Effects of experimental colitis on the expressions of calcitonin gene-related peptide and vanilloid receptor 1 in rat spinal cord sensory neurons

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Abstract: To study the acute and long-term effects of local gut inflammation on the sensitivity of the spinal sensory neurons, the expressions of vanilloid receptor 1 (VR1) and calcitonin gene-related peptide (CGRP) in the colon-innervated primary sensory neurons in dorsal root ganglia (DRG) were examined in rats with trinitrobenzenesulfonic acid (TNBS)-induced experimental colitis. The neurons projecting to the distal colon were identified by DiI(3) retrograde labelling. Macroscopic examination, mean damage score and myeloperoxidase (MPO) activity were determined to assess the inflammatory status of the colon tissue. The number of CGRP and VR1 immunoreactive neurons at different stages of inflammation (on days 7, 21 and 42 after TNBS treatment) were compared. On day 7 after TNBS treatment, macroscopic damage of the mucosa could be easily detected and the percentage of colon-innervated DRG neurons expressing CGRP and VR1 increased nearly two folds respectively [(95.38±9.45)% vs (42.86±5.02)% for CGRP, (89.23±8.21)% vs (32.54±4.58)% for VR1]. When the colon inflammatory reaction was resolved on days 21 and 42 after TNBS treatment, the percentage of colon-innervated DRG neurons expressing CGRP and VR1 were still higher than that in the control group [(86.25±8.21)%, (68.28±7.12)% vs (42.86±5.02)% for CGRP; (67.22±6.52)%, (56.25±4.86)% vs (32.54±4.58)% for VR1]. These results suggest that the local gut inflammation increases the expressions of CGRP and VR1 in gut-innervated DRG sensory neurons. More importantly, this abnormal status persists even after the gut inflammatory reaction has been resolved for certain time.

Key words: inflammation; dorsal root ganglia; calcitonin gene-related peptide; vanilloid receptor1
Nearly one third of patients suffering from functional gastrointestinal diseases (FGID) could be retrospected for the antecedent history of gut infection[1]. For these patients visceral hypersensitivity to different stimuli in the gut (such as cold or stretch) was confirmed by electrophysiological methods, although no biochemical or pathological abnormality was found in the gut[2]. In the experimental colitis animal model, the release of many inflammatory mediators in the gut increased[3]. Moreover, electrophysiological studies suggest that the myenteric or submucosal neurons exhibited higher sensitivity to stimuli in the gut, not only for a short term, but also after the inflammation had been resolved for a long time[4,5]. All of the previous researches indicated the role of inflammation in the pathogenesis of gut hypersensitivity at the bowel level. But at the spinal cord level, where the primary sensory neurons exist in the special structure as dorsal root ganglia (DRG), the role of inflammation in the sensitivity of the sensory neurons is little understood.

Capsaicin, a lipophilic vanilloid substance found in Capsicum peppers that produces the sensation of heat, has a prominent excitatory action on the primary afferent neurons. This neuronal action, which is mediated through vanilloid receptor 1 (VR1) coupled to a non-selective cation conductance, is associated with nociception and localized release of proinflammatory substances[6]. Calcitonin gene-related peptide (CGRP) is an important sensory neurotransmitter and is released by a variety of noxious stimuli, including VR1 activation, distension, and acidosis[7]. VR1 and CGRP immunoreactivities are highly co-localized in trigeminal ganglia[8]. The VR1 agonists capsaicin and anandamide evoke CGRP release from nerve terminals in the dorsal spinal cord[9]. In the present study we used trinitrobenzenesulfonic acid (TNBS) to induce experimental colitis in rat distal colon. By examining CGRP and VR1-immunoreactive DRG neurons projecting to the distal colon at different stages after the inflammation induction, we aimed to study the acute and long-term effects of local colitis on the sensitivity of the spinal sensory neurons.

1 MATERIALS AND METHODS

1.1 Animals
Sprague-Dawley rats of either sex weighing 180-250 g from Shandong University were housed at room temperature and fed rat chow. Tap water was given ad libitum and every 6 rats were housed in one cage. Animals were divided randomly into the control saline group, day 7 group, day 21 group and day 42 group. For each group the same volume of 50% ethanol was given to exclude the effect of ethanol. Animal protocols were approved by the Animal Care Institution of Shandong University.

1.2 DRG retrograde labeling and animal model
DRG neurons projecting to distal colon were identified by retrograde labeling following injection of retrograde tracer DiI(3) (DiIC18, Molecular Probes, Eugene, OR) into the distal colon[10]. The rats were anesthetized with pentobarbital sodium (45 mg/kg body weight, i.p.). The distal colon was exposed under sterile condition, and 70 µL of DiI(3) (25 mg in 0.5 mL methanol) was injected into the smooth muscle of the distal colon (8 cm to anus) through a 30-gauge needle. The surgical incision was closed and the animal recovered to permit DiI(3) to be transported back to the cell soma of colon sensory neurons. On day 3 after dye injection, experimental colitis was induced by administration (8 cm to anus) of an intrarectal enema of 30% TNBS in 50% ethanol at a dose of 80 mg/kg body weight under 5% halothane anesthesia. Animals in the control saline group were given an equivalent normal saline enema. To exclude the effect of ethanol, the same volume of 50% ethanol was given as above.

1.3 Assessment of TNBS-induced colitis
1.3.1 Macroscopic assessment
The animals treated with TNBS and ethanol were killed humanly by stunt and exsanguinations on days 7, 21 and 42 after treatment. Rats in the control saline group were killed on day 7. The distal colon was removed from each animal to permit DiI(3) to be transported back to the cell soma of colon sensory neurons. By examining CGRP and VR1-immunoreactive DRG neurons projecting to the distal colon at different stages after the inflammation induction, we aimed to study the acute and long-term effects of local colitis on the sensitivity of the spinal sensory neurons.

1.3.2 Myeloperoxidase (MPO) activity assay
MPO is a granule-associated enzyme primarily contained in neutrophiles, and measurement of it has been widely used as a marker of intestinal inflammation[12]. Colon samples were obtained from the same position of the inflammatory region in each group. The samples were immediately frozen in liquid nitrogen, stored at -70 °C, and assayed with the method demonstrated by Buyse et al[13]. Briefly, intestinal tissue samples (50-100 mg) were homogenized on ice by using a Polytron (13 500 r/min for 1 min) in a solution of 0.5% hexadecyl trimethylammonium

bromide (Sigma Chemicals) in 50 mmol/L potassium phosphate buffer (pH 6.0, 1 mL/50 mg tissue). The resulting homogenate was subjected to three rapid freezing (-70 °C) and thawing (immersion in warm water, 37 °C) cycles. The samples were then centrifuged (4 000 r/min, 15 min, 4 °C) to remove insoluble material. The MPO-containing supernatant (0.1 mL) was assayed spectrophotometrically after the addition of 2.88 mL of phosphate buffer (50 mmol/L potassium, pH 6.0) containing 0.167 mg/mL o-dianisidine hydrochloride (Sigma Chemicals) and 0.0005% hydrogen peroxide. The kinetics of absorbance changes at 470 nm was measured. MPO activity was reported in units per milligram of wet tissue, where one unit of MPO was defined as the quantity of the enzyme ablbing to convert 1 μmol of hydrogen peroxide to water in 1 min at room temperature.

1.4 DRG dissection and immunohistochemistry
DRG neurons projecting to the distal colon were mainly in the spinal level of L3-S3. After rats were sacrificed, DRG from bilateral spinal levels of L3-S3 were removed. Dissections were carried out in phosphate-buffered saline (PBS). Any excess connective tissue and spinal roots were carefully dissected away before the ganglia were placed in 4% paraformaldehyde and stored at 4 °C for 2-4 h to fix. DRG were then rinsed in PBS before cryoprotection in sucrose (30%, 4 °C) overnight. Excess liquid was removed from each DRG, which were then snap frozen by brief immersion into 2-methylbutane (-30 °C) and subsequently stored individually at -70 °C. DRG were sectioned at -30 °C in a cryostat and four to eight DRG sections (16 μm) were cut and placed on individual slides. The sections from each ganglion were thaw-mounted non-serially onto six slides, ensuring each slide contained sections taken at least 50 μm lower in the ganglion than the previous sections. This reduced the probability that any cell would appear on the same slide more than once. Standard techniques were used for immunohistochemical labelling for CGRP and VR1. The sections were stained on the slides. Briefly, sections were covered in the primary polyclonal antisera (VR1: R18, goat, 1:50, Santa Cruz; CGRP: rabbit, 1:1 500, Peninsula) and left for 24 h in humid chambers at room temperature. Sections were subsequently incubated in donkey anti-goat AMCA (1:500) and donkey anti-rabbit FITC (1:100) for 1 h at room temperature. Prior to each incubation, sections were rinsed several times in PBS. Tissue sections were examined under a fluorescent microscope with appropriate filters. For each section, the number of Dil-labelled cells (only cells with nuclei), CGRP/Dil or VR1/Dil double-labelled cells, and CGRP/VR1/Dil triple-labelled cells were counted. All sections in a set of slides were examined.

1.5 Chemicals and reagents
TNBS, ethanol, o-dianisidine hydrochloride, hexadecyl trimethylammonium bromide and sucrose were purchased from Sigma Chemical Company. MPO activity assay kit was purchased from Shenzhen Kangshengbao Biotechnology Co. Ltd., China.

1.6 Statistical Analysis
For each animal, the number of Dil-labelled cells, CGRP/Dil or VR1/Dil double-labelled cells, and CGRP/VR1/Dil triple-labelled cells in L3-S3 DRG were added up to a single value for that animal. The values from 6 rats per group were then used for statistical analysis. The data were presented as the percentage of double- or triple-labelled cells for CGRP/Dil, VR1/Dil and CGRP/VR1/Dil in total Dil-labelled cells (means±SEM). Data were analysed using Student’s t test for repeated measures. P<0.05 was considered statistically significant.

2 RESULTS

2.1 Macroscopic examination of TNBS-induced colitis
After administration of TNBS, the acute inflammation in the distal colon was obvious on day 7. Visual inspection of the mucosa revealed erosion, adhesion, hyperemia and occasional petechial hemorrhage in all animals treated with TNBS. On day 21, the acute inflammatory reaction was resolved with some thickening pale mucosa left. On day 42, the mucosa seemed completely normal without border to the normal gut. In the control saline group, there wasn’t any damage in the colon mucosa. Damage score in day 7 group was markedly increased (3.96±0.45 vs 0.72±0.06), while on days 21 and 42 the damage scores decreased to the same level as that in the control saline group (1.12±0.10, 0.84±0.07 vs 0.72±0.06) (Fig.1). Ethanol had no damage to the mucosa at different time after enema (Fig.2).

2.2 MPO activity
MPO activity of the inflammatory tissue at different time after TNBS treatment was shown in Fig.1. On day 7 (the acute phase of the inflammation), MPO activity increased more than two folds compared with that in the control saline group [(7.82±0.81) vs (2.48±0.26) U/mg tissue]. In day 21 group, MPO activity was a little higher than that in the control saline group without statistical difference [(3.48±0.42) vs (2.48±0.26) U/mg tissue]. MPO activity on day 42 returned to the same level as that in the control
saline group [(2.52±0.34) vs (2.48±0.26) U/mg tissue].

Ethanol had no effect on MPO activity at different time after enema (Fig.2).

### 2.3 CGRP/VR1 immunohistochemistry

In all TNBS-treated groups, the percentages of CGRP/ DiI-, VR1/DiI- and CGRP/VR1/DiI-labelled neurons in L3-S3 DRG increased significantly compared to those in the control saline group. At the acute stage of the colitis (i.e., 7 d after TNBS treatment), the percentage of DRG neurons expressing CGRP and VR1 increased nearly two folds respectively [(95.38±9.45)% vs (42.86±5.02)% for CGRP, (89.23±8.21)% vs (32.54±4.58)% for VR1]. While in day 21 group, the percentage of DRG neurons expressing CGRP and VR1 still increased [(86.25±8.21)% vs (42.86±5.02)% for CGRP, (67.22±6.52)% vs (32.54±4.58)% for VR1]. In day 42 group when the colitis had completely been resolved, the percentage of DRG neurons expressing these two markers were still higher than that in the control saline group [(68.28±7.12)% vs (42.86±5.02)% for CGRP, (56.25±4.86)% vs (32.54±4.58)% for VR1] (Fig.3). Ethanol had no effects on the expressions of CGRP and VR1 in DiI-labelled neurons at different time after enema (Fig.2). There was co-expression of CGRP and VR1 in the colon-innervated DRG neurons. In the control saline group, 66% of VR1-positive neurons were also CGRP-positive. While in CGRP-positive neurons, 55% were VR1-positive (Fig.4). Moreover these CGRP/VR1 double-immunoreactive neurons increased not only at the acute stage of colitis [(76.52±8.56)% vs (22.16±3.25)%, but also on days 21 and 42 after TNBS treatment [(48.56±5.22)%, (42.45±4.65)% vs (22.16±3.25)%] (Fig.3). Ethanol had no effects on the expressions of CGRP and VR1 in DiI-labelled neurons at different time after enema (Fig.2).

Fig. 1. Mean damage score and MPO activity in the distal colon at different time after TNBS treatment. Mean damage score and MPO activity in day 7 group increased significantly. *P<0.05 vs control group. n=6.

Fig. 2. Mean damage score, MPO activity and CGRP or VR1 expression in the distal colon at different time after ethanol treatment. There was no difference between the control saline group and ethanol group. n=6.

Fig. 3. Percentage of CGRP- or VR1-positive neurons in the distal colon-innervated DRG neurons at different time after TNBS treatment. Neurons immunoreactive for CGRP, VR1, or double-positive for CGRP/VR1 were increased significantly on days 7, 21 and 42 after TNBS treatment. *P<0.05 vs control group. n=6.
3 DISCUSSION

The gut afferent pathway includes at least three levels of sensory neurons, i.e., enteric neurons, spinal neurons and cortical neurons. All kinds of afferent nerve fibers connecting these three levels of neurons are also indispensable to form a sensation. Damages to any part of the afferent pathway would lead to abnormal sensitivity including increased or decreased reaction to the physiological stimuli in the gut.

Previous studies have confirmed the role of infection in sensitizing the local gut tissue. Many kinds of neuropeptides such as substance P (SP), vascular intestinal peptide (VIP) and CGRP were released from stimulated primary afferents via axon reflexes to influence local cellular function as secretion and sensory-motor dysfunction[14]. The important significance for the present research was to study the effect of local gut inflammation on afferent transduction at the spinal cord level because sensitization occurred at the level of spinal cord, selectively increasing postsynaptic ascending messages[15]. It was indicated that the local colitis could sensitize the DRG afferent neurons innervating this area. The increased expressions of visceral nociceptor VR1 and primary afferent neurotransmitter CGRP persisted not only at the acute stage, but also after the local inflammation had been resolved for certain time. These results may be useful to explain the mechanism for FGID patients who suffer from the gut hypersensitivity without any biochemical or pathophysiological abnormality in the gut.

Many neuropeptides are also important inflammatory mediators which are released in the inflammation process[8]. These inflammatory mediators could influence afferent neurons including DRG and nodose ganglia neurons (which project to the upper gut), indicating a complex reciprocal relationship between the sensory neurons and inflammatory tissue in which they lie. Neuropeptides such as CGRP are released in response to noxious stimuli such as VR1 activation, acidosis or distension[15] and is thought to provide tissue protection by increasing blood flow to damaged areas[15]. The co-expression of CGRP and VR1 in DRG neurons indicated that there must be some relationship between VR1 activation and CGRP release.

Since the central nervous system is also involved in the afferent pathway, any damage to the cortical sensory neurons would result in abnormal sensibility to the gut stimuli. In clinical practice functional magnetic resonance imaging technique (fMRI) has been used to study the abnormal sensibility for FGID patients[16]. There were really some differences in the cortical areas being activated between the patients and normal controls subjected to gut stimuli under threshold. These results indicated that an abnormal gut sensation could also result from the cortical level. And any factor including infection, stress or neoplasia could cause the dysfunction of the cortical neurons.

Although studies including ours indicated that the antecedent local inflammation could change the sensitivity at the bowel and spinal cord levels which may lead to the long-term effect of visceral hyperalgesia in FGID patients, in clinical practice about two thirds of patients with gut inflammatory history did not develop into FGID. The answer for this question may be that we are not sure what severity of the gut inflammation would lead to the gut abnormal sensitivity through the whole afferent pathway. The results for the nerve damage and reparation was influenced by a lot of factors. Another reason is that symptoms of FGID are subjective rather than objective, which are closely related with patient’s recognition about pain or discomfort. The increased sensitivity at the spinal level does not mean the final increased sensitivity at the cortical level because the reciprocal relationship between the peripheral and cen-
tral nervous systems is also very complicated.

In conclusion, our study has shown that the local gut inflammation may disturb the gut sensory transduction pathway at the spinal cord level and that under certain circumstances this abnormal sensitivity may persist even after the gut inflammatory reaction has been resolved.

REFERENCES


