Original Article

Antioxidant Treatment with α-Tocopherol Improves Erectile Function in Hypertensive Rats

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There is no known treatment for erectile dysfunction (ED) in hypertensive patients. We tested whether or not antioxidative therapy improves ED in the setting of hypertension. Spontaneously hypertensive rats (SHRs) were treated with a control chow or an α-tocopherol–enriched chow (12 or 24 mg/100 g chow) for 8 weeks. The isometric tension of corpus cavernosum strips from these SHRs was recorded. nNOS and HO-2 gene expression and NOx, cGMP, thiobarbituric acid–reacting substance (TBARS), and superoxide dismutase (SOD) activity levels were determined in serum and tissue. Relaxation in response to electrical field stimulation (EFS) in the corpus cavernosum increased after the administration of α-tocopherol at a dose of 24 mg/100 g chow. This effect was inhibited by a nitric oxide synthase (NOS) inhibitor and by a heme oxygenase (HO) inhibitor. nNOS and HO-2 gene expression and NOx concentrations in the corpus cavernosum were similar between 24 mg α-tocopherol–fed SHRs and controls. Tissue cGMP levels were greater in α-tocopherol–fed SHRs than in controls. Treatment with 24 mg α-tocopherol decreased TBARS levels and increased SOD activity in the serum and corpus cavernosum. Relaxation in response to acetylcholine chloride in the corpus cavernosum was improved with α-tocopherol treatment at each dose. These results suggest that α-tocopherol treatment increases the diminished relaxation in the corpus cavernosum of SHRs by improving neuronal or endothelial function related to nitric oxide and carbon monoxide. This, in turn, indicates that antioxidant therapy may play a role in treatment for ED in hypertensive patients. (*Hypertens Res* 2008; 31: 1007–1013)

***Key Words*: erectile dysfunction (ED), hypertension, spontaneously hypertensive rat (SHR), nitric oxide (NO), carbon monoxide (CO)**

Introduction

Penile erection is evoked by the elevation of pressure in the corpus cavernosum resulting from the relaxation of the cavernous smooth muscles and its arterioles. The relaxing substance has been found to be mainly nitric oxide (NO), which is liberated from neurons and endothelium in the corpus cavernosum (*1*). Erectile dysfunction (ED) often occurs in the elderly and in patients with hypertension or diabetes mellitus (*2*). We (*3*) and others (*4*) recently demonstrated that increased oxidative stress in spontaneously hypertensive rats (SHRs) decreases the bioavailability of NO, resulting in ED through reduction of relaxation depending on NO in the corpus cavernosum of SHRs (*3*). However, it remains unknown whether or not suppression of oxidative stress improves ED in hypertension. We therefore used SHRs as a model of hypertension to elucidate whether or not 1) treatment with an antioxidant, α-tocopherol, improves erectile function in corpus cavernosum of SHRs; 2) the neuronal or endothelial NO sys-

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tem in the corpus cavernosum is involved in such an action of an antioxidant; and 3) another relaxing substance system, that of carbon monoxide (CO), helps to increase pressure in the corpus cavernosum of SHRs.

Methods

Animals and Treatment with Antihypertensive Drugs

We used 14-week-old male SHR/Izm as a model of hypertension (Funabashi Farms, Funabashi, Japan). The animals were housed under a controlled temperature of 24°C, with automatic lighting that provided a 12 h on-off cycle. They had free access to laboratory rat chow and tap water. The rats were maintained on either a control chow or a rat chow containing α-tocopherol (12 or 24 mg/100 g chow) for 8 weeks from the age of 6 weeks.

Reagents

The control rat chow (α-tocopherol 6.7 mg/100 g chow) and a special chow, to which α-tocopherol was added at 12 or 24 mg/100 g chow, were purchased from Oriental Yeast, Tokyo, Japan. Guanethidine sulphate (guanethidine), atropine sulphate (atropine), phenylephrine hydrochloride (PhE), papaverine (PAP), acetylcholine chloride (Ach), sodium nitroprusside (SNP), and N^{ω}-nitro-L-arginine (L-NNA) were obtained from Sigma Chemical (St. Louis, USA). Zinc protoporphyrin-IX (ZnPP) was obtained from Porphyrin Products (Logan, USA). ZnPP was dissolved in a small volume of 0.5 mol/L NaOH and 95% ethanol. Subsequently, the solutions were diluted with saline immediately before use, as described previously (*5*). All other reagents were dissolved in distilled water.

Measurement of Blood Pressure

Systolic blood pressure (SBP) and heart rate (HR) in control and α-tocopherol–fed rats were measured without anesthesia by the tail-cuff method using a plethysmograph (PS-600; Riken Kaihatsu, Yokohama, Japan) before and weekly after the initiation of α-tocopherol treatment.

Preparation

Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (0.4 mg/kg) and killed by bleeding from the carotid arteries. Their penises were rapidly removed. The isolated corpus cavernosum (approximately 3 × 12 mm) were set in a Magnus chamber filled with 10 mL Krebs solution, and isometric tension was measured, as described previously (*3*). The initial resting tension applied to each strip was adjusted to 2 g. At the end of the experiments, PAP (100 μmol/L, final concentration) was added to attain the maximal relaxation of the corpus cavernosum.

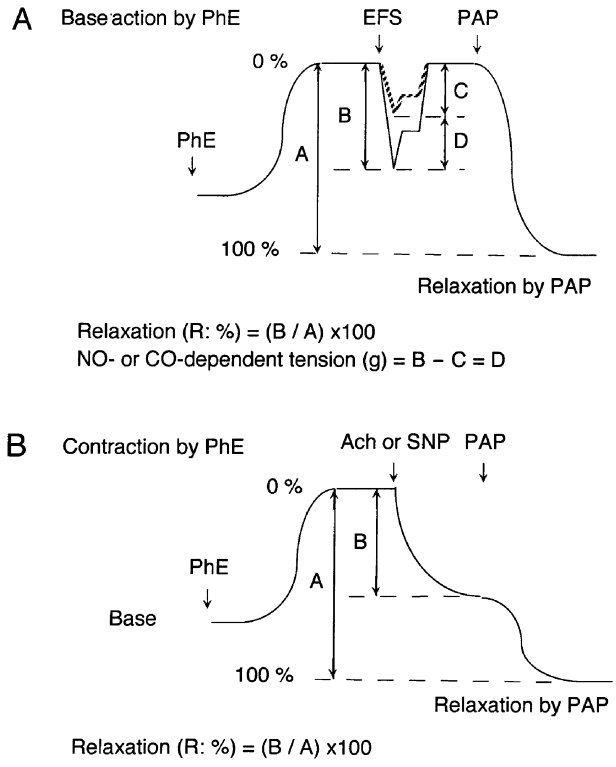

Fig. 1. *Calculation of relaxation in response to EFS, Ach, and SNP. A: Calculation of relaxation in response to EFS in SHR corpus cavernosum. "C" is relaxation to EFS after treatment with N^{ω}-nitro-L-arginine or zinc protoporphyrin-IX. B: Calculation of relaxation to Ach or SNP in corpus cavernosum of SHR. At the end of the examinations, corpus cavernosum was treated with papaverine (PAP). EFS, electrical field stimulation; PhE, phenylephrine; Ach, acetylcholine; SNP, sodium nitroprusside.*

Experimental Design

Measurements of Relaxation in Response to Electrical Field Stimulation

The corpus cavernosum was equilibrated with Krebs solution containing guanethidine (5 μmol/L, final concentration) and atropine (1 μmol/L, final concentration) for 90 min to deplete endogenous norepinephrine and block cholinergic endothelial relaxation. After contraction by adding PhE (10 μmol/L, final concentration) for 10 min, the strips were subjected to relaxation in response to electrical field stimulation (EFS) using sequential frequencies of 1, 2, 4, 8, 16, and 32 Hz delivered as 10-s trains (50 V, 0.8 ms) at 2-min intervals. After the strips were washed for 30 min in fresh medium containing guanethidine and atropine, L-NNA (nitric oxide synthase [NOS] inhibitor; 10^{-5} mol/L, final concentration) (*6*) was added for the examination of NO-dependent relaxation. On

Table 1. SBP (mmHg) in α-Tocopherol–Fed SHR

	0	1 month	2 months
Control	131.2±2.4	160.0±2.0	168.2±2.9
12 mg	134.4±2.9	160.1±2.4	171.4±2.3
24 mg	127.8±1.9	155.4±2.4	166.3±2.0

SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; 12 mg, α-tocopherol 12 mg/100 g chow; 24 mg, α-tocopherol 24 mg/100 g chow. *$p<0.05$ *vs*. control SHR.

the other hand, to examine CO-dependent relaxation, the strips were equilibrated for 20 min after treatment with L-NNA and for 40 min in the dark after treatment with ZnPP (heme oxygenase [HO] inhibitor; 3×10^{-5} mol/L, final concentration) (*7*). After contraction by PhE, the strips were stimulated by EFS as described above. Any strips that failed to show less than 4% relaxation to EFS at 16 Hz were discarded to remove unsuccessful preparations. Relaxation, NO-dependent relaxation, and CO-dependent relaxation were calculated as shown in Fig. 1A.

Measurement of Endothelium-Dependent and -Independent Relaxation

The treated corpus cavernosum strips were equilibrated in Krebs solution containing guanethidine (5 μmol/L, final concentration) for 90 min. After contraction by adding PhE (10 μmol/L, final concentration) for 10 min, relaxation in response to Ach (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, or 10^{-1} mol/L, final concentration) or SNP (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, or 10^{-1} mol/L, final concentration) was determined. The relaxation induced by Ach or SNP was calculated as shown in Fig. 1B.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Using the acid guanidium isothiocyanate–phenol–chloroform method, total RNA was extracted from corpus cavernosum tissue to which EFS was not applied. Gene expression of neuronal NO synthase (nNOS) and the HO-2 gene in the corpus cavernosum of 14-week-old SHRs ($n=6$ each) was determined by RT-PCR using rat nNOS- and HO-2–specific primers, as described previously (*8*, *9*). PCR for the comparison of nNOS, HO-2 transcripts was normalized for the presence of β-actin.

NOx Levels in Corpus Cavernosum Stimulated by EFS

Levels of nitrate/nitrite (NOx) in corpus cavernosum tissues obtained from control and α-tocopherol–fed SHRs ($n=8$ each) were measured after stimulation by EFS of 16 Hz using a Nitrate/Nitrite assay kit (Cayman Chemical, Ann Arbor, USA). The NOx concentration was normalized to the weight of a strip of the corpus cavernosum and expressed as nmol/mg weight.

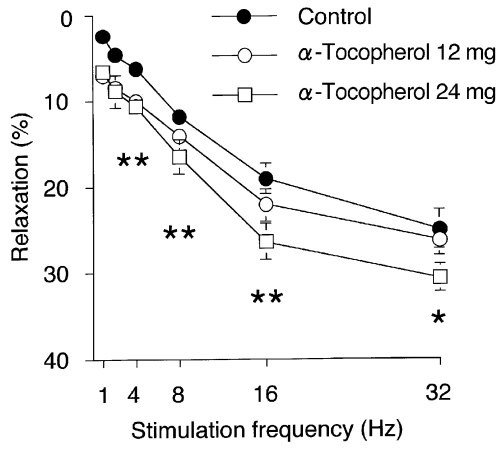

Fig. 2. *Effect of α-tocopherol on relaxation in response to EFS in corpus cavernosum of SHRs. Comparison among different doses of α-tocopherol (6.7 mg 100 g chow in controls, 12 and 24 mg/100 g chow in experimental groups, $n=10$ each). *$p<0.05$, **$p<0.01$ α-tocopherol–fed SHRs (24 mg/100 g chow) vs. controls. Values are reported as means±SEM.*

Measurement of cGMP Levels

After stimulation by EFS of 16 Hz, levels of guanosine 3′,5′-monophosphate (cGMP) in these tissues were measured by a cGMP enzyme immunoassay system (Amersham Pharmacia Biotech, Franklin Lakes, USA).

TBARS Levels and SOD Activity in Serum and Corpus Cavernosum

Thiobarbituric acid–reacting substance (TBARS) levels in the serum and corpus cavernosum were measured by the thiobarbituric acid assay method of Yagi as described previously (*10*). Superoxide dismutase (SOD) activity in serum and corpus cavernosum was measured by a modified Oyanagni assay method as described previously (*11*). TBARS levels and SOD activity in the corpus cavernosum were normalized to the weight of a strip (unit: nmol/g wet tissue or U/g wet tissue).

Statistical Analysis

Results were expressed as means±SEM. Statistical analysis was performed using paired or unpaired two-tail *t*-test or two-way analysis of variance (ANOVA). When ANOVA was used and indicated significance (at the 0.05 level), post hoc test analysis (unpaired two-tail *t*-test) was used to determine which conditions were significantly different from each other. Differences were considered statistically significant at $p<0.05$.

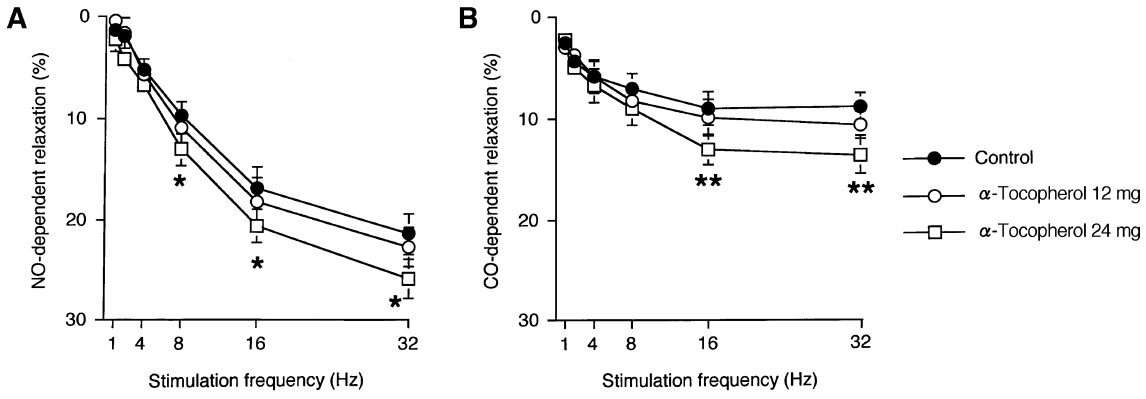

Fig. 3. *Effect of α-tocopherol on relaxation induced by neuronal NO and CO in corpus cavernosum of SHRs. Comparison is made between control SHRs and α-tocopherol–fed SHRs (12 or 24 mg/100 g chow). A: NO-dependent relaxation and B: CO-dependent relaxation is shown. *$p<0.05$, **$p<0.01$ α-tocopherol–fed SHRs (24 mg/100 g chow) vs. controls. Values are reported as means±SEM.*

Results

Treatment with α-Tocopherol Did Not Affect SBP in SHRs

SBP in SHRs fed the α-tocopherol–enriched chow diet (12 or 24 mg/100 g chow) was not different from that of controls fed the standard diet after 8 weeks (Table 1).

Relaxation of Corpus Cavernosum in Response to EFS Was Improved with α-Tocopherol Treatment in SHRs

Feeding with α-tocopherol at 24 mg/100 g chow, but not at 12 mg/100 g chow, significantly improved the impaired relaxation to EFS in the corpus cavernosum of SHRs (Fig. 2).

α-Tocopherol Treatment Augmented the Relaxation in the Corpus Cavernosum of SHRs by Neuronal NO and CO Systems

We examined the mechanisms underlying the α-tocopherol–induced improvement of erectile dysfunction in SHRs. When the corpus cavernosum was preincubated with L-NNA or ZnPP-added L-NNA, both NO-dependent relaxation (Fig. 3A) and CO-dependent relaxation (Fig. 3B) were greater in SHRs fed α-tocopherol at 24 mg/100 g chow than in control SHRs.

Expression of nNOS and HO-2 in Corpus Cavernosum of Control SHRs Was Similar to That of α-Tocopherol–Fed SHRs

Bands of an 879 bp RT-PCR product of nNOS and a 230 bp RT-PCR product of HO-2 were detected in corpus cavernosum obtained from SHRs fed the control or the α-tocopherol–enriched chow diet (24 mg/100 g chow), when EFS was not applied (Fig. 4A). Levels of nNOS and HO-2 gene expression were similar between control and α-tocopherol–fed SHRs (Fig. 4B).

α-Tocopherol Increased cGMP Levels, but Not NOx Levels, in EFS-Applied Corpus Cavernosum

When EFS was applied to the corpus cavernosum, NOx levels increased to the same extent in control and α-tocopherol–fed SHRs (Fig. 5A, $p>0.1$). However, cGMP levels in the corpus cavernosum were significantly higher in α-tocopherol–fed SHRs than in the controls (Fig. 5B, $p<0.05$).

α-Tocopherol Improved Levels of TBARS and SOD Activity in Corpus Cavernosum and Serum

When EFS was not applied to the corpus cavernosum of SHRs, levels of TBARS were lower in the corpus cavernosum or serum obtained from SHRs fed α-tocopherol at 24 mg/100 g chow than in controls (Table 2). However, although serum SOD activity was not different between the two groups, SOD activity was higher in the corpus cavernosum of SHRs fed α-tocopherol at 24 mg/100 g chow than in that of controls (Table 2).

α-Tocopherol Improved Endothelium-Dependent, but Not -Independent, Relaxation in the Corpus Cavernosum of SHRs

As illustrated in Fig. 6A, relaxation in response to Ach in the corpus cavernosum of α-tocopherol–fed SHRs at either dose (12 or 24 mg/100 g chow) was augmented in a dose-depen-

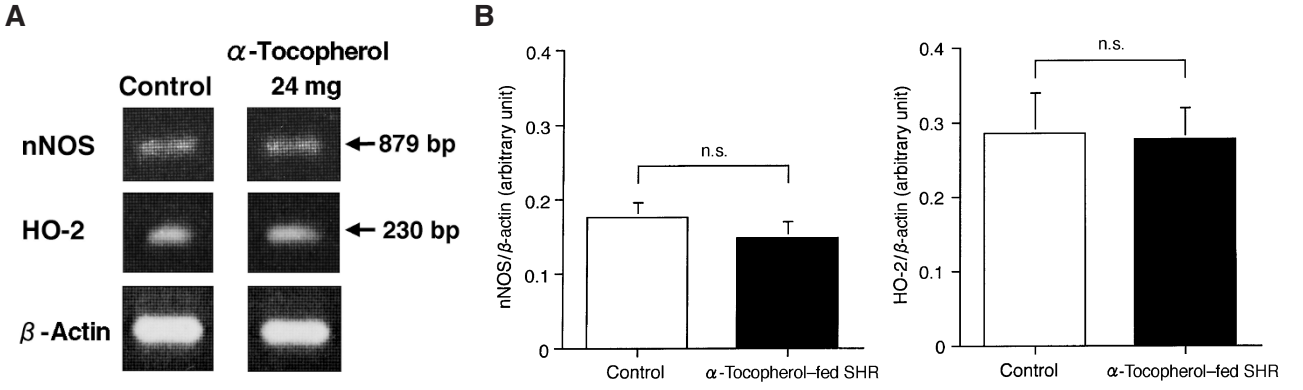

Fig. 4. *nNOS and HO-2 gene expressed in corpus cavernosum of control SHRs and α-tocopherol–fed SHRs (24 mg/100 g chow). Total RNA was extracted from corpus cavernosum of both strains, and RT-PCR was performed using specific primers for nNOS and HO-2. A: The top panel is nNOS. The middle panel is HO-2. The bottom panel is β-actin. B: Relative mRNA levels (normalized to β-actin mRNA) are shown in a graphic form.*

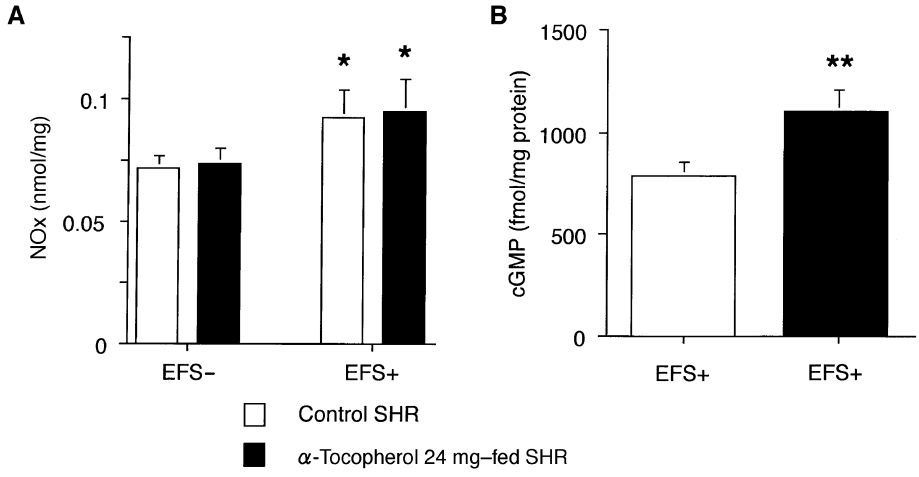

Fig. 5. *Comparison of NOx and cGMP levels in corpus cavernosum of SHRs. A: When 16 Hz EFS was applied (EFS+) or was not applied (EFS−) to corpus cavernosum of SHRs, NOx levels in homogenized tissues were measured. *$p < 0.05$: significantly different vs. EFS−. B: When 16 Hz EFS was applied (EFS+), cGMP levels in homogenized tissues were measured. **$p < 0.01$: vs. controls. Open bars, controls; closed bars, α-tocopherol–fed SHRs (24 mg/100 g chow). Values are reported as means ± SEM.*

dent manner, as compared with control SHRs. α-Tocopherol treatment did not affect relaxation in response to SNP in these tissues (Fig. 6B).

Discussion

Penile erection is impaired not only in the elderly and diabetics but also in hypertensives (*2*). We have reported that ED in hypertension may result from an impairment of neuronal relaxation, especially NO-dependent and CO-dependent relaxation, in the corpus cavernosum of SHRs (*3*). We have also indicated that inhibition of oxidative stress causes this impairment in the corpus cavernosum of SHRs (*3*). In the present study, we examined the effects of antioxidation by α-tocopherol on impaired erectile function of SHRs. Treatment with α-tocopherol dose-dependently increased EFS-induced relaxation in the corpus cavernosum of SHRs. This increase was recorded under conditions excluding Ach-induced NO release from the endothelium by atropine, indicating that neuronal relaxation of the corpus cavernosum is improved with an antioxidant. α-Tocopherol treatment improved NO-dependent relaxation and CO-dependent relaxation in response to EFS in SHRs, but did not affect nNOS gene expression or NOx levels in the corpus cavernosum. These observations suggest that α-tocopherol might improve neuronal NO action in the corpus cavernosum of SHRs, but would not increase

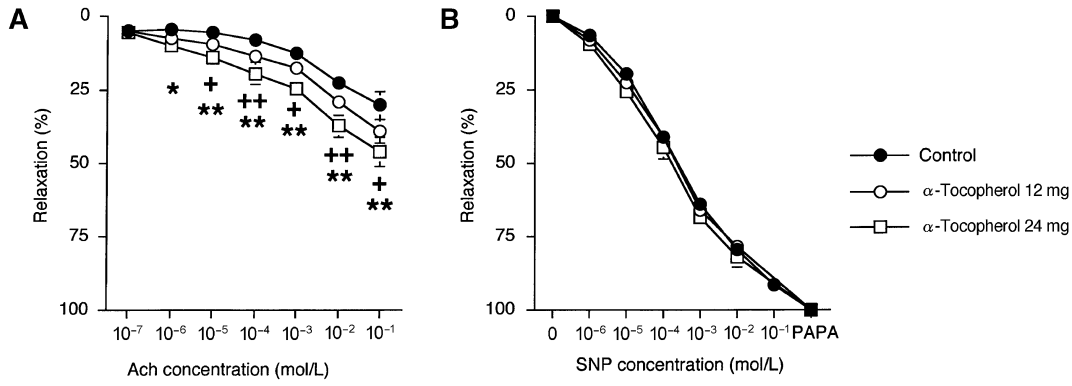

Fig. 6. *Effect of α-tocopherol treatment on endothelium-dependent or -independent relaxation in corpus cavernosum of SHRs. Dose-response curves of relaxation in corpus cavernosum of SHRs in response to acetylcholine (Ach: A) or sodium nitroprusside (SNP: B) are shown in controls and α-tocopherol–fed SHRs (12 or 24 mg/100 g chow). *$p<0.05$, **$p<0.01$ for α-tocopherol–fed SHRs (24 mg/100 g chow) vs. controls. $^{+}p<0.05$, $^{++}p<0.01$ for α-tocopherol–fed SHRs (12 mg/100 g chow) vs. controls. Values are reported as means±SEM.*

Table 2. α-Tocopherol–Induced Changes in Levels of TBARS and SOD Activity in Serum and Corpus Cavernosum of SHRs

	TBARS		SOD activity	
	Serum (nmol/mL)	Tissue (EFS−) (nmol/g wet tissue)	Serum (U/mL)	Tissue (EFS−) (U/g wet tissue)
Controls	2.23±0.11	52.7±3.1	20.1±1.4	262.1±3.3
α-Tocopherol 12 mg/100 g chow	2.18±0.11	52.0±9.4	21.0±0.5	286.0±25.2
α-Tocopherol 24 mg/100 g chow	1.53±0.10**	42.2±4.2*	19.5±0.7	338.1±26.0**

Values are mean±SEM. $n=8$, each. TBARS, thiobarbituric acid–reacting substance; SOD, superoxide dismutase; SHR, spontaneously hypertensive rat; EFS, electrical field stimulation. *$p<0.05$, **$p<0.01$ *vs*. control SHRs.

NO production or release. Compared to controls, α-tocopherol–fed SHRs showed decreased TBARS levels, increased SOD activity, and elevated cGMP response in response to EFS in the corpus cavernosum. These findings indicate that in SHRs, which are reported to have increased oxidative stress (*4*), antioxidant treatment increases NO bioavailability, thereby improving relaxation mainly attributed to neuronal NO in the corpus cavernosum. These findings are supported by reports that α-tocopherol scavenges peroxyl radicals and hydroxyl radicals but does not to react directly with NO (*12*). These reactions to α-tocopherol were independent of a blood pressure lowering effect, because α-tocopherol did not affect SBP in SHRs after 8 weeks of treatment. To our knowledge, these findings are the first of their kind.

In addition, we demonstrated that α-tocopherol improves endothelium-dependent relaxation in response to Ach, but not in response to SNP, in the corpus cavernosum of SHRs. Therefore, α-tocopherol augments the neuronal and endothelial relaxation in the corpus cavernosum of SHRs together.

Vascular iNOS expression is thought to be compensatory with HO-1 expression (*13*). In the nervous system, the relationship between the NO and CO systems is unclear. We therefore examined a neuronal relaxation factor, CO, in the corpus cavernosum of SHRs. α-Tocopherol treatment improved CO-dependent relaxation in response to EFS. These results may suggest that the relationship between the NO and CO systems of the nervous system in the corpus cavernosum is not compensatory, because treatment with α-tocopherol increases both NO- and CO-dependent relaxation together in response to EFS of SHRs. To our knowledge, these findings are the first of their kind. The increase in CO-dependent relaxation in response to EFS by α-tocopherol might not be attributable to increased CO production, because α-tocopherol did not affect HO-2 gene expression in the corpus cavernosum of SHRs. The exact mechanism by which α-tocopherol increases CO-dependent relaxation in response to EFS in the corpus cavernosum of SHRs remains to be clarified.

In summary, antioxidant treatment with α-tocopherol in SHRs improves the impairment of relaxation in the corpus cavernosum by improving neuronal NO and CO action as well as endothelial function. Antioxidant therapy may be helpful in treating ED associated with hypertension.

References

1. Burnett AL, Lowenstein CJ, Bredt DS, Chang TSK, Snyder SH: Nitric oxide. A physiologic mediator of penile erection. *Science* 1992; **257**: 401–403.
2. Feldman HA, Goldstein I, Hatzichristou DG, Krane RJ, McKinlay JB: Impotence and its medical and psychosocial correlates: results of the Massachusetts Male Aging Study. *J Urol* 1994; **151**: 54–61.
3. Ushiyama M, Morita T, Kuramochi T, Yagi S, Katayama S: Erectile dysfunction in hypertensive rats results from impairment of the relaxation evoked by neurogenic carbon monoxide and nitric oxide. *Hypertens Res* 2004; **27**: 253–261.
4. Newaz MA, Nawal NN: Effect of alpha-tocopherol on lipid peroxidation and total antioxidant status in spontaneously hypertensive rats. *Am J Hypertens* 1998; **11**: 1480–1485.
5. Maines MD, Trakshel: Differential regulation of heme oxygenase isozymes by Sn- and Zn-protoporphyrins: possible relevance to suppression of hyperbilirubinemia. *Biochim Biophys Acta* 1992; **1131**: 166–174.
6. Okamura T, Ayajiki K, Toda N: Monkey corpus cavernosum relaxation mediated by NO and other relaxing factor derived from nerves. *Am J Physiol* 1998; **274**: H1075–H1081.
7. Kim YC, Davies MG, Marson L, Hagen PO, Carson CC 3rd: Lack of effect of carbon monoxide inhibitor on relaxation induced by electrical field stimulation in corpus cavernosum. *Urol Res* 1994; **22**: 291–293.
8. EI-Skka AI, Hassan MU, Selph C, Perinchery G, Dahiya R, Lue TF: Effect of cavernous nerve freezing on protein and gene expression of nitric oxide synthase in the rat penis and pelvic ganglia. *J Urol* 1998; **160**: 2245–2252.
9. Liu H, Mount DB, Nasjletti A, Wang W: Carbon monoxide stimulates the apical 70-pS K^+ channel of the rat thick ascending limb. *J Clin Invest* 1999; **103**: 963–970.
10. Yagi K: A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem Med* 1976; **15**: 212–216.
11. Oyanagui Y: Reevaluation of assay methods and establishment of kit for superoxide dismutase. *Anal Biochem* 1984; **142**: 290–296.
12. Hogg N, Singh RJ, Goss SP, Kalyanaraman B: The reaction between nitric oxide and alpha-tocopherol: a reappraisal. *Biochem Biophys Res Commun* 1996; **224**: 696–702.
13. Cheng PY, Chen JJ, Yen MH: The expression of heme oxygenase-1 and inducible nitric oxide synthase in aorta during the development of hypertension in spontaneously hypertensive rats. *Am J Hypertens* 2004; **17**: 1127–1134.