Basic fibroblast growth factor causes urinary bladder overactivity through gap junction generation in the smooth muscle

Masaaki Imamura,1,2 Hiromitsu Negoro,1,2 Akihiro Kanematsu,1 Shingo Yamamoto,3 Yu Kimura,2 Kentaro Nagane,2 Toshinari Yamasaki,1 Isao Kanatani,1,2 Noriyuki Ito,1 Yasuhiko Tabata,2 and Osamu Ogawa1

1Department of Urology, Graduate School of Medicine, and 2Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan; and 3Department of Urology, Hyogo Medical College, Nishinomiya, Japan

Submitted 23 March 2008; accepted in final form 16 April 2009

Am J Physiol Renal Physiol 297: F46–F54, 2009. First published April 27, 2009; doi:10.1152/ajprenal.90207.2008.—Overactive bladder is a highly prevalent clinical condition that is often caused by bladder outlet obstruction (BOO). Increased coupling of bladder smooth muscle cells (BSMC) via gap junctions has been hypothesized as a mechanism for myogenic bladder overactivity in BOO, although little is known about the regulatory system underlying such changes. Here, we report the involvement of basic fibroblast growth factor (bFGF) and connexin 43, a bladder gap junction protein, in bladder overactivity. BOO created by urethral constriction in rats resulted in elevated bFGF and connexin 43 levels in the bladder urothelium and muscle layer, respectively, and muscle strips from these bladders were more sensitive than those from sham-operated controls to a cholinergic agonist. In vitro bFGF treatment increased connexin 43 expression in cultured rat BSMC via the ERK 1/2 pathway. This finding was supported by another in vivo model, where bFGF released from gelatin hydrogels fixed on rat bladder walls caused connexin 43 upregulation and gap junction formation in the muscle layer. Bladder muscle strips in this model showed increased sensitivity to a cholinergic agonist. These findings suggest that bFGF from the urothelium could induce bladder hypersensitivity to acetylcholine via gap junction generation in the smooth muscle, thereby contributing to the myogenic overactivity of obstructed bladders.

bFGF; bladder smooth muscle cell; connexin 43; ERK; hypersensitivity

OVERACTIVE BLADDER IS A HIGHLY PREVALENT CLINICAL CONDITION that is characterized by urgency and urinary frequency (1, 27, 41, 43). The etiology of bladder overactivity is diverse, including impaired innervation, inflammation, or bladder outlet obstruction (BOO) (27, 43). Of these, BOO associated with prostate hypertrophy is one of the most common conditions in elderly men and constitutes a major cause of overactive bladder (27). However, the precise mechanism of overactivity in the obstructed bladder has not yet been elucidated. Bladder overactivity in BOO is believed to result from neurogenic changes in detrusor innervation or myogenic changes in detrusor excitability (3, 23, 41). Recent investigations of the mechanisms of neurogenic changes have suggested nerve growth factor, neurotransmitters, and c-fiber activity as candidate mediators of the process (6, 41, 42). In contrast, however, little mechanistic evidence has been reported for myogenic changes.

Coupling of smooth muscle cells is a specific structural change commonly observed in overactive bladders and is considered to be a possible cause of myogenic overactivity (3, 6, 10). It has been suggested that gap junctions play an important role in the coupling of bladder smooth muscle cells (BSMC). Gap junctions consist of channels in the cell surface that connect neighboring cells by allowing the movement of molecules smaller than 1,000 Da, thereby sustaining and transferring chemical and electrical excitation between cells (10, 23). Connexins are the principal structural components of gap junctions (22). Connexin family proteins comprise of approximately 20 different types and have various physiological functions including cell growth and differentiation (17, 30, 39). Each connexin is located in specific cell types, and its function depends on the expressed cell type (21, 39). Cell-cell communication mediates these functions in a gap junction-dependent manner, but sometimes connexins directly regulate the binding protein activity to induce these functions in a gap junction-independent manner (17, 21). Gap junctions in the detrusor smooth muscle are reported to contain connexin 43 and connexin 45 (10, 13, 26). The differential roles of these two isoforms in the detrusor are not completely elucidated as yet. In the normal bladder, connexin 43 is mainly located in interstitial cells around muscle bundles (34). However, in overactive bladders under obstruction, connexin 43 is upregulated in the detrusor smooth muscle, as reported by Christ and colleagues and other groups (5, 11, 23, 25), strongly suggesting a correlation between them. In contrast, connexin 45 is reported to locate predominantly between smooth muscle cells (18, 34), but the expression level remains unchanged in obstructed bladders (18, 34).

There are two unresolved issues concerning connexin 43 in the obstructed bladder. One is the mechanism of connexin 43 upregulation and the other is the functional involvement of connexin 43 in detrusor hypersensitivity. Regarding the former issue, our previous study demonstrated a regulatory effect of basic fibroblast growth factor (bFGF) on BSMC remodeling in the obstructed bladder with specific alterations of collagen production and BSMC proliferation (14). Since bFGF has been reported to stimulate connexin 43 expression in cardiac myocytes and fibroblasts (7, 8), we postulated that the same mechanism that regulates collagen production and cell proliferation could also regulate connexin 43 expression in the obstructed bladder. For the latter issue, we hypothesized that...
differential regulation of connexin 43 could change the sensitivity to acetylcholine, a postsynaptic agonist of the detrusor (9, 29), since increased acetylcholine sensitivity has been reported to underlie myogenic overactivity secondary to BOO (2, 3). There are two possible mechanisms in this increased sensitivity. One is hyperactivity in smooth muscle cells (3), and the other is upregulated release of acetylcholine from the urothelium (12). We speculated that upregulation of connexin 43 could affect the former mechanism, hyperactivity.

First, we explored bFGF and connexin 43 expression as well as bladder muscle sensitivity to a cholinergic agonist in a rat BOO model. Next, the regulatory role of bFGF on connexin 43 expression in BMSC was investigated using an in vitro BMSC culture system. Finally, the physiological effects of bFGF on bladder smooth muscle were explored in our original in vivo animal model, where a gelatin hydrogel incorporating bFGF was employed as a carrier for sustained release (14, 15, 32, 35, 36).

MATERIALS AND METHODS

Reagents. Dispase was purchased from Godo Shusei (Tokyo, Japan), collagenase type IV and α-glycyrrhetinic acid (GA) from Sigma (St. Louis, MO), PD98059 from Calbiochem (San Diego, CA), Immobilon-P membranes from Millipore (Bedford, MA), diamino-benzidine tetrahydrochloride from Dojindo Laboratories (Kumamoto, Japan), and calcein AM from Molecular Probes (Eugene, OR). Recombinant human bFGF was provided by Kaken Pharmaceutical (Tokyo, Japan).

Antibodies were purchased from the manufacturers described below: anti-calponin, β-actin (Sigma), bFGF (Upstate, Lake Placid, NY), connexin 43 (Zymed, San Francisco, CA), connexin 45 (Chemicon, Temecula, CA), horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Pierce, Rockford, IL), biotinylated anti-rabbit antibodies (Vector Laboratories, Burlingame, CA), and FITC-conjugated anti-mouse antibodies (Dako, Glostrup, Denmark). Alexa Fluor 555-conjugated streptavidin was purchased from Molecular Probes.

A Bio-Rad Protein Assay Kit was purchased from Bio-Rad Laboratories (Hercules, CA), SuperSignal West Pico Chemiluminescent Substrate from Pierce, and a Vectastain Elite ABC Kit (Mouse IgG) from Vector Laboratories.

Cell culture. BMSC were isolated from 9-wk-old female Sprague-Dawley rats (Japan Slc, Shizuoka, Japan) using a procedure described previously (14, 19). Cells after two passages were seeded at 2 × 10^4 cells/ml in six-well plates for Western blot analysis or on chamber slides for immunofluorescence, and then serum-deprived in DMEM containing 0.5% FCS for 48 h before each assay. Cells were treated with bFGF over 4 days (0, 10, and 50 ng/ml) and 10 μg/ml anti-bFGF antibody or 5 μM PD98059 over 2 days (n = 3). To investigate pathways downstream of bFGF and the function of gap junctions stimulated with bFGF, cells were treated with 10 ng/ml bFGF plus 10 μg/ml anti-bFGF antibody or 5 μM PD98059 over 2 days (n = 3). Connexin 43 expression was evaluated by Western blot analysis and immunofluorescent studies. The function of gap junctions in the cells was evaluated by fluorescence recovery after photobleaching (FRAP) studies.

Animals. Seven-week-old female Sprague-Dawley rats, weighing 170–190 g, were purchased from Japan Srl. Animals were treated in accordance with National Institutes of Health animal care guidelines, and all animal experiments were approved by the Kyoto University Animal Experiment Committee.

Rat BOO model. Partial BOO was created in six rats using methods described elsewhere (37, 38). Briefly, the proximal urethra was freed from the vaginal wall. A longitudinal incision was made in the wall of 2-mm-long PE-200 polyethylene catheters (BD Intramedic, Sparks, MD). The open catheter was placed around the proximal urethra. Six sham-operated rats underwent similar procedures, but the catheter was removed before closing of the abdominal incision. After 4 wk, bladders were removed from all rats and weighed to confirm hypertrophy. Muscle strips were prepared from the bladders, and contractile responses of the muscle strips to carbachol were measured. Immunohistochemistry was performed to evaluate bFGF expression. Immunofluorescent studies and Western blot analysis were performed to evaluate connexin 43 expression.

In vivo effect of bFGF on rat bladders using gelatin hydrogels as release carriers. Gelatin hydrogel sheets were made as previously described (14, 35). Sheets were freeze-dried, cut in rectangles (8 × 5 mm), and impregnated with an aqueous solution containing bFGF to obtain gelatin hydrogels incorporating bFGF. Rats were anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine, and bladders were exposed via a midline incision. A gelatin hydrogel containing bFGF (0, 1, 5, and 10 μg/site, n = 12) was fixed over the ventral side of each bladder with four 8-0 nylon sutures. These sutures were utilized as marking sutures during specimen retrieval. Sham-operated rats (n = 12) did not receive the gelatin hydrogel treatment. Rats in each group were killed after 14 days. For six rats in each group, muscle strips were prepared from the region of the bladders where the hydrogels were fixed, and contractile responses of the muscle strips to carbachol were measured. Connexin 43 and connexin 45 expression in the same region of the bladders was evaluated by Western blot analysis. Connexin 43 expression was also evaluated by immunofluorescent studies. Gap junction formation in the detrusor layer was evaluated by transmission electron microscopy (TEM) studies. The other six rats in each group underwent filling cystometry.

Immunoblotting. Whole cell lysates from bladder tissue and cultured cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. The protein content of the cell lysates was measured using the Bio-Rad Protein Assay Kit. Cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P membrane. The membranes were incubated with antibodies against connexin 43 (1:200), connexin 45 (1:500), and β-actin (1:2,000) as an internal control. After incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies, immunoreactive proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate. Each band was quantified by densitometry using Image J 1.33 software.

Immunohistochemistry. Bladder specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Sections were heated in 0.01 M citrate buffer (pH 6.0) for 20 min for antigen retrieval. All samples were incubated with antibodies against bFGF (1:100) for 12 h at 4°C. Negative control sections were incubated without primary antibodies. Antibody binding was detected using the Vectastain Elite ABC Kit (mouse IgG). Sections were visualized following incubation with diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

Immunofluorescent study. For cell culture experiments, cells were washed twice with PBS and fixed with 4% paraformaldehyde. For animal experiments, bladder specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Sections were heated in 0.01 M citrate buffer (pH 6.0) for 20 min for antigen retrieval. All samples were incubated with antibodies against connexin 43 (1:100) or calponin (1:1,000) for 12 h at 4°C. Negative control sections were incubated without primary antibodies. Connexin 43 antibody binding was detected using biotinylated anti-rabbit secondary antibodies and Alexa Fluor 555-conjugated streptavidin. Calponin antibody binding was detected using FITC-conjugated anti-mouse secondary antibodies. Sections of bladder specimens were counterstained with 4,6-diamidino-2-phenylindole (DAPI).

FRAP study. Cells were incubated with 2 μM calcein AM in PBS for 30 min at room temperature and rinsed twice with PBS. A cell
GAP JUNCTIONS AND bFGF IN BLADDER SMOOTH MUSCLE

adjacent to other cells was selected, and its fluorescence was photo-bleached by strong laser pulses (488 nm and 40 iterations). The images of the cell were captured by a Fluoview FV300 confocal microscope (Olympus, Tokyo, Japan). The fluorescent emission was analyzed before bleaching, immediately after bleaching, and 4 min after bleaching by Fluoview image-analysis software 5.0 (Olympus). The recovery rates were measured using the method described elsewhere (23). Briefly, the fluorescence intensity before bleaching and immediately after bleaching was set as 100 and 0%, respectively. The recovery rates were calculated based on the fluorescence intensity 4 min after bleaching. The intensity was normalized against the intensity in one isolated unbleached cell.

TEM study. Samples were fixed with 1% glutaraldehyde and 1.44% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4°C. After washing in 0.1 M phosphate buffer, the samples were postfixed with 1% OsO4 and 0.1 M sucrose in 0.1 M phosphate buffer for 2 h. After dehydration with graded concentrations of ethanol, the samples were embedded in epon resin. Ultrathin 80-nm sections were cut with a microtome (Dosaka, Kyoto, Japan), placed on mesh copper grids, stained with uranyl acetate followed by lead citrate, and examined at 80-kV acceleration voltage using an H7650 electron microscope (Hitachi, Tokyo, Japan).

Muscle strip experiment. Force measurement was performed as described previously (14, 20). Bladder samples were trimmed in strips 2–3 mm wide with urothelium. The strips were fixed to a strip holder and placed in an organ bath filled with Krebs solution at 37°C. After equilibration for 1 h with a passive force of 0.5 g, strips were subjected to cholinergic stimulation. Other strips treated with 10 μg bFGF/site were prepared for stimulation in the presence of α-GA to inhibit gap junction activity. These strips were washed three times with Krebs solution after the regular stimulation, placed in Krebs solution containing 30 μM α-GA, and then subjected to cholinergic stimulation. Each strip was weighed at the end of the experiment. The contractile force was expressed as grams tension per 100 milligrams of tissue weight. The initial dose-response curve (0.01–100 μM) revealed that maximum tension occurred at 10 μM carbachol. The muscle contractile force with 10 μM carbachol was set as 100% for the calculation of percentage of maximum response. The EC50 value for each strip was estimated with two-parameter logistic equations using SAS 8.2 software (SAS Institute, Cary, NC) and then converted into negative logarithms as the pEC50 value.

Filling cystometry. Filling cystometries were performed as described previously (14, 20). Saline was infused into rat bladders at the rate of 2.8 ml/h under 900 mg/kg urethane anesthesia. Micturition was recorded for at least 2 h in each experiment. Micturition and residual urine volume were measured at the time of final contraction by aspiration of urine with a syringe. Bladder capacity was defined as micturition volume plus residual urine.

Statistical analysis. All data are presented as means ± SD. Data were analyzed with an unpaired Student’s t-test or Dunnett’s test using SAS 8.2 software. P < 0.05 was accepted as significant.

Fig. 1. Basic FGF (bFGF) expression, connexin 43 expression, and hypersensitivity in the obstructed bladder. A: bFGF and connexin 43 expression in the obstructed rat bladder. Bladder sections (5-μm thickness) were treated with a primary antibody against bFGF (1:100) and stained with diaminobenzidine (DAB; n = 6). The brown color indicates bFGF immunoreactivity. bFGF was prominently expressed in the urothelium of bladder outlet obstruction (BOO) rats at higher levels than in sham-operated rats. Scale bars = 50 μm. B: immunofluorescent studies for connexin 43 expression in the obstructed rat bladder. Bladder sections (5-μm thickness) were double-labeled with primary antibodies against connexin 43 (1:100) and calponin (1:1,000; n = 6). Top: staining of connexin 43 (red), calponin (green), and merged images, respectively. Bottom: connexin 43 (red) with 4,6-diamidino-2-phenylindole (DAPI) counterstaining of nuclear (blue) at higher magnification. Connexin 43 was expressed in the detrusor layer of BOO rats at higher levels than in sham-operated rats. Scale bars = 50 μm. C: Western blot analysis for connexin 43 in the obstructed rat bladder. Cell lysates were retrieved from the obstructed bladders (n = 6). Connexin 43 expression was significantly upregulated in the obstructed bladders compared with sham-operated bladders. D and E: muscle strip test for BOO rats. The contractile force of muscle strips was examined under carbachol stimulation for BOO rats and sham-operated rats (n = 6). The dose-response curve for BOO rats showed a leftward shift compared with sham-operated rats (D). pEC50 values in BOO rats showed a significant increase compared with sham-operated rats (sham 0.21 ± 0.09, BOO 0.61 ± 0.13; E). *Statistically significant difference (P < 0.05).
RESULTS

Obstructed bladders show increased urothelial bFGF expression, muscular connexin 43 upregulation, and hypersensitivity to cholinergic stimulation. BOO rats had hypertrophied bladders with significantly increased bladder weight (sham 66.5 ± 14.3 mg, BOO 227.7 ± 37.9 mg, P < 0.05). Immunohistochemistry showed that bFGF was more intensely expressed in the urothelial layer of obstructed bladders than sham-operated bladders (Fig. 1A). Immunofluorescent studies also showed more marked upregulation of connexin 43 in BOO rats than in sham-operated rats. Most connexin 43-positive cells were also positive for calponin, indicating that connexin 43 was expressed in the detrusor layer (Fig. 1B). Western blotting revealed that connexin 43 expression was significantly upregulated in obstructed bladders compared with sham-operated bladders (Fig. 1C).

Leftward shifts of the dose-response curve and increased pEC\textsubscript{50} values were considered evidence of myogenic hypersensitivity on the basis of previous studies (3, 33). The muscle strip test demonstrated that the dose-response curve of obstructed bladders was shifted to the left compared with sham-operated bladders (Fig. 1D). pEC\textsubscript{50} values for obstructed bladders also increased significantly compared with sham-operated bladders (sham 0.21 ± 0.09, BOO 0.61 ± 0.13) (Fig. 1E).

bFGF upregulates connexin 43 expression in rat BSMC through the ERK1/2 pathway. On Western blotting, the anti-connexin 43 antibody detected multiple bands: nonphosphorylated (41 kDa) and phosphorylated connexin 43 (43–46 kDa). Western blotting of cell cultures revealed that total connexin 43 expression in BSMC was significantly upregulated 2 and 4 days after treatment with 10 and 50 ng/ml bFGF. Phosphorylation of connexin 43 was also upregulated in the same manner (Fig. 2A). To assess the downstream signals mediating this effect of bFGF in rat BSMC, ERK1/2 signaling was pharmacologically blocked by a specific inhibitor. Western blotting revealed that upstream blockade of ERK1/2 with the MEK1 inhibitor PD98059, as well as treatment with an anti-bFGF neutralizing antibody, significantly suppressed connexin 43 upregulation (Fig. 2B). Immunofluorescent studies also showed that connexin 43 expression in BSMC was upregulated 2 days after treatment with 10 ng/ml bFGF. This effect was blocked by PD98059 and the anti-bFGF antibody (Fig. 2C). FRAP studies showed that the recovery rate significantly increased 2 days after treatment with 10 ng/ml bFGF (sham 22.3 ± 9.3%, bFGF 49.6 ±

**Fig. 2.** Connexin 43 expression in bladder smooth muscle cells (BSMC) under bFGF stimulation. A and B: Western blot analysis for connexin 43 expression in BSMC. Cell lysates were retrieved at the indicated time points from BSMC (n = 3). Anti-connexin 43-positive bands correspond to multiple isoforms: nonphosphorylated (41 kDa; N) and phosphorylated (43–46 kDa; P). Total connexin 43 expression in BSMC treated with 10 and 50 ng/ml bFGF at days 2 and 4. Phosphorylation of connexin 43 was also induced in BSMC treated with bFGF (A). PD98059 (ERK1/2 inhibitor) and neutralizing bFGF antibodies significantly inhibited bFGF-induced connexin 43 upregulation at day 2 (B). C: immunofluorescent study for connexin 43 expression in BSMC. BSMC were incubated in each indicated condition for 2 days (n = 3). Top: staining of connexin 43 (red), calponin (green), and merged panels, respectively. bFGF treatment upregulated connexin 43 expression in BSMC. PD98059 and neutralizing bFGF antibodies inhibited bFGF-induced upregulated connexin 43 expression at day 2 (B). D: FRAP study. BSMC were incubated in each indicated condition for 2 days (n = 3). Fluorescence of one cell adjacent to other cells was photobleached by strong laser pulses (arrowheads). Top: status before bleaching, immediately after bleaching, and 4 min after bleaching, respectively. Bottom: bFGF treatment increased the recovery rate of fluorescence in BSMC (sham 22.3 ± 9.3%, 10 ng/ml bFGF 49.6 ± 10.8%). PD98059 and neutralizing bFGF antibodies inhibited bFGF-induced fluorescence recovery. Scale bars = 20 μm. *Statistically significant difference (P < 0.05).
10.8%). This effect was also blocked by PD98059 and the anti-bFGF antibody (PD98059 18.7 ± 7.7%, anti-bFGF antibody 25.1 ± 6.3%) (Fig. 2D).

**bFGF induced myogenic hypersensitivity through gap junctions in rat bladders.** The muscle strip test demonstrated that dose-response curves were shifted to the left in bladders treated with 1, 5 (data not shown), and 10 μg bFGF/site compared with sham-operated bladders (Fig. 3A). pEC50 values in bladders treated with 10 μg bFGF/site increased significantly compared with sham-operated bladders (sham 0.22 ± 0.09, bFGF 0.64 ± 0.22) (Fig. 3B). To investigate whether gap junctions mediate the observed myogenic hypersensitivity, both sham-operated bladders and bladders treated with 10 μg bFGF/site were stimulated with carbachol in the presence or absence of α-GA as a gap junction inhibitor. α-GA reversed the leftward shift of the dose-response curve in bladders treated with 10 μg bFGF/site but did not affect the dose-response curve of sham-operated bladders (Fig. 3C). α-GA significantly decreased pEC50 values in bladders treated with 10 μg bFGF/site (without α-GA 0.56 ± 0.24, with α-GA 0.12 ± 0.06) (Fig. 3D). There were no effects of α-GA on the maximum contractile force in both sham-operated bladders and bladders treated with 10 μg bFGF/site (data not shown).

**bFGF induced connexin 43 expression and gap junction formation in rat bladders.** Based on the results of the muscle strip test, connexin expression was evaluated in bladders treated with 10 μg bFGF/site. Western blotting revealed that connexin 43 expression was significantly upregulated in bladders treated with 10 μg bFGF/site compared with sham-operated bladders, but connexin 45 expression remained unchanged (Fig. 4A). Immunofluorescent studies revealed that smooth muscle connexin 43 expression was upregulated after treatment with 10 μg bFGF/site compared with the sham-operated group (Fig. 4B). TEM studies showed formation of gap junctions between smooth muscle cells in bladders treated with 10 μg bFGF/site (Fig. 4C).

**bFGF induced some features of detrusor overactivity in rat bladders.** Cystometry showed that treatment with 10 μg bFGF/site induced some features of detrusor overactivity compared with the sham-operated group (Fig. 5A). The frequency of micturition in the bFGF-treated group was significantly higher than in the sham-operated group (Fig. 5B). The bladder capacity in the bFGF-treated group was significantly lower than that of the sham-operated group (Fig. 5C). Residual volume was negligible in all groups (data not shown). Maximum voiding pressure in the bFGF-treated group was significantly lower than in the sham-operated group (Fig. 5D). Maximum voiding pressure in the bFGF-treated group compared with the sham-operated group (Fig. 5E).

**DISCUSSION**

In this study, we show that upregulation of connexin 43 in urinary bladder smooth muscle cells by basic fibroblast growth factor may underlie hypersensitivity of the bladders to cholinergic stimuli. We speculate that this process could underlie bladder overactivity observed in outlet obstruction based on the following evidence: 1) obstructed rat bladders showed in-

![Fig. 3. Gap junction-mediated hypersensitivity in bladders stimulated with bFGF. A and B: muscle strip test for bladders stimulated with bFGF.](image-url)
Fig. 4. Gap junction expression in bladders stimulated with bFGF. 

A: Western blot analysis for connexin 43 and connexin 45 expression in bladders. Cell lysates were retrieved at day 14 from whole bladders treated with bFGF (n = 6). Total connexin 43 expression was significantly upregulated in bladders treated with 10 µg bFGF/site. Phosphorylation of connexin 43 was also induced in bladders treated with bFGF. In contrast, there was no difference in connexin 45 expression between groups.

B: immunofluorescent study for connexin 43 expression in bladders. Rat bladders were treated with 10 µg bFGF/site for 14 days. Top: staining of connexin 43 (red), calponin (green), and merged images, respectively. Bottom: staining of connexin 43 (red) with DAPI counterstaining of nuclear (blue) at higher magnification. bFGF treatment upregulated connexin 43 expression in the smooth muscle layer. Scale bars = 50 µm.

C: ultrastructural studies for gap junctions in bladders. Bladders treated with bFGF showed formation of gap junctions in the smooth muscle layer (arrowheads), which was not observed in control bladders (sham and 10 µg bFGF/site). Note irregular alignment of smooth muscle cells compared with sham-operated rats. Scale bars = 500 nm. *Statistically significant difference (P < 0.05).
Fig. 5. Filling cystometry for bladders stimulated with bFGF. A: representative traces of cystometry. Cystometry was performed for sham-operated group, 0 µg bFGF/site group, and 10 µg bFGF/site group. B: frequency of micturition. bFGF-treated group (10 µg/site) showed a significant increase in micturition times compared with sham-operated group (sham 8.7 ± 1.6/h, bFGF group 20.0 ± 4.4/h). C: bladder capacity. bFGF-treated group (10 µg/site) showed a significant decrease in capacity compared with sham-operated group (sham 0.34 ± 0.05 ml, bFGF group 0.18 ± 0.03 ml). D: maximum voiding pressure. bFGF-treated group (10 µg/site) showed a significant decrease in maximum voiding pressure compared with sham-operated group (sham 15.6 ± 1.8 mmHg, bFGF group 11.4 ± 2.2 mmHg). E: compliance. bFGF-treated group (10 µg/site) showed no significant decrease in compliance compared with sham-operated group (sham 0.022 ± 0.005 ml/mmHg, bFGF group 0.016 ± 0.005 ml/mmHg). *Statistically significant difference (P < 0.05).
increased urothelial bFGF expression concurrently with hypersensitivity and connexin 43 upregulation in BSMC (Fig. 1); 2) bFGF treatment upregulated connexin 43 both in vitro and in vivo (Figs. 2 and 4); 3) bFGF treatment induced hypersensitivity to a cholinergic agonist in rat bladder muscle strips that was reversed by blockade of gap junction activity (Fig. 3); and 4) bFGF treatment induced some features of bladder overactivity in rat bladders in vivo (Fig. 5).

A major finding in this study is that bFGF induces hypersensitivity to a cholinergic stimulus in the bladder via altered expression of connexin 43, the gap junction protein (Fig. 3). Our finding is in accordance with previous study showing involvement of gap junctions in bladder overactivity observed in outlet obstruction (5). Hypersensitivity to acetylcholine is reported to be a component of BOO pathophysiology (33) and may play a major role in myogenic enhancement of detrusor excitability, although the regulatory mechanism remains relatively unknown. The involvement of bFGF presents a novel mechanistic link between obstruction and hypersensitivity.

This regulation of connexin 43 in BSMC by bFGF has two interesting features. First, the present data showed that ERK1/2 was an intracellular signal mediating bFGF and connexin 43 expression in BSMC (Fig. 2, A–C). Since we have shown that the ERK1/2 pathway is the principal conduit for other bFGF effects in BSMC (14), the present findings reconfirm its importance in functional bladder remodeling. These findings are also in accordance with a report that connexin 43 expression is mediated by MAPK pathways in other kinds of cells (4, 16). Additionally, connexin 43 was hardly observed in BSMC in the presence of bFGF (Figs. 2C and 4B). In normal bladders, connexin 43 is expressed mainly in lamina propria myofibroblasts (10, 13, 26). However, in pathological bladders cell-cell communication via gap junctions increases in parallel with connexin 43 expression in the smooth muscle (10, 13, 26). It is notable that in our in vivo release model, bFGF treatment upregulated connexin 43 expression in the muscle layer in parallel with enhanced BSMC proliferation (14); this could be interpreted as a shift toward a myofibroblast-like phenotype.

Gap junctions in the detrusor smooth muscle also contain connexin 45, another kind of connexin. Connexin 45 expression was also observed but not affected with bFGF treatment in bladders (Fig. 4A). Functionally, the involvement of connexin 45 for detrusor hypersensitivity seems to be less significant, since α-GA inhibited hypersensitivity in bladders treated with bFGF but did not change the sensitivity of sham-operated bladders (Fig. 3, C and D). This finding further supports that gap junctions do not have a significant role in normal bladder sensitivity but that BSMC upregulation of connexin 43 could generate gap junction channels, which can significantly alter muscle sensitivity. These data are also in accordance with a previous study that gap junctions formed by connexin 45 are small and sparse, and consequently its conductance is smaller than that of connexin 43 (10, 13, 26). In addition, the effect of α-GA may also suggest that hypersensitivity caused by bFGF is mediated predominantly by gap junction formation, but not by other putative mechanism, such as the altered response of urothelium to a cholinergic stimulus (12).

Gap junction biosynthesis involves several coordinated steps (9, 29). First, connexins are synthesized in the endoplasmic reticulum and assemble into oligomeric hemichannels termed connexons. Next, connexons are delivered to the plasma membrane, where they dimerize to form gap junction channels. Finally, the gap junction channels aggregate into functional plaques in the plasma membrane. bFGF may initiate these steps by promoting connexin 43 expression. Furthermore, bFGF might play an additional role in gap junction formation. It has been reported that connexin 43 in gap junctions becomes resistant to solubilization by phosphorylation (31). bFGF not only increased connexin 43 expression but also induced its phosphorylation (Figs. 2, A and B, and 4A), which may have contributed to stabilization of gap junctions.

The functional results obtained with the in vivo release model corroborate these molecular mechanisms. Bladders stimulated by bFGF in vivo showed ex vivo hypersensitivity to acetylcholine (muscle strip data, Fig. 3, A–D), and in vivo bladder overactivity (cystometric data, Fig. 5, A–E). These results are in accordance with a recent clinical study that defined overactive bladder as a hypersensitive disorder (40). Our in vivo release model consistently reproduced two major features of overactive bladder: increased micturition frequency and decreased bladder capacity. Interestingly, bladders treated with bFGF showed a significantly lower maximum voiding pressure than the sham-operated group (Fig. 5D); we did not expect this finding because we have shown increased muscle strip contractility in the same model (14). The most possible explanation for this discrepancy is that detrusor overactivity in this model was caused by premature micturition without a significant decrease in compliance (demonstrated in Fig. 5E). Premature micturition is associated with myogenic changes and indicates an unstable but not low-compliant detrusor in overactive bladder (24, 28). Electrical coupling of BSMC allows transmission of stimuli to adjacent cells and consequently enhances the reactivity of the bladder to weaker stimuli (10, 13).

The present study and our previous study describing hypertrophic effects of bFGF complement each other (14). It is notable that a single bFGF-ERK pathway underlies two typical changes observed in the obstructed bladder, namely, hypertrophy and hypersensitivity. However, it should also be noted that the divergence of bFGF-ERK signaling in two different directions might present a similar dilemma, as seen with current therapeutic modalities that target cholinergic receptors (2). Anticholinergics block overactivity but also reduce the contractile force of the bladder, so they are contraindicated for obstructed bladders. Similarly, blockade of bFGF-ERK in the obstructed bladders may reduce overactivity but also reduce the contractile force. Consequently, inhibition of overactivity without reducing bladder contractility should be explored to provide a radical cure for BOO patients with overactive bladder. Pharmacological inhibition of gap junction activity might present an ideal treatment strategy, since our data show that blockade of gap junction activity inhibits hypersensitivity in bladders treated with bFGF (Fig. 3, C and D) but does not affect the maximum contractile force.

In summary, our data suggest that bFGF could be a pivotal signal for inducing detrusor overactivity in obstructed bladders through gap junction expression in BSMC via the ERK pathway. This study may provide a molecular basis for new therapeutic approaches to overactive bladder.
ACKNOWLEDGMENTS

The authors thank Dr. Devang Thakor and Dr. Kanji Nagahama (Kyoto University) for valuable technical support and advice.

GRANTS

This work was supported by a Grant-in-Aid for Scientific Research (nos. 19591846 and 20390425) from the Japan Society for the Promotion of Science.

REFERENCES