Life Sciences 116 (2014) 37–42

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Relationship between connexin43-derived gap junction proteins in the bladder and age-related detrusor underactivity in rats

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ARTICLE INFO

Article history:
Received 8 June 2014
Accepted 9 August 2014
Available online 20 August 2014

Keywords:
Connexin43
Detrusor underactivity
Aging

ABSTRACT

Aims: To confirm the mechanisms of age-associated detrusor underactivity (DU), we examined the differences in bladder activity and connexin-43 (Cx43)-derived gap junctions in the bladders of young and old rats.
Main methods: Female Sprague–Dawley rats aged 3 months (young) and 12 months (old) were used. Continuous cystometry was performed under urethane anesthesia in both ages of rats. In addition, isovolumetric cystometry was performed in young rats during the intravesical application of carbenoxolone, a gap junction blocker, to confirm the role of gap junction proteins in the bladder. Western blotting analyses were performed to assess Cx43 protein expression in the bladders of both groups of rats. Bladders were also analyzed using Masson's trichrome staining and immunostaining for Cx43.
Key findings: Cystometric evaluations revealed that compared with young rats, bladder contractility was reduced by 27% and residual urine volume was significantly increased in old rats. However, the intercontraction intervals did not differ between the two groups. Under isovolumetric conditions, bladder contraction was suppressed after the intravesical application of carbenoxolone. In the bladders of old rats, increase of smooth muscle cell hypertrophy and fibrous tissue was observed compared with young rats. In association with these findings, immunostaining for smooth muscle Cx43 and its protein level were decreased by 28% compared with young rats.
Significance: These results suggest that age-related DU might be caused by the downregulation of gap junctional intercellular communication in the bladder. Consequently, the normal signals that contribute to voiding function might not be transported between detrusor muscles.

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Introduction

Aging is commonly associated with lower urinary tract symptoms that affect geriatric health and independence (Siroky, 2004). Detrusor underactivity (DU) is defined by the International Continence Society as contractions of reduced strength and/or duration, resulting in prolonged or incomplete emptying of the bladder in the absence of urethral obstruction (Abrams et al., 2002). Aging contributes to this process, but the underlying mechanism has not been well understood. The proper management of DU is focused on preventing upper tract damage, avoiding overdistension, and decreasing residual urine (Miyazato et al., 2013). Treatment primarily comprises clean intermittent catheterization, although muscarinic receptor agonists, acetylcholinesterase inhibitors, and alpha-1 blockers have also been therapeutically used, but with minimal efficacy. However, DU has surprisingly received little clinical and research attention.

During aging, a distinct pattern of smooth muscle morphology has been described in human and animal bladders (Elbadawi et al., 1993; Lowalekar et al., 2012). Gap junctions are intercellular channels that allow the direct movement of ions, molecules, and neurotransmitters less than 1.2 kDa between neighboring cells (Bruzzone et al., 1996). Connexin-43 (Cx43) is a gap junction protein that is the best-characterized connexin in rat and human bladder smooth muscle cells (Christ et al., 2003; Haferkamp et al., 2004; Neuhaus et al., 2002). Previous reports from our and other laboratories reported the increased expression of Cx43 after bladder outlet obstruction in rats and in patients with detrusor overactivity (Christ et al., 2003; Haefliger et al., 2002; Haferkamp et al., 2004; Miyazato et al., 2009). It has been also reported that the circadian oscillation of Cx43 is associated with the biological clock, and contributes to diurnal changes in bladder capacity in mice (Negoro et al., 2012). In addition, gap junctions play a crucial role in regulating cell growth, development, and differentiation in multicellular organisms (Trosko et al., 1993). Therefore, we hypothesized that age-related DU might be associated with the malfunction of Cx43 in the bladder.

In the present study, we investigated the differences in bladder activity and Cx43 levels by measuring the expression of Cx43 protein as well as its immunoreactivity in the bladders of young and old rats. We

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http://dx.doi.org/10.1016/j.lfs.2014.08.009
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also assessed the effect of intravesical application of carbenoxolone, a gap junction blocker, to confirm the role of gap junction proteins in the bladder.

Materials and methods

Animal model

A total of 48 female Sprague–Dawley rats aged 3 months (young) and 12 months (old) were used in this study. The Institutional Animal Care and Use Committee of the University of Ryukyus approved the study protocol.

Continuous cystometry in young and old rats (n = 12 each)

Rats were anesthetized by the intraperitoneal and subcutaneous injection of urethane (1.2 g/kg). A polyethylene catheter (PE-50, Becton, Dickinson and Company, USA) was inserted into the bladder from the urethra and connected to a pressure transducer and an infusion pump through a three-way stopcock. Physiological saline at room temperature was infused into the bladder at a rate of 0.05 ml/min via the catheter, and bladder activity was monitored. Cystometry was continued for at least 60 min, and the intercontraction interval (ICI), pressure threshold (PT), baseline pressure (BP), and voiding bladder contraction pressure (VC) were measured during the final 30 min. At the final bladder contraction, bladder infusion was stopped, and the residual urine volume (RV) was measured by withdrawing intravesical fluid through the catheter using gravity and bladder compression.

Isovolumetric cystometry before and after the intravesical application of a gap junction blocker in young rats (n = 8)

We performed isovolumetric cystometry with the concurrent intravesical application of carbenoxolone (a gap junction blocker, Sigma-Aldrich, USA) to confirm the role of gap junctions in bladder contraction. A PE-50 was inserted into the bladder through the urethra, and the residual urine was removed under urethane anesthesia (1.2 g/kg). The urethra was ligated to the catheter near the external urethral meatus to produce isovolumetric conditions in the bladder. A lower abdominal incision was made, and the ureters were then transected at the level of the aortic bifurcation, and the distal ends were ligated. The bladder was filled with physiological saline (0.05 ml/min) to above the threshold volume to induce rhythmic isovolumetric contractions. After the bladder contractions had become stable for over 30 min, bladder activity was assessed for 30 min (the interval, baseline pressure, and amplitude of bladder contractions) as a control. The bladder was then emptied, and 3×10^{-3} M carbenoxolone dissolved in saline was instilled at a rate of 0.05 ml/min until rhythmic isovolumetric contractions were recorded. Bladder activity was then recorded and averaged for 30 min after the intravesical application of carbenoxolone, and the results were compared with the control recordings. In two of the eight rats, carbenoxolone was washed out, and the bladder was filled with saline until rhythmic isovolumetric bladder contractions were induced; these results were then compared with those of the control.

Western blotting for Cx43

Young and old rats different from those used in the cystometry experiments were sacrificed, and the whole bladder was excised (n = 4 each). The samples were then homogenized in cold lysis buffer (1 M Tris, 1% sodium dodecyl sulfate, and protease inhibitor cocktail; Complete, Roche Diagnostics, pH 7.4), and supernatants were obtained after centrifuging at 4 °C and 13,000 rpm for 10 min. The protein levels of the supernatants were determined using the Bradford method (Bradford, 1976). Equal amounts of protein from each sample (30 μg) were loaded onto 10% polyacrylamide gels, and separated by electrophoresis. After separation, proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were then incubated in phosphate buffer containing 0.1% Tween 20 and 0.5% nonfat milk at 4 °C overnight. The membranes were then incubated with rabbit polyclonal antibodies against Cx43 (Invitrogen, USA) and anti-GAPDH (loading control; HyTest, Turku, Finland) at room temperature for 1.5 h. Next, the membranes were incubated with peroxidase-conjugated anti-mouse antiserum (1:2500, Sigma-Aldrich) at room temperature for 1 h. The bands on the western blots were visualized using enhanced chemiluminescence (ECL; GE Healthcare Bio-sciences, USA). The intensity of each band was quantified by densitometry using a scanner (Fujifilm Luminescent Image Analyzer LAS4000 System, Japan), and the densities of the connexin bands were analyzed and quantified using software (Multi gauge, Fujifilm).

Masson's trichrome stain and immunohistochemical staining with Cx43 antibody

Next, bladders were removed from the remaining young and old rats (n = 4 each) to analyze the changes in histology and Cx43 protein expression. Sections were fixed in 10% buffered formalin, embedded in paraffin, and cut into 5-μm longitudinal sections. Slides were then deparaffinized, immersed in Mayer's hematoxylin, washed with water for 5 min, and immersed in Masson's trichrome stain for 2 min. Finally, they were dehydrated and cleared using ethanol and xylene, respectively.

For immunohistochemistry, the harvested bladders were frozen in liquid nitrogen, and 10-μm-thick sections were cut using a microtome and thaw-mounted on to gelatin-coated slides. Tissue sections were treated with 0.3% H_2O_2 for 10 min. They were then incubated for 10 min at room temperature in normal goat serum (Nichirei, Tokyo, Japan), followed by an overnight incubation at 4 °C with rabbit anti-Cx43 polyclonal antibody (Chemicon, USA) at a 1:250 dilution. Sections were subsequently incubated with biotinylated anti-rabbit secondary antibody for 1 h, followed by streptavidin–biotin–peroxidase complex reagent (Nichirei) at room temperature for 30 min. They were then immersed in eosin for 2 min, and stained with 3,30-diaminobenzidine (Wako, Osaka, Japan) for 10 min. Finally, the tissue sections were dehydrated in a graded ethanol series, cleared in xylene, mounted with permount, and placed under coverslips. A negative control was prepared using normal goat serum (Nichirei).

Statistical analysis

Data are presented as mean ± SE. Statistical comparisons were performed using paired or unpaired *t*-tests where appropriate, and $p < 0.05$ was considered statistically significant.

Results

The differences between young and old rats (n = 12 each)

The body weight of old rats was significantly increased (531.3 ± 38.1 g, $p < 0.01$) compared with that of young rats (263.9 ± 4.5 g). The weight of the removed bladder in old rats was also significantly increased (178 ± 10.0 mg, $p < 0.01$) compared with that in young rats (103 ± 5.3 mg).

Continuous cystometric parameters in young and old rats (n = 12 each) (Table 1)

In young rats, ICI (15.4 ± 1.5 min), VC (37.5 ± 3.5 cm H_2O), BP (5.7 ± 0.6 cm H_2O), and PT (7.3 ± 0.5 cm H_2O) became stable 1 h after the start of continuous cystometry (Fig. 1A). Dribbling overflow incontinence (i.e., urinary retention) was observed in four of 12 old rats (Fig. 1B). VC (27.2 ± 2.8 cm H_2O) was significantly lower ($p < 0.05$) in the remaining eight old rats than in young rats (Fig. 1B). BP (10.8 ±

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