

Experimental Evidence for Endothelium Dependent Relaxation and Neuronal Nitric Oxide in Corpus Cavernosum

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It is known that penile erection is mediated primarily through the release of a nonadrenergic noncholinergic (NANC) neurotransmitter which has been recently identified as nitric oxide (NO). To evaluate whether the endothelium is involved in neurally mediated relaxation in corpus cavernosum, we determined electrical field stimulation (EFS) induced relaxation in both the presence and absence of endothelium, and we tested the effect of an inhibitor of NO synthase, N^G-nitro-L-arginine (NOARG), in the absence of endothelium to examine if de-endothelialized tissue can still generate NO. Isolated corpus cavernosal strips from New Zealand White rabbits were used for isometric tension study using organ chambers. The endothelium was removed through denuding tissue. After the tissue was contracted with norepinephrine, EFS was performed at frequencies of 5, 15 and 40 Hz in the presence of guanethidine and atropine to evaluate NANC-selective neural relaxation. The relaxation induced by EFS was observed after preincubation with NOARG (10^{-4} M) for 30 minutes. L-arginine (10^{-3} M) was then added for 30 minutes in the presence of NOARG before a second set of EFS studies were performed. Following norepinephrine precontraction, EFS relaxed corporal strips in both the intact and de-endothelialized strips. However, deendothelialization significantly impaired EFS induced relaxation ($p < 0.05$). NOARG attenuated relaxation induced by EFS and the addition of L-arginine reversed the inhibitory effect of NOARG in the strips with endothelium. In the strips without endothelium, NOARG still inhibited EFS induced relaxation. This relaxation was reversed by the addition of L-arginine. From these results, NO may be released by the neurons of corpus cavernosum and NANC neurally mediated relaxation of corpus cavernosum has an endothelium dependent component. Therefore, the impairment in the function of endothelium in the penile sinusoidal spaces could cause an insufficient cavernosal relaxation and produce an erectile dysfunction.

Key Words: Impotence, Endothelium, Nitric oxide, Corpus cavernosum

The endothelium is a confluent monolayer of thin, flattened, rhomboid-shaped cells lining the

intimal surface of blood vessels and its importance in all aspects of cardiovascular physiology and homeostasis has been realized (Rubanyi, ed. 1991; Ryan and Rubanyi, eds. 1992; Davies and Hagen, 1993). The recent discovery that endothelium-derived relaxing factor (EDRF) is nitric oxide (NO) and the role of NO in mammalian physiology has placed further emphasis on the endothelium (Ignarro, 1992; Moncada and Palmer, 1992). In penile physiology, the endothelium in the sinusoidal spaces has been shown to have an important role in the relaxation of corpus cavernosum, a crucial event for penile erection (Saenz de Tejada *et al.* 1988; Kim *et al.* 1991;

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Azadzoi *et al.* 1992).

NO, which is a free radical gas, has been considered a principal messenger molecule which modulates smooth muscle function and serves as a neurotransmitter in both the central and peripheral nervous system. NO elevates cyclic GMP by binding to the iron of the heme molecule at the active site of soluble guanylyl cyclase. This results in smooth muscle relaxation (Moncada *et al.* 1991). In the penis, NO has recently been identified as a primary non-adrenergic noncholinergic (NANC) neurotransmitter mediating the relaxation of corpus cavernosal smooth muscle (Burnett, *et al.* 1992; Rajfer *et al.* 1992). Two possible sources of NO can be suggested in the corpus cavernosum: one is the endothelium and the second is neurons innervating in corpus cavernosum. If the endothelium has a role in penile erection and NO is generated by the endothelium, the relaxation of corpora cavernosa should be impaired after de-endothelialization. To test this hypothesis, we determined electrical field stimulation (EFS) induced relaxation in both the presence and absence of endothelium to evaluate whether the endothelium is involved in neurally mediated relaxation in corpus cavernosum. In addition, the effect of an inhibitor of NO synthase, N^G-nitro-L-arginine (NOARG) 10^{-4} M, on EFS induced relaxation was determined in both endothelialized and de-endothelialized tissues to evaluate if there was NO generation in the corpus cavernosum after endothelial removal.

MATERIAL AND METHODS

Preparation of tissue strip

Male New Zealand White rabbits weighing 2~2.5 kg were used. Cavernosal tissue procurement was performed under general anesthesia induced with xylazine, 30 mg/kg SQ (Rompun, Mobay Corp., shawnee, KS) and ketamine hydrochloride, 50 mg/kg SQ (Bristol Laboratories, Syracuse, NY). The penis was excised en block and placed in warm Krebs solution while the corpora cavernosa were sharply dissected from the tunica albuginea and sectioned transversely producing two strips (approximately $0.2 \times 0.2 \times 0.4$ cm) from each corpus. Sutures (4-0

silk) were ticed to each end of the strip for mounting in the tissue baths. The rabbit was then sacrificed with an intravenous overdose of sodium phenobarbital (30 mg/kg). Care was taken throughout the procedure to minimize tissue manipulation. Animal care complied with the "Principles of Laboratory Animal Care" as formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animal" issued by the National Institute of Health (U.S. Department of Health and Services, NIH publication no. 80-23, revised 1985). Each cavernosal strip was placed in a 25 ml tissue bath (Kent Scientific Corp., Litchfield, CT) tying one end to a tissue holder and the other to a force transducer (FTO3, Grass Instruments, Quincy, MA) for determination of isometric tension. The tissue was suspended in Krebs physiologic salt solution (PSS) at 37°C oxygenated with 95% O₂ at pH7.4. The millimolar composition of the PSS was NaCl 122, KCl 4.7, MgCl₂ 2.5, NaHCO₃ 15.4, KH₂PO₄ 1.2, glucose 5.5.

The removal of endothelium was achieved through mechanically denuding corporal tissue of endothelium by gentle rubbing between two gloved fingers for 30 seconds. The adequacy of de-endothelialization was examined by assessing the relaxation of acetylcholine on precontracted cavernosal tissues. If acetylcholine response was present, strips were rubbed repeatedly for 15 seconds until there was no acetylcholine mediated response.

Isometric tension studies

Optimal tension determination: After suspension in the tissue chambers, the tension applied to the tissue was periodically adjusted until the tissue equilibrated at 0.5 g (usually 1~2 hours). After equilibration, the resting tension was adjusted in 0.5 g increments and the maximal response to a modified oxygenated Krebs solution containing 60 mM KCl, 66.7 mM NaCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 15.4 mM NaHCO₃, 1.2 mM KH₂PO₄ and 5.5 mM glucose was measured at each resting tension to obtain a length-tension relationship. Optimal preload tension was defined as the level of preload after which a further increase in tension failed to generate an increase in active tension (total tension minus

resting tension) of at least 10%. All subsequent testing was then performed at the determined optimal resting tension for each strip. Tension was monitored with a four channel polygraph (Grass 7D, Grass Instruments, Quincy, MA).

EFS induced relaxation: After the tissue was contracted with norepinephrine (2×10^{-5} M), EFS was performed with 10 volt square wave of 0.5 msec duration in 10 second trains at frequencies of 5, 15, 40 Hz. The electrical field was generated using an electrical stimulator/generator (SD-9, Grass Instrument) connected to a current amplifier/Splitter (Stimu-Splitter II, Med-Lab Instrument, Loveland, CO). Relaxation was transient and allowed to resolve before the next frequency was tested. Response to the three frequencies was measured in the presence of guanethidine (5×10^{-6} M, 20 minutes of preincubation) and atropine (5×10^{-6} M, 20 minutes of preincubation) to evaluate NANC-selective neural relaxation. The relaxation induced by EFS was determined after preincubation with NOARG (10^{-4} M) for 30 minutes. L-arginine (10^{-3} M) was then added for 30 minutes in the presence of NOARG before a second set of EFS studies were performed.

All compounds were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in distilled water.

Statistical analysis

Relaxation in response to EFS are expressed as a percentage of active tension generated by norepinephrine at the optimal tension. Data are expressed as means \pm S.E.M with "n" representing the number of animals from which strips were obtained. The tissue response to EFS, in the presence and absence of endothelium were compared by two way analysis of variance with repeated measures and with Dunnett's post-hoc analysis for individual comparisons to control maintaining an experimentwise alpha level < 0.05 . Statistical significance was considered when $p < 0.05$.

RESULTS

EFS induced relaxation in the presence and absence of endothelium

Following norepinephrine (2×10^{-5} M) precon-

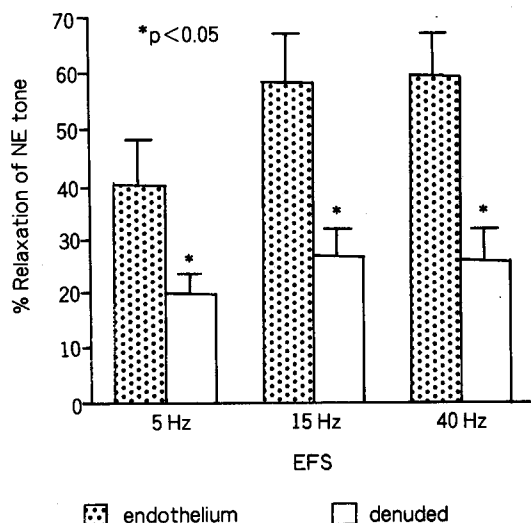


Fig. 1. Following norepinephrine (NE) precontraction, electrical field stimulation (EFS) at three frequencies (5, 15 and 40 Hz with 10V square waves of 0.5 msec duration in 10 sec trains) in the presence (endothelium) and absence (denuded) of endothelium. Relaxation induced by EFS was significantly attenuated at the three frequencies tested in the endothelium-denuded endothelium.

traction, EFS relaxed corporal tissue in both the endothelialized and the de-endothelialized strips. EFS produced relaxation of 40 ± 8 , 58 ± 9 and 59 ± 8 of norepinephrine precontraction at 5, 15 and 40 Hz in the presence of endothelium, respectively. In contrast, de-endothelialization significantly impaired EFS induced relaxation ($p < 0.05$): 20 ± 4 , 27 ± 5 and 26 ± 6 % of norepinephrine precontraction at 5, 15 and 40 Hz in the absence of endothelium, respectively ($n=8$, Fig 1).

The inhibitory effect of NOARG in EFS induced relaxation

NOARG attenuated relaxation induced by EFS in the strips with intact endothelium (40 ± 8 vs 15 ± 2 % of norepinephrine precontraction at 5 Hz, 58 ± 9 vs 24 ± 4 % at 15 Hz and 59 ± 8 vs 26 ± 6 % at 40 Hz. The addition of L-arginine reversed the inhibitory effect of NOARG (15 ± 2 vs 39 ± 5 % of norepinephrine precontraction at 5 Hz, 24 ± 4

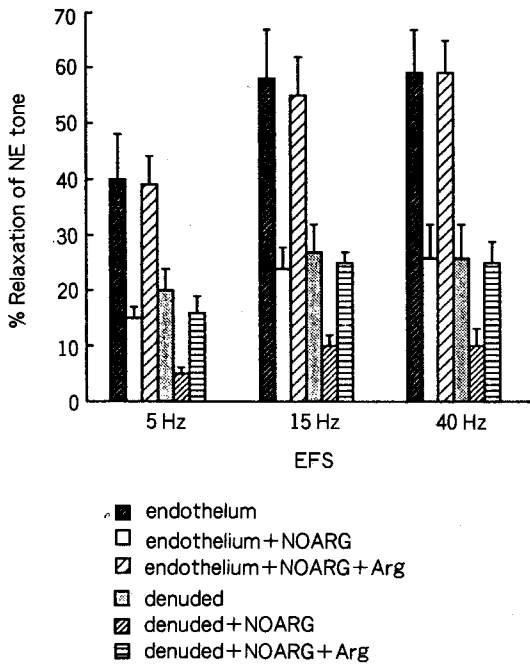


Fig. 2. The effect of the N^G -nitro-L-arginine (NOARG, an inhibitor of nitric oxide synthase, $10^{-4}M$) and L-arginine (Arg, $10^{-3}M$) on electrical field stimulation (EFS) induced relaxation. NOARG attenuated relaxation induced by EFS (endothelium+NOARG) and the addition of L-arginine reversed the inhibitory effect of NOARG (endothelium+NOARG+Arg) in the presence of endothelium (endothelium). In those strips without endothelium (denuded), EFS induced relaxation of precontracted tissue by norepinephrine (NE) was still inhibited by NOARG (denuded+NOARG) and reversed by the addition of L-arginine (denuded+NOARG+Arg) at three frequencies (5, 15, and 40 Hz with 10V square waves of 0.5 msec duration in 10 sec trains).

vs $55 \pm 7\%$ at 15 Hz and 26 ± 6 vs $59 \pm 6\%$ at 40 Hz). In the strips without endothelium, NOARG still inhibited EFS induced relaxation which was reversed by the addition of L-arginine: 20 ± 4 , 5 ± 1 and $16 \pm 3\%$ of norepinephrine precontraction at 5 Hz; 27 ± 5 , 10 ± 2 and $25 \pm 2\%$ at 15 Hz; 26 ± 6 , 10 ± 3 and $25 \pm 4\%$ at 40 Hz in the control, pretreatment of NOARG and the addition of L-arginine, respectively ($n=8$, Fig. 2).

DISCUSSION

Nitroglycerin and the organic nitrates have been used chronically to treat angina pectoris since the late 1800s. However, their mechanisms of action on a molecular basis were not clarified until 1970s. These compounds produce relaxation of blood vessels when they are metabolized to NO. NO, which has been known as an air pollutant, is now believed to be one of the most important messenger molecule in vessels, white blood cells and within the brain (Moncada *et al.* 1991; Bredt and Snyder, 1992). It has also been shown to have a crucial role in smooth muscle relaxation required for penile erection (Rajfer *et al.* 1992).

The relaxation of sinusoidal corpus cavernosum is controlled locally by three neuroeffector systems. These mechanisms include cholinergic, adrenergic and NANC pathways. Acetylcholine has been considered as the main neurotransmitter involving penile erection. However, intracavernosal injection of acetylcholine has failed to produce erection in vivo and EFS has been shown to relax the corpus cavernosum in the presence of atropine and guanethidine, suggesting that NANC neurotransmission may be a more predominant neural pathway (Bowman and Gillespie, 1983). Many substances have been suggested as modulators inducing relaxation through the NANC neurotransmitter system. Recently, NO has been identified as a major neurotransmitter modulating the relaxation of corpus cavernosum in the penis (Burnett *et al.*, 1992; Rajfer *et al.* 1992). Since NOARG, an inhibitor of nitric oxide synthase, attenuated EFS induced (neurally mediated) relaxation in the corpus cavernosum and L-arginine reversed the inhibitory action of NOARG in the current study, NO or NO containing molecule(s) most likely are mediators in penile erection.

The endothelium is a layer of cells which line the intimal surface of all blood vessels. After Furchgott and Zawadzki (1980) demonstrated the phenomenon that the relaxation of blood vessels caused by acetylcholine no longer occurred when the endothelium was removed from the vessel, a new era on the function of en-

endothelium was opened. The endothelium plays an important role in smooth muscle and platelet functions in the vessels, producing relaxing and constricting factors. NO or closely related derivative was proposed to account for the action of EDRF. Eventually, it has been demonstrated that NO can be synthesized from L-arginine by porcine aortic cells in culture and EDRF was found to be identical to NO (Palmer *et al.* 1988; Ignarro, 1992; Moncada and Palmer, 1992). Endothelium has an important role in penile erection as well. An intact endothelium is required in the relaxation of strips of corpus cavernosum induced by exogenous acetylcholine in humans (Saenz de Tejada *et al.* 1988) and Azadzi *et al.* (1992) demonstrated endothelium-dependent relaxation of corporal smooth muscle in the rabbit. We observed that EFS induced relaxation was significantly attenuated in the absence of endothelium, suggesting that NANC neurally mediated relaxation of corpus cavernosum is endothelium dependent. Therefore, the impairment in the function of the endothelium in the penile sinusoidal spaces could cause an insufficient cavernosal relaxation and produced erectile dysfunction.

In addition to the roles of NO in blood vessels and white blood cell, NO has been proven to serve as a messenger molecule in neuron (Bredt and Snyder, 1992). In synaptic function, NO synthase needs Ca^{++} and calmodulin to convert arginine to citrulline. NO synthase has been shown to be present in neuron within the body including penis. However, the content of NO synthase is different in the various organs (Snyder and Bredt, 1992). In the penis, Kim *et al.* (1991) suggested that a diffusible NO-like factor is also released by the penile autonomic nerves and Burnett *et al.* (1992, 1993) demonstrated that NO synthase can be localized to the penile neuron innervating the corpora cavernosa and to the neural plexuses in the adventitial layer of deep penile arteries in the rat and human. Thus, the results in the present study suggests that NO may be released in the neurons of corpus cavernosum, because NOARG inhibits EFS induced-NANC relaxation in the endothelium-denuded strips of corpus cavernosum, erectile tissue in the penis.

In summary, NANC mediated relaxation of corpus cavernosum was endothelium dependent

and neuronal NO may be generated in corpus cavernosum. Therefore, the dysfunction of or damage to the endothelium in diseases which affect the endothelium may cause impairment of the function of corpus cavernosum and development of sexual dysfunction. The supplementation with L-arginine may be a therapeutic modality in those patient with erectile dysfunction (Kim *et al.* 1994).

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