Estrogen modulates the expression of myosin heavy chain in detrusor smooth muscle

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Sanchez-Ortiz, Ricardo F., Ze Wang, Chandra Menon, Michael E. DiSanto, Alan J. Wein, and Samuel Chacko. Estrogen modulates the expression of myosin heavy chain in detrusor smooth muscle. Am J Physiol Cell Physiol 280: C433–C440, 2001.—The effect of low serum estrogen levels on urinary bladder function remains poorly understood. Using a rabbit model, we analyzed the effects of estrogen on the expression of the isoforms of myosin, the molecular motor for muscle contraction, in detrusor smooth muscle. Expression of myosin heavy chain (MHC) isoforms, which differ in the COOH-terminal (SM1 and SM2) and the NH2-terminal (SM-A and SM-B) regions as a result of alternative splicing of the mRNA at either the 3'- or 5'-ends, was analyzed in age-matched female rabbits that were sham operated, ovariectomized (Ovx), and given estrogen after ovariectomy. These changes were assessed by measuring total mRNA in Ovx, control, and estrogen-treated rabbits, respectively (P < 0.04), which was reversed by estrogen replacement (P < 0.02). MHC content, as a proportion of total milligram of protein in the bladder tissue extracted, was also increased in estrogen-treated Ovx rabbits. Quantitative competitive RT-PCR revealed 1.72-, 2.63-, and 5.82 × 105 copies of MHC mRNA/100 ng total mRNA in Ovx, control, and estrogen-treated rabbits, respectively (P < 0.01). RT-PCR analysis using oligonucleotides specific for the region containing the SM1/SM2 MHC alternative splice sites indicated a lower SM2-to-SM1 ratio in estrogen-treated compared with control and Ovx rabbits (P < 0.05). Similarly, SDS-PAGE analysis of extracted myosin from estrogen-treated rabbits revealed a significantly lower SM2-to-SM1 isoform ratio compared with control and Ovx rabbits (P < 0.05). Expression of the SM-A and SM-B isoforms was not affected. These results indicate that myosin content is increased upon estrogen replacement in Ovx rabbits and that the abundance of SM1 relative to SM2 is greater in estrogen-treated rabbits compared with normal and Ovx rabbits. These data suggest that estrogen affects alternative splicing at the 3'-end of the MHC pre-mRNA to increase the proportion of SM1 vs. SM2.

The presence of estrogen receptors in the human, baboon, and rabbit urinary bladder and urethra is well established (4, 17). Studies with rabbit, rat, and guinea pig urogenital tissues have demonstrated that estrogen increases bladder and urethral weight as well as urogenital tissue perfusion via vascular smooth muscle relaxation and modulates the cholinergic receptor density in the urinary bladder (3, 29). However, the precise effect of estrogen on urinary bladder contractility and cholinergic innervation remains controversial (11, 22). In an analysis of bladder function after ovariectomy (Ovx), Longhurst et al. (23) found an increased responsiveness to nerve stimulation, ATP, carbachol, and KCl in estrogen-treated rats compared with Ovx rats. On the other hand, Batra and Andersson (3) reported that prolonged estrogen treatment in Ovx rabbits resulted in a decrease in muscarinic receptor density in the urinary bladder, consistent with another study in which bladders were analyzed after 1, 4, and 8 wk of hormone treatment (29). However, this decrease in receptor density was not reflected in significant differences between estrogen-treated and non-estrogen-treated rabbits in contractile responses after field stimulation or stimulation with carbachol, although a difference in contractility was found when muscle strips were stimulated with K+ depolarization (23). Estrogen has been shown to display Ca2+ antagonist and K+ channel opening activities, leading to relaxation of bladder smooth muscle in guinea pigs (30, 37). These studies suggest that alterations in contractile machinery or in ion channel protein expression can mediate changes in contractility. Although several studies had evaluated contractile protein expression in uterine smooth muscle (6–9, 15, 16), no studies had evaluated the effects of estrogen on the expression of myosin isoforms in urinary bladder smooth muscle.

Although there is only one smooth muscle myosin gene, alternative splicing of the pre-mRNA produces myosin isoforms that differ at the COOH-terminal or the NH2-terminal region (1, 2). The COOH-terminal region isoforms, SM1 and SM2, differ in the presence of a 39-nucleotide insert in the 3'-end coding region of the myosin heavy chain (MHC) mRNA (2). Because this insert contains a stop codon in its sequence, the mRNA transcript containing the insert, SM2, is translated.
into a smaller size protein (200 kDa) than its noninserted counterpart, SM1 (204 kDa). Two additional MHC isoforms are also formed by alternative splicing in the 5′-end coding region of the MHC mRNA transcript: SM-B, encoded by an mRNA containing a 21-nucleotide insert in the 5′-end coding region, which translates a seven-amino-acid sequence in the NH2-terminal globular head of the myosin molecule near the ATP-binding region; and the SM-A, which lacks this seven-amino-acid insert (1).

The functional significance of MHC isoform expression is now beginning to be understood. Work by Kelley et al. (18) showed that smooth muscle myosin containing predominantly SM-B has higher actin-activated ATPase activity and moves actin filaments in the in vitro motility assays at a faster rate than the noninserted SM-A isoform. Smooth muscle tissues from aorta (12) and the corpus cavernosum penis (13), which exhibit high basal tone, contain predominantly the SM-A isoform, whereas the phasic bladder smooth muscle contains predominantly the SM-B isoform. The actin-activated ATPase activity of myosin isolated from small muscular arteries, containing predominantly SM-B, is two-fold higher than that of myosin isolated from aorta, which is composed of SM-A (12). Moreover, the maximum shortening velocity of smooth muscle that contains predominantly SM-A is about two-fold less than that of smooth muscle containing mainly SM-B (12). However, a difference in maximum velocity of shortening (Vmax) in colonic muscle was not associated with a change in the relative abundance of SM-B isoforms (31). Using SDS-PAGE, photoaffinity labeling, and immunoelectron microscopy, Cai et al. (6) showed that a 13-amino-acid peptide, mimicking a region of the tail unique to the SM1 isoform bound to the S2-light meromyosin hinge region of myosin, and the presence of this peptide inhibited the shortening velocity and calcium sensitivity of skinned Taenia-Coli. Rovner et al. (27), using recombinant SM1 and SM2, showed that the SM1 has a lower critical concentration for myosin filament assembly and that filaments formed by SM1 are more stable than those formed by SM2. In addition, a positive correlation between active stress generation and SM2 isoform content has been reported (16). Thus data from in vitro experiments suggest that the composition of both COOH-terminal (16, 27) and NH2-terminal isoforms (12) in a smooth muscle source may influence its biochemical characteristics. However, the exact role of these isoforms in regulating contractile activity remains to be established.

Using the rabbit animal model, we investigated the effect of estrogen withdrawal and replacement on the expression of myosin isoforms in urinary bladder wall smooth muscle (detrusor). Here, we report a decrease in total myosin expression in the detrusor after estrogen depletion by ovariectomy and a reversal of this decrease and an upregulation of the SM1 myosin isoform upon estrogen replacement. Relative overexpression of SM1 may have an effect on the assembly of myosin molecules into stable myosin filaments in myocytes in the bladder wall of estrogen-treated animals. Part of the data from this study were presented at the Annual Meeting of the American Urological Association (28).

METHODS

Rabbits. Twelve age-matched adult female New Zealand White rabbits were divided into the following three groups: sham operated plus placebo, ovariectomized (Ovx) plus placebo, and Ovx plus a 21-day release of 250 mg 17β-estradiol pellet (Innovative Research of America) as previously described (23). Placebo pellets contained all components except the active product. Surgical procedures were performed under anesthesia with pentobarbital sodium (25 mg/kg iv) and ketamine-xylazine (8.2 mg/kg im) through a lower midline abdominal incision. Pellets were placed subcutaneously in the interscapular region. Nubain (0.1 mg/kg iv as needed) was given for analgesia. Rabbits were killed 3 wk after surgery with an injection of beuthanasia-6 solution (0.5 ml iv). Urinary bladders were cut transversely at the level of the ureteral orifices and the bladder body was frozen immediately in liquid nitrogen for analysis. All experiments performed in this study were approved by the Institutional Animal Care and Use Committee.

Protein extraction. Frozen smooth muscle tissue from the bladder body was pulverized while immersed in liquid nitrogen using a mortar and pestle and homogenized using a minipolytron (Brinkmann, Westbury, NY) in extraction buffer [10 mM Tris-HCl (pH 6.8), 50% glycerol, 1% SDS, 50 mM dithiothreitol (DTT), and 0.02% bromphenol blue]. Muscle residue in the pellet was reextracted to ensure complete extraction of muscle proteins. The muscle homogenate was boiled for 2–3 min and centrifuged at 15,000 g for 10 min, and the supernatant was collected. SDS was removed from the homogenate by dialysis in buffer containing 10 mM imidazole, 0.002% NaN3, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 1 µM antipain to avoid interference with the protein determination. Total extractable protein was measured by the Lowry method (24) using BSA as standard.

SDS-PAGE and Western blot. MHC isoforms SM1 and SM2 were separated by electrophoresis as described (20) except that the SDS-PAGE were highly porous (4.5% acrylamide and 0.065% bisacrylamide). Except for quantitative analysis, gels were usually stained in 0.5% Coomassie brilliant blue (R-250) at 80°C for 30 min and destained with a 30% methanol-7.5% acetic acid solution. For quantitative analysis, gels were stained overnight at room temperature with 0.5% Coomassie brilliant blue to ensure saturated dye binding and destained with 30% methanol-7.5% acetic acid solution. Band intensities for SM1 and SM2 were quantified by densitometric scanning using a standard curve produced with purified pig urinary bladder myosin. Western blot analysis was performed to confirm the presence of smooth muscle MHC isoforms using an antibody (catalog no. M7786; Sigma Chemical, St. Louis, MO) specific for smooth muscle MHC.

DNA quantitation. Total DNA content was determined according to Burton (5). Frozen bladder tissue was weighed and homogenized in ice-cold water, and 50% TCA was added to obtain a 7% final concentration. After centrifuging the mixture at 10,000 rpm for 15 min, the pellet was resuspended in 4 ml of ice-cold 5% TCA, centrifuged again, and resuspended in 3 ml of 95% ethanol. After centrifugation, the pellet obtained was resuspended in 3 ml of 5% TCA and heated at 90°C for 15 min. The mixture was centrifuged again, 1.0 ml of supernatant was mixed with 2 ml of diphe-
nylamine reagent (1.5 mg diphenylamine in 100 ml glacial acetic acid, 1.5 ml concentrated sulfuric acid, and 0.1 ml of 1:6% aqueous acetaldehyde) and incubated 15–17 h at 25°C, and absorbance was read at 600 nm. A standard curve was generated with known amounts of salmon sperm DNA.

**RNA isolation and RT-PCR.** Bladder tissue samples were weighed, pulverized in liquid nitrogen, and homogenized, and RNA was extracted by the guanidinium thiocyanate method with a kit by Stratagene (La Jolla, CA) as described (10). Total RNA was isolated from urinary bladder tissue from control, Ovx, and Ovx plus estrogen-treated rabbits. RNA concentrations were measured spectrophotometrically. Total RNA (3 μg) was reverse-transcribed by incubation at 37°C with 200 units Superscript II RNase H−RT (GIBCO-BRL, Rockville, MD), 0.5 μg oligo(dT) (15) primer (Promega, Madison, WI), 0.01 M DTT, 1× first strand buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl2], 5 units RNase inhibitor (GIBCO-BRL), and 0.5 mM dNTP mix. After inactivating the RT by brief heating at 90°C, 2 μl of the cDNA was amplified by PCR in a 100-μl reaction mixture containing the following: 0.1 μg each of upstream and downstream primers for the 3′ (P3 and P4, respectively)- or 5′ (P1 and P2, respectively)-end regions encoding the myosin COOH-terminal tail and NH2-terminal head regions of the myosin (see Fig. 1, A and B), 2.5 units of ampli Taq DNA polymerase (Perkin-Elmer, Norwalk, CT), 1× PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, and 0.001% (wt/vol) gelatin), and 0.1 mM dNTPs. Cycle conditions were as follows: 50 s at 94°C, 90 s at 55°C, and 150 s at 72°C for 30 cycles with a final 7-min extension period at 72°C. PCR products were electrophoresed in a 2% agarose gel prepared in 1× Tris-Acetate EDTA buffer, stained with ethidium bromide (0.5 μg/ml), and quantified by densitometric scanning.

**Quantitative competitive RT-PCR.** A competitive internal standard for quantitative determination of rabbit smooth muscle MHC transcript was generated as described (13). The competitor RNA contained the target sequence present in SM1 and SM2 transcripts and the 90-nt caldesmon intron fragment (see mutant smooth muscle MHC in Fig. 3A). Total RNA was extracted from urinary bladder tissue from control, Ovx, and Ovx estrogen-treated rabbits as described above. Next, seven to nine aliquots containing a constant amount of rabbit total RNA (0.1 μg) were coreverse-transcribed with varying concentrations (determined by ultraviolet spectrophotometry) of competitor cRNA (26). RT reactions were terminated by heating the samples at 90°C for 5 min. The resulting cDNAs were immediately subjected to competitive PCR (see Fig. 3B). PCR mixtures contained 20 μl of reverse-transcribed product (of 100 μl total volume), 1× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM MgCl2, and 0.01% gelatin), 0.1 mM dNTPs, 0.1 μl each of upstream and downstream primers (as shown in legend for Fig. 3), and 2.5 units of Taq DNA polymerase (Perkin-Elmer). Cycle conditions were as described above except that 25 cycles were used. PCR products were run in a 2% agarose gel and stained with ethidium bromide (0.5 μg/ml), and band intensities were quantified as described above. The absolute copy number of MHC mRNAs for each sample as determined by quantitative competitive (QC) RT-PCR (see Fig. 3C), together with the data from relative RT-PCR were used to determine the absolute copy number of individual MHC mRNAs (e.g., a bladder sample containing 2 × 10^6 mRNA copies/100 ng total RNA, as determined by QC RT-PCR, and containing 50% SM1, as determined by relative RT-PCR, would contain 1 × 10^6 mRNA copies of SM1 and 1 × 10^6 copies SM2 mRNA/100 ng total RNA). The cycle numbers for RT-PCR and QC PCR were selected to fall within the logarithmic range of amplification.

**Statistical analysis.** Values are given as means ± SD, with the number of observations in parentheses. Differences between sham-operated, Ovx, and Ovx estrogen-treated rabbits were compared using one-way ANOVA. *P < 0.05 was considered significant.

## RESULTS

**Effects of estrogen on body weight, bladder wet weight, and DNA content.** Estradiol levels measured at the time of death were 33.8 ± 4.2 pg/ml for sham-operated, 15.1 ± 1.4 for Ovx, and 372.1 ± 59.7 for Ovx estrogen-treated rabbits (n = 4 in each group; P < 0.01). Baseline rabbit body weights were not significantly different between groups (control: 3.04 ± 0.07 kg, Ovx: 3.01 ± 0.12 kg, Ovx estrogen-treated: 3.06 ± 0.16 kg). At the time of death, there were no statistically significant differences in body weight between groups or within the same groups before and after treatment (control: 3.10 ± 0.10, Ovx: 3.12 ± 0.09, Ovx estrogen-treated: 3.21 ± 0.15 kg). As shown in Table 1, mean bladder body wet weight in Ovx estrogen-treated rabbits (2.64 ± 0.25 g) was significantly greater than in controls (1.56 ± 0.07 g) or Ovx rabbits (1.61 ± 0.04 g; P < 0.02). The total DNA per wet milligram bladder muscle (24.9 ± 0.8 μg/mg) in Ovx animals was signif-

### Table 1. Summary of biochemical and molecular analyses

<table>
<thead>
<tr>
<th>Determinations</th>
<th>Control</th>
<th>Ovariectomy</th>
<th>Ovariectomy Plus Estrogen</th>
<th>Statistical Significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder wet weight, g</td>
<td>1.56 ± 0.07</td>
<td>1.61 ± 0.04</td>
<td>2.64 ± 0.25</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Total RNA/bladder wet weight, ng/mg</td>
<td>125.3 ± 8.7</td>
<td>75.4 ± 2.4</td>
<td>187.4 ± 9.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total RNA/total protein, ng/μg</td>
<td>3.24 ± 0.8</td>
<td>3.23 ± 0.11</td>
<td>5.51 ± 0.12</td>
<td>&lt;0.04*</td>
</tr>
<tr>
<td>MHC mRNA/cell weight, copies/mg</td>
<td>208.9 ± 8.9</td>
<td>228.12 ± 7.9</td>
<td>310.6 ± 13.2</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>MHC mRNA copies/100 ng total mRNA</td>
<td>2.63 × 10^6 ± 0.3</td>
<td>1.72 × 10^6 ± 0.2</td>
<td>5.82 × 10^6 ± 0.06</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SM2-to-SM1 mRNA ratio</td>
<td>2.54 ± 0.08</td>
<td>2.51 ± 0.06</td>
<td>1.23 ± 0.04</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Total protein/bladder wet weight, mg/g</td>
<td>38.6 ± 3.8</td>
<td>28.7 ± 3.6</td>
<td>34.0 ± 2.2</td>
<td>NS</td>
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<tr>
<td>MHC protein/cell wet weight, mg/g</td>
<td>3.20 ± 0.45</td>
<td>1.71 ± 0.27</td>
<td>5.15 ± 0.62</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MHC protein/total protein, mg/mg</td>
<td>0.082 ± 0.002</td>
<td>0.059 ± 0.003</td>
<td>0.15 ± 0.08</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SM2-to-SM1 protein ratio</td>
<td>2.81 ± 0.07</td>
<td>3.38 ± 0.05</td>
<td>1.66 ± 0.05</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

Values are means ± SD. MHC, myosin heavy chain; SM, smooth muscle. *No statistically significant differences were seen between the control and ovariectomy groups. NS, not significant.
significantly lower than in control (35.4 ± 5.4 μg/mg) or Ovx estrogen-treated rabbits (36.6 ± 6.0 μg/mg; P < 0.04).

**Effect of estrogen on MHC mRNA expression.** The relative ratios of the mRNAs for SM1 and SM2 (formed by alternative splicing at the 3′-end) and for SM-A and SM-B (alternatively spliced at the 5′-end) were determined by RT-PCR using the oligonucleotide primers shown in Fig. 1A, which specifically amplify the regions containing these splice sites. As shown in Fig. 2A, the relative ratio of SM2-SM1 transcripts was similar for Ovx and control rabbits (lanes 2 and 3), whereas this ratio was altered in Ovx estrogen-treated rabbits (lane 1). Quantification of band intensities in the ethidium bromide-stained gels indicated a significantly lower MHC SM2-to-SM1 ratio in Ovx estrogen-treated rabbits (1.23:1 ± 0.04) compared with control (2.54:1 ± 0.08) and Ovx rabbits (2.51:1 ± 0.06; P < 0.01). No differences were found in the expression of SM-A and SM-B mRNAs: transcripts in all three groups contained ~100% myosin with the 21-nt insert (Fig. 2B).

There were significant differences in the amount of total RNA isolated per milligram bladder tissue. Ovariectomy resulted in an overall decrease in total RNA per bladder tissue (75.4 ± 2.4 ng/mg bladder tissue) compared with sham-operated rabbits (125.3 ± 8.7 ng/mg bladder muscle). As shown in Table 1, estrogen supplementation reversed this change (187.4 ± 9.8 ng/mg bladder muscle; P < 0.05). When total RNA was expressed per total protein, estrogen supplementation resulted in a significant increase in total RNA per protein (5.51 ± 0.12 ng total RNA/mg total protein; P < 0.04) compared with controls (3.24 ± 0.8 ng total RNA/mg total protein) and Ovx rabbits (3.23 ± 0.11 ng total RNA/mg total protein).

Absolute concentrations of the mRNAs encoding the various myosin isoforms were measured by QC RT-PCR using a mutant cRNA that contained a 90-nt region of intron 7 of mouse caldesmon as competitor (Fig. 3A). A representative gel and the corresponding data analysis for one sample are shown in Fig. 3, B and C. As shown in Fig. 4, A and B, MHC mRNA expression in Ovx rabbits (1.72 ± 0.2 × 10^6 mRNA copies/100 ng total RNA) was lower than controls (2.63 ± 0.3 × 10^6 mRNA copies/100 ng total RNA; P < 0.05). Estrogen supplementation reversed this change, and SM-MHC mRNA expression (5.82 ± 0.06 × 10^6 copies/100 ng total RNA) was significantly higher than in Ovx rab-
bits and controls ($P < 0.01$). Abundance of both SM1 and SM2 mRNA transcripts was decreased in Ovx rabbits, and estrogen replacement increased the expression of both transcripts compared with the control and Ovx animals. Moreover, the relative amount of SM1 mRNA transcript was higher in Ovx estrogen-treated rabbits (Fig. 4B), consistent with the RT-PCR data (Fig. 2).

**Effect of estrogen on MHC protein expression.** The SM1 and SM2 isoforms were separated by SDS-PAGE (Fig. 5A), and protein bands were identified by Western blotting (Fig. 5B). Consistent with the findings from analyses of MHC mRNA expression, ovariectomy caused a decrease in the amount of MHC in bladder smooth muscle ($1.71 \pm 0.27$ mg/g) compared with controls.

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**Fig. 3.** Quantitative competitive (QC)-PCR analysis of MHC mRNA. A: mutant cRNA containing a portion of the sequence for rabbit smooth muscle MHC (SMMHC) present in SM1 and SM2 transcripts plus a 90-nt caldesmon intron fragment were transcribed (13). B: known amounts of mutant cRNA were mixed with total RNA, and the mixture was used to perform QC RT-PCR with appropriate upstream (5'-TG-GCCACAGAGCGCAG-3') and downstream (5'-TCAGCTTCTCCCTCT-3') primers that amplify the region between residues 5402 and 5659, a region that is common to smooth muscle MHC SM1, SM2, SM-A, and SM-B. Representative ethidium bromide-stained agarose gel from a PCR performed on bladder smooth muscle using upstream and downstream primers. C: bands in B were scanned using a densitometer, and the data were plotted. The cDNA produced from the competitor cRNA possessed exactly the same primer binding sites as the endogenous SMMHC. The cycle number was kept low (25 cycles) to ensure amplification in the logarithmic range. At the point at which the endogenous and the competitor cDNA amplify equally log(mutant (m) SMMHC/SMMHC) = 0, the endogenous SMMHC is equal to the mSMMHC. Because the amount of cRNA used to obtain equal amplification of the endogenous and the mutant cDNA is known, the copy number of the endogenous mRNA will be the same as the copy number of the cRNA.

**Fig. 4.** Quantitative determination of total MHC mRNA and the ratios of mRNAs for MHC isoforms in bladder smooth muscle tissues. A: MHC mRNA expression in Ovx rabbits ($1.72 \pm 0.2$ relative mRNA copies/100 ng total RNA) was lower than in controls ($2.63 \pm 0.3$ copies/100 ng total RNA; $P < 0.02$). Estrogen (ET) supplementation of Ovx rabbits reversed this change and induced significantly higher expression of MHC mRNA ($5.82 \pm 0.6$ copies/100 ng total mRNA) than in Ovx rabbits ($P < 0.01$) and controls ($P < 0.05$). B: SM2 and SM1 mRNA copy numbers were determined from data derived from gels in Fig. 2A (for SM2-to-SM1 ratio) and Fig. 3B (for total copy number). Relative SM2-to-SM1 mRNA ratios in control (N) and Ovx rabbits were similar (2.54:1 and 2.51:1 respectively, $P > 0.05$), but the SM2-to-SM1 ratio for ET (1.23:1) differed significantly ($P < 0.01$).
trols (3.20 ± 0.45 mg/g; P < 0.02; Fig. 5C and Table 1). Estrogen replacement in Ovx animals reversed this change, significantly increasing the amount of myosin per gram of tissue above that of controls (5.15 ± 0.62 mg/g; P < 0.02). These differences also persisted when the amount of bladder smooth muscle MHC protein was expressed as a function of total extractable protein. There was a significant decrease in the amount of bladder smooth muscle MHC per total protein in Ovx rabbit bladders (0.059 ± 0.003 mg MHC/mg total protein) compared with sham-operated rabbits (0.082 ± 0.002 mg MHC/mg total protein). Estrogen replacement reversed this change (0.15 ± 0.08 mg MHC/mg total protein; P < 0.05).

Quantification of the SM1 and SM2 bands in the SDS-PAGE gel of myosin extracted from Ovx estrogen-treated rabbits revealed an SM2-to-SM1 isoform ratio of 1.66:1 ± 0.05 compared with 2.81:1 ± 0.07 for controls and 3.38:1 ± 0.05 for Ovx rabbits (P < 0.05; Fig. 5D). Thus estrogen treatment of Ovx rabbits induced the relative overexpression of SM1 at both the mRNA (Fig. 4B) and protein levels (Fig. 5D). These data are summarized in Table 1.

**DISCUSSION**

Estrogen has been suggested to modulate urogenital tissue perfusion, as well as cholinergic and adrenergic innervation of the urinary bladder (3, 17, 29). In the present study, we analyzed the effect of ovariectomy and ovariectomy plus estrogen supplementation on the expression of smooth muscle myosin isoforms. The mean wet weight of the bladder body for Ovx estrogen-treated rabbits was significantly higher than that of Ovx and control rabbits. The total DNA per milligram wet bladder tissue in Ovx rabbits was decreased compared with controls and Ovx estrogen-treated rabbits, suggesting a decrease in cell number in response to ovariectomy. In this context, Tompkinson et al. (36) showed that loss of estrogen induces apoptosis of osteocytes in rats. The observed reversal of the total DNA decrease in rabbit bladder tissue by estrogen is consistent with the known effect of estrogen on DNA synthesis (35). Ovariectomy also led to an overall decrease in the total RNA per milligram bladder tissue, which was reversed by estrogen supplementation (from 75.4 ng to 187.4 ng/mg wet weight of the bladder tissue). The increase in total RNA after estrogen treatment of Ovx rabbits was also evident when total RNA was expressed per total extractable protein. Stimulation of RNA synthesis has been shown to parallel nuclear translocation of the estrogen-receptor complex in rat pituitary glands (34).

QC RT-PCR analyses revealed lower levels of MHC mRNA in Ovx rabbits than in normal animals (1.72 ± 0.2 vs. 2.63 ± 0.3 × 10⁶ copies/100 ng total RNA).

**Fig. 5. MHC isoform protein expression in rabbit bladder smooth muscle. A: SDS-PAGE analysis of total protein extracts from urinary bladder smooth muscle, using a 4.5% highly porous SDS polyacrylamide gel. Lanes 1–3 correspond to normal female rabbit, Ovx, and Ovx estrogen-treated (ET), respectively. SM1 and SM2 were separated electrophoretically as described in METHODS and visualized by Coomassie brilliant blue staining. B: Western blot analysis of smooth muscle myosin SM1/SM2 using an antibody against smooth muscle myosin heavy chain. C and D: level of MHC protein expression and SM2-to-SM1 ratios as determined by densitometric scanning of the gel in A in control (N), Ovx, and Ovx-ET rabbits, respectively. Relative SM2-to-SM1 protein ratios in the three groups (N: 2.81:1, Ovx: 3.38:1, and ET: 1.66:1) differed significantly (P < 0.05).**
Estrogen supplementation of Ovx rabbits induced about a threefold increase in MHC mRNA/100 mg total RNA (Fig. 4, A and B). The mRNAs for both SM1 and SM2 were increased, although the rise in SM1 mRNA transcript was greater than that of the SM2 (5-fold vs. ~2.5-fold). There was no change in the expression of SM-B or SM-A isoforms, formed by alternative splicing at the 5’-end of the MHC pre-mRNA, in either Ovx or in Ovx estrogen-treated rabbits.

The action of estrogen in other systems is known to be mediated through the interactions of the ligand-receptor complex with DNA hormone response elements of target genes (25); however, very little is known about the transcriptional regulation of myosin isoforms. The increase in the SM1-to-SM2 isoform ratio might reflect the effect of estrogen on alternative splicing at the 3’-end of the MHC pre-mRNA. Alternatively, estrogen may induce the proliferation and/or differentiation of a population of smooth muscle cells that inherently expresses more SM1 than SM2. In that case, a decrease in SM1 only would be expected in Ovx rabbits. However, Ovx bladders showed a decrease in both SM1 and SM2, and the ratio of SM2 to SM1 was similar (2.54:1 vs. 2.51:1, respectively, in normal and Ovx rabbits compared with that in Ovx estrogen-treated rabbits (1.23:1; Fig. 4B and Table 1), with a preponderance of SM1. Further experiments using antibodies specific for SM1 and SM2 may shed light on this question.

Overexpression of SM1 was evident at both the mRNA and protein levels. Such relative overexpression of SM1 protein in estrogen-treated rabbits may have an effect on the myosin filament assembly, consistent with recent studies using recombinant SM1 and SM2 isoforms (6, 27). The abundance of the SM1 isoform might induce the assembly of more stable myosin filaments to form the thick filaments in the contractile apparatus that could optimize contractility of the bladder smooth muscle. Indeed, a correlation between the composition of the COOH-terminal isoform and mechanical parameters has been reported in rat myometrium (16).

Ovx rabbits showed a decreased SM1-to-SM2 ratio in the myometrium, and this effect was reversed after estrogen treatment (7). However, in contrast to the findings in bladder smooth muscle, where we found no significant change in the ratio of SM1 to SM2 mRNA between normal and Ovx and an increase (from ~0.4 to 0.9) in the SM1-to-SM2 ratio in the estrogen-treated Ovx, the ratio of SM1 to SM2 in myometrium of Ovx estrogen-treated rabbits was similar to that of control animals. The SM1-to-SM2 protein ratio was also high in the estrogen-treated rabbit bladders compared with Ovx and normal. This difference in the expression of myosin isoforms might rest in the use of different sources of smooth muscle. As in our present study, Capriani et al. (8) found no change in the expression of the NH2-terminal isoforms, SM-A and SM-B. By contrast, Calovini et al. (7) reported a downregulation of SM-A in the myometrium after ovariectomy, and estrogen supplementation reduced SM1-B to an undetectable level while the total SM1 (both SM1-B and SM1-A) remained constant. Those investigators suggested that estrogen increases SM1-A and decreases SM1-B to similar extents. Again, the source of the smooth muscle may account for a lack of effect on the expression of SM-B/SM-A isoforms in our study.

As suggested by the low level of mRNA transcript for SM-A isoforms, there was no difference in the SM-B isoforms after Ovx and estrogen supplementation (Fig. 2B). If the SM-B heavy chain is the major determinant for the $V_{\text{max}}$ in the detrusor (32), then this parameter is not expected to be affected by Ovx or estrogen treatment. In fact, no significant difference in the unloaded shortening velocity was found after maximal thio-phosphorylation of permeabilized hypertrophied colonic smooth muscle, despite a 5- to 10-fold decrease in SM-B isoform levels when compared with normal colon (31). Moreover, studies have shown that the 17-kDa light chain (LC17) isoform composition (16), myosin light chain kinase and phosphatase activities (33), actin (19), and caldesmon (14, 21) affect the contractile properties of smooth muscles from various sources. Future studies are needed to assess the direct or indirect effect of these proteins on $V_{\text{max}}$.

The effect of estrogen on the expression of smooth muscle myosin isoforms may be direct or indirect, by activating factors that in turn induce increased expression or stabilization of SM1 mRNA. The total MHC mRNA per gram wet bladder tissue in Ovx estrogen-treated rabbits was greater than that in normal or Ovx animals. Although the total myosin and SM1 per gram bladder tissue was also increased in response to estrogen treatment, a greater increase was observed in the mRNA than in the protein levels. It remains unclear whether the overexpression of SM1 results from enhanced transcription or from altered message stability and translation. The precise effect of the estrogen-induced increase in SM1 abundance on bladder function awaits further study.

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