Changes of muscarinic receptors and connexin-43 expression as a mechanism of overactive bladder in ovariectomized rats

Keon-Cheol Lee

Abstract

**Purpose** After menopause, the bladder is known to become overactive. To investigate the mechanisms involved in these changes, we examined the muscarinic receptors M2, M3 and gap junction protein connexin-43 in an ovariectomized rat bladder.

**Methods** Twenty 10-week-old female SD rats were used. Ten rats were ovariectomized, (Ovx group) and 10 rats received a sham operation (Con group). Four weeks after the operation, urodynamic tests were performed to verify overactive bladder, and the animals were killed. The body, bladder and uterus weights were measured. The bladder specimens were prepared for immunohistochemical staining for muscarinic receptors M2, M3 and connexin-43. Western blotting was also used for the same protein measurement (M2, M3 and connexin-43). A t test with a p value of 0.05 was considered significant, and SPSS 12.0 for Windows was used to analyze the data.

**Results** The mean body weight of the Ovx group (315.8 ± 18.1 g) was heavier than the Con group (270.0 ± 23.6 g) (p = 0.009). The mean uterus weight of the Ovx group (260.4 ± 186.8 g) was lighter than the Con group, (600.6 ± 175.9 g) (p = 0.028) and the mean bladder weight of the Ovx group (80.2 ± 15.9 g) was lighter than the Con group (97.4 ± 10.6 g) (p = 0.041). The mean bladder contraction of the Ovx group (5.5 ± 2.3/10 min) was more frequent than that of the Con group (3.2 ± 2.8) (p < 0.05). The expressions of M2 and M3 were not different between the Ovx and the Con group, but the expression of connexin-43 in the Ovx group was more intense than in the Con group in immunohistochemical staining. These findings were also confirmed by Western blotting results.

**Conclusions** Ovariectomized rats showed frequent bladder contraction and increased connexin-43 expression without changes in M2 and M3 receptor expression. These results imply that ovariectomy-induced overactive bladder may be due to an altered gap junction protein function rather than muscarinic receptor modification.

**Keywords** Menopause · Ovariectomy · Receptor · Muscarinic M2, M3 · Connexin 43

Introduction

Estrogen has an important role in bladder and urethral function, and estrogen deficiency occurring after menopause is related to many lower urinary tract symptoms [1]. After menopause, the bladder becomes overactive. The hormonal deficiency causing bladder dysfunction seems consistent [2], but the detailed process related to this dysfunction is not known. The inhibition of muscarinic receptors is a common treatment method for overactive bladder, and the muscarinic receptors could play some role in post-menopausal overactive bladder.

In animal models of post-menopausal overactive bladder, ovariectomy-induced bladder dysfunction was reported to be related with increased muscarinic receptors [3]; however, in other studies, no changes of muscarinic receptors were reported [4]. It is unclear whether the effect of menopause modifies muscarinic receptor properties to make the bladder overactive.

Gap junction proteins are substances that coordinate cell-to-cell communication, and detrusor cell contractions are synchronized by way of the gap junction protein.
Connexin-43 is the predominant connexin expressed on rat detrusor muscle cells [5] and is expressed in the suburothelial layer as well as the detrusor [6, 7]. Connexin-43 was found to increase in overactive neurogenic detrusor [8] and in ovariectomized rat bladders [9]. In a partial bladder outlet obstructed rat model, total connexin-43 increased, but cell membrane connexin-43 was reported to decrease [10]. Knowledge about the changes in connexin-43 after ovariectomy is lacking and needs further investigation.

We investigated the changes of muscarinic receptors M2 and M3 in relation to the change in connexin-43 to clarify their role in overactive bladder using an ovariectomized rat bladder model.

Materials and methods

Animals

Twenty 10-week-old female Sprague Dawley rats were used. All experimental animal procedures were approved by the Ilsanpaik Hospital Institutional Animal Care and Use Committee and were performed in accordance with institutional guidelines. After cystometry, animals were killed using cervical dislocation for tissue harvest.

Surgical procedures

Intramuscular ketamine (80 mg/kg) and xylazine (8 mg/kg) were used for anesthesia. Bilateral 2-cm lateral abdominal incisions were made vertically to expose the rat ovaries, which are located in the flank area. In 10 rats, both ovaries were removed. In the other 10 rats, just identification of ovaries was made (sham operation control). The abdominal incision was closed, and buprenorphine and gentamicin were given for 3 days postoperatively for pain and infection control, respectively. Four weeks later, a 1.5-cm low midline abdominal incision was made to expose the bladder under urethane anesthesia. A PE-50 polyethylene catheter was implanted through the bladder dome and exited through the abdominal wall. The wound was closed, and cystometry was recorded under the same anesthesia. Then, the animals were killed for tissue harvest, and the body weight and bladder and uterus weight were measured. The specimen of the bladder was frozen in liquid nitrogen and stored at −80 °C for Western blotting.

Cystometry

After placement of the PE-50 catheter into the bladder, a stabilizing time of 30 min was permitted before infusion. Under the urethane anesthesia, warm saline was infused at a rate of 0.1 ml/min using a Harvard 11 plus syringe pump (Harvard Apparatus, Holliston, MA) and pressure changes were recorded using the Biopac MP36 (Biopac Systems, Goleta, CA).

Immunohistochemistry

Immunohistochemistry was performed using the standard methods. Formalin-fixed, 4-µm-thick, paraffin-embedded tissue sections were deparaffinized and subjected to antigen retrieval (immersed in 10 mM citrate buffer (pH 6.0) and microwaved for 25 min). Immunohistochemical staining was performed on a Ventana Autostainer (Ventana Medical System, Inc., AZ). The tissue sections were incubated (30 min, room temperature) with primary antibodies for M2 (Sigma-Aldrich, St. Louis, MO, USA); M3 (Sigma-Aldrich, St. Louis, MO, USA) and connexin-43 (Sigma-Aldrich, St. Louis, MO, USA). Primary antibodies were detected using Vectastain Elite ABC kit reagents (Vector Laboratories, Inc., Burlington, CA). Secondary antibodies were applied at a dilution of 1:200. The slides were developed using 3,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA). The sections were counterstained with Mayer’s hematoxylin.

Western blot analysis

The bladder tissue was homogenized and lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 1 %, Triton X-100, 1 mM phenylmethylsulfonylfluoride). Total protein concentrations were measured using an advanced protein assay reagent (Bio-Rad, Hercules, CA). An aliquot of 30 µg of proteins was electrophoresed on 12 % SDS-polyacrylamide gels (Telco Co., Tokyo, Japan) under denaturing conditions. Protein samples were fractionated by gel electrophoresis run at 90 V and 100 V at 4 °C during the stacking and separation steps, respectively. Afterward, the membrane was washed and then blocked with 5 % skim milk in TBS-T for 1 h. Membranes were hybridized using a primary antibody (anti-M2 and M3 from rabbit; Sigma-Aldrich, St, Louis, MO) and anti-connexin-43 antibody (monoclonal anti-Cx43 from mouse; Sigma-Aldrich, St, Louis, MO) in TBS-T, including 5 % skim milk for overnight at 4 °C. After the membranes were washed with TBS-T three times for ten minutes, they were incubated with appropriate secondary antibodies coupled with horse radish peroxidase (HRP) for 1 h at room temperature. Specific signals were detected using the ECL solution (Amersham, Buckinghamshire, UK).

Results

Four weeks after the operation, the body weights of ovariectomized rats (315.8 ± 18.1 g) were significantly increased compared with those of the sham-operated rats.
The uterus weight of the ovariectomy group (260.4 ± 186.8 g) was decreased compared with that of the control group (600.6 ± 175.9 g) (p = 0.028). The bladder weights of ovariectomized rats (80.2 ± 15.9 g) were decreased compared with those of the sham-operated rats (97.4 ± 10.6 g) (p = 0.041).

Cystometry

The mean bladder contractions per 10 min in the ovariectomy group (5.5 ± 2.3) were more frequent than those in the control group (3.2 ± 2.8) (p < 0.05) (Fig. 1). The cystometric variables measured in each group are summarized in Table 1.

Table 1 Cystometry variables of ovariectomized rat (Ovx) and sham-operated control (Con)

<table>
<thead>
<tr>
<th>Group</th>
<th>Contraction frequency (in 10 min)</th>
<th>Baseline pressure, cm H2O</th>
<th>Maximum pressure, cm H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>3.2 ± 2.8</td>
<td>6.8 ± 2.4</td>
<td>32.5 ± 14.2</td>
</tr>
<tr>
<td>Ovx</td>
<td>5.5 ± 2.3*</td>
<td>7.2 ± 2.3</td>
<td>39.4 ± 19.2</td>
</tr>
</tbody>
</table>

* p < 0.05

Immunofluorescent analysis

Both expression intensities of M2 and M3 receptors of the ovariectomized rat bladder were not different from those of the sham-operated rat bladder. Only the expression of Cx-43 of the ovariectomized rat bladder was significantly increased compared with that of the sham-operated rat bladder (Fig. 2).

Western blotting

The intensity of M2 of the Ovx group (0.54 ± 0.04) was not different from that of the Con group, (0.57 ± 0.04) and the intensity of M3 of the Ovx group (0.28 ± 0.05) was
also not different from that of the Con group (0.25 ± 0.03). But, the intensity of connexin-43 of the Ovx group (0.37 ± 0.05) was significantly higher than that of the Con group (0.25 ± 0.04) \((p = 0.00)\) (Fig. 3).

### Discussion

Ovariectomized rats have increased body weight and less bladder weight with a shrunken uterus. The bladder of an ovariectomized rat is known to become overactive [11]. Unlike another well-known experimental OAB model of bladder outlet obstructed rats, no bladder wall thickening occurred, at least during the early period after ovariectomy, and in our study the bladder weight decreased. The reduced bladder weight after ovariectomy might be the result of decreased vascularity and muscle mass with resultant fibrotic change. Ovariectomized rat bladder seems to progress to fibrotic change without the compensatory hypertrophic period seen in bladder outlet obstructed model. This could mean that post-menopausal bladder has emptying problem as well as storage concern.

Several assumptions could be possible about the bladder function of ovariectomized rats in terms of decreased vascularity [12]. But, the exact mechanisms are still unknown.

In this study, we investigated the effects of ovariectomy on the bladder and examined the changes of possible candidate materials. Ovariectomy induced more frequent bladder contraction in rats. Regarding the changes of candidate proteins explaining the mechanisms of overactive bladder, muscarinic receptor M2 and M3 showed no difference. Muscarinic receptors might be in the center of the pathophysiology of overactive bladder, but it was not the main cause of the changes caused by ovariectomy. Other possible pathways for the explanation of ovariectomy-induced overactive bladder could be purinergic receptors [13], vanilloid receptors, tyrosine kinase receptors, neurokinin receptors [14] or gap junction proteins discussed in this study.

It is well known that detrusor muscle cell can evoke meaningful contraction only through the coupling of each cells, and in that process, connexin-43 could be the core channel [15]. Connexin-43 contributes to the propagation and amplification of the calcium signaling triggered by acetylcholine in the cells expressing the muscarinic receptors.

Increased connexin-43 can lead to easy coupling of muscle cells and can result in the functional augmentation of muscarinic receptor despite no changes in the expression of muscarinic receptors. If increased connexin-43 of the bladder is related with bladder overactivity, normalization of increased connexin-43 could be related with improvement of overactivity. Okada et al. [9] studied the effect of restoration of hormone status on the connexin-43 and bladder function in ovariectomized rats reporting that dietary estrogen-like compounds could restore estrogen status and was followed by the reduction of increased connexin-43 and improvement of overactive bladder patterns.

Altered connexin-43 expression may be explained as a possible mechanism of many forms of overactive bladder conditions. Connexin-43 was found to increase in overactive neurogenic detrusor [8]. As increased connexin-43 provokes easy coupling and firing of detrusor, decreased connexin-43 could be related with underactivity. Oshiro et al. [16] reported decreased connexin-43-derived gap junction proteins in detrusor underactivity of the aged rat bladder. Therefore, up- or down-regulation of connexin-43 seems to be translated as over- or underactivity of the bladder, and treatment of overactive bladder could normalize connexin-43 expressions.

However, in another recent study, ovariectomy had no effect on the connexin-43 expression of the bladder, and furthermore, it was suggested that hypoestrogenism might prevent the connexin-43 up-regulation observed in the bladder outlet obstruction [17]. It suggests that the connexin-43 expression is not simple and affected by other confounding
factors. The changes of bladder connexin-43 after ovariectomy are still controversial.

Ovariectomy of a virgin rat is a well-established post-menopausal model and can eliminate confounding factors such as delivery or advancing age and make it possible to study only the menopausal effect [18]. Rat cystometry parameters are greatly affected by the infusion rate. We used a bladder filling rate of 0.1 ml/min, and in that filling rate, up to 4 times contraction in 10 min is regarded as normal voiding patterns, while contraction frequencies of >4 times in 10 min as detrusor overactivity [19].

A limitation of this study is that specimens were not divided as detrusor and mucosa or submucosa. The expression patterns or changes of muscarinic receptors and connexin-43 could be different in those two layers. The mucosal or submucosal expressions of those proteins might be minorities in the total amount, and further functional studies regarding the effects of afferent signaling are needed.

Conclusions

Ovariectomized rats showed frequent bladder contraction and increased connexin-43 expression without changes of M2 and M3 receptor expression. These results imply that ovariectomy-induced overactive bladder may be due to altered gap junction protein function rather than muscarinic receptor modification.

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Conflict of interest Keon-Cheol Lee received the grant from Inje University in 2009, and this work was supported by the grant. Otherwise, Keon-Cheol Lee does not have any conflict of interest.

Ethical standards In this research, all experimental animal procedures were approved by the Ilsanpaik Hospital Institutional Animal Care and Use Committee and were performed in accordance with institutional guidelines. And Keon-Cheol Lee followed the Good Publication Practice Guidelines for Medical Journals and Guidelines on Good Publication.

References
