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Effect of estrogen on molecular and functional characteristics of the rodent vaginal muscularis

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Abstract

Introduction—Vaginal atrophy is a consequence of menopause however little is known concerning the effect of a decrease in systemic estrogen on vaginal smooth muscle structure and function. As the incidence of pelvic floor disorders increases with age, it is important to determine if estrogen regulates the molecular composition and contractility of the vaginal muscularis.

Aim—The goal of this study was to determine the effect of estrogen on molecular and functional characteristics of the vaginal muscularis utilizing a rodent model of surgical menopause.

Methods—3–4 month old Sprague Dawley rats underwent sham laparotomy (Sham, n=18) or ovariectomy (Ovx, n=39). Two weeks following surgery, animals received a subcutaneous osmotic pump containing vehicle (Sham, OvX) or 17- β estradiol (Ovx). Animals were euthanized one week later and the proximal vagina was collected for analysis of contractile protein expression and in vitro studies of contractility. Measurements were analyzed using a one-way ANOVA followed by Tukey's post hoc analysis ($\alpha = 0.05$).

Main Outcome Measures—Protein and mRNA transcript expression levels of contractile proteins, in vitro measurements of vaginal contractility

Results—Ovariectomy decreased the expression of carboxyl-terminal myosin heavy chain isoform SM1 and *h*-caldesmon and reduced the amplitude of contraction of the vaginal muscularis in response to KCl. Estradiol replacement reversed these changes. No differences were detected in the % vaginal muscularis, mRNA transcript expression of amino terminal MHC isoforms, *I*-caldesmon expression and maximal velocity of shortening.

Conclusion—Systemic estrogen replacement restores functional and molecular characteristics of the vaginal muscularis of ovariectomized rats. Our results indicate that menopause is associated

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with changes in the vaginal muscularis, which may contribute to the increased incidence of pelvic floor disorders with age.

Introduction

Although it is well known that decreases in ovarian hormone levels with menopause lead to vaginal atrophy, the molecular and functional changes of the vaginal wall in response to ovarian hormone deprivation are only partially described. In order to further delineate the effect of ovarian hormones on vaginal structure and function, studies have been conducted with ovariectomized animals as a model of surgical menopause. Results of these studies have demonstrated changes in collagen distribution (1;2), abundance of nonvascular smooth muscle (3;4), steroid hormone receptor expression (5) and innervation (6) within the vaginal wall. Physiological changes such as reduced vaginal blood flow (7), decreased contractility of the distal vaginal muscularis (8;9), and altered biomechanical properties of the vagina (10) have also been demonstrated. These studies provide compelling evidence that vaginal changes with menopause may compromise pelvic organ support and female sexual function. Despite evidence of molecular changes within the vaginal muscularis of women with pelvic organ prolapse (POP), little is known regarding the effect of ovarian hormone loss on contractile protein expression within this non-vascular smooth muscle layer.

Myosin, the molecular motor of contraction, is a hexamer consisting of two heavy chains (myosin heavy chain: MHC), two phosphorylatable 20kDa light chains and two 17 kDa essential light chains. Four isoforms of MHC are formed by alternative splicing of a single gene (11–13). Alternative splicing at the 5' end of pre-mRNA forms two isoforms (SM-A, SM-B) of MHC that differ in their amino terminal sequence (11;13;14). The SM-B isoform contains a seven amino acid insert near the ATP binding site of the myosin head and has been shown to correlate with a higher rate of shortening velocity (an estimate of myosin ATPase activity) as compared to the SM-A isoform which lacks this insert (15–19). Alternative splicing of pre-mRNA at the 3' end results in the formation of isoforms that contain either a 9 amino acid (SM2) or 43 amino acid (SM1) carboxyl terminus sequence (12;20;21). Expression of SM1/SM2 within bladder, uterine and aortic smooth muscle has been reported to be regulated by estrogen (22–25). As smooth muscle demonstrates tissue heterogeneity, it is necessary to also determine the effect of ovarian hormone loss on MHC isoform expression within the vaginal muscularis. The functional significance of SM1/SM2 expression is unresolved (16;26–31) and it is therefore difficult to determine the consequence of hormonally mediated changes in MHC.

Expression of the thin filament associated protein, caldesmon, has also been shown to be hormonally regulated (32). Caldesmon exists as two isoforms formed by alternative splicing of a single gene. *h*-Caldesmon (high MW, 120–150 kDa) is a smooth muscle specific isoform and *l*-caldesmon (low MW, 70–80 kDa) is an isoform expressed in most vertebrate cells (33;34). Lin et al. (32) reported decreases in *h*-caldesmon expression within detrusor smooth muscle of ovariectomized rabbits, with time dependent changes in expression following estrogen replacement. Pregnancy has also been shown to alter *h*-caldesmon expression within the myometrium of both mice (35) and humans (36–38), lending further evidence of sex steroid regulation of this contractile protein.

Smooth muscle fibers from the vaginal wall have been demonstrated to be a component of the endopelvic fascia that attaches the vagina to the levator ani (39). This structural feature of vaginal smooth muscle and vaginal smooth muscle tone have been hypothesized to play a role in pelvic organ support. Human studies using biopsies obtained from the vaginal cuff of patients with POP have shown a decrease in the fractional area of smooth muscle within the vaginal muscularis (40–43) and altered contractility in response to KCl stimulation (40).

Interestingly, changes in caldesmon gene and protein expression within the vaginal muscularis of POP patients have also been reported although the results are conflicting (42;44;45). It is important to note that age, parity, and hormonal status are risk factors for POP. It is therefore possible that the discrepancy in results could be related to the difficulty in controlling for these factors in human studies. Further research is needed to clarify the effect of POP and menopause on the vaginal muscularis.

Regional differences have been reported between the proximal and distal vagina (18;46–50). Importantly, animal models of female sexual arousal have illustrated *in vivo* regional differences vaginal contractility, and histological studies have demonstrated that the vaginal muscularis is more abundant in the proximal vagina (18;51). Although ovarian hormone regulation of vaginal blood flow and distal vaginal contractility has been described, the effect of ovarian hormones on proximal vaginal contractility has yet to be determined. The goals of this study were to determine the molecular and functional changes of the proximal vaginal muscularis in a rodent model of surgical menopause and the efficacy of systemic estrogen replacement in reversing changes associated with the loss of ovarian function. We have focused our study on the ovarian hormone, estrogen, as it is the currently FDA approved hormone for treating vulvovaginal atrophy with menopause. Results obtained from this study will further our understanding of the effect of menopause on the female sexual response and pelvic organ support.

Materials and Methods

Animals

Animal use and the experimental protocol were approved by the Institutional Animal Care and Use Committee of Drexel University College of Medicine. Sham-operated (sham) and bilaterally ovariectomized (ovx) female Sprague-Dawley rats (3–4 months old, 250–300 grams) were obtained from a commercial supplier and housed in a temperature (25 °C) and light-controlled (12h light/12h dark) room, with free access to food and water. Two weeks post-surgery, an osmotic pump (Alzet, Model 2002) was placed subcutaneously between the scapulae containing either 0.9% saline (sham, ovx) or cyclodextran-encapsulated 17 β -estradiol (ovx). 17- β estradiol was replaced at a delivery rate of 10 μ g/kg/day. One week following pump placement, animals were heavily sedated with ketamine (75mg/kg) and xylazine (10mg/kg), the thoracic cavity was exposed, and blood was collected from the heart for analysis of serum levels of 17- β estradiol by RIA (Cornell University, Animal Health Diagnostic Center). Animals were then euthanized by exsanguination and the abdominopelvic cavity was exposed. Ovariectomy was confirmed visually and the uterus was dissected and weighed. The vagina was dissected and cleaned of connective tissue for molecular and physiological studies.

Vaginal Tissue Preparation

For histological procedures, the vagina was placed in Histochoice fixative (Amresco, Solon, OH) and paraffin embedded. For physiological and molecular studies, the vagina was cut open longitudinally and the proximal vagina (upper 2/3) was dissected from the distal vagina as outlined by Basha et al. (18). Proximal vaginal segments were either snap frozen in liquid nitrogen and stored at –80 °C or placed in ice-cold MOPS-buffered physiological salt solution (PSS) for same-day physiological studies. The PSS solution contained (in mM) 140 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.6 CaCl₂, 1.2 Na₂HPO₄, 2.0 3-(N-morpholino) propanesulfonic acid, 5.0 D-glucose and 0.02 Na₂-EDTA.

Histology

Cross sections of 5- μ m thickness were taken from the proximal end of the paraffin embedded vaginal tube (n=3 animals/group). Images of Masson's trichrome stained (MTS) sections were visualized with an Olympus BX60 microscope (Olympus America, Melville, NY) and captured with an Olympus DP70 camera (Olympus, America, Melville, NY).

Reverse Transcriptase and Polymerase Chain Reaction

RNA was extracted from frozen vaginal tissue segments (n=5 animals/group) and quantified as previously described (18). 1.0 μ g of RNA was reverse transcribed with oligo (dT) primer (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) at 37°C for 60 minutes. The reaction was stopped by heating to 90° C. PCR was performed with 1 μ l of cDNA in 49 μ l reaction mixture containing the following: upstream and downstream primers, 10 $\times$ PCR Buffer (Applied Biosystems, Foster City, CA), dNTP (Roche Applied Science, Germany) and *Taq* polymerase (Perkin-Elmer, Foster City, CA). The 5' to 3' sequences of primers were as follows:

SM-A/SM-B: CCA CAA GGG CAA GAA AGA CAG C (upstream); TCC GGC GAG CAG GTA GAA GA (downstream)

SM1/SM2: AGC AGG CAG AGA AAG GAA ACA CCA (upstream); GAA GTC TGA GTC CCG AGC GTC CAT (downstream)

Western Blot Analysis

Protein was extracted from 50–100 mg of frozen proximal vaginal segments (n=5 animals/group) and quantified as previously described (18). Samples were separated by SDS-PAGE and protein was transferred to Immobilon-P membranes (Millipore, Bedford, Massachusetts). Membranes were blocked with 5% non-fat milk for one hour in 0.1% PBST prior to antibody incubations. Membranes were washed 3 times for 5 minutes in 0.1% PBST following each antibody incubation. For analysis of MHC, 5 μ g of protein was separated using a 7.5% gel and the membrane was incubated for one hour in a 1:50,000 dilution of a polyclonal MHC antibody generated in rabbits against bovine aorta (gift of Dr. R. Adelstein, NIH, Bethesda, MD), followed by a one hour incubation in a 1:3,000 dilution of secondary antibody (NA934V, GE Healthcare, Piscataway, NJ). As an internal control, the membrane was incubated for one hour in a 1:5,000 dilution of β -actin primary antibody (A5316, Sigma, St. Louis, MO), followed by a one hour incubation in a 1:3,000 dilution of secondary antibody (NA931V, GE Healthcare, Piscataway, NJ). For analysis of caldesmon, 10 μ g of protein was separated using a 10% gel and the membrane was incubated in a 1:10,000 dilution of primary antibody (C0297, Sigma, Saint Louis, MO) for 2 hours, followed by a one hour incubation in a 1:3,000 dilution of secondary antibody (NA931V, GE Healthcare, Piscataway, NJ). As an internal control for caldesmon, the membrane was incubated for one hour in a 1:100,000 dilution of α -actin primary antibody (A2547, Sigma, Saint Louis, MO), followed by a one hour incubation in a 1:5,000 dilution of secondary antibody (NA931V, GE Healthcare, Piscataway, NJ). MHC, caldesmon, β -actin, and α -actin were detected using an enhanced chemiluminescence kit (RPN2109, GE Healthcare, Piscataway, NJ). Resultant autoradiograms were analyzed by scanning densitometry with quantitation software (Quantity One, Bio-Rad, Hercules, CA).

Coomassie staining for visualization of carboxyl terminal isoforms (SM1 and SM2) of MHC

SM1 and SM2 isoforms of MHC were separated electrophoretically and then visualized by Coomassie staining. Briefly, up to 100 μ g of protein was separated overnight by SDS-PAGE using a large format (16 $\times$ 16 cm) 7.5% gel. SM1/SM2 bands visualized by Coomassie blue

staining were then analyzed by scanning densitometry with quantitation software (Quantity One, Bio-Rad, Hercules, CA).

Isometric Force Measurements

Longitudinal strips of proximal vagina measuring $\sim 1.5 \text{ mm} \times 6 \text{ mm}$ were cut from the proximal vagina ($n=5$; each strip obtained from a different animal from each group). Each strip was mounted between a force transducer (Grass model FT.03) and a glass rod and immersed in water-jacketed organ baths containing PSS (37°C , aerated with 100% O_2). Strips were equilibrated for 90 minutes, during which time they were stretched to optimal length of contraction (L_o) as previously described (18). Following measurement of peak force to 110 mM KCl (equimolar substitution for NaCl), strips were placed in Histochoice fixative and embedded in paraffin for determination of cross-sectional area of muscle and tissue as follows. Cross-sections (5 μm thickness) were obtained from the center of each longitudinal strip. Images of MTS cross-sections were viewed and captured as described above and analyzed for muscle cross-sectional area and total cross sectional area using Image J software. The amplitude of each contraction at peak force was normalized to cross-sectional area of muscle. The % vaginal muscularis was calculated as the ratio of cross sectional area muscle/ cross sectional area strip.

Measurement of V_{max}

Longitudinal strips measuring $\sim 1.5 \text{ mm} \times 6 \text{ mm}$ were cut from the proximal vagina of ovariectomized rats ($n=3$; each strip was obtained from a different animal). Each strip was mounted on one end to a micrometer, via a plastic clip, for control of muscle length. The other end was attached to an aluminum foil tube connected to a servo-lever (Model 300H, Cambridge Technology) interfaced to a Linux operating system-based computer. The preparation was immersed in a water-jacketed muscle chamber containing PSS at 37°C and aerated with 100 % O_2 . V_{max} was calculated at the point of peak force generation in response to 110 mM KCl stimulation of tissue strips as previously described (18). Briefly, strips were stimulated with 110 mM KCl and subjected to a series of isotonic quick releases to afterloads equaling 5,10,15 and 20% of the force at the time of release. Isotonic shortening velocity at each afterload was determined by the change in length versus time 100 ms after release. The velocities of shortening calculated at these afterloads were used to extrapolate velocity at zero load for estimation of V_{max} .

Statistical analysis

All data are expressed as means $\pm$ S.D. Statistical analysis was performed using Graph Pad Prism software, ver 5 (GraphPad Software Inc., San Diego, CA). Measurements were analyzed using one-way ANOVA followed by post hoc analysis (Tukey's) when results of the one-way ANOVA indicated significance ($\alpha = 0.05$).

Results

Ovariectomized rats exhibited a significant increase in body weight, decrease in uterine weight, and decrease in serum 17- β estradiol concentrations as compared to sham animals. One week of estrogen replacement 14 days following ovariectomy resulted in uterine weights that were similar to sham animals, and partially returned body weight to normal. Serum 17- β estradiol concentrations of estrogen-replaced rats were not significantly different than sham animals (Table 1), indicating that our protocol resulted in physiological doses of estrogen. Qualitative assessment of MTS cross-sections of the proximal vagina verified that our animal model of menopause resulted in vaginal atrophy, with an overall thinning of the proximal vaginal wall. Estrogen replacement reversed this atrophy and wall thickness appeared similar to sham animals (Figure 1).

No significant differences were detected in the amount of MHC protein, or the ratio of amino-terminal isoforms of MHC (SM-A and SM-B) transcript expression within the proximal vagina of ovariectomized rats as compared to sham animals (data not shown). In contrast, analysis of mRNA transcript and protein expression for carboxyl terminal MHC isoforms demonstrated that the %SM1, quantified as $[SM1 / (SM1 + SM2)]$, was decreased in ovariectomized rats. Systemic administration of estrogen reversed the decrease in %SM1 (Figure 2 and Figure 3). Ovariectomy also had a significant effect on the expression of the thin filament associated protein, *h*-caldesmon with a two-fold reduction in *h*-caldesmon: α -actin ratio, an effect reversed by estrogen replacement (Figure 4). The antibody utilized for analysis of protein expression of caldesmon detected both *h*- and *l*-caldesmon isoforms. Bands for the *l*-isoform were detected with longer film exposures. No significant differences were detected in *l*-isoform expression amongst groups.

Morphometric analysis of the tissue strips used for physiological studies indicated that the total cross sectional area of the tissue strips, muscle cross-sectional area of the tissue strips, and percentage of smooth muscle within the proximal vagina were not significantly different amongst experimental groups (Table 2). Ovariectomy resulted in a significant reduction in peak force of 110 mM KCL induced contractions (Figure 5). A significant reduction in peak force was detected when the amplitude of contraction was expressed as raw peak force ($P < 0.05$), peak force/cross sectional area of the strips ($P < 0.01$), and peak force normalized to cross sectional area of muscle ($P < 0.001$). Estrogen replacement resulted in a return of these values to that of strips obtained from sham operated animals (Figure 6). Ovariectomy had no effect on myosin ATPase activity as estimated by V_{max} of the proximal vaginal strips. Values obtained using muscle strips from ovariectomized animals were similar ($0.10 \pm .01$ muscle lengths/sec) to our previous report of V_{max} of the proximal vagina from control rats (0.11 ± 0.04 muscle lengths/sec) using identical procedures (18).

Discussion

The results of this study demonstrate an estrogen dependence of contractile protein expression and functional characteristics of the rodent proximal vaginal muscularis. We report a significant effect of ovariectomy on carboxyl-terminal MHC isoform and *h*-caldesmon expression within the vagina. Additionally, physiological studies indicated a decreased force of contraction of the vaginal muscularis of ovariectomized rats. Systemic administration of physiological doses of 17- β estradiol reversed molecular and functional changes. These findings suggest that menopausal vaginal atrophy includes both molecular and functional changes of vaginal smooth muscle.

We report that ovariectomy resulted in thinning of the vaginal wall (Figure 1) with no detectable differences in MHC protein expression and α -actin protein expression across treatments. Estimation of cross sectional area of muscle and % vaginal muscularis indicated a slight reduction of smooth muscle within tissue strips obtained from the proximal vagina of ovariectomized animals although these changes were not significant (Table 2). Taken together these findings suggest that vaginal atrophy induced by decreases in ovarian hormones includes a decrease in the vaginal muscularis that is proportional to decreases in other layers of the vaginal wall resulting in little change of the calculated percentage of the wall composed of smooth muscle. Our results are in support of previous studies of the rabbit 4 and 12 weeks following ovariectomy (4;8) and 4 week ovariectomized rodents (51). Our results are also in support of findings obtained with human tissue as it has been shown that MHC mRNA transcript expression was not altered within the vaginal muscularis of postmenopausal compared to premenopausal women (45). On the other hand, MHC isoform expression of the vaginal muscularis was altered by estrogen status in our study. and our findings of a decrease in the %SM1 following ovariectomy (Figure 2 and Figure 3) with no

effect on N-terminal MHC isoform expression (SMA, SMB) corroborate the results obtained in studies utilizing smooth muscle from uterus (22;52), aorta (25), and bladder (23). Alterations in smooth muscle carboxyl-terminal isoform expression have also been reported in animal models of bladder outlet obstruction (53;54), diabetes (53), atherosclerosis (54) and hypertension (55). Despite reports of changes in carboxyl terminal isoform expression in both physiological and pathological conditions, the physiological relevance of the relative expression of SM1/SM2 isoforms is unresolved (16;26–31;56). Some (26;27), but not all (16;28;29), studies have demonstrated a positive correlation between the SM1 isoform and unloaded shortening velocity. Results are also conflicting regarding a role of carboxyl terminal isoforms on filament assembly (29;30;57). A recent study using SM2 knockout mice shows SM (+/–) male mice with relatively high ratio of SM1/SM2 are predisposed to develop urinary tract obstruction and hypercontractility of bladder smooth muscle upon aging (56). Thus, there is evidence that suggests a relationship between changes in MHC isoform expression and smooth muscle function in this study.

Consistent with a previous report showing a reduction of *h*-caldesmon within the detrusor muscle of 3 week ovariectomized rabbits (32), we detected a 2-fold decrease in the *h*-caldesmon : α -actin ratio within the proximal vagina of ovariectomized animals; an effect reversed by estrogen replacement. However, our finding that 1-week estrogen replacement restored *h*-caldesmon expression (Figure 4) is at odds with Lin et al. (32) who reported that 1-week estrogen replacement reduced *h*-caldesmon expression below that of control animals. This discrepancy may due to species differences, differences between the vaginal muscularis and detrusor muscle, or differences in dose of estrogen. The estrogen replacement protocol utilized by Lin et al. (58) resulted in estradiol levels > 5-fold higher than control animals compared to physiological doses in our present study. Ovarian hormone regulation of caldesmon expression has also been demonstrated in uterine smooth muscle with an upregulation of myometrial *h*-caldesmon expression reported late in gestation in both animal (35;59) and humans (36–38). As *h*-caldesmon has been shown to inhibit myosin ATPase activity (see (60) for review), upregulation of this protein has been postulated to be a molecular mechanism to maintain quiescence of the myometrium during gestation.

Alterations in caldesmon expression within the vaginal muscularis of women with POP have been reported although results are conflicting, with both an increase in *h*-caldesmon mRNA transcript and protein expression (44;45) and a decrease in *h*-caldesmon mRNA transcript expression (42) detected within the vaginal muscularis. An increase in *h*-caldesmon expression has also been reported within the uterosacral ligament of women with POP (61). Given the results reported herein, it is likely that the discrepancy is related to the difficulty in controlling for hormonal status, age, and parity in human studies. Indeed, Bortolini et al. (45) demonstrated that caldesmon mRNA transcript expression is downregulated in vaginal cuff biopsies obtained from healthy postmenopausal women compared to healthy premenopausal women. Our result of decreased vaginal *h*-caldesmon protein expression in ovariectomized rats, and reversal of this down regulation within the vaginal muscularis following estrogen replacement, supports and expands upon this finding by demonstrating estrogenic regulation of *h*-caldesmon protein expression. Upregulation of *h*-caldesmon mRNA transcript expression has been reported in vaginal cuff biopsies obtained from postmenopausal POP patients compared to postmenopausal control subjects (45), with no differences detected between premenopausal POP patients and control subjects. This study sheds light on the finding of Northington et al. (40) who failed to detect differences in KCl induced contractile force (force/ mm² muscle) of the vaginal muscularis of premenopausal POP and control subjects. Taken together, these findings highlight the need to control for hormonal status when conducting studies of vaginal changes in patients with POP and suggest that the pathophysiology of POP may be distinct between premenopausal and postmenopausal women.

It remains to be determined if changes in *h*-caldesmon expression within the vagina is a cause or a consequence of prolapse. It is unclear if increased *h*-caldesmon expression may be a compensatory response to POP in postmenopausal women or representative of a decompensated state of the vaginal muscularis. There is evidence that mechanical stretch may regulate *h*-caldesmon expression (53;62) and phosphorylation of caldesmon, which modulates caldesmon's inhibitory effect on myosin ATPase activity (38;63). Therefore, future studies determining the effects of stretch on the vaginal muscularis in intact and ovarian hormone deprived animals may help to further elucidate the relationship between prolapse, menopause, *h*- caldesmon, and *h*-caldesmon phosphorylation.

It has been reported that ovariectomy decreases contractility of detrusor smooth muscle (58) and the distal rat vagina (9) with supraphysiological replacement of estrogen increasing force of contraction of these tissues. In light of regional differences between the proximal and distal vagina, we focused our investigation on the proximal vagina to facilitate comparison of results obtained with human biopsies obtained from the vaginal cuff. We report a decreased peak force, peak force/cross-sectional area of tissue strip, and peak force/cross-sectional muscle area of the proximal vagina of ovariectomized rats in response to KCl stimulation, with physiological estrogen replacement returning force of contraction to sham values (Figure 5 and 6). Our finding of decreased force normalized to cross sectional area of muscle demonstrate that molecular changes within the smooth muscle layer contribute to compromised contractility of the proximal vagina of ovariectomized animals. However, as our current report describes changes in more than one contractile protein, it is difficult to identify the impact each molecular change has upon vaginal smooth muscle function. Nevertheless, our findings provide evidence that menopause may be associated with compromised contractility of the proximal vaginal muscularis due both to a reduction in the smooth muscle layer and to changes in its molecular composition. As animal models of female sexual arousal have demonstrated contraction of the proximal vagina in response to pelvic nerve stimulation (64;65), results of this study indicate that menopause induced changes in the proximal vaginal muscularis may contribute to increased female sexual dysfunction with age. Ongoing studies in our laboratory are investigating the effect of ovarian hormones on sympathetic and parasympathetic agonist induced force of the rodent vaginal muscularis.

There are limitations to our study. Vaginal tissue from all sham-operated animals was collected one week following vehicle replacement in order to be consistent with the time point of tissue collection from ovariectomized and estrogen replaced animals. We therefore did not control for stage of estrus cycle in sham animals, which may have contributed to variability in our data from the control group.

In summary, our results demonstrate an estrogen dependence of contractile protein expression and contractility within the rodent proximal vaginal muscularis. Our novel finding of estrogenic regulation of *h*-caldesmon within the vaginal muscularis is of particular clinical significance as altered *h*-caldesmon expression has been reported within the vaginal muscularis of patients with POP (42;44). Future studies should determine the efficacy of vaginal estrogen replacement on reversing ovariectomy or age-dependent changes within vaginal smooth muscle in light of recommendations for local delivery of estrogens for vulvovaginal atrophy with menopause and dyspareunia (66;67). In addition, as previous studies have demonstrated androgen receptor expression within the vaginal muscularis (5), and changes in vaginal muscularis structure following androgen replacement (51), further research determining androgenic regulation of vaginal smooth muscle is warranted.

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Figure 1.

Representative images (200×) of Masson's trichrome stained cross sections of the rat proximal vagina obtained from sham operated (sham); ovariectomized (Ovx) and ovariectomized with 1-week 17- β estradiol treatment (Ovx + E). Ovariectomy resulted in an overall thinning of the vaginal wall, which was reversed by 17- β estradiol replacement. VM, vaginal muscularis; LP, lamina propria; E, epithelium.

Figure 2.

RT-PCR analysis of mRNA transcripts for the carboxyl terminal isoforms (SM1 and SM2) of myosin heavy chain in the proximal vagina of sham operated (Sham), ovariectomized (Ovx) and 17- β estradiol treated ovariectomized (Ovx + E) rats. A: Ethidium-bromide stained agarose gel of SM1 and SM2 amplification. B: Statistical analysis of the ratio of the OD of SM1/ (SM1 + SM2) using a one-way ANOVA indicated a significant effect of experimental groups ($P < 0.05$). Post hoc analysis demonstrated a significant decrease in the % SM1 in OvX rats compared to sham rats. The effect of ovariectomy on carboxyl terminal mRNA transcripts was reversed by estrogen replacement. Values are means $\pm$ S.D.; $n=5$.

Figure 3.

Analysis of protein expression of the COOH terminal isoforms (SM1 and SM2) of myosin heavy chain (MHC) in the proximal vagina of sham operated (sham, n=5), ovariectomized (ovx, n=5) and 17- β estradiol treated (Ovx + E, n=5) rats. A. Representative Coomassie stained gel demonstrating the separation of SM1 (upper band) and SM2 (middle band) isoforms of MHC. The lower band represents non-muscle myosin. B. Statistical analysis of the OD of SM1/(SM1 + SM2) using a one-way ANOVA indicated a significant effect of experimental groups ($P < 0.001$) and was followed by post hoc analysis. Ovariectomy resulted in a significant decrease in the % SM1 ($P < 0.001$), which returned to sham levels following one week of 17- β estradiol treatment. Values are means $\pm$ S.D.; n=5.

Figure 4.

Western blot analysis of *h*-caldesmon expression within the proximal vagina of sham operated (Sham), ovariectomized (Ovx) and ovariectomized with one week of 17-β estradiol treated (Ovx + E, n=5) rats. A. Representative membrane of *h*-caldesmon, *l*-caldesmon and α-actin. B. Post hoc analysis of a significant one-way ANOVA indicated that ovariectomy significantly decreased the *h*-caldesmon/α-actin compared to sham operated rats (P<0.01). *h*-caldesmon protein expression returned to sham levels following one week of 17-β estradiol replacement to ovariectomized animals. No significant differences were detected in *l*-caldesmon expression. Values are means ± S.D.; n=5.

Figure 5.

Representative tracings of the contractile response of rodent proximal vaginal tissue strips to 110 mM KCl. Strips were obtained from the proximal vagina of Sprague-Dawley rats that were sham-operated (Sham), ovariectomized (Ovx), and ovariectomized with 1-week 17- β estradiol replacement (Ovx + E).

Figure 6.

Peak force in response to 110 mM KCl stimulation of proximal vaginal tissue strips obtained from rats that underwent a sham operation (sham, n=5), ovariectomy (ovx, n=5) or ovariectomy with one week of 17- β estradiol replacement (Ovx + E, n=5). A. Peak force (mN) B. Peak force normalized to cross-sectional area of tissue strips (mN/mm²) C. Peak force normalized to cross-sectional area of muscle of tissue strips (mN/mm²). Ovariectomy resulted in a significant decrease in the amplitude of contraction compared to sham operated animals when peak force was expressed as mN, mN/mm² tissue strip and mN/mm² muscle, an effect reversed by estrogen replacement. Values are means $\pm$ S.D.; n=5.

Body weights, uterine weights and serum 17-β estradiol levels of Sprague Dawley rats that underwent one of three experimental protocols: sham operation and vehicle treatment (Sham), ovariectomy and vehicle treatment (Ovx), ovariectomy and 17-β estradiol treatment (Ovx + E).

Table 1

Experimental Group	Day 1:Surgery	Day14:Osmotic Pump	Day 21: Tissue and Blood Collection		
			Body Weight (g)	Uterine Weight (g)	Serum Estradiol (pg/mL)
Sham	Sham-operation	0.9 % Saline	285 ± 22	0.536 ± 0.142	45 ± 17
Ovariectomized	Ovariectomy	0.9 % Saline	327 ± 18 ***	0.162 ± 0.020 ***	27 ± 1 *
Estrogen replacement	Ovariectomy	17-β estradiol (delivery rate:10 ug/kg/day)	306± 21 *	0.608 ± 0.114	48 ± 14

Values are means ± SD. Rat weights and uterine weights were recorded for all animals (N=57). Serum estradiol was measured for a subset of animals (n=6 for each group) to verify estrogen replacement at a physiological dose.

*** P < 0.001 compared with sham operated animals.

* P < 0.05 compared with sham operated animals.

Table 2

Estimated total cross-sectional area of tissue strips, cross-sectional area of vaginal muscularis, and calculated percentage of muscle of tissue strips obtained from the proximal vagina of female Sprague –Dawley rats that were sham-operated (Sham) ovariectomized (Ovx), and ovariectomized with 1-week 17- β estradiol treatment (Ovx +E). Values are mean $\pm$ S.D.; n=5.

Experimental Group	Cross-sectional Area Tissue Strip, mm ²	Cross-sectional Area Muscle, mm ²	Cross-Sectional Area Muscle/Cross-sectional Area of Strip, %
Sham-operated	1.570 $\pm$ 0.389	0.148 $\pm$ 0.029	9.65 $\pm$ 1.68
Ovariectomized	1.516 $\pm$ 0.248	0.132 $\pm$ 0.023	8.82 $\pm$ 1.29
Ovariectomized + Estradiol	1.718 $\pm$ 0.485	0.147 $\pm$ 0.037	8.69 $\pm$ 1.05
	N.S.	N.S.	N.S.