly influence biological activity of the enzymes. Vitamin E, which is hydrophobic and located within the cell membrane,^{36–38} could alter cell membrane-associated NADPH oxidase by inhibiting or interrupting complex formation of the NADPH oxidase subunits. On the other hand, vitamin C is located in the cytoplasmic and mitochondrial compartments, which are superoxide dismutase-rich regions.^{36,38} In addition to the direct actions of vitamin C, some of its effects could be mediated via vitamin E, which can act as a pro-oxidant or an antioxidant.^{36,38} Vitamin C prevents the pro-oxidant activity of vitamin E by decreasing the activity of α -tocopheroxyl radical to α -tocopherol, thereby acting as a co-antioxidant and further contributing to increased total antioxidant status and reduced oxidative stress.

Findings from our study confirm the role of redox-sensitive processes in the pathogenesis of hypertension in salt-loaded SHRSP. However, it is still unclear whether elevated amounts of free radicals initiate the development of hypertension or whether they are a consequence of the disease process itself. Furthermore, it may be possible that salt itself could influence vascular redox status. We can also not exclude the possibility that some of the vascular effects of vitamins C and E observed in our model may be caused by blood pressure-lowering effects and not necessarily by direct actions of treatment. Elucidation of these aspects await clarification.

In summary, we have demonstrated that chronic treatment with vitamins C and E prevents progression of blood pressure elevation in severely hypertensive rats. These effects are associated with improved endothelium-dependent vasodilation, decreased vascular hypertrophy, and increased plasma antioxidant concentration. In addition, we have identified that vitamins C and E modulate activity of NADPH oxidase and superoxide dismutase, which could contribute, at least in part, to decreased vascular O_2^- and improved antioxidant status. Our data support an important role for oxidative stress in the pathogenesis of hypertension in salt-loaded SHRSP.

Acknowledgments

This work was supported by grants 13570 and 14080 from the Canadian Institutes of Health Research (CIHR; previously the Medical Research Council of Canada) and by a grant from the Fonds de la recherche en sante de Quebec. R.M.T. received a scholarship from the Canadian Hypertension Society/CIHR.

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