Increased visceral sensitivity to capsaicin after DSS-induced colitis in mice: spinal cord c-Fos expression and behavior

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During acute and chronic inflammation visceral pain perception is altered. Conflicting data exist, however, on visceral pain perception in the postinflammatory phase. The aim of the present study was to investigate whether visceral pain perception is altered after resolution of dextran sodium sulfate (DSS)-induced inflammation of the colon. Visceral sensory function in mice was assessed by monitoring behavioral responses to intracolonic capsaicin instillation. Two hours later the number of c-Fos-positive neurons in lamina I/II and X of spinal cord neuronal c-Fos activation were effective readouts for determining the disease activity index score. Animals developed a transient colitis and had recovered at day 49. At this time point, cytokine levels and colon length were similar to control animals. Importantly, after resolution of DSS-induced colitis the behavioral response to intracolonic capsaicin was increased compared with control mice. Moreover, capsaicin-induced spinal cord neuronal c-Fos expression was significantly increased. Interestingly, after colitis animals also exhibited referred somatic hyperalgesia as measured with von Frey hairs on the abdominal wall. We conclude that postinflammatory hyperalgesia can persist after resolution of an acute inflammation. A recent study in rats demonstrated that TNBS-induced colitis, which is characterized by a transient symptomatology, caused increased sensitivity to colorectal distension up to 17 wk after induction of colitis (17). Moreover, infection with the nematode Trichinella spiralis led to an increase in visceral sensitivity to pain for several weeks after the inflammation had subsided (1). However, the development of postinflammatory hyperalgesia after a transient inflammation in mice was not observed with TNBS, acetic acid, capsacin, or mustard oil (20, 23). It is not known yet whether DSS-induced colitis also causes postinflammatory visceral hyperalgesia.

The above-mentioned studies all used the visceral motor response (VMR) of the abdominal wall muscles to colorectal distension to assess visceral hyperalgesia. This VMR is used as a pseudo-affective response to study visceral nociceptive responses. Recently, a new model has been described to assess visceral sensitivity to pain (22). In this model, visceral sensitivity is measured by determining behavioral pain responses to intracolonic instillation of capsaicin. Capsaicin activates the transient receptor potential cation channel, subfamily V, member 1 (TRPV1) that is expressed on peripheral terminals of visceral neuronal afferents (32). Activation of the TRPV1 receptor induces neuronal activation, causing pain sensation. Several inflammatory mediators such as CCL3 (34), PGE2, nerve growth factor, and ATP can increase the sensitivity of the TRPV1 receptor on somatic afferents (reviewed in Refs. 7, 15, 28).

The aim of the present study was to test the hypothesis that after resolution of DSS-induced colitis increased behavioral pain responses to intracolonic capsaicin instillation are detectable compared with control animals. Moreover, we investigated whether changes in the behavioral pain responses are associated with changes in the level of spinal cord neuronal activation, as determined by c-Fos staining. Finally, we assessed the presence of referred somatic hyperalgesia after colitis using von Frey hairs.

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MATERIALS AND METHODS

Animals

Twelve- to 14-wk-old female C57/Bl6 mice were bred and housed with four to six animals per cage in the Central Animal Facility of the University of Utrecht. Animal experiments were performed in accordance with international guidelines and approved by the Experimental Animal Committee of the University Medical Center Utrecht.

Experimental Design

Animals of 12–14 wk were randomly assigned to the naive or colitis group and all analyses were run in parallel for naive animals and animals postcolitis. To induce colitis, animals received drinking water with 1% DSS for 7 days (see Ref. 12; molecular weight 40,000; ICN Biomedicals, Eschwege, Germany). From day 8 onward all animals received normal drinking water. Body weight, stool consistency and fecal blood loss (using Hemocult tests, Beckman Coulter, Eschwege, Germany) were recorded daily. The disease activity index (DAI) was calculated as described in Table 1.

At day 9 a group of animals was euthanized to determine disease activity-related parameters at the peak of colitis. At day 49 both naïve animals and animals postcolitis were randomly assigned to two groups that were subjected to the following procedures.

Group 1. Group 1 animals received capsaicin treatment and measurement of the capsaicin-induced behavioral response from 5 until 25 min after capsaicin treatment. At 2 h after capsaicin treatment the animals were euthanized, and spinal cord was collected for c-Fos staining.

Group 2. We determined referred hyperalgesia in group 2 animals using von Frey hairs. Subsequently, animals were euthanized and the colon was collected. After determination of colon length, colons were longitudinally divided into two samples. One was fixed in 4% paraformaldehyde, paraffin embedded, and stained with hematoxylin and eosin for histological examination. The other half was used for RNA and protein extraction.

Capsaicin-Induced Pain Responses

Behavioral responses to intracolonic capsaicin were determined based on Laird et al. (22). Briefly, animals were habituated to a raised wire mesh (5 × 5 mm apertures) under a clear plastic box (30 × 20 × 15 cm) for 30 min; 30 μl of capsaicin (0.1% wt/vol in 10% ethanol-10% Tween 80–80% saline) or vehicle was administered intracolonically under halothane anesthesia (3% in N2-O2 1:1). Petroleum jelly staining.

Reflected Hyperalgesia

Withdrawal responses to the application of von Frey hairs (Stoelting, Wood Dale, IL) to the abdomen were examined as a measure of referred hyperalgesia. Prior to capsaicin instillation, animals were habituated to a raised wire mesh (5 × 5 mm apertures) under a clear plastic box (10 × 10 × 5 cm) for 30 min. Next, von Frey hairs were applied up through the wire mesh to the lower to mid abdomen of the freely moving animals, avoiding external genitalia, three times each for 5 s in ascending order of force with an interstimulus interval of 10 s. Care was taken not to stimulate the same point twice in succession, to avoid “windup” effects or desensitization. A withdrawal response was defined as 1) sharp retraction of abdomen, 2) immediate licking or scratching of site of application of hair, or 3) jumping. The threshold was defined as the force (grams) that was represented by the von Frey hair that elicited three consecutive responses. Hairs used were 8, 20, 40, 70, 160, 400, and 600 mg.

c-Fos Staining

Spinal cord was postfixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, embedded in optimal cutting temperature freezing medium (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, The Netherlands), and frozen. Transverse free-floating sections of 35 μm were cut (CM3050; Leica Microsystems, Rijswijk, The Netherlands) and incubated with rabbit-anti-c-Fos antibody (1:20,000; Vector Laboratories, Burlingame, CA) followed by biotinylated donkey-anti-rabbit IgG (1:1,500; Jackson ImmunoResearch Laboratories, Suffolk, UK) and stained with the ABC nickel-enhanced diaminobenzidine method (Vector Laboratories). The number of c-Fos-positive cells was counted in the superficial lamina of the dorsal horn (lamina I/II) and lamina X of at least three slices per spinal segment per animal (T12/L3–S1).

Cytokine Expression

Total colonic RNA was extracted by use of Trizol (Invitrogen, Breda, The Netherlands). RNA concentration was determined spectrophotometrically and quality was assessed after agarose gel electrophoresis. cDNA was synthesized with Superscript RNase H-Reverse Transcriptase (Invitrogen) with 2.5 μM random hexamers (Invitrogen). Quantitative real-time PCR was performed using SYBRgreen probe and I-Cycler IQ5 (Bio-Rad, Alphen a/d Rijn, The Netherlands). Data were normalized using average β-actin and GAPDH mRNA expression. See Table 2 for list of primers used.

MPO

MPO activity was determined as described (29). Samples were centrifuged (15 min, 13,000 g, 4°C). Supernatants were diluted 1:5 in 10 mM HEPES, pH 8.0 containing 10 mM citrate (Merck, Darmstadt, Germany), pH 5.0 with 0.22% hexadecyltrimethylammoniumchloride (Sigma). Reaction buffer consisted of 3 mM 3’,3’,4’,4’-tetramethylbenzidine (Sigma), 120 μM resorcin (Aldrich), and 2.2 mM H2O2 in distilled water. Samples were 1:2 diluted in reaction buffer. Reaction was stopped with 150 μl of 2 M H2SO4 and absorbance was read 450 nm.

Western Blot

Membrane fractions from colons were obtained by homogenizing colons with a polytron tissue disruptor (Janke and Kundel, Staufen, Germany) in ice-cold lysis buffer (20 mM Tris, 2 mM EDTA, 1 mM DTT) supplemented with protease inhibitors (tissue protease inhibitors; Sigma, St. Louis, MO) and 1 mM PMSF. Unbroken cells and proteins were removed by centrifugation (10 min, 13,000 g, 4°C). Supernatants were diluted 1:5 in 10 mM HEPES, pH 8.0. Western blotting was performed using the I-Cycler IQ5 (Bio-Rad, Alphen a/d Rijn, The Netherlands).

Table 1. Disease activity index

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight Loss</th>
<th>Stool Consistency</th>
<th>Fecal Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>1–5%</td>
<td>Loose stool</td>
<td>Hemoccult +</td>
</tr>
<tr>
<td>2</td>
<td>5–10%</td>
<td>Loose stool</td>
<td>Hemoccult +</td>
</tr>
<tr>
<td>3</td>
<td>10–15%</td>
<td>Diarrhea</td>
<td>Gross Bleeding</td>
</tr>
<tr>
<td>4</td>
<td>15–20%</td>
<td>Diarrhea</td>
<td>Gross Bleeding</td>
</tr>
</tbody>
</table>

The disease activity index is the combined scores of weight loss, stool consistency and bleeding, leading to a maximum DAI of 12. Normal stool, firm and well-formed pellets; loose stool, pasty and semiformal stools that do not stick to the anus; diarrhea, liquid stools that stick to the anus.

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nuclei were pelleted by centrifugation (800 g for 5 min) and discarded. The supernatant was then centrifuged (48,000 g for 20 min at 4°C) and the membrane pellet was resuspended in ice-cold lysis buffer (20 mM HEPES, 1% Triton X-100, 2 mM EDTA, 1 mM DTT) with protease inhibitors (Sigma) and sonicated; 40 μg protein was separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA) by electroblotting. Blots were stained with rabbit anti-TRPV1 (1:1,000; Abcam, Cambridge, UK) and goat anti-β-actin (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was detected by enhanced chemiluminescence (Amersham, Roosendaal, The Netherlands). Band density was determined via a GS-700 Imaging Densitometer (Bio-Rad).

Statistical Analysis

Data are means ± SE. Statistical analysis of behavioral data was performed using Friedmann’s test or Kruskal-Wallis test with post hoc Dunn’s test as appropriate. C-Fos data were analyzed by repeated-measures ANOVA or two-way ANOVA and post hoc least significant difference test (LSD). Cytokine data were analyzed by one-way ANOVA and post hoc LSD. For correlational analysis, Spearman rank correlation test was used. Mechanical stimulation thresholds were log-transformed and analyzed by Kruskal-Wallis test with post hoc Dunn’s test. P < 0.05 was defined as statistically significant.

RESULTS

Behavioral and Neuronal Responses to Intracolonic Capsaicin in Control Mice

Behavioral responses. To verify that capsaicin instillation induced acute spontaneous pain behaviors in naive mice, we compared behavioral pain responses to intracolonic instillation...
of capsaicin or vehicle. Five minutes after capsaicin or vehicle instillation, spontaneous behaviors were recorded for 20 min. The frequency of pain-related behaviors (abdominal licking, abdominal contractions, and squashing of the abdomen to the bottom of the cage) was scored during four periods of 5 min. Intracolonic administration of only vehicle induced some spontaneous pain behaviors, consisting mostly of abdominal licking, which was clearly different from normal grooming activities. Intracolonic administration of capsaicin induced significantly more spontaneous pain behaviors than vehicle treatment (Fig. 1A; \(p < 0.001\)). The frequency of pain behaviors was most pronounced during the first 15 min of observation and significantly declined during the last 5 min of observation, i.e., 20–25 min after capsaicin treatment (Fig. 1A). As expected, untreated animals (without any intracolonic instillation) did not show any of these behaviors.

Neuronal responses. The superficial lamina of the spinal cord dorsal horn (lamina I/II) and lamina X at the spinal segments T12/13 to S1 is known to receive nociceptive input from the colon (26). To test whether a painful stimulus in the colon induces neuronal activation in these areas, we determined the number of c-Fos-positive cells in the superficial lamina of the dorsal horn and in lamina X from spinal segment T12/13 to S1 at 2 h after intracolonic instillation of capsaicin or vehicle. The results in Fig. 1 show that capsaicin induced a significant increase in the number of c-Fos-positive cells in both lamina I/II and X compared with vehicle alone in all spinal segments examined (Fig. 1, B–D; \(p < 0.001\)).

The highest absolute number of capsaicin-induced c-Fos-positive cells were observed at L4 in both lamina I/II and X (Fig. 1, B and D). In addition, in lamina I/II the relative increase in c-Fos-positive cells in capsaicin-treated compared with vehicle-treated animals was most pronounced from spinal segments L3 to S1, with maximal increases at segments L4 and L6. In lamina X, the relative increase in c-Fos-positive cells was most pronounced from segments T12 to L5 with a maximum at L4.

Compared with untreated animals, vehicle alone induced a small but statistically significant increase in the number of c-Fos-positive cells in lamina I/II of segments L2 and L3 (Fig. 1B). In lamina X, the number of c-Fos-positive cells was similar in vehicle-treated and untreated control animals (Fig. 1D). To test whether the behavioral pain response was directly related to the magnitude of neuronal activation in the superficial lamina of the dorsal horn, we performed a correlational analysis of the total number of pain behaviors recorded in 20 min and the total number of c-Fos-positive cells in the superficial lamina of the dorsal horn (segments T12/13–S1) from each animal. The number of c-Fos-positive cells in the superficial lamina of the dorsal horn in the spinal cord correlated highly with the number of pain behaviors (Fig. 1E; \(r = 0.836; p < 0.001; n = 14\)). Similarly, a high correlation between the number of c-Fos-positive cells of lamina X with the number of pain behaviors was present (data not shown; \(r = 0.788; p < 0.001; n = 14\)).
DSS-Induced Colitis

To address the question whether animals express visceral hyperalgesia after resolution of colonic inflammation, we first determined the course and signs of DSS-induced colitis. Weight, stool consistency, and blood loss were scored to calculate a DAI as described in Table 1. The first manifestations of the disease became apparent at days 2–3. Disease activity peaked on days 8–9. From day 9 onward, some loose stool was the only clinical sign left, which was present in 43% of the animals. In a parallel group of animals, we observed at the peak of the disease (day 9) a significant reduction in colon length as a result of the inflammatory process (Fig. 2B; P < 0.001). At day 49 after induction of colitis, colon length had completely normalized again (Fig. 2B). In addition, MPO activity was determined at day 49 in the colon of DSS-treated animals as a measure of the number of granulocytes in the colon. At day 49, MPO activity was not different in colons from DSS-treated vs. control animals (data not shown).

To further determine whether the colonic inflammation had resolved at day 49, we also measured expression of the proinflammatory cytokines IFN-γ, IL-1β, and TNF-α and the anti-inflammatory cytokine IL-10 in the colon. In line with data in the literature, we observed that during the active inflammatory phase of DSS-induced colitis (day 9) colonic IFN-γ, IL-1β, TNF-α, and IL-10 levels were increased (Fig. 2) (11). However, at 49 days after induction of colitis expression of IFN-γ, IL-1β, TNF-α, and IL-10 mRNA in colons from DSS-treated animals did not differ from healthy controls (Fig. 2, C–F).

Histological examination of the colons of DSS-treated mice at day 49 after disease induction also revealed no signs of inflammation, whereas at day 9 signs of inflammation were present (Fig. 3). In general the crypts were well organized at day 49 and the base of the tubular glands reached the muscularis mucosae. Sporadically, the colons of DSS-treated mice revealed modest signs of increased proliferation. No obvious cellular infiltration was found in the submucosa at day 49 (Fig. 3F), whereas at day 9 cellular infiltration was clearly present (Fig. 3D). At day 49, the epithelial cell layer on the surface of the mucosa of DSS-treated animals was intact (Fig. 3, E and F).

On the basis of these results, we decided to use day 49 as the time point for postinflammatory pain measurements.

Postinflammatory Hyperalgesia: Behavioral Responses

To determine whether hyperalgesia develops after colonic inflammation has resolved, we compared the behavioral pain responses to intracolonic capsaicin of mice at day 49 after colitis and of healthy control mice. Animals that received intracolonic capsaicin postcolitis showed a higher number of spontaneous pain-related behaviors than control animals without previous inflammation of the colon (Fig. 4A; P < 0.05). In the vehicle-treated groups, there was also a significant difference in the number of spontaneous pain-related behaviors of
animals postcolitis compared with healthy control animals (Fig. 4A; P < 0.05).

It has been described before that the proportion of time animals spend freezing increases with increasing intensity of the painful stimulus (22). In line with these reports, we observed that capsaicin administered postcolitis induced not only more pain-related behaviors but also more freezing behavior than in control animals treated with capsaicin (Fig. 4B; P < 0.05).

Postinflammatory Hyperalgesia: Neuronal Responses

To address the question whether the development of postinflammatory visceral hyperalgesia can also be determined at the level of neuronal activation, we compared the capsaicin-induced neuronal c-Fos activation in mice at day 49 after DSS-induced colitis with that of healthy control mice. The total number of c-Fos-positive cells in the superficial lamina of the dorsal horn from T12/L3–S1 was increased by 40% after capsaicin stimulation in mice that had recovered from colitis compared with healthy control animals (Fig. 5, A and B; P < 0.001). All individual spinal segments measured, except spinal segment L6, showed a significant increase in c-Fos expression. Moreover, spinal segments L4 and L5 showed the highest relative increase compared with healthy control animals (Fig. 5A). Healthy control and postcolitis animals did not differ in the c-Fos neuronal response to vehicle administration (Fig. 5B).

In lamina X of the spinal cord, the number of capsaicin-induced c-Fos-positive cells was also significantly increased postcolitis (Fig. 5, C and D; P < 0.05). The overall increase in the number of c-Fos-positive cells in lamina X was mainly caused by the increase in the lower thoracic and higher lumbar level of the spinal cord (T12–L2) (Fig. 5C). After colitis, the number of lamina X c-Fos-positive cells in vehicle-treated animals showed only a trend toward an increase in c-Fos-positive cells compared with vehicle instillation in animals without previous inflammation (Fig. 5D; P = 0.06).

**Response Threshold to von Frey Hairs of the Abdominal Skin After Inflammation**

We determined possible changes in sensitivity toward mechanical stimulation of the abdomen by means of von Frey hair stimulation of the abdominal wall of healthy control mice and mice postcolitis. Interestingly, postcolitis, thresholds to von Frey stimulation of the abdominal skin of mice were significantly decreased compared with threshold levels in healthy control animals (Fig. 6A; P < 0.01). These data indicate that after inflammation of the colon referred hyperalgesia can be detected on the abdominal wall.

**TRPV1 Expression in the Colon**

To test whether the increased visceral sensitivity to capsaicin stimulation after resolution of colitis was associated with increased TRPV1 receptor expression, we compared TRPV1 protein expression in the colon of mice at day 49 after colitis with that of control animals. As a positive control we also determined TRPV1 expression at day 9 during colitis. The results in Fig. 6 clearly show that colonic TRPV1 protein expression of animals after resolution of colitis at day 49 (Fig. 6C) did not differ from healthy controls, whereas during active disease TRPV1 expression was increased (Fig. 6B; P < 0.05).

**DISCUSSION**

We demonstrate here for the first time that after resolution of DSS-induced colitis the behavioral and neuronal c-Fos responses to intracolonic capsaicin were increased, showing that DSS-induced colitis in mice induces long-lasting visceral hyperalgesia. Moreover, we show that a significant correlation existed between the behavioral pain responses to rectal capsaicin and the c-Fos expression in the dorsal horn of the spinal cord, implying that capsaicin-induced c-Fos expression is a good and objective parameter to determine visceral hyperalgesia.

Recently, it has been described that during the acute and late phase of ongoing DSS-induced colitis in male C57Bl/6 mice the mechanosensitivity to rectal distension was similar to that of healthy animals (24). Another recent study, however, has shown that during acute DSS-induced colitis, male mice did develop increased responses toward noxious colonic distension (31). Moreover, in chronic DSS-induced colitis, induced by repetitive cycles of DSS administration to male mice, increased visceral perception did not occur (31). Despite the conflicting results whether visceral hypersensitivity occurs during colitis, our present data clearly demonstrated that after resolution of DSS-induced colitis the visceral pain response to intracolonic capsaicin is increased.
Other inflammatory models of colitis, such as TNBS or ethanol-induced colon inflammation in rats and colonic infection with *Trichinella spiralis*, have also been used to study postinflammatory hyperalgesia (1, 17, 20).

Comparable to the different outcomes on visceral hyperalgesia during DSS-induced colitis (24, 31), the occurrence of postinflammatory hyperalgesia after a transient inflammation does not seem to be a general phenomenon as well, since transient inflammation with TNBS or acetic acid, capsaicin, or mustard oil in mice did not produce long-lasting changes in visceral sensitivity (20, 23). In the above-mentioned studies, the change in visceral hyperalgesia was determined by measuring the response to colorectal distension, which is thought to be regulated via changes in the sensitivity of low-threshold
mechansensitve visceral afferents that encode distending pressures into the noxious range (14). The pain response to capsaicin administration described in this paper, however, depends on the binding of capsaicin to TRPV1 receptors. TRPV1 receptors are expressed on chemosensitive visceral afferents and recent evidence suggests that TRPV1 receptors are also involved in mechansensitve responses (19). It should also be noted that there are a number of other differences between our study and studies performed previously during or after colitis, including the method to induce colitis, sex, strain, species, and age of the animals, which may all contribute to outcome.

A striking observation in our study was that referred somatic hyperalgesia on the level of mechanical sensitivity of the abdominal wall was present after DSS-induced colitis, implying that somatic and visceral thresholds were decreased after colonic inflammation. This sensitization process most likely occurs centrally since neurons that innervate the abdominal wall and the colon are somatotopically organized in the spinal cord (3). These data also suggest that the increased visceral sensitivity to pain is not specific for capsaicin but rather represents a generalized neuronal sensitization process. Moreover, the increased vehicle-induced behavioral response observed postcolitis also supports our hypothesis of a more general neuronal sensitization process that is not specific for capsaicin.

Although almost half of the animals showed slight changes in stool consistency at day 49 (suggesting ongoing colitis), colon length, MPO level, and colon histology were normal at this time point after induction of colitis. Moreover, we could not detect differences in colonile 1B, IFN-γ, TNF-α, and IL-10 of healthy control animals and animals postcolitis. Therefore, we conclude that the increased visceral response to colonic inflammation at this time point. However, on the basis of the above-mentioned data it may well be that not the number of receptors but the coupling of the TRPV1 receptor to intracellular signaling molecules is altered, e.g., by increased TRPV1 receptor phosphorylation after resolution of colitis. The change in the phosphorylation state of the TRPV1 receptor may then lead to changes in the sensitivity of the receptor, a process that may partially contribute to the observed visceral hypersensitivity postcolitis.

In conclusion, we propose that DSS colitis is a good model to study postinflammatory visceral hyperalgesia. Stimulation of visceral afferents with capsaicin after the inflammation had subsided led to increased pain behaviors and increased spinal cord c-Fos activation. Further investigations addressing the regulatory mechanisms involved in the sensitization of TRPV1 receptors on peripheral colonic afferents may open up novel therapeutic strategies to ultimately treat postinflammatory pain in patients with inflammatory bowel diseases or irritable bowel syndrome.

**REFERENCES**


