Increased basal phosphorylation of detrusor smooth muscle myosin in alloxan-induced diabetic rabbit is mediated by upregulation of Rho-kinase β and CPI-17

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METHODS

Diabetic rabbit model. All animal studies were approved by the University of Pennsylvania Animal Care and Use Committee. Diabe-
tes was induced by injection of alloxan (100 mg/kg body wt) into the ear vein of 12-wk-old male New Zealand White rabbits weighing ~6 lbs. The blood samples were collected under normal conditions with food and water available for experimental animals. The blood glucose levels were checked 1 wk after the alloxan injection (using a glucometer) and again before euthanasia after 6 mo. Rabbits that maintained blood glucose levels of 300 mg/dl or higher over the 6-mo period were used for this study. Age-matched normal rabbits were given 5% sucrose in their drinking water to serve as diuretic controls.

Force measurements. Longitudinal strips of DSM (~50 mg and 3 × 10 mm) were suspended in 15 ml of Tyrode’s buffer at 37°C as previously described (4). After a 30-min equilibration, the length of optimal force development (L₀) was determined by increasing the length of each strip in 1.5-mm increments until maximal contractile force to electric field stimulation (80 V, 32 Hz, 1 ms) was achieved. After being washed three times with Tyrode’s buffer, the strips were equilibrated at L₀, for an additional 15 min to allow stabilization of the muscle at the resting level. The detrusor muscle strips were then stimulated to contract by adding increasing concentrations of endothelin-1 (ET-1) and norepinephrine (NE). Maximal contractile forces were measured by increasing the length of each strip in 1.5-mm increments until maximal contractile force to electric field stimulation (80 V, 32 Hz, 1 ms) was achieved. The detrusor muscle strips were then subjected to electric homogenizer. The tissue homogenate was centrifuged at 10,000 g for 20 min. The supernatant was applied to mini-IEF apparatus. The gels were then removed and subjected to second-dimension electrophoresis on 14% SDS-PAGE gels. The gels were then stained with Coomassie blue, and the spots corresponding to phosphorylated-MLC20 and unphosphorylated-MLC20 were identified based on their previously established migratory positions in this gel. The ratio of phosphorylated MLC20 to total MLC20 was determined by Bio-Rad GS-800 Calibrated Densitometry.

RNA extraction and RT. RNA was extracted from frozen bladder body of normal, diuretic, and diabetic rabbits using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, pieces of tissue (~50 mg) were crushed to a fine powder using a liquid nitrogen precooled mortar and pestle, mixed with 1 ml TRIzol reagent, and then homogenized with a mini-electric homogenizer. After 5-min incubation at room temperature, 0.2 ml of chloroform was added and vortexed vigorously for phase separation. After 15 min of centrifugation, 0.5 ml of isopropyl alcohol was added to the aqueous phase to precipitate RNA. The RNA quality and quantity were measured by UV spectrophotometry. M-MLV reverse transcriptase (Invitrogen) was used to synthesize first-strand cDNA. First, a 12-μl reaction was prepared containing 1 μl Oligo (dT) (Promega, Madison, WI), 3.5 μg RNA, and distilled water. Then this mixture was incubated at 70°C for 10 min. After slow cooling, the following contents (4 μl 5× buffer, 1 μl dNTP, 1 μl 0.1 M DTT, and 1 μl M-MLV) were added to make a final reaction volume of 20 μl. After 37°C for 1 h followed by a 90°C 5-min incubation, the cDNA was used as a template in PCR.

Real-time PCR. Real-time PCR was performed using the Light Cycler (Roche). Basically, a mastermix of the following reaction components was prepared to the indicated end-concentration: 1 μl forward primer (0.4 μM), 1 μl reverse primer (0.4 μM), 4 μl 5× PCR buffer (BD Biosciences Clontech, Palo Alto, CA), 2 μl dNTP (100 μM), 0.4 μl DMSO, 2 μl SYBR Green I (Sigma), 11 μl water, and 0.6 μl Titanium Taq DNA polymerase (BD Biosciences Clontech). Then 19 μl of the mastermix were filled into the LightCycler glass capillaries and 1 μl cDNA (produced as described above) was added as the PCR template. Capillaries were closed, centrifuged, and placed into the Light Cycler rotor. The following experimental protocol was used: denaturation (95°C for 30 s) followed by an amplification program repeated 20–35 cycles (95°C for 5 s then 68°C for 20 s) using a single fluorescence measurement. Also, a melting curve program (60–95°C with a heating rate of 0.1°C per s with continuous fluorescence measurement) was run and finally a cooling step to 40°C. The specificity of each PCR product was verified by the melting curve analysis and gel electrophoresis. The standard amplification curves were constructed using known amounts of purified PCR product. The expression of Rho-kinase β and CPI-17 was determined by the PCR efficiency and the cycle number of crossing threshold.

Protein extraction and Western blot analysis. Frozen pieces of bladder tissue (~50 mg) were ground into fine powder as described above. Total extractable protein was isolated and protein concentration was determined by Bio-Rad DC Protein Assay. Then 20 μg of total protein of each sample were separated by 12% SDS-PAGE on large-format (16 × 16 cm) gels and transferred to an Immobilon-P membrane (Millipore, Bedford, MA) overnight at 30 V (Bio-Rad mini-transfer unit) in buffer (25 mM Tris, 192 mM glycine, 20% methanol). After being blocked with 5% nonfat milk for 1 h in PBS containing 0.1% Tween 20 (PBST), the membrane was incubated with primary antibodies in PBS for 2 h at room temperature with a dilution of 1:1,000 for monoclonal Rho-kinase β antibody (Transduction Laboratories, Lexington, KY), 1:5,000 for monoclonal smooth muscle α-actin (Sigma, St. Louis, MO), and 1:2,000 for rabbit anti-CPI-17 antibody (Anti-CPI-17, Upstate, Charlotteville, VA). The membrane was then washed three times with PBST and further incubated with secondary horseradish peroxidase–linked antibody (1:3,000 dilution of anti-mouse IgG for Rho-kinase β, and 1:5,000 dilution of donkey anti-rabbit for anti-CPI-17; Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. Membranes were then washed five times with PBST, and protein expression was detected using an enhanced chemiluminescence kit (ECL) from Amersham Biosciences. The amount of Rho-kinase β and CPI-17 was determined by reflectance scanning. All reactions were kept in the linear range. Identical gels were run for all samples and stained with Coomassie blue to confirm that proteolytic breakdown of the samples had not occurred and also to confirm equal protein loading of gels. Smooth muscle α-actin was used as internal control to normalize Western blot data.

Immunohistochemistry. Cross-sections (5 μM) of bladder from diabetic and age-matched rabbits were made from the paraffin blocks. Tissue sections were deparaffinized and put into descending grades of alcohol and then blocked 30 min in 1% BSA solution. After blocking, sections were incubated for 2 h at room temperature with the antibodies specific for Rho-kinase β and CPI-17 (which are described above) at a 1:200 dilution. After washing three times in PBST, the sections were then treated with secondary antibody (anti-mouse IgG-FITC for Rho-kinase β and anti-rabbit IgG-FITC for CPI-17; Sigma) at a dilution of 1:400 for 1 h, washed again three times with PBST, and then mounted with a drop of mounting medium (Aqua-Mount, Lerner Labs, Pittsburgh, PA). Sections were viewed under a Nikon (Melville, NY) Eclipse E800 fluorescence microscope, and images were captured using a RT Slider SPOT camera (Diagnostic Instruments, Sterling Heights, MI) and Image-Pro Plus software from Media Cybernetics (Silver Spring, MD). A negative control, in which only the secondary antibody was added, was performed for all samples.

Statistical analysis. All data are expressed as means ± SE with P < 0.05 considered statistically significant. ANOVA was applied using SigmaStat Version 2.03 (SPSS, Chicago, IL). Each n refers to a set of age-matched normal, diuretic control, and diabetic rabbits.

RESULTS

Blood glucose level in normal, diuretic, and diabetic animals. Alloxan treatment induced a significantly high level of blood glucose in the rabbits (Fig. 1). The average blood
The maximum contractions of DSM strips were (Fig. 3). The phosphorylation level for diabetic sample dropped to 9.2 from 28% stimulated by adding increasing concentration of bethanechol similar to the normal sample, whereas diabetic DSM strips with Y-27632, and the relaxation of diuretic DSM strips was very easier to relax with Y-27632 than diabetic DSM (Fig. 4). Normal DSM relaxation. We found that normal DSM was much easier to relax with Y-27632, a highly selective Rho-kinase inhibitor. Five minutes after Y-27632 was added, the basal MLC phosphorylation level for diabetic sample dropped to 9.2 from 28% (Fig. 3).

Relaxation of bethanechol-precontracted DSM strips by Y-27632. The maximum contractions of DSM strips were stimulated by adding increasing concentration of bethanechol (0–250 μM). Then 10 μM Y-27632 was used to induce DSM relaxation. We found that normal DSM was much easier to relax with Y-27632 than diabetic DSM (Fig. 4). Normal DSM strips achieved ~50% relaxation in 3 min after adding Y-27632, and the relaxation of diuretic DSM strips was very similar to the normal sample, whereas diabetic DSM strips required 6 min to reach 50% relaxation.

Expression of Rho-kinase β in normal, diuretic, and diabetic DSM. We found that diabetes induced upregulation of Rho-kinase β at both mRNA and protein levels (Fig. 5). Real-time PCR is a very sensitive method to detect any change at mRNA level. A standard curve of target gene can be used to calculate its PCR efficiency and confirm the PCR amplification is in a linear range (Fig. 5A). A small PCR cycle number of crossing threshold means a high copy number of target transcript to start with. Our result showed the cycle number of crossing threshold was ~27.3 for normal sample, 26.9 for diuretic control, and 23.5 for diabetic sample (Fig. 5B). This significantly reduced cycle number revealed 10.3-fold higher expression of Rho-kinase β at the mRNA level in diabetic detrusor compared with normal and diuretic control. However, there was no significant difference between diuretic and normal detrusor. Western blot analysis confirmed the upregulation of Rho-kinase β in diabetic detrusor at the protein level. Smooth muscle α-actin was used as an internal control to normalize the Western blot. The Rho-kinase band was remarkably stronger in diabetic samples than in normal and diuretic controls (Fig. 5C). The overexpression of Rho-kinase β at the protein level revealed about a 2.1-fold increase in diabetic detrusor compared with normal and diuretic samples (Fig. 5D).

Expression of CPI-17 in normal, diuretic, and diabetic DSM. We also found that the expression of CPI-17 was significantly increased in diabetic detrusor at both mRNA and protein levels (Fig. 6). Real-time PCR showed that there was no significant difference in the required PCR cycle numbers between normal and diuretic controls, about 31.4 for normal samples and 31.6 for diuretic controls. However, it was significantly reduced to 26.1 cycles for diabetic samples, which meant ~12.5-fold more CPI-17 mRNA in diabetic DSM (Fig. 6B). Western blot also showed much stronger bands of CPI-17 for diabetic DSM samples compared with that of controls (Fig. 6C). Diabetes induced ~2.5-fold higher protein expression of CPI-17 in DSM (Fig. 6D).

Immunohistochemical localization of Rho-kinase β and CPI-17 in bladder from diabetic and normal rabbits. The representative immunostaining slides showed the localization of Rho-kinase β and CPI-17 in bladder tissue from normal and diabetic rabbits (Fig. 7). The intensity of immunofluorescent signals (FITC) indicated the expression of Rho-kinase β and CPI-17, which was much brighter in the bladder from diabetic rabbits (Fig. 7, A and C) compared with that from normal rabbits (Fig. 7, B and D). It can also be observed that both Rho-kinase β and CPI-17 are mainly expressed within smooth muscle bundles in the bladder. In conclusion, immunostaining confirmed that diabetes remarkably upregulated Rho-kinase β and CPI-17 in DSM.

Table 1. Body weight and bladder weight from normal, diuretic, and diabetic animals

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<th>Body Weight, kg</th>
<th>Bladder Weight, g</th>
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<tbody>
<tr>
<td>Normal</td>
<td>3.67±0.28</td>
<td>2.11±0.14</td>
</tr>
<tr>
<td>Diuretic control</td>
<td>3.88±0.31</td>
<td>2.19±0.22</td>
</tr>
<tr>
<td>Diabetic</td>
<td>2.83±0.15*</td>
<td>2.81±0.11*</td>
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Values are means ± SE. *P < 0.01.
DISCUSSION

Urinary bladder dysfunction is a common complication of diabetes mellitus, and the mechanisms of diabetes-induced bladder dysfunctions are not well understood. Therefore, the treatment strategies for bladder dysfunction in diabetes are very limited. DSM contractility is crucial for normal bladder function. In this study, we examined the effects of diabetes on the regulation of MLC phosphorylation, which is a key factor for smooth muscle contraction. We demonstrated that MLC phosphorylation was higher in DSM from diabetic animals compared with normal and diuretic controls (Fig. 2). This result is consistent with a recent study on diabetes showing...

Fig. 2. Selected areas from 2-dimensional (2D) gel showing basal MLC\textsubscript{20} phosphorylation in normal, diuretic, and diabetic detrusor smooth muscle (DSM). A: normal. B: diuretic. C: diabetic. D: bar graph showing the average values of myosin light chain (MLC) phosphorylation. Phosphorylated MLC\textsubscript{20} (P-MLC\textsubscript{20}) runs slightly higher and more toward the acidic side than unphosphorylated MLC\textsubscript{20} (UP-MLC\textsubscript{20}) in the gel. The basal phosphorylation of MLC\textsubscript{20} was −18% in normal DSM. Diuretic control had a very similar level (19.2%) of MLC\textsubscript{20} phosphorylation. However, the phosphorylation level was significantly increased to 28% in diabetic detrusor. *Significant difference between samples (n = 3, P < 0.05).

Fig. 3. MLC\textsubscript{20} dephosphorylation induced by selective Rho-kinase inhibitor Y-27632 in diabetic detrusor. A: 2D gel for diabetic detrusor. B: 2D gel for diabetic detrusor with Y-27632 incubation. C: bar graph showing the decreased MLC phosphorylation induced by Y-27632. The phosphorylation level of diabetic detrusor MLC\textsubscript{20} was significantly decreased from 28 to 9.2% at 5 min after adding Y-27632. *Significant difference between samples (n = 3, P < 0.05).
decreased detrusor contraction with increasing levels of MLC phosphorylation (25). Moreover, we found that basal MLC phosphorylation can be reduced by incubation with Rho-kinase inhibitor Y-27632 (Fig. 3) and that DSM relaxation occurred more slowly in diabetic animals (Fig. 4). These data suggest that Rho-kinase is involved in the regulation of MLC phosphorylation and there is more Rho-kinase activity in diabetic DSM, which contributes to higher basal MLC phosphorylation and slower relaxation. We confirmed the upregulation of Rho-kinase at both mRNA and protein level (Figs. 5 and 7).

Rho-kinase is activated by a small GTPase RhoA and then activated Rho-kinase phosphorylates MP, which leads to an inhibition of MP activity (12). There are two isoforms of Rho-kinase, α and β, and our previous study showed the overexpression of Rho-kinase β in diabetic corpus cavernosum smooth muscle (4). Using the same experimental model for this study, we found that diabetic DSM also overexpressed Rho-kinase β. Our findings reveal that diabetes induces up-regulation of Rho-kinase in more than one type of urological smooth muscle. There are also reports on the upregulation of RhoA/Rho-kinase in cavernosal tissue isolated from the penis of STZ-diabetic rats (2), increased RhoA translocation in renal cortex of diabetic rats (20), and overexpression of RhoA in the basilar artery from diabetic rats (21). CPI-17 is a PKC-catalyzed phosphorylation-dependent inhibitory protein for MP, and it is mainly expressed in smooth muscle (8). It is well documented that diabetes induces overexpression of PKC (9, 11). We directly measured the effect of diabetes on CPI-17, the downstream substrate of PKC, and found overexpression of CPI-17 in detrusor from diabetic animals (Figs. 6 and 7). To our knowledge, this is the first report of upregulation of CPI-17 in DSM. The upregulation of CPI-17 and Rho-kinase provides a logical explanation for decreased MP activity without any change in MP content (25). Both CPI-17 and Rho-kinase play an important role in the calcium sensitization pathway by regulating DSM contractility through inhibition of MP activity (26). Therefore, the upregulation of Rho-kinase β and CPI-17 contributes to the high basal MLC phosphorylation level and slow relaxation in DSM from diabetic animals.

A recent study showed the overexpression of two thin filament-associated proteins, caldesmon and calponin, in DSM from diabetic rabbits (19). Both caldesmon and calponin suppress actin-myosin interaction and act as a brake for smooth muscle contraction. The overexpression of caldesmon and calponin might necessitate higher levels of MLC phosphorylation to overcome the effect of these inhibitory proteins on force generation. This might be one of the reasons why DSM from diabetic animals has impaired contraction, despite higher basal MLC phosphorylation.

Fig. 4. Relaxation of bethanechol-precontracted DSM strips from normal, diuretic, and diabetic animals by Y-27632. Muscle strips were stimulated to contract in response to 0 to 250 μM of bethanechol. After the muscle developed maximal force, Y-27632 was added to the muscle bath to obtain a final concentration of 10 μM and the relaxation of muscle strips was calculated.

Fig. 5. Expression of Rho-kinase β at both mRNA and protein level. A: real-time PCR standard curve for Rho-kinase β. B: average of required PCR cycle numbers to reach crossing threshold. The required PCR cycle numbers was ~27.3 for normal samples, 26.9 for diuretic controls, and 23.5 for diabetic samples. A significantly lower number of PCR cycles for the diabetic sample indicated that diabetic DSM sample had more copies of Rho-kinase β transcript. C: Western blot for Rho-kinase β and smooth muscle α-actin. D: bar graph showing the average of relative protein expression level. There was almost a 2.1-fold higher protein expression of Rho-kinase β in diabetic detrusor compared with normal and diuretic samples. *Significant difference between samples (n = 4, P < 0.01).
However, it is not clear whether this alteration is compensatory or pathological. The hyperglycemia-induced oxidative stress is also likely to alter the contractile protein by modification of the myosin active sites. Additional studies are required to evaluate the role of oxidative stress in reducing the DSM contractility in diabetes.

In summary, our results showed that the high basal MLC phosphorylation level, slow relaxation, and upregulation of Rho-kinase and CPI-17 only occurred in diabetic animals, not in diuretic controls. Thus the downstream effects of hyperglycemia might be the primary reason for all these changes. The upregulation of Rho-kinase and CPI-17 may play an important role in the adaptation of the smooth muscle to high blood glucose. Further studies on the pharmacological effects of Rho-kinase and CPI-17 may provide novel strategies for the treatment of diabetes-induced bladder dysfunction.

Fig. 6. Expression of CPI-17 at both mRNA and protein level. A: standard curve of real-time PCR for CPI-17. B: average of the PCR results. The required PCR cycle numbers was significantly decreased in diabetic DSM samples compared with normal or diuretic controls. It was $-31.4$ cycles for normal sample, $31.6$ cycles for diuretic controls, and $26.1$ cycles for diabetic samples. C: Western blot for CPI-17. D: bar graph showing the relative expression of CPI-17 at the protein level. There was almost a 2.5-fold higher expression of CPI-17 at the protein level in diabetic detrusor compared with normal and diuretic samples. *Significant difference between samples ($n = 4$, $P < 0.01$).

Fig. 7. Immunohistological localization of Rho-kinase β and CPI-17. Representative bladder paraffin sections from age-matched normal and 6-mo diabetic rabbits were prepared for immunofluorescence microscopy. The immunofluorescent staining was remarkably brighter in the bladder from diabetic rabbits (B and D) compared with the bladder from normal rabbits (A and C), which indicated that both Rho-kinase and CPI-17 proteins were overexpressed in response to diabetes. It can also be concluded from A-D that both Rho-kinase and CPI-17 are mainly expressed in the smooth muscle cells within DSM bundles (black arrowheads). The blood vessels (white arrows) also react with these antibodies as expected, but not the collagenous matrix (*)
ACKNOWLEDGMENTS

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GRANTS

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REFERENCES