Taiwanese Journal of Obstetrics & Gynecology 56 (2017) 196–203

Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

A promising protein responsible for overactive bladder in ovariectomized mice

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ARTICLE INFO

Article history:
Accepted 7 November 2016

Keywords:
estradiol
ovariectomy
overactive bladder
tight junction
urothelium

ABSTRACT

Objective: Ovariectomy (OVX) in mice is a model mimicking a neuro-electronic proof of an overactive bladder in postmenopausal women. Overactive bladder (OAB) was recently found to be due to an altered gap junction protein in a rat model. Thus, this study was conducted to evaluate changes in cell junction protein expression and composition in the bladder of OVX mice.
Materials and Methods: Thirty-six virgin female mice were randomized into three groups: mice with a sham operation only (control), OVX mice without estradiol (E2) replacement, and OVX mice with E2 replacement (OVX + E2). Cystometry assessment was conducted and cell junction-associated protein zonula occludens-2 (ZO-2) expression was measured after 8 weeks. Voiding interval values (time between voids) were assessed in mice under anesthesia. After measurements, the bladders were removed for proteomic analysis using the label-free quantitative proteomics and liquid chromatography–mass spectrometry technology. Lastly, immunohistochemistry (IHC) and Western blot were used to confirm the location and level, respectively, of ZO-2 expression.
Results: We identified 73 differentially expressed proteins in the bladder of OVX mice. The OVX mice showed significantly lower voiding interval values. Voiding interval values were significantly higher in the OVX + E2 group than in the OVX group. Urothelial thicknesses in the bladder were also significantly lower in the OVX group than in the control group. E2 replacement reversed the urothelium layers. Additionally, the expression of ZO-2, a tight junction protein, was the most affected by OVX treatment. IHC and Western blot confirmed the downregulation of ZO-2 in the bladder of OVX mice. Expression of ZO-2 protein was significantly increased in OVX + E2 group compared with OVX group.
Conclusion: This exploratory study estimated changes in protein expression and composition in the bladder of OVX mice. These changes may be associated with the molecular mechanisms of OAB.

Introduction

Overactive bladder (OAB) is a common condition affecting elderly people. The prevalence of OAB increases with advancing age, with up to 40% of postmenopausal women being affected [1–3]. According to the recent definition of the International Urogynecological Association and International Continence Society, OAB is a urological disorder defined by bothersome symptoms of urgency with or without urgency urinary incontinence, typically accompanied by frequency and nocturia, in the absence of proven infection or other obvious pathologies [4,5]. With an overall prevalence of around 12% [6], OAB has a significant negative impact on quality of life [7].

There are many theories behind the etiology of OAB, but it is generally accepted that it is caused by a combination of myogenic and neurogenic alterations [8]. It is also believed that mucosal sensory systems also contribute to the disorder [9]. The urothelium, a specialized lining of the urinary tract, functions as an integral part of a sensory web which receives, amplifies, and transmits information regarding its external milieu. Nocchi et al [10] showed that

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http://dx.doi.org/10.1016/j.tjog.2016.11.006

induction of oxidative stress causes functional alterations in mouse urothelium, suggesting possible mechanisms by which oxidative stress causes physiological alterations in the bladder, which may also occur in other organs susceptible to aging [11,12].

Urothelial tight junctions play an important role in the formation of the blood–urine barrier. Tight junctions are essential for normal function of epithelia, restricting paracellular diffusion and contributing to the maintenance of cell surface polarity. Superficial cells of the urothelium develop tight junctions, the basis for the paracellular permeability barrier of the bladder against diffusion of urinary solutes. Rickard et al [13] showed that the tight junctions-associated proteins zonula occludens (ZO-1, ZO-2, and ZO-3) were localized at cell margins. The damage to urothelial tight junctions causes the disruption of barrier function, which induces urinary tract diseases [14]. More recently, ovariectomized rats were found to exhibit frequent bladder contraction and increased connexin-43 expression without changes in muscarinic receptor expression. These results imply that ovariectomy (OVX)-induced OAB may be due to an altered gap junction protein function rather than muscarinic receptor modification [15].

The relative contribution of each of these hypothetical pathways in the development of OAB remains unknown. The bladder and its surrounding structures are rich in estrogen receptors, and physiological and anatomical changes occur around and immediately after menopause [16,17]. After lifestyle changes and bladder retraining, antimuscarinic drugs combined with local estrogens constitute the first-line treatment in postmenopausal women with OAB [18,19]. Although treatments with 17β-estradiol (E2) result in increasing voiding interval, the mechanism of estrogen against OAB is unclear. Due to the limited availability of human tissues, animal models are an important adjunct in improving our understanding of the effects of estrogen on OAB.

Proteomic approaches to identify and quantify the entire protein content of a tissue at a given time may provide insights into disease mechanisms [20]. In this study, we sought to understand the molecular mechanism of OVX-related OAB using label-free quantitative proteomics and nano-liquid chromatography–mass spectrometry (LC-MS/MS) technology. We evaluated changes in protein expression or composition in the bladders of mice treated with and without OVX. This information could offer interesting clues regarding the pathogenesis of OVX-related OAB, and may suggest several avenues for novel research and potential new therapies.

Materials and methods

OVX-induced OAB model in mice

Thirty-six virgin female C57BL/6 mice, aged 6–8 weeks, were randomized into three groups (n = 12, respectively): Group 1—mice with sham operation only (control), Group 2—OVX mice without estradiol (E2) replacement [placebo with vehicle (250 μL subcutaneously; 10% dimethyl sulfoxide, 90% corn oil; Sigma-Aldrich, St. Louis, MO, USA)], and Group 3—OVX mice with E2 replacement (5 μg/kg subcutaneously; Sigma-Aldrich). Four weeks after OVX, mice received either E2 or placebo for 4 weeks. The mice undergoing OVX or sham operation were anesthetized with 1.5% isoflurane. In Group 1, midline longitudinal abdominal incision was excised and then closed with 2-0 silk. In Groups 2 and 3, both ovaries were excised through midline longitudinal abdominal incision that was then closed with 2-0 silk [21]. In the pilot experiments, the time at which the maximum effect on OAB was observed was after Day 56 (8 weeks). The experiments were performed on the 8th week after OAB or sham operation. All experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University (Reference number: 104-43-N).

Cystometry assessment

The surgical procedure was carried out under urethane (1 g/kg, intraperitoneal, Sigma-Aldrich) anesthesia according to the methods as previously described [22–25]. A suprapubic tube (SPT) implantation (PE-10 tubing, Clay Adams, Parsippany, NJ, USA) was implanted in the bladder. Key points of the operation include: (1) a midline longitudinal abdominal incision was made, 0.5 cm above the urethral meatus; (2) a small incision was made in the bladder wall, and PE-10 tubing with a flared tip was implanted in the bladder dome; and (3) the purse-string suture of 8-0 silk was tightened around the catheter, which was tunneled subcutaneously to the neck, where it exited the skin.

The bladder catheter was connected to both a syringe pump and a pressure transducer. Pressure and force transducer signals were amplified and digitized for computer data collection at 10 samples per second (PowerLabs, ADInstruments, Bella Vista, Australia). The mice were placed supine at the level of zero pressure while bladders were filled with room temperature saline at 20 μL/min through the bladder catheter. After a 30-minute equilibration period, intravesical pressure was recorded for 30 minutes. Voiding interval refers to the time between voids is measured from the start of a voiding contraction to the start of the next [26].

After confirmation with cystometry, the mice were sacrificed, and the bladders were removed for proteomics, Western blot analyses (n = 6 for each group), and for histological examination, immunohistochemistry (IHC) staining (n = 6 for each group), respectively.

Protein preparation

Frozen tissue sample were pulverized with a liquid nitrogen-chilled mortar and pestle. Tissue powder was then homogenized in buffer (16 mM potassium phosphate, pH 7.8, 0.12 mol/L NaCl, 1 mM ethylene diamine tetra-acetic acid, Sigma-Aldrich) containing a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Berlin, Germany), and then centrifuged at 10,000g. The supernatant was removed, and the previous homogenization step was repeated after resuspending the remaining tissue pellet in basic buffer. After removal of the second supernatant, the remaining tissue pellet was suspended in urea buffer (6M, Sigma-Aldrich). The samples were centrifuged (13,000g for 30 minutes), and the supernatant was removed. Protein concentration was determined using bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) [27].

Protein identification and label free quantitative proteomics by nanoLC-MS/MS analysis

The nanoLC-MS/MS was performed with a nanoflow UPLC system (UltiMate 3000 RSLCnano system, Dionex, Amsterdam, The Netherlands) coupled with a captive spray ion source and hybrid Quadrupole Time-of-Flight mass spectrometer (maXis impact, Bruker, San Antonio, TX, USA). The sample was injected into a tunnel-frit trap column [C18, 5 μm, 100 Å, packed length of 2 cm, 375 μm outer diameter (od) × 180 μm inner diameter (id)] with a flow rate of 8 μL/min and a duration of 5 minutes. The trapped analytes were separated by a commercial analytical column (Acclaim PepMap C18, 2 μm 100 Å, 75 μm × 250 mm, Thermo Scientific, Waltham, MA, USA) with a flow rate of 300 nL/min. An acetonitrile/water gradient of 1–40% within 90 minutes was used for peptide separation. For MS/MS detection, peptides with charge 2^+, 3^+, or 4^+ and the intensity >20 counts were selected for data