Alteration in expression of myosin isoforms in detrusor smooth muscle following bladder outlet obstruction


Alteration in expression of myosin isoforms in detrusor smooth muscle following bladder outlet obstruction. Am J Physiol Cell Physiol 285: C1397–C1410, 2003. First published July 30, 2003; 10.1152/ajpcell.00513.2002.—Partial urinary bladder outlet obstruction (PBOO) in men, secondary to benign prostatic hyperplasia (BPH), induces detrusor smooth muscle (DSM) hypertrophy. However, despite DSM hypertrophy, some bladders become severely dysfunctional (decompensated). Using a rabbit model of PBOO, we found that although DSM from sham-operated bladders expressed nearly 100% of both the smooth muscle myosin heavy chain isoform SM-B and essential light chain isoform LC$_{17a}$, DSM from severely dysfunctional bladders expressed as much as 75% SM-A and 40% LC$_{17b}$ (both associated with decreased maximum velocity of shortening). DSM from dysfunctional bladder also exhibited tonic-type contractions, characterized by slow force generation and high force maintenance. Immunofluorescence microscopy showed that decreased SM-B expression in dysfunctional bladders correlated with overexpression of SM-A and LC$_{17b}$.

Increased urethral resistance during micturition (compensation), whereas other rabbits show bladder dysfunction (decompensation), despite smooth muscle (SM) hypertrophy (26, 56). Physiological studies using muscle strips from hypertrophied DSM from decompensated bladder show a decrease in force in response to bethanechol or field stimulation (32). Furthermore, in vitro whole bladder studies have shown that the rate of emptying is significantly reduced and the time required to completely empty is significantly increased (32, 33). The molecular mechanism(s) responsible for these changes in contractility and emptying remain largely unknown.

Myosin II is the molecular motor for contraction in all types of muscles. In general, the velocity of force generation by the muscle is determined by the ATPase activity of the myosin II when it interacts with actin (7). SM myosin is a type II myosin that is composed of a pair of myosin heavy chains (MHCs) and two pairs of myosin light chains (MLCs). The MHCs wrap around the actin filament to produce force (1). Unlike skeletal and nonmuscle myosin, the mRNAs that encode the four vertebrate SM MHC isoforms identified to date originate from a single SM MHC gene through two alternative splice mechanisms. Two MHC isoforms, SM1 (204 kDa) and SM2 (200 kDa), which are different at the COOH-terminal region, are formed by alternative splicing at the 3’ end (5). Previous studies by us and others have shown that the ratio of SM2 to SM1 switches from ∼1.7:1 in the normal rabbit bladder DSM to ∼1:1 in the DSM from decompensated bladder (11, 61). A recent study shows that increasing SM1/SM2 ratio in bladder has a significant effect on contractile function (49), possibly by stabilizing the thick filament through tail region interaction with the head of an adjacent myosin molecule (51).

Alternative splicing at the 5’ end of the pre-mRNA also produces two MHC isoforms, SM-A and SM-B, which are different at the NH$_2$-terminal region (4). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: S. Chacko, 3010 Ravdin-Courtyard, HUP, Univ. of Pennsylvania, 3400 Spruce St., Philadelphia, PA 19104 (E-mail: chackosk@mail.med.upenn.edu).

http://www.ajpcell.org 0363-6143/03 $5.00 Copyright © 2003 the American Physiological Society

C1397
C1398  MYOSIN ISOFORM CHANGES IN SMOOTH MUSCLE HYPERTROPHY

SM-B isoform contains a 7-amino acid insert near the ATP-binding site (encoded by a 21-nucleotide insert in the mRNA), and this insert is absent in the SM-A isoform (3, 29). The presence of SM-B in the avian gizzard SM myosin correlates with increased actin-activated Mg\(^{2+}\)-ATPase activity and an increased velocity of movement of actin filaments in vitro compared with aortic SM myosin, which lacks this insert (29). The presence of SM-B isoform in small muscular arteries has also been correlated with increased maximum shortening velocity of SM (13). Furthermore, Babu et al. (6) reported that the absence of SM-B isoform in SM-B knockout mouse leads to decreased shortening velocity of the bladder SM.

In general, more SM-B is found in SMs that show a strong phasic-like response to stimulation (e.g., urinary bladder, gastrointestinal SM, etc.), whereas greater amounts of SM-A are present in SMs that are more tonic (e.g., aortic SMs) (13, 25, 29, 55). The SM-B isoform content increases as the external iliac artery branches into small muscular distributing arteries concomitant with increased maximum velocity of shortening in these arteries (13). Tonic SMs show slow rates of force activation and relaxation and slow speeds of shortening (V\(_{\text{max}}\)) but high force maintenance, whereas phasic SMs show low force maintenance but have fast V\(_{\text{max}}\) and rapid rates of force activation and relaxation (for review, see Ref. 19).

One of the pairs of MLCs, known as the essential light chains, also exist as two different isoforms generated via alternative splicing of a single gene (30, 43). These two isoforms, known as LC17a and LC17b, are identical except for five of the last nine carboxyl-terminal amino acids (21). Because a greater ratio of LC17b to LC17a is found in tonic-type muscles (e.g., aorta) than in more phasic muscles (e.g., bladder), it has been proposed that the LC17a/LC17b isoform composition in a particular SM may determine its tonicity. However, conflicting data exist as to whether it is the essential light chain composition or the SM-A/SM-B ratio that has a more important functional role.

Hasegawa and Morita (20) showed that by increasing the amount of LC17b in aortic SM myosin, they could lower the actin-activated ATPase activity of aortic SM myosin. Similarly, Matthew et al. (40) found that replacing the predominant isoform of the urinary bladder (LC17a) with LC17b caused a significant decrease in the unloaded shortening velocity of nonphosphorylated, slowly cycling cross bridges. Also, Morano et al. (41) showed that although no SM-B myosin isoform could be identified in either the normal or the hypertrophied myometrium, LC17a expression increased from ~25% in normal to 44% in hypertrophied myometrium. Because both the maximum force of isometric contraction of skinned myometrial fibers and the apparent maximum shortening velocity were increased approximately twofold in the hypertrophied myometrium compared with normal, this study also suggested that the essential light chain composition may be more important than the SM-A/SM-B myosin isoform composition in determining the contractile properties of the SM muscle. Finally, Huang et al. (23) showed that overexpressing either the LC17a or LC17b isoform in single chicken aortic or gizzard cells could alter the rate of force activation.

In contrast to the above studies, Kelley et al. (29) have shown that replacing the essential light chains of turkey aorta myosin (approximately equal amounts of LC17a and LC17b) with gizzard essential light chain (LC17a) did not affect the average velocity of movement of actin filaments. Also, Sweeney et al. (57), using recombinantly expressed SM myosin proteins, showed that they could modulate ATPase activity up to twofold by altering the amino acids in the SM-B insert region while keeping the 17-kDa isoform composition the same. Using a baculovirus/insect cell expression system, Rovner et al. (52) also showed that SM myosin with the insert possessed twofold greater in vitro motility and twofold higher enzymatic activity. Also, the SM-B knockout mice described above showed no changes in LC17 isoform composition but a decrease in the velocity of shortening, supporting a functional role for SM-A/SM-B composition independent of LC17 composition (6). Finally, Eddinger and Meer (16), using isolated single SM cells, found that the unloaded shortening velocity correlated positively with SM-B expression, whereas this same group found no correlation between LC17 isoform composition and unloaded shortening velocity (15, 54).

The goal of the present study was to determine whether the alteration in contractile characteristics of the partially obstructed bladders that are decompensated, despite DSM hypertrophy, is due to changes in the composition of the isoforms of myosin II. We have shown that 1) PBOO-induced hypertrophy of the DSM in decompensated bladders is associated with an overexpression of NH\(_{2}\)-terminal SM MHC isoform SM-A and essential MLC isoform LC17b; 2) overexpression of SM-A and LC17b correlates with decreased void volume and increased voiding frequency; 3) decrease in the SM-B isoform is not due to an emergence of cells that lack SM-B; and 4) DSM hypertrophy and increases in SM-A and LC17b isoforms that occur in decompensated bladders return toward normal upon removal of the obstruction. Thus the slow force generation and increased duration of force maintenance in the DSM from decompensated bladder are associated with a reversible increase in the expression of SM-A and a partially reversible increase in the expression of LC17b isoforms.

MATERIALS AND METHODS

Partial Bladder Outlet Obstruction and Reversal

The use of rabbits, including the surgical procedure, was approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and Children’s Hospital of Philadelphia. PBOO is created in adult male New Zealand White rabbits (weight 2.5–3.0 kg) by a modification of the procedure by Malkowicz et al. (38). After sedation with ketamine (35 mg/kg im) and shaving, deep anesthesia was induced with 4% isoflurane by face mask with a 1/min flow of oxygen. Next, 20 mg/kg gentamycin and 0.075–1.0 mg/kg
buprenorphine were given via intramuscular injection once anesthesia was induced. Betadine prep was carried out over the perineum, the bladder was catheterized with an 8-F coude tip catheter, and sterile drapes were applied. A 3-cm lower abdominal incision was made, the rectus fascia was divided in the midline, and the perirethral space was entered. The peritoneum was not violated during this approach and was swept up superiorly with a retractor blade, providing good exposure of the bladder neck and urethra. Exposure was enhanced with a self-retaining retractor system. At this point, a right angle clamp was used to encircle the proximal urethra just below the bladder neck. Great care was taken to separate the seminal vesicles from the posterior aspect of the bladder. At this point, a 2-0 silk suture was passed around the urethra. An extra section of 8-F catheter was placed outside the urethra, and the silk suture was tied down over both the urethra (containing an intraluminal 8-F catheter) and the external 8-F catheter. The use of these two catheters helps to standardize the grade of obstruction. Once the silk suture was tied down, the abdomen was closed with a running 3-0 Vicryl suture. Upon completion of the procedure, a fentanyl patch (25 mg/16 h) was applied to a shaved area on the animal’s neck to provide for postoperative pain control. Survival rates have exceeded 90%. Sham surgery was identical in all aspects, except that the silk suture was cut and removed after it had been tied down. For reversal surgery, the animals were anesthetized as before, a small incision was made parallel to the original incision, and the ligature was removed. The bladder was then catheterized, and the urine was removed. The incision was closed, and the rabbit was allowed to recover.

Five days after surgical obstruction, obstructed, sham-operated, and normal rabbits were kept in metabolic cages (Kent Scientific, Torrington, CT) for two days to monitor the voiding pattern noninvasively. After one day for adjustment of the animals to the metabolic cages, voided urine was monitored by collection in a container kept on digital scales interfaced to a computer. Scales were monitored at 2-min intervals. Data were transferred to a spreadsheet for analysis. Frequency data were expressed as voids per 24 h, and average voided volume was expressed in milliliters per void. For experiments reported in this article, we used five types of metabolically defined bladders: 1) normal bladders from age- and size-matched male rabbits; 2) bladders from age- and size-matched sham-operated male rabbits (sham); 3) 7-day obstructed bladders that showed decapsulation, based on increased urinary frequency (43 ± 12 voids per 24 h compared with 4 ± 3 voids per 24 h), decreased voided volumes (2.5 ± 1 ml/void compared with 26 ± 16 ml/void) and increased bladder mass (18.8 ± 0.7 g compared with 2.3 ± 0.4 g); 4) bladders from 7-day obstructed rabbits that showed bladder decapsulation before reversal surgery but recovered 7 days after surgery; and 5) bladders from 7-day obstructed bladders that showed bladder decapsulation before reversal surgery but recovered 14 days after surgery. The urinary frequency of the group 5 animals returned to 5 ± 4 voids per day after reversal surgery. Sham-operated and/or normal rabbits served as controls. For our correlation studies, a group of 12 bladders randomly selected from 7-day obstructed rabbits was used.

Tissue Preparation

Urinary bladder SM layers were obtained from the five groups of rabbits listed. The bladder body was separated from the base at the level of the ureters, and then the mucosal and serosal layers were removed. Only the bladder body (detrusor) was used for this study. Physiological studies were always completed the same day the tissue was harvested. For biochemical analyses, the cleaned tissue was either used immediately or frozen and stored in liquid nitrogen until used. Tissue stored for more than 3 wk was not used for this study.

Force Measurement

Strips of bladder muscle (~2 × 10 mm, ~50 mg) were suspended in a longitudinal manner in 10 ml of Tyrode buffer at 37°C, and the length of optimal force development (Lo) was determined as previously described (25). The strips were always obtained from the ventral midbody region (between the dome and base) of the bladder and were cut longitudinally from dome to base. These longitudinal strips of bladder SM layer were used for depolarization with high-KCl solution (125 mM) to evaluate tonic and phasic properties. Force was measured as described (25).

RNA Extraction and RT-PCR

RNA was isolated and reverse transcribed, and PCR was carried out to analyze the expression of SM MHC and the 17-kDa essential light chain isoforms as previously described (13). The PCR primer pairs and the predicted sizes of their amplified cDNAs were as follows: SM-A/SM-B (upstream: 5′-TACAGGAGCATGTGCTG-3′; downstream: 5′-TGCGGAG-TGCTCGTGA-3′; size: 337/358 bp) and LC17a/LC17b (upstream: 5′-GACCGTGCCAGAAGACAA-3′; downstream: 5′-CAGCCATTCCAGCACCATC-3′; size: 232/276 bp). Aliquots (20 μl) of the PCR products were electrophoresed on a 2% agarose gel and visualized after ethidium bromide staining. Band intensities were quantitated by reflectance scanning of the corresponding photograph of the gel using a Bio-Rad GS-700 imaging densitometer (Hercules, CA) and subsequent analyses using the Bio-Rad Quantity One program. The identity of the PCR products was confirmed by either Southern blot analysis or sequencing. The linear range of amplification was determined for all PCR primer pairs, and only reactions that fell into this range were used for quantitation.

Quantitative Competitive RT-PCR

A competitive internal standard was prepared by using an RT-PCR Competitor Construction Kit (Ambion, Austin, TX). The competitor was designed from a region common to all four SM MHC isoforms (SM1/SM2/SM-A/SM-B) but contained an internal deletion that was produced using a PCR strategy (25). Briefly, an upstream primer was designed with the sequence 5′-CGCTATAACGACTICTATAGGGAGGAGG-AGGGCCTCAAAAGTCAGTTCAGCAGAAGGAGAAAGAGGAGG-3′, where nt 1–3 serve as a GC clamp, nt 4–20 represent a core T7 promoter sequence, and nt 31–50 and 51–70 correspond to nt 4806–4825 and 4851–4871, respectively, of the rabbit uterus SM MHC full-length cDNA coding sequence. In vitro transcripción of the PCR product produced using the above primer and an appropriate downstream primer pair yields a competitor RNA molecule spanning from nt 4806 to nt 4871 but lacking 25 residues from nt 4826 to nt 4850. A second primer pair was designed, in which the upstream primer 5′-GGCGGTTAAATGCGATAGTGTTGC-3′ corresponds to only nt 4806–4825 of the rabbit SM MHC and the downstream primer 5′-CTCCATCCGCTTATGTATG-3′ corresponded to nt 5004–5023. Amplification of the competitor using this second primer pair in the presence of rabbit SM cDNA produced two bands of 266 bp (endogenous myosin) and 241 bp (competitor myosin).
Thus, to determine SM MHC copy number, total RNA was extracted from the experimental groups as described in RNA Extraction and RT-PCR. Six aliquots of each sample of rabbit total RNA (400 ng) were co-reverse transcribed with various concentrations (determined by measuring \[^{32}\text{P}\]ATP incorporation) of competitor RNA, and PCR was carried out. The exact number of SM MHC copies was determined by graphical analysis by determining the exact point at which equal amounts of PCR product are produced from competitor and endogenous RNA target, from which, since we know the exact amount of exogenous cDNA inputted at that point, we can then calculate the endogenous SM MHC mRNA level (10).

**Antibodies**

A 7-amino acid peptide with the sequence (QGPSLAY) identical to that deduced from the 21-nt insert in the 5' end of the rabbit MHC mRNA was conjugated to keyhole limpet hemocyanin (KHL), and a polyclonal antibody was raised in BALB/c mice as previously described (14). Immunoblotting and immunofluorescence studies on aortic and bladder SMs have confirmed the specificity of this antibody (14). An antibody against the whole MHC and secondary antibodies were purchased from Sigma Chemical (St. Louis, MO).

**Gel Electrophoresis and Western Blot Analysis**

Bladder tissue was ground in a mortar precooled in liquid nitrogen, and a fine powder was made without allowing the tissue to thaw. The powder was then mixed with the extraction buffer (0.05 M Tris, pH 6.8, 20% glycerol) and protease inhibitors (10) at a ratio of 100 mg powder/ml buffer and then homogenized on ice with an Ultra-Turrax T8 minihomogenizer (IKA Works, Wilmington, NC) at top speed twice for 30 s. The mixture was then brought to 1% SDS, 25 mM dithiothreitol, and 0.003% bromphenol blue and boiled for 5 min. The extracted muscle proteins present in the supernatant were separated from cell membrane and connective tissue fractions by centrifugation (13,000 g for 20 min). The MHCs SM1 and SM2 were separated by electrophoresis of the supernatant on large (16 × 16 cm) 4.5% SDS-polyacrylamide slab gels (1 mm thick) as previously described (60). Coomassie brilliant blue-stained proteins were quantified by scanning densitometry of the gel using the Bio-Rad GS-700 imaging densitometer and Bio-Rad Quantity One program described above. Standard curves were constructed for myosin absorbance to ensure that the sample loadings produced values in the linear response range of Coomassie blue staining. The relative ratio of SM2 to SM1 heavy chains was then estimated from the areas under the peaks of SM2 and SM1.

**Immunofluorescence Microscopy**

Paraffin sections were prepared from formalin-fixed (10% phosphate buffered) 7-day obstructed bladder wall. Sections (5 µm) were deparaffinized and processed for immunofluorescence microscopy using antibody specific to the 7-amino acid insert (present only in SM-B myosin isoform) as described below. After two washes with PBS, the slides were reacted with FITC-labeled anti-mouse IgG raised in goat. Sections stained for immunofluorescence microscopy were analyzed using a Leica TCS SP2 laser scanning confocal microscope.

Isolated cells were prepared from 7-day obstructed DSM by mincing the tissue into small pieces (2 × 2 mm) and then treating them overnight with collagenase (0.1 mg/ml) in nutrient medium (M199 with 1% penicillin/streptomycin without fetal calf serum). After treatment with collagenase (for 12–14 h overnight), the tissue pieces were flushed several times in a Pasteur pipette until the pieces disappeared. Dissociated cells were collected by centrifugation (3,000 rpm for 10 min) in a refrigerated centrifuge (Beckman). The cell pellet was disaggregated in nutrient medium and centrifuged again. The final pellet was resuspended in nutrient medium containing 10% fetal calf serum and a high concentration (15 mM) of thymidine to prevent cell division. Glass coverslips were coated with collagen (a drop of 1 mg/ml rat tail collagen in 5% acetic acid; Sigma Chemicals) and kept in 60-mm tissue culture dishes overnight under UV light to polymerize the collagen. These dishes were rinsed with balanced salt solution to remove the acid, and the cells were plated in the culture dishes and fed with nutrient medium containing 10% fetal calf serum and 15 mM thymidine and incubated overnight. The next day, cells were attached on the collagen substratum coated on the coverslip. These cultures were fixed in 5% buffered formalin, kept in the refrigerator for 4 h, and processed for immunofluorescence microscopy.

Fixed cells were washed three times with PBS for 2 min each time, and incubated in blocking solution (1% BSA in PBS) for 30 min. Cells were then incubated for 1 h at room temperature with the primary antibody against the rabbit SM MHC 7-amino acid insert (QGPSLAY) at a dilution of 1:50. The slides were then washed three times with PBS and reacted with anti-mouse IgG made in goat labeled with FITC. The slides were then washed three times in PBS and reacted with antibody specific to the whole SM myosin (Sigma Chemicals) labeled with Texas red. Cells were then rinsed three times for 5 min each time with PBS, mounted on glass slides using Vectashield mounting medium (Vector Laboratories), and viewed under a fluorescence microscope (Leitz Orthoplan-2). The number of cells staining positive for antibody against the 7-amino acid peptide was determined. Negative controls were prepared by replacing the primary antibody with preimmune serum.
**Statistical Analysis**

Data are given as means ± SE compared with one-way analysis of variance (ANOVA) and analyzed using Dunn's test of multiple comparisons vs. control groups. Differences were considered significant at \( P < 0.05 \). Pearson’s product moment correlation coefficient was determined to assess the relationship between voiding frequency and myosin isoform expression by using SigmaStat for Windows version 2.03 (SPSS Science, Chicago, IL). The relationship between void volume and myosin isoform composition was analyzed by the logistic model using multiple linear regression analysis, also using SigmaStat after the bladders were grouped on the basis of void volume.

**RESULTS**

**Relative mRNA Expression of MHC Isoforms**

We analyzed the expression of SM-A and SM-B in the DSM from the five groups of bladders defined. The relative expression of the mRNA transcript for the SM-A and SM-B MHC isoforms obtained from PCR analysis is shown in Fig. 1. The majority (~89%) of the mRNA expressed in DSM from sham was SM-B (containing the 21-nt insert near the ATP-binding region), and thus ~11% was of the SM-A type. The expression of the SM-A MHC isoform in the detrusor from normal (nonoperated) bladders was similar to that of the sham (data not shown). The detrusor from 7-day obstructed decompensated bladder showed a significant increase (from ~10 to ~80%) in the expression of SM-A. Upon reversal of the obstruction, the high expression of the mRNA for the SM-A isoform decreased, and by 14 days, only ~20% of the mRNA transcript was SM-A. Average SM-A expression data are shown graphically in Fig. 1B.

**Quantitative Expression of SM MHC mRNA**

A representative ethidium bromide-stained agarose gel obtained for quantitative competitive RT-PCR analysis of SM MHC mRNA expression for each of the different groups analyzed is shown in Fig. 2A, and the graphical analysis of the averaged data is shown in Fig. 2B. The detrusor from decompensated bladder showed a significantly \((P < 0.05)\) decreased expression of SM MHC mRNA \((1.57 ± 0.19 \times 10^8 \text{ copies/100 ng total RNA})\), compared with sham-operated bladders \((2.04 ± 0.15 \times 10^8 \text{ copies/100 ng total RNA})\) and 14-day reversed bladders, in which the bladder function returned close to normal after reversal of the obstruction \((2.28 ± 0.18 \times 10^8 \text{ copies/100 ng total RNA})\) (Fig. 2C). Although the 7-day reversed bladder was significantly different from the sham (data not shown), the 14-day reversed bladder was not significantly different. Copies of mRNAs for SM-B and SM-A isoforms (Fig. 2D) were estimated from the relative amounts of cDNAs for the two isoforms in the ethidium bromide-stained gel determined in Fig. 1.

**Expression of SM-B at Protein Level**

**Western blot analysis.** The relative expression of SM-B isoform was determined at the protein level by Western blotting, using antibody against the 7-amino acid insert. In Fig. 3, equal amounts of MHC from sham, decompensated, and reversed (after reversal surgery and regression of the hypertrophy and return of function) detrusors (determined by Coomassie blue staining) were loaded onto SDS-polyacrylamide gels and electrophoresed. After Western transfer, the membranes were immunoreacted, either with antibody that reacts with both SM-A and SM-B (Sigma) or with antibody against the 7-amino acid peptide, which reacts only to SM-B. As shown in Fig. 3 (compare A and B), the reaction with SM-B-specific antibody was decreased in Western blots of myosin isolated from 7-day obstructed, decompensated bladder (by ~75 ± 14%) compared with those of sham-operated and 7-day obstructed bladders in which the obstruction had been removed and the animals allowed to recover for 14 days. Although equal amounts of total myosin were loaded in Fig. 3B, there was a slight decrease (~15%) in the expression of SM-B isoform.
in the amount of total SM myosin in the 7-day obstructed lane compared with the sham-operated and reversed lanes. This decrease may be due to an overexpression of nonmuscle myosin B, which we have shown occurs in the rabbit bladder in response to PBOO (62). However, this decrease in total SM myosin was much less than the decrease in SM-B reactivity, indicating that SM-A is overexpressed at the protein level in the detrusor from decompensated bladder.

**Immunohistochemistry.** Representative confocal micrographs showing detrusor SM bundles reacted with antibody specific to SM-B are shown in Fig. 4A. All the SM cells in the DSM from sham (Fig. 4AA), decompensated (Fig. 4AC) and reversed (Fig. 4AE) rabbits reacted with the SM-B-specific antibody, whereas the connective tissue (Fig. 4A, stars) did not react with this antibody. Also, the intensity of the immunofluorescent staining for SM-B was greater in the DSM from sham-operated bladder than in decompensated bladder. Values are means ± SE. *P < 0.05 compared with sham.

**Fig. 2.** Quantitative competitive (QC) RT-PCR for total SM MHC mRNA transcript determination. A: representative ethidium bromide-stained agarose gel from QC-RT-PCR performed on bladder total RNA from sham (top), 7-day obstructed decompensated (middle), and 7-day obstructed/14-day reversed rabbits (bottom). The competitor (C) fragment runs below the fragment produced from the endogenous message (EM) SM MHC cDNA because it has an internal 25-bp deletion. The absolute number of competitor mRNA copies used is shown above each lane. A total of 400 ng of total endogenous RNA was amplified in each lane. Lane at far left contains a 100-bp DNA ladder as a marker. B: gels prepared as in A were scanned and analyzed with a densitometer, and the data were plotted to determine the exact number of SM MHC copies, which is the same as the mutant SM MHC competitor copy number when the intensities of the 2 bands are equal (n = 3). C: bar graph showing average SM MHC copy number obtained for each group. SM MHC copy number in 100 ng of total RNA was 2.25 ± 0.18 × 10^6 in obstructed bladder reversed for 14 days, 2.04 ± 0.15 × 10^6 in sham-operated bladder, and 1.57 ± 0.19 × 10^6 in decompensated bladder. D: bar graph showing average SM-A and SM-B isoform copy numbers obtained for the sham and decompensated groups. SM-A copy numbers in 100 ng of total RNA were 0.23 ± 0.02 × 10^6 in sham-operated bladder and 1.22 ± 0.12 × 10^6 in decompensated bladder. Copy numbers for SM-B were 1.81 ± 0.17 × 10^6 in sham bladder and 0.35 ± 0.03 × 10^6 in decompensated bladder. Values are means ± SE.

**Fig. 3.** Expression of SM-B MHC isoform at the protein level. The SM1 and SM2 SM MHC isoforms were separated by highly porous 4.5% SDS-PAGE as described in MATERIALS AND METHODS, and then Western blot analysis was performed using an antibody against the NH2-terminal 7-amino acid insertion to quantitate SM-B expression (A). Lanes 2–4 are bladder SM from sham-operated, 7-day obstructed (7O), and 7-day obstructed/14-day reversed rabbits (7O14R), respectively. B: an identical gel to that described in A was transferred and reacted with myosin antibody that recognizes all SM MHC isoforms. The position of a 200-kDa marker is shown in lane M.
operated (Fig. 4AA) compared with decompensated (Fig. 4AC) rabbits, and upon reversal of the obstruction for 14 days (Fig. 4AE), the level of intensity increased closer to the that in the DSM sham. The specificity of this antibody for SM-B is shown in the negatively stained (antibody replaced with preimmune serum) sections in Fig. 4, AB, AD, and AF.

To determine whether overexpression of SM-A isoform in the detrusor from decompensated bladder is due to generation of myocytes that cease to express SM-B isoform, we isolated cells from 7-day obstructed DSM and cultured them overnight under conditions (15 mM thymidine) that prevented DNA synthesis, cell proliferation, and subsequent dilution of this isoform. Cells were processed for double immunofluorescence microscopy as described in MATERIALS AND METHODS using SM-B-specific antibody and antibody that reacts with the whole myosin. Isolated cells dissociated from the SM layer of the intact tissue are also shown in Fig. 4B. The double immunofluorescence demonstrates that the cells in Fig. 4BD are myocytes, because they reacted with antibody specific to SM myosin (Fig. 4BA). All the myocytes in the field also reacted with antibody against the 7-amino acid insert (Fig. 4BB). Although only ~20 dissociated cells are shown in this slide, we randomly selected 10 microscopic fields and counted over 200 cells in preparations from 3 obstructed bladders and obtained similar results. Thus the SM-B isoform is expressed in all the myocytes, even when the SM-A isoform is overexpressed in the decompensated detrusor.

Relative Expression of LC17 Isoforms

The data from RT-PCR analysis of the relative expression of LC17 isoform is shown in Fig. 5. Because the mRNA that encodes the LC17b isoform contains a 44-nt insert not found in LC17a, the cDNA generated from it during the RT-PCR has a larger molecular size than that of LC17a. As shown in Fig. 5A, DSM from sham-operated animals contained <10% LC17b (not statistically significant from normal animals) with the predominant LC17 mRNA coding for LC17a. However, the DSM from the 7-day obstructed decompensated bladder possessed, on average, >35% mRNA transcript for the LC17b isoform. Similar to the SM-A heavy chain isoform, the expression of the LC17b isoform returned toward normal levels by 14 days after removal of the obstruction. However, statistical analysis revealed that the percentage of LC17b remained significantly higher than that of the sham at both 7 and 14 days postreversal. Average LC17b expression data are shown graphically in Fig. 5B. The overexpression of LC17b isoform is also evident at the protein level in decompensated bladders, as shown in the 2-D gels in Fig. 5 (compare C and D).

Correlation of Myosin Isoform Expression With Bladder Function

Myosin isoform expression and in vivo bladder function (assessed by metabolic cage monitoring) was determined for each of a group of 22 rabbits consisting of 5 normal rabbits, 5 sham-operated rabbits, and 12 rabbits that had been obstructed for 7 days. In this experiment we specifically did not select for badly decompensated bladders because we wanted to see a range of SM-A/LC17b expression. The relative expression of the SM-A (Fig. 6A) and LC17b (Fig. 6B) isoform correlated positively with voiding frequency with r values of +0.720 and +0.669, respectively. Using the Pearson’s product moment correlation method, we found both of these differences to be significant with P values < 0.05.

The relationship between myosin isoform composition and void volume did not appear to be represented by a single linear relationship. Therefore, using the logistic model, we divided the bladders into two groups (void volumes >20 ml and void volumes <20 ml) and performed multiple linear regression analysis. The analysis revealed that these two groups were statistically different (P < 0.05) with respect to both their SM-A (r = −0.611) and LC17b (r = −0.560). Thus bladders from rabbits with void volumes >20 ml have a statistically lower expression of SM-A and LC17b compared with those with voids <20 ml. These correlation coefficients were higher than for SM1/SM2 (data not shown).

Contractile Characteristics of DSM Strips

The maximum force developed as a function of time by muscle strips from these three groups of bladders is shown in Fig. 7. Muscle strips from all three groups developed maximum force 60–90 s after stimulation with KCl (125 mM). The force at 15 s was significantly lower for the strips from decompensated bladders compared with that generated by sham and reversed bladders. However, ~85% of the maximum force was maintained in the strips from decompensated bladder 300 s after stimulation. Statistical analysis showed that the 180, 240, and 300 s time points were significantly different for the 7-day obstructed bladders compared with the sham or reversed bladders. The typical force profile of DSM strips from a decompensated bladder is compared with that of sham in Fig. 8. The rate of force development is decreased in the DSM strips from decompensated bladders (Fig. 8B) compared with sham (Fig. 8A), whereas at 14 days after removal of the obstruction, the rate of force development appears more similar to that of the sham (Fig. 8C). These data are summarized in Fig. 7. In addition, the same strips from decompensated bladders showed increased amplitude of spontaneous activity compared with sham-operated rabbits, and this spontaneous activity was decreased by 14 days after removal of the obstruction.

DISCUSSION

Previous studies on the rabbit model for bladder outlet obstruction have identified several changes in whole bladder function (35, 36), contractile perfor-
mance of the muscle strip (32), and innervation (38). In the present study, we have analyzed the molecular changes in SM myosin expression in the decompensated detrusors that fail to return to normal function, despite significant compensatory hypertrophy to overcome the outlet resistance. Furthermore, utilizing detrusor from rabbits after reversal surgery and return of in vivo function, we have demonstrated that the molecular changes in myosin expression associated with bladder decompensation are partially reversible when bladder function is returned to near-normal levels. As expected, our data from RT-PCR and Western blotting of DSM from sham-operated bladders reveal expression of the SM-B isoform predominantly. The ~10% expression of the SM-A isoform in the sham-operated DSM could be from the blood vessels in the muscular layer, which are hard to completely remove. However, PCR analysis of liver (which contains small
Electrophoresis was performed on rabbit bladder protein extracts as described in MATERIALS AND METHODS. Intimations from at least 5 different rabbits. The products were 232 bp for LC17a and 276 bp for LC17b.

A 2-dimensional (2-D) gel electrophoresis.

Fig. 4. Localization of SM-B myosin in tissue sections and dissociated cells from hypertrophied decompensated bladder.

Myosin isoform changes in smooth muscle hypertrophy

arteries) did not show any amplification of SM-A under the conditions used for this study (data not shown). Furthermore, we have previously shown that the smaller arteries express predominantly SM-B (13). Thus the 10% SM-A mRNA is probably from the bladder SM itself, but because there is no antibody specific for SM-A, we do not know whether this isoform is expressed at the protein level in normal bladder.

Fig. 5. Analysis of essential myosin light chain (MLC) mRNA transcripts and protein expression using RT-PCR and 2-dimensional (2-D) gel electrophoresis. A: a pair of primers described in MATERIALS AND METHODS was used to amplify across the region of mRNA containing the essential MLC alternative splice site. The expected sizes of the RT-PCR products were 222 bp for LC17a and 276 bp for LC17b. Lanes 1–4 were amplified from urinary bladder SM total RNA obtained from sham-operated, 7-day obstructed (7O), 7-day obstructed/7-day reversed (7O/7R), and 7-day obstructed/14-day reversed rabbits (7O/14R), respectively. Lane M is a 100-bp DNA ladder run as a standard. B: bar graph showing the average expression of LC17b in the different samples. Values are means ± SE obtained from separate determinations from at least 5 different rabbits. *P < 0.05 compared with sham; +P < 0.5 compared with 7O group. 2-D gel electrophoresis was performed on rabbit bladder protein extracts as described in MATERIALS AND METHODS. In the sham-operated bladder (C), only the LC17a isoform is shown after 2-D gel electrophoresis, even when the gel is overloaded. In severely decompensated bladders, expression of LC17b was significantly increased (D). With reversal for 14 days, the level of LC17b again returned to near normal levels (data not shown). The positive (+) and negative (−) ends of the first dimension are indicated as well as the position of a 20-kDa prestained marker run in the second dimension.

Fig. 6. Localization of SM-B myosin in tissue sections and dissociated cells from hypertrophied decompensated bladder. A: confocal micrograph of 5-μm paraffin sections processed for immunofluorescence microscopy after reaction with the antibody specific to 7-amino acid (SM-B-specific antibody). Each antibody stained section was examined using a confocal microscope (Leica TCS SP2 laser scanning confocal microscope). AA, AC, and AE: sections of detrusor smooth muscle (DSM) layers from normal, decompensated, and reversed (by removal of the ligature for 14 days after 7-day obstruction) rabbit bladders, respectively, immunostained with antibody specific to SM-B myosin. AB, AD, and AF: negative staining for AA, AC, and AE, respectively. Negatively stained sections were treated with preimmune serum, instead of the antibody against SM-B. The background staining for all the sections is similar. DSM bundles are interspersed with interstitial connective tissue (indicated by stars), which shows only the background reaction. Notice that the adjacent myocytes in the muscle bundles react with SM-B-specific antibody in all sections. The muscle bundles are larger with less connective tissue in the section from decompensated bladder. The intensity of the fluorescence is higher for the DSM from normal (containing predominantly SM-B) compared with that of decompensated (containing both SM-B and SM-A). B: cells dissociated from the DSM from hypertrophied decompensated bladder were grown for 24 h on collagen-coated glass coverslips in regular culture medium in the presence of 15 mM thymidine to inhibit DNA synthesis and cell division during culturing. Cells were processed as described in MATERIALS AND METHODS for double immunofluorescence microscopy by using Texas red-labeled antibody specific to SM myosin, followed by antibody specific to the 7-amino acid peptide that reacts only with SM-B myosin and anti-mouse IgG labeled with FITC. Cells were examined with a ×10 objective (Leitz Orthoplan-2 microscope) under fluorescence (FITC and Texas red filters) or regular light (for phase contrast). BA: reaction to SM-specific myosin (which reacts with both SM-A and SM-B isoform). BB: reaction of the same cells to antibody against 7-amino acid insert. BC: combined fluorescence showing colocalization of the 2 antibodies. BD: photographs of the myocytes under phase-contrast optics. Magnification is the same for all images. Cells in BD appear smaller because flattened areas of the cells are not clearly visible under the phase, due to poor contrast. Cells in BB are identified as myocytes because they react with myosin-specific antibody (BA). All these cells also contain SM-B isoform (BB), and the reactions with both antibodies are colocalized in the cytoplasm and cytoplasmic fibrils. The nuclear region shows very little reaction with both antibodies. For all images, bar = 100 μm.
The DSM undergoes compensatory hypertrophy required to overcome increased outlet resistance for the increased force production. Quantitative competitive RT-PCR showed a small, but significant, decrease in the expression of SM MHC mRNA in the DSM of decompensated bladders (Fig. 2). To our knowledge, the absolute mRNA copy number of SM MHC has not been reported by others. Our value of \( \frac{108}{10000} \) copies/100 ng total extractable RNA may not be unreasonable. For example, using a SM cell line that we derived from urinary bladder SM (62), we obtained \( \frac{15}{100000} \) pg of total RNA per cell. This number is in the range of the 5–25 pg of total RNA per cell reported for different cell types (18, 45). Thus, if we use 15 pg of total RNA per cell, then 100 ng of total RNA would be from \( \frac{2 \times 10^{10}}{6666} \) cells. We found \( \frac{2 \times 10^{10}}{6666} \) copies of SM MHC per 100 ng of total RNA, so \( \frac{2 \times 10^{10}}{6666} \) copies/6,666 cells equals \( \frac{30,000}{100} \) copies.

Fig. 6. Determination of average void volume and voiding frequency by metabolic cage monitoring. After standardized partial urinary bladder outlet obstruction (PBOO) of male New Zealand White rabbits, the animals were divided into 3 groups: normal (n = 5; ●), sham operated (n = 5; ○), and obstructed (n = 12; ▽). Note that, for this study, data from all obstructions were plotted, and not just the data obtained from severely dysfunctional bladders (decompensated). Urine output and voiding frequency were monitored as described in MATERIALS AND METHODS. For each rabbit, relative mRNA expression of SM-A was plotted as a function of voiding frequency per 24 h (A) and average void volume (C). Similarly, for each rabbit, relative mRNA expression of LC17b was plotted as a function of voiding frequency per 24 h (B) and average void volume (D). The r values and statistical determinations were performed as described in MATERIALS AND METHODS.

Fig. 7. Force produced in response to KCl over time. The average amount of force generated in response to 125 mM KCl by DSM strips obtained from sham-operated, 7-day obstructed decompensated (7 Obs), and 7-day obstructed decompensated/14-day reversed bladders (7 Obs/14 Rev) is plotted. Data are expressed as percentages of the maximal contraction reached by that particular muscle at various time points post-KCl stimulation. Experiments were performed in duplicate for each bladder, and values are means ± SE of at least 5 separate animals. *P < 0.05, 7 Obs compared with sham and 7 Obs/14 Rev.

Fig. 8. Tracing of force generated by DSM strips in response to KCl. Graphs represent typical force tracings obtained for DSM strips from sham-operated (A), 7-day obstructed (B), and 7-day obstructed/14-day reversed (C) bladders. Notice that the obstructed bladder SM reaches maximal contraction more slowly but exhibits an increased ability to maintain the contraction compared with the bladders from either sham-operated or reversed rabbits. Arrows indicate exact point of KCl administration.
copies of SM MHC per cell. Superabundant genes such as myosin have been reported to occur at levels of around 15,000 mRNA transcripts per cell (47).

A second approach to validate the SM MHC mRNA copy number that we obtained is to start with the fact that an average cell has been reported to have between ~360,000 and 1,000,000 mRNA molecules (2, 46). Thus 30,000 copies/360,000 cells or 30,000 copies/1,000,000 cells means that between 3 and 8.3% of the mRNAs in the cell would be SM MHC. Given that it has been reported that myosin accounts for ~2–5% of the protein in a SM cell, the finding of 10^6 copies of SM MHC per 100 ng of total protein seems reasonable. We understand that a lot of other factors (e.g., translational efficiency) and assumptions would affect this number. To our knowledge, there have been no published studies examining the turnover of SM myosin.

A major molecular change that occurred in the DSM from decompensated bladders was an overexpression of the SM-A SM myosin isoform (Fig. 1). This overexpression of SM-A isoform was accompanied by a decrease in the expression of SM-B as shown in Fig. 1 and also in the immunostained DSM tissue sections shown in Fig. 4 (compare AA and AC), and this decreased expression of SM-B appears to be reversed in response to removal of the obstruction for 14 days (AE). The increased expression of SM-A appears to not be the result of a new population of SM cells that do not express SM-B, because we showed that all of the SM cells in the obstructive bladder continued to express SM-B (Fig. 4BB). Despite this adaptive change in the motor domain of the myosin molecule, the bladder function failed to return to a normal level in decompensated bladders. However, reversal of the obstruction and subsequent regression of the hypertrophy in decompensated bladders led to return of the SM-B and SM-A expression close to normal levels (Fig. 1). The recovery of bladder function to near-normal levels upon reversal of the obstruction suggests that the expression of the NH2-terminal SM MHC isoform is correlated with alteration in bladder function. This suggestion is further supported by our metabolic cage data showing a correlation between SM-B isoform expression and bladder function. The SM-A isoform shows a positive correlation with voiding frequency of 0.720 (considered highly correlated on the basis of our Pearson's analysis) and a negative correlation (−0.611) with average void volume (Fig. 6, A and C). Thus an overexpression of the SM-A SM MHC isoform is the hallmark of bladder outlet obstruction in the rabbit model for PBOO.

The SM-A isoform is the predominant isoform in SMs that exhibit tonic contractile characteristics (e.g., aorta and esophageal sphincter). DSM strips from sham-operated bladder showed the contractile characteristics of a phasic muscle with fast generation of force and fast relaxation (Figs. 7 and 8). The detrusor muscle strips from decompensated bladder, on the other hand, generated force more slowly, and the force achieved was maintained for a longer period, similar to tonic-type SMs (Figs. 7 and 8). At 14 days after removal of the obstruction from decompensated hypertrophied bladders, both the rate of force development and the spontaneous contractility of the DSM were more similar to that of the DSM from sham-operated rabbits.

Also apparent in the decompensated bladder is an increased amplitude of the spontaneous contractile activity observed in the absence of agonist (Fig. 8). This observation was made in the majority of decompensated bladders and supports the study by Kato et al. (27), who found that in vitro spontaneous contractions of whole cat bladders were increased in response to bladder outlet obstruction. The clinical relevance of this observation is supported by Levin et al. (34), who found that spontaneous activity in the in vivo bladder could be induced by increasing bladder pressure. Moreover, this finding may be related to the bladder overactivity seen in a large percentage of patients with bladder outlet obstruction (42).

In general, it appears that phasic-type SMs generally exhibit more spontaneous contractility than tonic-type SMs (58). Because phasic-type SMs generally have more SM-B and more LC17a, it would appear that the expression of these two isoforms would correlate with spontaneous contractility, which would be in contrast to the findings of the present study. We have demonstrated that the obstructed decompensated bladders express more of the isoforms generally associated with tonic-type SM (SM-A and LC17b) than that they exhibit increased spontaneous contractility compared with the DSM from normal and sham-operated rabbits that express SM myosin isoforms generally associated with phasic-type SM (SM-B and LC17a). However, there are reports in the literature that show that tonic-type SMs do exhibit spontaneous contractility. For example, Hypolite et al. (24), Levin et al. (31), and Sarioglu et al. (53) have demonstrated spontaneous contractions in corpus cavernosum smooth muscle, a SM that is considered a tonic type and that we have demonstrated expresses predominantly the SM-A isoform (14). Also, Kawano et al. (28) found that the tonic-type myometrium exhibits spontaneous contractility, even though this SM was found to express no SM-B (41). The increase in spontaneous contractility was only slightly decreased 14 days after removal of the obstruction, suggesting that the spontaneous contractility is not directly related to the SM myosin isoform composition.

As expected, the alteration in the contractile characteristics from phasic to tonic is associated with a change in the alternative splicing at the 5' end of the pre-mRNA that encodes MHC. This results in an overexpression of the SM-A isoform, which lacks the 7-amino acid insert that is spliced in. Interestingly, the splicing mechanism can be modulated by cellular events that follow obstruction-induced hypertrophy and regression of hypertrophy by reversal of the obstruction. Surprisingly, virtually nothing is known about the transcriptional regulation of SM myosin isoforms. Further studies on the DSM from decompensated bladders and bladders that return to near-normal isoform composition might be a good model to
study transcriptional regulation of myosin isoforms, since myosin isoform expression can be modulated by surgery. Furthermore, the organ function can be correlated with the molecular events.

In addition to the modulation in the expression of the NH2-terminal MHC isoform in response to PBOO, our data show that there is also an overexpression of the LC17b (essential) light chain isoform as well. The LC17 isoform composition has also been proposed to play a role in the contractile characteristics (i.e., phasic or tonic) of SM (22, 39). The relative amount of LC17a, which differs from that of LC17b by only five of the last nine COOH-terminal amino acid residues, is likely to affect the ATP hydrolysis by the myosin because it is located near the tail-head junction hinge region of the myosin head. Substitution mutations in the essential light chain of nonmuscle myosin from Dictyostelium lower the physiologically relevant actin-activated ATPase activity and motor function, as assessed by in vitro motility assay (12).

An inverse relationship between LC17b content of SMs and both the Vmax of shortening of skinned SM fibers (39) and actin-activated myosin ATPase activity (22) has been reported. In contrast, exchanging the LC17a isoforms on fully inserted SM-B myosin from intestine with the LC17b isoform had no effect on the LC 17 isoform-specific effects on the mechanical properties of SM cells (23) and muscle strips (40) have also been reported. Similar to the SM MHC isoform SM-A, expression of the essential light chain isoform LC17b shows a (+0.669) positive correlation with voiding frequency and a negative (−0.560) correlation with average void volume (Fig. 6, B and D).

In addition, as described in the Introduction, the SM MHC is also alternatively spliced at the COOH-terminal region to produce the SM1 and SM2 isoforms. Urinary bladder SM contains predominantly the COOH-terminal isoform SM2, which has a lower molecular size than SM1. The ratio of SM2 to SM1 is decreased after obstruction-induced hypertrophy of the DSM (11, 61) and returns to normal upon reversal of the obstruction and subsequent regression of the hypertrophy (61). Recent studies using recombinant SM1 and SM2 have shown that myosin filaments formed by SM1 are more stable than those of SM2 (51). This finding suggests that the overexpression of SM1 in DSM remodeling after outlet obstruction may be an adaptation to increase the efficiency of the contractile apparatus in myocytes to compensate for the increased outlet resistance. Indeed, a recent study using smooth muscle from transgenic mice overexpressing SM1 provides evidence for a difference in force production due to a change in the composition of COOH-terminal isoform (49). In addition to enhanced filament assembly, the difference in force production associated with overexpression of SM1 has also been suggested to result from a conformational change in the myosin S2 due to interaction with the tail region of the SM1 heavy chain isoform in the assembled myosin filament (9). Although not examined in this study, changes in the relative expression of the SM1 and SM2 isoforms may also contribute to the altered voiding parameters observed in response to PBOO.

In summary, our data on the expression of the SM MHC and LC17 essential light chain isoforms in the DSM show, for the first time, a coordinated modulation of the alternative splicing mechanism responsible for the expression of SM myosin in response to PBOO. Although the alteration in bladder function and the overexpression of SM-A MHC and LC17b MHC isoforms may be directly or indirectly related, these isoforms might serve as good molecular markers for decompensated bladders. It will be interesting to examine in subsequent studies whether these changes in myosin isoforms also occur in humans with PBOO resulting from BPH. Because not all men regain their urinary bladder function even after removal of the obstruction by the enlarged prostate (8), it will be interesting to determine whether the myosin isoform composition fails to return toward normal values in these nonresponding men.

We thank Xianqun Luan for help in performing statistical analysis.

DISCLOSURES

This study was supported by the University of Pennsylvania O’Brien Urology Center P50 Grant from the National Institute of Diabetes and Digestive and Kidney Diseases.

REFERENCES


Downloaded from http://ajpcell.physiology.org/ by IP: 10.220.32.247 on August 14, 2017


