Original Article

Erectile Dysfunction in Hypertensive Rats Results from Impairment of the Relaxation Evoked by Neurogenic Carbon Monoxide and Nitric Oxide

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Erectile dysfunction (ED) with aging and diabetes mellitus is caused by impairment of the relaxation evoked by nitric oxide (NO) of penile cavernous smooth muscles and arterioles. However, the mechanism of ED in hypertension is unknown. Carbon monoxide (CO), which is produced by heme oxygenase (HO)-2 in the neuronal system is a neurotransmitter and vasodilator. We examined the neurogenic role of CO in penile erection and the neurogenic mechanisms of ED in hypertension, using spontaneously hypertensive rats (SHR) or Wistar-Kyoto rats (WKY). The isometric tension of corpus cavernosum tissues from both strains was recorded after guanethidine and atropine treatment. Relaxation in response to electrical field stimulation (EFS) in WKY was suppressed dose-dependently by HO inhibitors both in the absence and presence of an NO synthase (NOS) inhibitor. Reverse transcription-polymerase chain reaction (RT-PCR) showed that the HO-2 gene was expressed in the corpus cavernosum. CO-saturated solution induced a concentration-dependent relaxation in WKY. The neurogenic relaxation to EFS in SHR was impaired as compared with that in WKY after the age of 5 weeks, when blood pressure began to be elevated, due to the attenuated relaxation in response to neurogenic NO and CO. In the corpus cavernosum of SHR, expression of the HO-2 and nNOS genes was similar, and NOx levels after EFS were similar to those of WKY. cGMP levels after EFS and the relaxation evoked by the NO donor was lower in SHR than WKY. Thiobarbituric acid-reacting substance (TBARS) levels were increased, and superoxide dismutase (SOD) activity was suppressed in SHR, as compared with those in WKY, suggesting that the increasing oxidative stress partially causes the impairment of NO-dependent relaxation. These findings suggest that CO regulates the relaxation evoked by EFS in the rat corpus cavernosum, and that ED in hypertension in rats results from an impairment of the relaxation induced by neurogenic CO and NO. (Hypertens Res 2004; 27: 253–261)

Key Words: hypertension, spontaneously hypertensive rat, neuronal systems, nitric oxide, carbon monoxide

Introduction

Penile erection is evoked by elevated pressure from the corpus cavernosum as a result of the relaxation of cavernous smooth muscles and arterioles. The relaxant substance has been clarified to consist mainly of nitric oxide (NO) that is liberated from neurons and endothelium in the corpus cavernosum (1). Erectile dysfunction (ED) often occurs in the elderly and in subjects with diabetes mellitus (2), and results...
from impaired relaxation of the corpus cavernosum, especially through an impairment of the NO system. Hypertension has also been thought to be a risk factor for ED (2). It has been reported that the incidence of atherosclerosis among patients with ED is approximately 40% (3), and that atherosclerotic vascular damage might be involved in the pathogenesis of hypertensive ED in animals (4). However, the neurogenic mechanisms of hypertensive ED remain to be clarified.

Like NO, carbon monoxide (CO) is a neurotransmitter (5) and a vasodilating agent (6) which is endogenously produced through heme by heme oxygenase (HO) (7). There are two isoforms of HO: HO-1 is the inducible-type and HO-2 the constitutive-type form. Previous studies have shown that HO-2 protein is localized in the major pelvic ganglion and the nerves distributing to the penis, urethra, bladder neck, vas deferens and prostate in mice (8). The neurotransmitters of these ganglions and neurons have been thought to be CO produced by HO (5). HO-2 distributes in a wide range of nervous systems in the genitourinary structure (8), but the role of CO in penile erection has not been examined.

Therefore, in this study we used spontaneously hypertensive rats (SHR) to examine whether 1) CO contributes to penile erection, and 2) relaxation in response to neurogenic CO or NO in the corpus cavernosum plays an important role in the pathogenesis of hypertensive ED.

**Methods**

These experiments were approved by the Animal Research Committee of Saitama Medical School.

**Animals**

We used male Wistar Kyoto rats/Izm (WKY) and SHR/Izm aged 5–16 weeks as a model of hypertension (Funabashi Farms, Funabashi, Japan). The animals were housed under a constant temperature of 24°C with a 12-h light/12-h dark cycle. They had free access to laboratory rat chow and tap water.

**Preparation**

After systolic blood pressure (SBP) was measured by the tail cuff method using a plethysmograph (PS-600; Riken Kaihatsu, Tokyo, Japan), rats were anesthetized by intraperitoneal injection of pentobarbital sodium (0.4 mg/kg) and killed by bleeding from the carotid arteries. The penis was rapidly removed, and the corpus cavernosum was isolated. The following examination methods were modified from those reported previously (9–11). Each strip of the corpus cavernosum (approximately 3–12 mm) was tied at each end with cotton threads and set in a Magnus chamber filled with 10 ml Krebs solution at 37°C bubbling with 95% O2 and 5% CO2 as described elsewhere (9–11). The upper end of the cotton thread of each strip was connected to an isometric force transducer (T7-15-240; Orientec Co., Saitama, Japan) linked to an amplifier (WGI-300C; Kyowa Electronic Instruments Co., Tokyo, Japan) and MacLab system (MacLab/8s; AD Instruments, Castle Hill, Australia). The initial resting tension applied to each strip was adjusted to 2 g. After strips were allowed to equilibrate for 1.5 h, Krebs solution was exchanged for a Krebs solution containing 124 mmol/l KCl. Each strip was depolarized for 5 min. The tension was allowed to return to the baseline by repeated washing for 60 min with Krebs solution containing 1 μmol/l atropine and 5 μmol/l guanethidine. After the strips were contracted by 10 μmol/l phenylephrine hydrochloride (PhE) for 10 min, they were subjected to relaxation in response to electrical field stimulation (EFS) using sequential frequencies of 1, 2, 4, 8, 16, 32 and 64 Hz delivered as 10-s trains (50 V, 0.8 ms) at 2 min intervals. This relaxation was defined as the control relaxation. Any strip that failed to show less than 4% relaxation by EFS at 16 Hz was discarded. At the end of the experiments, 100 μmol/l papaverine (PAP) was applied to attain the maximal relaxation, and the weight of each strip removed from the organ bath was measured.

**Determination of Relaxation in the Corpus Cavernosum**

The control relaxation of the corpus cavernosum to EFS was measured in WKY and SHR of different ages (5, 8, 14 and 16 weeks old, n = 10 each). After measurement of the control relaxation, the corpus cavernosa obtained from 16-week-old WKY or SHR were washed three times by Krebs solution for 30 min. Then, either 100 μl of zinc protoporphyrin-IX (ZnPP) (11) (final concentration: 10⁻⁶, 10⁻⁵ or 3 × 10⁻⁵ mol/l; n = 20 for each concentration) or tin protoporphyrin-IX (SnPP; final concentration: 3 × 10⁻⁵ mol/l; n = 10) and/or Nω-nitro-L-arginine (L-NNA) (12) (final concentration: 10⁻⁵ mol/l; n = 10) was added. Strips were equilibrated for 40 min in the dark after treatment of ZnPP or SnPP, or for 20 min after treatment of L-NNA. After contraction to PhE, the strips were stimulated by EFS in the dark. Exposure to CO was achieved by adding increasing volumes (40, 80, 160 and 320 μl) of a CO-saturated Krebs solution, which was obtained from a calcium-free Krebs solution at 37°C under bubbling with 95% CO and 5% N2 as described previously (13), or by adding a calcium-free Krebs solution as vehicle, to an organ bath containing the corpus cavernosa of 16-week-old WKY (n = 6). One microliter of a CO-saturated Krebs solution contains 30 ng of CO gas (13). To make a dose-response curve, sodium nitroprusside (SNP) at a concentration of 10⁻⁶, 10⁻⁵, 10⁻⁴, 10⁻³ and 10⁻² mol/l was applied to the corpus cavernosa of 16-week-old WKY and SHR (n = 12 each) after contraction in response to PhE.
Calculation

Three parameters were calculated to analyze the relaxation in response to EFS as shown below (Fig. 1).

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\text{Relaxation (%) = } \left( \frac{B}{A} \right) \times 100
\]

\[
\text{CO- or NO-dependent relaxation (%) = } \left( \frac{B - C}{A} \right) \times 100 \]

Percentage of CO- or NO-dependent relaxation (\%) = \( \left( \frac{B - C}{A} \right) \times 100 \)

where A is the maximum relaxation between the contraction by PhE and the relaxation by PAP, B is the relaxation in response to EFS or SNP, and C is the response to EFS in the presence of ZnPP/SnPP or L-NNA.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total RNA was extracted from a corpus cavernosum that had not been subjected to EFS using the acid guanidium isothiocyanate-phenol-chloroform method. HO gene and neuronal NO synthase (nNOS) gene expression in the corpus cavernosa from 16-week-old WKY and SHR (n = 6 each) were determined by RT-PCR using rat HO-1-, HO-2- or nNOS-specific primers, as described previously (14–16). PCR for comparison of HO-1, HO-2 or nNOS transcripts was normalized for the presence of \( \beta \)-actin.

**NO\textsubscript{x} Levels in the Corpus Cavernosum**

Levels of NO\textsubscript{x} in the corpus cavernosa of SHR (n = 8) and WKY (n = 8) were measured using a nitrate/nitrite assay kit (Cat No.780001; Cayman Chemical Co., Ann Arbor, USA) before or after stimulation by EFS at 16 Hz. Levels of NO\textsubscript{x} were normalized to the weight of a strip of the corpus cavernosum (unit: nmol/mg weight).

**Guanosine-3\textsubscript{5} monophosphate (cGMP) Levels in the Corpus Cavernosum**

Before or after stimulation by EFS at 16 Hz, levels of cGMP in these tissues obtained from SHR (n = 8) and WKY (n = 8) were measured using a cGMP enzyme immunoassay system (Amersham Pharmacia Biotech Inc., Piscataway, USA).

**Thiobarbituric Acid-Reacting Substance (TBARS) Level and Superoxide Dismutase (SOD) Activity in the Corpus Cavernosum**

Levels of TBARS in the corpus cavernosum of SHR (n = 8) and WKY (n = 8) were measured by the thiobarbituric acid assay method of Yagi, as described previously (17). SOD activity in the corpus cavernosum of SHR (n = 8) and WKY (n = 8) was measured according to the method of Oyanagui (18). The TBARS level or SOD activity was normalized to the weight of a strip of the corpus cavernosum (unit: nmol/g wet tissue or U/g wet tissue).

**Reagents**

ZnPP and SnPP were obtained from Porphyrin Products Inc. (Logan, USA). Guanethidine sulfate, atropine sulfate, PhE, PAP, L-NNA, SNP and all other reagents used were obtained from Sigma Chemical Co. (St. Louis, USA). ZnPP and SnPP were 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 (19). All other reagents were dissolved in distilled water.

**Statistical Analysis**

Results were expressed as the mean \( \pm \) SEM. Statistical analysis was performed using a paired or an unpaired two tail t-test or a one-way analysis of variance (ANOVA). When ANOVA was used and when this analysis indicated significance (at the 0.05 level), a post-hoc test (Fisher’s protected least significant difference) was used to determine which conditions were significantly different from each other. Differences of \( p < 0.05 \) were considered to be statistically significant.

**Results**

**Relaxation of the Corpus Cavernosum in Response to EFS was Suppressed by HO Inhibitors**

In all series of experiments, the relaxation of the corpus cavernosum of rats in response to EFS increased in a frequency-dependent manner up to 16 Hz, and showed a plateau at more than 16 Hz (Fig. 2A). ZnPP (HO inhibitor: \( 10^{-6} \) to \( 3 \times 10^{-5}\)mol/l) suppressed the EFS-induced relaxation in strips obtained from WKY in a concentration-dependent manner (Fig. 2A). This suppression was statistically significant over the 2 to 64 Hz EFS range. To determine the specificity of the effect of ZnPP on the EFS-induced relaxation, strips from
WKY were treated with SnPP, another HO inhibitor. SnPP decreased the relaxation by EFS significantly over the 2 to 64 Hz EFS range (Fig. 2A). Since ZnPP and SnPP have been reported to inhibit NOS (20), we blocked NOS using L-NNA in the corpus cavernosum strips of WKY. In the presence of L-NNA, ZnPP suppressed the EFS-induced relaxation significantly over the 16 to 64 Hz EFS range when compared with that observed in ZnPP-untreated strips (Fig. 2B). The suppression by ZnPP was different from that by L-NNA, as L-NNA significantly suppressed the EFS-evoked relaxation over the 2 to 64 Hz EFS range when compared with that observed in control strips (Fig. 2A).

The HO Gene Was Expressed in the Corpus Cavernosum of WKY

Bands of a 230 bp RT-PCR product, HO-2, were detected in the corpus cavernosum of WKY on an ethidium bromide-stained agarose gel (Fig. 3). However, HO-1 mRNA was not detected as a band of 512 bp RT-PCR product (Fig. 3).

CO-Saturated Solution Relaxed the Corpus Cavernosum of Rats

Cumulative application of a CO-saturated Krebs solution induced a concentration-dependent relaxation in the corpus cavernosum obtained from WKY (Fig. 4). The maximum relaxation after the final dose (320 µl) was 76.7 ± 4.3%. There was no response to control Krebs solution (n = 6, data not shown).
Relaxation in Response to EFS Was Impaired in the Corpus Cavernosum of Hypertensive SHR

SBP was significantly elevated in SHR as compared with WKY after the age of 5 weeks (WKY vs. SHR: 5 weeks, 111.0 ± 2.3 vs. 116.0 ± 6.1 mmHg; 8 weeks, 104.4 ± 1.7 vs. 141.9 ± 2.2 mmHg; 14 weeks, 117.8 ± 2.5 vs. 168.2 ± 2.9 mmHg; 16 weeks, 112.5 ± 1.6 vs. 175.6 ± 2.5 mmHg; p<0.01 except at 5 weeks). Dose-response curves of strip contraction by PhE were not different between 16-week-old SHR and WKY (data not shown). Figure 5A illustrates the relaxation in response to EFS in the corpus cavernosum of WKY and SHR at different ages. The EFS-induced relaxation in SHR was reduced as compared with that in age-matched WKY after the age of 8 weeks. This reduction was statistically significant at more than 2 Hz EFS (Fig. 5B).

CO- and NO-Dependent Relaxation, and the Percentage of CO- and NO-Dependent Relaxation Were Impaired in the Corpus Cavernosum of SHR

The levels of CO- and NO-dependent relaxation were suppressed in SHR as compared with WKY (Fig. 6A). NO-dependent relaxation was greater than CO-dependent relaxation in both strains. As shown in Fig. 6B, the percentage of NO-dependent relaxation over the 16 to 64 Hz EFS range in the corpus cavernosum of SHR was greater than that of WKY, but such a difference was not observed in the percentage of CO-dependent relaxation.

The Response of cGMP to EFS and the Relaxation in Response to an NO Donor Were Impaired in the Corpus Cavernosum of SHR

Basal cGMP levels in the corpus cavernosum were significantly lower in SHR than WKY (Fig. 7A). Although EFS resulted in a significant increase in cGMP levels in WKY, there was no increase in SHR (Fig. 7A). The dose-response curve of the relaxation in response to SNP was shifted to the right in the corpus cavernosum of SHR (Fig. 7B).

The Expression of HO-2 and nNOS in the Corpus Cavernosum of SHR Was Similar to Those of WKY

RT-PCR revealed that the HO-2 and nNOS genes in the corpus cavernosum were detected as bands of 230 and 879 bp RT-PCR products, respectively, with a similar level between WKY and SHR (Fig. 8; p>0.1).

Levels of NOx in the Corpus Cavernosum of SHR Were Similar to Those of WKY

The basal levels of NOx in SHR were similar to those in WKY (Table 1; p>0.1). EFS increased the levels of NOx in the corpus cavernosum of SHR to an extent similar to that in WKY (Table 1).

Oxidative Stress Was Increased in the Corpus Cavernosum of SHR

TBARS levels were increased (Table 1; p<0.05), and SOD activities were decreased (Table 1; p<0.05) in SHR compared with WKY, indicating an increased oxidative stress in SHR.
Discussion

Penile erection is a hemodynamic process involving relaxation of the smooth muscles of the corpus cavernosum and associated arterioles. Relaxing substances, such as NO, of the smooth muscles are synthesized in parasympathetic nerve terminals and endothelial cells lining blood vessels and lacuna spaces in the corpus cavernosum (1, 21). In the present study, we examined the relaxation evoked by EFS in the corpus cavernosum of rats. We revealed that the HO-2 gene is expressed in the rat corpus cavernosum, that relaxation of the rat corpus cavernosum in response to EFS is suppressed dose-dependently by HO inhibitors, and that dilatory response to exogenous CO is increased dose-dependently. Although a previous report demonstrated that CO is a neurotransmitter of HO containing nerves and ganglions (5), to our knowledge, this is the first report to demonstrate that neurogenic CO produced through HO-2 relaxes the corpus cavernosum of rats. On the other hand, Burnett et al. revealed that male HO-2 knockout mice have the same mounting latency and erection following electrical stimulation of cavernous nerves as wild-type mice (8). Kim et al. showed that treatment with zinc deuteroporphyrin, an HO inhibitor, did not affect relaxation by EFS in the corpus cavernosum of rabbits (11). These reports suggest that the role of CO in penile erection may have species specificity. Further studies will thus be needed to investigate the species specificity of the relaxation elicited by CO in the corpus cavernosum. It has been described that different neurotransmitters may be released from nerves in response to a change in stimulation frequencies (22). The present study also revealed that ZnPP suppressed the relaxation evoked by high EFS frequencies of more than 16 Hz, but L-NNA suppressed the relaxation evoked by EFS at lower frequencies (Fig. 2A), indicating that the frequency-dependence of the relaxation in response to CO released from nerves may be different from that to NO.

Hypertension has been thought to be a risk factor for ED (2). Vascular damage, including atherosclerosis, is reported

Fig. 6. Comparison of the relaxation in response to neurogenic CO and NO in the corpus cavernosum between SHR and WKY. A: CO- and NO-dependent relaxation in response to EFS of 1, 2, 4, 8, 16, 32 and 64 Hz in rats aged 16 weeks. Values are the means ± SEM. * p<0.05 and ** p<0.01 vs. WKY. B: The percentage of CO- and NO-dependent relaxation in response to EFS of 1, 2, 4, 8, 16, 32 and 64 Hz in rats aged 16 weeks. Values are the means ± SEM. * p<0.05 vs. WKY.
to cause hypertensive ED (3, 4). However, the pathophysiological role of the nervous system in these conditions has not been reported. We therefore examined the role of the neurological CO and NO systems in hypertensive ED using SHR. To block relaxation by acetylcholine-dependent NO release from the endothelium in response to EFS, we treated the corpus cavernosum with atropine (10, 12). In this condition, the relaxation in response to EFS in SHR above the age of 5 weeks was reduced as compared with that in the age-matched WKY. We revealed that NO- and CO-dependent relaxation of the corpus cavernosum in response to EFS was diminished in SHR as compared with WKY, indicating that hypertensive ED in rats may be caused by an impairment of neurogenic relaxation which depends on the NO and CO systems in the corpus cavernosum. Since the nNOS gene and NO levels, whether EFS was applied or not, were similar between the corpus cavernosum of SHR and that of WKY, it is suggested that the impaired relaxation in SHR may not be caused by a decrease in NO production. Relaxation of the corpus cavernosum in response to NO has been considered to be mediated through an NO-dependent increase in cGMP. In the present study, the response of cGMP to EFS in the corpus cavernosum of SHR was diminished when compared with that of WKY. In addition, the relaxing response to the NO donor was also decreased in SHR. Previous studies have demonstrated that oxidative stress is increased in hypertensive animals and humans (23, 24).

Therefore, we determined TBARS levels and SOD activity as a marker for oxidative stress. Augmented TBARS levels and diminished SOD activity were observed in the pres-
ent study, suggesting that an increased oxidative stress in SHR (23, 24) decreases the bioavailability of NO, resulting in an NO-dependent reduction of relaxation in the corpus cavernosum of SHR. Of interest is the observation that the relaxation induced by EFS was similar between SHR and WKY at the age of 5 weeks, when the blood pressure of SHR was not elevated. NO production and release from vessels of SHR were reported to be diminished along with blood pressure elevation and to be restored by hypertensive agents (25, 26). Taken together, our results suggest that hypertensive ED in SHR occurs in parallel with blood pressure elevation, leading to an increased oxidative stress.

The mechanisms of impaired CO-dependent relaxation in the corpus cavernosum of SHR remain unknown from the present study. Since the HO-2 gene expression was similar in the corpus cavernosum of SHR when compared with that of WKY, the impaired CO-dependent relaxation in SHR may result from a diminished HO-2 activity, although we did not determine HO-2 activity. Our previous study showed increased levels of HO gene expression in organs such as the aorta, left ventricle, and kidney in SHR and L-NNA-treated WKY, which had an impaired NO system (27), suggesting that the HO/CO system might take over the role of the NO system when the latter is impaired. However, in regard to hypertensive ED, crosstalk between the neurogenic NO and CO systems may not be compulsory. Such a difference may result from the different frequency-dependence for the release of CO or NO from nerves, since high frequencies of EFS suppress the relaxation induced by neurogenic CO more strongly than the relaxation induced by neurogenic NO, based on the calculated the percentage of NO-dependent relaxation (Fig. 6B).

In summary, penile erection is at least partially regulated by CO, and is impaired in SHR as the result of a decrease in not only neurogenic NO-induced relaxation but also neurogenic CO-induced relaxation in the corpus cavernosum. These results may be helpful in considering the pathogenesis and treatment of ED in hypertension.

References


