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TRPA1 mediates bladder hyperalgesia in a mouse model of cystitis

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Abstract

Urinary bladder pain is a primary symptom associated with interstitial cystitis/painful bladder syndrome. We employed systemic injections of cyclophosphamide (CYP), an alkylating anti-neoplastic agent, to induce cystitis and examine the roles of two channels previously shown to be required for inflammatory visceral hyperalgesia: transient receptor potential vanilloid-1 (TRPV1) and ankyrin-1 (TRPA1). Injection of CYP (100 mg/kg, i.p.) every other day for five days was accompanied by bladder edema and urothelial ulceration, but without significant plasma extravasation or infiltration of neutrophils. Toluidine blue staining showed a significant increase in the number of degranulated bladder mast cells following CYP treatment. Despite this mild pathology, CYP-treated mice exhibited bladder hyperalgesia one day following the final injection that persisted seven days later. Although many previous studies of visceral hyperalgesia have reported changes in dorsal root ganglion neuron TRPV1 expression and/or function, we found no change in bladder afferent TRPV1 expression or sensitivity, based on the percentage of bladder afferents responsive to capsaicin, including at sub-maximal concentrations. In contrast, the percentage of bladder afferents expressing functional TRPA1 protein (i.e., those responsive to mustard oil) increased ~2.5-fold one day after CYP treatment, and remained significantly elevated seven days later. Moreover, bladder hyperalgesia was reversed by acute treatment with the TRPA1 antagonist, HC-030031 (300 mg/kg, i.p.). Our results indicate that CYP-induced bladder hyperalgesia can be induced without robust inflammation or changes in primary afferent TRPV1. However, significant changes were seen in TRPA1 expression, and blockade of TRPA1 alleviated CYP-induced bladder hyperalgesia.

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Introduction

Pain originating from the urinary bladder is a cardinal symptom associated with interstitial cystitis/painful bladder syndrome (IC/PBS). The etiology of IC/PBS appears to be multifactorial, with associated sensory alterations resulting from both peripheral and central changes. The impact of peripheral signaling in generation of bladder pain is underscored by the reduction in pain reported by IC patients following intravesical treatment with lidocaine or botulinum toxin A [38; 45].

Transient receptor potential vanilloid-1 (TRPV1) and ankyrin-1 (TRPA1) are ligand-gated, Ca^{2+} -permeable cation channels that are requisite mediators of visceral hypersensitivity via regulation of both efferent and afferent function of primary afferent neurons [24; 48; 56]. Both channels are expressed in neuronal and non-neuronal lower urinary tract tissues in both humans [14; 41] and rodents [3; 16; 17; 51; 57; 59]. Their functional importance has been characterized for bladder function, e.g., micturition [6; 9; 34]. However, their roles in regulation of *in vivo* bladder nociception *per se* have not.

In contrast to bacterial cystitis, IC characteristically occurs in the absence of ongoing infection; it may, however, be accompanied by neurogenic inflammation, mastocytosis, and increased cytokines, of which TRP channels may be upstream mediators or downstream targets [47; 48; 58]. The goal of our experiments was to evaluate the roles of TRPV1 and TRPA1 in bladder afferent sensitization and persistent hyperalgesia. Using a mouse model of CYP-induced cystitis, we quantified and characterized the relationship between changes in visceral nociceptive behavior and TRPV1/TRPA1 channel expression and function in dorsal root ganglion (DRG) bladder afferent neurons. The conclusion from the data presented here is that changes in TRPA1 expression and function account for bladder hyperalgesia in the absence of changes in TRPV1.

Materials & Methods

Cyclophosphamide-induced cystitis

Female C57BL/6 mice (8–12 wks; The Jackson Laboratory) were randomly divided into control and experimental groups. Cystitis was induced by intraperitoneal injection of the alkylating anti-neoplastic agent cyclophosphamide (CYP; Sigma-Aldrich). CYP (100 mg/kg) or vehicle (sterile saline) was administered every other day for a period of five days. Mice were sacrificed 24 hrs (1d post-CYP) or one week (7d post-CYP) after the third CYP injection; control mice were always sacrificed 24 hrs after the third vehicle injection.

Myeloperoxidase assay, toluidine blue staining, immunohistochemistry, and histology

Myeloperoxidase (MPO) activity was quantified to assess neutrophil-based inflammation in bladder tissue. Mice were isoflurane-anesthetized and transcardially perfused with saline. Bladders were dissected and weighed. The assay (Calbiochem) was performed as previously described [48]. Reactions between tissue samples and MPO standards with O-dianisidine dihydrochloride (Sigma-Aldrich) were read at 460 nm every 20 sec for 15 min. The slope for each standard reading was calculated and the slope of those values was used to calculate MPO units/tissue weight.

Toluidine blue staining was performed on cross sections of bladder tissue taken from isoflurane-anesthetized mice following transcardial perfusion with 4% paraformaldehyde. Whole bladders were post-fixed for 4 hr, cryoprotected overnight in 25% sucrose, embedded and rapidly frozen in medium. Sections taken from the medial rostrocaudal bladder were stained with Toluidine Blue O (Sigma) dissolved in 70% ethanol with 1% NaCl. The total number of mast cells and number of degranulated mast cells were quantified in five sections of tissue per mouse.

Paraffin-embedded cross sections (30 μ m) of tissue taken from the medial rostrocaudal bladder were used for qualitative immunohistochemical and histological evaluation. Tissue sections were stained with protein gene product 9.5 (PGP9.5) to examine changes in innervation. Tissue sections were washed, blocked (5% normal horse serum and 0.25% Triton X-100 in 0.1M phosphate buffer), incubated in rabbit anti-PGP9.5 primary antibody (1:1000, UltraClone) overnight at room temperature, washed, incubated in donkey anti-rabbit Cy3 secondary antibody (1:500, Jackson ImmunoResearch) for 2 hrs at room temperature, washed, dehydrated, and coverslipped with DPX mounting media. Additional sections from the same bladder tissue were stained with hematoxylin and eosin to assess the presence/absence of edema, petechial hemorrhage, and mucosal ulceration. All sections were viewed and photographed on a LEICA DM 4000B microscope (Leica Microsystems) using Leica Application Suite software.

Evans blue extravasation

Mice were isoflurane-anesthetized and injected with an intra-arterial bolus (50 μ l) of Evans blue (1% in saline). Fifteen min later, mice were transcardially perfused with saline. Bladders were dissected, blotted dry, weighed, and placed in 1 ml of formamide overnight (55°C). Absorbance was measured spectrophotometrically (600 nm) and the concentration of extracted Evans blue was determined by standard curve.

Whole ganglia real-time polymerase chain reaction

Mice were isoflurane-anesthetized and transcardially perfused with saline. Lumbosacral (L6-S1) dorsal root ganglia were removed, frozen on dry ice, and homogenized in Qiagen Buffer RLT (Qiagen, Valencia, CA) using a Polytron tissue grinder. RNA was extracted using RNeasy columns (Qiagen), treated with DNase (Sigma-Aldrich), and reverse-transcribed using SuperScript II (Invitrogen). cDNA was used as template in 25 μ l real-time SYBR green (Life Technologies, Grand Island, NY) PCR reactions run on an ABI Prism 7000 system (Applied Biosystems, Foster City, CA). Values were normalized to β -actin and quantified using the Pfaffl method [43]. Primer sequences are listed in Table 1.

Retrograde labeling of urinary bladder afferents

Mice were isoflurane-anesthetized. The bladder was isolated via laparotomy. Three injections (12 μ l total volume) of Alexa Fluor-conjugated cholera toxin- β (CT β) were made into the bladder wall. Abdominal incisions were sutured and mice recovered for 4 days.

Cell dissociation

Mice were isoflurane-anesthetized and transcardially perfused with cold $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution (HBSS; Invitrogen). Bilateral L5-S1 DRG were dissected into cold HBSS and dissociated as previously described [31]. Cells were plated in DMEM-F12 media (Invitrogen) containing 10% fetal bovine serum and antibiotics (penicillin/streptomycin, 50 U/ml). Coverslips were flooded with media 2 hr later and used within 8–10 hr.

Calcium imaging

Ca^{2+} imaging was performed on retrogradely labeled bladder afferent neurons. Neurons were incubated in 2 μM Ca^{2+} indicator Fura-2 AM ester (Invitrogen) with 0.02% Pluronic F-127 (Invitrogen) in normal bath solution (in mM: 130 NaCl, 5 KCl, 1.5 CaCl_2 , 0.9 MgCl_2 , 20 HEPES, 5.5 glucose, 0.5 KH_2PO_4 , 0.5 Na_2HPO_4 , pH adjusted to 7.4 with NaOH, osmolality adjusted to 325 mOsm with sucrose) containing 5 mg/ml BSA (Sigma-Aldrich) for 20 min at 37°C. Coverslips were mounted on an inverted microscope stage (Olympus) with continuously perfused normal bath (1 ml/min). Perfusion rate was controlled with a gravity flow and rapid-switching local perfusion system (Warner Instruments). All solutions were maintained at 30°C using a heated stage and in-line heating system (Warner Instruments). Firmly attached, refractile $\text{CT}\beta^+$ cells were identified as regions of interest. A ratio (R) of fluorescence emission at 510 nm in response to excitations at 340 and 380 nm was acquired at 1 Hz (Lambda DG-4 and 10-B SmartShutter, Sutter Instruments) via camera (ORCA-ER, Hamamatsu Corporation) and saved to computer using HImage (Hamamatsu Corporation). The concentration of intracellular calcium ($[\text{Ca}^{2+}]_i$) was determined following *in situ* calibration experiments according to the following equation:

$$[\text{Ca}^{2+}]_i(\text{nM}) = K_d \left(\frac{S_{f2}}{S_{b2}} \right) \left(\frac{R - R_{\min}}{R_{\max} - R} \right)$$

where K_d is the dissociation constant for Fura-2, S_{f2}/S_{b2} is the fluorescence ratio of the emission intensity excited by 380 nm signal in the absence of Ca^{2+} , and $R_{\min}$ and $R_{\max}$ are the minimal and maximal fluorescence ratios, respectively. The procedure for determination of these variables has been described previously [25]. First, 50 mM K^+ was applied; unresponsive cells were not used in data analyses. Following a 5 min recovery period, the percentage of cells exhibiting TRPV1- and TRPA1-mediated responses was assessed by application of capsaicin (CAP; 1 μM) or mustard oil (MO; 100 μM), respectively. The magnitude and decay of evoked Ca^{2+} transients were determined by peak evoked change ($[\text{Ca}^{2+}]_i$) and time to 50% of the peak (T_{50}), respectively.

Visceral nociceptive behavior

Visceromotor reflexes (VMRs), quantified by abdominal muscle electromyography (EMG), were assessed as previously described [37]. Briefly, mice were isoflurane-anesthetized and silver wires were inserted into the left oblique abdominal musculature for differential amplification of myoelectric activity. A 24-gauge angiocatheter was passed transurethrally into the bladder for delivery of compressed air and measurement of intravesical pressure.

Anesthesia was then reduced until flexion reflexes were present but spontaneous escape behaviors absent. Two 60 mmHg urinary bladder distensions (UBDs) were administered to overcome an initial period of sensitization [8] and were followed by graded distensions (15, 30, 45, 60 mmHg; 10 s; 2 min inter-stimulus interval). EMG responses were recorded using Spike2 software (Cambridge Electronic Design Ltd.). Rectified EMG activity was normalized as: (evoked EMG – baseline EMG)/baseline EMG. Data are presented as a percent of the maximal response of the vehicle-treated control group. The TRPA1 antagonist, HC-030031 (Tocris Bioscience, Bristol, UK), was reconstituted in DMSO (100 mM) and administered at a dose of 300 mg/kg (i.p.); DMSO was administered (i.p.) as vehicle in control experiments.

Statistical analyses

Data are expressed as mean $\pm$ SEM. Statistical analyses were performed using Systat (Systat Software, Inc). MPO activity, mast cell counts, and Evans blue extravasation were analyzed using *t*-tests. The percentage of cells responding to CAP and MO were compared by Chi-square and Fisher's exact tests with Holm's correction [23]. $[Ca^{2+}]_i$ and T_{50} were compared by ANOVA and post-hoc *t*-tests between groups as indicated. EMG responses to graded UBD were compared by repeated-measures ANOVA and post-hoc *t*-tests with Holm's correction [23] at each pressure. P values of ≤ 0.05 were considered significant.

Results

CYP produces bladder edema and mild inflammation

Qualitative examination of bladder histology showed time-dependent changes in bladder morphology after CYP treatment (Fig. 1A–C). In control mice, an intact urothelium and normal muscularis were apparent with no signs of edema. Signs of cystitis, including submucosal edema and epithelial ulceration, were evident 24 hr after three CYP injections (1d post-CYP), but had resolved by one week later (7d post-CYP). Staining for PGP9.5 immunoreactivity indicated no obvious signs of hyperinnervation or loss of innervation within any area of bladder tissue among Control, 1d post-CYP, and 7d post-CYP mice (Fig. 1D–F).

MPO activity, the presence of mast cells, and Evans blue plasma extravasation were used to assess the degree of bladder inflammation. There was a significant effect of treatment on bladder weight (Fig. 2A; $F_{(2,24)}=16.271$; $p<0.01$); compared to control mice, bladder weight was significantly greater in 1d post-CYP ($t_{(18)}=5.604$, $p<0.01$) and 7d post-CYP ($t_{(19)}=4.536$, $p<0.01$) mice. Despite a significant overall increase in plasma extravasation in bladder tissue from 1d post-CYP ($2.41 \pm 0.27 \mu\text{g}$) versus control mice ($1.28 \pm 0.08 \mu\text{g}$; $t_{(4)}=3.185$, $p<0.05$), there was no difference after controlling for bladder weight ($t_{(4)}=-0.833$, $p=0.452$; Fig. 2B). Analysis of bladder MPO activity indicated no neutrophil-based inflammation as a result of CYP treatment ($t_{(4)}=-0.472$, $p=0.662$; Fig. 2C); however, there was a significant increase in the number of degranulated mast cells in 1d post-CYP mice compared to controls ($t_{(10)}=-3.526$, $p<0.01$; Fig. 2D) as well as a trend toward increased total number of bladder mast cells ($t_{(5,134)}=-2.445$, $p=0.057$; Fig. 2D *inset*).

CYP increases depolarization-evoked intracellular Ca^{2+} concentration and functional TRPA1 expression in lumbosacral bladder afferents

Ca^{2+} imaging was performed to assess changes in the regulation of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) and the percentage of bladder neurons responsive to capsaicin (Cap) and mustard oil (MO), agonists for TRPV1 and TRPA1, respectively. $\text{CT}\beta^+$ neurons were identified using epifluorescence (Fig. 3A) and their cell body diameter was measured under bright field illumination (Fig. 3B). The median cell body diameter of all $\text{CT}\beta^+$ neurons studied was 21.5 μm , with 17.38 μm and 25.63 μm as 25th and 75th percentiles, respectively. The diameter of cells responsive to either Cap or MO was significantly smaller than Cap- and MO-unresponsive cells ($20.67 \mu\text{m} \pm 0.20$ versus $22.27 \mu\text{m} \pm 0.21$; $p < 0.05$). Soma diameter of cells from CYP-treated mice did not differ from those from control mice.

Cytoplasmic Ca^{2+} exerts regulatory influence over many neuronal properties, including function of membrane-bound channels, neurotransmitter release, and transcriptional control of gene expression. As such, $[\text{Ca}^{2+}]_i$ and T_{50} of depolarization-evoked Ca^{2+} transients in labeled bladder afferents were compared. Despite no change in resting $[\text{Ca}^{2+}]_i$ (Fig. 3C; $F_{(2,194)} = 2.237$, $p = 0.109$), there was a significant effect of CYP on peak response to application of 50 mM K^+ ($F_{(2,194)} = 6.173$, $p < 0.01$) and decay of that response, measured as the time to 50% of peak (T_{50} ; $F_{(2,194)} = 8.332$, $p < 0.01$). Compared to neurons from control mice, those from 1d post-CYP mice exhibited larger magnitude (Fig. 3D; $t_{(187)} = -3.118$, $p < 0.01$), and those from 7d post-CYP mice exhibited both larger magnitude and longer decay (Fig. 3D and 3E; $t_{(87)} = -2.405$, $p < 0.05$ and $t_{(87)} = -3.849$, $p < 0.01$, respectively).

Ca^{2+} imaging results from experiments utilizing TRP channel agonists are shown in Fig. 4. Overall, a greater proportion of bladder afferents from control mice responded to Cap (Fig. 4A) than MO (Fig. 4C). We observed no effect of CYP on the percentage of Cap-responsive neurons (Fig. 4A; $\chi^2_{(3)} = 1.651$, $p = 0.6479$). Because post-translational modification of TRPV1 protein by phosphorylation has been shown to play a role in its sensitization, we performed a series of Cap dose-response experiments to address the potential for increased sensitivity of TRPV1 in neurons constitutively expressing it. We found no significant shift in neuronal sensitivity to Cap in bladder afferents from CYP-treated mice in comparison to those from control mice (Fig. 4B) at concentrations of 500 nM ($\chi^2_{(1)} = 0.04$, $p = 0.8508$), 200 nM ($\chi^2_{(1)} = 0.003$, $p = 0.9522$), or 100 nM ($\chi^2_{(1)} = 0.03$, $p = 0.8577$). Despite a lack of change in bladder afferent TRPV1, the percentage of afferents responsive to MO, the prototypic TRPA1 agonist, was significantly increased ($\chi^2_{(3)} = 12.35$, $p = 0.0063$) in 1d post-CYP (Fisher's exact test, $p < 0.01$) and 7d post-CYP (Fisher's exact test, $p < 0.01$) conditions (Fig. 4C). Because of this robust increase in *de novo* expression of TRPA1, we did not include a dose-response analysis of MO although it is possible that TRPA1 is upregulated the small percentage of bladder afferents expressing it in normal conditions. We did, however, confirm in independent experiments the observed increase in MO-responsive neurons via application of an additional TRPA1 agonist, cinnamaldehyde (Cinn; 100 μM). The percent of bladder neurons responding to CINN also increased, from $25.95 \pm 2.4\%$ to $67.19 \pm 9.8\%$ (Fig. 4C). Analysis of MO-evoked $[\text{Ca}^{2+}]_i$ and T_{50} indicated no significant effect of CYP on these measures ($F_{(2,32)} = 1.111$, $p = 0.342$ and $F_{(2,32)} = 2.893$, $p = 0.070$, respectively).

Quantitative real-time PCR on whole lumbosacral (L6-S1) DRG was used in a further attempt to identify potential upregulation of TRP channels based on mRNA content in bladder afferent neurons. Any observed changes could reflect either *de novo* expression in neurons not previously expressing TRPV1 or TRPA1 (as seen in Ca^{2+} imaging experiments), or increased expression in neurons already constitutively expressing them. However, we found no significant changes in either TRPA1 or TRPV1 mRNA expression between control and CYP treatment groups (Table 2). Given the relatively small proportion of DRG neurons that innervate the bladder [10], it is not surprising that we failed to detect any changes in whole ganglia. In addition, it is possible that if changes in expression did occur, it was on the protein, and not mRNA level.

CYP induces TRPA1-dependent bladder nociceptive hypersensitivity

The VMR, quantified via abdominal EMG responses, is a 'pseudoaffective' reflex [49] that serves as a reliable index of bladder nociception in mice [37] (Fig. 5). The first set of experiments compared responses of mice at the 1d and 7d post-CYP time points with those of vehicle-treated mice. Repeated measures ANOVA indicated a significant effect of treatment ($F_{(2,24)}=8.032$; $p<0.01$) and significant treatment $\times$ distension pressure interaction ($F_{(6,72)}=4.874$; $p<0.01$). Post-hoc analyses revealed that EMG responses in the 1d post-CYP group were potentiated at noxious distension pressures (30 mmHg: $t_{(19)}=-2.360$, $p<0.05$; 45 mmHg: $t_{(19)}=-3.277$, $p<0.01$; 60 mmHg: $t_{(19)}=-3.590$, $p<0.01$), and that 7d post-CYP EMG responses were significantly potentiated at all distension pressures (15 mmHg: $t_{(15)}=-3.624$, $p<0.01$; 30 mmHg: $t_{(15)}=-4.147$, $p<0.01$; 45 mmHg: $t_{(15)}=-4.700$, $p<0.01$; 60 mmHg: $t_{(15)}=-5.200$, $p<0.01$). There were no differences in magnitude of responses between 1d and 7d post-CYP mice (15 mmHg: $p=0.258$; 30 mmHg: $p=0.486$; 45 mmHg: $p=0.410$; 60 mmHg: $p=0.465$).

To determine whether the changes in bladder primary afferent TRPA1 function we observed *in vitro* were pertinent to behavioral hyperalgesia following CYP, we performed a second set of experiments examining responses of CYP-treated mice injected with the TRPA1 antagonist HC-030031 (300 mg/kg, i.p.) or vehicle (DMSO) one hr prior to VMR testing (Fig. 5). Again, repeated measures ANOVA indicated a significant effect of treatment ($F_{(1,10)}=24.613$; $p<0.01$) and a significant treatment $\times$ distension pressure interaction ($F_{(3,30)}=16.639$; $p<0.01$). Post-hoc analyses showed that 1d post-CYP mice injected with vehicle prior to testing had significantly greater EMG responses than those injected with the antagonist, at 45 mmHg ($t_{(10)}=6.259$, $p<0.01$) and 60 mmHg ($t_{(10)}=7.083$, $p<0.01$); i.e. there was no effect on the observed hyperalgesia. In contrast, EMG responses of 1d post-CYP mice injected with the antagonist prior to testing were not different from those of the control group; i.e., inhibition of the TRPA1 channel was able to completely reverse established hyperalgesia. Intravesical administration of dimethyl sulfoxide (DMSO), the HC-030031 vehicle, is known to have mild analgesia effects and is an FDA-approved treatment for interstitial cystitis. As such, the responses of 1d post-CYP + vehicle (i.e., DMSO) mice were compared to those of the control group and the 1d post-CYP group to evaluate any potential effects of DMSO. Responses of 1d post-CYP + vehicle were significantly greater than those of control mice at 45 mmHg ($t_{(11)}=-3.420$, $p<0.01$) and 60 mmHg ($t_{(11)}=-3.797$, $p<0.01$), and did not differ from those of 1d post-CYP mice (15 mmHg: $t_{(5.155)}=0.986$, $p=0.368$; 30

mmHg: $t_{(9.994)}=1.325$, $p=0.215$; 45 mmHg: $t_{(6.319)}=1.411$, $p=0.205$; 60 mmHg: $t_{(5.407)}=1.268$, $p=0.257$).

Discussion

The present study is the first to report changes in bladder afferent TRPA1 expression and function in a CYP-induced model of experimental cystitis. The observed changes in bladder afferent TRPA1 paralleled the development of robust bladder hyperalgesia, in that once changes in nociceptive behavior and TRPA1 were established, they persisted for up to a week despite a lack of robust bladder inflammation. The histological findings of submucosal edema and vasodilation, normal muscularis, and a variable presence of immune cells are consistent with characteristic histological findings in IC patients [35]. The most clinically relevant finding of these studies is that even after bladder hyperalgesia is established, a specific antagonist of TRPA1 could completely block pathological, pain-like behavior.

Previous studies have shown that the majority of visceral afferents, including those innervating the bladder, express TRPV1 [12; 28; 30; 48]. On the basis of this observation and in conjunction with the requisite role of TRPV1 in inflammatory pain, we predicted that CYP treatment would be accompanied by increased expression and function of TRPV1. We also anticipated that increased expression and function of TRPA1 might occur, given its role as it is expressed in a subset of TRPV1-expressing neurons. To our surprise, the expected changes were seen only in TRPA1. Despite this, a salient role for TRPA1 in our studies could have been anticipated. For example, TRPA1 has been shown to play a direct role in visceral neurogenic inflammation [15; 48] and had been demonstrated repeatedly to be involved in transduction of noxious stimuli [36; 39; 40]. In addition, a gain of function mutation in TRPA1 appears to be the basis of a familial episodic pain syndromes in humans triggered by cold, fatigue or fasting [7; 26]. However, recent studies also indicate that TRPA1 function may be context dependent. Andersson et al., 2011 [2] showed that intrathecal cinnamaldehyde (used here to confirm increases in the number of TRPA1-responsive neurons) was anti-nociceptive and that metabolites of the analgesic paracetamol are TRPA1 agonists. That TRPA1 agonists like cinnamaldehyde can be both pro- and anti-nociceptive was recently confirmed in studies of CHO cells that showed that a low concentrations cinnamaldehyde increased TRPA1 currents but at higher concentrations produces fast, but reversible current inhibition [1].

Although delineation of the mechanism(s) underlying CYP-induced upregulation of TRPA1 is not yet known, a selective role for changes in TRPA1 could also be a consequence of its direct activation by acrolein [5; 15] (also see [11; 15]), a toxic metabolic byproduct of CYP that accumulates in urine. However, with respect to clinical relevancy, it should be noted that acrolein is not solely the result of metabolism of an exogenous compound like CYP, and TRPA1 can be activated by a variety of stimuli including mustard oil, formalin, 4-hydroxynonenal, and reactive carbonyl species (that include acrolein). Some of these molecules can be generated by oxidative stress as a result of endogenous inflammation or exogenous environmental pollutants [4; 5; 29; 32; 33; 53; 54].

Another clinically relevant mechanism through which TRPA1 may mediate the induction and maintenance of bladder afferent hypersensitivity and hyperalgesia is via regulating the peripheral release of the neuropeptides calcitonin gene-related peptide (CGRP) and substance P. It has been demonstrated previously that TNBS-induced colitis in mice results from increased colonic neuropeptide release that is mediated by TRPA1 [15]. An increase in the number of substance P positive nerve fibers has been reported in IC patients [42], and the presence of substance P is reduced following epidural local anesthetic blockade [52]. Treatment with intravesical botulinum toxin A, which decreases neuropeptide release and bladder nociception in a rodent model of cystitis [13], is associated with symptom improvement in IC patients [27; 44; 45; 50].

We found that CYP treatment was associated with significant changes in depolarization-evoked Ca^{2+} transients in bladder afferent neurons. Of particular importance is the fact that these changes were persistent, and paralleled the time course of TRPA1 upregulation and behavioral hyperalgesia, despite the absence of robust inflammation and resolution of changes in bladder morphology post-treatment. Under normal conditions, cytoplasmic Ca^{2+} regulates many cell processes, including neuronal excitability, neurotransmitter release and gene expression [20; 22; 46]. Changes in the kinetics of depolarization-evoked intracellular Ca^{2+} could be mediated directly through upregulation of TRPA1 which is Ca^{2+} -permeable, or via influx through voltage-gated channels or endoplasmic reticulum Ca^{2+} -induced Ca^{2+} release [19], or a combination thereof. Because gene expression can be regulated in an activity-dependent fashion via modulation of intracellular Ca^{2+} [18; 19], it is possible that changes in $[\text{Ca}^{2+}]_i$ handling like the ones we observed in bladder afferents contribute to the persistence of CYP-induced hyperalgesia.

All animal models of visceral pain have caveats with respect to severity and/or time course of their induced pathology when compared to the human condition. However, one of the strengths of the CYP model used here is that it revealed significant and long-lasting changes in visceral afferent function in the absence of robust or persistent inflammation. Moreover, the resulting hyperalgesia occurred in the absence of changes in primary afferent TRPV1 expression or function, whereas dramatic changes were seen in TRPA1 and, in fact, TRPA1 antagonism was sufficient to reverse bladder hyperalgesia. This suggests that there may be a stepwise recruitment of these two channels such that mild injury, like that we observed leading to cystitis, activates mechanisms that rely on TRPA1 plasticity while more severe injury engages TRPV1-dependent processes.

Regardless of the long-term changes in both the bladder and bladder afferents that are likely to be associated with chronic pain in interstitial cystitis/painful bladder syndrome, the efficacy of the TRPA1 antagonist suggests that, at least in our model, changes in TRPA1 are sufficient for the maintenance of bladder hyperalgesia. This is a remarkable finding, given the difficulty in managing interstitial cystitis/painful bladder syndrome-related bladder pain. Recent clinical trials with TRPV1 antagonists were extremely promising until subjects reported impaired noxious heat detection with the potential to result in thermal injuries (scalding and cutaneous burns) [55] and hyperthermia [21]. The more restricted nature of TRPA1 expression, combined with its relatively minor role in detection of noxious stimuli, make pursuing TRPA1 antagonists an attractive therapeutic direction.

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Fig. 1.

CYP treatment (100 mg/kg) every other day for five days alters urinary bladder morphology.

A–C: Hematoxylin and eosin-stained sections of urinary bladder were taken one day following 3rd vehicle injection (Control; **A**) or CYP injection (1d post-CYP; **B**), or seven days later (7d post-CYP; **C**). Epithelial ulceration (arrowheads) and edema (arrows) between the submucosa and transitional epithelium were observed 1d post-CYP mice. By 7d post-CYP, edema was resolved and ulcerations were absent. **D–F:** PGP9.5 staining of bladder nerves in control mice (**D**), 1d post-CYP (**E**) and 7d post-CYP (**F**). Despite significant changes in the edema seen 1d post-CYP, there are not obvious differences in the number or density of large nerve bundles (arrows) or small clusters of nerve fibers (arrow heads). Transitional epithelium (epi) is not seen in **E** because the extent of edema increased the bladder wall thickness to the point that it could not be included at this magnification. Epithelium (**D&F**) exhibited non-specific, edge artifact staining. Calibration bar = 400 μ m in panels **A–C**; 200 μ m in panels **D–F**.

Fig. 2.

CYP treatment (100 mg/kg, i.p.) every other day for five days induced mild bladder inflammation. After the final CYP injection, bladder weight was significantly increased one day and seven days later compared to controls (**A**), but there was no change in extravasation of Evans blue (**B**) or neutrophil-based myeloperoxidase activity (**C**). There was a greater number of degranulated mast cells in bladder tissue from CYP-treated mice (**D**), and a trend toward an increase in the total number of bladder mast cells (**D**, *inset*). $n=3-4$ mice/group; * indicates $p<0.05$; NS indicates not significant.

Fig. 3.

CYP treatment (100 mg/kg) changes the kinetics of depolarization-evoked Ca^{2+} transients in lumbar (L6-S1) bladder afferents. Retrogradely labeled, healthy, dissociated urinary bladder afferents were identified using bright field (**A**) and fluorescence (**B**) microscopy. Despite no change in baseline intracellular Ca^{2+} ($[Ca^{2+}]_i$) (**C**) at either 1 day (1d post-CYP) or seven days (7d post-CYP) following three injections of CYP, the change in intracellular Ca^{2+} ($\Delta[Ca^{2+}]_i$) in response to application of 50 mM K^+ was significantly greater in both 1d post-CYP and 7d post-CYP treatment groups compared to control (**D**). The decay of evoked Ca^{2+} transients (T_{50}) was significantly longer in the 7d post-CYP group (**E**). $n=8-12$ mice/group; * indicates $p<0.05$ versus control; NS indicates not significant. Calibration bar = 20 μm .

Fig. 4.

CYP treatment (100 mg/kg) increases the percentage of lumbosacral (L6-S1) bladder afferents expressing functional TRPA1, but not TRPV1. The number of TRPV1- and TRPA1-expressing bladder afferents was determined using Ca^{2+} imaging. Expression of TRPV1, as determined by percentage of cells exhibiting Ca^{2+} transients in response to application of capsaicin (Cap; 1 μM ; $n=4-7$ mice/group), did not change either one or seven days following CYP treatment (**A**). Dose-response experiments (**B**) further indicated no change in sensitivity to Cap (500, 200, or 100 nM; $n=1-4$ mice/group). In contrast, there was a significant increase in the percentage of bladder afferents responsive to application of the TRPA1 agonist, mustard oil (MO; 100 μM ; $n=4-8$ mice/group) at both post-treatment time points (**C**); this increase was confirmed via an additional TRPA1 agonist, cinnamaldehyde (Cinn; 100 μM ; $n=2$ mice/group). The magnitude and decay of MO-evoked Ca^{2+} transients did not significantly change. * $p<0.05$ indicates 1d post-CYP versus control; # indicates $p<0.05$ 7d post-CYP versus control; NS indicates not significant.

Fig. 5.

CYP treatment (100 mg/kg) induced robust urinary bladder hyperalgesia. The visceromotor reflex (VMR), quantified by abdominal muscle electromyography (EMG), was used to assess nociceptive responses to bladder distention. Compared to control mice, CYP-treated mice exhibited augmented EMG responses when tested one day (1d post-CYP) and seven days (7d post-CYP) following the 3rd CYP injection. Bladder hyperalgesia was completely blocked by pre-treatment with the TRPA1 antagonist, HC-030031 (300 mg/kg, i.p.), one hr prior to testing. $n=6-8$ mice/group; * indicates $p<0.05$ 1d post-CYP versus control; # indicates $p<0.05$ 7d post-CYP versus control; + indicates $p<0.05$ versus 1d post-CYP + vehicle.

Table 1

Primer sequences for real-time PCR quantification of TRP channel mRNA in lumbosacral (L6-S1) dorsal root ganglia.

Gene	Forward Primer	Reverse Primer	Accession No.
TRPA1	5' GCAGGTGGAACTTTCATACCAACT 3'	5' CACTTTTGCGTAAGTACCACAGAGTGG 3'	NM_177781
TRPV1	5' TTCCTGCAGAGAGCAAGAAGC 3'	5' CCCATTGTGTCAGATTGAGCAT 3'	NM_001001445
GAPDH	5' ATGTGTCCGTCGTGGATCTGA 3'	5' ATGCCTGCTTCACACCTTCTT 3'	NM_008084

CYP treatment did not significantly change mRNA expression of TRPA1 or TRPV1 in whole lumbosacral (L6-S1) dorsal root ganglia relative to control expression at one day post-treatment, when behavioral hyperalgesia peaked.

Table 2

Treatment Group	mRNA Expression Ratio	
	TRPA1	TRPV1
Control	1.164 ± 0.56	0.720 ± 0.22
1d post-CYP	1.204 ± 0.75	0.871 ± 0.52
p value	0.901	0.479