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Experimental colitis triggers the release of substance P and calcitonin gene-related peptide in the urinary bladder via TRPV1 signaling pathways

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Abstract

Clinical data provides evidence of high level of co-morbidity among genitourinary and gastrointestinal disorders characterized by chronic pelvic pain. The objective of this study was to test the hypothesis that colonic inflammation can impact the function of the urinary bladder via activation of TRPV1 signaling pathways followed by alterations in gene and protein expression of Substance P (SP) and calcitonin gene-related peptide (CGRP) in sensory neurons and in the bladder. Inflammation was induced by intracolonic instillation of trinitrobenzene sulfonic acid (TNBS, 12.5 mg/kg) and desensitization of TRPV1 receptors was evoked by intracolonic resiniferatoxin (RTX, 10^{-7} M). mRNA and protein concentrations of CGRP and SP were measured at 3, 5 and 30 days. RTX instillation in the colon caused 3-fold up-regulation of SP mRNA in the urinary bladder at day 5 ($n=7$, $p \leq 0.05$) followed by 35-fold increase at day 30 ($n=5$, $p \leq 0.05$). Likewise, TNBS colitis triggered 15.8-fold up-regulation of SP mRNA one month after TNBS ($n=5$, $p \leq 0.05$). Desensitization of colonic TRPV1 receptors prior to TNBS abolished SP increase in the urinary bladder. RTX led to 4.3-fold increase of CGRP mRNA at day 5 ($n=7$, $p \leq 0.05$ to control) in the bladder followed by 28-fold increase at day 30 post-RTX ($n=4$, $p \leq 0.05$). Colitis did not alter CGRP concentration during acute phase, however, at day 30 mRNA level was increased by 17.8 ± 6.9 fold ($n=5$, $p \leq 0.05$) in parallel with 4-fold increase in CGRP protein ($n=5$, $p \leq 0.01$) in the detrusor. Protein concentration of CGRP in the spinal cord was diminished by 45–65% ($p \leq 0.05$) during colitis. RTX pretreatment did not affect CGRP concentration in the urinary bladder, however, caused a reduction in CGRP release from lumbosacral DRG neurons during acute phase (3 and 5 days post-TNBS). Our results clearly demonstrate that colonic inflammation triggers the release of pro-inflammatory neuropeptides SP and CGRP in the urinary bladder via activation of TRPV1 signaling mechanisms enunciating the neurogenic nature of pelvic organ cross-sensitization.

Keywords

inflammation; cross-sensitization; neuropeptides; pelvic pain

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Introduction

High level of co-morbidity among urinary and gastrointestinal disorders suggests an existence of pathways connecting and concurrently coordinating the function of several pelvic organs. Functional pelvic disorders are distinguished by chronic pelvic pain of unknown etiology and may develop as a result of cross-sensitization in the pelvis (Malykhina, 2007). Our previous studies have demonstrated an association between inflammation in the pelvis, hyperexcitability of extrinsic sensory neurons and development of pelvic organ cross-sensitization (Malykhina et al., 2006; Qin et al., 2005). The mechanisms underlying visceral organ “cross-talk” are still unclear but suggest involvement of convergent neural pathways in the central and autonomic nervous systems (Brumovsky and Gebhart, 2009; Malykhina, 2007).

Little is known about the modulatory factors which contribute to pain detection and transmission resulted from pelvic organ cross-sensitization. It is well established that release of neuropeptides from afferent terminals leads to the occurrence of neurogenic inflammation at the peripheral sites composed of degranulated mast cells, local plasma extravasation and arteriolar vasodilation (Wesselmann, 2001). Substance P (SP) and calcitonin gene-related peptide (CGRP) are the main neuropeptides released from peripheral axons of sensory neurons upon noxious stimulation and are synthesized in primary sensory neurons projecting to the pelvic organs (Bueno and Fioramonti, 2002; Candenas et al., 2005). The majority of bladder afferents in the rat lumbosacral spinal cord are also SP and CGRP-positive (Hwang et al., 2005). It is well established that SP and CGRP release is stimulated by direct inflammatory insult (Avelino et al., 2002; Domotor et al., 2005), however, their role in the development of cross-organ sensitization in the pelvis is unclear and was investigated in this study.

Substance P and CGRP-positive DRG neurons often co-express transient receptor potential vanilloid (TRPV1) channel (Avelino et al., 2008; Gabella and Davis, 1998; Smet et al., 1997). TRPV1 is one of the key components of nociceptive signal transduction pathways (Caterina et al., 1997) and is widely distributed throughout both the gastrointestinal tract and genitourinary system. In humans, TRPV1 mRNA was detected in the prostate, testis, penis, bladder, and extrinsic sensory neurons innervating these organs (Stein et al., 2004). In the urinary bladder, TRPV1 is expressed mostly in the urothelium, detrusor smooth muscle, and interstitial cells (Birder et al., 2001; Ost et al., 2002) as well as on blood vessels (Gabella and Davis, 1998). Sensitivity to TRPV1 agonist capsaicin, or its more potent analog resiniferatoxin (RTX), is considered to be a “hallmark” of nociceptive neurons. Capsaicin application triggers membrane depolarization due to activation of a non-selective cation current followed by an increase in calcium influx, altered action potential kinetics, and the release of modulatory neurotransmitters and neuropeptides (Caterina et al., 1997; Holzer and Maggi, 1993).

Activation of TRPV1 receptor by potent agonists has a biphasic effect (Caterina and Julius, 2001). Primary response includes an increase in action potential firing along with enhanced sensitivity to noxious stimuli followed by a prolonged refractory period of receptor desensitization. The ability of TRPV1 agonists to cause a prolonged desensitization of sensory neurons to subsequent noxious stimulations has been demonstrated in animal studies (Avelino et al., 2006; Tang and Nakata, 2008) and laid a foundation for initiating clinical trials. Intravesical instillations of vanilloids were tested for the treatment of overactive bladder and pain relieve in patients diagnosed with interstitial cystitis/painful bladder syndrome (IC/PBS). However, RTX administered intravesically did not improve overall symptoms (pain, frequency, urgency, nocturia) in IC/PBS group (Chen et al., 2005; Payne et al., 2005) which may suggest the complexity of the mechanisms and pathways activated by TRPV1 and provided a warrant for the present investigation.

This study tested the hypothesis that activation of TRPV1 signaling pathways by direct inflammatory insult in one of the pelvic organs could trigger the release of proinflammatory neuropeptides SP and CGRP in adjacent pelvic organs via convergent afferent “wiring” in the nervous system. Specifically, the goals of our study were: a) to determine the effects of TRPV1 receptor desensitization in the colon on the release of SP and CGRP in the urinary bladder; b) to follow time-dependent changes in SP and CGRP gene and protein expression in the nervous system as well as in the pelvic viscera after experimentally induced colitis; and c) to test whether desensitization of intracolonic TRPV1 receptors prior to the induction of colonic inflammation could affect the release of pro-inflammatory neuropeptides in the urinary bladder.

Materials and Methods

Animals and experimental groups

Male Sprague–Dawley rats (N=71, Charles River Laboratories, Malvern, PA, 170–200 g, 9–10 weeks of age) were used in this study. Rats were housed two per cage, with free access to food and water and maintained on a 12-h light/dark cycle. All protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and adhered to the guidelines for experimental pain in animals published by the International Association for the Study of Pain. Animals were divided into four experimental groups: 1- control; 2 – resiniferatoxin (RTX, TRPV1 agonist) instillations in the colon; 3 - colonic inflammation induced by TNBS (2,4,6-trinitrobenzene sulfonic acid); 4 - RTX treatment (colon) followed by the induction of colonic inflammation. Control group of rats received saline enema, RTX group received 10^{-7} M of RTX in saline, and TNBS group included TNBS containing enema (colon). Rats in the fourth experimental group were pretreated with RTX followed by TNBS instillation 2 days later. Animals from each group were sacrificed at 3, 5, and 30 days after the last treatment. Tissue samples from the distal colon, urinary bladder (detrusor), lumbosacral L6-S2 spinal cord and L6-S2 dorsal root ganglia (DRG) were isolated from each animal and snap-frozen in liquid nitrogen for myeloperoxidase (MPO) assay, RNA and protein isolation.

TNBS model of experimental colitis

Colonic inflammation was induced by administration of TNBS in 50% ethanol solution. The TNBS solution was prepared fresh before the instillation procedure. For 1 ml of the final solution 0.25 ml of TNBS (5 % w/v, Sigma) 0.25 ml of water and 0.5 ml of ethanol (C_2H_5OH , Sigma) were mixed. The final concentration of TNBS was 12.5 mg/ml. Rats were fasted for 24 hours before instillation procedure to provide better access to the colonic lumen. Animals were briefly anesthetized with isoflurane (VEDCO Inc., St. Joseph, MO), a 7–8 cm long catheter made of polyethylene tubing and attached to a 1 cc syringe was inserted into the rat colon for enema administration. After instillation procedure, an animal was held by the tail to avoid any spill of instilled liquid. To assess the severity of developed inflammation, the daily Disease Activity Index (DAI) was calculated followed by MPO assay as previously described (Malykhina et al., 2006).

MPO assay

The MPO assay was based on the method adopted from (Pothoulakis et al., 1994). Briefly, tissue samples (colon and urinary bladder) were homogenized in 2 ml of phosphate buffer (PB, pH=6.0, 50 mM) with HTAB (hexadecyltrimethylammonium-bromide, 0.5 % Sigma, St. Louis). 1 ml of each homogenate was transferred to eppendorf tubes and underwent 3 cycles of freeze-thawing followed by sonication for 10 s. After 15 min of centrifugation at $12000\times g$ ($4^{\circ}C$), supernatant was collected and used to determine the total protein concentrations for all control and experimental groups. The total protein used for the assay was 200 $\mu g/ml$ for all tissues. The assay was started in a 96-well microplate using human MPO (Alpeco, Salem, NH) as a standard, 25 μl of total protein from each sample (200 $\mu g/ml$, colon and urinary bladder)

and 25 μ L of 3,3',5,5'-tetramethylbenzidine (TMB; dissolved in DMSO, 1.6 mM), and incubated at 37°C for 5 min. 100 μ L H₂O₂, dissolved in PB (0.05 M Na₃PO₄, 0.5% HETAB, pH 5.4) in a final concentration of 0.003% v/v was added, and the plate was incubated at 37°C for 5 min. The reaction was stopped by adding 100 μ L of H₂SO₄ (4 M). The optical density value of each sample was read at 450 nm on a Multiscan EX spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and converted into MPO values by using curves obtained from a standard sample of human MPO. Fold increase in MPO activity corresponded to the level of developed inflammatory reaction. MPO levels were compared for each organ separately between control and all experimental groups by two-way ANOVA followed by group comparison between control and experimental groups using Bonferroni's t test method (Systat Software Inc., San Jose, CA). The data is expressed as the mean $\pm$ standard error of the mean (S.E.M).

RNA and Protein Extraction

Total RNA was extracted from tissue samples using Trizol reagent and following the protocol from Invitrogen (Carlsbad, CA). Frozen tissue samples were homogenized in Trizol, diluted by chloroform and then centrifuged for 15 min at 4°C. Aqueous phase containing RNA was transferred to the fresh Eppendorf tube, and RNA was precipitated with isopropyl alcohol (0.5 ml per 1 ml of TRIZOL), incubated at room temperature for 10 min and centrifuged at 12 000 $\times$ g for 10 min. Supernatant was removed, RNA was washed with 75% ethanol and the pellet was dissolved in RNase-free water and stored at -80°C. Concentration of RNA was determined using the Eppendorf BioPhotometer (Thermo Fisher Scientific, Waltham, MA).

Proteins were separated from DNA fraction after adding ethanol to the organic phase followed by centrifugation, and protein precipitation in acetone. The pellet was washed with guanidine hydrochloride in ethanol, and dissolved in 1% sodium dodecyl sulfate (SDS). Protein samples were incubated at 50°C then centrifuged to sediment any insoluble proteins. Total protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Bovine serum albumin (BSA) was used to generate the standard curve. Each protein sample was diluted 1:20 with 1% SDS. All standards and samples were run in duplicate. The absorbance was measured at 562 nm on the Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT) and data analysis was performed using Gen5 Microplate Data Collection & Analysis Software (BioTek Instruments, Winooski, VT).

Quantitative RT-PCR

First strand cDNA was synthesized from 2 μ g of the total RNA with 200 U of the superscript III reverse transcriptase (Invitrogen, #18080-051) in the presence of 40 U RNaseOUT, 10mM DTT, dNTP mix at 10 mM and 50 μ M of Oligo (dT)₂₀. Real time PCR was run on 7500 Fast Real Time PCR system (Applied biosystems, Foster City, CA). Each reaction contained 2 μ L (1:10 dilution) sample of the cDNA product mixed with SYBR-Green Mix (SABiosciences, #PA-012) up to total volume of 25 μ L. Primer sets for CGRP, SP and GAPDH were designed based on the sequences in the GeneBank (NCBI) database using Sci Tools Primer Quest (Integrated DNA Technologies) and UCSC In-Silico PCR and synthesized by integrated DNA Technologies. Primer sequences are presented in Table 1 and primers' specificity was confirmed on 2% agarose gel by RT-PCR (Figure 1). Each PCR amplification consisted of heat activation for 2 min at 50°C, and for 10 min at 95°C, followed by 40 cycles 95°C for 15 s, 60°C for 1 min. Dissociation stage was added at 95°C for 15s, 60°C for 20 s, 95°C for 15 s and 60°C for 15 s. All samples were run in duplicate. The PCR product (double stranded DNA) was measured during the extension step of each cycle as a fluorescent signal coming from SYBR Green interchelated into the double stranded DNA. For each sample (control and experimental groups) the Ct (threshold) value was detected. The fidelity of the product was assessed by checking the melt curve and confirmed by electrophoresis at the end of the reaction

(data not shown). Results were analyzed using the obtained Ct values for all tissue samples (Livak and Schmittgen, 2001). For example, the Ct value for CGRP in control group was subtracted from that of GAPDH (housekeeping gene) to obtain the Δ Ct value. The same subtraction was done in all treated groups to obtain the Δ Ct. To compare the changes in expression levels of CGRP and SP between control and experimental tissues these two values were subtracted to obtain the $\Delta\Delta$ Ct. The fold change was measured as $2^{-\Delta\Delta\text{Ct}}$.

ELISA

The levels of SP in harvested tissues were measured using a rat Substance P ELISA kit (MD Biosciences Inc., St. Paul, MN) according to the manufacturer's instructions. The concentration of the total protein for SP assay was 200 μ g/ml. Briefly, a 96-well microplate was loaded with 25 μ l of primary antibody specific for rat SP. 50 μ l of each sample and 50 μ l of the standard SP dilutions (as a control) mixed in the assigned wells in duplicate followed by the addition of biotinylated SP into each well except the Blank. The plate was incubated for 2 h at RT, and then washed 6 times with provided in the kit wash buffer. Subsequently, 100 μ l of biotinylated anti-SP antibody solution was added, incubated for 1 h, and then washed four times. 100 μ l of streptavidin-horseradish peroxidase conjugate solution was added to each well except chromogen blank, incubated for 1 hour, and washed again. After that, 100 μ l of substrate solution provided in the kit was added to each well and the plate was incubated for 1 hour at room temperature. The reaction was stopped with 2N HCl and the optical density values were read at 450 nm using a Biotek Synergy 2 plate reader (BioTek Instruments Inc., Winooski, VT).

Tissue CGRP levels were measured using CGRP Enzyme Immunoassay (EIA) kit for rats (ALPCO Diagnostic, Salem, NH) according to the manufacturer's instructions. For CGRP assay the total protein concentration was 400 μ g/ml for the colon and bladder samples and 300 μ g/ml for the spinal cord and DRG. Briefly, a 96-well microplate was coated with 100 μ l of antibody specific for rat CGRP mixed with 100 μ l of EIA buffer provided in the kit plus 100 μ l of standard and sample aliquots. Each sample and standards were run in duplicate. Plate was incubated for 16–20 h at RT followed by the aspiration of the samples and washing them 3 times with wash buffer. Subsequently, 200 μ l of Ellman's reagent was added to each well incubated for 30–60 min in the dark and then read between 405 and 414 nm using a Biotek Synergy 2 plate reader (BioTek Instruments Inc., Winooski, VT). The protein concentrations of both SP and CGRP were statistically analyzed using one-way repeated measures ANOVA followed by group comparison between control and experimental groups using Bonferroni's t test method (Systat Software Inc., San Jose, CA). All data are expressed as the mean $\pm$ standard error of the mean (S.E.M).

Immunohistochemistry

Cross-sections (5 μ M) of the bladder and colon from rats (control and RTX treated group) were made from paraffin blocks. Tissue sections were put into xylene to remove paraffin and descending concentrations of alcohol (100%, 95%, 70%, 30% and 1X PBS) before antigen retrieval treatment (10 mM sodium citrate, pH 6.0, incubated at 95°C for 10 minutes). The sections were kept in blocking solution, 3 % bovine serum albumin (BSA), for 30 minutes. After blocking, the sections were incubated with primary antibody for CGRP (Sigma, St. Louis, MO) or SP (Sigma, St. Louis, MO) at the dilution of 1:400 overnight at 4°C. Sections were washed three times in PBS and treated with secondary anti-rabbit Alexa Fluor 488 antibody (Invitrogen, Carlsbad, CA) at a dilution of 1:400 for 1 hour. Before starting double staining, sections went through 3 times PBS washing and then were kept for other primary antibody incubation, monoclonal neuronal marker protein gene product (PGP) 9.5 (Genway Biotech Inc., San Diego, CA) at a dilution of 1:300 for 2 hours. After washing with PBS, the sections were incubated with secondary antibody anti-mouse IgG-cy3 (Sigma, St. Louis, MO) for 1

hour at a dilution of 1:400. At last, sections were mounted on slides with a drop of mounting medium (Vector Laboratories, Burlingame, CA). Slides were viewed under a Confocal Laser Scanning Biological Microscope (Olympus, Center Valley, PA). Images were captured and analyzed using Fluoview FV1000 software (Olympus, Center Valley, PA). A negative control to confirm the specificity of immunostaining was prepared using non-immune rabbit and mice serum in place of the primary antibody.

Chemicals

TNBS and RTX were purchased from Sigma Aldrich (St. Louis, MO). Trizol kit for RNA/protein isolation and SuperScript III Reverse Transcriptase were bought from Invitrogen (Carlsbad, CA). SP ELISA kit was purchased from MD Biosciences (St. Paul, MN) and CGRP ELISA kit from ALPCO Diagnostics (Salem, NH). BCA kit was obtained from Thermo Fisher Scientific (Waltham, MA).

Results

Desensitization of TRPV1 receptors prior to the induction of experimental colitis prevents the development of inflammation in the distal colon

MPO assay is a widely used method of quantification of the severity of inflammatory insult and is based on the assessment of an enzyme found mostly in neutrophils which are tremendously increased in number in the inflamed tissues (Krawisz et al., 1984). Desensitization of colonic TRPV1 receptors did not affect MPO activity in the pelvic organs. However, acute colitis caused 2.7-fold increase in MPO level at day 3 post-TNBS ($p \leq 0.05$ to control) followed by 7.6-fold increase at day 5 ($p \leq 0.05$, Fig. 2A) with recovery to control values one month after TNBS. RTX treatment prior to the induction of experimental colitis completely suppressed the development of colonic inflammation during the entire period of the study (Fig. 2A). Since neither RTX nor TNBS affected the MPO level in the urinary bladder, it can be concluded that the urinary bladder did not develop any signs of acute inflammatory reaction (Fig. 2B).

Colonic administration of TRPV1 agonist triggers Substance P gene and protein expression in the urinary bladder

This set of experiments was conducted to address the question whether desensitization of TRPV1 receptors in the distal colon could affect the release of SP in the urinary bladder. Colonic administration of RTX did not affect mRNA nor protein expression during the entire period of observation in the distal colon except 2-fold increase in SP mRNA at day 30 (Fig. 3A, $n=4$, $p \leq 0.05$). Unlike in the colon, RTX caused 3-fold up-regulation of SP mRNA in the urinary bladder at day 5 ($n=7$, $p \leq 0.05$) followed by more prominent increase of 35-fold at day 30 (Fig. 3C, $n=5$, $p \leq 0.05$).

We performed immunohistological staining to identify the major sites of SP release in the bladder after RTX treatment. Colonic RTX increased the intensity of SP labeling in the urothelium, lamina propria and smooth muscle bundles of the urinary bladder compared to the control group (data not shown). To test the hypothesis whether the sites of SP release are located close to the nerve fibers coursing in the bladder wall, we co-stained the tissues for SP and neuronal marker protein gene product (PGP) 9.5. PGP 9.5 is a developmentally regulated neuron- and neuroendocrine cell-specific ubiquitin carboxy-terminal hydrolase (UCHL1) expressed throughout the mammalian central and peripheral nervous systems (Day and Thompson, 2009). Panel D in Fig. 4 shows the results of SP and PGP9.5 co-localization and a number of cell bodies along with fibers which were stained for both markers (accented by arrows).

Activation of TRPV1 receptors by RTX in the distal colon affected SP content not only in the pelvic organs but also in the lumbosacral spinal cord. mRNA level was increased at day 3 by 54% (Fig. 5A, $n=4$, $p\leq 0.05$) followed by an increase in protein expression by day 30 (60%, $n=5$, $p\leq 0.05$, Fig. 5B). No significant changes were observed in SP mRNA nor protein level in L6-S2 DRG neurons after RTX administration (Fig. 5C and D).

Experimental colitis stimulates persistent release of SP in the nervous system and affected pelvic organs

In the colon, TNBS-induced colitis led to up-regulation of SP mRNA by more than 2-fold from day 5 ($n=7$, $p\leq 0.05$) to day 30 (Fig. 3A, $n=5$, $p\leq 0.05$). Protein expression of SP was also significantly elevated during acute (3 and 5 days) colonic inflammation followed by significant down-regulation one month after inflammatory insult (Fig. 3B). In the urinary bladder, experimental colitis triggered 15.8-fold up-regulation of SP mRNA one month after TNBS ($p\leq 0.05$, Fig. 3C) whereas an increase in SP protein was observed during acute phase (3 and 5 days) followed by a decrease at 30 days post-TNBS (Fig. 3D, $p\leq 0.05$ to control at all time points).

Acute experimental colitis increased both mRNA and protein expression level in the lumbosacral spinal cord by day 3 post-TNBS ($n=5$, $p\leq 0.05$, Fig. 5A and B) with recovery to control levels at later time points. In primary sensory neurons, the amount of SP protein was increased from 1.33 ± 0.36 ng/ml (control) to 4.31 ± 0.42 ng/ml (3 days post-TNBS, $p\leq 0.05$, Fig. 5D) and stayed elevated almost 2-fold by day 5 ($p\leq 0.05$, Fig. 5D). One month after TNBS-induced colitis protein concentration of SP in L6-S1 DRG neurons was significantly decreased to almost unidentifiable level (Fig. 5D).

Desensitization of colonic afferents with RTX prevents the release of SP in the bladder triggered by experimental colitis

In the colon, pretreatment with RTX did not eliminate the effects of TNBS-induced colitis on SP content compared to TNBS group (Fig. 3A and B). However, in the urinary bladder, RTX administration before the induction of experimental colitis decreased mRNA concentration of SP from 2.39 ± 0.9 fold to 0.75 ± 0.3 fold at day 5 ($p\leq 0.05$ to TNBS group) followed by an increase to TNBS levels at day 30. RTX pretreatment completely prevented an increase in SP protein in the urinary bladder with SP levels close to control during the entire period of the study (Fig. 3D).

In the lumbosacral spinal cord, both mRNA and protein levels of SP were still high in the RTX/TNBS group at day 3 ($n=5$, $p\leq 0.05$, Fig. 5A and B) and then returned to control levels at later time points. In primary sensory neurons, mRNA level of SP was not affected by RTX pretreatment. However, at the protein level double treatment significantly reduced inflammation-related increase in the SP protein during acute inflammation (3 and 5 days) reaching control levels ($p\leq 0.05$ to TNBS group, Fig. 5D).

RTX instillation in the rat colon up-regulates gene and protein expression of CGRP in the urinary bladder

Desensitization of TRPV1 receptors by RTX led to a significant up-regulation of CGRP mRNA in the distal colon. Thus, at 5 days after the treatment the level of CGRP mRNA was increased by 95% ($n=7$, $p\leq 0.05$ to control) and reached 2.2-fold by day 30 (Fig. 6A, $n=5$, $p\leq 0.05$). The concentration of CGRP protein in the colon was not changed by RTX at days 3 and 5, but was elevated at day 30 (14.5 ± 3.0 pg/ml in the control group vs 23.03 ± 2.0 pg/ml in experimental group, Fig. 6B, $p\leq 0.05$). Surprisingly, the effects of colonic RTX on both gene and protein expression of CGRP in the urinary bladder were more substantial than in the colon. At day 5 mRNA level of CGRP was enhanced by 4.3-fold (Fig. 6C, $n=7$, $p\leq 0.05$ to control) followed

by 28-fold increase at day 30 post-RTX ($n=4$, $p\leq 0.05$). The protein concentration of CGRP in the urinary bladder was also increased one month after RTX application reaching 69.1 ± 4.9 pg/ml vs 11.7 ± 1.4 pg/ml in the control group ($p\leq 0.01$, Fig. 6D).

Immunohistochemical staining for CGRP and PGP9.5 in the urinary bladder after RTX treatment showed the pattern similar to one observed for SP (Fig. 7). A number of single cells along with fibers located mostly in lamina propria were co-stained for both PGP 9.5 and CGRP (pointed by arrows). RTX application did not cause any changes in CGRP expression at both gene and protein level in the lumbosacral spinal cord and DRG (Fig. 8).

Increase in CGRP expression in the pelvis occurs after recovery from acute colonic inflammation

TNBS induced colitis did not affect CGRP mRNA in the colon (Fig. 6A). However, protein concentration of CGRP in the colon followed phasic pattern and included a decrease at day 3 post-TNBS (8.06 ± 1.04 pg/ml vs 17.1 ± 2.7 pg/ml in control group, $p\leq 0.05$) with recovery to control values at day 5 and then a significant increase up to 33.8 ± 4.6 pg/ml (vs 14.5 ± 3.0 pg/ml in the control group, $p\leq 0.05$, $n=4$, Fig. 6B) at day 30 post-TNBS. Colonic inflammation did not affect gene nor protein expression of CGRP in the urinary bladder during acute colitis (3 and 5 days post-TNBS). However, at day 30 mRNA level was increased by 17.8 $\pm$ 6.9 fold (Fig. 6C, $n=5$, $p\leq 0.05$ in parallel with 4-fold increase in CGRP protein (Fig. 6D, $n=5$, $p\leq 0.01$).

Acute experimental colitis also triggered a 2-fold increase in CGRP gene expression at day 3 post-TNBS in the lumbosacral spinal cord (Fig. 8A, $n=7$, $p\leq 0.05$) followed by 60% decrease at day 5 compared to control values ($n=7$, $p\leq 0.01$). Protein concentration of CGRP in the spinal cord has been significantly lower by 45–65% within one month after the onset of colonic inflammation (Fig. 8B). mRNA level of CGRP in primary sensory neurons fluctuated during the entire period of observation without significant changes, however, protein expression was substantially diminished during acute inflammation (3 and 5 days, Fig. 8) followed by an increase at day 30 (170.7 ± 7.8 pg/ml vs 113.3 ± 18.3 pg/ml in age-matched control group, $p\leq 0.01$, Fig. 8D).

Desensitization of TRPV1 receptors in the distal colon prevents the release of CGRP from the spinal cord and DRG without changes in the urinary bladder

Desensitization of TRPV1 receptors in the colon prior to the induction of acute colitis reduced mRNA by 59% (Fig. 6A, $p\leq 0.01$) and protein (from 17.1 ± 2.7 pg/ml to 6.2 ± 1.7 pg/ml, Fig. 6B, $p\leq 0.05$ to control) concentrations of CGRP in the distal colon at day 3 after TNBS instillation. One month after TNBS-induced colitis both parameters returned to control levels. Unlike in the colon, RTX pretreatment did not cause any changes in mRNA nor protein levels of CGRP in the urinary bladder during acute experimental colitis (days 3 and 5, Fig. 6C and D). However, at day 30 post-TNBS both mRNA and protein concentration of CGRP were elevated reaching the same values as in the TNBS group (Fig. 6C and D). This may suggest that RTX pretreatment cannot prevent the release of CGRP in the urinary bladder and other mechanisms should be considered.

In the lumbosacral spinal cord RTX pretreatment led to 68% decrease in CGRP mRNA at day 5 (Fig. 8A, $p\leq 0.01$ to control group, n/s to TNBS group) followed by recovery to control values at later time point. However, at the protein level, RTX pretreatment completely abolished a decrease in CGRP protein induced by both acute and subsided colitis ($p\leq 0.05$ to TNBS group for all time points, Fig. 8B). CGRP mRNA level in DRG was not affected by double treatment during both acute and subsided inflammation (Fig. 8C). At the protein level, pretreatment with RTX caused a reduction in CGRP release from the DRG neurons during acute phase of

experimental colitis (3 and 5 days) followed by subsequent increase of CGRP protein at day 30 (172.6 ± 23.1 pg/ml vs 113.3 ± 18.3 in control group, $n=5$, $p \leq 0.05$).

Discussion

We have performed an extensive evaluation of the effects of colonic inflammation with and without desensitization of colonic TRPV1 receptors on gene and protein expression of pro-inflammatory neuropeptides SP and CGRP in the urinary bladder, lumbosacral spinal cord and dorsal root ganglion neurons concurrently over a period of one month. The results of our study provide evidence that cross-sensitization in the pelvis induced by colonic inflammation is conveyed, at least in part, via TRPV1-related pathways and includes the development of neurogenic inflammation in the pelvis.

One of the major findings of our study was an up-regulation of SP and CGRP in the urinary bladder after colonic application of RTX. The changes in neuropeptide mRNA were observed starting from day 5 whereas protein expression was increased one month after the treatment. Direct effects of TRPV1 activation by potent agonists on the release of tachykinins and CGRP from the afferent terminals are well documented. Systemic administration of SP has been shown to induce dose-dependent increases in vascular permeability in various segments of the lower urinary tract - bladder dome and neck, proximal urethra, ureters (Abelli et al., 1989). Intravesical application of RTX also caused a significant decrease in the number of CGRP and SP immunoreactive fibers in the muscular layer and the mucosa by 20% (Avelino et al., 2002). Delay in protein expression following mRNA changes observed in our experiments could be due to several reasons. First, gene expression could be affected and detected earlier than alterations in protein concentration. Second, there is a possibility that initial massive release of neuropeptides in the pelvis occurred within first 3 days after RTX application and was missed due to our experimental design. Subsequently, release of SP/CGRP from afferent terminals triggered mRNA up-regulation followed by secondary increase in expression and/or release of SP/CGRP in the urinary bladder at later time. Third, the sources of neuropeptides in the colon and urinary bladder are quite different. In the colon, extensive network of enteric nervous system includes large number of intrinsic sensory neurons which synthesize SP and CGRP (Miampamba and Sharkey, 1998; Pothoulakis et al., 1994) along with extrinsic DRG neurons, whereas in the urinary bladder the major source of neuropeptides is their release from the primary afferents due to limited number of intrinsic sensory neurons (Birder et al., 2010; Saban et al., 2008).

Gene and protein expression of SP and CGRP may be also affected by translation processes occurring in the distal parts of peripheral axons in the bladder wall. A number of recent findings established that axons of sensory neurons contain mRNAs (Zheng et al., 2001; Willis et al., 2005) and small proteins (e.g. Staufen2 and FMRP) that bind mRNA and transport them to the peripheral sites (Price et al., 2006; Price and Flores, 2007). Translation in axons has been a controversial issue and the modern view is very different from the classical theory summarized in (Alvarez et al., 2000; Giuditta et al., 2002; Kiebler and Bassell, 2006; Lin and Holt, 2008; Steward and Schuman, 2003). Some studies suggest that axons are capable of about 10% of the protein synthesis capability of cell bodies, and this axonal synthesis is largely of cytoskeletal proteins (Zheng et al., 2001). In addition, there is a significant overlap of signaling pathways activated by released neuropeptides that affect TRPV1 channels and *vice versa*. For example, at the cellular level, SP release is associated with the activation of MAP kinases, PKC and COX-2 in cultured DRG neurons (Tang et al., 2007). Likewise, activation of PLC, protein kinase A (PKA), PKC, the IP3-dependent calcium release, and COX is involved in the sensitization and activation of TRPV1 (Chuang et al., 2001; Premkumar and Ahern, 2000; Tang et al., 2004; Tang et al., 2006; Tang and Nakata, 2008). Additional studies are warranted to clarify the mechanisms of neuropeptide/TRPV1 cross-talk and its role in afferent signaling.

Unlike in the pelvic viscera, intracolonic RTX did not influence gene nor protein expression of neuropeptides in the lumbosacral segments of the spinal cord and respective DRG except an increase of SP in the spinal cord. It is clear that there is a time lapse between activation of TRPV1 on peripheral terminals and translation in the cell bodies of primary and secondary sensory neurons. In our experiments pelvic organs and neural tissues were extracted simultaneously from the same animals starting at day 3 and some points of phasic changes in neuropeptide content could have been missed if occurred earlier or between the selected time points. Additionally, the afferent signal from the colon and bladder was probably “diluted” in the lumbosacral spinal cord and DRG by irrelevant neurons receiving input from other visceral and large somatic fields that send projections to the same segments of the spinal cord and DRG.

We performed double immunohistochemical staining of the bladder for SP and CGRP with neuronal/neuroendocrine marker PGP 9.5 to determine the main sites of neuropeptide release induced by RTX. In addition to labeled afferent fibers, a number of double labeled cells with neuron-like morphology and clearly visible nuclei were detected in the lamina propria of the bladder (Fig. 4 and 7). Afferent fibers identified by neuropeptide immunoreactivity for CGRP and SP were previously detected throughout the bladder wall (Gabella et al., 1997) from the serosal layer to the lamina propria, including a dense suburothelial (Fowler et al., 2008) and muscle (Avelino et al., 2002) plexuses. Intravesical instillation of RTX also decreased the number of PGP 9.5 and TRPV1 immunoreactive nerve fibers in the human bladder with higher intensity of staining in patients with spinal neurogenic detrusor overactivity (Brady et al., 2003). Based on the published results, PGP 9.5 does not label immune cells like macrophages, neutrophils or mast cells (Day and Thompson, 2009; Thompson et al., 1983; Wilson et al., 1988; Barreau et al., 2008). We suggest that the labeled cells have either neuronal or neuroendocrine origin and may include interstitial cells of Cajal (McCloskey, 2010) or intrinsic sensory neurons. Immunohistochemical studies of the human urinary bladder and bladder neck determined that up to 54% of bladder intrinsic neurons are positive for CGRP and SP (Smet et al., 1996). One report showed that PGP9.5 can label fibroblasts (Olerud et al., 1998) and a number of publications mentioned the presence of myofibroblasts in lamina propria of the bladder (Blyweert et al., 2004; Eyden, 2009; Wiseman et al., 2003). Further studies need to be performed to clarify the nature of the cells expressing SP/CGRP in the urinary bladder.

TNBS-induced colitis triggered a substantial increase in gene and protein expression of SP and CGRP in the urinary bladder during both acute and recovery phases. In general, the observed effects of colonic inflammation were similar to those evoked by RTX administration but more prominent, especially, at the levels of the spinal cord and DRG. This provides evidence that transmission of nociceptive signal during inflammation occurs, at least in part, via TRPV1 signaling pathways. Our results are in line with the previously published data which showed that the TRPV1 receptor initiates and maintains colonic hypersensitivity induced by neonatal colon irritation in rats (Winston et al., 2007) and plays a role in mechanical and chemical visceral hyperalgesia following experimental colitis (Miranda et al., 2007). The underlying mechanisms include decrease of SP protein after experimental colitis in L4–S4 DRG neurons projecting to the colon (directly inflamed) (Reinshagen et al., 1995) and also significantly increased expression of CGRP in L1 and S1 bladder DRG neurons by 23% and 11%, respectively (directly unaffected by inflammatory insult) (Qiao and Grider, 2007). Our data concur with the results that peripheral inflammation is associated with enhanced release of SP in the dorsal horn of the spinal cord. Studies using *in vivo* microdialysis with a highly sensitive radioimmunoassay to monitor SP immunoreactivity in the dorsal horn showed that perfusion of the microdialysis probe with capsaicin induced a significant increase of SP in microdialysate (Warsame et al., 2004). One of the suggested mechanisms is that capsaicin stimulates SP release in a concentration-dependent manner from spinal cord synaptosomes (Schmid, 1998). Although antinociception is triggered by the initial release of SP, it is not dependent on a persistent decrease in SP release or content (Lin et al., 2007).

Desensitization of TRPV1 receptors in the colon prior to the onset of experimental colitis had differential effects on gene and protein expression of SP and CGRP. Release of SP protein in the bladder, spinal cord (30 days) and DRG triggered by TNBS was significantly reduced by pre-treatment with RTX whereas the fluctuations in CGRP content after double treatment were substantially eliminated in the spinal cord and DRG compared to the pelvic organs. There is a number of important factors that convey the neuropeptide effects including content, transport to nerve terminals (peripheral), release, rate of peptide metabolism after release, positive/negative feedback on synthesis and/or release (Lundberg et al., 1992). It was shown that at the subcellular level CGRP is often co-stored with tachykinins in large dense cored vesicles (Gulbenkian et al., 1986) providing explanation for parallel changes in neuropeptides upon noxious stimulation. However, SP does not have the storage capacity in peripheral terminals and the continual activation may be evident as a partially depleted terminal. Unlike SP, CGRP seems to be more metabolically stable than the co-released tachykinins (Le, Greves et al., 1989) and CGRP release from the central branches of afferents in spinal cord slices is Ca²⁺-dependent (Lundberg et al., 1992). These may explain the fact that the effects of RTX pretreatment do not cause the same alterations for both neuropeptides at the same time points. Protective effect of RTX pretreatment before the development of acute inflammation could be due to desensitization of peripheral afferents and internalization of TRPV1 receptors which makes the neurons less sensitive to subsequent noxious stimulation (Rigoni et al., 2003; Tang et al., 2006; Tang and Nakata, 2008).

Summarizing the effects of pelvic inflammation and desensitization of TRPV1 receptors in the pelvis, the main question is - how the activation of TRPV1 receptors in one of the pelvic organs can trigger the release of pro-inflammatory neuropeptides from the afferents innervating the neighboring structure? Release of SP and/or CGRP in the urinary bladder after either desensitization of TRPV1 receptors in the colon or TNBS-induced colonic inflammation is conveyed mainly via convergent neural circuits that are well addressed and summarized in several recent reviews (Brumovsky and Gebhart, 2009; Malykhina, 2007; Ustinova et al., 2010). The first route includes direct activation of a convergent bladder-colon sensory neuron in DRG followed by the signal transmission via a collateral capsaicin-sensitive terminal to the urinary bladder (axon-reflex mechanism). We previously characterized a subpopulation of DRG neurons innervating both the colon and the urinary bladder and showed that visceral inflammation causes prolonged hyperexcitability of these convergent cells (Malykhina et al., 2006). A combination of tracing and multiple color immunofluorescence revealed that 69% of rat DRG innervating the urinary bladder express the vanilloid receptor TRPV1. 50–60% of TRPV1-positive DRG neurons express SP and CGRP (Hwang et al., 2005; Price and Flores, 2007).

The second mechanism by which the release of neuropeptides from afferent terminals in the bladder includes retrograde activation of a capsaicin-sensitive afferent neuron innervating the colon that has a projection to the convergent second order neuron in the dorsal horn of the spinal cord. Further, nociceptive information from the convergent neuron in the spinal cord could be transmitted to a bladder projecting DRG neuron via dorsal root reflexes (DRR mechanism) (Lin et al., 1999; Rees et al., 1995) Li, D et al (Li et al., 2008) recently studied the release of CGRP in the periphery driven by DRR following activation of TRPV1. This effect was partially but significantly inhibited by dorsal rhizotomy. Other studies determined that application of TRPV1 antagonist can significantly inhibit both capsaicin-induced CGRP release from slices of rat dorsal spinal cord as well as the contraction of isolated guinea-pig and rat urinary bladder, and also reduce plasma extravasation in mouse urinary bladder (Rigoni et al., 2003). In addition to the aforementioned neural mechanisms underlying “neuropeptidergic” cross-talk between the colon and urinary bladder non-neural pathways involving recruitment of serum factors and/or inflammatory cells can also potentially enhance

local neurogenic inflammation in the pelvis, however, these mechanisms are yet to be investigated.

In summary, our study provides evidence that cross-sensitization is conveyed, at least in part, via TRPV1-related pathways and includes the development of neurogenic inflammation in the pelvis due to release of pro-inflammatory neuropeptides SP and CGRP after recovery from acute inflammatory insult. Understanding the molecular physiology of cross-sensitization in the pelvis has the potential to advance the generation of new pharmacological therapies for the treatment of functional pelvic disorders characterized by chronic pelvic pain and warrants further studies on the mechanisms underlying co-morbidity between genitourinary and gastrointestinal dysfunctions.

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Figure 1. Primers' specificity for SP, CGRP and GAPDH was confirmed on the agarose gel (2%) for the colon and spinal cord tissues. All primer sets yielded single bands (see methods for details).

Figure 2. Concentration of myeloperoxidase enzyme in the pelvic organs. A, MPO level in the control and three experimental groups in the distal colon. B, Concentration of MPO in the urinary bladder. *-statistical significance to control group ($p \leq 0.05$), # - $p \leq 0.001$ to control.

Figure 3.

Substance P gene and protein expression levels in the pelvic organs. A, mRNA level of SP in the colon in the control and three experimental groups measured at 3, 5 and 30 days after the last treatment (see methods for details). B, Bar chart shows the concentration of SP protein in the colon after acute and subsided colonic inflammation along with RTX group and combined treatment group. C, mRNA level of SP in the urinary bladder in the control and three experimental groups. D, Protein concentration of SP in the urinary bladder at three time points after RTX, TNBS and double treatment of the colon. *-statistical significance to control group ($p \leq 0.05$), # - $p \leq 0.05$ to TNBS group.

Figure 4.

Immunofluorescent staining of the urinary bladder 5 days after intracolonic application of RTX (60X). A, SP immunoreactivity (green). B, UCHL1/PGP9.5 neuronal marker staining (red) shows single neurons and their fibers in laminae propria of the urinary bladder. C, DAPI staining (nuclear localization), D, Overlap of SP and UCHL1/PGP9.5 immunoreactivity, E, overlap of all three channels (SP, UCHL1/PGP9.5 and DAPI). The arrowheads point to the cells with double labeling for SP and UCHL1/PGP9.5. SM – smooth muscle, LP – laminae propria, U- urothelium. Scale bar 50 μ m.

Figure 5.

Substance P gene and protein expression levels in neural tissues. A, mRNA level of SP in the spinal cord in the control and three experimental groups measured at 3, 5 and 30 days after the last treatment (see methods for details). B, Bar chart shows the concentration of SP protein in the spinal cord after acute and subsided colonic inflammation along with RTX group and combined treatment group. C, mRNA level of SP in lumbo-sacral DRG neurons in the control and three experimental groups. D, Protein concentration of SP in lumbo-sacral DRG at three time points after RTX, TNBS and double treatment of the colon. *-statistical significance to control group ($p \leq 0.05$), # - $p \leq 0.05$ to TNBS group.

Figure 6.

Calcitonin gene-related peptide gene and protein expression levels in the pelvic organs. A, mRNA level of CGRP in the colon in the control and three experimental groups measured at 3, 5 and 30 days after the last treatment (see methods for details). B, Bar chart shows the concentration of CGRP protein in the colon after acute and subsided colonic inflammation along with RTX group and combined treatment group. C, mRNA level of CGRP in the urinary bladder in the control and three experimental groups. D, Protein concentration of CGRP in the urinary bladder at three time points after RTX, TNBS and double treatment of the colon. *- statistical significance to control group ($p \leq 0.05$), # - statistical significance to control group ($p \leq 0.001$), ^ - $p \leq 0.05$ to TNBS group.

Figure 7.

Immunofluorescent staining of the urinary bladder 5 days after intracolonic application of RTX (60X). A, UCHL1/PGP9.5 neuronal marker staining (red) shows single neurons and their fibers in laminae propria of the urinary bladder. B, CGRP immunoreactivity (green). C, DAPI staining (nuclear localization), D, Overlap of CGRP and UCHL1/PGP9.5 immunoreactivity, E, overlap of all three channels (CGRP, UCHL1/PGP9.5 and DAPI). The arrowheads point to the cells with double labeling for CGRP and UCHL1/PGP9.5. DM – detrusor muscle, LP – laminae propria, U- urothelium. Scale bar 50 μ m.

Figure 8.

CGRP gene and protein expression levels in neural tissues. A, mRNA level of CGRP in the spinal cord in the control and three experimental groups measured at 3, 5 and 30 days after the last treatment (see methods for details). B, Bar chart shows the concentration of CGRP protein in the spinal cord after acute and subsided colonic inflammation along with RTX group and combined treatment group. C, mRNA level of CGRP in lumbosacral DRG neurons in the control and three experimental groups. D, Protein concentration of CGRP in lumbosacral DRGs at three time points after RTX, TNBS and double treatment of the colon. *-statistical significance to control group ($p \leq 0.05$), # - statistical significance to control group ($p \leq 0.001$), ^ - $p \leq 0.05$ to TNBS group.

Table 1

Primers for CGRP, SP and GAPDH for real time RT-PCR

Gene	Primer	Sequence	Position	Product (bp)	GeneBank access number
SP	Forward	GTTTGCAGAGGAAATCGGTGCCAA	150–173	168	M34184
	Reverse	GCATCCCGTTTGCCCATTAATCCA	294–317		
α -CGRP	Forward	AGAAGAGATCCTGTGCAACACTGCCA	244–267	94	M11597
	Reverse	GGCACAAAAGTTGTCTTCACCCACA	314–337		
GAPDH	Forward	TCCGTTGTGGATCTGACA	1564–1581	138	NM_017008
	Reverse	GAAGTCACAGGAGACAACC	1683–1701		