Penile erection requires association of soluble guanylyl cyclase with endothelial caveolin-1 in rat corpus cavernosum

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Erectile dysfunction, the persistent inability to achieve or maintain an erection sufficient for satisfactory sexual performance, affects 30 million men in the United States (5), and its incidence increases sharply with age (18, 51). A pivotal role for nitric oxide (NO) as the central component of a major signal transduction system that acts in the penis to mediate the erectile response is widely accepted (2, 9, 32). NO released upon nerve stimulation or by exogenous cGMP. Further- more, sGC and caveolin-1, the major coat protein of caveolae, were colocalized in rat corpus cavernosum sinusoidal endothelium. Electron microscopy indicated caveolae disruption in corpus cavernosum treated with methyl-β-cyclodextrin. In summary, our results provide evidence of compartmentalization of sGC in the caveolae of cavernosal endothelial cells contributing to NO signaling mediating smooth muscle relaxation and erection.

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The enzyme NO synthase responsible for NO production in the endothelium (eNOS) has been shown to be targeted to caveolin-1, the major coat protein of caveolae (1, 19, 21, 41). Caveolae exist in most cell types and are particularly abundant in endothelial cells and smooth muscle cells (20, 48). The absence of this organelle impairs NO production, causing aberrations in endothelium-dependent relaxation (7, 14). Caveolae have been observed in the corpus cavernosum of mice and rats (4, 8). Caveolin-1 expression, the protein marker of caveolae, is decreased in the penile tissue of aged rats (4), a model of sexual dysfunction. Furthermore, two other models of sexual dysfunction, hypercholesterolemic and diabetic rats, when treated with an herbal medicine, present enhanced erectile response associated with increased caveolin-1 expression (3, 39).

Our group (30) recently showed that downstream molecules in the NO cascade, such as the sGC, are associated with caveolin-1 in rat aortic endothelium, suggesting that colocalization of components in this signal transduction cascade appear to be a critical determinant of signaling efficiency by eNOS activation. We therefore hypothesized that endothelial caveolae are the platform for sGC activation contributing to corpus cavernosum smooth muscle relaxation and erection.

MATERIAL AND METHODS

Animals. Adult male Sprague-Dawley rats (220–350 g; Harlan Laboratories, Indianapolis, IN), maintained on a 12:12-h light-dark cycle with rat chow and water ad libitum, were used in these studies. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia.

In vivo measurement of erectile response in rats. Rats were anesthetized with intramuscular ketamine (87 mg/kg) plus xylazine (13 mg/kg) and maintained on supplemental ketamine as needed. Mean arterial pressure (MAP) and intracavernosal pressure (ICP) were continuously and simultaneously monitored via pressure transducers (Grass; Astro-Med Industrial Park, West Warwick, RI), as previously described (12). Briefly, the left carotid artery was cannulated for MAP recording. For monitoring of ICP, the shaft of the penis was denuded of skin and fascia, and a 30-gauge needle was inserted into the right corpus cavernosum. The left corpus cavernosum was cannulated with a 30-gauge needle attached to a microsyringe via a short length of PE-10 tubing and used for drug administration (intracavernosal injection). The abdominal cavity was opened, exposing the right major pelvic ganglion (MPG). Stainless steel bipolar electrodes connected to a 30-gauge needle attached to a microsyringe via a short length of PE-10 tubing and used for drug administration (intracavernosal injection). The abdominal cavity was opened, exposing the right major pelvic ganglion (MPG). Stainless steel bipolar electrodes connected to

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absence of methyl-β-cyclodextrin (MβCD) injection of methyl-β-cyclodextrin (~15–20 μl; 0.3 mol/l stock solution). A resting period of 5 min was allowed between MβCD stimulations.

In vitro measurement of isometric force generation in cavernosal strips. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and killed by pneumothorax, and the penis was excised as previously described (12). After the ventral corpus spongiosum, dorsal vein, and surrounding connective tissue were removed, the right and left corpus cavernosal strips were obtained. Each tissue strip was longitudinally mounted in an organ chamber for isometric tension recordings and bathed in physiological salt solution (PSS) of the following composition (in mmol/l): 130.0 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.6 CaCl2, 14.9 NaHCO3, 0.03 EDTA, and 5.5 glucose, which was maintained at 37°C and bubbled with 95% O2-5% CO2. The cavernosal strips were stretched to 250–300 mg of passive tension. Under this tension, optimal contractile responses were previously observed in rat corpus cavernosum. Changes in isometric force were recorded using a PowerLab/8SP data acquisition system (Chart software, version 5.0; AD Instruments, Colorado Springs, CO). After a 1-h equilibration period in the presence of indomethacin (10 μmol/l; Sigma Chemicals, St Louis, MO), the strips were contracted with phenylephrine (10 μmol/l; Sigma Chemicals). Two cumulative concentration-response curves to sodium nitroprusside (SNP; 30 mmol/l to 1 mmol/l; Sigma Chemicals) were obtained in rat corpus cavernosum preparations precontracted with phenylephrine (10 μmol/l) in the absence of and after 60 min in the presence of methyl-β-cyclodextrin (10 mmol/l; Sigma Chemicals).

Cumulative concentration-response curves to 3-(5’-hydroxy-2’-furyl)-1-benzyl indazole (YC-1; 10 nmol/l to 30 μmol/l; Sigma Chemicals) and 8-bromoguanosine 3’,5’-cyclic monophosphatase (8-BrcGMP; 0.1 μmol/l to 0.5 mmol/l; Sigma Chemicals) were obtained in rat corpus cavernosum preparations precontracted with phenylephrine (10 μmol/l) in the presence of methyl-β-cyclodextrin (10 mmol/l; added 60 min before phenylephrine).

In another set of experiments, electrical field stimulation (EFS) was applied to cavernosal strips placed between platinum pin electrodes attached to a stimulus splitter unit (Stimu-Splitter II), which was connected to a Grass S88 stimulator (Astro-Med Industrial Park). EFS was conducted at 50 V, 1-ms pulse, and trains of stimuli lasting 10 s at varying frequencies (1–32 Hz). The strips were exposed to bretyllium tosylate (30 μmol/l; Sigma Chemicals) to inhibit norepinephrine release from sympathetic nerve endings. EFS was performed in precontracted strips with phenylephrine (10 μmol/l) before and after treatment with methyl-β-cyclodextrin (10 mmol/l) for 60 min.

Control responses were obtained in experiments in which methyl-β-cyclodextrin was replaced with vehicle (PSS). The contractile response induced by phenylephrine did not differ significantly after treatment with methyl-β-cyclodextrin (maximal force development: 108 ± 14 mg, n = 6, control vs. 109 ± 8 mg, n = 6, after methyl-β-cyclodextrin; P > 0.05).

Immunohistochemistry. Corpora cavernosa obtained from separate rats were isolated in PSS and embedded in OCT compound (Sakura Finetek USA, Torrance, CA). Fresh-frozen sections (8 μm thick) were thaw-mounted onto precleaned glass slides (Fisher Scientific) and kept overnight in a desiccator at 4°C, as previously described for rat aorta (30). After a wash in phosphate-buffered saline (PBS) and fixation in acetone, slides were blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS for 30 min at room temperature. The slides were then incubated with mouse monoclonal anti-caveolin-1 (final dilution 1:500; Research Diagnostics, Flanders, NJ) and rabbit polyclonal anti-sGC (β3-subunit; final dilution 1:500; Cayman Chemical, Ann Arbor, MI). After a wash in PBS, the fluorescent secondary antibodies goat anti-mouse IgG AlexaFluor 488 and goat anti-rabbit AlexaFluor 594 (final dilution 1:1,000; Molecular Probes, Eugene, OR) were applied and incubated for 1 h at room temperature. After a wash in PBS, the slides were coverslipped with anti-fading mounting medium (Gel/Mount medium; Biomedica, Foster City, CA) and allowed to desiccate overnight at 4°C. Sections were viewed using confocal microscopy (Zeiss Confocal).

Electron microscopy. Cavernosum segments treated with methyl-β-cyclodextrin (10 mmol/l) or vehicle (PSS) for 1 h were fixed overnight at 4°C in a solution consisting of 2% glutaraldehyde, 2% paraformaldehyde dissolved in sodium cacodylate buffer (0.1 mol/l) containing sucrose, as previously described for rat aorta (30). Samples were washed with sodium cacodylate buffer (0.1 mol/l) and then post-fixed in 4% osmium tetroxide for 1 h. Samples were dehydrated through a graded series of alcohol and embedded in Epon 812 with Araldite. Ultrathin sections were double stained with alcoholic uranyl acetate and lead citrate and examined with a JEOL-1010 transmission electron microscope.

Data analysis. In vivo erectile responses were expressed as the ratio of ICP (mmHg) to MAP (mmHg), measured during the flaccid state and during the plateau phase of the erectile response at each voltage applied. Isometric force generation data in vitro were measured as contraction (mg) or as a percentage of the maximal changes in phenylephrine-induced contraction, which was taken as 100%. Data are expressed as means ± SE and were analyzed using Student’s t-test for paired comparisons. Additional statistical analyses were performed using one-way ANOVA followed by the Student-Newman-Keuls post hoc test for multiple comparisons. A value of P < 0.05 is considered statistically significant.

RESULTS

Methyl-β-cyclodextrin inhibits erectile responses. ICP and MAP under resting conditions in anesthetized rats averaged 17 ± 3 and 119 ± 11 mmHg, respectively (n = 4). Electrical stimulation of the MPG induced voltage-dependent (1–5 V) and sustained increases in ICP, accompanied by transient decreases in MAP. The ratio of ICP to MAP was used to provide an index of erectile activity (Fig. 1). The erectile
response induced by electrical stimulation of the ganglion 2 min after methyl-β-cycloextrin was not significantly different from control responses (data not shown), whereas the erectile responses evoked by the electrical stimulation of the ganglion 10 min after the intracavernosal injection of methyl-β-cycloextrin were significantly decreased (n = 4) (Fig. 1).

**Methyl-β-cycloextrin disrupts cavernosal caveolae.** The effects of methyl-β-cycloextrin (10 mmol/l) on the structure of cavernosal membrane were evaluated using transmission electron microscopy. Micrographs of sinusoidal endothelial cell membrane (6, 40, 49) in control vessels (Fig. 2A) clearly show the presence of caveolae that are disassembled in samples treated with methyl-β-cycloextrin (Fig. 2B). Smooth muscle caveolae also were disrupted by methyl-β-cycloextrin (data not shown).

**Methyl-β-cycloextrin does not inhibit EFS-induced relaxation in isolated cavernosal segments.** In cavernosal smooth muscle strips precontracted with phenylephrine (10 μmol/l) in the presence of bretylium tosylate, EFS (1–32 Hz) produced frequency-dependent relaxations, with maximal relaxation of 54 ± 8% (Fig. 3). Methyl-β-cycloextrin (10 mmol/l) had no effect on EFS-induced relaxation (maximal relaxation: 60 ± 8%, n = 6; P > 0.05).

**Methyl-β-cycloextrin inhibits relaxation responses to SNP and YC-1 but not to 8-BrcGMP in isolated segments.** The NO donor SNP (30 mmol/l to 0.1 mmol/l) induced relaxation of phenylephrine-precontracted cavernosal smooth muscle strips in a concentration-dependent manner [pD2 (− log EC50): 4.4 ± 0.09, maximal relaxation: 99 ± 0.3%, n = 8, P > 0.05]. The relaxation induced by SNP was significantly impaired in the presence of methyl-β-cycloextrin (10 mmol/l) (pD2: 5.4 ± 0.15, maximal relaxation: 46 ± 5%, n = 10; P < 0.05) (Fig. 4A).

YC-1-induced relaxation was concentration-dependent in rat corpus cavernosal strips precontracted with phenylephrine in the presence and absence of methyl-β-cycloextrin. The sensitivity to YC-1 was not changed by methyl-β-cycloextrin (pD2: 4.58 ± 0.14, n = 5, control vs. 4.57 ± 0.04, n = 5, after methyl-β-cycloextrin; P > 0.05). However, the relaxation induced by YC-1 was significantly impaired in the presence of methyl-β-cycloextrin (Fig. 4B). The relaxation induced by YC-1, at the concentration of 1 μmol/l, was 28.5 ± 1.7 and 16.9 ± 2.3% in the absence and presence of methyl-β-cycloextrin, respectively (n = 5; P < 0.05).

8-Br-cGMP induced a concentration-dependent relaxation of rat corpus cavernosal strips precontracted with phenylephrine in the absence and presence of methyl-β-cycloextrin (Fig. 4C). The sensitivity to 8-Br-cGMP was not changed in the presence of methyl-β-cycloextrin (pD2: 4.5 ± 0.2, n = 4, control vs. 4.3 ± 0.2, after methyl-β-cycloextrin, n = 4; P > 0.05). Methyl-β-cycloextrin had no effect on the maximal relaxation induced by 8-Br-cGMP (87.6 ± 3.9%, n = 4, control vs. 87.7 ± 2.0, n = 4, after methyl-β-cycloextrin; P > 0.05).

**Colocalization of caveolin-1 with sGC in rat corpus cavernosum.** Localization of caveolin-1 with sGC is shown by confocal-quality immunofluorescence microscopy in corpus cavernosum sections (Fig. 5). sGC was visualized using a red fluorescent secondary antibody, whereas caveolin-1 was visualized using a green fluorescent secondary antibody. A wide distribution of sGC and caveolin-1 was observed in both corporal smooth muscle fibers and in the endothelium lining
the cavernosal sinusoids of rats. Colocalization of the two proteins was confirmed by the overlay of pseudocolored red and green images, resulting in a yellow signal at sites of colocalization. The yellow signal was found in the endothelial lining of corpus cavernosum, suggesting the presence of these two proteins in the endothelium. The signal obtained by incubation with the red (AlexaFluor 594) and green (AlexaFluor 488) secondary antibodies alone, representing tissue autofluorescence (background), is also shown in Fig. 5.

DISCUSSION

The aim of this study was to investigate whether endothelial caveolae are the platform for NO signaling pathway in the rat corpus cavernosum contributing to smooth muscle relaxation and erection. The cholesterol sequester methyl-β-cyclodextrin disassembled endothelial corpus cavernosal caveolae. Intracavernosal administration of methyl-β-cyclodextrin impaired erectile responses in rats in vivo. In vitro rat corpus cavernosum smooth muscle relaxation induced by the NO donor SNP and by the sGC activator YC-1, but not by EFS or 8-Br-cGMP, was impaired in the presence of methyl-β-cyclodextrin. Colocalization of sGC and caveolin-1 was observed in corpus cavernosal sinusoidal endothelium. These findings lead us to conclude that sGC targeted to caveolae is involved in rat corpus cavernosum smooth muscle relaxation and erection.

The functional state of the penis, flaccid or erect, is controlled by smooth muscle tone. Substantial evidence supports NO as the central component of a major transduction system that acts in the penis to mediate the erectile response (2, 32). Despite its direct action to induce relaxation of corpus cavernosum, NO also can inhibit smooth muscle vasoconstriction (11, 45, 50). NO constitutively produced and released during NANC neurotransmission, as well as from the endothelium lining the sinusoids, is the mediator of corpus cavernosum smooth muscle relaxation (9, 24, 33). It has been shown that in vivo sinusoidal endothelium disruption abolishes the erectile response to acetylcholine, whereas the relaxation induced by electrical stimulation is only partially inhibited (46). On the basis of this study, one can suggest that the erectile response observed upon electrical stimulation of the MPG in vivo in our studies represents nerve-derived NO inducing smooth muscle relaxation with subsequent release of NO from the sinusoidal endothelium in response to stretch and possibly to shear stress. On the other hand, in vitro rat corpus cavernosum smooth muscle relaxation induced by EFS is mainly due to nerve-derived NO. In support of this view are the studies, among others, showing poor relaxation induced by muscarinic agonists in rat corpus cavernosum and a lack of effects of inhibitors of NO synthase on passive tone (13, 23, 45), ruling out a significant role for endothelium-derived NO under these experimental conditions where stretch (observed in vivo) is absent.

It has been reported that there is a decreased expression of caveolin-1, the caveolar marker protein, in the penile tissue of aged rats, a model of sexual dysfunction (4). It also has been shown that hypercholesterolemic and diabetic rats, two other models of erectile dysfunction, present enhanced erectile response and increased caveolin-1 expression after treatment with an herbal medicine (3, 39). Altogether, these data suggest that increased caveolin-1 expression may counterbalance the
harmful effects of vascular diseases on the smooth muscle and endothelial cells, reversing the erectile dysfunction observed in these animals. Furthermore, it also has been shown that in hypercholesterolemic rabbits, endothelial dysfunction is associated with a decrease in the amount of caveolae in the endothelium (14). Caveolae are small invaginations in the plasma membrane, rich in cholesterol, that are involved in signal transduction by ensuring the compartmentalization of several signaling proteins (15, 31, 37). Cholesterol-rich membrane domains may possibly be considered as physical platforms for the coding of intracellular signals. Cyclodextrin is a membrane-impermeable molecule that depletes cellular cholesterol content through solubilization of the plasmalemmal cholesterol (28). This modifies cholesterol interactions with other plasmalemmal components, consequently causing the caveolar components to disassemble. This cholesterol-binding agent has been used widely as a pharmacological tool to study the role of caveolae in vascular reactivity (17, 26, 27, 30). Indeed, in our experiments we observed, using electron microscopy, that methyl-β-cyclodextrin disassembled endothelial sinusoidal caveolar structures in rat cavernous tissue. Moreover, impaired erectile responses in rats in vivo following intracavernosal administration of methyl-β-cyclodextrin were observed, supporting a role for caveolae in the erectile response. These data suggest that erectile dysfunction associated with endothelial dysfunction may be due to a decreased number of endothelial caveolae.

Smooth muscle caveolae also were disrupted by methyl-β-cyclodextrin. However, this effect does not seem to contribute to the erectile response, because the contractile responses induced by phenylephrine were not affected by methyl-β-cyclodextrin. These data suggest that methyl-β-cyclodextrin impairs erectile responses by specifically targeting the NO cascade in the endothelium.

It has been shown that the enzyme eNOS is targeted to caveolae (7, 21). Furthermore, caveolar cholesterol depletion is associated with a marked decline in acetylcholine-induced eNOS activation (7). Supporting these data, our group (30) and others (14, 27) have found that caveolar disruption by methyl-β-cyclodextrin inhibits acetylcholine-induced relaxation in vascular tissues. In the present study we observed that methyl-β-cyclodextrin had no effect on corpus cavernosum smooth muscle relaxation in rats in vitro induced by EFS, where the source of NO is neuronal. These data suggest that methyl-β-cyclodextrin affects the production and/or the actions of NO produced by the endothelium and not by neuronal sources.

It is well established that NO activates sGC, increasing intracellular cGMP levels, leading to relaxation of smooth muscle of the corpus cavernosum (32). Consistent with this concept, we observed corpus cavernosum smooth muscle relaxation induced by the NO donor SNP, by the sGC activator YC-1, and by 8-Br-cGMP. The natural receptor for NO, sGC has been detected in association with the plasma membrane interacting with caveolin-1 in the endothelium (30). Because translocation of sGC to caveolar domains seems to be a necessary step to be sensitized by NO (52), we hypothesized that the possible target of sGC to endothelial caveolae in rat corpus cavernosum would contribute to corpus cavernosum smooth muscle relaxation and erection. Indeed, in the present study we observed that relaxation induced by the NO donor
SNP and by the sGC activator YC-1 was inhibited in the presence of methyl-β-cyclodextrin in rat corpus cavernosum segments.

Considering that NO from any source activates sGC, a consideration regarding the reason why methyl-β-cyclodextrin had no effect on the relaxation evoked by EFS (neuronal NO) could be raised. The neuronal origin of NO in the relaxation induced by EFS has been previously demonstrated (43, 44). Furthermore, EFS-induced relaxation of corpus cavernosum is independent of NO produced by eNOS (36). On the other hand, SNP, despite being able to induce smooth muscle relaxation independently of the presence of endothelium, also increases cGMP in endothelial cells (47). Moreover, in guinea pig trachea, the relaxations induced by nitroglycerin and by EFS were differently affected by a sGC inhibitor (38). Altogether, these data suggest that selective activation of sGC in a target tissue may vary according to the NOS isoform activated: neuronal NO activates sGC in the smooth muscle, whereas SNP activates sGC in both smooth muscle and endothelial cells, resembling NO produced by eNOS activation.

Despite the well-accepted model of diffusion of NO produced in the endothelial cells to the adjacent smooth muscle cells to activate sGC (34), there is increasing evidence for sGC localization and cGMP production in endothelial cells (10, 16, 22, 25, 30, 42, 47) that would facilitate endothelial NO actions. Venema et al. (47) reported two different pools of sGC: cytosolic and membrane associated. Their study also demonstrates that the prevalence of each pool may vary between smooth muscle and endothelial cells: whereas smooth muscle cells present predominantly the cytosolic pool, the pool associated to the plasma membrane is found more abundantly in endothelial cells. These data further support our hypothesis that sGC may be compartmentalized in the endothelial caveolae. As for protein composition, caveolin-1 is considered the most associated to the plasma membrane is found more abundantly in endothelial cells. These data further support our hypothesis that sGC may be compartmentalized in the endothelial caveolae. As for protein composition, caveolin-1 is considered the most important protein involved in the structure and function of caveolae (1). Using immunohistochemistry, we observed colocalization of sGC and caveolin-1 in rat corpus cavernosum sinusoidal endothelium. These findings established the compartmentalization of sGC to caveolae because of its association with caveolin-1 in the endothelium, introducing a potential therapeutic strategy for sexual dysfunction.

cGMP generated upon sGC activation in the microdomain underlying endothelial caveolae may activate PKG. Recently, our group (30) showed that the relaxation induced by 8-Br-cGMP was impaired in endothelium-intact rat aortic rings previously treated with methyl-β-cyclodextrin. Colocalization of PKG and caveolin-1 also was reported in these studies. To investigate whether downstream molecules to cGMP in the NO pathway were compartmentalized in the cavernosal caveolae, we tested in the present study the effects of methyl-β-cyclodextrin on the relaxing effects induced by 8-Br-cGMP in rat corpus cavernosum strips. The lack of effect of methyl-β-cyclodextrin on 8-Br-cGMP-induced relaxation of rat corpus cavernosum smooth muscle suggests that once produced in the endothelial caveolae, cGMP effects occur in other intracellular sites different from caveolae in rat corpus cavernosum.

In summary, we propose that sGC is compartmentalized in cavernosal endothelial caveolae as a necessary spatial organization to facilitate NO actions. Our results provide new insights by which the endothelium mediates cavernosum smooth muscle relaxation and erection.

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