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Evidence for the role of mast cells in colon–bladder cross organ sensitization

Jocelyn J. Fitzgerald^{a,*}, Elena Ustinova^{a,b}, Kevin B. Koronowski^a, William C. de Groat^a, and Michael A. Pezzone^a

^aDepartment of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, 200 Lothrop Street, W1340 Biomedical Science Tower, Pittsburgh, PA 15260, USA

^bDepartment of Medicine, University of Pittsburgh School of Medicine, 200 Lothrop Street, W1340 Biomedical Science Tower, Pittsburgh, PA 15260, USA

Abstract

This study examined the contribution of mast cells to colon–bladder cross organ sensitization induced by colon irritation with trinitrobenzene sulfonic acid (TNBS-CI). In urethane anesthetized rats 12 days after TNBS-CI, the voiding interval was reduced from 357 s to 201 s and urothelial permeability, measured indirectly by absorption of sodium fluorescein from the bladder lumen, increased six-fold. These effects were blocked by oral administration of ketotifen (10 mg/kg, for 5 days), a mast cell stabilizing agent. TNBS-CI in wild type mice produced a similar decrease in voiding interval (from 319 s to 209 s) and a 10-fold increase in urothelial permeability; however this did not occur in Kit^{aW}/Kit^{aW-v} mast cell deficient mice. Contractile responses of bladder strips elicited by Compound 48/80 (50 µg/ml), a mast cell activating agent, were significantly larger in strips from rats with TNBS-CI (145% increase in baseline tension) than in control rats (55% increase). The contractions of strips from rats with TNBS-CI were reduced 80–90% by pretreatment of strips with ketotifen (20 µM), whereas contractions of strips from control animals were not significantly changed. Bladder strips were pretreated with SLIGRL-NH₂ (100 µM) to desensitize PAR-2, the receptor for mast cell tryptase. SLIGRL-NH₂ pretreatment reduced by 60–80% the 48/80 induced contractions in strips from rats with TNBS-CI but did not alter the contractions in strips from control rats. These data indicate that bladder mast cells contribute to the bladder dysfunction following colon–bladder cross-sensitization.

Keywords

Bladder; Bowel; Mast cell; PAR-2; Cross-sensitization; Trinitrobenzene sulfonic acid; Compound 48/80; Afferent nerves

1. Introduction

Women experiencing chronic pelvic pain often exhibit overlapping symptoms of interstitial cystitis (IC) and irritable bowel syndrome (IBS) (Mathias et al., 1996). Much of this overlap is attributable to central and peripheral neural mechanisms of pelvic organ cross-sensitization (Malykhina, 2007). Specifically, the bladder and colon receive part of their afferent innervations from the same subpopulation of lumbosacral dorsal root ganglion

Conflict of interest

The authors declare that they have no conflict of interest.

(DRG) cells (Christianson et al., 2007), an anatomical organization that could allow afferent activity arising in one organ to be transmitted by antidromic action potentials to the other co-innervated organ. In addition, afferent activity from both organs carried by the shared DRG neurons should be received by a common population of second order neurons in the spinal cord. Thus, activation of nociceptive afferents in one organ could sensitize central sensory pathways or induce firing in peripheral afferent pathways in another organ to release afferent neurotransmitters such as substance P that trigger neurogenic inflammation (Pan et al., 2010). These mechanisms could lead to recruitment and activation of mast cells, release of inflammatory mediators, plasma extravasation and smooth muscle contraction.

The juxtaposition of substance P positive nerve fibers and mast cells (Bauer and Razin, 2000) may play an important role in pelvic organ cross-sensitization by amplifying afferent signaling. Intravenous administration of Compound 48/80, a mast cell activating and degranulating agent, which depletes mast cells of their inflammatory mediators, significantly attenuated and/or abolished ovalbumin-induced maximal bladder contractile response and bladder plasma protein extravasation in a rat model of bladder overactivity induced by sensitization with ovalbumin (Ahluwalia et al., 1998). Furthermore, mast cell deficient mice exhibit reduced pelvic pain behavior and bladder permeability changes to noxious bladder stimuli (Bauer and Razin, 2000) and do not develop pseudorabies virus induced inflammation of the bladder (Jasmin et al., 2000). Our previous studies in rats with bladder overactivity following trinitrobenzene sulfonic acid-colon irritation (TNBS-CI) revealed increased numbers of mast cells in the bladder (Ustinova et al., 2007) and that colon-bladder cross-sensitization is dependent upon capsaicin-sensitive C-fiber afferents (Ustinova et al., 2006; Christianson et al., 2007; Ustinova et al., 2007).

The goal of the present study was to further elucidate some of the major contributors to colon-bladder cross-sensitization, focusing on the role of the mast cell and its downstream targets. Specifically, we determined if mast cells are an essential link in the pathway for colon-bladder cross-sensitization by the treating the animals with ketotifen fumarate, a mast cell stabilizing agent, after TNBS-CI. We subsequently examined bladder function, including voiding interval and urothelial permeability as measures of end-organ change. In addition, we tested the mast cell activating agent, Compound 48/80, on in vitro smooth muscle strip preparations to determine if the responses to mast cell mediators were altered in the model. Finally, we further examined the role of mast cells in cross-sensitization by inducing TNBS-CI in the Kit^W/Kit^{W-v} mast cell-deficient mice.

2. Materials and methods

2.1. Animal model

Virgin, female Sprague Dawley rats (200–250 g, n=66) purchased from Hilltop Labs (Scotdale, PA) were housed in polypropylene cages with ad-libitum access to food and water in the University of Pittsburgh animal facility. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. We also studied Kit^W/Kit^{W-v} mast cell-deficient mice (n=42) and control wild-type (WT, Kit^{+/+}) mice (n=34) purchased from Jackson Labs (Bar Harbor, Maine) as complete deletion of the c-kit gene to eliminate mast cells is a lethal mutation in rats (Waskow et al., 2004). All experimental procedures requiring anesthesia were done under isoflurane unless otherwise stated. Animals were euthanized using CO₂ asphyxiation, followed by cervical dislocation.

2.2. Trinitrobenzenesulfonic acid (TNBS) colitis model of cross-sensitization (CS)

The protocol for the subacute colon inflammation and subsequent changes in the physiologic parameters of bladder function in the TNBS-CI model have been well described in previous

publications for both the rat (Ustinova et al., 2007) and mouse models (Lamb et al., 2006). The methods were the same in rats and in mast cell-deficient Kit^W/Kit^{W-v} mice (Tsai et al., 2002) and their wild type littermates. Under isoflurane anesthesia, 50 mg/ml TNBS dissolved in 50% ethanol was administered transanally at a volume of 0.4 ml with a catheter placed 6 cm and 4 cm from the anal verge in rats and mice, respectively. The perineum, vagina, and urethra were protected with surgical lubricant while the animal remained supine to protect from TNBS leakage. Control animals in all experiments were given a saline enema vehicle of equivalent volume. Animals were then housed for 7–12 days to fully develop colitis, which has been previously defined functionally and histologically (Ustinova et al., 2007).

2.3. Ketotifen fumarate administration

On day 7 after instillation of TNBS into the colon when maximal bladder mastocytosis occurs (Ustinova et al., 2007), ketotifen fumarate salt (Sigma, St. Louis, MO), a mast cell membrane stabilizer was administered at a dose of 10 mg/kg/day via drinking water at a concentration of 0.1 mg/ml for 5 consecutive days, taking into account that, on average, a 200 g rat drinks 30 ml of water per day. Water intake in each rat was monitored to determine if variation in drinking had an influence on drug effect, though all animals drank $\pm 10\%$ of the average volume. The dose of ketotifen was selected based on efficacy in comparable studies (Serna et al., 2006). Cystometric parameters and urothelial permeability were measured after 12 days and compared to measurements in intracolonic saline vehicle controls.

2.4. Mast cell histologic quantification

We also validated the development of bladder mastocytosis in the TNBS-CI model using histological methods described in our earlier studies (Ustinova et al., 2007). The urinary bladders of four groups of animals (control, ketotifen alone, 12 days of TNBS-CI, 12 days of TNBS-CI treated from day 7 to day 12 with ketotifen) were excised and dissected longitudinally along their midline. Both halves were embedded in OCT medium and frozen. Using a cryostat (Microm HM 505 E, Mikron Instruments, San Marcos, CA), multiple sections were obtained at a thickness of 5 μm . Serial sections were mounted and stained with hematoxylin/eosin and Giemsa stains. Mast cells were counted independently by two investigators under a 1000 $\times$ -power field as previously described (Spanos et al., 1997). Ten to twenty fields per bladder section were examined from each animal ($n=4$ per group), and the average number of mast cells per field was calculated.

2.5. Cystometry

On day 12 after the induction of TNBS-CI, cystometry was performed in urethane-anesthetized (1.2 g/kg sc, Sigma, St. Louis, MO) rats as previously described (Ustinova et al., 2006). Room-temperature saline was infused into the bladder constantly using PE-20 tubing (Fisher Scientific, Hanover Park, IL) at a rate of 0.05 ml/min during continuous open cystometry. In urethane anesthetized (1.2 g/kg sc) mice the bladder was constantly infused (25 $\mu\text{l}/\text{min}$) with saline using PE-10 tubing. A Transbridge transducer amplifier (World Precision Instruments, Sarasota, FL) was used to amplify the signal from the pressure transducer, which was processed using the PowerLab 8 unit data acquisition system. After an initial equilibration period multiple cystometrograms ($n=4-6$) were performed to determine the average voiding interval in each animal.

2.6. Urothelial permeability

To determine if urothelial permeability was increased as a result of cross-sensitization, absorption of sodium fluorescein, a well-documented methodology for assessing biological barrier function (Natah et al., 2009) was used in rats as well as Kit^W/Kit^{W-v} and Kit^{+/+} mice.

Briefly, sodium fluorescein was infused into the bladder, and then blood levels were measured to estimate absorption and in turn urothelial permeability. On day 12 after TNBS-CI, the bladder was emptied, the urethra was clamped and then the bladder was refilled for 15 min with sodium fluorescein (10 mg/ml in Sorenson buffer, pH 7.4) (0.5 ml in rats and 0.1 ml in mice). After 15 min, a blood sample (1 ml in rats and 0.1 ml in mice) was collected from the jugular vein. Blood samples (1 ml) were centrifuged (3000 rpm/5 min) and deglucuronidated by incubating 100 μ l serum aliquots in 200 μ l of 0.2 M acetate buffer (pH 5.1) and 50 μ l of glucurase (5000 U/ml) for 30 min at 37 ° C. Samples were adjusted to 2 ml with 0.2 M carbonate buffer (pH 9.15) and fluorescein levels in the blood were measured spectrofluorometrically (emission 494 nm and excitation 516 nm) using known standards.

2.7. Bladder strip experiments

In the 7–12 day time period after TNBS-CI, the bladders were removed from rats under isoflurane (4% in O₂) anesthesia, and bladder strips were prepared as described previously (Artim et al., 2009). Briefly, the bladder was placed in warm Krebs solution (composition in mM: NaCl 118, KCl 4.7, CaCl₂ 1.9, MgSO₄ 1.2, NaHCO₃ 24.9, KH₂PO₄ 1.2, dextrose 11.7, pH 7.4 bubbled with 95% O₂, 5% CO₂) and cut into four longitudinal strips (~1.5 mm×8–10 mm). Strips were tied with a fine thread at each end, mounted in a vertical double-jacketed organ bath in oxygenated Krebs solution (15 ml volume) and kept at 37 ° C via a circulating warm water bath. The tissue was stretched one time at the beginning of the experiment to achieve a uniform baseline tension of 1 g and allowed to equilibrate for 1–2 h prior to drug testing (Kullmann et al., 2011). Amplitude of phasic contractions was measured for a 3 min period before and for two 3 min periods at 3 and 15 min after the application of 50 μ g/ml of Compound 48/80 (Sigma), a mast cell activating agent. In this paper the term phasic contraction refers to transient (less than 30 s duration) increases (greater than or equal to 0.1 g) in strip tension. Bladder activity was measured during the maximal effect of the drug; amplitude during that period was measured from the minimum to the maximum amplitude of the spontaneous contractions. Data were recorded and analyzed using Windaq software (DATAQ Instruments, Inc. Akron, OH). The concentrations of Compound 48/80 were selected based on previous studies (Bytautiene et al., 2004; Paterson et al., 2007), which reported a maximal contractile response of esophageal and uterine tissues at 100 μ g/ml, which we also found to elicit a maximal response in the bladder. 50 μ g/ml was chosen as a sub-maximal concentration so that a possible enhancement by TNBS treatment would be detectable.

To determine if the contractile responses to Compound 48/80 were elicited by activation of mast cells, responses to Compound 48/80 were also recorded in the presence of ketotifen fumarate salt, a mast cell stabilizer. To determine if contractile responses to mast cell activation involved the protease activated receptor-2 (PAR-2), 100 μ M of the PAR-2 peptide agonist, SLIGRL-NH₂ (synthesized by University of Pittsburgh Peptide Synthesis Facility) was applied in some experiments for 30 min to desensitize the receptor prior to the administration of 50 μ g/ml of Compound 48/80 (Soh et al., 2010). Preliminary experiments showed that this concentration of SLIGRL-NH₂ elicited contractions of the bladder strips as reported by other investigators (Nakahara et al., 2004) and that the first application of SLIGRL-NH₂ produced a marked reduction in the response to a second application after a 30 min interval.

2.8. Drugs

Compound 48/80 (Sigma), a mast cell activating agent, is a multimer of C₁₁H₁₅NO. Ketotifen fumarate (Sigma), C₁₉H₁₉NOS·C₄H₄O₄, is a mast cell stabilizing agent. SLIGRL-NH₂, a PAR-2 activating peptide, was synthesized by the University of Pittsburgh Peptide Synthesis facility and has been well described (Hollenberg et al., 1997).

2.9. Statistical analysis

Reported values throughout the text are presented as mean $\pm$ S.E.M. Data were analyzed in Graph Pad Prism (La Jolla, CA). One-way ANOVA was used to analyze data from experiments with multiple comparisons between treatments but only one measured endpoint, i.e. the measurement of cystometric voiding interval in four different treatment groups (vehicle control, TNBS-CI, ketotifen treated vehicle control and ketotifen treated TNBS-CI). One-way ANOVA was followed by a Dunn's multiple comparison test to determine significance between the groups. Two-way ANOVA was used to analyze data from the bladder strip experiments in which responses to a particular drug in the four different treatment groups were measured at two different time points (3 and 15 min). The two-way ANOVA was followed by a Bonferroni post-test. Data were considered significant if $p < 0.05$.

3. Results

3.1. Effect of oral ketotifen on mast cell numbers in bladders of TNBS-CI

Using our previously published methods, we confirmed that mast cell numbers in the bladders of rats 12 days after TNBS-CI increased seven-fold over the numbers in control rats (one way ANOVA, $p < 0.05$, Fig. 1) (Ustinova et al., 2007). The histological analysis of 69 microscopic fields in $n=4$ rats with TNBS-CI treated with ketotifen (10 mg/kg, p.o.) for 5 days starting on day 7 also showed significant bladder mastocytosis compared to control ($p < 0.05$, Fig. 1) and was not different from the TNBS-CI group ($p > 0.05$, average 13.6 cells/20 $\times$ field compared to 15 cells/20 $\times$ field in $n=4$ untreated rats with TNBS-CI).

3.2. Effect of oral ketotifen treatment on voiding interval in TNBS-CI

Cystometry was performed on four groups of urethane anesthetized rats ($n=3$ per group): (1) intracolonic saline control, (2) saline control with ketotifen treatment, and (3) TNBS-CI, (4) TNBS-CI with ketotifen treatment. Voiding intervals, which averaged 357 ± 39 s in controls, decreased to 201 ± 63 s in rats with TNBS-CI (one way ANOVA, $p < 0.05$). In the group of rats with TNBS-CI treated with ketotifen, voiding interval was 340 ± 82 s, which was not different from controls ($p > 0.05$, Fig. 2). Ketotifen treatment did not change baseline voiding intervals in control rats (358 ± 58 s, $p > 0.05$).

3.3. Effect of oral ketotifen treatment on urothelial permeability in TNBS-CI

Five groups of rats ($n=4$ per group): (1) intracolonic saline control, (2) intracolonic saline control with ketotifen treatment 10 mg/kg on days 7–12 (3) TNBS-CI, (4) TNBS-CI with ketotifen treatment 10 mg/kg on days 7–12 and (5) TNBS-CI with ketotifen treatment 10 mg/kg on days 5–12, were evaluated for changes in urothelial permeability. Control animals had an average value of 1.87 ± 0.62 μ g/ml sodium fluorescein detectable in their plasma, and animals with TNBS-CI showed a six-fold increase (one way ANOVA, $p < 0.05$) in plasma sodium fluorescein to 11.11 ± 0.67 μ g/ml (Fig. 3). Administration of ketotifen for 5 days starting at day 7 after TNBS-CI reduced the plasma sodium fluorescein concentration to 1.97 ± 1.34 μ g/ml, a value not significantly different than that after treatment with ketotifen alone (3.0 ± 0.34 μ g/ml, $p > 0.05$). Administering ketotifen for 7 days starting at day 5 after TNBS-CI also normalized (3.25 ± 0.46 μ g/ml) the plasma sodium fluorescein concentration (Fig. 3).

3.4. Enhancement of the responses of bladder smooth muscle strips to Compound 48/80 after TNBS-CI

Bladder smooth muscle strips from control rats ($n=9$ strips from 8 animals) exhibited low amplitude phasic activity (0.67 ± 0.08 g, Fig. 4a), while strips from rats with TNBS-CI ($n=9$

strips from 8 animals) exhibited significantly larger amplitude phasic activity ($1.06 \pm .15$ g, one way ANOVA, $p < 0.05$, Fig. 4b). Compound 48/80 (50 $\mu\text{g/ml}$), a mast cell activator, elicited contractions of bladder smooth muscle strips from control rats and rats with TNBS-CI evident as an increase in baseline tone and/or an increase in phasic contractions (Fig. 4). In contrast to the rapid onset contractions produced by carbachol or KCl, the maximal response of a bladder strip to Compound 48/80 was delayed and reached a maximum between 8 and 15 min after application (Figs. 4 and 5). Due to the variability of basal contractile activity between preparations, the calculation of percent change from baseline before drug to responses at 3 and 15 min after drug was used to normalize data between preparations. As shown in Figs. 4a, b, and 5, the amplitude of phasic contractions in response to Compound 48/80 in the TNBS-CI rat bladder increased 56% at 3 min (compared to 8% in control, two way ANOVA, $p < 0.05$) and 145% at 15 min (compared to 47% in control, $p < 0.05$). These changes were reversible with washout over a period of approximately 1 h. A second application of Compound 48/80 after full recovery elicited smaller maximal responses.

3.5. Effect of ketotifen on the responses of bladder smooth muscle strips to Compound 48/80

Bladder strips from either control or TNBS-CI rats ($n=8$ and $n=7$ strips, from 2 and 3 animals respectively) were pretreated with ketotifen fumarate for 30 min before applying Compound 48/80 to determine if mast cells were involved in the excitatory effect of Compound 48/80. Previous studies (Schoch, 2003) showed that ketotifen at concentrations ranging from 10^{-10} to 10^{-4} M reduced the mast cell release of tryptase by 90% or more, and that the clinically effective concentration is 10^{-4} M. Our pilot study, which was designed to evaluate the ketotifen concentration–response curve and to determine an appropriate concentration that could be used to selectively block contractile activity induced by the release of mediators from mast cells, revealed that concentrations 18 μM in strips from control animals did not alter the spontaneous phasic contractions of the bladder or the contractile response to KCl (50 mM). In strips from control animals, these concentrations also did not significantly reduce the maximal excitatory response to 50 $\mu\text{g/ml}$ of Compound 48/80 at 15 min after application. However higher concentrations (25 μM) reduced the responses.

In strips from rats with TNBS-CI, a concentration–response study revealed that 20 μM ketotifen was the highest concentration that did not change spontaneous contractions or affect the KCl response. However this concentration of ketotifen significantly suppressed the responses to Compound 48/80 (50 $\mu\text{g/ml}$). Ketotifen reduced the effect of Compound 48/80 measured at 3 and 15 min after drug application from a 67% to 5% increase (two way ANOVA, $p < 0.05$) and from a 172% to 14% increase ($p < 0.05$), respectively (Figs. 4c and 5). In strips from control rats, this concentration of ketotifen did not change the response to Compound 48/80 ($p > 0.05$, Fig. 5).

3.6. Effect of SLIGRL-NH₂, a PAR-2 agonist, on the responses of bladder smooth muscle strips to Compound 48/80

The experiments with Compound 48/80 and ketotifen suggested that factors released from mast cells, such as mast cell tryptase, which acts on a G-protein coupled receptor (PAR-2), have a role in cross-sensitization. This mechanism could be tested by determining if the enhanced responses to Compound 48/80 in strips from rats with TNBS-CI are blocked by a PAR-2 antagonist. Unfortunately, PAR-2 antagonists are not available and therefore we have used prolonged exposure of bladder strips to a PAR-2 agonist, SLIGRL-NH₂, which is known to mimic the effects of mast cell tryptase (Nakahara et al., 2004). Repeated application of SLIGRL-NH₂ desensitizes PAR-2 (Soh et al., 2010) and reduces responses of

smooth muscle to activation of mast cells. SLIGRL-NH₂ elicits contractions in normal bladder strips (Nakahara et al., 2004) and in our study, at a concentration of 100 μ M, elicited transient contractions of strips from rats with TNBS-CI. The SLIGRL-NH₂ effect returned to basal phasic activity prior to the application of Compound 48/80 and was not different from control phasic activity 30 min after application (one way ANOVA, $p>0.05$). A second application of SLIGRL-NH₂ to the same strip elicited a very small response suggesting desensitization. SLIGRL-NH₂ did not change the effect of Compound 48/80 in strips from control rats ($n=4$ strips from 3 animals) at 3 or 15 min after application ($p>0.05$). However, in strips from TNBS-CI rats ($n=8$ strips from 6 animals), SLIGRL-NH₂ (100 μ M) pretreatment for 30 min reduced the effect of Compound 48/80 from a 145% to 56% increase at 15 min after drug application but did not reduce the effect at 3 min after application (two way ANOVA, $p<0.05$, Figs. 4d and 5).

3.7. Kit^W/Kit^{W-v} mouse cystometry

Cystometry was performed in four groups of mice ($n=5$ per group): (1) untreated Kit^{+/+}, (2) untreated Kit^W/Kit^{W-v}, (3) Kit^{+/+}+TNBS-CI and (4) Kit^W/Kit^{W-v}+TNBS-CI. Voiding intervals in untreated Kit^{+/+} animals (309 ± 18 s) were not different from untreated Kit^W/Kit^{W-v} (312 ± 9 s, one way ANOVA, $p>0.05$, Fig. 6). However TNBS-CI treatment in Kit^{+/+} mice significantly (one way ANOVA, $p<0.05$) decreased voiding interval to 209 ± 16 s but did not alter the voiding interval (319 ± 24 s) in Kit^W/Kit^{W-v} mice.

3.8. Kit^W/Kit^{W-v} mouse urothelial permeability

The same method described above for the studies in rats was used to measure urothelial permeability in four groups of mice: (1) untreated Kit^{+/+} ($n=5$), (2) untreated Kit^W/Kit^{W-v} ($n=3$), (3) Kit^{+/+}+TNBS-CI ($n=4$) and (4) Kit^W/Kit^{W-v}+TNBS-CI ($n=4$). Sodium fluorescein solution (0.1 ml, 10 mg/ml) was infused into the bladder and after 15 min blood samples were drawn to measure plasma concentration of the tracer. The plasma concentrations of sodium fluorescein were not significantly different in untreated Kit^{+/+} and Kit^W/Kit^{W-v} mice (one way ANOVA, $p>0.05$, Fig. 7). However TNBS-CI produced an almost ten-fold increase in the plasma concentration in Kit^{+/+} mice ($p<0.05$) but did not change the concentration in the untreated Kit^W/Kit^{W-v} mice indicating that the increase in urothelial permeability induced by colon-bladder cross-sensitization was blocked in mast cell deficient mice.

4. Discussion

The present studies provided evidence that mast cells have an important role in the generation of bladder overactivity (cross-sensitization) following TNBS-induced colonic irritation. TNBS-CI-treated rats exhibited increased voiding frequency, increased urothelial permeability and increased numbers of mast cells in the bladder. Oral administration of ketotifen, a mast cell stabilizing agent, normalized bladder capacity and bladder permeability in TNBS-CI rats but did not alter these parameters in normal rats. In mice, TNBS-CI elicited a similar increase in bladder activity and permeability in wild type animals, but not in mast cell deficient animals. TNBS treatment also increased contractile responses of rat bladder strips to Compound 48/80, a mast cell activating agent. The effects of Compound 48/80 were reduced by ketotifen or by desensitization of PAR-2 with prolonged administration of SLIGRL-NH₂, a PAR-2 agonist. These results indicate that mast cells in the bladder contribute to bladder dysfunction in the TNBS-CI model (Fig. 8).

The location of mast cells in close proximity to afferent nerve endings has prompted the speculation that bidirectional chemical communication occurs between these two types of cells (Fig. 8) (Purcell and Atterwill, 1995; He, 2004). Previous studies showed that systemic

cap-saicin pretreatment, which depletes afferent neuropeptide stores or destroys capsaicin-sensitive C-fibers, prevents the bladder mastocytosis associated with TNBS-CI (Ustinova et al., 2007) suggesting that chemical signals from afferents influence the recruitment or proliferation of mast cells. Conversely, mast cells release chemicals that increase afferent excitability (Bauer and Razin, 2000).

Stimulation or sensitization of bladder afferents directly or via cross-sensitization can elicit a decrease in bladder capacity and/or induce bladder overactivity during filling (de Groat and Yoshimura, 2009; Asfaw et al., 2011). This commonly occurs following intravesical administration of various agents that activate nociceptive C-fiber bladder afferents and was clearly evident during cystometry in both rats and wild type mice after TNBS-CI. Ketotifen significantly reversed the decrease in voiding interval in rats with TNBS-CI but did not affect the voiding interval in untreated rats (Fig. 2). These data suggest that mast cells are activated to release mediators, which in turn modulate afferent firing and voiding in rats with TNBS-CI but not in normal rats.

Co-activation of mast cells and C-fiber afferent nerves commonly occurs during neurogenic inflammation (Barnes, 1991; Purcell and Atterwill, 1995). To determine if inflammation in the TNBS-CI model was dependent on mast cell activation, we evaluated urothelial permeability by measuring the plasma concentration of sodium fluorescein after intravesical administration in normal rats and rats with TNBS-CI. Rats with TNBS-CI had a significantly increased bladder permeability, which was reversed by oral ketotifen (Fig. 3), indicating a role for either enhanced release or actions of mast cell mediators. The downstream targets of the mediators could be the urothelium or afferent nerves that in turn release neuropeptides that target the urothelium (Birder, 2011) (Fig. 8).

To further validate the role of mast cells in the functional bladder changes in the TNBS-CI model, we studied the $\text{Kit}^W/\text{Kit}^{W-v}$ mouse, a mast-cell deficient animal. Compared to the prominent sensitivity of the bladder in their $\text{Kit}^{+/+}$ littermates to TNBS-CI, the untreated $\text{Kit}^W/\text{Kit}^{W-v}$ mouse did not exhibit the decrease in voiding interval after TNBS-CI (Fig. 6). In these experiments the $\text{Kit}^{+/+}$, untreated $\text{Kit}^W/\text{Kit}^{W-v}$ and $\text{Kit}^W/\text{Kit}^{W-v}$ with TNBS-CI mice all had normal voiding intervals, whereas the $\text{Kit}^{+/+}$ mice with TNBS-CI had a significantly reduced voiding interval suggestive of bladder afferent irritation.

In addition to cystometry, bladder permeability measurements revealed that uptake of sodium fluorescein from the bladder lumen was almost ten-fold greater in $\text{Kit}^{+/+}$ TNBS-CI mice than in the mast cell deficient mice which had plasma levels of the marker similar to those in the $\text{Kit}^W/\text{Kit}^{W-v}$ mice without TNBS-CI. While these findings provide strong support for the essential role that mast cells play in functional pathophysiological changes of the bladder after TNBS-CI, further investigations are needed to determine if the mutations resulting in mast-cell deficiency could be more complex than originally understood. We draw these conclusions cautiously as there is evidence that animals with this particular knockout mutation have secondary phenotypic abnormalities such as loss of interstitial cells of Cajal that could also influence colon-bladder cross-sensitization (Wershil, 2000).

The release of mast cell mediators can affect bladder activity not only by targeting afferent nerves or urothelium but also by affecting bladder smooth muscle (Fig. 8). To evaluate the effects of mast cells on the contractility of bladder smooth muscle we examined the effect of Compound 48/80, a mast cell activator on the phasic contractions and baseline tone of bladder smooth muscle strip preparations in vitro. Compound 48/80 increased the amplitude of phasic contractile activity and this effect was significantly greater in strips from TNBS-CI rats than in strips from control rats (Figs. 4a–b and 5). To lend support to our in vivo studies, we also applied Compound 48/80 to strips that had been pretreated with 20 μM ketotifen

fumarate, and showed that this concentration, which did not change spontaneous contractile activity of the bladder or responses to KCl, did suppress the contractile responses to Compound 48/80 in TNBS-CI strips. In these preparations where the numbers of mast cells are much greater and large changes in mast cell activity are apparent, ketotifen had a prominent attenuating effect on the response to Compound 48/80. While the effect of chronic oral administration of ketotifen in vivo on reflex bladder activity and effect of acute application of ketotifen to bladder strips in vitro do not have to be mediated by the same mechanisms, these findings provide support for the view that Compound 48/80 targets mast cells and that mast cells play a more significant role in the overactive bladder under cross-sensitization conditions than under control conditions.

There are various mechanisms that might contribute to the enhancement of the response to Compound 48/80 in TNBS preparations including: (1) increased numbers of mast cells in the bladder, (2) increased content and/or release of mast cell mediators, (3) increased sensitivity of target cells to the mediators. The first approach in attempting to establish a mechanism would be to identify the major mediators released from bladder mast cells. Mast cell tryptase, which is the most abundant mediator stored in mast cell granules has been used as a marker of mast cell degranulation (Payne and Kam, 2004). The major target of mast cell tryptase is PAR-2 (Fig. 8), a G-protein coupled receptor known to be involved in inflammation and visceral hyperalgesia (Coelho et al., 2002) and to mediate contractions of the rat urinary bladder (Shimizu et al., 2007).

To determine if the enhanced contractile activity of the bladder strip preparations required PAR-2, we desensitized PAR-2 by a prolonged 30-min application of the PAR-2 peptide agonist, SLIGRL-NH₂, followed by application of Compound 48/80. SLIGRL-NH₂ alone produced a weak contraction of the bladder strips that returned to basal activity after 30 min. PAR-2 desensitization significantly attenuated the contractile response to Compound 48/80 in strips from TNBS rats, indicating that the contractile activity induced by Compound 48/80 is mediated at least in part by PAR-2. Other investigators have shown that vasodilation induced by mast cell activation by Compound 48/80 is absent in the PAR-2 knockout mouse (Palmer et al., 2007). In our experiments PAR-2 desensitization did not significantly change the response to 48/80 in strips from control animals (Fig. 5), indicating that the role of PAR-2 and mast cells is more important in pathological conditions. However, mast cells synthesize, release, and respond to different mediators depending on the microenvironment (Bauer and Razin, 2000) and inflammatory conditions (He, 2004); therefore activation of PAR-2 by tryptase may be only one of the mechanisms contributing to cross-sensitization.

5. Conclusions

In summary, the present study provides considerable support for the hypothesis that mast cells in the bladder, which are present in significantly increased numbers after TNBS-CI, play a role in the changes in bladder function that occur in this pathological model. Pharmacological experiments indicate that the release of mediators from mast cells is an essential contributor to the decreased voiding interval, increased bladder permeability and increased amplitude of spontaneous contractions in TNBS-CI.

Mast cells release a variety of mediators that could act on many targets in the bladder including afferent nerves, urothelium, smooth muscle, or even mast cells (Fig. 8). Thus the mechanisms underlying the pathologically-induced functional changes are likely to be complex involving the interaction between multiple cell types (Malykhina et al., 2006). Our studies indicate that mast cell tryptase which acts on PAR-2 is involved in the facilitation of smooth muscle contractile activity induced by mast cell activation after TNBS-CI. Our

preliminary experiments (Fitzgerald et al., 2011) indicate that PAR-2 expression in the bladder is increased in the TNBS-CI model and studies in other laboratories have revealed that PAR-2 expression is increased in cyclophosphamide models of bladder inflammation (Nakahara et al., 2004; Shimizu et al., 2007). Clearly, more experiments are necessary to evaluate the roles of tryptase and PAR-2 in other aspects of colitis- or cystitis-induced bladder dysfunction. Nevertheless, based on the effects of ketotifen and Compound 48/80, it is still reasonable to conclude that communication of the mast cell-afferent nerve unit (Fig. 8) with its downstream targets is a vital part of the mechanism underlying pelvic organ crosstalk. When this mechanism is fully elucidated it could lead to the development of new therapies for the treatment of visceral pain disorders such as IBS and IC.

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Fig. 1.

The number of mast cells (per 20× field) in the bladder is significantly increased 12 days after initiation of TNBS colitis. Ketotifen (10 mg/kg/day) administered for 5 days in control animals (n=4) did not alter mast cell numbers (one way ANOVA, $p>0.05$). Ketotifen administered for 5 days between days 7 and 12 after TNBS-CI (n=5) did not significantly ($p>0.05$) reduce the increased numbers of bladder mast cells after TNBS treatment (n=4). Both TNBS treatment groups had significantly increased numbers of mast cells compared to the control (vehicle/no treatment) group ($p<0.05$).

Fig. 2.

Effect of TNBS-CI and ketotifen 10 mg/kg/day on voiding interval during continuous infusion cystometry in urethane anesthetized rats. Four groups of animals (n=3 per group) consisting of control (vehicle/no treatment), ketotifen treated controls, TNBS treated and TNBS treated with ketotifen from day 7 to day 12 of TNBS-CI were used in this study. Ketotifen administered for 5 days did not alter voiding in controls but normalized hyperactive voiding measured on day 12 in rats with TNBS-CI (one way ANOVA, $p = 0.05$). Voiding interval in TNBS animals was significantly different from control animals (*) and from TNBS-CI animals treated with ketotifen (#) which were not different from controls (* $p < 0.05$ vs. control, # $p < 0.05$ vs. TNBS-CI+Ketotifen).

Fig. 3.

Treatment with ketotifen 10 mg/kg/day administered for 5 days starting on day 7 or for 7 days starting on day 5 after TNBS-CI normalizes bladder urothelial permeability measured on day 12 in rats with TNBS-CI. Urothelial permeability was evaluated by measuring the plasma concentration of sodium fluorescein after intravesical administration. Experiments were conducted on five groups of animals (n=4 animals per group): controls (vehicle/no treatment), controls treated with ketotifen for 5 days, TNBS-CI for 10–12 days, TNBS-CI treated with ketotifen for 5 or 7 days. Plasma concentration of sodium fluorescein was significantly increased 12 days after TNBS-CI (one way ANOVA, * $p < 0.05$ vs. control) but not after TNBS+ketotifen (# $p > 0.05$ vs. control).

Fig. 4.

Compound 48/80 (50 $\mu\text{g/ml}$) produces a small increase in the contractile activity of bladder smooth muscle strips from control rats (a), but this effect is enhanced in strips from rats with TNBS-CI. (b). The effect of Compound 48/80 in strips from rats with TNBS-CI is suppressed by pretreatment with ketotifen (20 μM) (c) or by desensitization of PAR-2 by 30 min pretreatment with SLIGRL-NH₂, (100 μM) a PAR-2 agonist (d). These recordings are from four separate experiments.

Fig. 5.

The effect of Compound 48/80 (50 $\mu\text{g/ml}$) to enhance phasic contractions of bladder smooth muscle strips was larger in preparations from rats with TNBS-CI ($n=9$ strips) than in preparations from control rats ($n=9$ strips, vehicle/no treatment, two way ANOVA, $*p<0.05$). In all preparations the effect of Compound 48/80 (50 $\mu\text{g/ml}$) is greater at 15 min compared to 3 min after administration. Pretreatment with the mast cell stabilizer ketotifen (20 μM) ($n=8$ strips) reduced the responses to Compound 48/80 at 3 and 15 min after drug application in strips from rats with TNBS-CI ($\#p<0.05$) but did not alter the response to Compound 48/80 in strips from control animals ($n=9$ strips). PAR-2 desensitization via SLIGRL-NH₂ (100 μM) also reduced the effect of Compound 48/80 at 15 min after drug application in strips from TNBS-CI rats ($@p < 0.05$) but did not alter the response in strips from control rats. Enhancement is expressed as percent change in amplitude of phasic contractions of bladder smooth muscle strips after administration of Compound 48/80 (50 $\mu\text{g/ml}$).

Fig. 6.

The effect of TNBS-CI on voiding interval in urethane anesthetized mast cell deficient (Kit^W/Kit^{W-v}) and wild type (Kit^{+/+}) adult mice. Voiding interval was measured during continuous infusion saline cystometry (25 μ l/min). Kit^{+/+} (n=5) and Kit^W/Kit^{W-v} (n=3) animals in which TNBS vehicle was instilled intrarectally 7 days prior to the experiment exhibited similar voiding intervals. TNBS-CI 7 days prior to the experiment significantly reduced the voiding interval (n=5 per group) in Kit^{+/+} mice but not in Kit^W/Kit^{W-v} mice, which have normal voiding intervals equivalent to those in wild type control littermates (one way ANOVA, $p < 0.05$). Voiding interval in Kit^{+/+} TNBS-CI is significantly different (* $p < 0.05$) from that in wild type Kit^{+/+} and significantly different (# $p < 0.05$) than that in Kit^W/Kit^{W-v} TNBS-CI mice.

Fig. 7.

The effect of TNBS-CI on urothelial permeability in urethane anesthetized mast cell deficient (Kit^W/Kit^{W-v}) and wild type (Kit^{+/+}) adult mice. To estimate urothelial permeability plasma concentration of sodium fluorescein was measured 15 min after infusion of the tracer solution (0.1 ml, 10 mg/ml) into the bladder. Wild type Kit^{+/+} (n=5) and untreated Kit^W/Kit^{W-v} (n=3) animals in which TNBS vehicle was instilled intrarectally 7 days prior to the experiment exhibited similar urothelial permeabilities. TNBS-CI 7 days prior to the experiment significantly increased urothelial permeability in wild type Kit^{+/+} mice (n=4) but not in mast cell deficient mice (Kit^W/Kit^{W-v}) which have normal urothelial permeability equivalent to that in wild type littermates (one way ANOVA, $p < 0.05$). Voiding interval in Kit^{+/+} TNBS-CI is significantly less (* $p < 0.05$) from that in Kit^{+/+} and significantly different (# $p < 0.05$) from that in Kit^W/Kit^{W-v} TNBS-CI mice.

Fig. 8.

Putative mechanisms involved in mast cell contribution to bladder dysfunction in the TNBS-CI model. Dorsal root ganglion (DRG) neurons co-innervating the colon and bladder are activated by TNBS colitis. Action potentials (AP) propagating from the colon to the bladder release neuropeptides (NP) that can activate receptors (neurokinin receptor, NKR) in smooth muscle (SM), urothelium (UT), mast cells and afferent nerve terminals. The stimulated mast cell release tryptase (tryp) that activates protease activated receptor-2 (PAR-2) in SM, afferent nerves and UT to induce a contraction ("?" in the figure implies that the UT induced SM contraction mechanism is unknown). Compound 48/80 releases Tryp from mast cells and SLIGRL-NH₂ activates and desensitizes PAR-2. See text for further description of chemical interactions between the various cell types.