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Basic Study

Bladder-colon chronic cross-sensitization involves neuro-glial pathways in male mice

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Abstract

BACKGROUND

Irritable bowel syndrome and bladder pain syndrome often overlap, and are both characterized by visceral hypersensitivity. Since pelvic organs share common sensory pathways, it is likely that those syndromes involve a cross-sensitization of the bladder and the colon. The precise pathophysiology remains poorly understood.

AIM

To develop a model of chronic bladder-colon cross-sensitization and to investigate the mechanisms involved.

METHODS

Chronic cross-organ visceral sensitization was obtained in C57BL/6 mice using ultrasound-guided intravesical injections of acetic acid under brief isoflurane anaesthesia. Colorectal sensitivity was assessed in conscious mice by measuring intracolonic pressure during isobaric colorectal distensions (CRDs). Myeloperoxidase,

used as a marker of colorectal inflammation, was measured in the colon, and colorectal permeability was measured using chambers. c-Fos protein expression, used as a marker of neuronal activation, was assessed in the spinal cord (L6-S1 level) using immunohistochemistry. CX3CR1^{gfp+} mice were used to identify and count microglia cells in the L6-S1 dorsal horn of the spinal cord. The expressions of NK1 receptors and MAPK-p38 were quantified in the spinal cord using western blot.

RESULTS

Visceral hypersensitivity to CRD was observed after the intravesical injection of acetic acid *vs* saline ($P < 0.0001$). This effect started one hour post-injection and lasted up to seven days post-injection. No increased permeability or inflammation was shown in the bladder or colon seven days post-injection. Visceral hypersensitivity was associated with the increased expression of c-Fos protein in the spinal cord ($P < 0.0001$). In CX3CR1^{gfp+} mice, intravesical acetic acid injection resulted in an increased number of microglia cells in the L6-S1 dorsal horn of the spinal cord ($P < 0.0001$). NK1 receptor and MAPK-p38 levels were increased in the spinal cord up to seven days after injection ($P = 0.007$ and 0.023 respectively). Colorectal sensitization was prevented by intrathecal or intracerebroventricular (ICV) injections of minocycline, a microglia inhibitor, by ICV injection of CP-99994 dihydrochloride, a NK1 antagonist, and by ICV injection of SB203580, a MAPK-p38 inhibitor.

CONCLUSION

We describe a new model of cross-organ visceral sensitization between the bladder and the colon in mice. Intravesical injections of acetic acid induce a long-lasting colorectal hypersensitivity to distension, mediated by neuroglial interactions, MAPK-p38 phosphorylation and the NK1 receptor.

Key Words: Cross-organ sensitization; MAPK-p38; Microglia; NK1 receptor; Pain; Visceral hypersensitivity

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Core Tip: A model of chronic cross-organ visceral sensitization in mice was developed using ultrasound-guided intravesical injections of acetic acid. Visceral hypersensitivity to colorectal distension was observed as early as one hour post-injection and lasted up to seven days. Visceral hypersensitivity was associated with an increased expression of c-Fos protein in the spinal cord. NK1 receptor and MAPK-p38 Levels were upregulated in the spinal cord seven days post-injection. Colorectal sensitization was prevented by intrathecal or intracerebroventricular (ICV) injections of minocycline, a microglia inhibitor, by ICV injection of CP-99994, a NK1 antagonist, and by ICV injection of SB203580, a MAPK-p38 inhibitor.

INTRODUCTION

Irritable bowel syndrome (IBS) and bladder pain syndrome (BPS) are two functional disorders that affect the gastrointestinal and the urinary tract, respectively[1,2]. Their prevalence in the general population is 4.6%[3] and 4.2%[4] respectively, and recent studies have evidenced a strong overlap between both syndromes[1,2]. Indeed, BPS is found in 40%-60% of IBS patients[5], and IBS is observed in 25.4%-38.6% of BPS patients[1,2]. Both syndromes are characterized by visceral mechanical hypersensitivity at the urinary tract level for BPS, and at the intestinal level for IBS[6,7]. The involvement of several mechanisms has been suggested in the onset and/or the maintenance of visceral hyperalgesia, including urothelial and/or intestinal epithelial permeability, mucosal immune activation and altered brain-gut interaction[8].

Despite increasing knowledge of the pathophysiology of IBS and BPS, limited mechanistic data is available in the context of BPS-IBS overlap. Based on the fact that pelvic organs share common sensory pathways, a few studies have offered evidence that cross-sensitization between the bladder and the colon may explain sensitization of both organs[9]. This may involve primary extrinsic afferents or central sensitization, both at the spinal and supraspinal levels[10]. All these studies involved acute sensitization models, however, often in anesthetized animals[11], and therefore, there is not yet any data regarding the chronicization of pelvic organ cross-sensitization.

The role of spinal glia has recently been highlighted in the sensitization of the bladder or colon[12], and even in cross-organ sensitization between both organs[13]. The role of spinal glia, however, has never been demonstrated in the maintenance of such sensitization using models of bladder-colon cross-sensitization induced chronic visceral hyperalgesia. The aims of our study were therefore to develop a model of chronic cross-organ sensitization between the bladder and the colon, and to investigate the mechanisms involved in the development and persistence of this cross-sensitization.

MATERIALS AND METHODS

Ethics

The experiments were carried out in accordance with the ethical guidelines of the International Association for the Study of Pain[14], and in accordance with the guidelines of the French Ministry of Agriculture and Fisheries (decree No. 874848). Our protocol was approved by the local Ethics Committee for Animal Experiments (CENOMEXA No: N/02-01-13/02/01-16).

Animals

Adult male wild type C57Bl/6 mice (Janvier Laboratories, Le Genest-Saint-Isle, France) and transgenic mice expressing the green fluorescent protein (GFP) on the fractalkine receptor (CX3CR1) of microglial cells (CX3CR1^{gfp+} mice; INSERM Laboratory U1239, Dr. David Vaudry Team, Mont Saint Aignan, France[15]), all eight weeks old on the day of the experiment (weight range: 22-26 g), were used. The animals were randomized by their weight in several cages, with five mice per cage, stabled on a 12 h light/dark cycle in an animal housing facility free of specific pathogenic organisms and maintained at room temperature (22 ± 2 °C). The animals received a standard diet (RM1 diet; SDS, Witham, Essex, United Kingdom). Drinking water and food were available *ad libitum*. Each manipulation or experiment took place after at least one week of acclimatization to the conditions of stabling. All animals were euthanized by cervical dislocation after anaesthesia with ketamine (100 mg/kg; Imalgene 1000, Merial, Lyon, France) and xylazine (10 mg/kg; Rompun® 2%, Bayer, Berlin, Germany), administered intraperitoneally before tissue collection.

Study design

The animal protocol was designed to minimize pain or discomfort to the animals. Eight series of mice were used in this work. Each set was comprised of different groups of mice, and each group was formed of 5-8 mice. The first series was used to develop the cross-organ sensitization model. Once the model was validated, a second series was used to analyse inflammatory parameters in the colon and the bladder, and to assess colonic and bladder permeabilities. We then focused on the central nervous system,

especially at lamina I and II of the dorsal horn at the L6-S1 segment, where pelvic extrinsic primary afferent neurons (EPANs) form synapses with spinal neurons. Our third series assessed the neuronal activity in this model using c-Fos expression, and inflammatory parameters were measured in the spinal cord of a fourth series. The model was then transposed on transgenic mice to assess changes in microglia cells. Finally, three different inhibitors/antagonists were used in the sixth, seventh and eighth series to gain a better understanding of which pathway could be involved in the cross-sensitization process.

Acetic acid sensitization

An injection of acetic acid (0.75%, 200 µL) or saline (NaCl 0.9%, 200 µL, Baxter, Deerfield, Illinois, United States) was made into the urinary bladder using ultrasound monitoring in mice under brief anaesthesia (isoflurane: 3% in 1.5 L/min of air, Iso-Vet®, Piramal Critical Care, Voorschoten, The Netherlands).

Colorectal distension and visceral sensitivity measurement

We measured visceral pain to colorectal distension (CRD) using a non-invasive method, as previously reported in mice[16]. Changes in intracolonic pressure, reflecting viscero-motor responses (VMR) induced by CRD (nociceptive stimulus), were used as a surrogate marker of colorectal sensitivity[16].

An infinitely compliant distension balloon (diameter 0.7 cm) was made using a polyethylene bag attached to a PE-50 catheter (Intramedics, France) drilled in its end and taped 2 cm below the pressure sensor of a miniaturized pressure transducer catheter (SPR-524 Mikro-Tip catheter; Millar Instruments, Houston, TX, United States). Polypropylene 4-0 Ligatures (Prolène®; Ethicon Inc., Somerville, NJ, United States) were covered with parafilm to prevent any air leak.

On experimental day, mice were briefly anaesthetized with isoflurane (3% in air), and the lubricated "balloon-pressure sensor" was introduced into the colorectum, so that the balloon was inserted 2 cm upstream of the anal margin into the colon. Each mouse was

placed in an adjustable mouse restrainer (30 mm diameter × 90 mm length, LE5016, In vivo research instruments, United States), and left to rest for 30 min before the CRD procedure. The balloon was then secured to the tail with tape and connected to an electronic barostat (Distender Series II, G&J Electronics Inc., Toronto, ON, Canada) to perform isobaric CRD. Our distension protocol consisted of a set of graded phasic distensions of 15, 30, 45, and 60 mmHg (two times each, 20 s duration, 4 min inter-stimulus interval) (Protocol Plus Deluxe, G&J Electronics, Toronto, Canada). Voltage output was converted digitally using CED digital-analogic converter (Micro 1401, Cambridge Electronic Design, Cambridge, United Kingdom) and Spike 2 software (CED, Ltd., Cambridge, United Kingdom). The pressure sensor allowed the assessment of visceral pain *via* a custom-made script which allowed signals to be specifically extracted from abdominal muscle contractions (excluding those from colonic contractions).

Colonic sensitivity was measured in awake mice at 60 min, 3 and 7 d following acetic acid urinary bladder injections.

Measurement of bladder and colonic paracellular permeabilities

After euthanasia, bladder and distal colon samples were removed at day 7. Samples were cut along the mesenteric border. Bladder and colonic permeabilities were assessed by measuring fluorescein isothiocyanate (FITC)-dextran (4 kDa) fluxes in Ussing chambers with an exchange surface of 0.07 cm^2 (Harvard Apparatus, Holliston, MA, United States) as previously described[17]. FITC-dextran (5 mg/mL) was loaded on the mucosal side. After three hours at 37 °C, the medium from the contralateral side (serosa) was removed and stored at -80 °C. The fluorescence level of FITC-dextran (excitation at 485 nm, emission at 535 nm) was measured in a 96-well black plate using spectrometer Chameleon V (Hidex Co, Turku, Finland). The results were converted to concentrations of FITC-dextran (mg/mL) for analysis.

Myeloperoxidase measurement

After euthanasia, bladder and distal colon samples were removed at day 7. Colonic and vesical tissues (around 50 mg) were washed in phosphate-buffered saline (PBS) and then homogenized (50 mg/mL) in 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, Steinheim, Germany) with 50 mmol/L of PBS (pH 6.0). They were frozen at -80 °C and thawed at 37 °C three times, then sonicated (Vibra Cell ultrasonic processor 75115, Bioblock Scientific, Illkirch, France) and finally centrifuged (14000 rpm at 4 °C for 15 min). Myeloperoxidase (MPO) was assayed in the supernatant by adding 1mg/mL of dianisidine dihydrochloride (Sigma-Aldrich, Steinheim, Germany) and 5 × 10^{-5}% of H_2O_2 (Sigma-Aldrich, Steinheim, Germany). The change in optical density was measured at 450 nm. One unit of MPO activity was defined as the amount that degraded 1.0 µmol of hydrogen peroxide per minute at 25 °C, and human neutrophil MPO (Sigma-Aldrich, Steinheim, Germany) was used as standard, as previously described[18,19].

c-Fos immunofluorescence

Seven days after the intra-bladder injection of acetic acid 0.75%, c-Fos immunohistochemistry was performed after 120 min of CRD at 45 mmHg (20 s of distension every 4 min for 120 min). Under ketamine (100 mg/kg i.p.)/xylazine (10 mg/kg) anaesthesia and upon thoracotomy, mice were perfused through a cardiac-aorta cannula with saline followed by 150 mL/mouse of ice-cold 4% paraformaldehyde and 14% saturated picric acid in a 0.1 M phosphate buffer solution (pH 7.2). After decapitation, the lumbo-sacral spinal cord (L6-S1) was post-fixed in the same fixative solution overnight at 4 °C and cryoprotected by immersion in 10% sucrose overnight, and transferred to 30% sucrose overnight. The spinal cords were then embedded in Tissue-Tek® optimal cutting temperature (OCT) compound (Sakura Finetek United States, Inc., Torrance, California, United States), snap-frozen and cut with cryostat. Fluorescent microscopy was used to identify activated neurons. The expression of c-Fos was assessed by immunofluorescence. We applied a first anti-c-Fos antibody (1:2000; Calbiochem, Darmstadt, Germany) overnight at 4 °C and then incubated Cy3-

conjugated goat anti-rabbit IgG (1:400; Fisher, Invitrogen, Carlsbad, CA, United States) for two hours at room temperature. Pictures were taken using a fluorescence microscope (DM5500 B, Leica Microsystem Ltd., Wetzlar, Germany) at magnification × 10 and the number of c-Fos immunoreactive cells in lamina I and II of the dorsal horn at the L6-S1 segment of the spinal cord was counted for each mouse. The average number of stained nuclei in three 20 μm thick slices for each mouse was used for analysis.

Semiquantitative polymerase chain reaction for the detection of TNF-α, IL-1β and IL-10 messenger RNA in the spinal cord

Total RNA from L6-S1 spinal cord segments was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). RNA was purified according to the manufacturer's instructions. Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, United States) to remove any contaminating DNA. DNase I was stopped with DNase inactivation reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The quality and quantity of total RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, United States). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA. A ratio of ≥ 2.0 was accepted for analysis.

After reverse transcription of 1.5 μg total RNA into complementary DNA (cDNA) by using 200 units of SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, United States), quantitative polymerase chain reaction (PCR) was performed using SYBR Green technology on a Bio-Rad CFX96 real time PCR system (Bio-Rad Laboratories, Marnes-la-Coquette, France). *GAPDH* gene was chosen as the reference gene. All samples were performed in duplicate in a single 96-well reaction plate. Serially diluted cDNA samples were used as external standards. The absolute quantification of messenger RNA (mRNA) was performed by converting the sample Ct values to concentration (copies per μL) based on standard curves. The identity and purity of the amplified product were assessed using melting curve analysis at the end of amplification. The technique was used to assay TNF-α, IL-1β, and IL-10 mRNA in the

spinal cord. The primer sequences for the targeted mouse genes are presented in Supplementary Table 1.

Analysis of microglia cells

CX3CR1^{gfp+} transgenic mice were perfused as described for c-Fos immunofluorescence, and the dorsal horn of L6-S1 spinal cord was embedded in Tissue-Tek® OCT compound, snap-frozen and cut with cryostat. Pictures were then taken with the Leica photonic microscope used for c-Fos experiments at magnification × 10 and the number of microglia cells expressing microglia GFP in lamina I and II of L6-S1 dorsal spinal cord was counted using NIH ImageJ software (version 2.0.0-rc-43/1.51u)[20]. The average of the number of stained nuclei per field in three 20 µm thick slices for each mouse was used for statistics.

Pharmacologic studies

Minocycline (2.5 mg/mL; 1.25 mg/kg, Sigma-Aldrich, Steinheim, Germany), a microglia blocker, CP 99994 (15 mg/mL; 7.5 mg/kg, Sigma-Aldrich, Steinheim, Germany), a NK1R antagonist, SB203580 (5 mg/mL; 2.5 mg/kg, Calbiochem, Merck, EMD Millipore Corp., Billerica, MA, United States), a MAPK-p38 blocker, or saline have been injected in the intracerebroventricular (ICV) region using a Hamilton syringe (Neuros™, Gastight, 1705, 33 gauge; Dutscher, France), one hour before injecting acetic acid (0.75%) into the bladder. Intrathecal (IT) injections of minocycline at the L6-S1 Level of the spinal cord were also performed to demonstrate that microglia activation occurred at that level.

Western blot analysis

After euthanasia, the L6-S1 segment of the mice spinal cord was removed on ice and quickly frozen in liquid nitrogen. After thawing on ice, the spinal cord samples were homogenized at 4 °C in a lysis buffer (100 µL of buffer A X2, 2 µL of 100 mmol/L dithiothreitol, 50 µL of 1% NP40, 1 µL of 1X P8340 protease inhibitors, 2 µL of 1X P2850

phosphatase inhibitors, for 200 mL of H_2O). Samples were displayed on ice for 15 min, then centrifuged at 12000 × g for 15 min at 4 °C. The protein-containing supernatant was collected and stored at -80 °C until analysis. The proteins (25 µg) were loaded on a 4%-20% gradient polyacrylamide gel (Bio-Rad, Marnes la Coquette, France), and transferred onto a nitro-cellulose membrane (GE Healthcare, Orsay, France). Membranes were then blocked for one hour at room temperature with 5% (w/v) non-fat dry milk in Tris buffered saline containing 0.05% Tween® 20 (Sigma-Aldrich, Steinheim, Germany). An overnight incubation at 4 °C was then performed with primary antibodies: anti-P-p38 (1:500; Cell Signaling Technology®, Leiden, The Netherlands; P/N 4511) or anti-NK1R (1:200; Atlas Antibodies, Bromma, Sweden; P/N HPA074573) from rabbit, or anti-GAPDH (1:5000; Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France) from goat. All antibodies were diluted in a blocking solution. After three washes, a one-hour incubation was performed with a peroxidase-conjugated IgG secondary antibody from goat anti-rabbit or from rabbit anti-goat (1:5000; Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France). After three additional washes, immunocomplexes were revealed using the ECL detection system (GE Healthcare Life Sciences, LittleChalfont, United Kingdom). Proteins were quantified by densitometry using ImageScanner III and ImageQuant TL software (GE Healthcare Life Sciences, LittleChalfont, United Kingdom), and standardized against the intensity of GAPDH.

Statistical analyses

The data was expressed as means ± SEM. Quantitative data was compared between groups using an unpaired *t*-test, with Welch's correction in case of unequal variances. Comparisons of multiple groups were performed using one-way analysis of variance (ANOVA, Kruskal-Wallis test) with post-hoc analysis (Dunn's multiple comparison post-test to compare all pairs of columns to each other or Dunnett's post-test to compare all pairs of columns to controls) to assess the difference among the groups, and two-way ANOVA with post-hoc analysis (Bonferroni's correction) was used to assess

groups with repeated measures. Individual data for visceral sensitivity in mice, especially the kinetics of visceromotor responses to increasing colorectal distensions, was visually assessed for each experimental group separately; tracings of animals with aberrant or outlier responses to distensions were excluded from the analysis. A P value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 9.3.1 for Windows (GraphPad Software Inc., San Diego, California, United States, www.graphpad.com). The statistical methods used in this study were reviewed by Fabien Wuestenberghs from CHU UCL Namur.

RESULTS

Development and validation of a mouse model of chronic bladder-colon cross-sensitization

Based on our previous rat study[9], we tested the intravesical administration of a 0.75% acetic acid solution in mice. A single intravesical injection of 0.75% acetic acid under ultrasound monitoring induced an increase of the colonic nociceptive response during CRD at 30 mmHg ($P < 0.05$), 45 mmHg ($P < 0.05$) and 60 mmHg ($P < 0.001$) one hour after injection (Figure 1). An increased colonic nociceptive response during CRD was still observed at 60 mmHg ($P < 0.05$) on the seventh day in the acetic acid group compared to the control group (Figure 1), confirming that colonic hypersensitivity persists up to seven days after the intravesical injection in our model. Further experiments were therefore designed to understand the mechanisms of the chronicization of cross-sensitization in this model. No serious adverse event occurred during the experiments.

Permeability and inflammatory parameters of bladder and colon

No differences were found at day 7 in either bladder or colon permeabilities, or MPO activities between animals treated with acetic acid or saline intravesically (Figure 2).

Neural activation in the dorsal horn of the spinal cord L6-S1

In the absence of CRD, the number of c-Fos-IR cells in lamina I and II of the L6-S1 level of the dorsal horn was 6.5 ± 0.7 per slice in mice treated with saline. The CRD performed on the seventh day in these mice treated with saline increased the number of c-Fos-IR cells to 12.4 ± 1.3 ($P < 0.05$). In mice treated with 0.75% acetic acid, the number of c-Fos-IR cells was 12.9 ± 1.3 in the absence of CRD, and increased to 23.2 ± 1.5 ($P < 0.05$ *vs* all other groups) after CRD (Figure 3).

Implication of central microglia from the dorsal horn of the spinal cord L6-S1 in bladder-colon cross-sensitization

The number of microglial cells has been observed in a transgenic mouse model which specifically expresses GFP associated with fractalkine receptors ($CX3CR1^{gfp+}$) (Figure 4A and B). Seven days after injection of a 0.75% acetic acid solution into the bladder, the number of microglial cells increased compared to mice injected with saline (113.4 ± 13.0 *vs* 102.9 ± 11.2 per field respectively; $P < 0.05$) (Figure 4C).

We administered minocycline, a microglial inhibitor, at the central level one hour before the intravesical injection of acetic acid or saline to demonstrate the involvement of microglia cells. Both IT and ICV injections of minocycline prevented the development of cross-sensitization induced by intravesical administration of acetic acid (Figure 5A and B). ICV injections were favoured for the following experiments because they are less traumatic and cause less stress to the animals.

Involvement of central NK1R in bladder-colon cross-sensitization

The ICV injection of CP 99994, a NK1R antagonist, one hour before the intravesical injection of acetic acid, prevented the development of bladder-colon cross-sensitization compared to mice treated intracerebroventricularly by saline (Figure 6A). The intravesical administration of acetic acid increased the phosphorylation of MAPK-p38, a microglial protein involved in chronic pain generation[21], compared to mice injected intravesically with saline, but the ICV injection of CP 99994 prevented this

phosphorylation, suggesting that microglial activation depends on the activation of NK1R (Figure 6B).

The ICV injection of SB203580, a MAPK-p38 inhibitor, one hour before the intravesical injection of acetic acid prevented the occurrence of bladder-colon cross-sensitization (Figure 7A). The intravesical administration of acetic acid induced an increase in NK1R expression in the posterior horn of the L6-S1 level of the spinal cord compared to the administration of saline (Figure 7B). Acetic acid-induced spinal overexpression of NK1R was blocked by prior ICV administration of SB203580 (Figure 7B), suggesting that the MAPK-p38 pathway is involved in the development of bladder-colon cross-sensitization.

Assessment of the expression of spinal pro- and anti-inflammatory transcripts

Levels of expression of IL-1β, TNF-α and IL-10 mRNAs did not differ between control and acetic acid groups (P = 0.22, 0.47 and 0.19 respectively) (Figure 8).

DISCUSSION

A mouse model of bladder to colon cross-sensitization with persistent visceral hypersensitivity in the colorectum has not been developed until now to our knowledge. Our study showed that pelvic visceral cross-sensitization involves central sensitization with microglia modulation following a peripheric inflammatory event, and is mediated by the NK1 and MAPK-p38 pathways.

The pathophysiology of colon-bladder cross-sensitization is complex, and probably involves both peripheral and central mechanisms: the sensitization of sensory nerve terminals in both organs, cross-sensitization of adjacent sensory primary afferent neurons within DRGs (involving satellite glia cells and macrophages), axon reflexes *via* primary sensory afferent neurons with dichotomizing axon explaining neurogenic inflammation if there is a convergence of sensory information from distinct organs to a single neuron and antidromic release of inflammatory mediators in the unaffected organ (pre-spinal convergence), the sensitization of second order spinal neurons in

which there is a convergence of inputs from both colon and bladder, involving spinal interneurons (convergence-projection theory, or dorsal root reflex), and supraspinal mechanisms (modified central processing of visceral stimuli in the amygdala, *etc.*)[10]. Our work adds to the current understanding that central sensitization in the spinal cord involves microglia modulation, and is mediated by NK-1 and MAPK-p38 pathways.

It is already known that an increase in proinflammatory factors within the bladder tissue can sensitize the colon[22-24]. Several mechanisms have been proposed to explain this inter-organ sensitization. These mechanisms include the increased mechanical sensitivity of visceral muscle afferents, a higher proportion of chemo-sensitive visceral afferents[23], brainstem neurons integrating somato-visceral messages after bladder irritation[25], and central sensitization resulting from stressful life events[26]. In our study, we used a mouse model of colonic visceral hypersensitivity induced by cross-organ sensitization to demonstrate that microglia plays a central role in the development of this hypersensitivity. Indeed, we showed that the ICV injection of minocycline, which is known to be an inhibitor of microglial cells, blocks the cross-sensitization process. It has already been shown that minocycline also induces a decrease in neuronal excitability by preventing phosphorylation of the ERK protein and the MAPK, which are expressed in the spinal cord[27]. The analgesic effect of minocycline is mediated by its action in both microglial activation and neuronal activation. Microglial cells have already been shown to be recruited during colonic sensitization induced by chronic stress in rats[12] but our study is the first to identify the crucial role of microglia in bladder to colon cross-sensitization, and in a mouse model. We showed that the role of microglia in the development of cross-sensitization from the bladder to the colon is mediated by the tachykininergic pathway, and more particularly involving NK1R. This receptor would activate, directly or indirectly, the MAPK-p38 protein *via* its phosphorylation (P-p38). Several studies have confirmed that MAPK-p38 is expressed only by microglial cells[21,28,29]. Its involvement in the course of chronic stress-induced colonic sensitization has already been demonstrated in rats[12]. Another study suggests that the activation of microglial MAPK-p38 protein at the ventromedial nucleus of the spinal cord (rostral

ventromedial medulla) is responsible for utero-colonic cross-sensitization in an acute model[30]. In our work, we found that visceral hypersensitivity induced by chronic stress was associated with the phosphorylation of MAPK-p38 at the microglial cell level in mice, and that this effect was inhibited by the ICV injection of a MAPK-p38 inhibitor (SB203580). Bradesi *et al*[12] also showed that CX3CL1 potentiates the development of visceral hypersensitivity *via* a chemokine function on NK1R. The activation of MAPK-p38 is known to be associated with the increased synthesis and secretion of several neurotransmitters of inflammation, such as COX2, IL-1β, inducible nitric oxide synthase, PLA2 and PGE2[31]. The mediators of inflammation involved in our model are probably different from those involved in trinitrobenzenesulfonic acid-induced colitis models, in which expression of IL-1β is upregulated in the spinal cord[32], since we did not demonstrate any changes at the TNF-α, IL-1β and IL-10 mRNA levels.

We therefore propose a mechanistic view of the molecular and cellular mechanisms underlying the development of colonic hypersensitivity by bladder-to-colon cross-sensitization in which the inflammatory reaction following irritation of the urothelium induces the activation of EPANs, some of them co-innervating the bladder and the colon, and giving rise to axon reflexes, while others innervating the colon are activated by paracrine interactions. Convergent neurons in the DRGs and the spinal cord, and those innervating the colon, secondarily sensitize the colon. Some activated EPANs relaying to other neurons in the dorsal horn of the spinal cord specifically secrete substance P, a member of the tachykinin family, which binds to NK1R, activating the MAPK-p38 by phosphorylation (P-p38), which in turn could either directly or indirectly induce the synthesis of mediators involved in neuroplasticity and neuroinflammation in the spinal cord.

A mini-invasive approach which specifically targets the bladder without inducing structural lesions in neighbouring areas (especially the colon and peritoneum) is a prerequisite for cross-sensitization. Indeed, abdominal surgery is known to induce stress, involving a hormonal stress response at the peripheral and central levels, and implicating CRF pathways[33], and can induce visceral sensitization in the absence of

signs of overt inflammation in mice[16]. Similarly, we decided to use measurements of the intra-colonic pressure as a surrogate marker of VMR induced by CRD to assess visceral sensitivity in our study, with this technique being an alternative to the measurement of the electromyographic activity of the abdominal muscles[34] and having the advantage of being minimally invasive.

When we planned our study, the available animal models for the study of bladder-bowel interactions included acute[11] and chronic[35,36] colon irritation with 2,4,6-trinitrobenzenesulfonic acid in mice[36] and in rats[11], and acute bladder irritation by cyclophosphamide, an antitumoral drug known to induce haemorrhagic cystitis, in mice[35] and by infusing protamine sulphate and potassium chloride in rats[11]. In those studies, colonic sensitivity was assessed on day 5 after bladder irritation in mice and on the day of the irritation in rats. The main limitation of our model is that the chronicity of the cross-organ sensitization was considered at day 7 following bladder irritation in mice. This period may seem short in view of clinical situations which evolve over several years, however, no other inflammatory model of cross-sensitization from the bladder to the colon published in the literature exceeds 48 h[35]. Furthermore, stress-induced chronic colonic sensitization models with 11 d of homotypic stress[12] were shown to be sufficient to induce changes in colonic tenderness and spinal microglia modulation. Our seven-day period in mice therefore seems to be long enough to attest the chronicity of the process. Further studies with a longer longitudinal assessment of visceral sensitivity are necessary to confirm our results. Our results can only be extrapolated to males because sex-dependent differences in the responses to the sensitization process[37,38] and microglia[39] have been reported. Since pelvic neuroanatomy is similar in other rodents and in humans, we could expect similar results in those species, which could explain in part the common overlap between BPS and IBS in the clinic.

CONCLUSION

In conclusion, we have developed the first model of cross-organ chronic visceral sensitization between bladder and colon in mice. Pelvic cross-sensitization involves central sensitization with microglia modulation and is mediated by the neurokinin-1 receptor pathway and MAPK-p38 activation.

ARTICLE HIGHLIGHTS

Research background

Limited mechanistic data is available in the context of overlap between bladder pain syndrome (BPS) and irritable bowel syndrome (IBS). Based on the fact that pelvic organs share common sensory pathways, a few studies have offered evidence that cross-sensitization between the bladder and the colon may explain sensitization of both organs. This may involve primary extrinsic afferents or central sensitization, both at the spinal and supraspinal levels.

Research motivation

The precise pathophysiology involved in cross-sensitization of the bladder and the colon remains poorly understood.

Research objectives

The objectives of this study were to develop a model of chronic bladder-colon cross-sensitization and to investigate the mechanisms involved.

Research methods

Chronic cross-organ visceral sensitization was obtained in C57BL/6 mice using ultrasound-guided intravesical injections of acetic acid under brief isoflurane anaesthesia. Colorectal sensitivity was assessed in conscious mice by measuring intracolonic pressure during isobaric colorectal distensions. Three different inhibitors/antagonists assessed to gain a better understanding of which pathway could be involved in the cross-sensitization process.

Research results

Visceral hypersensitivity to colorectal distension was observed after the intravesical injection of acetic acid. This effect started one hour post-injection and lasted up to seven days post-injection. Colorectal sensitization was prevented by intrathecal or intracerebroventricular (ICV) injections of minocycline, a microglia inhibitor, by ICV injection of CP-99994 dihydrochloride, a NK1 antagonist, and by ICV injection of SB203580, a MAPK-p38 inhibitor.

Research conclusions

We describe a new model of cross-organ visceral sensitization between the bladder and the colon in mice lasting up to seven days. Intravesical injections of acetic acid induce colorectal hypersensitivity to distension, mediated by neuroglial interactions, MAPK-p38 phosphorylation and the NK1 receptor.

Research perspectives

A bladder-colon chronic cross-sensitization mouse model using intravesical injections of acetic acid can be used as a preclinical model of overlap between BPS and IBS.

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