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Vitamins Reverse Endothelial Dysfunction Through Regulation of eNOS and NAD(P)H Oxidase Activities

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Abstract—Antioxidant vitamins C and E have protective properties in genetic hypertension associated with enhanced oxidative stress. This study investigated whether vitamins C and/or E modulate vascular function by regulating enzymatic activities of endothelial nitric oxide synthase (eNOS) and NAD(P)H oxidase using thoracic aortas of 22-week-old male spontaneously hypertensive rats (SHR) and their matched normotensive counterparts, Wistar-Kyoto rats (WKY). SHR aortas had impaired relaxant responses to acetylcholine but not to sodium nitroprusside, despite an ≈2-fold increase in eNOS activity and NO release. The levels of superoxide anion (O$_2^-$), a potent NO scavenger, and NAD(P)H oxidase activity were also 2-fold higher in SHR aortas. Mechanical but not pharmacological inactivation of endothelium (by rubbing and 100 μmol/L L-NAME, respectively) significantly abrogated O$_2^-$ in both strains. Treatments of SHR aortas with NAD(P)H oxidase inhibitors, namely diphenyleneiodonium and apocynin, significantly diminished O$_2^-$ production. The incubation of SHR aortas with different concentrations of vitamin C (10 to 100 μmol/L) and specifically with high concentrations of vitamin E (100 μmol/L) improved endothelial function, reduced superoxide production as well as NAD(P)H oxidase activity, and increased eNOS activity and NO generation in SHR aortas to the levels observed in vitamin C- and E-treated WKY aortas. Our results reveal endothelial NAD(P)H oxidase as the major source of vascular O$_2^-$ in SHR and also show that vitamins C and E are critical in normalizing genetic endothelial dysfunction through regulation of eNOS and NAD(P)H oxidase activities. (Hypertension. 2003;41:534-539.)

Key Words: nitric oxide ■ endothelium ■ enzymes ■ antioxidants ■ hypertension, experimental ■ vitamins

The endothelium plays pivotal roles in the maintenance of vascular tone and blood pressure by regulating the release of several vasoactive substances, including nitric oxide (NO). However, its characteristics change in pathological conditions leading to a phenomenon called “endothelial dysfunction,” which is characterized by impaired endothelium-dependent relaxation. Endothelial dysfunction has been reported in conduit and resistance arteries of both essential hypertensive patients and various animal models of essential hypertension.

Current data as to the pathogenesis of hypertensive endothelial dysfunction are extremely diverse. Diminished production of NO caused by either inefficient utilization of substrate L-arginine or impaired expression/activity of endothelial NO synthase (eNOS) and impaired endothelium-derived hyperpolarizing factor–mediated hyperpolarization of resistance vessels have been implicated in this process. However, a single unifying mechanism has yet to emerge.

NO, generated within vascular endothelium by eNOS, is considered the most important endothelium-derived vasodilator in conduit arteries. Although eNOS is constitutively expressed, many pathophysiological stimuli may regulate its expression. Indeed, sex hormones elicit an increase in eNOS gene expression, whereas hypoxia downregulates its expression. The current data on the molecular regulation of eNOS in the cardiovascular system in essential hypertension are rather conflicting. Increased, decreased, and unaltered levels of eNOS expression have thus far been reported in SHR.

In recent years, exaggerated vascular production of reactive oxygen species (ROS), in particular O$_2^-$ by NAD(P)H oxidase and/or uncoupled state of eNOS as a result of its activation at suboptimal concentrations of its cofactor tetrahydrobiopterin (H$_4$ B) or diminished metabolism of ROS by antioxidant enzymes or impaired regeneration of reduced antioxidants, has been associated with the pathogenesis of this phenomenon in hypercholesterolemia and stroke prone SHR (SHRSP).

ROS modulate vascular function by scavenging NO and producing peroxynitrite (OONO$^-$) in the process. OONO$^-$, another ROS, oxidizes proteins and elicits both breakage in DNA structure and shortage of intracellular antioxidants such as glutathione and cysteine. ROS are also involved in the oxidative modification of LDL.

Several recent studies have shown the beneficial effects of treatments with antioxidants such as allopurinol and drososoluble coenzyme Q10 in patients with diabetes mellitus and insulin-resistant hypertension, respectively. An accumulating body of evidence has also demonstrated that anti-
oxidant vitamins C and E are also implicated in the improvement of endothelial function in human subjects and in animal models of human diseases including hypertension. Indeed, vitamin C alone or in combination with vitamin E has been shown to enhance NO generation and reduce blood pressure in hypertensive animals. Moreover, dietary supplementation of vitamin E has been shown to enhance total antioxidant status and reduce blood pressure in SHR.

Although both antioxidant vitamins are known to stimulate NO generation in endothelial cells and have ROS-scavenging effects, the underlying mechanisms of action are unknown.

The purpose of the present study was therefore to investigate whether vitamins C and E improve endothelial function in SHR aortas by alteration of the redox state through regulation of the enzymatic activities of eNOS and NADPH oxidase and therefore release of NO and O$_2^-$, respectively.

**Methods**

**Animals and Thoracic Aorta Preparation**

The studies were performed with thoracic aortas obtained from 20- to 22-week-old male SHR and their age- and gender-matched normotensive counterparts, Wistar-Kyoto rats (WKY). Thoracic aortas were removed from anesthetized rats (100 U heparin IV) under deep sodium pentobarbital anesthesia (100 mg/kg body IP) and carefully cleaned of adhering tissue. The aortas were then either cut into rings or cut longitudinally before placing into culture flasks. The aortas were incubated in medium containing 10% fetal calf serum, 10% newborn calf serum, 250 U/mL penicillin, 250 µg/mL streptomycin, 1.25 µg/mL amphotericin B, 50 µg/mL gentamicin, and different concentrations (10 to 100 µmol/L) of vitamins C (L-ascorbic acid, Sigma) or E (Ephynal; injectable form – tocopherol acetate at the concentration of 100 mg/2 mL, Roche). All other reagents were purchased from Gibco BRL. Flasks that did not contain vitamins served as controls. After 24 hours, media were removed; the tissues were washed with PBS and homogenized before measurements of enzyme activities and NO or O$_2^-$ levels.

All investigations were performed in accordance with the UK “Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.”

**Vascular Reactivity Studies**

Rings were equilibrated for 90 minutes under 2 g resting tension in organ baths filled with Krebs buffer [in (mM/L); NaCl 118.3, KCl 4.7, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.22, CaCl$_2$ 2.5, NaHCO$_3$ 25, glucose 11.1, pH 7.4] and constantly gassed with carbogen (95% O$_2$/5% CO$_2$). Changes in isometric tension were detected and recorded by a force transducer and an 8-channel transducer data acquisition system (LE-TR201 and PowerLab/8S, ADInstruments), respectively.

After the initial equilibration period, ordinary Krebs solution was replaced with fresh solution containing 1 µmol/L indomethacin to prevent the relaxant effects of prostanooids. All experiments were performed in the latter solution.

At the beginning of each experiment, dose-response curves to phenylephrine (PE, 0.003 to 10 µmol/L) were obtained. Rings were then washed and equilibrated before contraction with submaximal concentration of PE before detection of endothelium-dependent and endothelium-independent relaxant responses to acetylcholine (ACh, 0.03 to 10 µmol/L) or sodium nitroprusside (SNP, 0.01 to 10 µmol/L), respectively. Only one vasorelaxant agent was used on each ring.

This experimental pattern was repeated for the studies in which either vitamin C or E was added to organ baths for 20 minutes before investigation of relaxant responses to ACh.

**Measurement of NOS Activity**

NOS activity was measured by the conversion of L-[3 H]-arginine to L-[3 H]-citrulline. Briefly, isolated aortic rings were homogenized on ice, in TRIS buffer (50 mmol/L, pH 7.4) containing leupeptin (0.2 µmol/L), pepstatin A (1.5 µmol/L), and phenylmethylsulfonyl fluoride (PMSF, 1 mM). Samples were incubated at 37°C for 30 minutes in the presence of calmodulin (30 mmol/L), NADPH (1 mM), H$_2$B (5 µmol/L), Ca$^{2+}$ (2 mM), L-valine (50 mmol/L), and a mixture of unlabelled (0 to 5 µmol/L) and L-[3 H]-arginine (10 µmol/L) (Amersham Pharmacia). To assess the contribution of iNOS (calcium-independent isofrom) to overall NOS activity, Ca$^{2+}$ was replaced with EGTA (1 mM). Reactions were terminated by the addition of 1 mL HEPES (20 mM/L, pH 5.5) containing EDTA (1 mM) and EGTA (1 mM/L). Newly formed L-[3 H]citrulline, neutral at pH 5.5, was separated from the incubation mixture by cation exchange resin (Dowex AG 50 W-X8, Bio-Rad) and quantified by a liquid scintillation counter. Results were expressed as picomoles of L-citrulline per milligram of protein per minute.

**Nitrite Detection**

Nitrite levels were measured in aortic homogenates by Griess reaction as index of NO generation after conversion of nitrate to nitrite by nitrate dehydrogenase. An aliquot of the supernatant was mixed with an equal volume of Griess reagent (sulfanilamide, 1% wt/vol; naphthylethylenediamine dihydrochloride, 0.1% wt/vol; and α-naphthol, 1% wt/vol). The reaction was terminated by the addition of 1 mL HEPES (20 mM/L, pH 5.5) containing EDTA (1 mM) and EGTA (1 mM/L). Newly formed L- or D-[3 H]nitrite, neutral at pH 5.5, was separated from the incubation mixture by cation exchange resin (Dowex AG 50 W-X8, Bio-Rad) and quantified by a liquid scintillation counter. Results were expressed as picomoles of L-citrulline per milligram of protein per minute.

**NADH/NADPH Oxidase Activity**

Aortic homogenates were prepared on ice in lysis buffer containing 1 mMOL/L EGTA, 20 µMOL/L monobasic potassium phosphate (pH 7.0), 0.5 µg/µL leupeptin, 0.7 µg/µL pepstatin, 10 µg/µL aprotinin, and 0.5 µMOL/L PMSF. Oxidase activity was measured by lucigenin-enhanced chemiluminescent detection of superoxide in a luminometer. The reaction was initiated by the addition of 200 µM of total protein to reaction buffer containing 1 mMOL/L EGTA, 150 mMOL/L sucrose, 5 µMOL/L lucigenin, and 1 mMOL/L NADH or NADPH. Luminescence was calculated as the rate of counts per mg protein after deduction of the counts obtained from a buffer blank.

**Detection of Vascular O$_2^-$ by Lucigenin-Enhanced Chemiluminescence**

Aortic segments were prepared as described above. Approximately 5-mm-long rings were transferred into tubes containing HEPES buffer and incubated at 37°C for 30 minutes before addition of 5 µMOL/L of lucigenin. Luminescence was measured as above.

**Statistical Analysis**

Data are expressed as mean±SEM. Numbers (n) indicated throughout the article denote the number of animals used in vascular reactivity studies or enzymatic assays. Dose-response curves were fitted by nonlinear regression with simplex algorithm. Relaxant responses were given as the percentages of PE precontraction. Comparisons of dose-response curves were evaluated by 2-way ANOVA for repeated measures. Probability values <0.05 were considered to be statistically significant.

**Results**

**General Data**

The systolic blood pressure and the heart weight/body weight ratio were significantly higher in SHR compared with WKY.
Levels of eNOS and NAD(P)H Oxidase Activity and NO and O$_2^-$ in WKY and SHR Aorta Before and After Treatments With Vitamins C and E

The activities of eNOS and NAD(P)H oxidase were found to be significantly higher in SHR aortic homogenates before quantification of O$_2^-$ production. However, there was no significant difference in body weight or basal heart rate between the 2 groups (Table 1).

Enzymatic and Cellular Sources of O$_2^-$ in WKY and SHR Aorta

Endothelial NOS activity was abrogated in SHR and WKY aortas through mechanical and pharmacological removal of the endothelium by rubbing and incubation with Nω-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L) for 1 hour before quantification of O$_2^-$ levels. Although the mechanical removal of endothelium significantly reduced the basal level of O$_2^-$ in both strains, the decrease was not significant in response to treatments with L-NAME in either strain (Table 3).

In contrast, the treatment of SHR aortas with 2 different inhibitors of NAD(P)H oxidase, namely apocynin and diphenyleneiodinium (DPI, 10 and 100 μmol/L), elicited a significant reduction in O$_2^-$ production in a dose-dependent manner, indicating this enzyme as the main source of vascular O$_2^-$ (Table 3).

Vasodilator Responses in Thoracic Aorta

In PE-precontracted aortic rings, although ACh produced a concentration-dependent relaxation in WKY, it generated contractile responses in SHR at higher doses, indicating a defect in vascular endothelium (Figure 1A). There was no difference in the relaxant responses of SHR and WKY rings to incremental concentrations of SNP, an endothelium-independent relaxant (Figure 1B).

Effects of Vitamins C and E on Vasodilation

To determine whether antioxidant vitamins potentiate the vasodilatory effects of ACh in SHR and WKY aortas, the rings from both species were treated with 4 different concentrations of these vitamins (10, 25, 50, and 100 μmol/L) for 20 minutes before investigation of relaxant responses. All concentrations of vitamin C elicited significant relaxation in SHR rings compared with WKY (Figure 2). In contrast, only the highest concentration of vitamin E (100 μmol/L) improved endothelial function in SHR suggesting a dose-dependency for the beneficial effects of vitamin E (Figure 3).

Discussion

The major conclusions to be drawn from this study are that the antioxidant properties of vitamins C and E are associated with decreased activation of NAD(P)H oxidase and hence generation of O$_2^-$ and enhanced activation of eNOS and therefore generation of NO. These findings provide crucial
evidence as to how antioxidant vitamins C and E may improve vascular endothelial function in addition to their well-established free radical–scavenging effects.

Vitamins C and E, along with the other low-molecular-weight antioxidants such as urate and thiols, constitute the first line of defense against oxidative stress in the extracellular environment. These antioxidants prevent oxidation of proteins and lipids by scavenging free oxygen radicals at the expense of being consumed in the process. It is therefore plausible to suggest that the levels of these antioxidants may reflect the severity of resistance to oxidative stress. A close association between the alleviation of hypertension and treatments with these vitamins has been shown in experimental models of hypertension, including SHR and salt-loaded SHRS. A consistent beneficial effect of vitamin C has been reported in human subjects in that acute intra-arterial administration of vitamin C to patients with diabetes mellitus improves endothelium-dependent vascular relaxation. Similarly, physiological and supraphysiological concentrations of vitamin C have been shown to reverse endothelial dysfunction in conduit arteries of patients with congestive heart failure and hypertension, respectively. However, clinical data pertaining to beneficial effects of vitamin E have been mixed. A randomized study, the Cambridge Heart Antioxidant Study (CHAOS), revealed a marked reduction in nonfatal myocardial infarction in patients randomly assigned to treatment with 400 to 800 IU of vitamin E per day compared with patients receiving placebo. Nevertheless, several subsequent studies including the Heart Outcomes Prevention Evaluation (HOPE) study have failed to confirm these findings. In the present study, vitamin C (10 to 100 μmol/L) and high concentrations of vitamin E (100 μmol/L) improved endothelial function in the thoracic aortas of 20- to 22-week-old male SHR to the levels that were observed in matching normotensive WKY. Putative mechanisms whereby antioxidant vitamins improve endothelial function may be due to (1) their direct free radical–scavenging ability; (2) their ability to regulate NO synthases that generate NO; (3) their ability to regulate enzymes such as NAD(P)H oxidase that generate free radicals; or (4) their ability to regulate antioxidant enzymes, including superoxide dismutases (SODs), which metabolize free radicals. The free radical–scavenging effects of antioxidant vitamins are well documented. The beneficial effects of dietary supplementation of vitamins C and E with regard to reduction of blood pressure has also been well documented in SHRS and DOCA-salt hypertensive rats, respectively. A recent study has linked these effects to the regulation of enzyme systems that generate free radicals such as NAD(P)H oxidase and SODs in vitamin C- and E-treated SHRS mesenteric arteries, which contribute to peripheral resistance and blood pressure regulation significantly. However, the current data as to regulation of eNOS in SHR cardiovascular system are contradictory, as increased, decreased, and unaltered levels of eNOS expression/activity have been reported. In the present study, we investigated the levels of eNOS activity and NO release in SHR thoracic aortas before and after treatment with vitamin C and E.
ies. Although higher percent increases in NOS activity and NO generation in SHR arter-
which were in keeping with the previous studies also showing eNOS activity and NO levels in both SHR and WKY aortas, thereby suggesting that vascular smooth muscle cells and adventitial fibroblasts may be alternative sources of O2− generation. However, the decrease in the O2− was not significant in response to treatments with L-NAME in both strains, indicating NAD(P)H oxidase as the main source of O2− in SHR at 20 to 22 weeks age, the well-established hypertensive stage. To further verify this hypothesis, we treated SHR aortas with specific inhibitors of NAD(P)H oxidase, for example, apocynin and DPI, and showed that both inhibitors reduced O2− production significantly in a dose-dependent manner (Table 3).

Although this study did not investigate either vascular SOD activity or total antioxidant status, a previous study showed significant elevations in both of these parameters in another model of genetic hypertension, salt-loaded SHRSP.22

The mechanisms by which vitamins C and E may modulate NAD(P)H oxidase or eNOS activities are unclear; it has been suggested that vitamins may be involved in both the transcription and posttranslational modification of NAD(P)H oxidase36 and perhaps eNOS. Vitamin E, a lipophilic antioxidant,36 may modulate the interactions between cytochrome b558, the membrane-bound part of NAD(P)H oxidase enzyme that is required for enzyme activity and stability as a whole, and the cytosolic components of this enzyme system that translocate to the plasma membrane on activation. Moreover, vitamin E is taken up by LDL particles and therefore improves endothelial function by reducing the sensitivity of LDL to in vitro oxidation in healthy subjects as well as type 2 diabetics.37 However, other studies have shown that in vitro or in vivo enrichment of LDL with α-tocopherol accelerates rather than inhibits LDL oxidation if there is a shortage of other antioxidants including vitamin C in the environment.38 This suggests that vitamin C may mediate some of its functions through vitamin E. Vitamin C, in addition to its direct ROS-scavenging effects, may also have other beneficial effects as the result of its ability to increase the availability of the eNOS cofactor H4B, thereby enhancing eNOS activity.39

Data from this study confirm the involvement of oxidative stress in the pathogenesis of genetic endothelial dysfunction and indicate a requirement for a relatively higher dose of vitamin E compared with vitamin C to improve vascular relaxation. It also sheds some light as to why discrepant results have been reported in response to treatments with vitamin E.

We have demonstrated in the present study that treatment with vitamins C and high doses of vitamin E reverse impaired endothelial-dependent vascular relaxation in a genetic
model of human essential hypertension. These effects are associated with enhanced generation of NO and activity of eNOS as well as diminished production of $\text{O}_2^-$ and activity of NAD(P)H oxidase. These findings provide important evidence for the use of adequate levels of antioxidant vitamins C and E in the treatments of pathologies that are associated with oxidative stress.

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