RhoA-Rho kinase mediates synergistic ET-1 and phenylephrine contraction of rat corpus cavernosum

Christopher J. Wingard, Shahid Husain, Jan Williams, and Sharita James

Departments of Physiology and Biochemistry, Medical College of Georgia, Augusta, Georgia 30912-3000

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Wingard, Christopher J., Shahid Husain, Jan Williams, and Sharita James. RhoA-Rho kinase mediates synergistic ET-1 and phenylephrine contraction of rat corpus cavernosum. Am J Physiol Regul Integr Comp Physiol 285: R1145–R1152, 2003.—Maintenance of the detumescent state of the penis is believed to involve the actions of several vasoconstrictors. However, our mechanistic understanding of any synergistic vasoconstrictor influences is extremely limited. We tested the hypothesis that a vasoconstrictor combination of endothelin (ET-1) and phenylephrine (PE) augments the constrictor responses in rat corporal cavernosal tissues by a mechanism involving the RhoA-Rho kinase pathway. Independently, ET-1 (1 nM–30 μM) and PE (100 nM–100 μM) both caused dose-dependent contractions of isolated rat cavernosal tissues. In combination, ET-1 (30 nM) augmented the contractile effect of PE and shifted the calculated EC50 for PE (90 ± 12 to 45 ± 5 μM). The active stress generated by cavernosal strips during the ET-1 + PE combined stimulation (4.9 ± 0.2 mN/mm2) was greater than the combined stress generated with ET-1 (0.4 ± 0.1 mN/mm2) or PE (3.3 ± 0.2 mN/mm2) stimulations alone. Blockade of ETα receptors (30 nM; A-127722) reversed the augmented stress generation and the Rho-kinase inhibitor Y-27632 differentially and dose-dependently relaxed the tissue. The combined constrictor effect was associated with a fourfold increase of RhoA in the membrane faction of the tissue homogenates. We conclude that the ET-1 + PE combination potentiates vasoconstriction through mutual activation of the RhoA-Rho kinase pathway. The interactions of these agonists likely play important roles in the maintenance of the flaccid state and contribute to some forms of erectile dysfunction.

penile erection; vasoconstrictor interaction; calcium sensitization; smooth muscle

Address for reprint requests and other correspondence: C. J. Wingard, Dept. of Physiology, Medical College of Georgia, 1120 15th St., Augusta GA, 30912 (E-mail: Cwingard@mail.mcg.edu).

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augment constrictor responses in rat corporal cavernosal tissues by a mutual activation of the RhoA-Rho kinase pathway.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (275–400 g), obtained from Harlan Laboratories (Indianapolis, IN), were used in these studies. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals established by the National Institutes of Health and approved by the Medical College of Georgia committee on the use of animals in research and education.

Tissue Collection and Preparation

Cavernosal tissue dissections were done under anesthesia induced by intramuscular injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). The penis was exposed by an incision at its base. Before harvest of the corpus cavernosum, the distal end of the penis, connective tissue, corpus spongiosum, and the dorsal vein were removed. The cavernosal strips were cleaned and washed in a 2.0 mM MOPS-based physiological saline solution (PSS) at 4°C, pH 7.4, and used within 1 h of dissection.

All tissues were attached to an isometric muscle bath system and equilibrated for at least 60 min at 37°C in a 20 ml organ bath chamber containing PSS of the following composition (in mM): 140.0 NaCl, 5.0 KCl, 1.6 CaCl₂, 1.2 MgCl₂, 1.2 Na₂HPO₄, and 5.6 d-glucose. Tissues were continuously bubbled with compressed air. Tissue resting force was set to 500 mg by a series of stretches and length releases, followed by a period of stress relaxation. Setting the tissue to a preset resting force of 500 mg was found to correlate to an optimal length force generation in response to maximal K⁺ depolarization (data not shown). All tissues were contracted by the addition of 109 mM K⁺ PSS. K⁺ PSS was prepared by stoichiometric substitution of KCl for NaCl in PSS. Tissues were depolarized for 10 min and then relaxed with repeated washes of PSS at 10-min intervals before the start of any experimental protocol.

Cumulative Dose Responses

Cumulative dose responses (CDRs) were constructed for each cavernosal preparation by the cumulative addition of drugs in log increments. Preliminary studies on rat corporal tissues affirmed the long-lasting and slowly reversible contractions characteristic of ET-1-induced response seen with other vascular tissues. Because of potential long-lasting effect of ET-1, CDRs after ET-1 exposure were not constructed on the same tissues. Thus either PE then ET-1 CDRs or PE and the combination of ET-1 and PE CDRs were constructed within the same tissue.

ET-1. ET-1 was added every 10 min in a cumulative fashion, resulting in bath concentrations of 1 nM–30 μM. The incubation time of 10 min for each concentration was chosen to allow achievement of a steady-state stress response.

PE. PE was added every 5 min in a cumulative fashion, resulting in bath concentrations of 0.3–100 μM.

Combination agonist effects. In these experiments, bath concentration of ET-1 was set at 30 nM. After 5 min, and in the continuous presence of ET-1, PE was added to each bath, every 5 min, in a cumulative fashion, achieving concentrations of 0.3–100 μM.

Blockade of NO and ET₄ receptors. In these experiments, tissues were preincubated with 10 μM N⁶-nitro-L-arginine methyl ester (L-NAME) for 10 min before a cumulative (PE or ET-1) dose-response experiment was initiated. For ET₄-receptor blockade, 30 nM A-127722 (25, 33) was introduced to each bath 10 min before the addition of ET-1 or PE.

Blockade of Rho kinase. Tissues were prestimulated with either 10 μM PE, 30 μM ET-1, or the 30 nM +10 μM PE combination and then relaxed with Y-27632 over the 1–30 μM concentration range.

Immunoblot Analysis of Cavernosal RhoA Protein Expression

RhoA was detected in cavernosal tissue homogenates by Western blotting. Cavernosal strips (cleaned of the corpus spongiosum and dorsal vein) were homogenized in cold immunodetection buffer, which contained 50 mM Tris·HCl (pH 7.4), 1.0% Nonidet P-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na₃VO₄, and 1 mM NaF. Samples were centrifuged (1,000 g, 4°C, 10 min), and the supernatant was collected for cytosolic and membrane fractionation. In control experiments, no RhoA immunoreactive band was found in the 1,000-g pellet. Homogenates were separated into cytosolic and membrane fraction by high-speed centrifugation (100,000 g, 4°C, 45 min). Supernatant was removed, and the pellet was resuspended in immunodetection buffer plus 1.0% Triton X-100.

Equal amounts of protein (15-μg total protein per lane) were loaded and resolved by 10% SDS-polyacrylamide gel electrophoresis (at room temperature, 3 h). Jurket cell lysate was run as a positive control, and prestained multimark (Invitrogen Life Technology) was run in parallel as protein molecular weight markers. Protein determination was accomplished by using a Micro BCA Protein assay (Pierce Biotechnology).

Proteins were transferred to a nitrocellulose membrane (0.22 μm Immobilon-P, Millipore) by using a Bio-Rad Mini-Protein III apparatus (2 h at 4°C) in the presence of 25 mM trizma base, 191.8 mM glycine, and 20% methanol. The nitrocellulose membrane was then blocked with 5% skimmed milk in phosphate-buffered saline (30 min, room temperature; Biorad, Hercules, CA). After blocking, the membrane was incubated with primary monoclonal antibody to RhoA (1:1,000 dilution; BD BioSciences) for 12 h at 4°C with gentle shaking. After membranes were washed, they were incubated with secondary antibody (horseradish peroxidase conjugated goat anti-mouse IgG at 1:3,000 dilution) for 1 h at room temperature. Immunoreactive bands were visualized by use of enhanced chemiluminescence reagent (Amersham Pharmacia, Piscataway, NJ) for 1 min and exposed to enhanced chemiluminescence hyper film (Kodak) for 1–2 min.

RhoA protein expression corresponded to a band of 25-kDa range. Blots were reprobed for β-actin staining to ensure equivalent protein loading.

Data Analysis and Statistics

Raw force responses were recorded digitally with POLYview data acquisition (Astro-Med, West Warwick, RI). Post-acquisition analysis included conversion of force (in g) to stress (mN/mm²). Force was converted to stress values (force/ cross-sectional area) by the following equation: \(|\text{force (mg)} \times \frac{0.0987}{\text{area}}| \div 1.055\), where area is calculated from wet weight (mg) ÷ length (mm). Dose-response profiles were constructed for the individual traces for each experimental
condition. The profiles were fit by Sigma Plot (SPSS Science, Chicago, IL), by using a Hill fit protocol, allowing for the report of the estimated EC$_{50}$ values. Data are presented as means ± SE. Statistical differences were determined by ANOVA followed by Bonferroni’s complementary analysis, where relevant, and Student’s t-test using the SigmaStat Analysis Program (SPSS Science). A P value < 0.05 was considered to be significant.

Materials

ET-1 was purchased from The American Peptide (Sunnyvale, CA). A-127722 was a gift from Abbott Laboratories and Dr. David Pollock. The Rho kinase inhibitor Y-27632 was a gift from the Mitsubishi Pharma Group (Osaka, Japan) or purchased from CALBIOCHEM (La Jolla, CA). PE and all other reagents were purchased from Sigma Chemical (St. Louis, MO). Antibodies were purchased from BD Biosciences (San Diego, CA).

RESULTS

Figure 1A displays the active stress generation response of cavernosal tissue strips to increasing concentrations of ET-1 and PE. Both agonists produced a graded contractile response that achieved a plateau in the micromolar concentration range. The observation that ET-1 had an equivalent constrictor effect, relative to PE, on isolated cavernosal tissue may reflect simultaneous activation of ET$_A$ and ET$_B$ receptors with subsequent generation of NO. To test this hypothesis, we inhibited NO generation with the NO synthase (NOS) inhibitor L-NAME (10 μM). Tissues were preincubated for 10 min with L-NAME before the construction of the ET-1 dose-response profile. As shown in Fig. 1B, L-NAME had a minor effect on the constrictive action of ET-1 in the rat cavernosal strips, which proved not significant at all concentrations examined (P > 0.065). The sensitivity of the tissue to ET-1 was unchanged with L-NAME pretreatment. The mean calculated EC$_{50}$ for ET-1 alone was 1.1 ± 0.2 μM (n = 4) and for ET-1 with L-NAME was 0.9 ± 0.2 μM (n = 4). No further increases in the constrictor response to ET-1 were seen with either 30 or 100 μM L-NAME preincubations (data not shown).

In an effort to test our hypothesis, we examined if ET-1 could act synergistically with the α-adrenergic agonist PE to increase the contractile response. In a separate set of tissues, a threshold concentration of ET-1 (30 nM) was applied 5 min before the cumulative addition of PE to the bath. This concentration was found, on average, to generate <5% of the stress a maximal PE response developed. In these experiments, L-NAME was included in one set of tissues to prevent any contribution of endothelium-derived NO. As plotted in Fig. 2, the addition of 30 nM ET-1 produced a marked increase in PE stress generation ability over most of the concentration range tested (Fig. 2A). Additionally, pretreatment with 30 nM of ET-1 shifted the EC$_{50}$ of the PE response profile to the left from 89.6 ± 12.0 μM (n = 10) and PE alone to 45.0 ± 5.0 μM (n = 5) and 36.5 ± 5.0 μM (n = 5), with and without L-NAME, respectively (Fig. 2B). This shift in EC$_{50}$ values with the presence of ET-1 with or without L-NAME was significantly different from calculated EC$_{50}$ for PE with P values of <0.016 and <0.021, respectively.

The ability of ET-1 to increase the contractile response to PE was believed to be manifest through its activation of the ET$_A$ receptor. We tested this hypothesis by use of the ET$_A$-receptor antagonist A-127722. As shown in Fig. 3, the preincubation of tissues with this antagonist for 10 min before the addition of 30 nM of ET-1 was capable of blocking ~90% of the ET-1-induced contraction, whereas, in paired tissues with vehicle controls, there was no reduction in the stress generated (Fig. 3). Additionally, A-127722 also blocked the stress generation induced by 300 nM ET-1 administrations by >80% (data not shown). Furthermore, A-127722 reversed the synergism of ET-1 (either 30 or 300 nM) on the 10 μM PE contraction (Fig. 3B). The measured stress generation in these experiments with
the combination of ET-1 and PE (7.2 ± 0.4 mN/mm²) was significantly different from the stress calculated by adding the separate PE and ET-1 stress responses together (6.0 ± 0.3 mN/mm²; P = 0.037).

In experiments aimed at elucidating the mechanism for the effect of ET-1 on a PE constriction, we wanted to block the Rho kinase activity in a test of the hypothesis that the synergism involves the calcium-sensitization pathway involving RhoA-Rho kinase. Y-27632 (1–30 μM) dose dependently relaxed the contraction induced by the combination of 30 nM ET-1 and 10 μM PE (Fig. 4). In these set of experiments, the stress generated with the agonist combination (4.9 ± 0.2 mN/mm²) was significantly larger than the total additive stress generated by ET-1 and PE (3.6 ± 0.3 mN/mm²; P = 0.002). Additionally, we investigated if there was any shift in responsiveness of the corpus cavernosal tissue to relaxation by the Rho kinase inhibitor Y-27632. As reported in Fig. 5, we observed a dose-dependent relaxation of prestimulated corpus cavernosum with Y-27632. Over the concentration range of 0.1–30 μM, Y-27632 could completely relax a maximal PE (10 μM) contraction while only producing 60% and 80% maximal relaxation effect on 30 μM ET-1 or the combination of 30 nM ET-1 + 10 μM PE, respectively (Fig. 5B). The mean calculated EC₅₀ of Y-27632-induced relaxation of the ET-1 + PE combination (7.4 ± 2.4 μM; n = 4) was not significantly different from that calculated for ET-1 (27.5 ± 10.4 μM; n = 4, P = 0.114) or for PE (9.0 ± 0.4 μM; n = 4, P = 1.0) alone. However, there was a statistical significance between PE and ET-1 calculated EC₅₀ values (P = 0.043).

Stimulation of the rat corporal cavernosal strips with 30 nM ET-1 or 10 μM PE for 10 min or their combination resulted in a significant increase of RhoA in the membrane fraction of tissue homogenates (Fig. 6). The combined stimulation was associated with a significantly larger amount of RhoA protein (4.3 ± 0.4 relative units) detected in the membrane fraction than that seen with either ET-1 (2.4 ± 0.1 relative units) or PE (2.7 ± 0.4 relative units) alone (P = 0.05).

![Fig. 2. Synergistic effect of threshold ET-1 concentration on PE dose response in rat corpus cavernosum.](image)

![Fig. 3. Effect of ET₁-receptor blockage on the ET-1 (30 nM) potentiation of the 10 μM PE-induced stress generation.](image)
DISCUSSION

The accumulated evidence in the literature suggests that ET-1 and \( \alpha \)-adrenergic stimulation have roles in the maintenance of penile flaccidity and detumescence. Whereas both agonists are hypothesized to act as direct regulators of smooth muscle tone, modulators of the constrictor effects of other agents, or phenotype modulators, the exact mechanisms involved have yet to be fully established (2). Whereas these actions are generally acknowledged as occurring for all penile vasculatures, there are limited data regarding the constrictor role of ET-1 in the rat corpora cavernosa (5, 8, 23). In an effort to elucidate the mechanism by which ET-1 impacts the constrictor response to \( \alpha \)-adrenergic stimulation, we set out to test the hypothesis that ET-1 acts to increase the adrenergic contractile response of rat cavernosal tissue via the RhoA-Rho kinase pathway.

We report here the first evidence that the mechanism of action for a synergistic effect of ET-1 on PE constriction in the rat corpus cavernosum occurs via \( \text{ET}_A \) receptors and subsequent activation of the RhoA-Rho kinase pathway. We observed that the ET-1 constrictor response was not significantly affected by NOS blockade, but was nearly abolished by an \( \text{ET}_A \)-receptor antagonism. In combination with PE, ET-1 induced a significant upregulation of the expression of RhoA in the membrane fraction of tissue homogenates, and the constrictor responses could be dose dependently blocked by the inhibitor to Rho kinase, Y-27632.

Several reviews have cited reports that concluded that ET-1 acts as a vasoconstrictor equal to or exceeding the ability of \( \alpha \)-adrenergic stimulation to generate force in human and rabbit tissues (2, 3). However, there had been no reported evaluation of the constrictor action of ET-1 in the rat corporal tissue. We were surprised by the moderate constriction and sensitivity of rat cavernosal strips to ET-1 in the concentration range reported to produce maximal constrictive responses in rabbit and human cavernosal tissues (Fig. 1). Our results differed from those that have reported a strong vasoconstrictor action of ET-1 over a similar concentration range in cavernosal tissue from other species (12, 13, 16, 26). Holmquist and coworkers (12) reported that ET-1 corporal force responses were approximately equivalent to a maximal \( K^+ \) depolarization, a condition only seen at a concentration of >10 \( \mu \)M. Whereas the response range of the rat to ET-1 was dissimilar from those reported in the literature (12), we were able to achieve a defined maximum with a higher concentration of ET-1. Care must be made in any direct comparison of these responses with that of other species, in particular from human tissues that may reflect

Fig. 4. The dose-dependent effect of Rho kinase inhibition on combined ET-1 and PE constricted corpus cavernosum. Reported are mean active stress levels for PE (10 \( \mu \)M), ET-1 (30 nM), and combined ET-1 and PE stimulations. Rho kinase inhibition was accomplished by cumulative additions of Y-27632 (Y; 0.3–30 \( \mu \)M) applied to the ET-1 + PE combination. Values are means ± SE of 4 separate tissues. *Statistical difference from the PE stress value; †statistical difference from the ET-1 + PE stress value: \( P < 0.05 \).

Fig. 5. The Y-27632 dose-dependent relaxation of rat corpus cavernosal tissues maximally contracted by PE, ET-1, or a combination of ET-1 and PE. A: mean active stress values for 10 \( \mu \)M PE (open circles), 30 nM ET-1 (shaded circles), and the ET-1 (30 nM) + PE (10 \( \mu \)M) (solid circles) to cumulative addition of Y-27632 (0.1–30 \( \mu \)M). B: normalized maximal stress relaxation response and shift in sensitivity to Rho kinase inhibition by the cumulative addition of Y-27632. Values are means ± SE of 4 separate tissues.
an unidentified underlying vascular pathology. Our observation is extremely important as it is the first reported broad range ET-1 dose-response relationship of rat corpus cavernosum at a time when more investigators are relying on the rat models for therapeutic studies on erectile dysfunction.

The moderate ET-1 constrictor response of rat cavernosal strips might have been due to release of vasoconstrictor substances, including NO from the endothelial cells lining the sinuses or blood vessels in the cavernosum (5). In these studies, the use of L-NAME to block endogenous NOS activity failed to significantly improve the constrictor response of ET-1. Such a result may suggest that any dilatory effect by ET-1 stimulation via NO production in the rat cavernosal strips was minor. The unchanged contractile response to ET-1 in the presence of L-NAME is not that surprising in light of the report from Bell that ET B receptors were not detected in normal rat cavernosal tissues (6). Alternatively, the lack of ET B receptor expression could also limit the constrictive response of this tissue. The ET A antagonist blocked most of the constrictive response to ET-1 but a small amount of stress remained (Fig. 3). This result could reflect stimulation of a small number of ET B receptors on the smooth muscle. A greater abundance of ET B receptors in human and rabbit cavernosal tissue relative to rat tissues would contribute to the powerful constrictive response and could account for the apparent difference in stress generation between species. Additionally, work published from our group found that administration of the ET B antagonist failed to show any significant effect on the vasoconstrictor action of ET-1 in the whole rat model of erectile function (8). Together, these results suggested that ET-1-dependent relaxation via ET B receptors and NO generation or a direct vasoconstrictive effect were not significant players in the response of the rat corpus cavernosum to ET-1. These results do not rule out the possibility that shear stress may still contribute to the activation of endothelial NOS and release of NO during erection.

Little is understood about the mechanisms responsible for the synergistic role of vasoconstrictors in regulating target organ blood flow. Subpressor concentrations of ET-1 are known to enhance agonist-induced vasoconstrictor responses in a variety of other vascular tissues (11, 24, 34). Several reports have demonstrated the involvement of ET-1 in augmenting the constrictive action of α 1-adrenergic stimulation in the vasculature of the rabbit penis (10, 12, 13, 16, 26). While in our hands the constrictor response to ET-1 was moderate, we suggested that ET-1 may influence the action of PE. Continued exposure to low concentrations of ET-1 both shifted the sensitivity to the α-adrenergic agonist PE and also elevated the stress response. These results agree with other studies that described a similar combinatorial effect of ET-1 on rabbit tissue (16) and support our hypothesis that low concentrations of ET-1 could augment PE constrictor responses. The exact mechanism of the combinatorial effect is not known, but one report hypothesized that the activation of separate α 1 and ET-1 membrane receptors have a common link downstream from receptor occupancy in the control of cavernosal tone (16). Several recent studies in other vasculatures have demonstrated that ET-1 and other agonists may modulate vascular smooth muscle tone through activation of the RhoA-Rho kinase pathway (20, 21, 27).

There has been growing evidence that receptor agonists may augment smooth muscle contraction by increasing Ca 2+ sensitivity (15, 28). The increased calcium sensitivity seen with agonist-induced contractions appears to be regulated by several cellular events, including the myosin light-chain phosphatase through the Rho-Rho kinase pathway (30). Thus a Rho kinase mechanism of calcium sensitization could serve as a common link for the synergistic effect of ET-1 on adrenergic-generated smooth muscle tone of the penis. Our results are the first to demonstrate that the synergism of ET-1 and PE in the corpus cavernosum was mediated by activation of RhoA and its downstream target Rho kinase, whereas Matsumura and colleagues (20) demonstrated the involvement of this pathway in the ET-1-induced augmentation of the noradrenalin response in rat mesenteric arteries. We also observed a differential relaxation with the Rho kinase inhibitor...
Y-27632 in maximally contracted tissues by PE or ET-1, suggesting that these two agonists may not rely equally on the RhoA-Rho kinase to exert their full constrictive effect. Caution is warranted in the interpretation because Y-27632 was capable of completely relaxing the PE-induced contraction and would suggest that the entire force maintenance during adrenergic stimulation relies exclusively on Rho kinase-mediated Ca$^{2+}$ sensitivity. Whereas this may be true, it is also likely that the inhibitor may also have some non-specific action at the highest concentrations examined.

As such, it is interesting to speculate on potential physiological implications of our observations. For one, these studies are the first in rat cavernosum to illustrate that ET-1 displays a moderate vasoconstrictor role and that ET-1-mediated NO production did not modulate cavernosal reactivity. Second, a relatively small concentration of ET-1 might be associated with significant changes in cavernosal smooth muscle tone during simultaneous adrenergic stimulation like those seen in human and rabbit corporal tissues (10, 12, 16). Third, the action of ET-1 may play a more profound vasoconstrictor role at the level of prepenile arteries, regulating blood flow into the penis, as suggested by Manabe et al. (19), who examined the resistance control of the rat penile vasculature. In studies by Ari et al. (5) and from our group (8, 23), ET-1 acted as a strong vasoconstrictor suppressing penile erection but, in light of the evidence presented here, would point to the prepenile arteries as being under a greater vasoconstrictor influence of ET-1 than the corpus cavernosum. Finally, and most intriguing is the first evidence for a common signaling mechanism responsible for the synergistic vasoconstriction induced by a combination of endothelin and PE that may activate the calcium-sensitizing pathway involving RhoA-Rho kinase in cavernosal tissues. The development of single agent that prevents the propagation of a signal downstream of the convergence point for two different agonist signaling pathways might prove to be an effective therapeutic approach in treating erectile dysfunction. This may be particularly useful in light of the varied success achieved at treating erectile dysfunction by targeting a single agonist’s receptor (17). Our data reveal a mechanism for how these constrictors may interact to play a role in the regulation of cavernosal tone in a variety of pathological states resulting in erectile dysfunction, as suggested in several recent papers (7, 30, 31).

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DISCLOSURES

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