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Original Article

Reverse mode of sodium/calcium exchanger subtype 1 contributes to detrusor overactivity in rats with partial bladder outflow obstruction

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Abstract: To investigate whether the reverse mode of sodium/calcium exchanger subtype 1 (NCX1) plays an important role in the excitability of detrusor cells in rats with partial bladder outflow obstruction (PBOO), PBOO was maintained for 6 weeks in forty female Wistar rats. Thirty of the animals exhibited non-voiding bladder contraction and comprised the DO group. An additional thirty sham-operated female Wistar rats were used as the control group. The expression levels of NCX1 were compared between the two groups by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), western blotting (WB), and double-label immunofluorescence. The contractions of detrusor strips in NCX reverse mode were measured in both groups using isometric tension. The role of NCX in the regulation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of smooth muscle cells was observed in reverse mode using confocal microscopy, and the current was evaluated in the presence of the antagonist KB-R7943 (5 μM and 10 μM) using the whole-cell patch-clamp technique. The expression of NCX1 was significantly higher in the DO group than in the control group, as assessed by qRT-PCR, WB analysis and immunofluorescence. The volume and rate of Ca^{2+} ion flux through the NCX, as well as the NCX currents, were higher in the DO group than in the control group in both modes. Increased NCX1 levels may contribute to the establishment of DO after PBOO by elevating $[Ca^{2+}]_i$ in reverse mode under depolarization, potentially inducing cell excitability.

Keywords: Sodium/calcium exchanger, smooth muscle cells, detrusor overactivity, calcium ion concentration, bladder

Introduction

Detrusor overactivity (DO) is characterized by involuntary spontaneous contractions during bladder filling, which can occur in some patients with overactive bladder (OAB) [1]. Smooth muscle contractile activity is associated with the regulation of $[Ca^{2+}]_i$. In addition, aberrant $[Ca^{2+}]_i$ has been observed in smooth muscle cells from human OAB patients [2].

A variety of channels, transporters, and pumps are involved in the mediation of Ca^{2+} regulation. The sodium/calcium exchanger (NCX) spans the plasma membrane and transports three Na^+ ions in exchange for one Ca^{2+} ion, thereby helping to maintain the balance of intracellular Ca^{2+} [3]. Recent evidence has indicated that the NCX, when operating in reverse mode, may cause imbalances of $[Ca^{2+}]_i$ in some pathophysiologic conditions, such as ischemia. Moreover, bladder smooth muscle contraction is increased in transgenic mice overexpressing NCX1.3 ($NCX1.3^{tg/tg}$) [4]. These findings indicate that NCX may play a critical role in bladder smooth muscle contraction disorders. Although three mammalian NCX isoforms have been verified based on amino acid sequence homology, the isoforms may play different roles in regulating $[Ca^{2+}]_i$. However, NCX expression and its role in DO remain unclear.

In our previous work, we reported that NCX subtype 3 is co-expressed with c-kit in bladder interstitial cells of Cajal (ICC) and contributes to bladder excitability [5]. Thus, in this study, we evaluated the functional role of NCX proteins, especially NCX1 and its reverse mode, in DO rats after partial bladder outflow obstruction (PBOO).

Materials and methods

Ethical approval and animals

This study was approved by the Research Council and Animal Care and Use Committee of the Third Military Medical University, China (approval no. SYXK20070002). The rats were used in accordance with the Guidelines on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research and the Guidelines for Animal Care. Seventy adult female Wistar rats (weighing 200-220 g) were used in this study. All rats were anesthetized with a peritoneal injection of pentobarbital sodium (40 mg/kg) for surgery and cystometry. All animals were sacrificed with an intravenous overdose of pentobarbital sodium after the completion of the studies.

PBOO surgery and group classifications

PBOO was performed and maintained for 6 weeks in 40 rats to establish DO. A small plastic catheter tube (1.0-mm outer diameter) was placed in the urinary bladder via the urethral orifice. The bladder neck was then tied with a 2/0 silk ligature with the tube inside to achieve partial obstruction, after which the tube was removed. Thirty sham-operated rats were classified as the control group and underwent the same procedures without the ligature, as we described previously [6]. After 6 weeks, the bladder was catheterized through the urethra by a human epidural catheter (2-F internal diameter), which was connected to urodynamic equipment (Dantec Menuet, Skovlunde, Denmark) via a three-way connector for both infusion and pressure recording. Cystometry was performed by infusing warm saline (37-38°C) at a rate of 0.2 ml/min, and the infusion was stopped when urine leakage was observed around the catheter. After PBOO surgery, thirty rats developed significant non-voiding detrusor contractions before the onset of micturition and were classified as the DO group [6]. Ten rats from the PBOO group failed to display significant non-voiding detrusor contractions and were eventually sacrificed with an intravenous overdose of pentobarbital sodium. The remaining thirty sham-operated rats with consistent bladder activity were classified as the control group.

Quantitative reverse transcriptase-PCR (qRT-PCR)

To measure the NCX1 mRNA levels in DO, four rats from each group were studied, and their detrusors were dissected to remove the mucosa. Total RNA was extracted with a commercial kit optimized for RNA purification (Tiangen, Beijing, China). Single-stranded cDNA was synthesized using a ReverTra Ace kit (Toyobo, Osaka, Japan) and oligo(dT)-20 primers. The cDNA was used for qRT-PCR, which was performed in a 7500 Fast RT-PCR system (Applied Biosystems, CA, USA) with Real Master Mix SYBR Green (Tiangen, Beijing, China). The samples were amplified simultaneously for 30 cycles in triplicate, with a single fluorescence measurement per replicate. qRT-PCR data were collected using the ABI Prism 7000 SDS software (Applied Biosystems, CA, USA). The primer sequences and the lengths of the PCR products were as follows: NCX1 forward, 5'-TGCGGCCAACGGGGAACAG-3'; NCX1 reverse, 5'-CACAGGAGCACAAACAGGGAAGA-3' (186 bp); NCX2 forward, 5'-GCTGGCCTTCTCGGTCACACTG-3' and GAPDH forward, 5'-ACGGGAAGCTCACTGGCATGG-3'; GAPDH reverse, 5'-GCCGCCTGCTTCACCACCTTCT-3' (124 bp). All of the primers were analyzed with the Basic Local Alignment Search Tool (BLAST) and were synthesized by Sangon (Shanghai, China).

NCX1 protein detection by western blotting (WB) and double-label immunofluorescence

Four rat bladders were harvested from each group, and the detrusors were dissected to remove the mucosa. Their protein concentrations were determined using a Bio-Rad (Bio-Rad Laboratories, CA, USA) protein assay kit following the manufacturer's instructions [5]. Equal amounts of protein (50 μg) were separated on 12% sodium dodecyl sulfate-polyacrylamide gels. The NCX1 proteins were detected with goat polyclonal anti-NCX1 (dilution 1:500, Santa Cruz Biotechnology, California, USA), followed by incubation with a secondary antibody (HRP-conjugated rabbit anti-sheep antibody, dilution 1:1000; Dako, California, USA). The immunoreactive protein was detected using enhanced chemiluminescence (ECL; Millipore, Massachusetts, USA) and scanned with a Bio-Rad image analyzer. Specimens were obtained

from three fresh rat bladders from each group to observe the protein localization of NCX1 by double-label immunofluorescence, as described previously [5]. The primary antibodies used included a goat polyclonal antibody for NCX1 and myosin (dilution 1:200; Abcam, London, UK); the specimens were incubated with the primary antibodies for 24 h and were imaged using a Leica TCS-SP5 confocal microscope (Leica, Solms, Germany). The images representing reconstructions of optical sections were prepared using a personal computer with the LAS AF Lite software (Leica, Solms, Germany).

Contractility analysis of detrusor strips

Eight rats from each group were used in this experiment. The bladder detrusor strips (2-3 mm wide and 5-7 mm long) were prepared with a razor blade after removal of the mucosa by blunt dissection, as previously described [7]. For isometric detrusor smooth muscle (DSM) tension recordings, an initial tension (~10 mN) was applied to the DSM strips. The Powerlab data acquisition system using the 'Chart' software (AD Instruments, New Hampshire, USA) was employed in this experiment. The strips were bathed in Krebs solution (118 mM NaCl, 11.7 mM D-glucose, 24.9 mM $NaHCO_3$, 4.7 mM KCl, 1.15 mM $MgCl_2$, 1.15 mM NaH_2PO_4 and 1.8 mM $CaCl_2$), equilibrated at 37°C, gassed with a 5% CO_2/95% O_2 mixture for 30 min, and pH-adjusted to 7.35-7.45. Low-Na^+ Krebs solution was generated by substituting Tris-Cl for NaCl and mixing 50/50 with Krebs's solution. To clarify the role of NCX in the reverse mode, strips were incubated in KCl (20 mM) and then bathed in Low-Na^+ Krebs solution before 10 μM KB-R7943 (Sigma-Aldrich, Missouri, USA) was added to block the exchanger. Changes in amplitude and frequency were observed.

Fluorescence measurement of $[Ca^{2+}]_i$ with preloaded flou-3AM

The intracellular Ca^{2+} ion exchange was compared when the smooth muscle cells experienced reverse-mode NCX activity. Six rats from each group were used in this test. Smooth muscle cells (SMCs) were isolated as described previously [5]. SMCs were loaded with 1 mM flou-3AM (Dojindo, Tokyo, Japan) in HBS (Gibco, New York, USA), incubated at 37°C and 5% CO_2/95% O_2 for 30 min, and perfused with Tyrode solution (118 mM NaCl, 6.1 mM glucose, 24 mM $NaHCO_3$, 4.0 mM KCl, 1.0 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 1.8 mM $CaCl_2$ and 5.0 mM sodium pyruvate, pH 7.35) for scanning (1 image/second) via a Leica TIRF dynamic image collection system within 1 h. To test the effect of the NCX in reverse mode, cells were challenged by caffeine (10 mM; Sigma-Aldrich, Missouri, USA) for 5 min to estimate endoplasmic reticulum Ca^{2+} content as previously described [8], and the cells were stimulated with a low-Na^+ solution with or without KB-R7943 (5 μM; Sigma-Aldrich, Missouri, USA) to reduce the Na^+ concentration from 147.4 mM to 87.4 mM as reported previously [5]. The background fluorescence of unloaded cells was negligible. The final data were normalized as the relative fluorescent intensity (RFI = F1/F0, where F1 = mean fluorescent intensity and F0 = baseline fluorescent intensity).

NCX current analysis using the whole-cell patch-clamp technique

Eight rats from each group were tested to analyze the NCX current. Using a previously published protocol, cells were perfused in an external solution containing 137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, and 10 mM D-glucose, pH 7.4. Glass pipettes (4-6 MΩ) (pH adjusted with NaOH to 7.4 and gassed with 100% O_2) were filled with 5 mM NaCl, 100 mM CsOH, 5 mM KCl, 2 mM $MgCl_2$, 20 mM TEA-Cl, 10 mM HEPES, 8 mM D-glucose, 1 mM Na_2ATP, 5 mM EGTA, and 4.94 mM $CaCl_2$, pH 7.2 [9]. We performed direct measurements of the NCX currents in freshly isolated bladder SMCs using the patch-clamp technique. The NCX currents were elicited by depolarization in 10-mV steps from a holding potential of -40 mV to test potentials from -100 to +60 mV at a frequency of 0.1 Hz for 300 ms. The NCX currents were measured before and after the application of the inhibitor, Ni^{2+} at 5 mM, as the total I_{ncx}, and KB-R7943 (5 μM and 10 μM) were subsequently added to test the effect of NCX inhibition in the reverse mode [10]. The experiments were performed at 23 ± 2°C. The current records were acquired and analyzed using the pClamp 10.0 software (Axon Instruments, California, USA).

Statistical analysis

All responses are reported as the means ± SD; n refers to the number of animals. Statistical

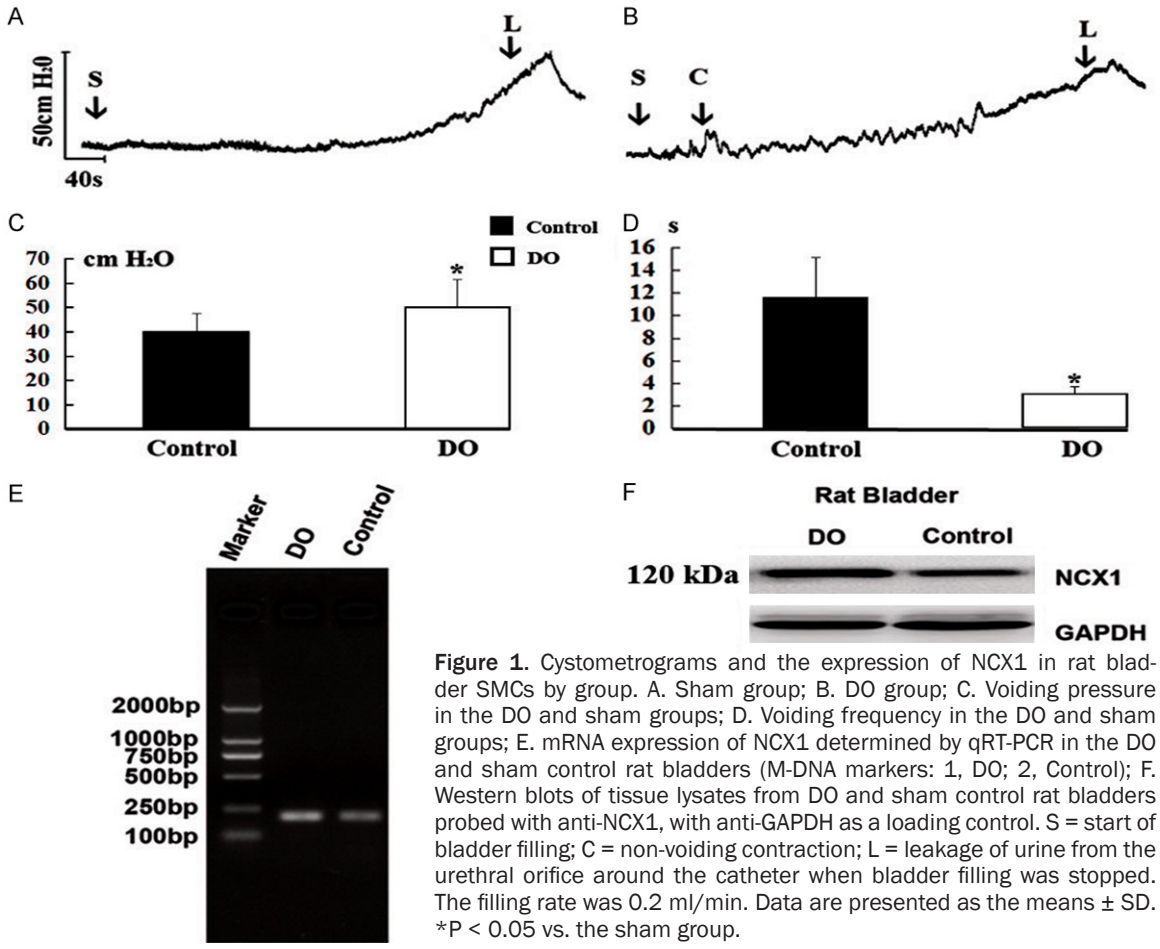

Figure 1. Cystometrograms and the expression of NCX1 in rat bladder SMCs by group. A. Sham group; B. DO group; C. Voiding pressure in the DO and sham groups; D. Voiding frequency in the DO and sham groups; E. mRNA expression of NCX1 determined by qRT-PCR in the DO and sham control rat bladders (M-DNA markers: 1, DO; 2, Control); F. Western blots of tissue lysates from DO and sham control rat bladders probed with anti-NCX1, with anti-GAPDH as a loading control. S = start of bladder filling; C = non-voiding contraction; L = leakage of urine from the urethral orifice around the catheter when bladder filling was stopped. The filling rate was 0.2 ml/min. Data are presented as the means ± SD. *P < 0.05 vs. the sham group.

comparisons were performed using Student's t-test. *P* values less than 0.05 were considered statistically significant.

Results

Filling cystometry & bladder weight

The mean voiding pressures and micturition intervals of the control animals were 40.04 ± 7.4 cmH_2O and 12.13 ± 3.77 min (n = 30), respectively (**Figure 1A**). The thirty PBOO-induced DO animals presented with frequent and high non-voiding contractions during the filling phase, with a mean voiding pressure of 50.26 ± 11.39 cmH_2O and a mean micturition interval of 3.09 ± 0.78 min (n = 30) (**Figure 1B**). The other ten rats, in stable micturition, were excluded from this study. The results of the cystometry test in the DO group were significantly different from those for the control group (P < 0.05), as shown in **Figure 1C** and **1D**. The wet weights of the bladders were 110.0 ± 7.55 mg in the control group (n = 30) and 480.5 ± 41.45 mg in the DO group (n = 30, P < 0.05).

NCX1 expression in the SMCs from the Wistar rat bladders

NCX1 expression in bladder SMCs was confirmed by qRT-PCR, WB and double-label immunofluorescence (**Figures 1** and **2**). Relative to the controls, the mRNA levels of NCX1 increased 2.43-fold (n = 4, P < 0.05) in the DO group (**Figure 1E**). The mean ratios of NCX1 protein between the two groups relative to GAPDH were 0.94 ± 0.12 and 0.66 ± 0.11, respectively (n = 4, P<0.05) (**Figures 1F** and S1). Double-label immunofluorescence with anti-NCX1 antibodies showed fluorescent staining at the cell surface membrane in the DO group (**Figure 2A** and **2B**).

Isometric measurement of NCX activity in bladder contraction

There was a statistically significant difference between the weights of the bladders in the DO

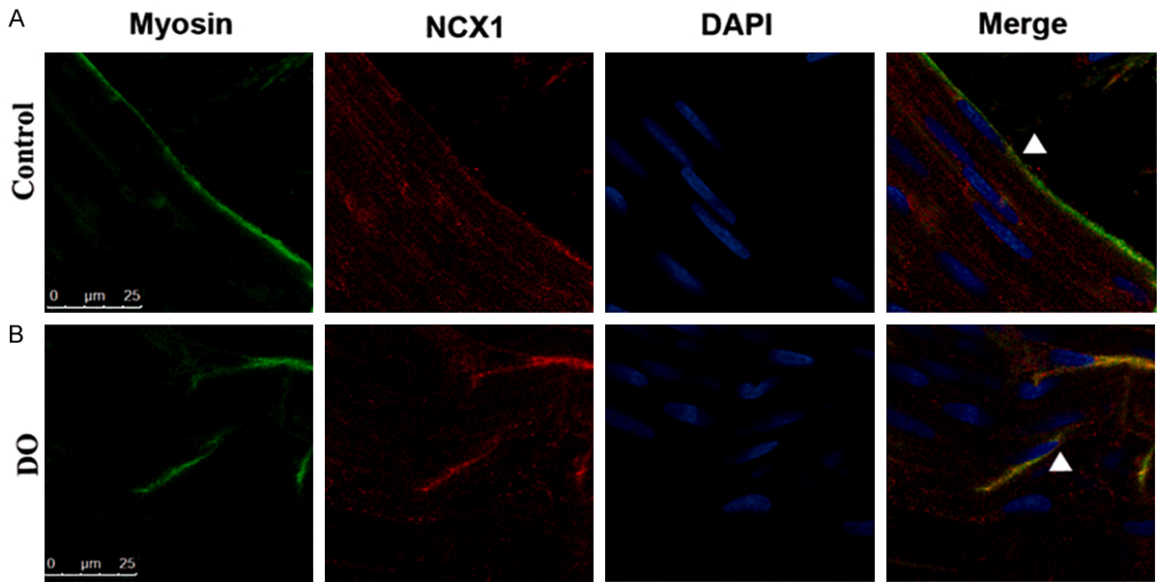

Figure 2. Double-label immunofluorescence for NCX12 and smooth muscle myosin (SMM) in rat bladders. A. NCX1 in the sham group; B. NCX1 in the DO group. The final image shows the merge of all fluorescence channels. Triangle: SMCs expressing NCX1. The scale bar represents 25 μm.

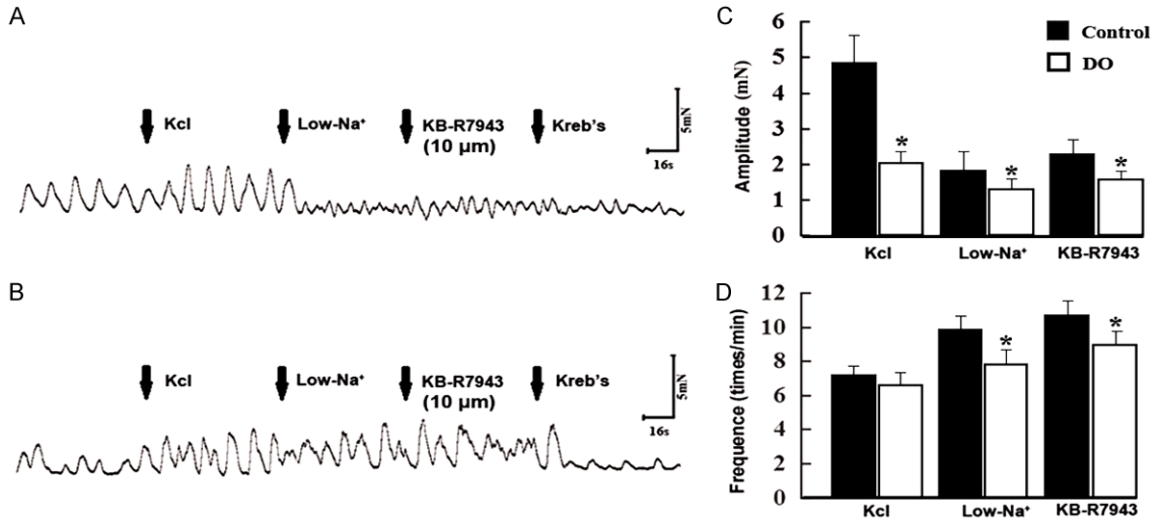

Figure 3. The effect of the reverse mode NCX on the control of bladder contraction in the DO and sham groups. A. The NCX reverse mode in the sham group (n = 8); B. The NCX reverse mode in the DO group (n = 8); C. The amplitude of contraction in the NCX reverse mode in the DO and sham groups; D. The frequency of contraction in the NCX reverse mode in the DO and sham groups. Data are presented as the means ± SD. *P < 0.05 vs. the sham group.

and control rats (P < 0.05), and the responses were normalized to the strip weights according to our previous report. The contraction of detrusor strips in reverse mode from both groups is presented in **Figure 3A** and **3B**. When the $[Na^+]_o$ was decreased from 144.4 mM to 87.22 mM, the amplitude of contractions changed from 1.75 ± 0.31 mN to 1.12 ± 0.27 mN in the DO group (n = 8, P < 0.05) and from 4.21 ± 0.69 mN to 1.57 ± 0.49 mN (n = 8, P < 0.05) in the control group (**Figure 3C**). The frequency in the DO group was enhanced from 5.77 ± 0.67/min to 6.83 ± 0.82/min (n = 8) compared with 6.26 ± 0.52/min to 8.63 ± 0.73/min (n = 8, P < 0.05) in the control group (**Figure 3D**). After KB-R7943 (10 μM) was used to block the reverse mode of

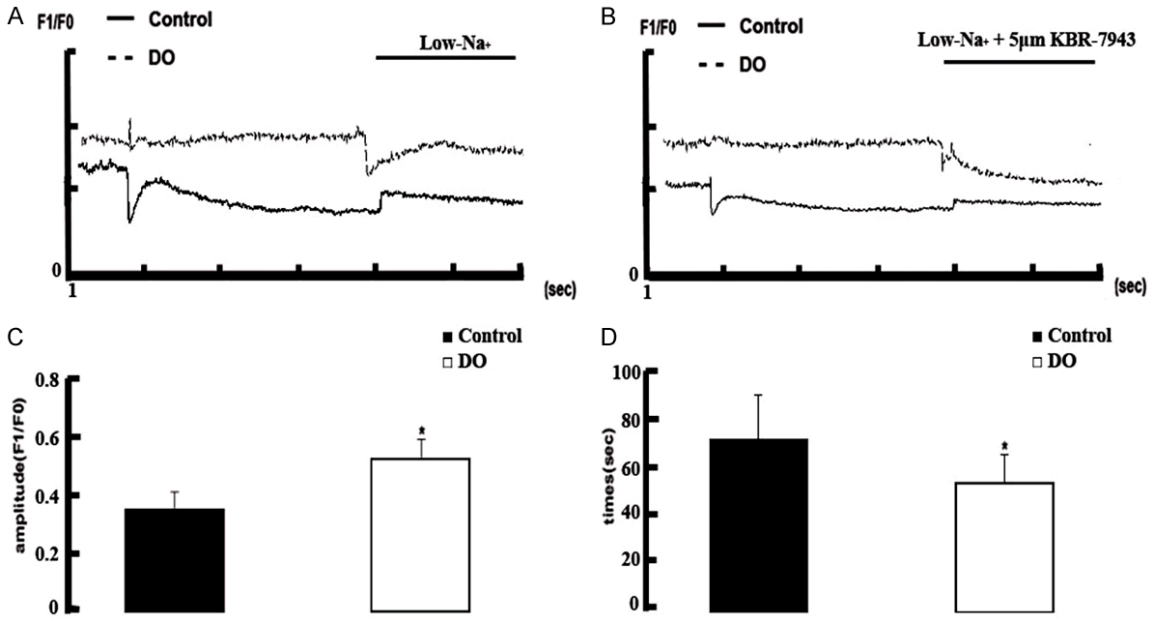

Figure 4. The effects of the NCX reverse modes on SMCs in the DO and sham groups. A. The effect of the NCX reverse mode on SMCs in the DO and sham groups; B. The amplitude of $[Ca^{2+}]_i$ in the NCX reverse mode; C. The decay time for $[Ca^{2+}]_i$; D. The effect of the NCX reverse mode on SMCs in the DO and sham groups. The data are presented as the means ± SD (N = 10 from 3 animals per group). *P < 0.05 vs. the sham group.

the NCX, the contraction amplitude changed to 1.35 ± 0.22 mN (n = 8, P < 0.05) and 7.85 ± 0.75/min (n = 8, P < 0.05) in the DO group and to 1.96 ± 0.39 mN (n = 8) and 9.34 ± 0.82/min (n = 8) in the control group (**Figure 3C** and **3D**).

Effect of $[Ca^{2+}]_i$ on the NCX reverse mode in rat bladder SMCs

In the experiment, the cells were stimulated with a low-Na^+ solution, and the amplitudes of the increase in $[Ca^{2+}]_i$ in the reverse mode of the NCX were 0.034 ± 0.008 (n = 10) and 0.046 ± 0.005 (n = 10, P < 0.05) in the DO and control groups, respectively, as presented in **Figure 4A**. The inhibitor KB-R7943 (5 μM) was used to block the NCX reverse modes in the DO and control groups to 0.014 ± 0.004 (n = 10) and 0.035 ± 0.007 (n = 1, P < 0.050), respectively, as presented in **Figure 4B**. The time spent in the NCX reverse mode for the control group was 33.1 ± 2.2 s (n = 10, P < 0.05), and the time for the DO group was 14.7 ± 4.5 s (n = 10) (**Figure 4A**).

NCX current is supported by Ni^{2+} and KB-R7943

The total NCX currents in both groups were verified using 5 mM Ni^{2+}. When the resting potential of the SMC was set to -100 mV, the density of NCX in the control group was 2.49 ± 0.55 pA/pF (n = 8) (**Figure 5E**), which differed significantly from that in the DO group (4.56 ± 0.68 pA/pF, n = 8, P < 0.05, **Figure 5F**). A 5-mM Ni^{2+} application decreased the I_{NCX} to 2.05 ± 0.43 pA/pF (n = 8) in the DO group (**Figure 5F**) and to 1.96 ± 0.4 pA/pF (n = 8, P < 0.05) in the control group (**Figure 5E**); the difference in these changes was significant. The NCX currents in both groups were also verified using KB-R7943 (5 μM and 10 μM) to test the effect NCX inhibition in the reverse mode. When the resting potential of the SMC was set at -100 mV, the density of the NCX in the control group was 2.29 ± 0.65 pA/pF (n = 8) (**Figure 6D**), which differed significantly from 3.72 ± 0.88 pA/pF (n = 8, P < 0.05) in the DO group (**Figure 6H**). KB-R7943 (5 μM) significantly decreased the I_{NCX} by 45% in the DO group (**Figure 6F**) compared with 31% in the control group (**Figure 6B**). Furthermore, 10 μM KB-R7943 significantly decreased the I_{NCX} by 75% in the DO (**Figure 6G**) group compared with 60% in the control group (**Figure 6C**). **Figure 6D** and **6H** present the traces and the current-voltage (I-V) relationship for the effect of KB-R7943 on the NCX currents in the bladder SMCs. The reversal potential of the NCX in SMCs was -35.71 ± 1.32 mV (n = 8) in

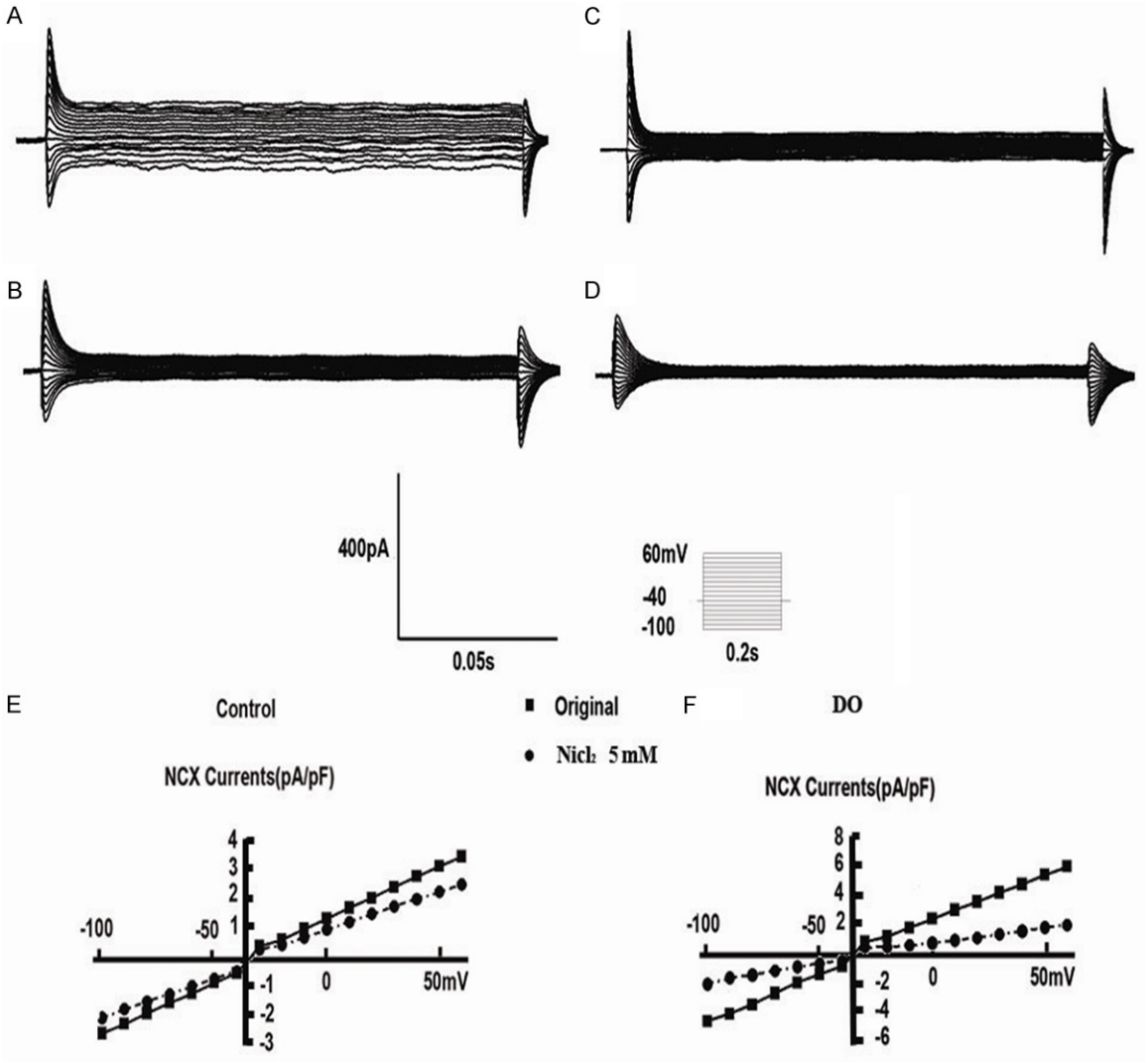

Figure 5. The traces and I-V relationships of the NCX currents before and after the administration of Ni^{2+} in the bladder SMCs from the DO and sham groups. A. The original NCX current values in the sham group; B. Ni^{2+}, 5 μM; C. The original NCX currents in the DO group; D. Ni^{2+}, 5 μM; E. The NCX current I-V relationship in the sham group (insets in the current traces indicate the various clamp protocols); F. The NCX current I-V relationships for the DO group (insets in the current traces indicate the various clamp protocols). The data are presented as the means ± SD (N = 7 from 8 animals). *P < 0.05 vs. the sham group.

the control group and -15.17 ± 0.41 mV in the DO group (n = 8).

Discussion

To our knowledge, this is the first study to provide morphological evidence, molecular and functional data, and cellular and pharmacological evidence of NCX1 activity in DO in rats. We assessed the differences in NCX function in detrusor strip contraction in the reverse mode between the two groups. Furthermore, we demonstrated that a significant change in the NCX currents and aberrant $[Ca^{2+}]_i$ obtained through this exchanger may be the underlying mechanisms responsible for DO. Cells and strips were also exposed to KB-R7943 to confirm inhibition in both groups.

In this study, chronic PBOO caused a significant increase in the bladder mass due to hypertrophy, resulting in a decreased bladder-emptying capacity [11], which may have been caused by hypoxia-dependent signaling pathways [12]. Bladder contraction is influenced by many factors [13]. Even more influential than these factors, the calcium ion channel exhibits a unique role in the contraction process. Under patho-

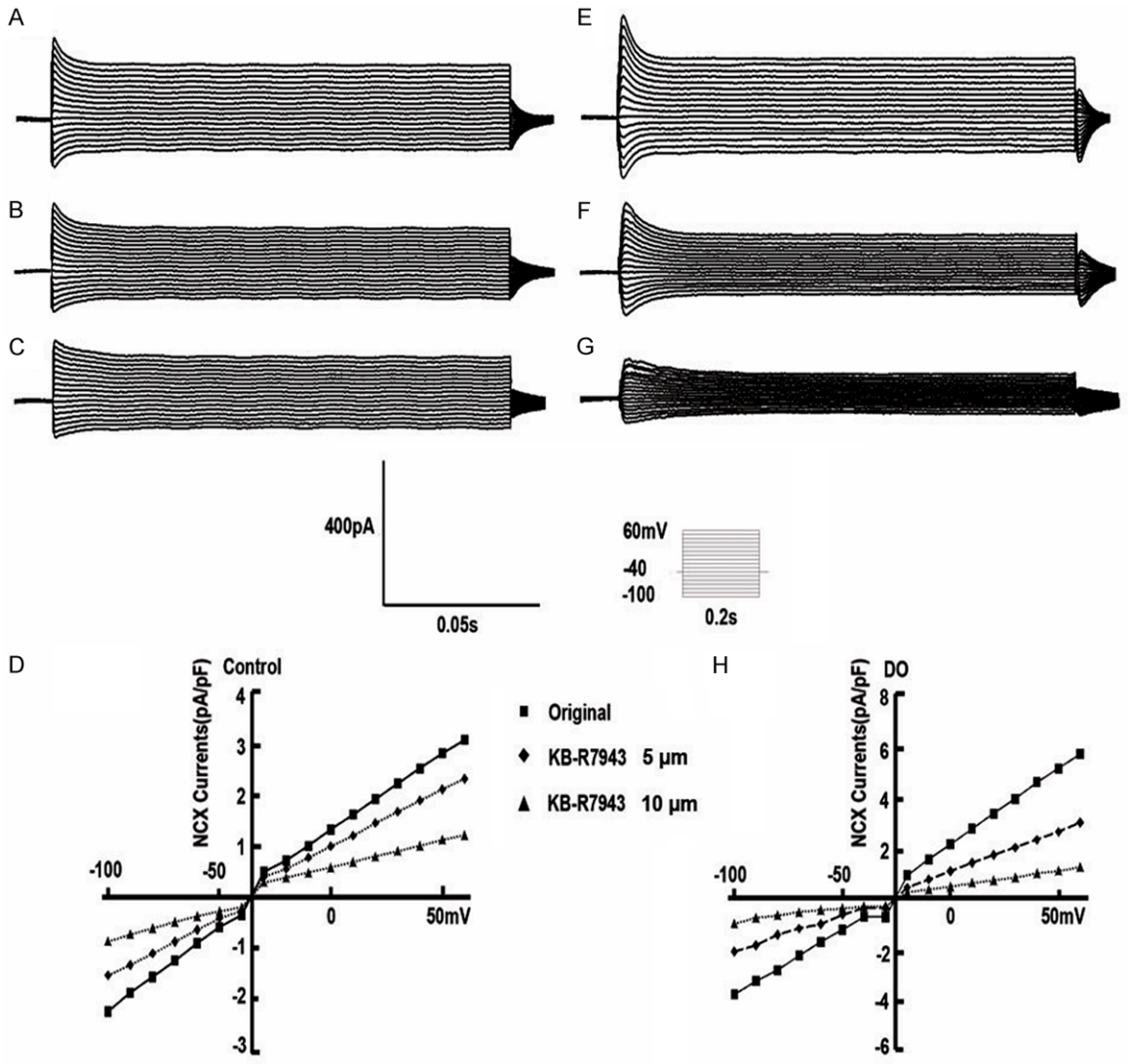

Figure 6. The traces and I-V relationships of the NCX currents before and after the administration of KB-R7943 in the bladder SMCs from the DO and sham groups. A. The original NCX current values in the sham group; B. KB-R7943, 5 μM; C. KB-R7943, 30 μM; D. The NCX current I-V relationship in the sham group (insets in the current traces indicate the various clamp protocols). E. The original NCX currents in the DO group; F. KB-R7943, 5 μM; G. KB-R7943, 30 μM; H. The NCX current I-V relationships for the DO group (insets in the current traces indicate the various clamp protocols). The data are presented as the means ± SD (N = 7 from 8 animals). *P < 0.05 vs. the sham group.

logical conditions, the SMCs exhibiting DO were found to possess aberrant $[Ca^{2+}]_i$ [2]. Traditional treatment with anti-cholinergic drugs usually leads to intolerable side effects [14]. Thus, for DO, aberrant $[Ca^{2+}]_i$ resulting from a variety of pathways may be a main therapeutic target.

The NCX has been observed in a variety of tissues, such as the heart, brain, and smooth muscle [15, 16]. The NCX plays an important role in cardiovascular pathophysiology, including ischemia-reperfusion injury and heart failure [17, 18]. In detrusor smooth muscle cells, NCX was first described in toad bladder in 1981 [19]. Because of its low currents under physiological conditions in guinea pig detrusor smooth muscle cells [20], the role of the NCX in the bladder remains controversial, especially in disease. In this study, we suggest that changes in NCX1 may disrupt the equilibrium of $[Ca^{2+}]_i$ and lead to irregular DO activity.

Although the NCX typically operates in the forward mode, leading to the exclusion of intracellular Ca^{2+} ions under physiologic conditions, the forward mode NCX has not received the same

attention as the reverse [21]. In reverse mode, NCX transports Ca^{2+} ions into the cell via the membrane when extracellular Na^{+} levels decrease. The NCX reverse mode is necessary to completely refill intracellular stores in the airway smooth muscle and to maintain blood pressure [22]. In SMC disease research, Ca^{2+} influx through the up-regulation of the NCX operating in reverse mode contributes to an overload of $[Ca^{2+}]_i$ and is important in idiopathic pulmonary arterial hypertension and airway hyper-responsiveness [23, 24].

There have been few reports concerning the NCX and its reverse mode in the bladder. The NCX has been reported to be able to load intracellular Ca^{2+} stores in guinea pig detrusor smooth muscle, but the level of resting $[Ca^{2+}]_i$ is not determined by the reverse mode of the NCX due to the small net flux through this exchanger [20]. Interestingly, NCX1.3 overexpression is associated with abnormal urination because of the enhanced Ca^{2+} influx via reverse mode NCX function, leading to prolonged and propagating spontaneous Ca^{2+} release events and the potentiation for spontaneous bladder SMC contraction [4]. Consistent with the previous experiment, our data indicate that the expression of NCX1 during DO development enhanced $[Ca^{2+}]_i$ and reduced the duration of influx through the reverse mode. In addition, the electrophysiological recordings also exhibited much higher NCX currents in reverse mode. Together, these data indicate that the NCX in the DO groups operating in reverse mode may partially lead to bladder SMC contraction instability under bladder-filling conditions.

At this time, three distinct NCX isoforms expressed in different tissues and cells have been cloned. It has been previously reported that the brain expresses high levels of all three isoforms [25]. In this study, the NCX1 isoform was overexpressed in the DO bladders. Although the NCX isoforms exhibit similar transport kinetics, NCX1 may play specific roles in different organs under physiological and pathophysiological conditions according to knockout mouse studies [26-28]. In this study, we used KB-R7943 to test the NCX function. Due to the lack of NCX subtype-specific inhibitors, KB-R7943 is the only NCX inhibitor widely applied to measure the NCX current [29]. According to previous research, KB-R7943 can block the reverse mode of the NCX at a low concentration [30]. Although the nonspecific inhibitor KB-R7943 has been reported to inhibit several ion transporters, it is appropriate for reverse mode inhibition at 5 or 10 μM [4, 31], which is consistent with oral administration to prevent overactive bladder in NCX1.3$^{tg/tg}$ mice [4].

In this study, the increased expression of NCX1 was confirmed in DO rat bladders after PBOO. The increase in NCX1 expression increased $[Ca^{2+}]_i$ in the reverse mode and accelerated the increase of $[Ca^{2+}]_i$ during the reverse mode, which enhanced the frequency of SMC contraction in the DO bladders. The roles of the NCX1 isoforms during SMC contraction in DO rats require further study.

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Disclosure of conflict of interest

None.

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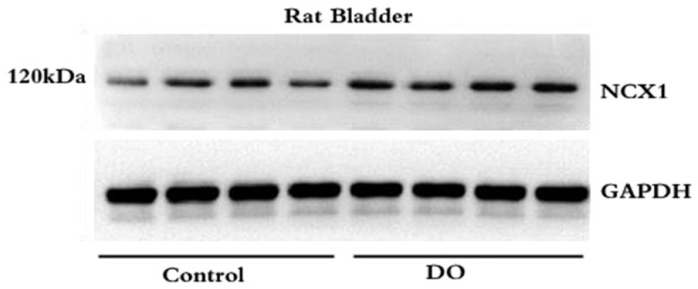

Figure S1. Western blots of tissue lysates from DO and sham control rat bladders probed with anti-NCX1, with anti-GAPDH as a loading control.