Role of p75NTR in female rat urinary bladder with cyclophosphamide-induced cystitis

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Klinger MB, Vizzard MA. Role of p75NTR in female rat urinary bladder with cyclophosphamide-induced cystitis. Am J Physiol Renal Physiol 295:F1778–F1789, 2008. First published October 8, 2008; doi:10.1152/ajprenal.90501.2008.—Previous studies demonstrated changes in urinary bladder neurotrophin content and upregulation of neurotrophin receptors, TrkA and the p75 neurotrophin receptor (p75NTR), in micturition reflex pathways after cyclophosphamide (CYP)-induced cystitis. p75NTR can bind nerve growth factor (NGF) and modulate NGF-TrkA binding and signaling. We examined p75NTR expression and the role of p75NTR in the micturition reflex in control and CYP-treated rats. p75NTR Immunoreactivity was present throughout the urinary bladder. CYP-induced cystitis (4 h, 48 h, chronic) increased (P ≤ 0.05) p75NTR expression in whole urinary bladder as shown by Western blotting. The role of p75NTR in bladder function in control and CYP-treated rats was determined using conscious cystometry and immunoneutralization or PD90780, a compound known to specifically block NGF binding to p75NTR. An anti-p75NTR monoclonal antibody or PD90780 was infused intravesically and cystometric parameters were evaluated. Both methods of p75NTR blockade significantly (P ≤ 0.05) decreased the intercontractual interval and void volume in control and CYP-treated rats. Intravesical infusion of PD90780 also significantly (P = 0.01) increased intravesical pressure and increased the number of nonvoiding contractions during the filling phase. Control intravesical infusions of isotype-matched IgG and vehicle were without effect. Intravesical injection of PD90780 significantly (P ≤ 0.01) reduced the volume threshold to elicit a micturition contraction in control rats (no inflammation) and CYP-treated in a closed urinary bladder system. These studies demonstrate 1) ubiquitous p75NTR expression in urinary bladder and increased expression with CYP-induced cystitis and 2) p75NTR blockade at the level of the urinary bladder produces bladder hyperreflexia in control and CYP-treated rats. The overall activity of the urinary bladder reflects the balance of NGF-p75NTR and NGF-TrkA signaling.

PD90780; conscious cystometry; Western blotting; bladder hyperreflexia; immunoneutralization

NERVE GROWTH FACTOR (NGF) is important in sensory and sympathetic neuronal development and maintenance (35); however, many recent studies suggest additional role(s) for NGF in painful somatic and visceral inflammatory conditions (1, 12, 28, 50). NGF upregulation occurs at sites of tissue injury and inflammation (40, 71) and changes in NGF levels in urine as well as urinary bladder have been documented in humans with painful bladder syndrome (PBS)/interstitial cystitis (IC) and rodents with bladder inflammation (43, 48, 49, 64). A number of addition and subtraction studies have begun to demonstrate the role(s) of NGF in inflammatory conditions of the urinary bladder. For example, intravesical infusion of intramuscular detrusor administration of exogenous NGF results in increased bladder activity, sensitization of bladder afferents (12, 20), increased expression of Fos protein in lumbosacral spinal cord in response to bladder distention, and increased expression of neuropeptides in lumbosacral spinal cord (73). Strategies to reduce NGF in micturition reflex pathways reduce or eliminate these effects (20, 34).

NGF signals through its specific receptor TrkA, as well as p75NTR. TrkA is a tropomyosin-related receptor tyrosine kinase receptor and p75NTR belongs to the tumor necrosis factor-α family of receptors (1, 4, 8). p75NTR binds all neurotrophins, including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. NGF-TrkA signaling is involved in some inflammatory effects of NGF (50), including sensitization of nociceptive afferents and the release of neuropeptides. TrkA expression is increased in bladder afferent cells in lumbosacral dorsal root ganglia (DRG) with acute and chronic cyclophosphamide (CYP)-induced bladder inflammation (51). In addition, p75NTR expression is also significantly upregulated in bladder afferent cells (2.4- to 2.8-fold) in lumbosacral DRG after CYP-induced cystitis (39).

The pan-neurotrophin receptor p75NTR exhibits a ubiquitous distribution and has many functions and multiple binding partners and ligands (4, 8). In the absence of Trk expression, p75NTR is suggested to be involved in apoptosis and regulation of neuronal growth (4, 27); however, in the presence of TrkA, p75NTR is suggested to function by enhancing Trk binding to neurotrophins (3, 4, 70). p75NTR expression has previously been identified in the urinary bladder (61, 66, 67) but few studies have examined the role of p75NTR in bladder function or in the context of urinary bladder inflammation (66). Although we do know that p75NTR expression is present in micturition reflex pathways in control rats and is regulated with CYP-induced cystitis (39), the potential role(s) of p75NTR in urinary bladder reflexes have not been explored. In this study, we determined 1) expression of p75NTR in the urinary bladder with immunohistochemistry and Western blot and regulation of p75NTR expression after CYP-induced cystitis, 2) the effect of immunoneutralization with an anti-p75NTR monoclonal antibody on bladder function in female rats without (noninflamed) and with bladder inflammation (48-h CYP treatment) using conscious cystometry, and 3) the effect of intravesical infusion of PD90780, a compound known to specifically block NGF binding to p75NTR (14, 55), in female rats without (noninflamed) and with bladder inflammation (48-h CYP treatment) using conscious cystometry.

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MATERIALS AND METHODS

CYP-Induced Cystitis: Acute, Intermediate, or Chronic

Chemical cystitis was induced in adult (200–225 g), female Wistar rats by CYP treatment (39, 63). CYP was administered in one of the following ways: 1) 4 h (150 mg/kg ip) before euthanasia of the animals to elicit acute bladder inflammation, 2) 48 h (150 mg/kg ip) before euthanasia to examine an intermediate inflammation, or 3) administered every third day for 9 days to elicit chronic bladder inflammation (75 mg/kg ip). All injections of CYP were performed under isoflurane (2%) anesthesia. Control animals were gender matched to the experimental groups and received a corresponding volume of saline (0.9%) or distilled water injected intraperitoneally under isoflurane (2%) anesthesia. Animals were euthanized by isoflurane anesthesia (5%) plus thoracotomy at the indicated time points, and the urinary bladder was harvested and weighed. The University of Vermont Institutional Animal Care and Use Committee approved all experimental procedures (protocol number 06–014) involving animal use. Animal care was under the supervision of University of Vermont College of Medicine’s Office of Animal Care in accordance with American Association for Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. All efforts were made to minimize animal stress/distress and suffering and to use the minimum number of animals.

Whole Mount Bladder Preparation and Immunohistochemistry

The urinary bladder from control (n = 5) and CYP treatment (n = 5) was dissected and placed in Krebs solution. The bladder was cut open along the midline and pinned to a Sylgard-coated dish. The bladder was incubated for 3 h at room temperature in cold fixative (2% paraformaldehyde + 0.2% picric acid), and the urothelium was removed as previously described (74). Urothelium and bladder muscle cultures were processed separately for p75NTR-immunoreactivity (IR). Control and CYP-treated tissues were incubated overnight at room temperature in rabbit anti-p75NTR antiserum [1:3,000; Ad- (IR)]. Control and CYP-treated tissues were incubated overnight at room temperature, followed by washing and coverslip-conjugated goat anti-rabbit; Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature in cold fixative (2% paraformaldehyde + 0.2% picric acid), and the urothelium was removed as previously described (74). Urothelium and bladder muscle cultures were processed separately for p75NTR-immunoreactivity (IR). Control and CYP-treated tissues were incubated overnight at room temperature in rabbit anti-p75NTR antiserum [1:3,000; Advanced Targeting Systems (ATS), San Diego, CA] in 1% goat serum and 0.1 M KPBS (0.1 M PBS with potassium) and then washed (3 × 15 min) with 0.1 M KPBS, pH 7.4. After being washed, the tissue was incubated in a species-specific secondary antibody (1:500; Cy3-conjugated goat anti-rabbit; Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature, followed by washing and coverslipping with Citifluor (London, UK). Control tissues incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining was observed. The specificity of the p75NTR antiserum was previously established (42). Some whole mount preparations were stained with the pan neuronal marker, protein gene product 9.5 (Abcam, Cambridge, MA; 1:15), to visualize nerve fibers in the suburothelial plexus and to demonstrate that suburothelial nerve fibers expressed p75NTR-IR.

p75NTR Localization in Urinary Bladder Sections After Intravesical p75NTR Infusion

Immediately after cystometric analyses, urinary bladders were harvested from rats that had received intravesical infusion of monoclonal antibody to p75NTR and those that had received intravesical infusion of protamine sulfate. Animals were deeply anesthetized with isoflurane (5%) and euthanized via thoracotomy. Bladders were quickly removed and postfixed in 4% paraformaldehyde overnight. Tissues were cryoprotected by immersion in 30% sucrose (in 0.1 M PBS) overnight. Bladders were sectioned (20 μm) on a cryostat and directly mounted on gelled (0.5%) microscope slides. Tissue was incubated in secondary antibody (Cy2-conjugated goat anti-mouse; Jackson ImmunoResearch) for 2 h and washed (3 × 15 min) at room temperature with 0.1 KPBS (pH 7.4). Slides were coverslipped with Citifluor.

Assessment of Positive Staining in Urinary Bladder

Staining observed in experimental tissue was compared with that observed from experiment-matched negative controls. Tissues exhibiting immunoreactivity that was greater than the background level observed in experiment-matched negative controls were considered positively stained.

Imaging and Visualization of Bladder Sections

Tissues were examined under an Olympus fluorescence photomicroscope (Optical Analysis, Nashua, NH) for visualization of Cy2. Cy2 was visualized with a filter with an excitation range of 470–490 and an emission range from 510 to 530. Images of bladder sections were captured through a video camera attachment to the microscope with the exposure time, brightness, and contrast being held constant.

Imaging and Visualization of Bladder Whole Mounts

Tissue was examined and optical sections were acquired using a Zeiss LSM 510 confocal scanning system attached to a Zeiss LSM 510 microscope using a plan Fluor ×20 or ×10 objective. An excitation wavelength of 543 nm was used for visualization of p75NTR. Bladder whole mount images were captured through a video camera attachment to the microscope with the exposure time, brightness, and contrast held constant.

Western Blotting for p75NTR Expression in Whole Urinary Bladder

Whole urinary bladders were homogenized separately in tissue protein extraction agent with protease inhibitors (T-PER; Roche, Indianapolis, IN), and aliquots were removed for protein assay. Samples (23 μg) were suspended in sample buffer for fractionation on gels and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and efficiency of transfer was evaluated. Membranes were blocked overnight in a solution of 5% milk, 3% bovine serum albumin in Tris-buffered saline with 0.1% Tween. Membranes were incubated in rabbit anti-p75NTR (1:2,000; ATS) overnight at 4°C. Washed membranes were incubated in a species-specific secondary antibody (1:7,000; goat anti-rabbit horseradish peroxidase) for 2 h at room temperature for enhanced chemiluminescence detection (Pierce, Rockford, IL). Blots were exposed to Biomax film (Kodak, Rochester, NY) and developed. The intensity of each band was analyzed, and background intensities were subtracted using Un-Scan It software (Silk Scientific, Orem, UT). Western blot analysis of erk1 and erk2 (1:2,000; Cell Signaling Technology, Danvers, MA) in samples was used as a loading control. The specificity of the p75NTR antiserum was previously established (42). In pilot studies, the concentration of p75NTR antiserum used for Western blotting studies of urinary bladder was titrated. The p75NTR antiserum concentration selected for experimentation did not result in saturation in control tissues and permitted changes in p75NTR expression with CYP treatment to be evaluated semiquantitatively.

Intravesical Catheter Placement

A lower midline abdominal incision was performed under general anesthesia with 2–3% isoflurane using aseptic techniques. Polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ) with the end flared by heat was inserted into the dome of the bladder and secured in place with a 6–0 nylon purse-string suture (33). The distal end of the tubing was sealed, tunneled subcutaneously, and externalized at the back of the neck, out of the animal’s reach. Animals were maintained for 72 h after surgery to ensure complete recovery.

Cystometry

Continuous cystometry. The effects of p75NTR blockade on bladder function in control (no inflammation) and CYP-treated rats (48 h) were evaluated by immunoneutralization with intravesical infusion of...
anti-p75NTR monoclonal antibody (ATS; 100 μg/ml) or PD90780 (Pfizer; 10–100 μM), known to specifically block NGF-p75NTR (14, 55) using conscious cystometry and continuous infusion of intravesical saline. Intravesical instillation of an isotype-matched IgG and saline (room temperature, 0.9%) were used as controls. Animals were placed conscious and unrestrained in recording cages with a balance and pan for urine collection and measurement was placed below (7, 34, 73). Intravesical pressure changes were recorded using a Small Animal Cystometry System (Med Associates). Saline at room temperature was infused at a rate of 10 ml/h to elicit repetitive bladder contractions. At least four reproducible micturition cycles were recorded after an initial stabilization period of 25–30 min. Voided saline was collected to determine voided volume. Intercontraction interval, maximal voiding pressure, pressure threshold for voiding, and baseline resting pressure were measured (44). The number of nonvoiding bladder contractions (NVCs) per voiding cycle during the filling phase was determined. For these studies, NVCs were defined as rhythmic intravesical pressure rises greater than 7 cmH2O from baseline pressure without a release of fluid from the urethra.

Exclusion Criteria

Rats were removed from study when adverse events occurred that included: ≥20% reduction in body weight postsurgery, a significant postoperative event, lethargy, pain, or distress not relieved by our IACUC-approved regimen of postoperative analgesics or hematuria in control rodents. In the present study, no rats were excluded from the study or from analysis due to any of these exclusion criteria. In addition, behavioral movements such as grooming, standing, walking, and defection rendered bladder pressure recordings during these events unusable (58). Experiments were conducted at similar times of the day to avoid the possibility that circadian variations were responsible for changes in bladder capacity measurements (21). Rats were euthanized at the conclusion of the study and urinary bladder was harvested as described above.

Isovolumetric cystometry. For isovolumetric cystometry, the bladder was exposed and cannulated as described above. The ureters were ligated and cut proximally, and the external urethral orifice was ligated to generate a closed urinary bladder system. The bladder cannula was connected to a pressure transducer and infusion pump via a three-way connector. The bladder was drained at the start of the study and saline was infused at a constant rate of 5 ml/h for up to 30 min per cycle. Saline was drained from the bladder between infusion cycles and the bladders were rested for 20 min before the start of the next infusion. The volume threshold for initiation of contraction and the effects of PD90780 (50 μM) on volume threshold were derived from these data in control (n = 4) and CYP-treated (48 h; n = 4) rats.

Drug Treatments

The effects of p75NTR blockade on bladder function in control (no inflammation) and CYP-treated rats (48 h) were evaluated by immunoneutralization with intravesical infusion of anti-p75NTR monoclonal antibody (ATS; 100 μg/ml) or PD90780 (Pfizer; 10–100 μM), known to specifically block NGF-p75NTR (14, 22, 55, 69) using conscious cystometry (continuous fill and isovolumetric). Immediately before cystometric analysis, rats were anesthetized (1–2% isoflurane) and bladder was manually emptied with the Credé maneuver. A solution of protamine sulfate (Sigma, St. Louis, MO; 10 mg/ml in sterile saline) was then infused into the bladder (≤1 ml) and maintained (45 min) in the bladder while the rat was anesthetized (isoflurane, 1–2%) to prevent voiding and expulsion of bladder contents (7, 15). The concentrations and duration of PD90780 infusion chosen for this study were based on previous experiments with PD90780 (14, 22, 55, 69). The concentration anti-p75NTR monoclonal antibody used for intravesical infusion was based on previous studies using anti-p75NTR or anti-NGF in intrathecal infusion or cell culture applications (22, 36, 47, 52, 53).

Figure Preparation

Digital images were obtained using a charge-coupled device camera (MagnaFire SP, Optronics; Optical Analysis) and LG-3 frame grabber attached to an Olympus microscope (Optical Analysis). Exposure times were held constant when acquiring images from control and experimental animals processed and analyzed on the same day. Images were imported into Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA) where groups of images were assembled and labeled.

Materials

All standard chemicals were obtained from Sigma or Fisher and were either analytical or laboratory grade. PD90780 was obtained through a compound transfer agreement with Pfizer (Groton, CT). PD90780 was made up as concentrated stock solutions stored in single use vials (to eliminate freeze thaw cycles) at −20°C until usage.

Statistical Analyses

All values represent means ± SE. Data were compared with one-or two-way ANOVA, where appropriate. For isovolumetric cystometry data, data were compared with repeated-measures ANOVA. When F ratios exceeded the critical value (P ≤ 0.05), the Dunnett’s post hoc test was used to compare group means.

RESULTS

p75NTR IR in Urinary Bladder

To determine the localization of p75NTR expression in the urinary bladder, whole mount preparations were prepared in control (Fig. 1, A–J) and 4-h (not shown) and chronic CYP-treated rats (not shown). p75NTR expression was present in the urothelium and neuronal fibers in the urinary bladder of control rats and CYP-treated rats. p75NTR-IR was seen in urothelial cells (Fig. 1, A, B, D), nerve fibers (Fig. 1, B–J) in the suburothelial plexus, and in nerve fibers in the detrusor in both control and CYP-treated rats. Nerve fibers adjacent to and encircling suburothelial vasculature also exhibited p75NTR-IR (Fig. 1, C, D, H–J). p75NTR-immunoreactive suburothelial nerve fibers and nerve fibers associated with the suburothelial vasculature also exhibited immunoreactivity for the pan neuronal marker, protein gene product (PGP9.5; Fig. 1, E–J). Given the widespread and overlapping nature of the p75NTR-IR in the defined structures, it was not possible to quantify changes in p75NTR-IR in individual structures with CYP-induced cystitis. Rather, we examined regulation of p75NTR protein expression in whole urinary bladder using Western blotting techniques.

p75NTR Protein Expression in Urinary Bladder with CYP-Induced Cystitis

p75NTR protein expression in whole urinary bladder as determined by Western blot analysis significantly (P ≤ 0.05) increased after 4-h (7.7-fold), 48-h (11.1-fold), or chronic CYP treatment (3.3-fold; Fig. 2, A and B).

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Fig. 1. p75<sup>NTR</sup> and pan-neuronal marker, protein gene product 9.5 (PGP9.5), immunoreactivity (IR) in urinary bladder whole mount preparations with the urothelium/suburothelium dissected from the detrusor smooth muscle. A: confocal image of p75<sup>NTR</sup>-IR in urothelial cells. B: epifluorescence image of p75<sup>NTR</sup>-IR suburothelial nerve fibers (yellow arrow) in close proximity to p75<sup>NTR</sup>-IR urothelial cells (white arrow). C: p75<sup>NTR</sup>-IR in suburothelial nerve fibers (white arrow) and vasculature (yellow arrows). D: p75<sup>NTR</sup>-IR in vasculature (yellow arrows), nerve fibers (white arrows), and urothelial cells out of the focal plane (*). p75<sup>NTR</sup>-immunoreactive suburothelial fibers (arrows E, H) also expressed PGP9.5-IR (arrows F, I). G and J: merged images of E, H and F, I, respectively. Calibration bar represents 100 μm.
Effects of Immunoneutralization of p75NTR on Cystometry in Rats with and without CYP-Induced Cystitis with Intravesical Instillation of Anti-p75 Monoclonal Antibody

Control (no inflammation). Intravesical infusion of an anti-p75NTR monoclonal antibody (100 μg/ml; Fig. 3, B and D) increased voiding frequency, with a decreased intercontraction interval (ICI; \( P \leq 0.001 \)) and decreased void volume \( (P \leq 0.001) \) compared with control rats (Figs. 3A and 4, A and B) without intravesical anti-p75NTR instillation. Intravesical infusion of a monoclonal antibody to p75NTR did not affect baseline, micturition, or threshold pressure or nonvoiding contractions during the filling phase (Table 1).

CYP treatment. As previously demonstrated, CYP treatment (48 h) decreased ICI \( (P \leq 0.001) \) and void volume \( (P \leq 0.001) \) and increased micturition \( (P \leq 0.001) \) and threshold pressure \( (P \leq 0.001) \); Table 1). Intravesical infusion of anti-p75NTR monoclonal antibody (100 μg/ml) in 48-h CYP-treated rats (Fig. 3D) resulted in an additional increase in voiding frequency with associated decreased ICI \( (P \leq 0.001) \) and decreased void volume \( (P \leq 0.005) \); Fig. 4, A and B, and Table 1). There were no significant changes in micturition pressure, threshold pressure, or nonvoiding contractions compared with rats with only 48-h CYP treatment (Fig. 3C; Table 1). Control experiments with intravesical infusion of an isotype-matched monoclonal IgG (100 μg/ml) or protamine sulfate (10 mg/ml) showed no effects on bladder function in control or CYP-treated rats (Figs. 3 and 4; Table 1) compared with rats without protamine sulfate or antibody infusion in control or CYP-treated rats. In pilot studies performed without the initial infusion of protamine sulfate, no changes in bladder function with subsequent intravesical infusion of the anti-p75NTR monoclonal antibody (100 μg/ml) were observed. Therefore, all subsequent experiments included protamine sulfate intravesical infusion. The duration of anti-p75NTR monoclonal antibody effects on all cystometric parameters evaluated in control and CYP-treated rats was 30–40 min.

p75NTR-IR in Urinary Bladder after Intravesical Instillation of Anti-p75NTR Monoclonal Antibody

p75NTR-IR was examined after intravesical instillation of anti-p75NTR monoclonal antibody by application of a species-specific secondary antibody to cryostat sections (20 μm). p75NTR-IR was present in urothelium and lamina propria (Fig. 5A). p75NTR-IR decreased in intensity with increasing distance from the bladder lumen. Urinary bladder sections

Fig. 2. A: representative Western blot of whole urinary bladder (23 μg) for p75NTR expression in control rats and those treated with cyclophosphamide (CYP) for varying duration. Erk1 staining was used as a loading control. B: histogram of relative p75NTR band density in all groups examined normalized to Erk1 in the same samples. p75NTR expression in whole urinary bladder is significantly increased with acute (4 h), intermediate (48 h), and chronic CYP treatment. *\( P \leq 0.05; n = 4 \) for all groups.

Fig. 3. Intravesical infusion of anti-p75NTR monoclonal antibody in control and in CYP-treated (48 h) rats increased voiding frequency (decreased intercontraction interval). Representative continuous cystometrogram recordings in rats treated with intravesical protamine sulfate in control rats (A), protamine sulfate and anti-p75NTR monoclonal antibody (100 μg/ml) in control rats (B), protamine sulfate in CYP-treated (48 h) rats (C), and protamine sulfate and anti-p75NTR monoclonal antibody (100 μg/ml) in CYP-treated (48 h) rats (D).
from rats not receiving intravesical instillation of anti-p75NTR monoclonal antibody exhibited no immunoreactivity above background levels (Fig. 5A).

Effects of NGF-p75NTR Blockade on Cystometry in Rats with and without CYP-Induced Cystitis with Intravesical Instillation of PD90780

Control (no inflammation) rats. PD90780, a compound that specifically blocks NGF binding to p75NTR (14, 55), was infused intravesically (10, 50, and 100 g/mL) in rats without CYP-induced cystitis and the effects on cystometric parameters using continuous instillation were determined (Fig. 6, A–D).

Intravesical instillation of PD90780 decreased void volume ($P < 0.002$) at all concentrations evaluated, and decreased ICI at 10 g/mL ($P < 0.05$; Fig. 7) and 50 g/mL ($P < 0.05$; Figs. 6, A and B, and 7). PD90780 instillation increased threshold pressure.

Table 1. Effects of intravesical infusion of an anti-p75NTR monoclonal antibody on cystometric parameters

<table>
<thead>
<tr>
<th>Intravesical Infusion</th>
<th>Threshold Pressure, cmH$_2$O</th>
<th>Micturition Pressure, cmH$_2$O</th>
<th>Baseline Pressure, cmH$_2$O</th>
<th>NVCs per Micturition Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no CYP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No antibody</td>
<td>15.1±0.9</td>
<td>38.5±1.3</td>
<td>15.1±0.9</td>
<td>0.7±0.3</td>
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<tr>
<td>Anti-p75NTR monoclonal antibody</td>
<td>14.2±1.0</td>
<td>39.5±2.0</td>
<td>15.0±0.6</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td>48-h CYP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No antibody</td>
<td>19.3±0.7*</td>
<td>46.7±1.4*</td>
<td>20.0±0.8*</td>
<td>0.2±0.1*</td>
</tr>
<tr>
<td>Anti-p75NTR monoclonal antibody</td>
<td>13.9±1.1</td>
<td>44.1±2.0</td>
<td>14.0±1.0</td>
<td>0.3±0.1</td>
</tr>
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</table>

Values are means ± SE. * $P < 0.001$ compared with isotype-matched IgG infusion in control rats; $n = 4–11$ rats in each group. Effects of intravesical infusion of an anti-p75NTR monoclonal antibody on baseline pressure, threshold pressure, micturition pressure, and numbers of nonvoiding contractions (NVCs) per micturition cycle are indicated. Threshold pressure and micturition pressure were significantly increased after 48-h cyclophosphamide (CYP) treatment. No effects of anti-p75NTR monoclonal antibody on threshold pressure, baseline pressure, or peak micturition pressure were observed. The no antibody group received intravesical infusion of protamine sulfate (PS; 10 mg/mL), which did not differ from rats receiving intravesical infusion of PS and isotype-matched IgG.
CYP treatment. Intravesical infusion of PD90780 in CYP-treated (48 h) rats significantly increased voiding frequency (Fig. 6, C and D) with a decreased void volume ($P \leq 0.002$; Fig. 8B) and ICI ($P \leq 0.002$; Fig. 8A) compared with rats with CYP treatment (48 h) alone. PD90780 (10, 50, and 100 $\mu$M) significantly ($P \leq 0.001$) increased threshold, baseline, and micturition pressure (Table 2). The numbers of nonvoiding contractions per micturition cycle during the filling phase were significantly ($P \leq 0.002$) increased at 10 and 50 $\mu$M PD90780 concentrations (Table 2). The duration of PD90780 effects for all concentrations examined for all cystometric parameters evaluated in control and CYP-treated rats was 30–40 min.

Effects of NGF-p75NTR Blockade on Volume Threshold in Rats with and without CYP-Induced Cystitis with Intravesical Instillation of PD90780 Using Isovolumetric Cystometry

Intravesical instillation of PD90780 (50 $\mu$M) significantly ($P \leq 0.01$) reduced the volume threshold to elicit a micturition contraction in control rats (no inflammation) in a closed urinary bladder system (Fig. 9, A, B, F). CYP treatment (48 h) reduced ($P \leq 0.01$) the volume threshold to elicit a micturition contraction compared with control rats (no inflammation; Fig. 9, C and F). Intravesical instillation of PD90780 (50 $\mu$M) further reduced the volume threshold in CYP-treated (48 h) rats (Fig. 9, D and F).

DISCUSSION

We recently demonstrated upregulation of p75NTR in dye-labeled lumbosacral bladder afferent cells in the DRG and in...
Table 2. Effects of intravesical infusion of PD90780 on cystometric parameters

<table>
<thead>
<tr>
<th>Intravesical Infusion</th>
<th>Threshold Pressure, cmH2O</th>
<th>Micturition Pressure, cmH2O</th>
<th>Baseline Pressure, cmH2O</th>
<th>NVCs per Micturition Cycle</th>
</tr>
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<tbody>
<tr>
<td>No CYP</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PS only</td>
<td>15.1 ± 0.9</td>
<td>38.5 ± 1.3</td>
<td>15.1 ± 0.9</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>10 μM PD90780</td>
<td>17.0 ± 1.3</td>
<td>47.1 ± 3.0*</td>
<td>18.4 ± 1.2*</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td>50 μM PD90780</td>
<td>19.4 ± 1.1*</td>
<td>47.1 ± 2.6*</td>
<td>21.5 ± 1.0*</td>
<td>1.6 ± 0.4</td>
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<tr>
<td>100 μM PD90780</td>
<td>19.1 ± 1.2</td>
<td>51.2 ± 2.1*</td>
<td>21.3 ± 1.4*</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>48 h CYP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS only</td>
<td>19.5 ± 0.7</td>
<td>46.7 ± 1.4</td>
<td>20.0 ± 0.8</td>
<td>0.2 ± 0.1</td>
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<tr>
<td>10 μM PD90780</td>
<td>25.1 ± 0.8*</td>
<td>57.8 ± 2.8*</td>
<td>24.3 ± 0.6*</td>
<td>1.1 ± 0.3*</td>
</tr>
<tr>
<td>50 μM PD90780</td>
<td>25.7 ± 0.8*</td>
<td>61.2 ± 2.5*</td>
<td>27.7 ± 1.3*</td>
<td>0.6 ± 0.2*</td>
</tr>
<tr>
<td>100 μM PD90780</td>
<td>24.0 ± 0.8*</td>
<td>56.1 ± 2.6*</td>
<td>24.3 ± 0.8*</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P ≤ 0.001 compared with PS (10 mg/ml) and saline infusion in control rats; n = 4–11 rats in each group. Effects of intravesical infusion of PD90780 on baseline pressure, threshold pressure, micturition pressure, and NVCs per micturition cycle are indicated. In control rats (no CYP treatment), intravesical infusion of PD90780 significantly increased micturition pressure and threshold pressure. In CYP-treated rats (48 h), PD90780 infusion significantly increased threshold pressure, micturition pressure, and NVCs.

Fig. 8. Summary histograms of the effects of intravesical infusion of PD90780 in CYP-treated (48 h) rats. Rats were treated with CYP 48 h before intravesical infusion of PD90780 and analysis with conscious cystometry. A: intravesical infusion of PD90780 (50 and 100 μM) significantly (*P ≤ 0.002) reduced intercontraction interval in rats treated with CYP (48 h). B: void volume in CYP-treated (48 h) rats was also significantly (*P ≤ 0.002) reduced at these same concentrations; n = 5–11 for all groups.

central micturition reflex pathways after CYP-induced cystitis (39). Few studies examined the regulation of p75NTR in rat urinary bladder after nerve injury or urinary bladder inflammation (23, 37, 65, 66). We demonstrated several novel findings with respect to the expression and regulation of p75NTR in urinary bladder with CYP-induced cystitis and the role of p75NTR in urinary bladder function in control (no inflammation) and CYP-treated rats. p75NTR is expressed throughout the urinary bladder and is present in nerve fibers of the detrusor smooth muscle, the suburothelial nerve plexus, urothelial cells, and nerve fibers associated with the suburothelial bladder vasculature. Total urinary bladder p75NTR expression increased after acute (4 h), intermediate (48 h), and chronic CYP-induced cystitis. p75NTR blockade via immunoneutralization and PD90780, known to block NGF-p75NTR interactions (14, 55), resulted in bladder hyperreflexia in control rats and further enhanced bladder hyperreflexia in CYP-treated (48 h) rats associated with decreased void volume, volume threshold, and ICI and an increased number of nonvoiding contractions for PD90780. These studies suggest that p75NTR interactions can affect overall bladder function. Cystometric effects of p75NTR blockade with an anti-p75NTR monoclonal antibody may be due to blockade of other neurotrophins in addition to NGF, BDNF, NT-3, and NT-4 may also bind p75NTR and are also present in urinary bladder, and expression is regulated by CYP-induced cystitis (64). However, the PD90780 compound, which specifically blocks NGF binding to p75NTR (14, 55), produced similar and broader cystometric changes. Thus, we suggest that the observed cystometric effects are attributable to blockade of p75NTR-NGF interactions.

NGF binds to two receptors, the p75NTR receptor, a member of the tumor necrosis factor family of receptors, and TrkA, a receptor tyrosine kinase (1, 3, 28). However, little information exists concerning the underlying interactions between neurotrophins, TrkA, and p75NTR receptors in mediating inflammatory-induced changes in micturition pathways. The majority of NGF-responsive cells express both p75NTR and TrkA (9) and TrkA is exclusively expressed with p75NTR in rat DRG (72). NGF selectively binds TrkA, whereas p75NTR can bind all neurotrophins with the same affinity (35). p75NTR also binds neurotrophin precursor molecules with high affinity, including proNGF (35). The ability of NGF to bind two very distinct receptors and the resulting signaling via this receptor complex has puzzled researchers (3). Much of the recent attention on p75NTR has been focused on its role in apoptosis and neuronal growth inhibition. However, the original function attributed to p75NTR was as an accessory protein that modulates Trk receptor affinity for their appropriate ligand (3, 10). The coexpression of TrkA and p75NTR in heterologous cells results in high-affinity NGF binding sites (4, 62). Thus, it has become a prevailing concept that p75NTR and TrkA combine to form a receptor complex that results in high-affinity NGF...
binding (10). However, a recent study (70) provides structural and mechanistic evidence for a ligand-passing model in which NGF rapidly associates with p75NTR and then is presented to TrkA (70). In the model presented, blocking NGF binding to p75NTR attenuates NGF binding to TrkA and subsequent TrkA activation (70). It is also suggested that convergent signaling pathways activated by p75NTR and TrkA could underlie the activation (70). It is well documented that NGF is important in a number of inflammatory conditions including urinary bladder, colon, and lung inflammation (18, 26, 56, 64). The exact contribution of NGF to bladder function is not known but a role for NGF in bladder hyperreflexia and bladder overactivity has been suggested (12, 13, 20, 30, 34, 48, 73). NGF or TrkA sequestration methods and Trk inhibitors reduce bladder hyperreflexia in rats with experimentally induced inflammation and improve animal well being (20, 34) (Vizzard et al., unpublished observations). Elevated levels of neurotrophins are detected in the urine of women with PBS/IC (49) or in the urothelium of individuals with neuropathic bladder (43). However, a recent study failed to demonstrate an association between increased urothelium/suburothelium NGF with detrusor overactivity or bladder sensation (6). More recently, it has been demonstrated that urinary NGF levels are increased in overactive bladder (OAB), urinary incontinence, and decreased in patients responding to botulinum toxin-A treatment (41, 42). Thus, NGF may be a potential biomarker for OAB.

Blockade of the NGF-TrkA pathway in respiratory inflammation models with k252a (45) or NGF blocking antibodies (30) decreased recruitment of inflammatory cells and bronchial hyperresponsiveness, respectively. Immunoneutralization of p75NTR in adult rat sensory neurons inhibits upregulation of substance P induced by NGF application (54). Studies using p75NTR knockout (KO) mice or immunoneutralization of p75NTR reduced bronchial hyperresponsiveness and substance P release in the airway (24, 38, 59). In contrast, the present studies demonstrate bladder hyperreflexia or enhanced hyperreflexia in control and CYP-treated rats following intravesical blockade of p75NTR by two different approaches. Immunoneutralization of p75NTR via intravesical instillation of an anti-p75NTR monoclonal antibody increased urinary bladder frequency (decreased ICI, decreased void volume) in both control (no inflammation) and CYP-treated rats, whereas intravesical pressures (baseline, micturition, threshold) were not affected. To confirm that the observed changes in bladder function were not associated with nonspecific actions of immunoglobulin, intravesical instillation of isotype-matched IgG was infused and was without effect on any cystometric parameter evaluated. Intravesical infusion of PD90780, known to block NGF-p75NTR interactions (14, 55), produced similar but also additional changes in the cystometric parameters evaluated. PD90780 increased voiding frequency (decreased ICI, decreased void volume) in control and CYP-treated rats, but also altered threshold, baseline, and micturition pressures; vehicle infusions were without effect. In continuous cystometry recording, voided volume and ICI may be affected by increased residual urine volume due to decreased voiding efficiency and changes in urethral outlet resistance. In a closed urinary bladder system, PD90780 significantly decreased the volume threshold to elicit a micturition contraction in both control (no inflammation) and CYP-treated (48 h) rats. Although the present studies cannot rule out the possibility of NGF-p75NTR actions at the urethra, the isovolumetric studies clearly demonstrate NGF-p75NTR actions at the level of the urinary bladder.

Intravesical infusion of PD90780 produced some differential effects between control (no inflammation) and CYP-treated rats in contrast to the effects produced by immunoneutralization of p75NTR that were similar in control and CYP-treated rats. In control rats treated with intravesical instillation of PD90780, there was evidence of incomplete voiding as rats exhibited small void volumes compared with infused volume. This mismatch between void and infused volumes was not observed in CYP-treated rats infused with PD90780 or in
control or CYP-treated rats infused with anti-p75NTR monoclonal antibody. This mismatch between void volume and infused volume may suggest PD90780 effects on the urethral outlet. Given the ubiquitous expression of p75NTR throughout the urinary bladder demonstrated in the present studies and the peripheral and central nervous system in general (27, 28, 65, 66), it would not be surprising that the urethra expressed p75NTR. Why the potential effects of PD90780 on the urethra are exhibited in control but not CYP-treated rats may be related to p75NTR and NGF expression in the urinary bladder with CYP-induced cystitis. Previous and present studies demonstrated significant increases in total p75NTR and NGF bladder expression with CYP-induced cystitis (34, 39, 64). In control rats with less p75NTR and NGF expression in the urinary bladder, infusion of PD90790 may exert bladder and urethral effects because the drug may be more available and have broader effects and affect NGF-p75NTR actions at the urethra. In contrast, with CYP-induced cystitis and increased p75NTR and NGF expression in the urinary bladder, effects of PD90780 infusion may be less available and restricted to the urinary bladder. In the future, urethral pull-through studies (68) or urethrometry (53, 60) studies may be designed to determine potential NGF-p75NTR actions at the level of the urethra. These future studies will necessitate changes in route of drug application from intravesical to systemic or intrathecal administration and the use of anesthetized rodents.

These studies demonstrate widespread p75NTR expression in many cell types in the urinary bladder and increased total p75NTR expression in the urinary bladder with CYP-induced cystitis. This makes it difficult to determine the exact site(s) of action in the urinary bladder of the p75NTR antibody. We do know that intravesical infusion of anti-p75NTR monoclonal antibody without infusion of protamine sulfate showed no cystometric effects. This likely indicates a site of p75NTR action deep to the urothelium, since protamine sulfate is used to disrupt cell-to-cell contact of urothelial cells (11, 57). Our demonstration of p75NTR-IR after intravesical infusion of anti-p75NTR supports this suggestion as p75NTR-IR is present in the lamina propria. Potential targets include the suburothelial nerve plexus, interstitial cells of Cajal, and myofibroblasts all distributed beneath the urothelium (2) as well as inflammatory nerve plexus, interstitial cells of Cajal, and myofibroblasts all distributed beneath the urothelium (2) as well as inflammatory cell infiltrates in bladders from CYP-treated rats and nerve fibers innervating the detrusor smooth muscle.

p75NTR receptor blockade at the level of the urinary bladder produced changes in ICI, void volume, changes in threshold and micturition pressure, and increases in the number of nonvoiding contractions during the filling phase. Changes in ICI and volume threshold to initiate micturition and changes in threshold pressure are suggestive of changes in the afferent limb of the micturition reflex. Changes in micturition pressure are thought to be dependent on the efficiency of neurotransmission of bladder efferents (44) or changes in the urethral outlet with continuous cystometry. Changes in nonvoiding contractions observed with PD90780 infusions in CYP-treated rats may have both myogenic and neurogenic components (29, 32, 58). For example, myogenic contractions could trigger afferent firing which then evokes a reflex efferent discharge that amplifies the myogenic contraction. Effects of intravesical anti-p75NTR infusion were limited to ICI and void volume without effects on intravesical pressures suggesting that the afferent limb of the micturition reflex is targeted. Greater concentrations of anti-p75NTR were not evaluated in the present study so it is not known whether additional cystometric changes would have emerged suggestive of effects on the efferent limb of the micturition reflex. In contrast, effects of PD90780 on ICI, volume threshold, void volume, and intravesical pressures suggest that both the afferent and efferent limbs of the micturition reflex are affected. In addition, changes in micturition pressures (e.g., baseline and peak micturition pressure) with continuous cystometry may suggest changes in the urethral outlet as a result of intravesical drug administration.

The present experiments show that p75NTR blockade at the level of the urinary bladder increases voiding frequency in control rats and further increases voiding frequency in CYP-treated rats. Thus, one function of p75NTR and NGF-p75NTR interactions in vivo may be to reduce bladder activity or to offset bladder hyperreflexia induced by CYP-induced cystitis. Whereas it is known that p75NTR expression can enhance TrkA-NGF binding in some systems (1, 9), p75NTR may also reduce NGF-TrkA signaling by sequestering NGF (19, 31, 46). In the latter instance, any decrease or perturbation in NGF/p75NTR binding may tip the homeostatic equilibrium, resulting in increased NGF bioavailability to enhance NGF/TrkA signaling. If NGF/TrkA signaling is favored, increased expression or function of ion channels such as the transient receptor potential (TRP) channels (e.g., TRPV1) and neuropeptides may contribute to urinary bladder hyperreflexia. A role for both TRPV1 expression in bladder afferents and urothelial cells in bladder hyperreflexia has been suggested (5). In addition, increased expression of TRPV1 in bladder nerves and urothelium has been demonstrated in neurogenic detrusor overactivity (5). Our previous studies demonstrated increased expression of substance P (63), calcitonin gene-related peptide (63), and pituitary adenylate cyclase activating polypeptide (PACAP) (7) in lower urinary tract tissues with CYP-induced cystitis. Furthermore, intravesical and intrathecal blockade of the PACAP receptor, PAC1, reduced bladder hyperreflexia induced by CYP treatment (7). These downstream regulatory events can be examined in TRPV1 null or PACAP null mice with p75NTR blockade. Conversely, if NGF/p75NTR signaling is favored, NGF/TrkA signaling in bladder function may be dampened. However, as p75NTR can partner not only with other Trk receptors but also with a large number of other membrane-associated proteins (4), such as TROY and sortilin, there may be multiple mechanistic intersections, the summation of which will determine bladder reflex responses.

Conclusions

The present studies suggest that one role of p75NTR and NGF-p75NTR interactions in vivo may be to reduce bladder activity or to offset bladder hyperreflexia induced by urinary bladder inflammation. Although perhaps less clinically relevant than OAB, detrusor overactivity, or bladder hyperreflexia, detrusor underactivity is a clinical problem that is seen most often in men, although women are also affected (16, 17). It consists of reduced muscle contraction, strength, or velocity and often results in incomplete voiding, postvoid residual, prolonged emptying, and reduced free uroflow (16, 17). Detrusor underactivity can be neurogenic, idiopathic, or a result of bladder outflow obstruction (16, 17). Whether blockade of
p75<sub>NTR</sub> receptors in this context would be beneficial or whether p75<sub>NTR</sub> receptors are regulated by detrusor underactivity awaits further study.

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