Nitric oxide is involved in the inhibitory neurotransmission and endothelium-dependent relaxations of human small penile arteries

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(Received 5 July/24 October 1996; accepted 28 November 1996)

INTRODUCTION
Dilatation of the cavernosal and helicine arteries with the resultant increase in penile blood flow and filling of the cavernous sinusoids is considered as a primary haemodynamic event in erection [1, 2]. However, the mechanisms of penile smooth muscle relaxation have been mainly investigated in isolated strips of corpus cavernosum, where the inhibitory neurotransmission leading to relaxation was shown to be atropine resistant [3, 4], and mediated by nitric oxide (NO) both in rabbits and man [5–8]. Moreover, studies *in vivo* have demonstrated that infusion of L-arginine analogues, which block NO synthase, were able to inhibit the tumescence and erection induced by nerve stimulation in several animal species [9–11]. Histochemical and immunocytochemical studies confirmed the presence of a nitrogic innervation to the trabecular smooth muscle of the corpus cavernosum in rat, rabbit and man.

Key words: electrical field stimulation, endothelium, nerves, nitric oxide, NG-nitro-L-arginine, small penile arteries.

Abbreviations: ACh, acetylcholine; EFS, electrical field stimulation; KPSS, potassium-rich physiological salt solution; NO, nitric oxide; L-NOARG, NG-nitro-L-arginine; PhE, phenylephrine; PSS, physiological salt solution; SNP, sodium nitroprusside.

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[12–16], and these studies evidenced numerous NO-containing nerves around penile small arteries or helicine arteries [12–14], which control the blood flow between the large deep penile arteries and the cavernous sinusoids [2]. An inhibitory non-adrenergic non-cholinergic neurotransmission was found earlier in bovine large dorsal penile arteries [17], and later shown to be inhibited in the presence of NO synthase inhibitors and haemolysate [18, 19]. In addition, we have recently reported that the inhibitory neurotransmission of intracavernous small penile arteries from horses is mediated by NO [20]. Although alterations in penile blood flow are thought to play an important role in impotence, information concerning the penile arterial circulation is scarce and has mostly concentrated on characterization of adrenergic and cholinergic receptors in large penile arteries (see [2]). Therefore, the aim of the present study was to investigate whether NO plays a role in the nerve-evoked relaxation of human small penile arteries, the helicine arteries, which are the terminal branches of deep penile arteries and dilate to produce penile erection.

NO is believed to account for the biological effects of the endothelium-derived relaxing factor [5] and NO synthase activity has recently been demonstrated in endothelial cells of helicine arteries and corpus cavernosum [12, 16]. Therefore, the role of endothelium-derived NO was also investigated in the present study by the use of a potent endothelium-dependent vasodilator such as acetylcholine (ACh).

METHODS

Tissue

Specimens of human corpus cavernosum were obtained from six different patients (19 or 47–72 years old). One of them was an organ donor who died due to a traffic accident, and the other five were impotent patients who had a prosthesis inserted. Three of these impotent patients had diabetes, one patient had diabetes and hypertension, and the last patient was impotent due to veno-occlusive dysfunction. Tissues were transported to the laboratory in ice-cold (4°C) physiological salt solution (PSS, see composition below). The procedure for obtaining erectile tissue was approved by the Ethics Committee, Hospital Clínico de San Carlos, Madrid, Spain, and prior consent was obtained from the patients, and in the case of the organ donor consent was obtained from the relatives.

Preparation of tissue for mechanical recording

Penile small arteries, helicine arteries (lumen diameter 200–700 μm), which are the terminal branches of deep penile arteries, were isolated from the corpus cavernosum by dissection. The adhering cavernous tissue was carefully removed and arterial rings segments with a length of 2 mm were transferred to thermostatically controlled (37°C) 5 ml organ baths or microvascular myographs containing PSS bubbled with a mixture of 5% CO2 in O2 [20, 21]. In the former, segments were mounted on two L-shaped wires, while in the microvascular myographs the arteries were mounted on two 40 μm wires. One wire was connected to a displacement unit and the other to an isometric transducer permitting recording of tension on a Grass polygraph. The preparations were successively stretched and stimulated with 10⁻⁶ mol/l phenylephrine (PhE) until maximum tension and a stable contraction was obtained. The endothelium was mechanically removed in a few preparations by introducing a horse hair or a wire into the lumen and then rubbing back and forth several times as described earlier [20]. The presence or absence of the endothelium was taken as the presence or absence of relaxation to ACh (10⁻⁵ mol/l) in PhE-contracted preparations before they were incubated with atropine. Guanethidine (10⁻⁵ mol/l) and atropine (10⁻⁷ mol/l) were present throughout the experiments with electrical field stimulation (EFS) to inhibit adrenergic and cholinergic neurotransmission, respectively.

EFS

EFS was performed through two electrodes mounted parallel to the vessel segment using a Cibertec stimulator (Barcelona, Spain) with constant current output. Square pulses of 0.3 ms duration in 20 s trains with varying frequency (0.5–32 Hz) were applied with 3–4 min intervals. A first frequency–response curve in PhE-contracted preparations was obtained. The preparations were washed, and then incubated either with NG-nitro-L-arginine (L-NOARG), L-arginine or tetrodotoxin. Finally, relaxation curves to sodium nitroprusside (SNP) were obtained and a maximum relaxation was induced with 10⁻⁴ mol/l papaverine.

Responses to ACh

Relaxations to ACh (10⁻⁹ to 10⁻⁵ mol/l) were examined in penile small arteries contracted with the thromboxane analogue, U46619 (3 × 10⁻⁶ mol/l), and in strips of corpus cavernosum mounted in 5 ml baths and contracted with PhE (10⁻⁶ mol/l). After construction of a first concentration–response curve the preparations were washed several times, and after a 1 h equilibration period they were incubated with L-NOARG (10⁻⁴ mol/l) for 30 min and a second concentration–response curve to ACh was obtained.

Solutions and drugs

PSS was of the following composition (mmol/l): NaCl, 119; NaHCO₃, 25; KCl, 4.7; CaCl₂, 1.5;
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MgSO₄, 1.18; KH₂PO₄, 1.17; EDTA, 0.026; glucose, 11. Potassium-rich PSS (KPSS) was equivalent to PSS but with NaCl exchanged with KCl on an equimolar basis.

The following drugs were applied in the present study: acetylcholine hydrochloride, atropine sulphate, guanethidine sulphate, L-arginine hydrochloride, SNP, tetrodotoxin and 9,11-methanoepoxy prostaglandin H₂ (U46619) from Sigma, St. Louis, MO, U.S.A., all dissolved in twice-distilled water. Stock solutions were prepared and stored at −20°C and further fresh dilutions were prepared daily. Stock solutions of guanethidine, atropine and PhE were prepared for each experiment.

Data and statistical analysis

Relaxant responses to EFS, SNP and ACh are expressed as a percentage of the contractile response to PhE or U46619. Sensitivity is expressed in terms of EF₅₀ and EC₅₀, which are the frequency and concentration causing half-maximal relaxation, respectively. pDᵢ₀ = −log(ECᵢ₀).

The results are expressed as means ± SEM, where n and N indicate, respectively, the number of preparations and patients studied in each set of experiments. Statistical evaluations were performed using the number of patients and when more than one vessel per patient was examined, an average value was taken. The frequency- or concentration-response curves before and after treatment were compared referring to number of patients by analysis of variance for repeated measures, and by paired t-test for comparisons of individual concentrations or frequencies. When multiple comparisons were performed with a single control, values were evaluated according to one-way analysis of variance and Bonferroni method as an a posteriori test [22]. Probability levels below 5% were considered significant.

RESULTS

Effect of L-NOARG on EFS

PhE (10⁻⁶ mol/l) induced a stable contraction of human small penile arteries of 5.2 ± 1.0 Nm⁻¹ (n = 10, N = 6), representing 64.9 ± 9.8% of the contractions to 123.7 mmol/l KPSS (8.7 ± 2.2 Nm⁻¹, n = 6, N = 6). In the presence of guanethidine and atropine, EFS (20 s trains with square pulse of 0.3 ms, 0.5–10 Hz) evoked frequency-dependent relaxations of small penile arteries contracted to PhE (Fig. 1a). The relaxations peaked at 15 s with an EF₅₀ of 1.4 ± 0.4 Hz (n = 10, N = 6) and a maximum relaxation was obtained at 32 Hz and averaged 63.3 ± 7.0% of the tone induced by 10⁻⁶ mol/l PhE. EFS induced comparable relaxations in endothelium-intact and -denuded preparations (Fig. 2). The absence of the endothelium was confirmed by a functional test before the addition of atropine. Thus, ACh (10⁻⁵ mol/l) relaxed endothelium-intact preparations precontracted with PhE, whereas this relaxation was reduced in preparations where the endothelium was mechanically removed (Fig. 2). The relaxations to EFS were reproducible in a second frequency–response curve.

In endothelium-intact human small penile arteries, L-NOARG (3 × 10⁻³ mol/l) increased the basal tension and the concentration of PhE was reduced to 5 × 10⁻⁷ mol/l to obtain contractions (5.1 ± 1.1 Nm⁻¹, N = 6) comparable to those of the control curves. The presence of L-NOARG inhibited the relaxations to EFS at the lowest frequencies, while slow-developing relaxations peaking 1 min after the onset of EFS were still observed at 16 and 32 Hz (Figs. 1b and 3). L-Arginine (3 × 10⁻³ mol/l) alone had no effect on the relaxations to EFS but significantly reversed the inhibition induced by L-NOARG.
Fig. 2. Effect of endothelial cell removal on EFS in human small penile arteries. 0, Endothelium-intact preparations; 0, endothelium-denuded preparations. ACh (10⁻⁶ mol/l) caused relaxations in endothelium-intact (+E), but not in endothelium-denuded (-E) vessels (see inset). The preparations were treated with 10⁻⁵ mol/l guanethidine and 10⁻⁷ mol/l atropine throughout the experiment. Relaxations are expressed as a percentage of the tension obtained by PhE (10⁻⁴ mol/l), and each point represents the mean ± SEM of four preparations from four patients.

Effect of L-NOARG on relaxations to ACh

In small penile arteries contracted to U46619, ACh (10⁻⁹ to 10⁻⁵ mol/l) induced concentration-dependent relaxations with a pD₂ of 7.31±0.18 and maximum relaxations of 82.8±6.1% (n=10, N=5). Incubation with L-NOARG induced significant inhibition of the relaxations to ACh in penile arteries. This inhibition of the relaxations by L-NOARG was most pronounced at high concentrations of ACh (Figs. 4a and 5a). pD₂ values and maximum relaxations were 7.72±0.21 and 75.5±14.6%, and 6.73±0.78 and 56.2±15.8% (P<0.01, paired t-test, N=4) in the absence and presence of L-NOARG, respectively.

ACh also induced potent relaxations of PhE-contracted strips of the corpus cavernosum, and these relaxations were markedly inhibited at all concentrations of ACh in the presence of the NO synthase inhibitor, L-NOARG (Figs. 4b, 5b). Thus, the pD₂-values and maximum relaxations were 7.26±0.47 and 76.3±7.5% in the absence, and 5.96±0.35 (P<0.05, paired t-test, N=4) and 44.7±9.3% (P<0.001, paired t-test, N=4) in the presence of 10⁻⁴ mol/l L-NOARG, respectively.

DISCUSSION

The finding that neurogenic relaxations to EFS in human small penile arteries are markedly reduced by the inhibitor of NO synthase, L-NOARG, provides functional evidence that NO derived from nerves can mediate these responses and thus contribute to the initial arterial dilatation observed in penile erection.

Nerve-derived nitric oxide

The present experiments were performed in the presence of guanethidine and atropine to obtain non-adrenergic non-cholinergic conditions. Thus, the neurogenic relaxations in human small penile arteries can be attributed to a non-adrenergic non-
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In the rat penis both the nerves and the endothelium of the small penile arteries displayed immunoreactivity for NO synthase [16]. However, in the present experiments mechanical endothelial cell removal did not affect the relaxations to EFS, indicating that the non-adrenergic non-cholinergic transmitter released by EFS is derived from the nerves and acts directly on the smooth muscle cells.

Immunocytochemical localization of NO synthase indicated the presence of nitrergic nerves in human small penile arteries [13]. In the present study, the inhibition of NO synthesis from L-arginine by means of L-NOARG markedly reduced the electrically induced relaxations in human small penile arteries and this inhibition was reversed in the presence of L-arginine. These results indicate that the arteries receive a functional nitrergic innervation, which confirms our recent findings in intracavernous arteries of the horse [20]. In clinical studies, injection of NO donors such as nitroglycerine, linsidomine chloride and SNP induced tumescence or erections in man [23-25]. The present finding that SNP induces potent relaxations in small penile arteries supports earlier observations showing relaxation to linsidomine chloride in human isolated cavernous arteries [26], and indicates that NO donors have a direct effect on the human small penile arteries.

In the present study, treatment with a high concentration of L-NOARG, which causes maximum inhibition of the NO/L-arginine pathway in horse penile arteries [20], did not completely abolish the responses to EFS in the human arteries. The residual relaxations were slow in onset and sensitive to tetrodotoxin indicating a neurogenic origin. The present results indicate the release of a non-adrenergic non-cholinergic inhibitory neurotransmitter different from NO, and probably of peptidergic nature, since the relaxations were resistant to L-NOARG preferentially at high frequencies of stimulation. In fact, immunocytochemical and in vivo studies have suggested several mediators of the vasodilatation associated with penile erection such as vasoactive intestinal polypeptide, calcitonin gene-related peptide, substance P and helospectin [2, 15, 26, 27], and further studies in animal models must clarify the nature of the transmitter released at high-frequency stimulation.

Fig. 4. Endothelium-dependent relaxations to ACh in the absence (left panel) and presence (right panel) of 10^{-4} mol/l L-NOARG in (a) a human small penile artery with a lumen diameter of 305 \mu m contracted to the thromboxane analogue, U46619 (3 \times 10^{-8} mol/l), and (b) trabecular smooth muscle contracted to PhE (10^{-6} mol/l). The preparations were isolated from the corpus cavernosum of the same patient. L-NOARG abolished the relaxations to ACh in the trabecular smooth muscle, while considerable residual relaxations were still observed in the penile artery. In the corpus cavernosum strips the highest dose of 10^{-4} mol/l ACh induced relaxation often followed by oscillations (b). Arrows indicate the frequencies or concentrations in log units applied, while the vertical bar shows force (mN) and horizontal bar time in min, and W indicates washout.
Endothelium-derived nitric oxide

The vasodilator ACh induced potent endothelium-dependent relaxations (see Fig. 2) of the small penile arteries and these relaxations were partially inhibited by the NO synthase inhibitor L-NOARG, indicating the release of endothelium-derived NO. In addition to NO, several L-NOARG-resistant factors have been characterized in studies of small arteries from different animals, and an endothelium-derived hyperpolarizing factor has been proposed to mediate the relaxations to ACh [28]. The inhibitory effect of L-NOARG in small penile arteries was less pronounced than expected from earlier studies performed in strips of human corpus cavernosum, where the relaxations to ACh were markedly abolished in the presence of other L-arginine analogues inhibiting NO synthase [29, 30]. In the present study, control experiments performed in preparations of corpus cavernosum from the same patients confirmed the latter studies and indicated a difference in the relative contribution of endothelial NO to the relaxations elicited by ACh in the penile small arteries and cavernous sinusoids, respectively. Thus, in the human small penile arteries, relaxations to ACh seem to involve both NO and a L-NOARG-resistant factor. These results agree with a recent study in human subcutaneous arteries [31], where ACh was also found to produce relaxation through release of NO and a factor resistant to inhibition of NO synthase. Differences between the pharmacological reactivity of corpus cavernosum strips and large deep penile arteries of man have been reported previously [32], and the present experiments provide evidence for functional differences in the endothelium of the corpus cavernosum and small penile arteries.

Pathophysiological implications

The L-arginine/NO-pathway has earlier been shown to be impaired in preparations of corpus cavernosum from diabetic men and hypercholesterolaemic rabbits [33, 34]. Most of the small penile arteries examined in the present study were isolated from impotent men who had a prosthesis inserted. The neurogenic responses in these arteries were quite similar in sensitivity and magnitude to those observed in normal tissue from horses [20]. However, the present study does not allow any conclusions to be drawn regarding the influence of disease on functionality of the arteries examined. Common risk factors associated with arterial insufficiency and erectile dysfunction include hypertension, hyperlipidaemia, cigarette smoking and diabetes mellitus [35]. Thus, further studies of human small penile arteries must show whether the neurogenic and endothelium-dependent responses are affected in these disease states. Moreover, development of new drugs for treatment of impotence should take into consideration their specific action on penile arterial smooth muscle, since there might be differences compared with the cavernous smooth muscle.

In summary, the present study suggests that relaxations of human deep penile arteries induced by non-adrenergic non-cholinergic nerve stimulation partially involve NO and also another inhibitory transmitter causing relaxations resistant to NO synthase blockade. In addition endothelium-dependent
relaxations in human deep penile arteries are mediated by both NO and a l-NOARG-resistant factor.

ACKNOWLEDGMENTS

This work was supported by project no. PM 92-0031 DGICYT and PR188/92-4163 UCM. U.S. was supported by a predoctoral grant from Universidad Complutense de Madrid. I.S.D.T. was supported by grants from the National Institute of Health (Rol-DK-39080 and DK-40487). The Spanish laboratory is part of the concerted action of the European Working Party on Resistance Artery Disease (EURAD).

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