SM2^{+/-} male mice are predisposed to develop urinary tract obstruction and hyper contractility of the bladder smooth muscle upon ageing

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Abstract

We previously showed that complete loss of smooth muscle myosin heavy chain isoform 2 (SM2) resulted in postnatal lethality, but in het mice a partial loss of SM2 (SM2^{+/-}) was accompanied by down-regulation of SM1 with unaltered SM2:SM1 ratio. To determine whether a normal bladder function would be maintained throughout its lifespan, we aged WT and SM2^{+/-} mice up to 18 months and analyzed a) SM2:SM1 ratio b) bladder smooth muscle structure and c) function in SM2^{+/-} het mice. A notable finding was that ~50% of 15–18 months old male SM2^{+/-} mice exhibited urinary retention in bladder with the distention of upper urethra. In SM2^{+/-} mouse bladder with urinary retention, the SM2:SM1 ratio was decreased but not in SM2^{+/-} mouse bladder that did not develop urinary retention. Interestingly in the distended bladder the expression levels of α -actin and tropomyosin remained unaltered despite a reduction in the number of myosin thick filaments. These distended bladders showed hypersensitivity to submaximal K⁺ depolarization and M3-receptor stimulation, without a significant increase in myosin light chain phosphorylation. We therefore suggest that a partial loss of SM2 may predispose male mice to develop lower urinary tract obstruction during ageing. In addition our data suggest that bladder obstruction can cause a further reduction in SM2 expression and SM2:SM1 ratio, and a hyper-contractility of the bladder smooth muscle.

Key words: gene knockout, SM2:SM1 ratio, thick filament

Introduction

Smooth muscle myosin heavy chains (SMHCs) are the primary structural proteins forming thick filament, which together with actin filaments constitute the mechanic apparatus of smooth muscle contraction (Rovner *et al.*, 1986; Loukianov *et al.*, 1997; Murphy *et al.*, 1997; Craig and

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Woodhead, 2006). Splicing at 3' ends of the SMHC gene has been found to produce SM1 and SM2 myosin isoforms. SM2 (200 kDa) contains a unique 9-amino-acid sequence at the carboxyl terminus, whereas SM1 (204 kDa) has a 43 amino acid non-helical tail region. These C-terminal amino acid sequences that specify SM1 and SM2 isoforms have been noted to be highly conserved across all vertebrates (Murphy *et al.*, 1997; White *et al.*, 1998; Babu *et al.*, 2000). However, the expression level of SM2 alters in a variety of pathological conditions. For example, decreased expression of SM2 has been reported in obstructed urinary bladder of rabbit and rat (Chacko and Longhurst, 1994; Wang *et al.*, 1995; Cher *et al.*, 1996; Gomes *et al.*, 2000; Lin *et al.*, 2000; DiSanto *et al.*, 2003; Austin *et al.*, 2004), in pulmonary arteries of primary pulmonary hypertension (Packer *et al.*, 1998; Itoh *et al.*, 2002), and in neointimal smooth muscle cells of injured or atherosclerotic vessels (Aikawa *et al.*, 1993). On the other hand, in myometrium of ovariectomized rabbits SM2 has been found to increase, yet such an alteration can be normalized by estrogen treatment (Capriani *et al.*, 1997). All these findings imply that SM2 myosin may play an important role in defining the functional property of smooth muscle cells in physiological and pathological condition.

The contraction of smooth muscle cells is produced by the cross-bridge involving thick and thin filaments that are composed of myosin and α -actin, respectively. In this process, each myosin molecule has been considered as a motor unit to generate force of cell contraction (Craig and Woodhead, 2006). However, SM1 and SM2 myosin isoforms may differ in force generation (Martin *et al.*, 1997; Eddinger and Meer, 2007). Recent attempts to over express SM1 or SM2 myosin in smooth muscle using transgenesis did not significantly alter total myosin content but it was shown to increase or to decrease mouse bladder smooth muscle contraction, respectively (Martin *et al.*, 1997; Martin *et al.*, 2007). To investigate the unique roles of SM2 and SM1 myosin isoforms we recently developed an SM2 isoform specific knockout mouse model. The SM2 knockout (SM2^{-/-}) mice died within 30 days after birth; and showed severe bladder distention and developed end-stage hydronephrosis. Loss of SM2 myosin was accompanied by a down-regulation of SM1, and a reduced number of thick filaments in SM2^{-/-} bladder smooth muscle. Bladder smooth muscle from SM2^{-/-} homozygous mice showed enhanced contraction to K⁺ depolarization and M3 receptor activation despite a significant decrease in total myosin and number of thick filaments (Chi *et al.*, 2008).

In comparison to SM2^{-/-} null mice, the SM2^{+/-} mice appeared normal, reproduced well and showed reduced total myosin but unaltered SM2:SM1 ratio. Although bladder smooth muscle from SM2^{+/-} neonate mice exhibited comparable contractile properties to control mice, the functional consequences of deleting one copy of the SM2 exon had not been studied throughout adult life. Unlike the SM2^{-/-} mice where there is a complete loss of SM2 myosin, the SM2^{+/-} mice provide a unique model where the SM2 myosin is partially decreased, with a concomitant reduction in SM1. Based on our recent findings, we hypothesize that maintaining the ratio of SM2:SM1 is more important than myosin content for force generation in bladder smooth muscle. Therefore the goal of this study was to determine whether the ratio of SM2:SM1 in bladders from SM2^{+/-} mice remains constant or is altered through their lifespan and how it would influence thick filament formation and smooth muscle contraction.

Methods

Animals and tissue preparation

The generation of SM2^{+/-} mice was previously described (Chi *et al.*, 2008). SM2^{+/-} and control mice were bred in the animal facility of the Ohio State University with free access to food and water. Mice were sacrificed by decapitation, which was in accordance with National Institutes of Health guideline and was approved by the institutional animal care use committee at The Ohio State University. For the preparation of muscle tissues, bladders were isolated and cut open, and the mucosa was removed by dissection under a binocular microscope in physiological salt solution (PSS) (Zhou *et al.*, 2003; Martin *et al.*, 2007). Functional studies were carried out using muscle strips. Muscles from the front wall were cut transversely into 1 mm wide and 2 mm long strips, which were further tied with thread filaments to form loops at both ends separated by a distance of 1 mm (Zhou *et al.*, 2003).

Analyses of protein expression

Protein expression was analyzed by western blotting as described elsewhere (Babu *et al.*, 2001). Briefly, crude myofibrillar proteins isolated from bladder were separated by electrophoresis on SDS-PAGE (5% for SM2 and SM1; 10% for α -actin and tropomyosin), and transferred to a nitrocellulose membrane. Proteins were sequentially probed with respective first and secondary antibodies. Anti-SM1 and Anti-SM2 antibodies were kindly provided by Dr. AF Martin. Anti- α -actin antibody was purchased from Sigma (St Louis, MO). In some experiments, gel was stained with Coomassie-blue to directly analyze the ratio of SM2:SM1 in total myosin content. All band densities were quantified and analyzed with a Biochemi System (UVP, Upland, CA).

Electron Microscopic examination

Bladders were expanded in situ in PSS (250–500 μ L) and first fixed with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Muscle strips cut from above treated bladders were further post-fixed in 2% OsO₄ for 2 hrs at room temperature, and were contrasted in saturated uranyl acetate for 4 h at 60°C (Moore *et al.*, 2004). Samples were embedded in Epon 812 and cut into sections, which were stained with uranyl acetate and lead. Images were detected and photographed with an electron microscope (EM 410; Philips Electron Optics, Mahwah, NJ). The number of thick filaments in a given field was counted and expressed in counts per μ m². In addition, the diameters of myosin thick filaments cross-section (both *x* and *y* axis) in chosen fields were measured by using image analysis software (Scion Image, version 4.0.2).

Analysis of bladder smooth muscle contraction

In vitro bladder smooth muscle contraction was analyzed as described previously (Zhou *et al.*, 2003; Martin *et al.*, 2007; Chi *et al.*, 2008). Briefly, muscle strips were attached with the loops to two tungsten wires in an organ bath (3 mL) filled with PSS maintained at 37°C. One wire was fixed, and the other was connected to a force transducer (AE801, Holten, Norway). Tissues were set at 1.5 times resting length (at which length the contractile response to 60 mM K⁺ was maximal and reproducible). After 30 min of equilibrium, muscle strips were intermittently exposed to 60

mM K⁺ for 10 min, and relaxed with PSS for an additional 10 min until two consecutive contractions of the same magnitude were obtained. After the final 60 mM K⁺-induced contraction was relaxed with PSS for 10 min, muscle strips were then stimulated with 30 mM K⁺ or carbachol (CCh) (1 μ M). The contraction to 30 mM K⁺ or carbachol (1 μ M) was expressed as a percentage of peak value of the force developed with 60 mM K⁺.

Measurement of MLC20 phosphorylation

Bladder muscle strips (1 $\times$ 2 mm²) were placed in 30 mM K⁺ for 5 min and the reactions were stopped with 90% acetone, 10% trichloroacetic acid and 10 mM dithiothreitol (DTT) chilled at -80°C . Proteins were extracted, separated on urea/glycerol gel (with the amount adjusted to achieve similar total MLC20 content), and transferred onto a nitrocellulose membrane as described previously (Zhou *et al.*, 1999). Phosphorylated and un-phosphorylated MLC20 were probed with anti-MLC20 antibody (Sigma, St Louis, MO), and quantified as in western blot analyses.

Statistics

For statistical evaluation, values were presented as mean $\pm$ SE. Comparison of two means was performed by unpaired Student's *t*-test. When more than two means were compared, 1-way or 2-way ANOVA followed by Dennett's or Bonferroni's post hoc test was used. $P < 0.05$ was considered to be statistically significant.

Results

The male SM2^{+/-} mice develop bladder distention upon aging

We previously reported that SM2^{-/-} null mice died within two weeks after birth but SM2^{+/-} mice lived well into adulthood without obvious pathology in urinary organs, including the bladder. To determine whether a normal bladder function would be maintained throughout its lifespan, we aged WT and SM2^{+/-} mice up to 18 months. We did not observe any pathology including bladder distention in aged WT male and female mice. In SM2^{+/-} mice, both male and female showed no evidence of pathology in the bladder body up to 14th month, but at around 15–18 months urinary retention in bladder was observed only in $\sim 50\%$ of male mice but not in female mice. In addition, in SM2^{+/-} mice with bladder urinary retention, both the bladder body and upper part of urethra were distended (Fig. 1A). However histology of these distended bladders did not reveal smooth muscle hypertrophy (Fig. 1, B and C). Furthermore, examination of other smooth muscle containing organs including the gastrointestinal system and aorta of SM2^{+/-} mice did not reveal any structural abnormalities (data not shown).

The ratio of SM2:SM1 is decreased in SM2^{+/-} bladder with urinary retention

Next, we determined whether the expression of SM2 and SM1 is altered with ageing. For this purpose, bladders from 2 month and 14 month-old male WT mice were examined. Immunoblotting showed that the level of SM1 and SM2 was not significantly altered between the 2- and 14-month-old WT mice, suggesting that aging does not induce a significant change in SM1 and SM2 myosin levels in the mouse bladder (Fig. 2A). Furthermore, we studied SM2 and SM1 expression levels

Fig. 1. Bladder morphology and histology; A: SM2^{+/-} male mice showing distended bladder and enlarged urethra. B: Histology of male WT bladder (HE staining (200x magnifications). C: Histology of male SM2^{+/-} distended bladder (200 x magnifications). Arrow indicates smooth muscle layer.

in age matched (15–18 months) WT, SM2^{+/-} bladders with and without bladder distention. As shown in Fig. 2, B and C, western blotting showed that SM1 and SM2 myosin levels were decreased by 29% and 41%, respectively in SM2^{+/-} mice with distention as compared to age matched WT mice. In addition, we carried out direct staining of the gel with Coomassie-blue showed that the ratio of SM2:SM1 was not significantly different in WT and SM2^{+/-} bladder without distention but decreased in aged SM2^{+/-} mice with urinary retention (0.83 ± 0.02 , 0.96 ± 0.034 , and 0.34 ± 0.06 , respectively) (Fig. 2D).

The number of myosin thick filaments is significantly decreased in SM2^{+/-} bladder with urinary retention

To determine how the above alteration in SM1 and SM2 expression influences thick filament formation, we examined smooth muscle ultra structure. Analyses with EM revealed that the number of thick filaments in SM2^{+/-} bladder was significantly decreased but the diameter of thick filament was comparable to wild type (Fig. 3A). In addition, the number of myosin filaments in bladders with urinary retention in a given field further decreased by ~50% as compared to SM2^{+/-}

Fig. 2. Myosin expression in WT and SM2^{+/-} male mice. A: Western blot analyses of SM1, SM2 in wild type bladders. B: SM1 expression in age matched SM2^{+/+} and SM2^{+/-} bladders with and without distention. C: SM2 expression in age matched SM2^{+/+} and SM2^{+/-} bladders with and without distention. The relative decrease in SM1 and SM2 was calculated by comparing to its levels in WT (100%). D: The ratio of SM2:SM1 was determined by Coomassie blue staining of SDS gel (upper panel). SM2:SM1 ratio determined by densitometry (lower panel).

with no phenotype ($16 \pm 1.4/\mu\text{m}^2$ and $32 \pm 3.2/\mu\text{m}^2$, respectively) (Fig. 3B). Further the diameter of thick filament cross section in SM2^{+/-} bladder with urinary retention appeared smaller compared to those with no phenotype (Fig. 3B, lower panel).

SM2^{+/-} bladder with urinary retention shows increased contractility

To determine how changes in thick filament structure and density affected bladder smooth muscle contractility, we studied isometric force development in bladder muscle strips. The strips were stimulated with high K⁺ (60 mM and 30 mM; submaximal) and 1 μM carbachol (CCh), a M3 receptor agonist. The contractile response at high K⁺ (60 mM KCl) depolarization was very similar in WT, SM2^{+/-} mice with and without bladder distention (100, Fig. 4, A and B). The contractile response to low K⁺ (30 mM KCl) and activation by 1 μM CCh was not significantly

Fig. 3. A: Electron micrographs of bladder smooth muscle cells. Upper panel: smooth muscle cells (6,000x Magnifications) from SM2^{+/+} mouse, middle panel: smooth muscle cells (6,000x magnification) from SM2^{+/-} mouse without bladder distention and lower panel: smooth muscle cells (6,000x magnifications) from SM2^{+/-} mouse with bladder distention. B: cross-section of myosin filaments (68,000x magnifications). Lower panel: enlarged images of square areas indicated in the upper panel. Arrows point to the myosin thick filaments; circles indicate the locations of dense bodies.

different between SM2^{+/-} mice (without distention) and age-matched WT mice (10.0 ± 2 vs. 10.7 ± 1.1 and 42.0 ± 7.1 vs. 43.5 ± 8 , respectively, $P > 0.05$). On the other hand, SM2^{+/-} mice with urinary retention showed enhanced contractility to 30 mM K⁺ and that to 1 μ M CCh (61.7 ± 2.3 and 123 ± 8.5 , respectively, $P < 0.001$, Fig. 4, A and B).

Phosphorylation of regulatory myosin light chain (MLC20) is not altered in SM2^{+/-} bladder with urinary retention

We further investigated whether increased contractility in SM2^{+/-} bladder with urinary retention was related to the rate of MLC20 phosphorylation, which is an important determinant of force development, and influenced by Ca²⁺ as well as phosphatase activity of the affiliated contractile apparatus. To this end, muscle strips from SM2^{+/-} bladder with urinary retention and those of WT mice were stimulated with 30 mM K⁺. As shown in Fig. 5, the ratio of phosphorylated to total MLC20 was not significantly different between SM2^{+/-} bladders with urinary retention and those of control WT mice, both at rest and upon stimulation with 30 mM K⁺ (Fig. 5A). Furthermore the expression level of thin filament contractile proteins, α -SM actin and tropomyosin were not altered in aged mice with urinary retention (Fig. 5B).

Fig. 4. Isometric contractions of bladder smooth muscle. A: Representative traces (taken from 5 experiments) showing isometric force generation of bladder strips in response to 30 and 60mM KCl. B: Response to 1 μ M carbachol, a muscarinic receptor (M3) agonist. Control (blue), SM2^{+/+} (red), and SM2^{+/-} with bladder distention (brown).

Discussion

Recently we showed that complete loss of SM2 resulted in post natal lethality (Chi *et al.*, 2008). All of the SM2^{-/-} mice died during the first week and we were unsure if the phenotype was due to a developmental defect or solely due to dysfunction of the bladder smooth muscle. On the other hand, SM2^{+/-} het mice live to full term and reproduce well with a significant alteration in SM2 myosin level. Therefore we took advantage of these mice to understand how a decrease in SM2 myosin hence an altered SM2:SM1 ratio would affect bladder smooth muscle physiology throughout their adult life. Therefore these studies allow us, for the first time to explore how an alteration in SM2 myosin affects 1) SM1 myosin expression, smooth muscle ultra structure; 2) thick and thin filament composition; 3) bladder contractility; and 4) lastly the effect of ageing in this context.

In this study we demonstrate that SM2^{+/-} mouse bladder, having decreased SM2 and SM1 myosin and reduced number of thick filaments but an unaltered ratio of SM2:SM1, maintained normal function till 14 months of age. However, ~50% male SM2^{+/-} mice aged to 15–18 months developed urinary retention with distention of both bladder and the upper part of urethra. Interestingly, we found that bladders from mice with urinary retention exhibited hypercontractility as evidenced by increased activation at sub-maximal K⁺ depolarization and 1 μ M CCh stimulation. Most importantly ~ 50% of 15–18 months old SM2^{+/-} mice which developed urinary tract obstruction had a further reduction of bladder SM2 levels alone resulting in a lower SM2:SM1 ratio, presumably in an effort to overcome the obstruction. The electron microscopy studies of the myofilament structure revealed that, not only was the number of thick filaments reduced, as seen

Fig. 5. MLC phosphorylation status. A: Detection of phosphorylated and unphosphorylated MLC20 in resting (PSS) and 30 mM K⁺ (K⁺) stimulated bladder smooth muscle of SM2^{+/+} (blank bar) and SM2^{+/-} with bladder distention (filled bar) (n = 4). B: Expression of α-SM actin and SM-tropomyosin is not altered.

in SM2^{+/-} without bladder distention, but their size was also decreased.

In addition thin filament contractile proteins including α-SM-actin and tropomyosin were not altered. Moreover, MLC phosphorylation in isolated bladder strips was not significantly different. These findings suggest that the primary defect is arising from myosin filaments. We propose that SM2 : SM1 ratio is the basis for adult smooth function and any alteration in SM2 will affect the composition and assembly of myosin filaments leading to altered myofilament activation, force development and sustenance during smooth muscle contraction. We thus conclude that SM2:SM1 ratio rather than the number of thick filaments or total myosin content appears to play a key role in maintaining normal bladder smooth muscle function.

An interesting finding is that only 50% of aged male SM2^{+/-} mice developed bladder dysfunction and the female counter parts do not show such a phenotype. A number of factors could have contributed to this phenotype a) due to anatomical differences in male and female urogenital organs b) environmental factors including urinary infection and obstruction of the urethra and c) individual variations in compensatory response to loss of SM2 myosin. The development of

urinary retention in SM2^{+/-} mice older than 14 months does not appear to result from malfunction of the bladder or the inability of the bladder to empty its contents. Also, we noted that in such mice both the bladder and the upper part of urethra were distended. Thus, the urinary retention in aged SM2^{+/-} bladder was rather due to the obstruction of distal part of urethra or the lower urinary tract. However, bladder urinary retention was not observed in aged Male WT mice. Therefore, the inherently low level of SM2 in the SM2^{+/-} mice is probably the major reason for the development of lower urinary tract obstruction in ageing, although the exact mechanism behind this remains to be further determined.

Interestingly, in SM2^{+/-} bladders with urinary retention, the expression level of SM2 is further reduced, leading to a decrease in SM2:SM1 ratio. However, this was not observed in any aged SM2^{+/-} mice that did not develop urinary retention. Thus, the further decrease of SM2 and altered SM2:SM1 ratio was not the reason, but rather the result of obstruction in lower urinary tract. Indeed, a decreased ratio of SM2 : SM1 has been previously found in bladders with partial obstruction of bladder outlet, and this has been considered as a phenotypic marker of smooth muscle hypertrophy (Cher *et al.*, 1996; Gomes *et al.*, 2000; Burkhard *et al.*, 2001; Hypolite *et al.*, 2001; Austin *et al.*, 2004). Thus, a further decrease of SM2 or reduced ratio of SM2:SM1 in SM2^{+/-} bladder with urinary retention could be an adaptive response resulting from lower urinary tract obstruction, and could be responsible for the increased bladder smooth muscle contraction, to overcome the increased urinary resistance. The finding that the basal rate of MLC20 phosphorylation and that induced by 30 mM K⁺ were not altered in muscle strips from SM2^{+/-} bladder with urinary retention, excludes the possible influences of altered Ca²⁺ and phosphatase activity known to regulate smooth muscle contraction. However in this study we were not able to address the dephosphorylation rate which could be modified by regulators of the phosphatase activity.

In summary, in the present study we demonstrate for the first time that while partial loss of SM2 may be well compensated in the bladder, it can lead to the development of lower urinary tract obstruction during ageing in male mice. In addition, we found that the development of urinary tract obstruction in SM2^{+/-} mice leads to a further reduction in SM2 or decreased ratio of SM2:SM1, resulting in hypercontractility of the bladder smooth muscle. These findings point out an important role for SM2:SM1 ratio in modulating bladder smooth muscle contraction both in normal physiology and under pathological conditions. Future studies are needed to explore the fundamental differences between SM1 and SM2 filaments and how they regulate contractile function in an intact muscle.

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