Altered regulation of bladder nerve growth factor and neurally mediated hyperactive voiding

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Clemow, David B., William D. Steers, Richard McCarty, and Jeremy B. Tuttle. Altered regulation of bladder nerve growth factor and neurally mediated hyperactive voiding. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1279–R1286, 1998.—Elevated bladder smooth muscle cell (BSMC) nerve growth factor (NGF) secretion and related neuroplasticity are associated with hyperactive voiding in spontaneously hypertensive rats (SHRs: hypertensive, behaviorally hyperactive), compared with control Wistar-Kyoto (WKY) rats. We used two inbred strains (WKHT: hypertensive; WKHA: hyperactive) to further investigate this phenomenon. WKHA BSMCs secreted higher basal levels of NGF than WKHT BSMCs. Antagonists did inhibit NGF output in WKHA but not WKHT cultures. Thus augmented basal secretion of NGF cosegregates with a hypertensive phenotype, whereas a lack of regulatory inhibition of NGF output cosegregates with a hyperactive phenotype. Bladder norepinephrine content paralleled NGF content, with WKHTs > SHRs > WKHAs > WKYs, providing evidence that a lack of inhibition is the greatest contributor to elevated bladder NGF and noradrenergic innervation. Protein Kinase C (PKC) agonists affected NGF production differentially depending on strain, suggesting that altered PKC signaling may contribute to strain differences in NGF secretion. Finally, 6-h voiding frequency differed between the strains, with SHRs > WKHTs = WKHAs > WKYs. Thus aspects of both the hypertensive and hyperactive phenotypes may be associated with elevated SHR bladder NGF and hyperactive voiding.

MATERIALS AND METHODS

Animals. SHR and WKY breeders were maintained from breeding stock originally purchased from Taconic Farms. WKHTs and WKHAs, derived from the same source as the SHR and WKY strains, were maintained from breeding stock...
originally provided by Dr. E. D. Hendley, University of Vermont College of Medicine. For all of the studies, age-matched SHR (300–350 g), WKY (400–450 g), WKHT (250–300 g), and WKHA (250–300 g) adult rats were screened for resting systolic blood pressure with tail-cuff plethysmography (10). An average blood pressure of at least 150 mm Hg was required for all hypertensive rats and of not more than 130 mm Hg for all normotensive rats. Animals were housed individually in wire cages and provided with laboratory chow and tap water ad libitum. The vivarium room was kept on a 12:12-h light-dark cycle, and the ambient temperature was maintained at 22 ± 1°C. Body and bladder weights were measured from eight rats of each strain.

Voiding studies. Rats used for voiding studies were housed in individual cages with free access to food and water (23–30 adult rats: ⅓ female, ⅔ male for each strain). Animals were kept on a 12:12-h light-dark cycle starting at 7:00 AM. Voiding studies were run in mid-light cycle (6-h time period: 10:00 AM–4:00 PM). For the measurement of voiding frequency, SHR and WKY rats were plated in individual metabolic caging (Nalgene) with unrestricted food and water. A graduated cup was attached to an FT03 force transducer (Grass) and positioned to collect all urine during the sample period. Void events were amplified using a model 7D amplifier and traced on a polygraph (Grass). The sensitivity of this method allowed the measurement of void frequency for voids ≥100 μl, with void volume measurements accurate to ±25 μl.

BSMC cultures. Bladders were dissected from rats after CO2 euthanasia and cultured as described previously (16, 20). For routine culture, we plated smooth muscle cells at a density of 5 × 10^3 cells/cm² and they were maintained in growth medium consisting of Dulbecco’s modified Eagle medium (GIBCO) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), penicillin (100 U/ml), and amphotericin B (250 ng/ml; antibiotic/antimycotic; Sigma). All cultures were maintained in a humidified chamber containing 5.0% CO2 at 37°C.

Cultures were made from six different primary cell lines (3 WKHA, 3 WKHT). Each cell line was derived from four bladders (2 male, 2 female rats). Each set of rats was the offspring of different breeders. There were no apparent differences derived from a particular strain/cell type. Only cultures between passages 3 and 6 were used. To confirm the smooth muscle character of the cultured cells, immunohistochemical staining with anti-smooth muscle α-actin specific antibody (Sigma) was used as previously described (5).

To study the pharmacological regulation of NGF secretion by smooth muscle cells, we plated BSMCs into 12-well tissue culture plates at a density of 5 × 10^3 cells/cm² and maintained the cultures in serum-containing medium until confluence (entire plate covered by cells such that no part of plate was visible, with cultures checked daily). Once confluent, the culture medium was changed to serum-free medium (SFM) consisting of growth medium without FBS, supplemented with insulin (1 U/ml), transferrin (5 μg/ml), and ascorbic acid (200 μmol/l; Sigma). Culture medium was changed 48 and 72 h after the initial change to SFM to which 1,250 μl fresh SFM was added, with or without pharmacological treatments (see RESULTS). Each culture well was then sequentially sampled (250 μl each time point from the same medium) at 4, 6, 8, and 24 h after treatment. Previous work has demonstrated no significant effect of extracellular proteolysis under these conditions (29). All pharmacological agents were obtained from Sigma, excluding platelet-derived growth factor (PDGF; Upstate Biotechnology). Each experimental paradigm consisted of six experiments, within which each experiment condition was performed in triplicate. After 24 h of treatment, the cultures were fixed and cell number determined as previously described (20).

Conditioned medium NGF determination. For the quantification of NGF, a two-site ELISA for NGF was used (20, 21). We calculated the rate of NGF secretion from cultured BSMCs for each sample period (0–4, 4–6, 6–8, and 8–24 h) by subtracting the total amount of NGF at the start of each time period (concentration of NGF in conditioned media multiplied by the volume of media) from that present at the end of each sample period and dividing by the time between samples. The ELISA was sensitive to 1.0 pg/ml of NGF. Single time point samples from each well were assayed in quadruplicate. Due to the potential effects of cell number on NGF secretion in a given experiment or culture well, rates of NGF secretion were expressed as femtograms NGF per hour per 100 cells.

Bladder tissue NGF determination. Bladders were dissected from male rats (n = 8 for each strain) and frozen at −80°C. Bladders were prepared based on a protocol developed by Zettler and colleagues (33). Frozen bladders were finely pulverized in liquid N2 and prepared for 1 min by Polytron tissue disruption at 1:40 (wt/vol) in a high-salt/high-detergent buffer (100 mM Tris·HCl, 1 M NaCl, 2% BSA, 4 mM EDTA, 2% Triton X-100, 0.02% sodium azide, 0.1 μg/ml pepstatin A, 5 μg/ml aprogin, 0.5 μg/ml antipain, 167 μg/ml benzamidine, 5.2 μg/ml phenylmethyl-sulfonyl fluoride) with the use of 6-ml polypropylene tubes. Samples were then base treated to pH 11 with the use of 4 M NaOH. After centrifugation at 13,000 g (4°C, 15 min), the supernatant was acid treated to pH 3 with the use of glacial acetic acid followed by centrifugation at 13,000 g (4°C, 30 min). The supernatant was then neutralized to pH 7 with the use of 10 M NaOH. After a final centrifugation at 13,000 g (4°C, 15 min) the supernatant was assayed for NGF. The samples were vortexed and left undisturbed for 5 min before each centrifugation. Bladder NGF levels determined from the newly developed acid-base NGF extraction protocol were compared with four protocols our laboratory has used in the past: 1) tissue homogenation in ELISA sample buffer, 2) Trizol (GIBCO) extraction according to the manufacturer’s guidelines, 3) a variation of the Trizol extraction protocol that uses dialysis of the phenol-ethanol supernatant, 4) homogenization in Trizol without further processing.

Norepinephrine analysis. For measurement of norepinephrine (NE) in tissue, male bladders (n = 6 each for WKY and SHR, 8 each for WKHT and WKHA) were removed from rats after CO2 euthanasia and snap frozen on dry ice. Frozen samples were weighed and homogenized in 4 × volume of cold 0.2 N perchloric acid. Fifty microliters of homogenate were removed for determination of protein according to manufacturer’s instructions using a Bio-Rad protein assay kit. The remaining homogenate was centrifuged at 15,000 rpm for 30 min at 4°C. The resulting supernatant was removed, and NE and other catecholamines were measured by batch alumina extraction followed by high-performance liquid chromatography with electrochemical detection (11).

Statistical analyses. For statistical purposes, the data were analyzed using a multivariate analysis of variance. Comparisons between two variables in different strains were made using Student’s t-test for independent samples. Values shown (both raw rates and percent control) represent the mean ± SE. For all comparisons, significance was set to P < 0.05.

RESULTS

Basal and drug-mediated BSMC NGF secretion. See Table 1 for a summary of drug effects on BSMC
cultures. Basal NGF secretion by WKHA BSMCs was significantly greater than WKHTs at all time points measured (Fig. 1, A and B). Basal NGF secretion for WKHA and WKHT BSMCs was relatively steady throughout the testing period. Vasoconstrictors are potent stimulators of vascular smooth muscle NGF secretion (29). Thus, to test any possible effect of bladder contractile agents (1, 2, 12, 21, 26, 28) on NGF output, we treated BSMC cultures with the cholinergic agonist carbachol (100 µM), the α-adrenergic agonist phenylephrine (10 µM in combination with 10 µM propranolol), the β-adrenergic agonist isoproterenol (10 µM), the purine analog αβ-methyl-ATP (100 µM), and the peptide neuropeptide Y (NPY; 1 µM). To examine the role of protein kinase A (PKA) signaling in NGF secretion, we treated cultures with 10 µM forskolin. Carbachol had no effect on NGF output. Phenylephrine, isoproterenol, αβ-methyl-ATP, NPY, and forskolin all inhibited NGF output in WKHA BSMCs but had no effect or increased NGF secretion in WKHT BSMCs (Fig. 1, A and B). PDGF, working via protein kinase C (PKC) signaling pathways, has been shown to be a potent stimulus for BSMC NGF output (16). We tested 350 pM PDGF on NGF production. PDGF increased NGF output in both WKHA and WKHT BSMCs (Fig. 2A). To examine the direct role of PKC signaling in NGF secretion, we treated cultures with the phorbol ester phorbol-12-myristate-13-acetate (PMA). The experimental paradigm consisted of either a 24-h pretreatment with 100 nM PMA and no PMA at the initiation of the experiment (PMA-pre), the addition of 10 nM PMA at the beginning of the test period (PMA-post), or both conditions (PMA-pre-post). The PMA pretreatment was used for the general downregulation of responsive PKC isozymes (16). The PMA-pre condition had no effect. The PMA-post condition increased NGF production in WKHA but not WKHT BSMCs (Fig. 2B). In the PMA-pre-post condition, PKC downregulation blocked subsequent increases in WKHA BSMC NGF output. To determine the effect of NGF-inhibiting agents on pharmacologically elevated NGF secretion, we added 750 µl of SFM or SFM with forskolin (10 µM), isoproterenol (10 µM), or phenylephrine (10 µM in combination with 10 µM propranolol) to WKHA and WKHT BSMC cultures. One hour later we added PDGF (350 pM) to the culture medium. The culture medium was sampled once, 6 h after PDGF administration. PDGF alone significantly increased NGF secretion in both WKHA and WKHT cultures. Forskolin had no effect on PDGF-induced increases in NGF secretion in WKHA cultures but further increased NGF secretion by WKHT BSMCs (Fig. 3). Isoproterenol completely blocked the effect of PDGF in WKHA cultures but only decreased the effect in WKHT cultures by one-half. Phenylephrine decreased the effects of PDGF on NGF secretion by one-half in WKHA cultures but had no effect on WKHT BSMCs (Fig. 3).

Table 1. NGF secretion by cultured bladder smooth muscle cells

<table>
<thead>
<tr>
<th>BSMCs</th>
<th>Carb</th>
<th>PE</th>
<th>Iso</th>
<th>ATP</th>
<th>NPY</th>
<th>Forsk</th>
<th>PDGF</th>
<th>PMA</th>
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<tbody>
<tr>
<td>WKHA</td>
<td>70%</td>
<td>63%</td>
<td>64%</td>
<td>47%</td>
<td>77%</td>
<td>241%</td>
<td>165%</td>
<td></td>
</tr>
<tr>
<td>WKHT</td>
<td>43%</td>
<td>43%</td>
<td>38%</td>
<td>36%</td>
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Summary of maximal drug effects on bladder smooth muscle cell (BSMC): –, no effect; ↓ decrease; ↑ increase over control (P < 0.05). Carb, carbachol; PE, phenylephrine; Iso, isoproterenol; NPY, neuropeptide Y; Forsk, forskolin; PDGF, platelet-derived growth factor; PMA, phorbol-12-myristate-13-acetate. Rat strains: WKHA, hyperactive; WKHT, hypertensive.

Fig. 1. Rate of nerve growth factor (NGF) secretion from Wistar-Kyoto (WKY) hyperactive rats (WKHA; A) and WKY hypertensive rats (WKHT; B) bladder smooth muscle cells (BSMCs) under confluent, serum-free conditions quantified from samples of conditioned medium taken 4, 6, 8, and 24 h from the start of each experiment. Basal/control rate of NGF secretion from WKHA BSMCs was significantly elevated during all time periods over WKHT cells, indicated by * (P < 0.05). Isoproterenol (10 µM) and phenylephrine (10 µM) decreased NGF secretion compared to controls. Significant differences from control within a strain are also shown. * Significant differences from control within a strain. Inset is provided so that trends in WKHT NGF secretion rates can be better visualized (different y-axis scale).
Tissue NGF levels and NE content. Homogenation of bladder tissue in ELISA sample buffer or Trizol without further processing and in Trizol followed by processing steps outlined in the manufacturer's guidelines revealed positive bladder NGF levels. However, the results using these extraction procedures were highly variable, such that no significant differences between strains were observed. Trizol extraction with dialysis resulted in a low recovery of NGF protein. Bladder NGF extraction using the newly developed acid-base methodology (33) revealed positive levels of NGF with little variability within a strain. Acid-base protein extraction also exposed significant differences between strains in bladder NGF content, WKHT > SHR > WKHA > WKY (Fig. 4A). Significant differences in bladder NE content also existed between strains, WKHT > SHR > WKHA = WKY (Fig. 4B).

 Voiding frequency and volume. Adult SHRs voided significantly more frequently than WKHTs and WKHAs, and these inbred strains voided significantly more frequently than WKYs (Fig. 5A). WKYs voided at a higher volume per void than the other strains (Fig. 5B). The only difference between strains in the total volume voided was that SHRs voided slightly more than WKHAs (Fig. 5C). Due to the design of the
The only difference in bladder-to-body weight ratios was that WKHAs had a lower ratio than each of the other three strains. This difference could be attributed to WKHAs also having a significantly lower bladder weight than the other three strains (Table 2).

**DISCUSSION**

WKHT (hypertensive) and WKHA (behaviorally hyperactive) rats were used in the present study to determine whether elevated bladder tissue, NGF, increased BSMC NGF secretion, and hyperactive voiding appear in association with the hypertensive or behaviorally hyperactive phenotype. Results from Zettler and colleagues (33) indicate that endogenous NGF concentrations have been underestimated in past studies, probably due to the inability of existing techniques to free NGF from neuronally derived binding proteins. The inability of our previous NGF extraction techniques to unmask differences in bladder NGF between strains or bladder regions (17) may be due to the association of NGF with a receptor or other binding site, hindering immunological detection of the protein. Use of the newly developed acid-base protocol for NGF extraction may have revealed both receptor-bound and free levels of NGF protein. On the basis of this new data, augmented bladder NGF, present in the parent strain SHRs, does not segregate with a particular phenotype, because SHR, WKHT, and WKHA bladder NGF is greater than control WKYs.

Smooth muscle hypertrophy triggers elevated NGF levels in obstructed bladders (25). Smooth muscle hypertrophy does not appear to cause the augmented NGF levels observed in SHR, WKHT, and WKHA bladders because no significant increases were observed in the bladder-to-body weight ratios of the various strains. Significant bladder hypertrophy should increase the ratio. The only difference between strains is a smaller ratio for WKHAs. Thus an unknown genetic characteristic(s) of both the WKHT and WKHA phenotypes may contribute to heightened levels of SHR bladder NGF.

Because elevated basal NGF secretion from SHR vascular smooth muscle cells (VSMCs) cosegregates with a hypertensive phenotype (3), we inferred that this would be the case for BSMCs as well. However, basal NGF secretion from BSMCs of the behaviorally hyperactive (WKHA) strain was significantly higher than that of the hypertensive (WKHT) strain. In contrast to our hypothesis, elevated basal levels of SHR

**Table 2. Bladder-to-body weight ratios**

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR</th>
<th>WKHT</th>
<th>WKHA</th>
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<tbody>
<tr>
<td>Body wt, g</td>
<td>430 ± 30</td>
<td>328 ± 20*</td>
<td>303 ± 8*</td>
<td>313 ± 12*</td>
</tr>
<tr>
<td>Bladder wt, mg</td>
<td>134 ± 8</td>
<td>100 ± 5*</td>
<td>89 ± 2*</td>
<td>63 ± 5*‡</td>
</tr>
<tr>
<td>Ratio of bladder to body wt</td>
<td>0.032</td>
<td>0.031</td>
<td>0.030</td>
<td>0.020*‡</td>
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</table>

Values of weights are means ± SE. WKHA, WKY hyperactive rats. Significant differences: *differences from Wistar-Kyoto rats (WKY); †differences from spontaneously hypertensive rats (SHR); ‡differences from WKY hypertensive rats (WKHT).
BSMC NGF were linked to the hyperactive phenotype, not the hypertensive. Although WKHA BSMCs secrete higher basal levels of NGF than do WKHTs, α- and β-adrenergic-, purinergic-, peptidergic-, and PKA-dependent stimulation all inhibit NGF secretion in WKHA cultures. In contrast, the same agents increase NGF output in WKHT cultures or have no significant effect. It could be that inhibitory mechanisms regulating NGF secretion are intact in WKHT BSMCs but that the level of secretion is already so low that further inhibition is not possible. To test this hypothesis, we determined the effect of NGF inhibiting agents in BSMC cultures in which NGF secretion had been elevated with the addition of PDGF. Whereas inhibitory effects were observed in WKHA cultures, one-half the inhibition, no inhibition, or a further increase in NGF secretion was observed in WKHT cultures. These findings support the interpretation that there is disturbed signaling in WKHT BSMCs such that there is a lack of inhibition and oversensitivity to NGF-regulating agents. Pharmacological inhibition of NGF output in WKHA but not WKHT BSMCs parallels results in the WKY/SHR comparison of VSMCs (21). β-Adrenergic and PKA-dependent signaling agents inhibit NGF output from WKY VSMC cultures, and the same agents increase or have no effect on NGF secretion in SHR VSMC cultures (21, 31). α- And β-adrenergic agonists inhibit NGF output in Wistar BSMCs (17), suggesting that this receptor-mediated inhibition is common to BSMCs from normotensive rats. This finding also lends evidence to the hypothesis that BSMCs from hypertensive animals have a defect(s) in signaling mechanisms controlling NGF production.

These observations suggest that there may be two mechanisms underlying the elevated tissue NGF content of SHRs. First, SHR vascular and bladder smooth muscle may secrete elevated basal levels of NGF. This defect in NGF output cosegregates with the hypertensive phenotype in VSMCs (3) but with the hyperactive phenotype in BSMCs. Second, regulation of NGF production in SHR smooth muscle appears defective, such that smooth muscle cell NGF output is not affected by inhibitory signaling pathways whereas excitatory signaling mechanisms are hyperresponsive. This receptor-mediated defect in NGF metabolism cosegregates with the hypertensive phenotype in both VSMCs (3) and BSMCs. Thus the overall regulation of NGF production is similar in bladder and vascular smooth muscle but displays subtle variations in relation to hypertensive and hyperactive characteristics. Moreover, a lack of regulatory inhibition and/or an oversensitivity to NGF agonists in vitro may be the best indicator of bladder NGF content in vivo, because WKHT bladder tissue NGF is significantly higher than that of WKHA bladder.

PKC is an important signaling molecule in the regulation of NGF secretion in both VSMCs and BSMCs (16, 17, 31). Differences in PKC expression and/or activation could underlie the differences between strains in smooth muscle NGF regulation. PDGF, working via PKC signaling pathways, has been shown to be a potent stimulus for BSMC NGF secretion in Wistar rats (16). There is a trend for WKHT BSMCs to respond to PDGF with a greater output of NGF than WKHA BSMCs. This trend is significant in the WKHT/WKHA VSMC comparison (3). In VSMC cultures, PKC activation by PMA increases NGF output in differing amounts in all four strains (3, 31). In BSMC cultures, under serum-free culture conditions, PMA-induced PKC activation increases NGF secretion in WKHA but not WKY, SHR (20), or WKHT cultures. The effect of PMA on WKHA BSMCs is blocked by downregulation of PKC before the start of the experiment, confirming a role for PKC in the signaling of NGF production. Under conditions of 5% horse serum, PMA significantly increases NGF output in Wistar BSMCs (17). Thus altered PKC signaling pathways may indeed underlie differences between strains in smooth muscle NGF output and may also account for differences between vascular and bladder smooth muscle NGF secretion. However, whereas PDGF stimulates NGF secretion by both WKHA and WKHT BSMCs in SFM, PMA stimulates NGF secretion only in WKHA cultures, suggesting that PDGF may also work through PKC-independent mechanisms. Moreover, culture conditions have an important influence on the regulation of NGF secretion. The effect of culture condition is most evident when VSMCs are considered. Under serum-free conditions, SHR VSMCs secrete an elevated level of NGF compared with WKY VSMCs (21). In contrast, under conditions with 10% FBS, no difference was detected (32).

The development of hyperactive voiding in rats with urethral obstruction (25) or inflammation (7) is characterized by increased bladder levels of NGF followed chronologically by altered patterns of innervation (22, 23) and micturition reflex activity (6, 24). In SHRs, higher NGF secretion rates by bladder smooth muscle are associated with hyperinnervation of the bladder and may be an underlying cause of hyperactive voiding (20). The effects of elevated NGF on bladder innervation and voiding frequency are understandable considering C-fiber afferents, Aδ- afferents, and sympathetic efferents are dependent on NGF for survival and functional maintenance (13, 19, 30). In the present study, because there are few if any differences between the strains in total volume of urine voided, differences between strains in voiding frequency are probably due to NGF-induced neurogenic alterations rather than changes in urine production. The slight difference in volume voided between SHRs and WKHAs may be due to artifact (overestimation of volume from an increased number of quantified small graph deflections).

The result that NE levels, a marker for the density of sympathetic innervation, are elevated in SHR and WKHT but not WKHA bladders is intriguing. SHR, WKHT, and WKHA bladders contain elevated levels of NGF and would be expected to be sympathetically hyperinnervated. These results imply that the noradrenergic hyperinnervation observed in SHR bladders cosegregates with a hypertensive phenotype despite the observation that increased bladder tissue NGF occurs in both hypertensive and hyperactive animals. Com-
pared with WKYs, the elevations in WKHA bladder NGF are significantly less than the elevations in either SHRs or WKHTs. Perhaps the increased levels of bladder NGF in WKHAs are not sufficient to affect sympathetic innervation but are elevated enough to affect the sensory control of micturition. The data presented do not indicate the exact change(s) responsible for increased voiding frequency in SHRs. However, our results do suggest that SHR hyperactive voiding may be directly or indirectly attributable to characteristics of both the hypertensive (e.g., sympathetic hyperinnervation) and behaviorally hyperactive genotypes. Attributes of both genotypes may act together to increase voiding frequency in SHRs because SHRs void more frequently than either WKHTs or WKHAs. Experiments designed to determine which aspects of these genomes affect NGF metabolism and voiding behavior could yield valuable information on the determinants of bladder circuit function.

In summary, defects in BSMC basal NGF production cosegregate with a behaviorally hyperactive phenotype. A lack of regulatory inhibition of NGF output cosegregates with a hypertensive phenotype. Thus aspects of both the hypertensive (WKHT) and hyperactive (WKHA) phenotypes appear to be associated with elevated smooth muscle NGF and thereby may contribute to hyperactive voiding in SHRs by inducing changes in bladder innervation. Further insight into these hyperactive voiding syndromes may lead to clinically relevant intervention for patients with voiding dysfunction.

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