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17Beta-Estradiol Inhibits Calcium-Activated Potassium Channel Expressions in Rat Whole Bladder

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Purpose: To investigate the effect of estrogen on the expression of calcium-activated potassium (K_{Ca}) channels in an overactive bladder rat model. To this end, mRNA and protein levels of K_{Ca} channel subtypes in the bladder of ovariectomized rats were measured by reverse transcription polymerase chain reaction and western blotting, respectively.
Methods: Ten-week-old female Sprague-Dawley rats were divided randomly into 3 groups: sham-operated control group (n=11), ovariectomy group (n=11), and the group treated with estrogen after ovariectomy (n=12). Rats in the last group were subcutaneously injected with 17β-estradiol (50 μg/kg) every other day for 2 weeks, whereas rats in the other 2 groups received vehicle (soybean oil) alone. Two weeks after treatment, the whole bladder was excised for mRNA and protein measurements.
Results: Protein levels of the large-conductance K_{Ca} (BK) channels in the ovariectomy group were 1.5 folds higher than those in the sham-operated control group. However, the protein levels of the other K_{Ca} channel subtypes did not change significantly upon bilateral ovariectomy. Treatment with 17β-estradiol after ovariectomy restored BK channel protein levels to the control value. In contrast, BK channel mRNA levels were not significantly affected by either ovariectomy alone or 17β-estradiol treatment. The small-conductance K_{Ca} type 3 channel (SK3) mRNA and protein levels decreased to 75% of control levels upon 17β-estradiol treatment.
Conclusions: These results suggest that 17β-estradiol may influence urinary bladder function by modulating BK and SK3 channel expression.

Keywords: Potassium Channel, Calcium Activated; 17β-Estradiol; Ovariectomy; Urinary Bladder

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• **Conflict of Interest:** No potential conflict of interest relevant to this article was reported.

INTRODUCTION

The high prevalence of lower urinary tract symptoms (LUTS) in the elderly is caused by age-related changes to the pelvic floor and lower urinary tract anatomy and function [1]. Overactive bladder (OAB) is one of the most annoying LUTS; it is characterized by urinary urgency with or without urge incontinence, usually associated with frequency and nocturia. Indeed, urinary urgency is the chief element of an OAB diagnosis [1]. Considering the extent of symptoms, drug side-effect profiles, incidence of coexisting diseases and existing drug regimens, OAB treatment must be personalized for each patient [1]. Muscarinic antagonists, the most commonly used remedy for OAB, have limited efficacy and dose-related side effects. Mirabegron, a selective androgen receptor (AR) 3 agonist, has been recently approved for OAB treatment in spite of having unresolved issues

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of efficacy and safety in weak elderly patients or upon prolonged therapy [2,3]. Combinations of therapeutic agents with different mechanisms of action, such as a muscarinic antagonist and an AR 3 agonist, offer new avenues for OAB therapy [1]. Therefore, there is growing demand for effective OAB therapeutics, either as alternatives to or in combination with existing drugs. LUTS are twice as frequent in women as in men. Estrogen deficiency caused by menopause is known to be involved in urinary dysfunctions, such as an OAB, urinary incontinence, and detrusor underactivity [4]. Although results from previous studies are controversial, they suggest that local application of estrogen through the vaginal route had a therapeutic effect on OAB [4,5]. Moreover, a combination of muscarinic antagonists and topical vaginal estrogen has been shown to have a synergistic effect on OAB in postmenopausal women [6]. However, the mechanism of action of systemic estrogen replacement and its effect on LUTS are still unclear.

Potassium (K^+) channels are involved in the regulation of nerve and muscle cell excitability in various tissues, including the urinary bladder, and may be regarded as attractive targets for OAB treatment. The calcium-activated potassium (K_{Ca}) channels are found in detrusor smooth muscle cells [7-10]. K_{Ca} channels are defined based on their single channel conductance as follows: large (BK), intermediate (IK), and small (SK1–SK3) channels. A recent review by Petkov [11] suggested that BK is the most important K^+ channel in the regulation of the human urinary bladder smooth muscle (UBSM) excitability. BK channels are activated by changes in membrane depolarization and increases in intracellular Ca^{2+}. They are composed of either a homotetramer of pore-forming α-subunits alone, or four α-subunits combined with tissue-specific regulatory β-subunits [11]. Since the pore-forming α-subunit of the BK channel is encoded by a single *Slo* gene, alternative splicing of *Slo* may explain the channel's functional diversity [11,12]. Several studies have shown that estrogen increases the BK channel's open probability, and hence its activity in various smooth muscle cells [13-15]. Additionally, Afeli et al. [16] demonstrated that SK channels were involved in the regulation of spontaneous and nerve-stimulated contraction of human UBSM cells. Therefore, a drug related to K_{Ca} channels could be a potential new candidate for OAB treatment, especially in women. At present, there is insufficient knowledge about the effect of estrogen on BK channel gene and protein expression in the whole urinary bladder because pharmacological studies were conducted on UBSM cells only.

The aim of the present study was to examine the effect of 17β-estradiol on the number of K_{Ca} channel proteins expressed in the bladder of female ovariectomized rats.

MATERIALS AND METHODS

Bilateral Ovariectomy and Tissue Preparation

All animal experiments were approved by the Animal Subjective Committee of Kyungpook National University. Ten-week-old female Sprague-Dawley rats (body weight [BW], 200–220 g; n = 34) were randomly divided into 3 groups: sham-operated controls (n = 11), bilateral ovariectomized rats (n = 11), and bilateral ovariectomized rats with estrogen replacement (n = 12). Bilateral ovariectomy was performed aseptically through a midline abdominal approach under general anesthesia with enflurane. Animals were treated with antibiotic (150 mg/kg/day ampicillin) for 3 days. Immediately after operation, estrogen replacement was performed by subcutaneous injection with 50 μg/kg of 17β-estradiol benzoate (Sigma Chemical Co., St. Louis, MO, USA) every other day for 2 weeks [17]. Rats were sacrificed 2 weeks after ovariectomy and the whole bladder was isolated and divided in two. One half was immediately minced for protein extraction. The other was quickly frozen in liquid nitrogen, and stored at −70°C for RNA extraction.

Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI, USA). cDNA was amplified with *Taq* polymerase (Finnzymes, Espoo, Finland) in a DNA thermal cycler (MJ research, Watertown, MA, USA). The following cycling conditions were applied: 30 cycles of 1 minute at 95°C, 45 seconds at the annealing temperature, and 1 minute at 72°C. Annealing temperatures were 50°C for connexin 43 (Cx43); 53°C for SK3; 55°C for BK, SK1, SK2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); 57°C for IK; and 59°C for connexin 26 (Cx26). One-tenth of each polymerase chain reaction (PCR) product was resolved on a 1% agarose gel containing 0.5 μg/mL of ethidium bromide, and quantified using Quantity One 1-D image analysis software (Bio-Rad, Hercules, CA, USA). Primers for IK (GenBank AF149250), Cx26 (GenBank X51615), Cx43 (GenBank: M19317), and GAPDH (GenBank NM_017008) were designed using Primer 3-software [18]. BK [19] and SK [20] primer sequences were designed on the basis of previous reports. The primer sequences were as follows: BK (312 bp), 5′-GGCTG-

GAAGTGAATTCTGTAG-3′ (forward) and 5′-TGAGTAAGTAGACACATTCCC-3′ (reverse); IK (233 bp), 5′-CTTGGGTGCTGTCTGTGG-3′ (forward) and 5′-GTGTTTCTCCGCCTTGTTG-3′ (reverse); SK1 (159 bp), 5′-CAGGCCCAGCAGGAGGAGTT-3′ (forward) and 5′-GGCGGCTGTGGTCAGGTG-3′ (reverse); SK2 (190 bp), 5′-TCCGACTTAAATGAAAGGAG-3′ (forward) and 5′-GCTCAGCATTGTAGGTGAC-3′ (reverse); SK3 (182 bp), 5′-GTGCACAACTTCATGATGGA-3′ (forward) and 5′-TTGACACCCCTCAGTTGG-3′ (reverse); Cx26 (220 bp), 5′-GCCCCCAGTTAAGGGTAAAG-3′ (forward) and 5′-CCATGCTCACATCACAAACC-3′ (reverse); Cx43 (627 bp), 5′-GACTGCTTCCTCTCACGTC-3′ (forward) and 5′-TAGGTGCATGTTCTGCAAGC-3′ (reverse); and GAPDH (230 bp), 5′-ATCAAATGGGGTGATGCTGGTGCTG-3′ (forward) and 5′-CAGGTTTCTCCAGGCGGCATGTCAG-3′ (reverse). A single PCR product of the expected size was detected for each sample. The intensity of each band was normalized to that of GAPDH.

Western Blot Analysis

Each bladder sample was homogenized in cold lysis buffer (150mM NaCl, 25mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein from each sample (40 μg) was separated by 10% SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and incubated overnight at 4°C with primary antibodies (1:500) against K_{Ca} channels (Alomone Labs, Jerusalem, Israel), Cx23 and Cx43 (both Santa Cruz Biotechnology, Dallas, TX, USA). Membranes were then washed and incubated with a secondary antibody conjugated to peroxidase (1:2,000, Santa Cruz Biotechnology). Immunoreactive signals were visualized on autoradiography film after an enhanced chemiluminescence reaction (Amersham Biosciences, Buckinghamshire, UK). Band intensities were quantified using a molecular imager and Quantity One 1-D image analysis software (Bio-Rad). Equal protein loading was confirmed by subsequently probing the membranes with an antibody against β-actin (1:3,000; Santa Cruz Biotechnology).

Data Analysis

Data are presented as means ± standard error of the mean. In each sample, mRNA expression was normalized to GAPDH and plotted as fold induction relative to the average of the control group. Protein levels were normalized to β-actin. Differences between groups were evaluated using Student t-test, Mann-Whitney test, or Kruskal-Wallis one-way analysis of variance with Tukey test for multiple comparison. $P < 0.05$ was considered significant.

RESULTS

Body and Uterine Weight

As previously reported [21], rats from the ovariectomy alone group gained more weight than those from the other groups. Baseline and final BWs of rats in the 3 groups were as follows: sham-operated control (n = 11) baseline BW, 191.4 ± 15.3 g; final BW, 211.4 ± 14.0 g; ovariectomy alone (n = 11) baseline BW, 201.7 ± 15.3 g; final BW, 235.4 ± 17.4 g; ovariectomy plus estrogen replacement (n = 12) baseline BW, 191.4 ± 18.2 g; final BW 196.4 ± 17.3 g. Uterine weight decreased significantly in the ovariectomy alone group compared with the other groups, as previously reported [21].

mRNA Expression of K_{Ca} and Gap Junction Channels in Rat Bladder

Semiquantitative PCR measurements revealed the presence of mRNAs for all subtypes of K_{Ca} channels, Cx26 and Cx43 (Fig. 1A). In the absence of any detectable SK1 channel protein, SK1 expression was not determined. Expression of BK, IK, Cx26, and Cx43 in the bladder of bilaterally ovariectomized rats did not differ significantly from that in the control, or estrogen replacement groups (Figs. 1B, C, 2A, C). At the same time, SK2 and SK3 expression decreased significantly after estrogen replacement, but not after ovariectomy alone (Fig. 1D, E).

BK and SK3 Protein Levels Decreased in Ovariectomized Rats Upon Estrogen Replacement

Protein levels of BK in the bladder of bilaterally ovariectomized rats increased by 1.5 folds compared with those in the sham-operated controls (Fig. 3A, B), but were restored by estrogen replacement (Fig. 3B). In addition, SK3 protein levels were unchanged by ovariectomy alone, but they decreased to 75% of control levels upon 17β-estradiol treatment for 14 days after surgery (Fig. 3E). Levels of IK, SK2, Cx26, and Cx43 did not change significantly under any condition (Figs. 2B, D, 3C, D).

DISCUSSION

The present study shows that the increase in BK protein levels

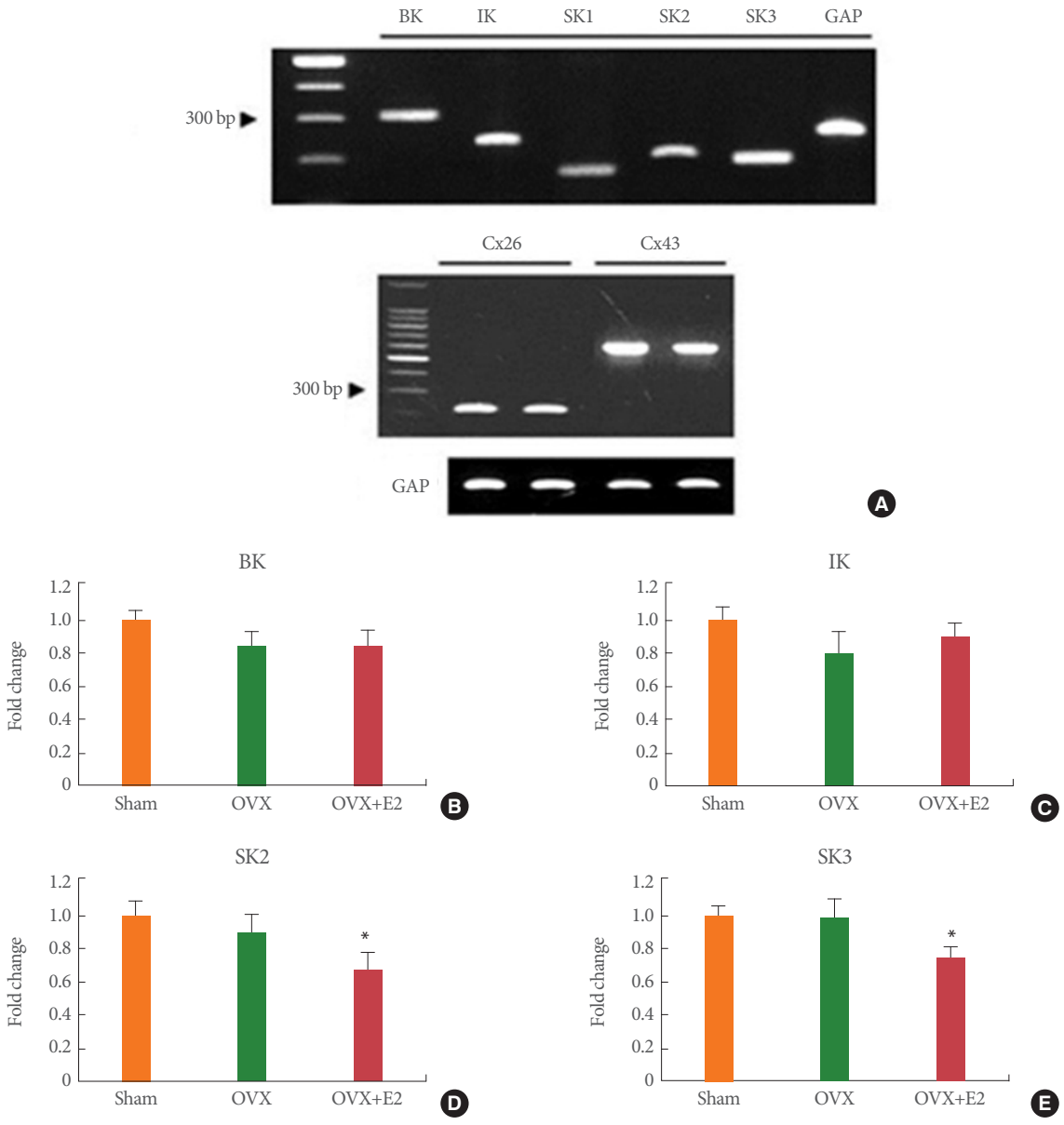

Fig. 1. (A) Representative ethidium bromide-stained gel showing the polymerase chain reaction (PCR) products from different calcium activated potassium (K_{Ca}) channel subtypes, connexin 26 (Cx26) and connexin 43 (Cx43), in normal rat bladder (B-E). Densitometric analysis of the PCR products from the K_{Ca} channel subtypes, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAP) Results are expressed as fold changes in mRNA (mean ± standard error of the mean, n = 11–12). BK, IK, and SK denote large, intermediate, and small conductance K_{Ca} channels, respectively. Sham, OVX, and OVX+E2 denote the 3 experimental groups: sham-operated control, ovariectomized (OVX), and ovariectomized, with 17β-estradiol (E2) replacement, respectively. *P < 0.05, vs. sham.

in rat bladder caused by ovariectomy can be reversed by concomitant estrogen administration. In addition, we found that SK3 mRNA and protein levels decreased significantly upon 17β-estradiol treatment after surgery, but were not affected by the procedure itself.

The absence of any significant differences between the BKα-subunits' mRNA levels in the three groups coincides with the results from a previous study on rat aortas [22]. The authors found that both α-and β-subunit mRNA levels of vascular BK channels were unaffected by ovariectomy alone or 17β-estradiol replacement. Various studies on smooth muscles have reported that both BK channel activity and smooth muscle relaxation

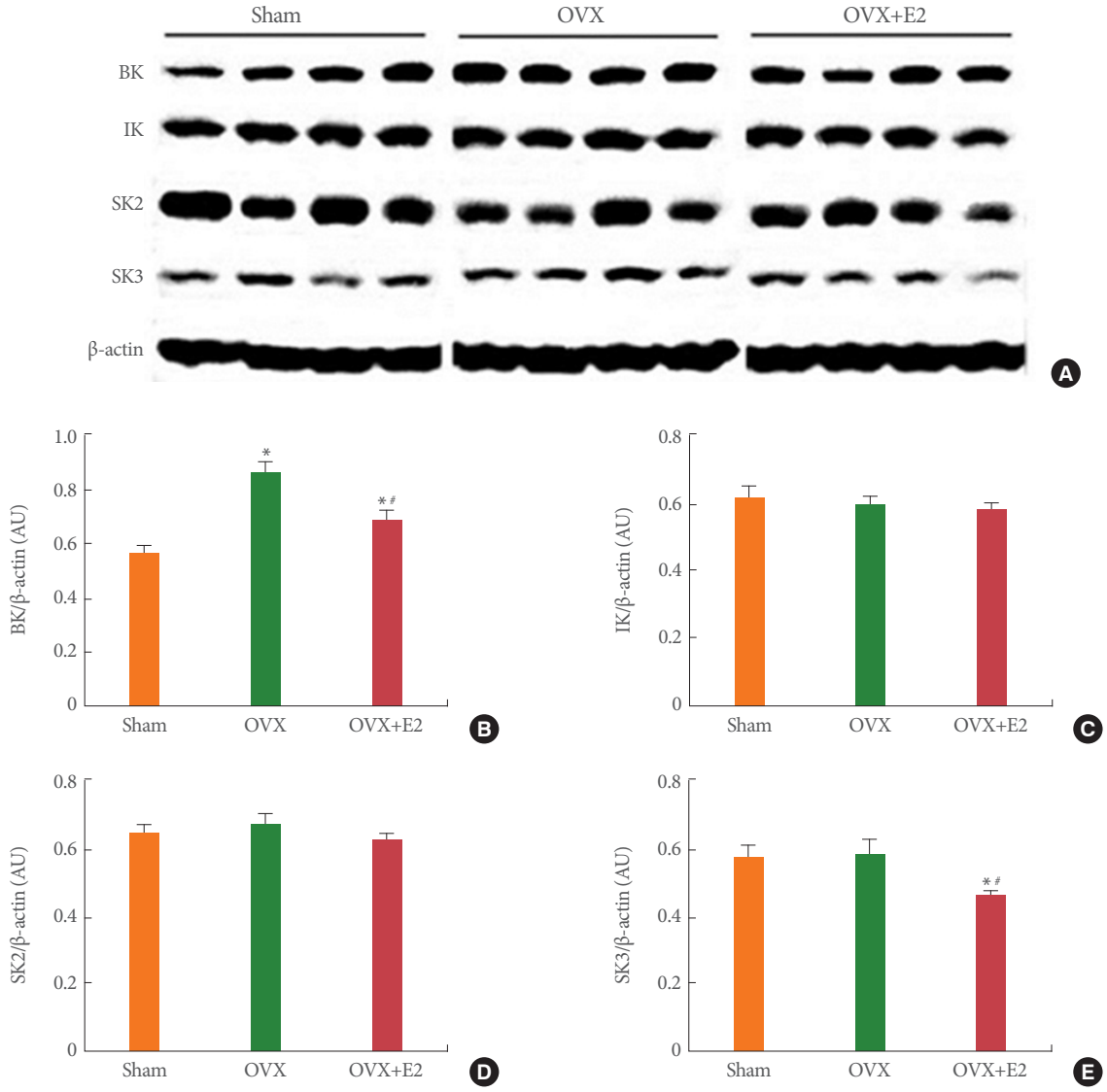

Fig. 2. (A) Representative results of western blot analysis for K_{Ca}α-subunits. (B–E) K_{Ca}α-ubunit protein levels normalized to β-actin. Values are shown in arbitrary units (AU) as mean ± standard error of the mean (n = 11–12). BK, IK, and SK denote large, intermediate, and small conductance K_{Ca} channels, respectively. Sham, OVX, and OVX+E2 denote the 3 experimental groups: sham-operated control, ovariectomized, and ovariectomized with 17β-estradiol (E2) replacement, respectively. *P < 0.05, vs. sham. #P < 0.05, vs. OVX.

were augmented by estrogen [13-15]. Thus, estrogen deficiency caused by ovariectomy would be expected to lower BK mRNA and protein levels. However, unexpectedly, the 1.5-fold BK protein increase was not accompanied by a change in mRNA expression. A possible explanation for this mismatch is the occurrence of posttranscriptional regulation by estrogen-regulated microRNAs (miRNAs). miRNAs are small, single-stranded, noncoding RNA molecules, whose main roles in posttranscriptional regulation are translational repression or mRNA degradation [23,24]. Vasudevan et al. [23] documented that miRNA increased the translation of target mRNAs during cell cycle arrest. A recent review by Klinge [24] has provided a comprehensive review of estrogen-regulated miRNAs and their role in modulating estrogen receptor expression and function. A recent study by Pietrzykowski et al. [25] has shown that the stability of BK mRNA splice variants is regulated posttranscriptionally by miRNAs, such as miR-9. In addition, a very recent study on osteosarcoma cells [26] has shown that miR-9 increases upon 17β-estradiol treatment. Thus, it is possible that estrogen-related miRNAs down-regulate BK translation and/or BK

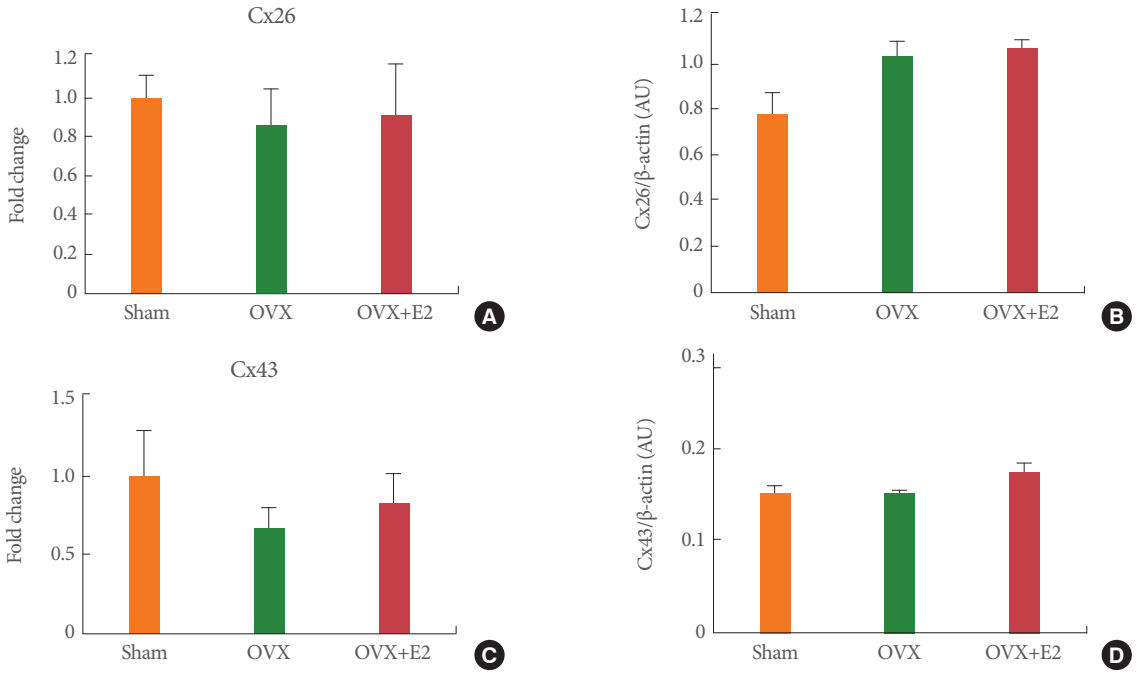

Fig. 3. Densitometric analyses of PCR products (A, C) and western blot analyses (B, D) for connexin 26 (Cx26; A, B), and connexin 43 (Cx43; C, D), normalized to glyceraldehyde-3-phosphate dehydrogenase and α-actin, respectively. Values are presented as mean ± standard error of the mean (n = 11–12). Protein levels are shown in arbitrary units (AU). Sham, OVX, and OVX+E2 denote the three experimental groups: sham-operated control, ovariectomized (OVX), and ovariectomized with 17β-estradiol (E2) replacement, respectively.

mRNA stability. It may also be possible that miRNAs affect BK channel expression by regulating the stability and translation of estrogen receptor mRNAs. As most studies on estrogen-regulated miRNAs were done in the context of breast cancer, there is limited data on its role in bladder pathophysiology. Similarly, studies on miRNAs and BK mRNA stability are also scarce. Therefore, further work is needed to investigate the hypotheses mentioned above.

Changes to intracellular trafficking could also explain the difference between BK mRNA and protein levels. A previous study by Eghbali et al. [27] demonstrated that BK channel protein accumulated in the perinuclear organelles of mouse myometrium under the control of sex hormones.

A previous study by Zhu et al. [12] revealed that alternative splicing of the *Slo* gene may be related to the lowering of BKα-subunit protein levels by 17β-estradiol treatment in the ovariectomized rat myometrium. Therefore, one possible explanation for the rise in BK protein levels under ovariectomy may be linked to the regulation of *Slo* gene splicing by 17β-estradiol in UBSM cells. Another possibility relates to changes in the stoichiometry of BK channel subunits. In reproductive arterial vascular smooth muscle of nonpregnant sheep, Nagar et al. [15] provided evidence that 17β-estradiol regulated the expression of the BKβ1 subunit and altered the function and estrogen responsiveness of the BK channel by modifying the stoichiometry of the $\alpha:\beta_1$ subunit. A previous study by Valverde et al. [28] suggested that the coassembly of BK channel α- and β-subunits in different tissues could explain its functional diversity upon estrogen modulation. Four β-subunits combine with the pore forming α-subunit tetramer to regulate BK channel function in various tissues [7,28]. The β-subunits are classified into 4 subtypes, β1 to β4. The β1-subunits are known to be major accessory subunits in smooth muscles and are abundant in UBSM cells [7,28]. In addition, Chen and Petkov [8] described the neuronal-specific β4-subunits in rat and mouse UBSM cells suggesting that they may have a role as accessory subunits and may modulate BK function in UBSM cells. Moreover, other researchers reported that, unlike β1-subunits, β4-subunits decreased Ca^{2+} sensitivity and slowed the gating kinetics of BK channels [29,30]. Finally, a recent study by Yan and Aldrich [31] has shown the existence of four new accessory subunits (γ1 to γ4), revealing the possibility of voltage-dependent BK channel

activation and tissue-specific mRNA expression. Although we are unable to confirm the exact changes, our findings suggest that 17β-estradiol treatment may alter the composition of BKα and BKβ1/BKβ4.

As reported previously in human whole bladder [15], we were able to detect the IK and SK channel proteins. Of these, only SK3 mRNA and protein levels were shown to decrease significantly upon 17β-estradiol treatment after ovariectomy. These findings are consistent with previous studies [15,32], whereby SK3 was shown to be the main IK/SK channel subtype in UBSM cells. Taken together, our findings indicate that SK3 channels may be an important modulator of UBSM excitability under estrogen control.

We also measured connexin Cx26 and Cx43 mRNA and protein levels in the bladder. Gap junctions are known to stimulate detrusor excitability and contractility in certain conditions such as in response to spinal transection and bladder outlet obstruction [33,34]. However, as reported in a recent study [35], connexin mRNA and protein levels failed to show any significant difference between the three groups (control, ovariectomy, and ovariectomy plus estrogen replacement).

In conclusion, the present study demonstrates that the protein levels of the BKα-subunit increase upon estrogen deficiency caused by ovariectomy, but can be reversed by 17β-stradiol treatment. In addition, protein levels of the SK3 channel decrease only upon 17β-estradiol treatment, suggesting that SK3 is the principal IK/SK channel subtype in rat UBSM cells to be under the control of estrogen. These findings may underlie why some estrogen replacement treatments targeted at improving LUTS fail in postmenopausal women.

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