Smooth muscle myosin expression, isoform composition, and functional activities in rat corpus cavernosum altered by the streptozotocin-induced type 1 diabetes

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Diabetes mellitus (DM) is a common chronic disease affecting 285 million people, corresponding to 6.4% of the world’s adult population in 2010 and expected increase to 7.9% by 2030 (40). The prevalence of erectile dysfunction (ED) is three times higher in diabetic men (28 vs. 9.6%) (17), occurs at an earlier age, and increases with disease duration, being ~15% at age 30 yr and rising sharply to 55% at 60 yr in diabetics (30, 31). DM may cause ED by affecting one or a combination of the following: psychological function, central nervous system function, androgen secretion, peripheral nerve activity, endothelial cell function, and smooth muscle (SM) contractility (15). For streptozotocin (STZ)-induced DM in an experimental rat model, decreased endothelial nitric oxide synthase- and neuronal nitric oxide synthase-mediated cavernous SM relaxation, along with increased cavernous muscle tone, was the primary mechanism for this DM-induced ED (8, 35). Recently, with STZ-induced rat and alloxan-induced rabbit diabetic models, we found testosterone deficiency and several biochemical alterations, including neuronal nitric oxide synthase and phosphodiesterase type 5 (PDE5) downregulation and Rho kinase-β upregulation (45, 52). Indeed, by using these and other diabetic models, DM-associated ED has been examined extensively. However, the majority of studies focused on corpus cavernosum (CC) SM (CCSM) relaxation pathways rather than the actin-myosin contractile apparatus. Moreover, PDE5 inhibitors (PDE5i), the first-line therapeutic agents, such as sildenafil, vardenafil, and tadalafil, are less efficacious in diabetic patients than in other subjects with ED (11, 44).

SM myosin (SMM) is the thick filament and motor molecule of the contractile apparatus, composed of a pair of myosin heavy chains (MHCs) and two pairs of myosin light chains (MLC) that are intimately intertwined (1). It has been shown that both the 3′ and 5′ ends of the MHC mRNA are alternatively spliced to generate COOH-terminal (SM1 and SM2) and NH2-terminal (SM-A and SM-B isoforms), respectively (4, 5). Also, the essential light chain MLC17 is alternatively spliced and has two 3′ isoforms known as MLC17a and MLC17b (24, 36). The SMM isoform composition has been demonstrated to affect force development (6) as well as force maintenance (42). The SM-B, MLC17a, and SM2 isoforms are associated with a faster, more phasic-type contraction, whereas the SM-A, MLC17b, and SM1 isoforms are associated with a slower, more tonic force generation (12, 14, 20–22). Regarding the CCSM (14), at the mRNA level we characterized the relative percentage of SM-B, MLC17b, and SM2 isoforms within rabbit CCSM to be ~31, 13, and 54%, respectively. Also, we found that human CC samples expressed mainly the SM-A isoform, and there was a predominance of SM2 isoform mRNA relative to SM1 with a mean of 63.8%, whereas the ratio of LC17b to LC17a was ~1:1 for all patients (22). At the functional level, most recently we found that blebbistatin (BLEB), a small cell-permeable selective myosin II inhibitor, potently relaxed both rat and human CCSM in vitro and demonstrated proerectile activity in vivo (51). Thus...
BLEB is a potential erectogenic agent for ED targeting at the CCSM contractile apparatus.

Alterations in SMM isoform composition have now been documented in a number of pathological conditions, including bladder outlet obstruction (13), megacolon (41), and pulmonary hypertension (34). In addition, a direct role for SMM isoform composition in altering physiological properties of SM has been demonstrated using SM-B knockout mice (6). To our knowledge, no study has characterized rat CC SMM isoforms. Moreover, no one has investigated the effect of DM on SMM composition and functional activity. The aim of the present study was to determine SMM expression and isoform composition in rat CCSM while emphasizing a role for altered SMM expression and isoform composition in the pathophysiological mechanism of DM-induced ED and the efficacy of BLEB.

MATERIALS AND METHODS

**Chemicals and tissues.** All chemicals were from Sigma (St. Louis, MO) except (+) for BLEB, which was from Tocris (Ellisville, MO). The racemic mixture (±) of BLEB was used in all studies since it was determined that the active (−) enantiomer form was equipotent to the racemic mixture (±) in the in vitro studies and that the inactive (+) form did not induce significant relaxation (16, 25, 43, 50). A stock solution of BLEB was made in dimethyl sulfoxide (DMSO); other substances were dissolved daily in double-distilled water. Control experiments showed that the final concentrations (1/1,000–3/1,000) of DMSO used in these studies did not significantly modify the relaxation response induced by (±) BLEB. Because of the known light sensitivity of BLEB, it was always kept in the dark in the refrigerator until just prior to usage, and during the experiment the organ bath chambers were kept covered. Human CC samples were obtained from patients undergoing penile prosthesis implantation (10 cases) with informed consent and the approval of the Institutional Review Boards of Montefiore Medical Center and the Albert Einstein College of Medicine. Male rat CC, urinary bladders, and aorta were obtained from adult male rats (Charles River, Raleigh, NC). All animal studies were approved by the Animal Institute Committee of the Albert Einstein College of Medicine.

**STZ rat model.** Male 3-mo-old Fischer 344 rats were used. As reported previously (9), rats were made diabetic by an intraperitoneal (ip) injection with 35 mg/kg STZ dissolved in citrate buffer (60 ml of 0.1 M citric acid and 40 ml of 0.2 M Na2HPO4, pH 4.6). Age-matched controls (AMC) received an injection of vehicle (citrate buffer). All animals were kept for 8 wk. Blood glucose was measured with the Ascensia Elite blood glucose meter (Bayer Health Care, Mishawaka, IN) at 1, 2, and 3 days immediately after the STZ injection as well as after intracavernous pressure (ICP) determination at the end of the 2-mo diabetic period. In addition, urine glucose was measured with the Diastix reagent strips (Bayer, Elkhart, IN) calibrated at levels of 100, 250, 500, 1,000, and 2,000 mg/dl for 3 consecutive days after STZ injection to confirm glycosuria. Only rats with confirmed initial glycosuria and blood glucose levels >250 mg/dl at the start and end of diabetes duration were used as “diabetic” samples for the study (9, 23, 27, 33, 37, 48). Rats (~10%) that were injected with STZ and showed no hyperglycemic symptoms such as weight loss, polydipsia, and blood glucose levels <250 mg/dl were not used for this study. Average blood glucose levels and body weights of AMC and STZ rats at euthanization are given in Table 1.

**In vitro organ bath studies.** ICP measurement was performed as described previously (32, 51, 53). Rats were anesthetized with pentobarbital sodium (35 mg/kg) via an intraperitoneal injection. An incision was made in the perineum. The right crura was exposed and perforated with a 28-gauge needle connected to a PE-50 tube for an ICP recording. Mean arterial pressure (MAP) via carotid artery and ICP were recorded through pressure transducers connected in line to a PowerLab 4/30 data acquisition system (ADInstruments, Colorado Springs, CO), which was connected in turn to a Dual-Core processor Pentium computer for real-time monitoring of pressure changes. Pressure transducers were calibrated to water prior to each experiment. ED was determined by ICP rise elicited by electrical stimulation (ES) of the cavernous nerve at varied currents in mA (0.75, 1, 2, 4, 8, and 10). Each ES lasted ~1 min, followed by a 2-min washout interval. By convention, we always stimulated the cavernous nerve on the right side. However, if the right side failed (rarely) we would then use the left side. Only one ES was performed for each current. For quantitation of the BLEB proerectile effect the left crura was also exposed, and intracavernous injection (ICI) of 50 µl of DMSO or increasing doses (50, 250, 500, and 1,000 mmol) of (±) BLEB were made, with 10-min intervals between washout (saline flush). The ICP rise elicited with ES or BLEB stimulation was quantified either by maximum ICP or by calculating the ratio of maximum ICP/MAP × 100. The maximum ICP is the maximal ICP rise, with MAP being the mean arterial pressure during the plateau phase.

**Table 1. Rat blood glucose and body weight**

<table>
<thead>
<tr>
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<th>Rats</th>
<th>Body Weight, g</th>
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<tr>
<td></td>
<td>Blood Glucose, mg/dl</td>
<td>Initial</td>
</tr>
<tr>
<td>AMC</td>
<td>129.50 ± 7.53</td>
<td>297.90 ± 2.41</td>
</tr>
<tr>
<td>STZ</td>
<td>403.26 ± 36.94*</td>
<td>308.23 ± 7.84</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 19. AMC, age-matched controls; STZ, streptozotocin. *P < 0.0001 vs. AMC; †P < 0.0001 vs. initial weight.

**In vivo studies.** The in vivo contractility studies were performed as described previously (10, 50, 51). Briefly, CC and detrusor strips were mounted longitudinally in a 4-ml organ bath (Multi-Myograph Model 810MS; Danish Myo Technology, Aarhus, Denmark). Aortic rings were mounted in a horizontal manner in a 5-ml Multi-Myograph Model 610M physiological force-measuring apparatus (Danish Myo Technology) by securing to the two pins. The myographs were connected in line to a PowerLab 4/30 Data Acquisition System (ADInstruments) and in turn to a Dual-Core processor Pentium computer for real-time monitoring of physiological force. The SM strips were equilibrated for ≥1 h in Krebs-Henseleit (Krebs) buffer at 37°C, with continuous bubbling of 95% O2 and 5% CO2. The buffer had the following composition (in mM): 110 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11 dextrose; it was changed every 15 min. The strips were continuously adjusted to a resting tension (2,000 mg for human CC, 500 mg for rat CC, 500 mg for bladder, and 700 mg for aorta), and isometric tension was recorded (38, 47, 51). After equilibration, rat CCSM was contracted with 60 mM KCl. This degree of contractile response was taken as 100%, and the force induced by different concentrations (10⁻⁸ to 10⁻⁴ M) of phenylephrine (PE) was expressed as a percentage of this value. Next, strips were precontracted with 3 µM PE (a dose that induces ~50% maximal contraction) and allowed to reach a stable tension, and then the relaxant effects of increasing doses of BLEB (1, 3, 6 µM) were evaluated. Human CC, bladder, and aorta strips were also contracted with 60 mM KCl and then stimulated with PE and carbachol.

**RNA extraction and cDNA synthesis.** Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, the tissue was ground into a powder using a mortar and pestle cooled in liquid nitrogen, without allowing the tissue to thaw. The powder was then homogenized immediately in denaturing buffer using a T8 Ultra-Turrax mini-electric homogenizer (IKA Works, Wilmington, NC), chloroform was added and mixed, the phases were separated by centrifugation,
and the RNA was precipitated by isopropanol and then washed with 70% ethanol and dissolved in RNase-free sterile water. The resulting RNA was quantitated by spectrophotometry at 260/280 nm. Total RNA (1 μg) then was reverse transcribed using 0.5 μg of oligo (dT)_{12-18} primer (Invitrogen), 500 μM dNTPs (Invitrogen), and 200 U of SuperScript II RNase H reverse transcriptase (Invitrogen) in a total volume of 20 μl for 50 min at 42°C.

**Competitive RT-PCR.** As reported previously (22), PCR was performed on 100 ng of the reverse-transcribed cDNA using 2 units of Red Taq DNA polymerase (Sigma), 200 ng each of upstream and downstream primer, and 200 μM dNTPs (Invitrogen). SM-A/SM-B, SM1/SM2, and MLC1/MLC2, alternatively spliced isoforms were amplified with competitive PCR, using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA). The primer sequences are shown in Tables 2 and 3. The cycling conditions were an initial 5 min at 94°C, followed by 35 cycles (30 s at 94°C, 30 s at 55°C, and 1 min at 72°C) using a single fluorescence measurement. SM MHC, myocardin, and nonmuscle (NM) MHC targeted genes were amplified using SYBR Green for amplicon detection, and primer sequences are shown in Table 2. For relative quantification, the efficiency of amplification for each individual primer pair was determined using cDNA target and the 2^{-ΔΔCt} method (26) in conjunction with the RQ Study Software version 1.23 (Applied Biosystems). Gene expression was normalized to expression of the ribosomal protein L19 housekeeping gene.

### Statistical analysis.

Results are expressed as means ± SE for n experiments. Statistical analysis was performed using either the Student t-test (when 2-sample treatments were being compared) or ANOVA when multiple means were compared. P < 0.05 was considered significant.

### RESULTS

The general profiles of our STZ-induced diabetic rats were described in Table 1, from which we can see that mean blood glucose increased significantly from 129.50 ± 7.53 to 403.26 ± 36.94 mg/dl (P < 0.0001). The body weight of AMC rats increased from 297.90 to 399.50 g, whereas the body weight of the STZ rats decreased from 308.23 to 169.01 g (P < 0.0001) over the same 2-mo diabetes period. In line with previous studies, 2-mo DM rats were found to exhibit significant ED, as demonstrated in Fig. 1, with the ICP rise elicited by ES of the cavernous nerve significantly attenuated in the STZ group at all stimulation currents compared with AMC. Even at the highest 10-mA stimulation, the STZ rats still generated only ~50% of the ICP/MAP for AMC rats.

The impaired erectile function can be attributed partially to increased CSMC tone (Fig. 2). Cavernosal strips from STZ animals displayed heightened contractile responses to cumulative doses of PE, reaching almost twice the force generation compared with the AMC at maximal PE stimulation. In addition, Fig. 2A demonstrates that the STZ rats exhibited a more phasic-type contractile profile with a lesser ability to maintain force compared with AMC rats. Also, as exhibited in Fig. 2A, spontaneous contraction was quite often observed for CSMC from STZ animals, whereas it seldom occurred in AMC rats.

Consistent with decreased tonicity, as demonstrated in Fig. 3, A and B, DM altered CC SMM isoform composition with the expression of LC17_a relative to LC17_b increased by ~20% (from ~46 to 65%), whereas relative expression of SM-B (from 68 to 80%) and SM2 (from 22 to 32%) also increased significantly, but to a lesser extent. Figure 3C summarized all

### Table 2. Primer sequences used to amplify rat target genes by PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>SM-A/SM-B</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-AAGCGCAAGAAGAAGACAGCACTCA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-AAAACGCCGAGATTTTCCAGA-3'</td>
</tr>
<tr>
<td>LC17_ab</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GACTGTGGCACAAGAA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CAGCATTCCAGACCATCAGG-3'</td>
</tr>
<tr>
<td>SM1/2</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GGCTGAAAGGCGCAGGAACTC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GGACACCTGTGTTTTTCAATA-3'</td>
</tr>
<tr>
<td>MHC</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-TTTGGCATTTGGCTTTAGG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GTTTACAAGGCGTGAAATCCA-3'</td>
</tr>
<tr>
<td>NMM</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-TGAGAAGCGCGCAGACATC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CAGCGGTCAGGAATGGA-3'</td>
</tr>
<tr>
<td>Myocardin</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-CAGATGCTAGTGCTTTGGAAG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-ATCAGTTCGCGAGCTGAC-3'</td>
</tr>
<tr>
<td>RPL19</td>
<td></td>
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<tr>
<td>Forward</td>
<td>5'-GGCTTCTGGCCTGGTGTA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GATTGGAGATTGGTGTT-3'</td>
</tr>
</tbody>
</table>

SM, smooth muscle; LC, light chain; MHC, myosin heavy chain; NMM, nonmuscle myosin; RPL19, ribosomal protein L19.

### Table 3. Primer sequences used to amplify human target genes by PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-A/SM-B</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GCGCTCTCTTCTGGCTGTC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TTTGGCGAAGATGTGAGATGTC-3'</td>
</tr>
<tr>
<td>LC17_ab</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GAGATGCGGCGCAAAGAAA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-AAGCTATGGCAGACATCAGC-3'</td>
</tr>
<tr>
<td>SM1/2</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GCCTGGAGGAGGAGGAGGAGTC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GAACGATATGCGATTTTCAATA-3'</td>
</tr>
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Fig. 1. Evaluation of erectile function. A: representative tracings of intracavernous pressure (ICP) measurements from the age-matched controls (AMC; top) and streptozotocin (STZ) group (bottom) in response to electrical stimulation (ES) of the cavernous nerve at various currents. The gray arrows indicate stimulation duration. B: bar graph of the average of maximal ICP normalized by mean arterial pressure (MAP). The maximal ICP is the highest pressure reached in response to stimulation, with MAP being the mean arterial pressure during the plateau phase. **$P < 0.01$ vs. STZ ($n = 6$ different rats for each group).

Data for myosin isoform composition and revealed statistically significant increases in the LC$_{17a}$, SM-B, and SM2 alternatively spliced isoforms. We further compared rat CC SMM isoform composition with human CC, rat bladder, and rat aorta. Rat aorta expressed exclusively SM-A and more LC$_{17b}$ and SM1 favoring a slow tonic contraction (Fig. 3, D and F), whereas rat bladder expressed 90% SM-B, 70% LC$_{17a}$, and 30% SM2 exhibiting a fast phasic contraction (Fig. 3, D–F). Rat CC contained more SM-B (68%), less SM2 (27%), and almost equal LC$_{17a}$ (46%) compared with their alternatively spliced counterparts, whereas in contrast human CC predominantly expressed SM-A (90%), more LC$_{17a}$ (66%), and nearly equal SM2 (44%) (Fig. 3, A–E). However, both human and rat CCSM exhibited intermediate tonicity between phasic bladder and tonic aorta with a rapid increase in force development but also with extended force maintenance (Fig. 3F).

To determine whether the altered SMM isoform composition in DM rat CC influences BLEB inhibitory ability, in vitro
organ bath studies were performed. As demonstrated in Fig. 4, BLEB strongly and dose-dependently relaxed rat CC strips from both AMC and STZ rats but exhibited more efficacy in relaxing STZ CCSM at all doses. At 3 μM, BLEB almost completely attenuated the PE-precontracted DM strips, whereas only ≈60% relaxation was observed for CC from AMC rats. When our in vitro study was extended to in vivo, ICI BLEB dose-dependently induced an increase in ICP (Fig. 5). Again, BLEB was demonstrated to be more proerectile for STZ rats, inducing a significantly greater increase in ICP at concentrations at or above 250 nmol.

Finally, because some have suggested BLEB to be more efficacious on nonmuscle myosin (NMM) II than SMM, as described in the INTRODUCTION, we performed real-time PCR to quantify the relative expression of SMM II, myocardin, and NMM II. As demonstrated in Fig. 6, MHC and myocardin
Fig. 3. Smooth muscle myosin (SMM) isoform composition and functional activity. A and B: representative GelStar-stained agarose gels of cDNA products resulting from competitive RT-PCR analysis of the smooth muscle (SM) myosin heavy chain (MHC) pre-mRNA of 5’ region containing the SM-A/SM-B alternative splice site, 17-kDa essential myosin light chain (MLC) pre-mRNA of 3’ region containing the light chain (LC17a/LC17b alternative splice site, and SM MHC pre-mRNA 3’ region containing the SM1/SM2 alternative splice site from AMC and STZ rats. C: averaged quantitative determination of smooth muscle myosin (SMM) pre-mRNA mean isoform percentages in rat corpus cavernosum determined by using the information gathered from gels as in A and B. Values are expressed as means ± SE. *P < 0.05 vs. AMC, **P < 0.01 vs. AMC (n = different animals for each group). D: representative GelStar-stained agarose gels of cDNA products resulting from competitive RT-PCR analysis of the SMM pre-mRNA of SM-A/SM-B, LC17a/LC17b, and SM1/SM2 from rat (top) and human tissues (bottom). E: averaged quantitative determination of SMM pre-mRNA mean isoform percentages in rat and human corpus cavernosum as well as rat bladder determined by using the information gathered from gels as in D. F: typical tracings of human and rat corpus cavernosum, rat bladder, and rat aorta SM contractions in response to various stimuli. The x-axis represents time (min), whereas the y-axis represents force (mg). White vertical lines in gel images in B and D denote intragen splicing for better comparison ability.
expression were significantly upregulated by approximately three- and sixfold, respectively, whereas NMM expression was found to be unchanged, thus effectively increasing the relative ratio of SMM II to NMM II.

DISCUSSION

For the first time, the present study demonstrated that 1) rat CCSM expressed mainly the SM-B and SM1 SM MHC isoforms but with relatively equal expression of the essential light chain isoforms (LC17a to LC17b) at the mRNA level, correlating with an intermediate contractile phenotype between fast phasic-type bladder contraction and the slow tonic-type aorta contraction at the functional level; 2) 2-mo diabetes led to a significant, relative overexpression of the SM-B and SM2 MHC isoforms and the LC17a essential light chain isoform, which generally favors a more phasic-type CCSM; and 3) STZ-induced diabetes also increased the efficacy of the SMM type II selective inhibitor BLEB, which potently relaxed rat CCSM both in vitro and in vivo.

The STZ-induced type 1 diabetic rat model was selected as the experimental DM model in the present study since it is well established and recapitulates relevant aspects of DM-related genitourinary disease in humans (32). Our DM rat model was validated by hyperglycemia (>400 mg/dl) and significant weight loss (Table 1). Consistent with previous reports, ED was confirmed in these diabetic rats with heightened CCSM contractile response to increasing doses of PE (49) and reduced maximum ICP induced by ES of the cavernous nerve (9).

With the exception of the actin-myosin contractile apparatus, diabetes-related ED has been studied extensively. However, refractory episodes to treatment, including PDE5i (the first line therapy for ED), are prevalent in these patients. Interestingly, we recently found that BLEB, a small cell-permeable selective myosin II inhibitor, can potently relax rat CCSM.

**Fig. 4.** Blebbistatin (BLEB) relaxing effect on rat corpus cavernosum strip contractility. 
* A: representative tracings of increasing doses of BLEB relaxing cavernosum SM strips precontracted with PE. Top: AMC group. Bottom: STZ group. The x-axis represents time (min), whereas the y-axis represents force (mg). B: summary graph for the data shown in A. Response to PE stimulus was taken as 100%, whereas the relaxation effect of BLEB was evaluated as a percentage of this response. Values are expressed as means ± SE. *P < 0.05 vs. AMC, **P < 0.01 vs. AMC (n = strips obtained from 37 different animals).
and human CCSM precontracted with a variety of potent agonists in vitro and induce rat ICP increase in vivo, suggesting the possibility of BLEB binding at myosin II as a therapeutic treatment for ED by targeting SM contractile pathways (51). Recent work also suggested that differences existed in BLEB efficacy for different SMM isoforms and/or SMM vs. nonmuscle myosin (NMM) (39). Therefore, whether DM has an effect on CC SMM isoform composition and whether STZ diabetes influences BLEB efficacy in relaxing CCSM are novel, highly intriguing questions that were in need of investigation.

Indeed, we thoroughly characterized rat CC SMM and found that it expressed mainly SM-B but less SM2 and LC17a compared with their alternatively spliced counterparts, which was in contrast to human CC containing mainly SM-A and more SM2 and LC17a (Fig. 3E). But human preparations were obtained from patients with various kinds of ED that might affect the SMM isoform composition. Compared with what we reported previously in normal rabbit CCSM, which expressed approximately SM-B (31%), LC17a (87%), and SM2/SM1 (1.2:1) (14), differences were found from both rat and human. Thus, there may be inherent species differences. However, at the functional level, both rat and human penis displayed an intermediate contraction phenotype between phasic bladder and tonic aorta (Fig. 3F).

Two-month DM significantly increased all rat CC SMM isoforms associated with phasic-type contraction with the expression of LC17a relative to LC17b upregulated by 20%, which favored not only a switch to a more phasic-type contraction but also an increase in force generation in response to cumulative

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**Fig. 5. ICP induced by intracavernous injection (ICI) of BLEB.** A: typical tracings of ICP rise induced by ICI of increasing doses (50–1,000 nmol) of BLEB. Top: AMC group. Bottom: STZ group. The x-axis represents time (min), whereas the y-axis represents pressure (cm H2O). B: summary graph for the data shown in A. Maximal ICP induced by 50 μl of DMSO and increasing doses (50–1,000 nmol) of BLEB are plotted. Each dose was observed for ≥10 min with a 2-min washout. Values are expressed as means ± SE. *P < 0.05 vs. AMC (n = tissue obtained from 4 AMC rats and 5 STZ-induced diabetic rats).
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Fig. 6. Expression of SM MHC, mycardin, and nonmuscle myosin (NMM) heavy chain in rat corpus cavernosum. The studied molecules were quantified by real-time RT-PCR. Expression is normalized to the RPL19 housekeeping gene. Values are expressed as means ± SE. *P < 0.05 vs. AMC (n = 6 different animals for each group).

PE stimulation (Fig. 2), as predicted from previous studies (12, 19, 28, 29). The resulting higher CCSM tone could make endogenous CCSM relaxation mechanisms less effective and contribute to diabetes-induced ED.

For most animals, penile erection lasts no more than 1 min. In particular, rat penile erection and ejaculation occur almost simultaneously, which is quite different from humans (2, 7). Hence, the faster, more phasic CC contraction observed in STZ-diabetic rats may quicken the time to penile detumescence and shorten the duration of penile tumescence.

Relative expression of the LC17a MLC isoform is higher in more phasic-type muscles such as in the bladder, where there is ~90% expression of LC17a in rabbit bladder (13, 28). Several studies have shown that LC17a seems to increase contraction (19, 29). In addition, it is known that the increased relative expression of LC17a correlates positively with the relative expression of SM-B (3, 13). We found a nearly 50% expression of LC17a relative to LC17b across all of the control CC samples, which correlated with our very high relative expression of SM-B. Accordingly, around 10% upregulation of SM-B in STZ CC was in line with a 20% increase in the expression of LC17a. Thus, upregulation of phasic SMM isoforms like SM-B and LC17a by DM could be involved in the mechanisms of ED in these STZ rats with shortened penile tumescence duration and heightened CCSM tone. Actually, we characterized human CC SMM isoforms and tried to determine the DM effect on them in a previous study. Because all samples were from ED patients with various kinds of diseases, we just compared non-DM patients with DM ones, and no significant difference was found (22). However, without a normal control, which is not possible in a human study, it is hard to justify these results. In addition, the STZ model used in the present study is a type 1 diabetic model, whereas most men with ED generally have T2D. Further studies with an increased number of human CC samples that can be segregated based upon comorbidities are clearly needed.

Interestingly, spontaneous contractions were quite often observed in CCSM from STZ rats, whereas they were seldom observed for AMC (Fig. 2A). The STZ rat penile tonicity switched toward a more phasic-type SM resembling urinary bladder, which exhibited a lot of spontaneous activity. It is likely that the change in SMM isoform composition is involved in these spontaneous contractions, generally occurring upon action potential discharge resulting in Ca2+ influx through L-type Ca2+ channels and associated Ca2+ transients (18). However, the exact mechanism underlying these physiological changes remains to be ascertained.

Since it has been suggested that BLEB efficacy may be impacted by the relative expression of the SM-A/SM-B SM MHC isoform composition (39), we compared the efficacy of BLEB at inhibiting CCSM from AMC with that from STZ rats in which we demonstrated an altered SM MHC isoform composition, including a 10% increase in the relative expression of the SM-B isoform. Our data revealed that the SMM type II selective inhibitor BLEB potently relaxed PE precontracted CCSM in vitro (Fig. 4) and increased ICP in vivo (Fig. 5) with sensitivity significantly increased for CCSM from rats with DM. Thus, BLEB would be predicted to be an effective potential new therapeutic for ED, which is especially important for ED of diabetic origin since there is a large population of men with DM-associated ED, and they are more refractory to conventional ED treatments such as PDE5i (11, 44). However, BLEB has the potential to inhibit other smooth muscles and other types of myosin II when administered systemically rather than by intracavernous delivery.

Finally, to further explore the fact that CCSM from STZ rats was more responsive to BLEB, the expression of SM MHC II and NMMHC II was examined with real-time RT-PCR. Our data revealed that SM MHC expression increased threefold, with no change in NMMHC expression (Fig. 6). Concomitant upregulation of myocardin expression, which is a master regulator of SM expression (46), confirmed the relative overexpression of SM MHC in STZ animals. Because BLEB has been found not to compete with ATP binding or inhibit MLC kinase (43) or alter SMM regulatory light chain phosphorylation levels (39), it thus appears that BLEB functions via binding to the myosin-ADP-Pi complex and blocking of the myosin II in an actin-detached state. Therefore, the threefold upregulation of SM MHC possibly contributed to the increased efficacy of BLEB for CCSM from STZ rats.

A limitation for the present study is that the protein levels of SMM isoforms were not determined, since SM-A/SM-B isoform-specific antibodies are not commercially available at the present time. However, previous studies demonstrated that SMM isoform mRNA levels correlated well with the protein expression (13).

In conclusion, we demonstrated for the first time that STZ CCSM contractility was increased and switched from an intermediate-type SM to a more phasic contractile phenotype with overexpression of SM-B, LC17a, SM2, and total MHC. Our novel data also showed that the myosin II selective inhibitor BLEB potently inhibited CCSM contraction with its efficacy increased significantly by diabetes, with the SM MHC playing an apparently more important role than NMMHC in modulating BLEB inhibitory ability. Importantly, with increased efficacy in STZ rats, BLEB could be therapeutically useful as a potential new agent for diabetic ED.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
AUTHOR CONTRIBUTIONS
X.Z. and N.D.K. performed the experiments; X.Z., N.D.K., and M.E.D. analyzed the data; X.Z. and M.E.D. interpreted the results of the experiments; X.Z., N.D.K., and M.E.D. prepared the figures; X.Z. and M.E.D. drafted the manuscript; X.Z., N.D.K., and M.E.D. edited and revised the manuscript; X.Z., N.D.K., A.M., and M.E.D. approved the final version of the manuscript; A.M. and M.E.D. did the conception and design of the research.

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