Experimental colitis in mice and sensitization of converging visceral and somatic afferent pathways

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Lamb, Kenneth, Fang Zhong, G. F. Gebhart, and Klaus Bielefeldt. Experimental colitis in mice and sensitization of converging visceral and somatic afferent pathways. Am J Physiol Gastrointest Liver Physiol 290: G451–G457, 2006. First published September 29, 2005; doi:10.1152/ajpgi.00353.2005.—Chronic pain syndromes affecting different organs often coexist. We hypothesized that sensitization of one afferent pathway may affect converging input from other areas of the body. We induced colitis in mice with 2,4,6-trinitrobenzenesulfonic acid (TNBS); control animals were treated with equal volumes of vehicle (50% ethanol) only. Visceromotor responses to graded colorectal distension, cystometrograms, and response thresholds to mechanical and thermal stimulation of both hind paws were determined on days 7 and 14. Inflammation of colon and bladder was assessed with validated histological markers and scores. TNBS caused significant colitis on day 7 that resolved by day 14; there was no evidence of bladder inflammation. There was a significant hypersensitivity to colorectal distension on day 7, which returned to normal on day 14. This was associated with bladder overactivity, as demonstrated by early onset of micturition and more frequent micturition on day 7 after TNBS administration. Colitis also significantly altered responses to mechanical and thermal stimulation of both hind paws on day 7 but not day 14. We conclude that cross talk between afferent visceral and somatic pathways may contribute to the coexistence of pain syndromes.

visceral hypersensitivity; micturition

MANY PATIENTS EXPERIENCE PAIN affecting multiple areas of the body, which is generally attributed to changes in central processing, such as hypervigilance or decreased descending inhibitory modulation (1, 2, 5, 10, 31, 57, 60, 62, 63). Several studies also indicate that peripheral mechanisms may contribute to the coexistence of some pain syndromes. Anatomic and functional data suggest that axons may dichotomize and send their peripheral terminals to anatomically distinct tissues (12, 32). More likely, because several primary sensory neurons converge onto second-order neurons in the spinal cord, sensitization of one afferent pathway can lead to heterosynaptic facilitation and alter processing of input from other neurons (40–42, 49). Cystitis affects responses to colorectal distension in rats and mice, and colitis alters the micturition reflex in rats, which is due at least in part to viscerovisceral convergence in the spinal dorsal horn (7, 47, 49). But this cross talk goes beyond the two neighboring pelvic organs. Experimentally induced cystitis decreases thresholds to stimulation of the hind paw (viscerosomatic sensitization), and inflammation in the lower extremities causes enhanced responses to colorectal distension (somato-rectal sensitization) (7, 36, 45). On the basis of these results, we hypothesized that sensitization of colon afferents during inflammation will affect convergent afferent pathways from somatic and visceral structures.

METHODS

Animals. All experiments were performed with adult male C57BL/6 mice (Taconic, Germantown, NY) weighing 20–30 g. The animals were housed in groups of four on wood shavings with free access to water and food. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Iowa and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Surgical preparations. Mice were pretreated with 40 μg/kg intraperitoneal atropine (Fujisawa, Deerfield, IL) 10–60 min before being anesthetized with ketamine (87.5–175 mg/kg) and xylazine (12.5–25 mg/kg ip) (Phoenix Pharmaceutical, St. Joseph, MO). To obtain electromyographic (EMG) recordings from the abdominal musculature, Teflon-coated stainless steel wires (Cooner Wire Sales, Chatsworth, CA) were sewn into the external oblique abdominal musculature proximal to the inguinal ligament. The wires were tunneled out through the neck as described previously (24).

Induction of localized inflammation. To induce colitis, we anesthetized mice as described above, and 5 mg/ml 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma; St. Louis, MO) in 50% ethanol (total volume 0.1 ml) was instilled into the rectum with a 1.2-mm polyethylene catheter (16). Control mice were treated with a similar volume of vehicle (50% ethanol) only. To ensure exposure of the distal colon, mice were held up by the tail for 3 min.

Evaluation of mucosal inflammation. To assess the severity of colitis, we used a previously validated grading scheme. After completion of cystometrograms (see below), mice were euthanized by an overdose of pentobarbital (Nembutal, 200 mg/kg ip; Abbott Laboratories, Abbott Park, IL), and the distal 3 cm of the colon and the bladder were removed on either day 7 or day 14 after induction of colon inflammation. The tissue was fixed in formalin, embedded in paraffin, cut in 10-μm sections, and stained with hematoxylin and eosin. Sections were analyzed by a person blinded to the treatment and graded with previously validated semiquantitative scoring systems (16, 52).

Mechanical sensory threshold. Mice were placed individually on an elevated metal grid, covered with clear plastic cages (10 × 10 × 13 cm), and allowed to acclimate for 1 h. Baseline withdrawal thresholds to punctate mechanical stimuli were determined by applying calibrated Semmes-Weinstein von Frey filaments from underneath the grid (openings 12 × 12 mm) to the glabrous skin of the right or left hind paw of an inactive mouse. To apply appropriate punctate pressure, care was taken to see bending of the filament and keep it in position for 1 s. All filaments between 1.65 and 4.74 mN were applied to each hind paw and tested 10 times each, with at least 10 s between applications. We chose 4.74 mN as the cutoff because the filament lifted off the paw in essentially 100% of the tests.

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Thermal sensory threshold. Mice were placed in plastic cages (10 × 10 × 13 cm) on top of a 6.0-mm-thick glass surface and allowed to acclimate for at least 1 h before testing. A radiant heat light source (setting: 25) was applied to the plantar surface of the right or left hind paw of an inactive mouse (IITC, Woodland Hills, CA). The latency to withdraw was measured to the nearest 0.1 s. All tests were performed in triplicate with at least 3 min between tests. To prevent thermal injury, the heat stimulus was automatically turned off at 30 s.

Visceromotor response to colorectal distension. To determine changes in colonic sensation, we measured the visceromotor response to distension (24). Mice were briefly sedated with halothane to introduce a small balloon catheter [3-cm polyethylene plastic balloon attached to polytetrafluoroethylene (PTFE)-24 tubing, Cole-Parmer Instrument, Vernon Hills, IL] into the rectum. The distal tubing was taped to the tail to secure proper placement. Mice were then placed into small restraining devices and allowed 30 min for recovery from sedation and acclimation before testing. With a custom-made pressure distension device, the colorectal balloon was distended for 20 s to 15, 30, 45, or 60 mmHg, separated by 4-min intervals. To determine the visceromotor response (VMR) to colorectal distension, the EMG activity of the external oblique muscle was amplified, rectified, and recorded 10 s before and during the stimulus. The EMG signal was integrated and normalized as change over baseline activity (Spike2 software; Cambridge Electronic Design, Cambridge, UK).

Cystometrogram. Mice were anesthetized with halothane (1-1.5% in 100% O2 at 2 l/min, ≈5 min; Halocarbon Laboratories, River Edge, NJ), and the urinary bladder was exposed through a suprapubic incision. A 30-gauge needle was inserted into the dome of the bladder and affixed with cyanoacrylate. After 15 min, the bladder was drained. Two minutes later, prewarmed (37°C) saline was infused into the bladder at a constant rate of 20 μl/min for 20 min. Micturitions were observed, and bladder pressure was continuously monitored with a pressure transducer. The time interval between the onset of bladder infusion and the first micturition (micturition onset) and the number and peak amplitude of micturition contractions were analyzed by a person blinded to the treatment.

Data analysis. All data are given as means ± SE. Results were analyzed with Mann-Whitney rank sum test or two-way ANOVA for repeated measures where appropriate, followed by Holm-Sidak’s or Dunn’s test for multiple comparisons if warranted. A value of *P < 0.05 was considered statistically significant.

RESULTS

Induction of experimental colitis. Instillation of TNBS caused macroscopic inflammation of the colon with wall thickening and some edema on day 7. On day 14, there was no difference between control mice and mice treated with TNBS. Visual inspection of the bladder did not show any differences on either day 7 or day 14. Consistent with this impression, we found an increase in the thickness of the lamina propria with microscopic signs of edema, mucin depletion, and inflammatory infiltrate with areas of transmural inflammation of the colon on day 7 after TNBS, but not vehicle, instillation. There was no difference between the two groups on day 14 (Fig. 1). Although we noted some engorged subepithelial blood vessels in the bladder at both time points in control and TNBS-treated
mice, there was no inflammatory infiltrate within the bladder wall (Fig. 1).

Effect of colitis on VMRs to colorectal distension. In control mice, graded colorectal distension triggered a significant increase in EMG activity above baseline when balloon pressures exceeded 30 mmHg. TNBS treatment shifted the stimulus response function to the left on day 7 \((F = 7.3, P < 0.01)\), and it returned to control levels on day 14 (Fig. 2).

Effect of colitis on micturition reflex. Constant infusion of prewarmed saline into the bladder triggered an initial micturition in controls within \(648 \pm 19\) s. Within the 20-min period of bladder infusion, control mice showed reflex voiding of the urinary bladder 6.3 \(\pm 0.2\) times, generating a peak micturition pressure of 11.9 \(\pm 0.4\) mmHg. As shown in Fig. 3, TNBS colitis significantly altered the micturition reflex when examined on day 7, significantly shortening the time to the initial onset of micturition and triggering significantly more frequent voiding compared with controls. On day 14, there was no difference between control and TNBS-treated mice (Fig. 3).

Effect of colitis on responses to hind paw stimulation. Increasing the force applied to the glabrous skin of the right hind paw triggered an increasing frequency of withdrawals in both control and TNBS-treated mice, with essentially all animals responding at 4.31 mN. Compared with control mice, TNBS-treated mice showed a significant shift in the stimulus-response curve to the left on day 7 (right hind paw: \(F = 6.6, P < 0.05\); left hind paw: \(F = 10.2, P < 0.01\)); there were no differences between groups on day 14 (Fig. 4).

In controls, withdrawal latencies of the right and left hind paws to the thermal stimulus were 13.1 \(\pm 0.9\) and 13.3 \(\pm 1.1\) s, respectively. As shown in Fig. 5, there was a significant decrease in the withdrawal latency in TNBS-treated mice on day 7, but not day 14, after induction of colitis.

**DISCUSSION**

Convergence of sensory pathways is responsible for the referral of visceral pain and may contribute to the coexistence of pain syndromes affecting different areas of the body (46, 47). The present study extends a recent study showing the effect of cystitis on responses to colorectal distension and mechanical and thermal stimulation of the hind paw (7). The key findings of the present study are that 1) TNBS colitis in mice produces a visceral hypersensitivity that does not persist after resolution of the inflammation, 2) bladder overactivity...
develops during active colitis, and 3) colitis is associated with reduced response thresholds for both mechanical and thermal stimulation of the hind paws bilaterally.

Consistent with prior investigations, rectal TNBS instillation caused colitis in C57BL/6 mice that essentially resolved within 14 days (20). Induction of colitis increased the VMRs to colorectal distension, documenting the presence of a visceral hypersensitivity, as was previously shown in rats (15, 54). Resolution of colitis on day 14 was associated with normalization in the responses to colorectal distension, whereas Diop et al. (15) observed enhanced responses 21 days after inducing TNBS colitis in rats. This apparent discrepancy could be due to differences in TNBS instillation (distal colon in the present study vs. proximal colon during laparotomy). Furthermore, the lower sensitivity of C57BL/6 mice to TNBS colitis compared with other mouse strains may decrease the development of hypersensitivity (58); we previously noted (44) a relationship between severity of gastric inflammation and persistence of gastric hypersensitivity.

In addition to enhanced responses to colorectal distension, colitis also triggered bladder overactivity in anesthetized mice, which is a well-established method to investigate the modula-

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**Fig. 4.** TNBS colitis induces reversible hypersensitivity to mechanical stimulation of hind paws. In control animals (n = 9 for day 7, n = 6 for day 14), increasing forces applied to the hind paw consistently triggered paw withdrawal when calibrated von Frey filaments exceeding 3.0 mN were used. There was a significant left shift in the stimulus response function for the right (A and C) and left (B and D) hind paw on day 7 (n = 9) but not on day 14 (n = 6) after TNBS instillation.
Latency on day 7, day 7 (of 13.1 s for the right (A) and 13.2 s for the left (B) hind paw. TNBS instillation in naïve rats (day 7, n = 9; day 14, n = 6) caused a significant decrease in withdrawal latency on day 7 but not day 14. *P < 0.05.

Fig. 5. Box plot showing that TNBS colitis induces reversible hypersensitivity to thermal stimulation of hind paws. In control animals (day 7, n = 9; day 14, n = 6) radiant heat application triggered paw withdrawal with a median time of 13.1 s for the right (A) and 13.2 s for the left (B) hind paw. TNBS instillation (day 7, n = 9; day 14, n = 6) caused a significant decrease in withdrawal latency on day 7 but not day 14. *P < 0.05.

Our findings confirm and expand recently published results showing that colonic irritation similarly changes reflex micturition in rats (47). Whereas Pezzone et al. (47) only examined the acute effects of intracolonic administration of ethanol and TNBS, we demonstrate here that these effects can be seen for at least 7 days after the initial insult and are associated with evidence of colon inflammation. Several mechanisms can account for cross-sensitization of two pelvic organs. First, transmural inflammation as seen during severe TNBS colitis may involve the bladder and thus lead to changes in bladder function. Considering the absence of macro- or microscopic signs of bladder inflammation, this seems an unlikely explanation for our results. Second, recent studies suggest the presence of dichotomizing axons extending peripheral processes to both bladder and colon, providing a mechanism for cross-sensitization as observed in the present study (12, 32). Third, convergence of primary afferents onto the same second-order neuron in the spinal cord is responsible for referral of visceral pain to somatic sites (41, 42). Although not studied intensely, viscerovisceral convergence has been observed in rats for different organs in the pelvis and thoracic cavity (6, 17, 49).

Increased peripheral input, perhaps coupled with release of target-derived factors such as neurotrophins, can alter the properties of second-order spinal neurons (central sensitization), augmenting responses to synaptic input (7, 11, 19, 25). At least two independent lines of evidence suggest that such enhanced responsiveness affects different inputs to the same second-order neurons (heterosynaptic facilitation). 1) Direct recording from spinal neurons showed increased responses to somatic stimulation of the site of convergence in animal models of visceral hypersensitivity (45, 51). 2) Using paradigms that induce long-term potentiation in the spinal cord, Klein et al. (26) showed perceptual changes in humans that support the importance of heterosynaptic facilitation as a mechanism of central sensitization.

Further support is provided by the changes in sensory thresholds in somatic areas of referral that have been reported in animal models of diseases affecting the urinary bladder, ureter, and uterus (8, 22, 23, 27, 28, 61). In humans, acidification of the esophagus acutely sensitized the esophagus and chest wall, but not a site distant from the referral area, to electrical stimulation (53). Similarly, inflammation, such as seen in appendicitis and symptomatic gallbladder disease, altered cutaneous sensitivity in the area of referral, but not the contralateral site (18, 55). We did not examine potential mechanisms underlying the likely changes in second-order spinal neurons. Using a different paradigm, Miranda et al. (36) recently demonstrated that ionotropic glutamate receptors play an important role in the cross-sensitization of visceral and somatic sensory pathways.

Although consistent with previous observations after sensitization of bladder afferents, the increased responses to cutaneous stimulation were seen outside of the dermatomes that overlap with visceral afferents innervating the distal colon, which project to the thoracolumbar (T13–L2) and lumbar-sacral (L6–S1) segments of the spinal cord (12, 33, 40, 59). Recordings from spinal neurons responding to colorectal or bladder distension performed in naive rats showed convergent cutaneous receptive fields in the medial thigh, perineum, or genitalia, with only a few cells being excited by stimulation of the hind paw (38, 39, 41). However, adjacent segments within the spinal cord are activated after irritation of the colon, thereby potentially contributing to an increase in the cutaneous referral area, which is also seen in patients with functional bowel disorders (21, 35, 37, 43). These experimental observations, including the lower response thresholds for mechanical and thermal stimulation of the hind paws noted bilaterally in the present study, suggest an involvement of supraspinal sites as well as contributions from convergent afferent input to viscerosomatic hypersensitivity. Interestingly, acute stimuli, such as visceral distension or luminal application of irritants, can cause opposing results such as longer withdrawal latencies and higher thresholds for mechanical stimulation in areas distant from the convergent somatic referral sites (9, 41, 48, 56). This hyposensitivity has been attributed to increased descending inhibitory modulation of nociceptive input (42, 48). Systemic or local effects of inflammatory mediators may contribute to the apparent discrepancies between results obtained after acute or more lasting (>1 day) injury, which may affect peripheral afferents as well as descending modulatory pathways (4, 14, 29, 30). The present results illustrate how cross-talk between sensory pathways innervating different viscera and/or viscera and cutaneous referral areas may provide a mechanism for the coexistence of different pain syndromes. However, we only noted changes in sensory function during the presence of...
inflammation, whereas many clinically relevant disorders are characterized by pain in the absence of identifiable structural abnormalities such as inflammation. In contrast to our results obtained in adult animals, induction of colitis during the early neonatal period can produce long-lasting, chronic colon hyper-sensitivity in rats (3). Future studies must examine whether such long-lasting visceral hypersensitivity is associated with sensory changes in areas of viscer-visceral and viscer-so-matic referral.

GRANTS

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