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Endothelin-1 Induces Contraction of Female Rat Internal Pudendal and Clitoral Arteries through ET_A Receptor and Rho-Kinase Activation

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Abstract

Introduction—Endothelin-1 (ET-1), a potent vasoconstrictor peptide, acts mainly through the Gprotein-coupled ET_A receptor (ET_AR). Increased vascular ET-1 production and constrictor sensitivity have been observed in various cardiovascular diseases, including hypertension, as well as erectile dysfunction. The internal pudendal artery (IPA) supplies blood to the vagina and clitoris. Inadequate blood flow through the IPA may lead to insufficient vaginal engorgement and clitoral tumescence.

Aim—Characterize the effects of ET-1 on the IPA and clitoral artery (CA).

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Methods—IPA and CA from female Sprague Dawley rats (225–250 g) were mounted in myograph chambers. Arterial segments were submitted to increasing concentrations of ET-1 (10⁻¹⁰–10⁻⁶ M). Segments were incubated with the ET_AR antagonist, atrasentan (10⁻⁸ M) or the Rho-kinase inhibitor, Y-27632 (10⁻⁶ M) 30 minutes prior to agonist exposure. All E_{max} values are expressed as % KCl-induced maximal contraction. ET_AR, RhoA, and Rho-kinase expression from IPA was evaluated by Western blot. mRNA of preproET-1, ET_AR, ET_BR, RhoA, and Rho-kinase were measured by real time PCR.

Main Outcome Measures—ET-1 constrictor sensitivity in IPA and CA, protein expression and messenger RNA levels of ET-1-mediated constriction components.

Results—ET-1 concentration-dependently contracted IPA (% Contraction and pD₂, respectively: 156 ± 18, 8.2 ± 0.1) and CA (163 ± 12, 8.8 ± 0.08), while ET_AR antagonism reduced ET-1-mediated contraction (IPA: 104 ± 23, 6.4 ± 0.2; CA: 112 ± 17, 6.6 ± 0.08). Pretreatment with Y-27632 significantly shifted ET-1 pD₂ in IPA (108 ± 24, 7.9 ± 0.1) and CA (147 ± 58 and 8.0 ± 0.25). Protein expression of ET_AR, ET_BR, RhoA, and Rho-kinase were detected in IPA. IPA and CA contained preproET-1, ET_AR, ET_BR, RhoA, and Rho-kinase message.

Conclusion—We observed that the IPA and CA are sensitive to ET-1, signaling through the ET_AR and Rho-kinase pathway. These data indicate that ET-1 may play a role in vaginal and clitoral blood flow and may be important in pathologies where ET-1 levels are elevated.

Keywords

Internal Pudendal Artery; Endothelin-1; Rho Kinase; Clitoral Artery

Introduction

Female sexual dysfunction (FSD) is a highly prevalent condition, affecting 25 to 63% of women in the United States [1]. Sexual dysfunctions are more commonly reported in women than men and can be characterized by disturbances in sexual desire, arousal, painful intercourse, and inability to achieve orgasm [1,2]. FSD is a multi factorial disease as several etiologies of FSD have been identified including, vasculogenic, neurogenic, hormonal/endocrine, and psychogenic components [3]. Advances in male sexual dysfunction treatment, such as the development of phosphodiesterase type 5 inhibitors [4], overshadow the relatively sparse treatment options for women with sexual dysfunction, whom have primarily been limited to psychological therapy [5]. Therefore, there is a great need to further our knowledge regarding FSD and to develop therapeutic options.

Endothelin-1 (ET-1) is a potent endogenous vasoactive peptide whose principal actions include vascular smooth muscle contraction and proliferation. ET-1 signals through the G protein-coupled ET_A receptor (ET_AR) and ET_B receptor (ET_BR) [6–8]. The ET_AR is found on various tissues and cells, including vascular smooth muscle and corpus cavernosum [9–11]. As ET-1 binds to the ET_AR, the peptide initiates a wide range of vasoconstrictive signaling cascades, including the activation of RhoA, a 20 kDa G-protein, which in turn stimulates Rho-kinase [12]. Rho-kinase is a principal component involved in calcium sensitization-mediated contraction of vascular smooth muscle cells, where contraction persists independent of increases in intracellular calcium, thus contributing to a prolonged and greater constricted state of the vasculature. Increased Rho-kinase protein and activity levels have been observed in the corpus cavernosum of diabetic rats demonstrating reduced erectile response via cavernosal nerve stimulation [13]. Additionally, Rho-kinase inhibition improved erectile responses in spontaneously hypertensive rats that displayed erectile dysfunction (ED) [14]. Therefore, the role of ET-1-signaling through Rho-kinase in the vasculature is important to improve our understanding of sexual dysfunctions.

Consistent evidence has shown that plasma and tissue ET-1 levels, as well as ET-1-mediated constrictor sensitivity, are elevated in hypertensive patients and hypertensive animal models [15–17]. Recently, our laboratory reported that male deoxycorticosterone acetate (DOCA)-salt hypertensive rats and mice displaying ED demonstrate augmented cavernosal contractile reactivity to ET-1 [18,19]. Current data suggest that ED may precede hypertension, diabetes, and cardiovascular diseases [20–23]. Recent studies have also demonstrated a correlation between FSD and hypertension [24,25]. The internal pudendal artery (IPA) is the primary supplier of blood to both male and female genitals in both human and rat. Diminished blood flow through the IPA has been shown to contribute to ED, as well as vaginal engorgement and clitoral erectile insufficiencies [26,27]. Therefore, a commonality between vasculogenic FSD and ED may be the elevated contractile state of the IPA, potentially mediated by ET-1.

During the normal female sexual arousal response, an increase in blood flow proximally through the IPA and more distally through the clitoral artery (CA) leads to vaginal and clitoral engorgement. Therefore, understanding vasoconstrictor sensitivity in these arteries could prove to be a fundamental advancement in furthering our understanding of the role of these key arteries in the female sexual arousal response. Thus, we hypothesized that ET-1 constricts IPA and CA through ET_AR activation, and downstream actions are mediated through activation of Rho-kinase.

Methods and Main Outcome Measures

Animals and Use

Mature female Sprague-Dawley rats (10–12 weeks of age, 230–250 g; Harlan, Indianapolis, IN, USA) were used in all studies. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education. The animals were housed four per cage on a 12-hour light/dark cycle and fed a standard chow diet and water *ad libitum*. Prior to sacrifice, vaginal smears taken to determine estrus status and samples were stained using methods previously described [28]. Animals were killed by carbon dioxide exposure, followed by diaphragm incision.

Arterial Isolation and Force Measurement Protocol

The IPA and CA were removed and placed in chilled physiological salt solution [(PSS, mM): NaCl, 130; NaHCO₃, 14.9; dextrose, 5.5; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄·7H₂O, 1.17 and CaCl₂·2H₂O, 1.6] and loose connective tissue and fat were carefully removed by dissection under a stereomicroscope. The arteries were then cut into 2 mm segments and mounted on a wire myograph (Danish MyoTech; Aarhus, Denmark) filled with 5 mL PSS and continuously gassed with 95% O₂–5% CO₂ while maintaining temperature at 37°C. Internal diameters of the IPA and CA were approximately 150 µm and 100 µm, respectively. The segments were stretched to a length resulting in a resting wall tension of 1.5 mN. The segments were allowed to equilibrate for a period of 60 minutes (buffer changes every 15 minutes). Arterial integrity was assessed by contracting the segments with a depolarizing concentration of potassium chloride (KCl, 120 mM) and phenylephrine (10^{−6} M) followed by relaxation with acetylcholine (ACh, 10^{−6} M). Cumulative concentration-response curves (CRCs) to ET-1 (10^{−10}–10^{−6} M) in the respective presence of vehicle (ddH₂O), atrasentan, an ET_AR antagonist (10^{−8} M) or Y-27632, a Rho-kinase inhibitor (10^{−6} M). Antagonists were administered 30 minutes before the start of CRCs.

Tissue Homogenization and Protein Expression

IPA were dissected, snap frozen in liquid nitrogen and stored at −80°C. Sample was homogenized in Radioimmunoprecipitation assay (RIPA) buffer containing protease

inhibitors (20 μ L/sample). The bicinchoninic acid (BCA) assay (Pierce Biotechnology; Rockford, IL, USA) was used to measure protein concentration. Protein (20 μ g) was loaded onto an SDS-PAGE 10% gel and transferred to a nitrocellulose membrane. Membranes were blocked with bovine serum albumin (5% in Tween 20/Tris-Buffered Saline [TTBS]) and subsequently probed with rabbit polyclonal anti-ET_AR (1:200; Alomone Laboratories), rabbit polyclonal anti-ET_BR (1:200; Alomone Laboratories), mouse polyclonal anti-RhoA (1:750; BD; Franklin Lakes, New Jersey, USA Transduction Laboratories), mouse polyclonal anti-Rho-kinase- α (1:250; BD Transduction Laboratories) or mouse polyclonal anti-Rho-kinase- β (1:250; BD Transduction Laboratories) overnight in at 4°C. The immunostaining was detected using horseradish peroxidase-conjugated anti-rabbit IgG (1:2,000; GE Healthcare) or anti-mouse IgG (1:2,500; GE Healthcare) for 1 hour at room temperature. Bands were revealed by the SuperSignal Substrate (Pierce Biotechnology) and quantified by densitometry imaging software. All densitometric measurements were normalized using an antibody against β -actin (1:20,000; Sigma Aldrich) as a loading control.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was extracted from IPA (N = 6) and CA (N = 3; each 'N' pooled from five animals, total of 15 animals) using the RNeasy kit (Qiagen Sciences, MD, USA). The quantity, purity, and integrity of all RNA samples were determined by NanoDrop spectrophotometers (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription was performed in IPA and CA (complete sample > 1 μ g) in a final volume of 50 μ L using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA), and single-strand cDNA was stored at -20°C. Primers for preproendothelin-1 (preproET-1) (N° Rn00561129_m1), ET_AR (N° Rn00561137_m1), ET_BR (N° Rn00569139_m1), RhoA (N° Rn005891722_m1), and Rho-kinase-2 (N° Rn00564633_m1) mRNA were obtained from Applied Biosystems. Real-time reverse transcriptase polymerase chain reaction (qPCR) reactions were performed using the 7500 fast Real-Time PCR system (Applied Biosystems) in a total volume of 20 μ L reaction mixture following the manufacturer's protocol, using the TaqMan fast universal PCR master mix (2 $\times$) (Applied Biosystems; Foster City, CA, USA), and 0.1 mM of each primer. Negative controls contained water in the place of first-strand cDNA. Each sample was normalized on the basis of its 18S ribosomal RNA content. The 18S quantification was performed using a TaqMan ribosomal RNA reagent kit (Applied Biosystems) following the manufacturer's protocol. Results were reported as Δ CT (change in cycle threshold), calculated by subtracting the average threshold cycle (CT) value of the transcript under investigation from the average CT value of the 18S rRNA control gene for each sample.

Data Analyses and Statistics

Arterial contractions recorded from the myograph were expressed as changes in the displacement from baseline in mN and were represented as percent maximum contraction to 120 mM KCl (% contraction). Agonist concentration-response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 4.0; GraphPad Software Inc., San Diego, CA, USA). $E_{\max}$ was determined by summation of all maximal contraction values to ET-1. Agonist potencies are expressed as pD₂ (negative logarithm of the molar concentration of agonist producing 50% of the maximum response). Constraining curve-fit parameters were used to fit a sigmoidal curve and to determine pD₂ values for vehicle, atrasentan and Y-27632 curves. Statistical analyses of $E_{\max}$ and pD₂ values were performed using Student's *t*-test. Values of *P* < 0.05 were considered statistically significant.

Results

ET-1 Reactivity in IPA and CA

In this set of experiments, ET-1-mediated contraction of IPA from female Sprague Dawley rats was evaluated. Percent maximal contractile response to ET-1 in IPA (Figure 1A, C: 156 ± 18 ; $pD_2 = 8.2 \pm 0.1$) and CA (Figure 1B, D: 163 ± 12 ; $pD_2 = 8.8 \pm 0.08$) were determined as a percentage of maximum contraction based on a reference concentration of KCl (120 mM). Pre-treatment of IPA and CA with an ET_AR antagonist, atrasentan (10^{-8} M), reduced maximal ET-1-mediated contraction, as well as produced a rightward shift in pD_2 values (Figure 1A, C: 104 ± 23 ; $pD_2 = 6.4 \pm 0.2$; Figure 1B, D: 112 ± 17 ; $pD_2 = 6.6 \pm 0.08$). Inhibition of ET_BR with the specific antagonist, BQ-788 (0.1 and 1.0 μ M, Tocris) did not reduce ET-1-mediated constriction (data not shown).

Addition of IRL-1620 (10^{-10} – 10^{-7} M), a specific agonist of ET_BR, did not result in vasorelaxation (following precontraction with phenylephrine, 10^{-6} M) nor vasoconstriction of IPA or CA (data not shown).

Rats were used during all phases of their menstrual cycle. Vaginal smears were taken from each animal. No differences were observed in ET-1 contraction during the different phases.

Y-27632, a Rho-Kinase Antagonist and ET-1-Mediated Contraction of IPA and CA

Using Y-27632, evaluation of Rho-kinase in ET-1-mediated contraction of IPA and CA from female Sprague Dawley rats was conducted. Maximal stimulation of IPA and CA with ET-1 was not significantly reduced in the presence of Y-27632 (10^{-6} M) (Figure 2A, C: 156 ± 17 vs. 108 ± 24 ; 2B and D: 158 ± 35 vs. 147 ± 58). Rho-kinase inhibition caused a significant rightward shift in pD_2 values in ET-1-mediated contraction in both IPA and CA (Figure 2E: 8.2 ± 0.1 vs. 7.9 ± 0.1 ; 2F: 8.8 ± 0.08 vs. 8.0 ± 0.25).

Protein Expression from IPA

In support of the observations made in the functional studies, Western blot analysis was utilized to determine and demonstrate protein expression of the ET_AR, ET_BR, RhoA and both isoforms of Rho-kinase from isolated IPA (Figure 3).

mRNA Expression from IPA and CA

qRT-PCR was used to determine mRNA expression of preproET-1, ET_AR, ET_BR, RhoA, and Rho-kinase from IPA and CA. The presence of preproET-1, ET_AR, ET_BR, RhoA and Rho-kinase was detected within IPA samples (Figure 4). Figure 5 shows detected preproET-1, ET_AR, and ET_BR mRNA from pooled CA. Data are expressed as Δ CT (targeted sample—18S sample).

Discussion

To date, the vascular contributions in FSD remain to be elucidated. The IPA and CA provide the main blood supply to the female genital tissue and enhancing our comprehension of the basic physiology of these arteries will increase our understanding of female sexual arousal response. This study demonstrates that the IPA and CA are sensitive to the potent vasoconstrictor peptide, ET-1, signaling through the ET_AR and activating Rho-kinase. In addition, our functional data are supported by protein analysis demonstrating the ET_AR, and ET-1-signaling components, RhoA and both Rho-kinase isoforms, α and β . Furthermore, IPA and CA contain mRNA message for both ET-receptor subtypes (ET_A and ET_B), the ET-1 precursor (preproET-1), RhoA and Rho-kinase. To the best of our knowledge, this is the first study to address vascular reactivity of the female IPA and CA to ET-1. Since ET-1

plays a role in ED [18] and a variety of cardiovascular diseases [29], it may be speculated that abnormal activation of the ET-1 system in the IPA and CA may contribute to the vasculogenic component of FSD.

The ET_AR is the primary receptor involved in ET-1-mediated contraction of the IPA, where as the ET_BR does not appear to play a role. This is not surprising in that ET_AR activation results in vascular smooth muscle contraction in several arterial beds [30]. We performed studies using an ET_BR antagonist (BQ-788, 0.1 and 1.0 μ M); however, we did not observe a reduction in ET-1-mediated effects, suggesting that ET-1 activates the ET_AR to induce contraction. This suggests that although ET_BR protein and mRNA was detected in these arteries (Figures 3–5), it does not play a role in ET-1-initiated contraction.

Rho-kinase, however is a key component in ET-1 signaling as our results demonstrated that inhibition of Rho-kinase in the IPA and CA reduced ET-1-mediated constriction (Figure 2). Rho-kinase inhibitors have been used in other female genital tissue investigations as well, most recently illustrating that ET-1-mediated activation of Rho-kinase negatively effected clitoral smooth muscle relaxation in female rabbits with experimentally induced overactive bladder [31]. This study further showed that ET-1 signaled through the ET_AR and inhibition of the ET_BR had no effect on ET-1-induced contraction, which is consistent with our observation in the IPA and CA. Other studies have also shown that Rho-kinase plays a role in vaginal wall and clitoral corpus cavernosum contractile state, both of which can be pharmacologically modulated by the Rho-kinase inhibitor, Y-27632 [32,33]. Taken together, ET-1 appears to contribute to smooth muscle contraction in genital vasculature, vagina and clitoris, and alterations in ET-1 signaling could have a deleterious effect on female sexual function.

The clitoris is an important sexual sensory organ found in both humans and animals. Parada et al. recently demonstrated that rats which received clitoral stimulation via delicate passing of a lubricated paintbrush resulted in increased conditioned place preference, a measure of the female rats ability to control the initiation/rate of copulation [34]. The clitoral stimulation was positively associated with activation of brain regions linked with reward and genitosensory input, suggesting that rat clitoral stimulation may indeed have similarities to human. The rat clitoris has been investigated in functional studies measuring clitoral relaxation mediated by a nitric oxide donor and cyclic guanosine monophosphate analog [35]. Additionally, clitoral morphological changes have been observed in spontaneously hypertensive rats [36], supporting clinical observations linking FSD and cardiovascular diseases, such as hypertension [23,37]. Clitoris from both rats and humans share similar innervation and vasculature [34], therefore studying rat sexual responses in these specific arteries is indeed a practical application for the study of FSD.

ET-1-mediated contraction via Rho-kinase throughout the systemic vasculature is well established [19,38–41] and supports our reported observations in the IPA and CA. We can appreciate that both clinically and in animal models, hypertension exacerbates both circulating and local levels, as well as constrictor sensitivity of ET-1 [42–45]. Therefore, we speculate that ET-1-mediated alterations in IPA and CA contractility may play a part in the vasculogenic component of FSD. Further experiments are necessary to elucidate the role of ET-1 in vasculogenic-mediated FSD, which require investigation of animal models of cardiovascular disease that display FSD phenotypes. Our study demonstrating the effect of ET-1 in the IPA and CA has initiated a new focus onto the genital vasculature that may be an effective therapeutic target for genital vascular-mediated FSD.

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Figure 1. Responses of internal pudendal (A) and clitoral (B) arteries to increasing concentrations of ET-1 (open circle = vehicle, closed circle = pretreatment with atrasentan 10⁻⁸ M). Maximal responses (E_{MAX} values) and potency (pD₂ values) for all conditions are represented as C–D and E–F, respectively. Data represent the mean ± SEM of N = 5. *P < 0.05 compared with vehicle.

Figure 2.

Responses of internal pudendal (A) and clitoral (B) arteries to increasing concentrations of ET-1 (open circle = vehicle, closed circle = pretreatment with Y-27632 10^{-6} M). Maximal responses (E_{Max} values) and potency (pD_2 values) for all conditions are represented as C–D and E–F, respectively. Data represent the mean $\pm$ SEM of $N = 6$ –8. $*P < 0.05$ compared with vehicle.

Figure 3. ET_AR, RhoA, Rho-kinase-α, and Rho-kinase-β protein expression in internal pudendal arteries. Densitometry values reported have been normalized to β-actin levels in all samples to account for differences in loading (N = 8).

Figure 4.

Messenger RNA expression of preproE-1, ET_AR, ET_BR, Rho-A and Rho-kinase determined by real time reverse-transcriptase polymerase chain reaction using total RNA extracted from internal pudendal arteries. Bar graphs show Δ CT values of preproET-1, ET_AR, ET_BR, Rho-A, and Rho-kinase. Values were normalized by the correspondent 18S rRNA of each sample. Results are mean $\pm$ SEM (N = 5).

Clitoral Arteries

Figure 5.

Messenger RNA expression of preproE-1, ET_A and ET_B receptors determined by real time reverse-transcriptase polymerase chain reaction using total RNA extracted from aorta (clear) and clitoral areteries (grey). Bar graphs show ΔCT values of preproET-1, ET_A and ET_B receptors. Values were normalized by the correspondent 18S rRNA of each sample. Results are mean $\pm$ SEM (N = 3; each 'N' pooled from 5 animals, total 15 animals).