FULL PAPER *Pharmacology*

Colonic Mast Cell Infiltration in Rats with TNBS-Induced Visceral Hypersensitivity

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(Received 28 June 2007/Accepted 2 August 2007)

ABSTRACT. Colonic mucosal mast cells are implicated in the pathogenesis of visceral hypersensitivity associated with irritable bowel syndromes. This study was designed to investigate the roles of mucosal mast cells in development of an experimental visceral hypersensitivity induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in rats. TNBS, when injected into the proximal colon through laparotomy, produced a significant decrease in pain threshold of the distal colon to mechanical distention, indicating a visceral hypersensitivity. In the proximal colon that was directly insulted by TNBS, mucosal necrosis and extensive inflammatory cell infiltration were observed with concomitant increase in tissue myeloperoxide (MPO) activity. In the distal colon where distention stimuli were applied, the number of mucosal mast cells significantly increased following TNBS treatment, although neither mucosal injury nor increase in tissue MPO activity was observed. In an organ culture, spontaneous release of a mucosal mast cell-specific protease (RMCP-2) from the distal colon tissue of TNBS-treated rats was significantly larger than that of sham animals. Furthermore, TNBS-induced visceral hypersensitivity was significantly suppressed by subcutaneous pretreatment with a mast cell stabilizer doxantrazole in a dose-dependent manner. These findings suggest that prominent colonic mast cell infiltration associated with an enhanced spontaneous mediator release is responsible, at least partly, for development of visceral hypersensitivity induced by TNBS in rats.

KEY WORDS: doxantrazole, IBS, mast cell, TNBS, visceral hypersensitivity.

J. Vet. Med. Sci. 69(12): 1223–1228, 2007

The irritable bowel syndrome (IBS) is the most common functional gut disorders characterized by abdominal pain or discomfort [5, 14, 21, 25]. Accumulated data have shown that colorectal sensory threshold to mechanical distention stimuli is markedly decreased in IBS patients, indicating a phenomenon referred to as visceral hypersensitivity [5, 25]. Recent studies have suggested the roles of immune cells in the pathogenesis of abdominal pain associated with IBS [1, 16, 26], and mucosal mast cells have been a focus of much attention in relation to the close proximity to the enteric sensory nerves and capability to release a wide variety of mediators [2–4, 22]. Indeed, mucosal mast cells locate throughout the gut, and degranulate in response to various mechanical and chemical stimuli to release histamine, tryptase, eicosanoids and cytokines that can excite the visceral sensory nerves [15, 23]. Also, it has been demonstrated that IBS patients show a prominent colonic mast cell infiltration associated with frequent degranulation and increased spontaneous release of mediators, such as histamine and tryptase [2, 8]. In contrast, other investigators have found neither change in the number of intestinal mast cells in IBS patients, nor significant correlation between the number of intestinal mast cells and the severity of IBS symptoms [24, 25]. Thus the role of mucosal mast cells in the visceral hypersensitivity is still a matter of controversy.

The association of mucosal mast cells with visceral pain has also been investigated in rats. Coelho *et al.* reported that an intraperitoneal injection of mast cell degranulators, such as lipopolysaccharide and BrX-537A, to rats provoked acute increase in the rectal sensitivity to mechanical distention, which was significantly suppressed by pretreatment with a mast cell stabilizer doxantrazole [10]. Also, it was demonstrated that visceral hypersensitivity in the late phase of acetic acid-induced colitis was associated with the higher degranulation rate of colonic mucosal mast cells in rats [18]. These findings suggest the key role played by activated mast cells in the development of visceral hypersensitivity in rats.

Recently, it was reported that an injection of 2,4,6-trinitrobenzene sulfonic acid (TNBS) into the proximal colon of rats resulted in significant, sustained decrease in sensory threshold of the distal colon in which neither histological tissue damage nor inflammatory response was observed [11–13]. Since TNBS-induced increase in colonic sensitivity was found to last for 21 days and occurred in the non-inflamed region of the gut, it is considered to provide a valuable experimental tool for elucidation of pathophysiological mechanisms underlying chronic functional gut disorders characterized by visceral hypersensitivity, including IBS. In the present study, in order to confirm and further extend the possible involvement of mucosal mast cells in IBS, we investigated 1) whether TNBS-induced visceral hypersensitivity is associated with increased number of colonic mucosal mast cells, and 2) the effect of a mast cell stabilizer dexantrazole on the experimental visceral hypersensitivity.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley (SD) rats (240–270 g body weight, Charles River Inc.) were purchased from Charles Rive Co. (Tokyo, Japan). They were kept under conditions of constant temperature (23 ± 2°C) and humidity (55 ± 10%) with a 12-hr light/dark conditions with free

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access to normal laboratory chow and tap water. All procedures employed in the experiments were approved by the institutional Animal Ethics Committees according to the Laboratory Animal Welfare guidelines.

TNBS-induced hypersensitivity: After 16–18 hr fasting, the animals were anesthetized by combined intramuscular administration of ketamine (40 mg/kg) and xylazine (6 mg/kg), abdominal laparotomy was made for an injection of TNBS (50 mg/1.5 m//kg) into the proximal colon (1 cm distal from the cecum). The sham-operated rats were prepared with the same surgical procedure, but received vehicle alone instead of TNBS. Measurement of visceral pain threshold was carried out on day 7 post-surgery as described previously [13]. In short, a 5-cm latex balloon (Okamoto, Japan) was inserted through the anus and placed in the distal colon at 5 cm from the anus. After 30-min acclimation, the balloon was progressively inflated from 0 to 70 mmHg, by 5 mmHg increments every 30 sec using the electronic barostat (G&J, Canada). The distention procedure was repeated twice with 10 min interval, i.e. the first one for a preliminary conditioning and the second one to determine the pain threshold. Pain threshold was defined as the pressure that was required to elicit a behavioral sign of pain, corresponding to the repeated waves of contraction of oblique musculature with inward turning of the hind limb, or to hump-backed position, or to squashing of the lower abdomen against the floor [28]. In some experiments, animals were treated with subcutaneous doxantrazole (1.25, 2.5 and 5 mg/kg) at 2 hr before colonic distention. The doses of doxantrazole was chosen based on its efficacy in preventing mast cell degranulation-induced rectal allodynia [9].

Histological study: On day 7 following TNBS injection, a segment was taken from the proximal colon (5 cm proximally from the cecum) and distal colon (5 to 10 cm from the anus), sectioned transversely in their entirety, and fixed overnight in 4% paraformaldehyde. The fixed tissues were processed in paraffin, cut into 5 μm sections, stained with hematoxylin-eosin (H&E) or toluidine blue (TB), and examined with light microscopy. All sections were masked to avoid any biases in histological examinations.

Determination of myeloperoxidase activity: Tissue samples were taken from the proximal and distal colon on day 7 post-TNBS, cut into ~3 mm^2 pieces, and homogenized in 1 m*l* of 0.5% hexadecyltrimethylammonium bromide per 100 mg of colon tissue. The homogenate was stored at –80°C in a deep freezer. Myeloperoxidase (MPO) activity was determined according to the method described in a previous report [10]. The results were expressed as MPO units per milligram (wet weight) of tissue.

Determination of rat mast cell protease-II: The distal colon tissue measuring approximately 3 × 50 mm was removed from each animal, rinsed with PBS, and the wet weight was measured. Each tissue was incubated in 3 m*l* of RPMI medium at 37°C for 180 min, and the released contents of RMCP-2 in the medium was measured by a commercially available ELISA kit (Rat Mast Cell Protease-II ELISA: Moredun, Midlothian, UK). Results were expressed as nanograms of RMCP-2 released per gram (wet weight) of tissue [7].

Compounds: 2,4,6-trinitrobenzene sulfonic acid was purchased from Fluka (Buchs, Switzerland) and dissolved in 30% ethanol at 33 mg/m*l* for intra-colonic injection. Doxantrazole was purchased from Sigma and dissolved in 2% dimethyl sulfoxide for subcutaneous injection. Ketamine/xylazine solution was purchased from Sigma Aldrich Co. (St. Louis. MO).

Statistical analysis: The pain threshold data are represented as medians and the 1st and 3rd quartiles that indicate the range of median values calculated by Prism Software (GraphPad, CA, U.S.A.). The statistical analysis was carried out using Kruskal-Wallis testing followed by individual Mann-Whitney U-test and P-values less than 0.05 were considered as statistical significance. The data of the number of mast cells and colonic MPO and RMCP-2 activities are represented as mean ± SE, and subjected to Student's *t*-test for statistical analysis.

RESULTS

TNBS-induced hypersensitivity: As shown in Fig. 1, there was no significant change between naïve and sham-operated rats in the distention threshold of the distal colon required to elicit a behavioral signs of abdominal pain. An injection of TNBS into the proximal colon resulted in a significant decrease in the colonic pain threshold on day 7 post-TNBS.

Histological changes: Light microscopic examination of H&E-stained tissue sections demonstrated that there were submucosal edema, erosion, partial necrosis and extensive polynuclear cell infiltration in the proximal colon mucosa that was directly insulted by TNBS. No tissue damage was observed in the distal colon where the mechanical distention

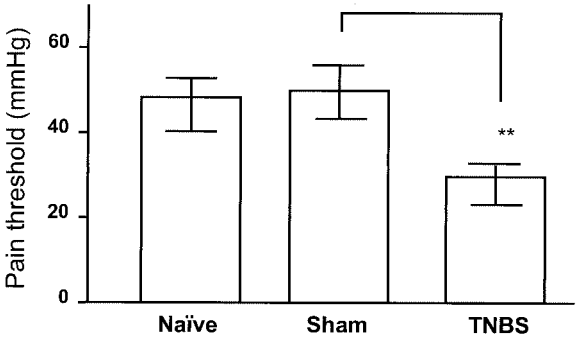


Fig. 1. Changes in the pain threshold of the distal colon in TNBS-treated rats on day 7 post-injection. Pain threshold is expressed as balloon pressure (mmHg) that was required for induction of an abdominal cramp in each group. Each group consists of 6 rats, and the data are represented as medians and the 1st and 3rd quartiles that indicate the range of median values calculated by Prism Software (GraphPad, CA, U.S.A.). The statistical analysis was carried out using Kruskal-Wallis testing followed by individual Mann-Whitney U-test. ** P<0.01 vs. sham-operated group.

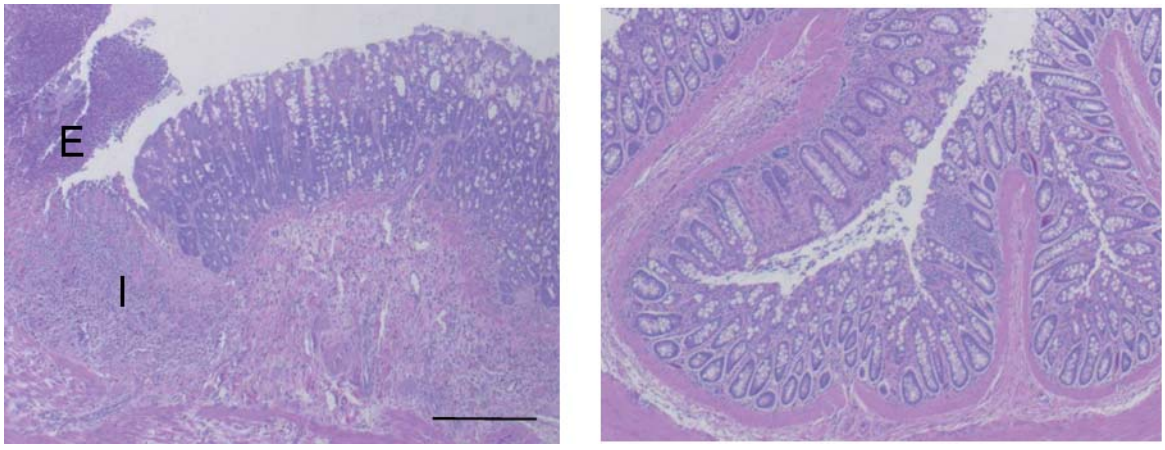


Fig. 2. Histological examination (Hematoxylin-Eosin stain) of the proximal (left) and distal (right) colon tissues of TNBS-treated rats on day 7 post-injection. Inflammatory cell infiltration (I) and erosion (E) were observed at the proximal colon. The bar in the left photograph indicates 200 μm.

stimuli were applied (Fig. 2). Also, no histological change was observed in the proximal and distal colon of sham-operated rats that received vehicle alone (data not shown).

Colon MPO activities: The changes of MPO activities in the proximal and distal colon of TNBS-treated rats on day 7 post-TNBS are summarized in Fig. 3. There was significant increase in the tissue MPO activity of the proximal colon that was directly insulted by an intra-colonic injection of TNBS, whereas MPO activity of the distal colon remained unchanged. The MPO activities of the proximal and distal colon tissues of sham rats were very low.

*Mast cell counts in the distal colo*n: The number of mast cells in the distal colon mucosa was counted by light microscopic examination of toluidine blue-stained sections. Figure 4 illustrates the changes in the number of toluidine-blue positive mucosal mast cells in the distal colon of rats on day 7 following an injection of TNBS into the proximal colon. Mucosal mast cell counts in the distal colon of TNBS-treated rats were significantly increased by 50% ($P<0.05$) compared to that of sham-operated control animals.

Colonic RMCP-II release: Spontaneous release of RMCP-2, a rat mucosal mast cell-specific protease, from the distal colon tissues was determined *ex vivo* as a specific marker of mucosal mast cell degranulation [19]. As shown in Fig. 5, RMCP-2 release from the distal colon of TNBS-treated rats was significantly increased by 200% ($P<0.05$) compared to that of sham-operated control animals.

Effect of doxantrazole on TNBS-induced hypersensitivity: Figure 6 shows the effect of subcutaneous treatment with doxantrazole, a mast cell stabilizer, on TNBS-induced visceral hypersensitivity in rats. This mast cell stabilizer suppressed the hypersensitivity at doses of 1.25, 2.5 and 5 mg/kg s.c. in a dose-dependent fashion, and the effect achieved statistical significance ($P<0.05$) at the highest dose.

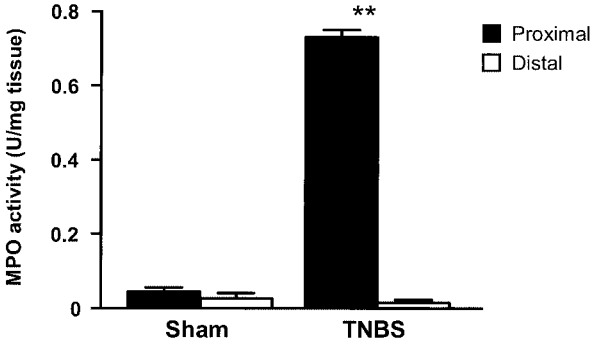
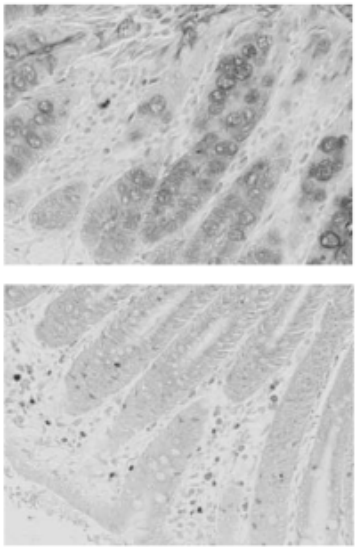


Fig. 3. Tissue myeloperoxidase activities in the proximal and distal colon of TNBS-treated rats on day 7 post-injection. Data are expressed as mean ± S.E. (n=6), and statistical analysis was carried out by Student's *t*-test. ** $P<0.01$ vs. sham-operated control group.

DISCUSSION

Due to the close proximity to the enteric sensory nerves and capability to release a wide variety of mediators that can excite enteric sensory nerves [2, 3, 22], colonic mucosal mast cells are considered to be implicated in the pathogenesis of visceral hypersensitivity associated with IBS. This study was designed to investigate the possible involvement of mucosal mast cells in the pathogenesis of an experimental visceral hypersensitivity induced by TNBS in rats.

TNBS, when injected into the rat proximal colon through laparotomy, produced a significant decrease in the pain threshold of the distal colon to mechanical distention stimuli, indicating development of a visceral hypersensitivity. This finding is in agreement with the earlier report of Diop *et al.* [13]. Histological examination showed that mucosal necrosis and extensive inflammatory cell infiltration with concomitant increase in tissue MPO activity were produced in the proximal colon that was directly insulted by TNBS.



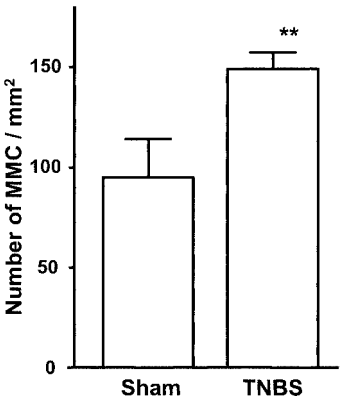


Fig. 4. Changes in the numbers of mucosal mast cells in the distal colon of TNBS-treated rats on day 7. Mucosal mast cells were stained by toluidine blue (Photo Upper: Sham-operated rat colon, Photo Bottom: TNBS-treated rat colon). The data are expressed as mean ± S.E. (n=4), and statistical analysis was carried out by Student's *t*-test. ** P<0.01 vs. sham-operated group.

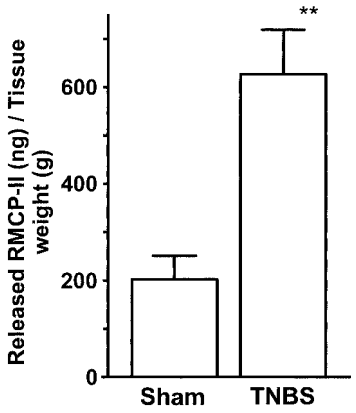


Fig. 5. Changes in the released rat mast cell protease-II (RMCP-2) in the distal colon of TNBS-treated rats on day 7. The data are expressed as mean ± S.E. (n=4), and statistical analysis was carried out by Student's *t*-test. **P<0.01 vs. sham-operated group.

On the other hand, neither mucosal injury nor increase in tissue MPO activity was observed in the distal colon, indicating that TNBS-induced visceral hypersensitivity was not caused by the local tissue damage or inflammatory cell infiltration in the region of the gut where distention stimuli were applied.

Our data also demonstrated that the number of mucosal mast cells in the distal colon was significantly increased following a TNBS injection into the proximal colon. In addition, it was found that the distal colon tissue of TNBS-treated rats spontaneously released RMCP-2 to a greater extent compared to that of sham-operated animals. Furthermore, the extent of increase in RMCP-2 release was 4-fold larger than that in the number of mucosal mast cells, suggesting that, in addition to the increased cell count, enhanced degranulation rate and mediator synthesis also contribute to the demonstrated increase in spontaneous mediator release from the distal colon of TNBS-sensitized rats. Based on these biochemical and histological data, it is conceivable that prominent mucosal mast cell infiltration associated with an enhanced spontaneous mediator release is responsible, at least in part, for the increased sensitivity of enteric sensory nerves after TNBS treatment. This view is further supported by the present finding that TNBS-induced visceral hypersensitivity was significantly suppressed by pretreatment with a mast cell stabilizer dexantrazole in a dose-dependent fashion [9]. This mast cell stabilizer has been reported to suppress the visceral hypersensitivity induced by mast cell degranulation at same doses as used in the present study, its effect on TNBS-induced hypersensitivity is considered to be attributable to inhibition of mediator release from mucosal mast cells.

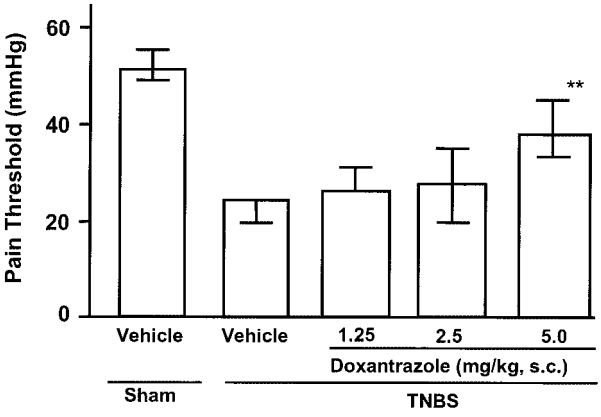


Fig. 6. Effect of a mast cell stabilizer doxantrazole on colonic pain threshold in TNBS-treated rats. Pain threshold of the distal colon is expressed as balloon pressure (mmHg) required for induction of an abdominal cramp in each group. Each group consists of 6 rats, and the data are represented as medians and the 1st and 3rd quartiles that indicate the range of median values calculated by Prism Software (GraphPad, CA, U.S.A.). The statistical analysis was carried out using Kruskal-Wallis testing followed by individual Mann-Whitney U-test. ** P<0.01 vs. sham-operated group.

The mechanisms underlying a prominent mast cell infiltration associated with increased degranulation rate after TNBS treatment are unknown in the present study. A few reports have described that colonic mucosal mast cell counts were increased in rats with TNBS-induced colitis [20, 29]. These studies, however, examined the intestinal tissues that was directly exposed to TNBS insult, and detected the

increase in mast cell counts in the late phase of the colitis with tissue repair. In the present study, we examined the colonic mast cells in the tissue that remained intact, but was with increased sensitivity of enteric sensory nerves. Since severe tissue damage and extensive inflammatory cell infiltration were observed in the proximal colon following TNBS injection, it seems likely that inflammatory mediators or cytokines released during the development of primary inflammation in the proximal colon may affect the distant region of the gut to induce mast cell infiltration with increased degranulation rate. Indeed, TNBS has been shown to produce an increase of various inflammatory mediators and cytokines, such as leukotriene B4, platelet activating factor, prostaglandins E_2 and $F_{2\alpha}$, and inducible nitric-oxide synthetase in the gastrointestinal tissues [17, 27]. Further investigation will be necessary to elucidate this hypothesis as to the pathological mechanisms of visceral hypersensitivity and colonic mast cell infiltration induced by TNBS.

In summary, the present study demonstrates that TNBS-induced visceral hypersensitivity was associated with increases in colonic mucosal mast cell counts and degranulation rate, and that it was significantly attenuated by pretreatment with a mast cell stabilizer dexantrazole. These data strongly suggest that enhanced spontaneous release of mucosal mast cell mediators is responsible, at least in part, for the increased sensitivity of colonic sensory nerves in TNBS-treated rats.

ACKNOWLEDGMENTS. We are grateful to Drs. Nigel Bunnett (Department of Surgery and Physiology, The University of California) and John Furness (Department of Anatomy and Cell Biology, The University of Melbourne) for continuous encouragement and helpful suggestions on this study.

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