Attenuation of acute experimental colitis by preventing NPY Y1 receptor signaling

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Neuropeptide Y (NPY), a 36-amino acid peptide, is involved in the regulation of several physiological processes, including energy balance, food intake, and nociception. Recently, we showed that activation of the NPY Y1 receptor is required for cutaneous neurogenic inflammation. Because neurogenic inflammation could participate in colitis, the aim of this study was to investigate the role of the NPY Y1 receptor in acute colitis using mice genetically deficient of NPY Y1 receptor. In addition, the Y1 receptor antagonist H409/22, was also investigated. Animals received 5% dextran sulfate sodium (DSS) in drinking water for 7 days. One group of animals also received the Y1 receptor antagonist, administered intraperitoneally twice daily. Disease activity was assessed daily for 7 days in all groups. DSS induced colitis in all animals resulting in weight loss, diarrhea, epithelial damage, crypt shortening, and inflammatory infiltration. However, clinical manifestation of the disease was markedly attenuated in Y1 null mutant mice as well as in mice receiving the Y1 antagonist. Histological analysis showed that tissue damage and ulceration were less severe in Y1-deficient animals. Consistent with the clinical and histological data, capsaicin-induced plasma extravasation was significantly reduced in the gut of Y1 null mutant animals compared with treated wild-type animals. These data indicate that NPY and Y1 receptor are involved in intestinal inflammation and suggest that inhibition of NPY Y1 receptor signaling may provide a novel therapeutic approach in the treatment of colonic inflammation.

neurogenic inflammation; dextran sulfate sodium; in vivo animal models

ULCERATIVE COLITIS AND CROHN’S disease constitute the two main forms of inflammatory bowel disease (IBD). This disease is characterized by a dysregulated immune response and chronic relapsing inflammation of the gastrointestinal (GI) tract, leading to diarrhea, hemoccult, abdominal pain, weight loss, and anemia. Despite intense research, the etiology of IBDs remains largely unknown. The development of IBD involves interactions among genetic, immunological, and environmental components (46). Other mediators implicated in the pathogenesis of colitis are neuropeptides released from enteric and sensory afferent neurons (37). These peptides have been shown to modulate different aspects of mucosal function, including blood flow and secretion, and may play a role in the recruitment of granulocytes and lymphocytes and in the modulation of mast cell activation (14, 18).

Stimulation and activation of capsaicin-sensitive sensory nerve endings produce an inflammatory response characterized by vasodilation and plasma extravasation (13). The inflammatory response is mediated by local release of sensory neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) (25). The inflammatory response, termed neurogenic inflammation, is well described in many peripheral organs including the skin, airways, and urogenital tract (25). However, the capability of sensory neurons in evoking a neurogenic inflammatory response in the GI tract is equivocal. There is evidence suggesting neuropeptides, such as SP and its receptors, play a significant role in initiating and modulating GI inflammation. A marked increase in both the colonic mucosal SP concentration and SP binding sites on mucosal blood vessels and lymphoid tissue in patients with ulcerative colitis (29, 47) has been observed. Other reports (35) have implicated SP in acute inflammation of the ileum induced by Clostridium difficile. In addition, recent experiments have shown that administration of a nonpeptide NK-1 receptor antagonist attenuates and reduces colonic inflammation in a rat model of chronic experimental colitis (40).

Neuropeptide Y (NPY), a 36-amino acid peptide, is involved in the regulation and modulation of multiple physiological processes in the body, including energy balance and feeding, anxiety, blood pressure, and nociception. The effects of NPY are mediated by the activation of at least five NPY-receptor subtypes that belong to the family of G protein-coupled receptors (44). NPY-immunoreactive nerve fibers are abundant in GI tract (22, 23). With the use of receptor autoradiography, NPY and peptide YY (PYY) binding sites have been localized to submucosal and myenteric plexi (30, 45). In the GI tract, NPY and PYY have been shown to be potent inhibitors of intestinal fluid, electrolyte secretion, and motility (36, 38). The receptors mediating these effects include Y1, Y2, Y4, and Y5 NPY receptors (11). We have recently reported that mice genetically deficient in NPY Y1 receptor display an absence of plasma extravasation and lack of increased SP release following capsaicin administration in skin (33), suggesting a role of Y1 receptor in the release of SP. Therefore, subsequent neurogenic inflammation in the skin/periphery of these mice is absent. The purpose of the present study was to determine the role of NPY and Y1 receptor in colonic inflammation in a well-described model of experimental colitis. Dextran sulfate sodium (DSS) given in water to rodents for 7 days induces a colitis that

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resembles ulcerative colitis in humans. Using DSS to induce colitis, we studied the role of the Y1 receptor using Y1 receptor-deficient mice.

MATERIALS AND METHODS

Animals and experimental design. Eight- to twelve-week-old female NPY Y1+/+ or NPY Y1−/− mice (33) of mixed SV 129 × Balb/c background, matched for weight, were bred and housed according to standard environmental conditions. Mice were fed on standard rodent chow and water ad libitum. At the start of the experiment, mice were weighed and housed, two by two, in each cage. Four groups of animals were used for experiments: controls received H2O, experimental animals received 5% (wt/vol) DSS (molecular weight 30,000–40,000; ICN Biomedicals) in drinking water, animals received 5% DSS in conjunction with the NPY Y1 receptor antagonist H409/22 (AstraZeneca) (27), and one group of animals received 5% DSS for 6 days followed by a period of 14 days during which they received H2O. Different lot number of the DSS results in different kinetics of development of acute experimental colitis, but within one lot number, the kinetics are consistent between experiments. All acute experiments were performed on one lot number, and the study on severity of DSS-induced colonic injury in Y1+/+ and Y1−/− mice after a period of recovery was performed on another lot number.

All groups of mice within each experimental setup received DSS from the same lot number. All animals administered with Y1 receptor antagonist were wild-type (WT; Y1+/+) mice and received a dose of 10 mg/kg body wt ip twice daily, starting simultaneously with DSS treatment. Control animals received vehicle (saline) injected in the same manner as the Y1 receptor antagonist group. After 7 days, animals receiving DSS were killed by CO2 asphyxiation, and their colons were immediately removed. The intestinal specimens were prepared as previously described (26), embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Assessment of colitis. Body weight, presence of occult (detected by Hemoccult strips; Sarstedt), or gross blood in feces and stools consistency were assessed daily for each animal. Each parameter was assigned a score and was used to calculate an averaged daily disease activity index (DAI) for each animal. The DAI for each animal is the combined score of weight loss, stool consistency, and bleeding divided by three, adapted from Cooper et al. (5). In addition, food and water consumption was recorded daily in all experiments. No differences in food and water consumption were seen (data not shown). To quantitate the extent of the colon ulceration, image analysis was performed on more severely affected sections of each colon with ulceration. With the use of National Institutes of Health 1.62 image-analysis software, the size of ulceration (μm) of each colon was measured as the distance between the two edges at the ulcer base and divided by the length of the entire tissue. Data are presented as percent ulceration.

Extravasation of Evans blue. Evans blue is a dye that binds to plasma proteins. It remains within the vasculature until gaps are formed between endothelial cells and it leaks out into tissues. The leakage of Evans blue into tissue can be employed to measure extravasation of plasma proteins. Mice were anesthetized subcutaneously with 2.4% averin (2,2,2-bromoethanol, dissolved in 2-methyl-2-buthanol, diluted in 0.9% saline; Sigma, Sigma-Aldrich, St. Louis, MO). Evans Blue (50 mg/kg, dissolved in 0.9% saline; Sigma, Sigma-Aldrich) was injected intravenously into the left jugular vein. After 5 min, 100 μl of a solution of 1% capsaicin (dissolved in 0.9% saline containing 5% Tween-80 and 5% ethanol; Sigma) were administered through a plastic cannula, the tip of which was 3 cm proximal to the anus (Y1+/+ mice, n = 6; Y1−/− mice, n = 7). Control mice (WT mice, n = 6; Y1−/− mice, n = 7) received vehicle solution only (0.9% saline containing 5% Tween-80 and 5% ethanol). The distal colon was exposed to capsaicin or vehicle for 10 min. Animals were killed, and the colonic tissue in front of the inserted tube (1 cm) was removed, gently opened, and cleaned. Tissues were weighed, and Evans blue was extracted by incubation in formamide at 55°C for 24 h. Evans blue was quantitated spectrophotometrically at 620 nm and expressed as micrograms per gram weight of the tissue.

Immunohistochemistry. Control and DSS-treated animals were killed by CO2 asphyxiation, and their colons were immediately removed. Tissues were immersed in 4% paraformaldehyde overnight. After cryoprotection in 20% sucrose for 48 h, tissues were mounted in OCT and sectioned (10 μm). Sections were incubated for 1 h in PBS, 0.3% Triton X-100, and 1% bovine serum albumin at room temperature. Antibodies that include rabbit anti-NPY serum (1:200; Peninsula Laboratories) were diluted in blocking buffer. Sections were incubated with primary antibodies at 4°C, washed in PBS, and incubated with secondary antibodies at room temperature. A Cy2-conjugated donkey anti-rabbit (1:200 to 1:500; Jackson Immunoresearch Laboratories) secondary antibody was used. Confocal images were taken using a Zeiss Axioplan LSM 510 confocal microscope.

Statistics. Statistical analysis of the results were analyzed by unpaired (between subjects) or paired (within subjects) two-tailed Student’s t-test when indicated. Data from the time course of changes in the DAI were analyzed by two-way ANOVA with repeated measurements (time, within subjects; and genotype between subjects). After main effect of ANOVA, individual comparisons between subjects were made by the Student’s t-test. Significance was assumed to occur at P < 0.05. All results are expressed as means ± SE.

RESULTS

Reduced disease activity of DSS-induced acute colitis in NPY receptor Y1−/− mice and by NPY Y1-receptor antagonist. Colitis was induced by oral administration of 5% DSS to WT (i.e., Y1+/+) and Y1 null mutant (Y1−/−) mice. The first clinical signs were observed at day 4, where Y1+/+ mice showed signs of hemoccult positive stools, gross bleeding, and loose stools. However, none of the above clinical findings were noted in Y1−/− mice at the same time point, although both groups exhibited weight loss (Table 1). The clinical signs became more severe in both groups over time. However, in Y1−/− mice, the symptoms of colitis were milder and delayed. Body weight was decreased in both groups but occurred more rapidly in Y1+/+ mice compared with Y1−/− mice. At day 7, weight loss in both groups was indistinguishable. Disease activity indexes for both groups indicated that the physical signs induced by 5% DSS were significantly less severe in Y1−/− mice and the clinical symptoms were attenuated compared with Y1+/+ mice. No physical findings were noted in Y1+/+ and Y1−/− mice receiving H2O (n = 5 for Y1+/+ and n = 6 for Y1−/−; data not shown). Also, no significant difference in food and water consumption was observed between the two experimental groups in the absence or presence of 5% DSS (data not shown).

To exclude that the ameliorating effects of the genetic ablation of the NPY Y1 receptor in experimental colitis is a consequence of developmental effects, we next induced experimental colitis by oral administration of 5% DSS to WT (i.e., Y1+/+) mice with or without injection of the NPY Y1 receptor antagonist H409/22. Initially, animals receiving either DSS alone or DSS together with H409/22 showed no clinical symptoms. The earliest clinical findings occurred on day 3, when Y1+/+ animals receiving DSS alone showed signs of hemocult (Table 2). Hemoccult-positive stools in Y1+/+ animals receiving DSS together with Y1 receptor antagonist, was noticed at day 4. Loose stools were observed on day 4 in animals receiving 5% DSS compared with the mice receiving
DSS together with Y1 receptor antagonist, who first showed signs of loose stools at day 5. The number of animals showing hemocult positive stools and loose stools increased in both groups. Diarrhea was noted at day 7 for all Y1+/+ animals receiving 5% DSS alone, whereas only 25% of the animals receiving DSS together with Y1 receptor antagonist were having diarrhea. Weight loss occurred in both groups, but it was significantly more severe in animals receiving DSS without any Y1 receptor antagonist. No significant difference in food and water consumption was observed between the two experimental groups in the absence or presence of H409/22. The calculation of DAI indicated that animals receiving only 5% DSS developed significant physical signs associated with DSS-induced colitis compared with the animals administered Y1 receptor antagonist. These animals showed a more attenuated response from day 3 and onward compared with animals receiving 5% DSS alone (Table 2). None of the mice receiving H2O instead of 5% DSS showed any physical signs of disease (n = 5 for all groups; data not shown).

Severity of DSS-induced colonic injury in Y1+/- and Y1-/- mice after a period of recovery. To examine the recovery from DSS-induced colonic injury in Y1+/- and Y1-/- mice, animals were orally administered 5% DSS for 6 days followed by a period of 14 days, in which animals received H2O. Similar to previous experiments, Y1-/- mice receiving 5% DSS exhibited attenuated clinical signs compared with Y1+/- mice (Fig. 1). During the period when the animals received H2O alone, Y1-/- mice displayed signs of a more rapid recovery than Y1+/- mice. Within a week following H2O administration, Y1-/- mice were almost free of clinical symptoms, whereas Y1+/- mice required another full week to recover to a similar degree. No physical findings were noted in Y1+/- mice and Y1-/- mice receiving H2O only (n = 5 for Y1+/- and Y1-/- animals, respectively; data not shown).

Reduction of tissue damage in Y1-/- mice. Our clinical data indicated an attenuated response to DSS-induced colitis in the absence of Y1 receptor signaling. We continued by examining histological samples from animals 7 days after treatment with 5% DSS. No morphological differences in colonic sections between Y1+/- and Y1-/- mice receiving H2O were observed (Fig. 2, A and B). Crypts showed a healthy architecture and goblet cell component with no sign of inflammatory infiltration. In contrast, both Y1+/- and Y1-/- animals treated with 5% DSS displayed damage characterized by disruption of the colonic mucosal architecture, and inflammatory infiltrate and ulcerations. Figure 2C represents a colonic mucosa of a Y1+/- mouse receiving 5% DSS for 7 days. Mucosal ulceration associated with an inflammatory infiltrate is shown. Histological analysis at day 7 in Y1-/- mice also showed inflammatory infiltration and damaged mucosal architecture classified as ulceration. However, the damages in these mice were less severe compared with Y1+/- mice. Y1-/- mice treated with 5% DSS demonstrated increased mucosal height associated with shortening of the crypts and inflammatory infiltration.
We quantitatively measured the length of ulcerations in colonic sections from both Y1+/H11001/H11001 and Y1+/H11002/H11002 mice consuming 5% DSS. A marked difference in ulceration between the two groups was observed. Y1+/H11002/H11002 mice showed significantly less ulceration than Y1+/H11001/H11001 mice (Fig. 3).

**Upregulation of NPY immunoreactivity during colitis in Y1+/+ and Y1−/− mice.** Immunoreactivity for the Y1 receptor ligand NPY in Y1+/+ and Y1−/− mice was examined before colonic inflammation and 7 days after DSS-induced colitis. A similar NPY immunoreactivity was observed in noninflamed colons of both groups of animals. NPY-positive nerve fibers were present in the myenteric and submucosa plexus (data not shown) and alongside crypts within the mucosal layer (Fig. 4, A and C). An increase in NPY immunoreactivity in both Y1+/+ and Y1−/− mice during DSS-induced colitis, compared with control mice was observed. An increased thickness of fibers was also seen and was similar in both Y1+/+ and Y1−/− mice in the myenteric plexus and mucosal layer in undamaged parts of the colon (data not shown). In addition, NPY-positive nerve fibers were observed at sites of damaged crypts. These fibers displayed a disorganized distribution (Fig. 4, B and D).

**Capsaicin-induced Evans blue plasma extravasation in colons of Y1+/+ and Y1−/− mice.** Local or systemic application of capsaicin, a ligand for the vanilloid receptor (VR1) (4), induces neurogenic inflammation (11). We have previously reported that cutaneous capsaicin-induced plasma extravasation is abolished in Y1−/− mice. The present data indicated that the
response of Y1−/− mice to DSS-induced colitis was attenuated. To determine whether a lack of neurogenic inflammation in the colon could be a mechanism for the resistance of Y1−/− mice to DSS-induced colitis, animals were treated with capsaicin. Capsaicin was administered to the colon of Y1+/+ and Y1−/− mice. A significant increase in Evans blue extravasation was detected in the distal colons of Y1+/+ mice following administration of capsaicin compared with vehicle-administered Y1+/+ mice. In contrast, we did not observe any plasma extravasation in the distal colons of Y1−/− mice after administration of capsaicin, compared with vehicle-injected Y1−/− mice (Fig. 5).

**DISCUSSION**

Recently, we have shown that NPY Y1 receptor activation is sufficient and required for SP release and the subsequent development of neurogenic inflammation and plasma leakage in the skin (33). In the present study, we examined the role of the NPY Y1 receptor in an experimental model of acute colitis using an NPY Y1 receptor antagonist as well as mice harboring a null mutation in the Y1 receptor. The major finding of this study is that the inflammatory response in the colon is attenuated in mice lacking the Y1 receptor. This attenuation was also observed when a selective NPY Y1 receptor antagonist was used. Our data strongly suggest that the absence of the Y1 receptor is the cause of this attenuation, because a lack or the inhibition of NPY Y1 receptor makes the animals less susceptible to damage caused by colonic inflammation as indicated by reduced clinical signs of colitis as determined by DAI as well as histological analysis. We observed a significant difference in the DAI of Y1−/− mice receiving DSS compared with that of Y1+/+ mice at days 3–7. Significant differences in DAI were also observed between Y1+/+ mice receiving or not receiving a Y1 antagonist at days 4–6 of the experiment. The DAI data from Y1−/− mice treated with DSS correlated well with morphological and histological findings of less ulceration and less severe damage to the epithelium observed in these animals after a 7-day period of DSS treatment compared with WT animals. Similar results could also be noted in Y1−/− mice receiving DSS followed by a period of recovery. These animals showed faster signs of recovery and returned to basal levels of...
clinical symptoms more rapidly compared with control mice. It is conceivable that the faster rate of recovery seen in Y1−/− mice might partly be due to the fact that these animals did not reach the same DAI as control mice during the time of DSS consumption. These results show that Y1-receptor activation participates in DSS-induced colitis, and elimination of the receptor, either by use of antagonists or gene targeting in mice, attenuates IBD.

Increasing evidence indicates that neuropeptides released from the sensory or enteric nervous system are involved in the pathogenesis of colitis. NPY via Y1 receptors tonically affect release of SP in the colon (12). An SP antagonist was shown to inhibit enterocolitis induced by Clostridium difficile toxin A in rats (35). Consistently, mice genetically deficient in the SP receptor were protected from the inflammatory effects of toxin A (3). In addition, surgical denervation of primary sensory neurons to a loop of intestine injected with toxin A inhibited and protected rats from enteritis (28). In another study, mice lacking the cell surface enzyme neutral endopeptidase that degrades SP exhibited an exacerbated inflammatory response to toxin A (17). Recent studies (6, 40) have shown that the administration of a nonpeptide SP receptor antagonist reduces colonic inflammation in a model of experimental colitis in rats. Further studies show that both neonatal capsaicin-denervated rats and normal adult rats treated with the VR1 antagonist capsazepine (16) exhibit reduced damaging effects after DSS administration, confirming the role of primary sensory neurons in mediating colonic inflammation. Collectively, these studies suggest the contribution of a neurogenic mediator of intestinal inflammation.

To elucidate possible mechanisms of the attenuation seen in Y1−/− mice and to test the existence of a neurogenic component involved in DSS-induced colitis, we treated both Y1+/+ and Y1−/− mice with capsaicin. Capsaicin stimulates the release of SP and CGRP from primary sensory nerve endings in the gut (24) and thereby elicits an inflammatory response characterized by vasodilation and plasma extravasation (13). Consistently, intravenous administration of SP in conscious rats stimulates the extravasation of plasma proteins in the duodenum, stomach, and pancreas (8, 34). We have previously demonstrated that the lack of cutaneous capsaicin-induced plasma extravasation in Y1−/− mice is caused by a failure of capsaicin-induced SP release (33). In the present study, administration of capsaicin into the distal part of the colon also failed to increase Evans blue extravasation in Y1−/− mice. The lack of capsaicin-induced extravasation in distal colon of Y1−/− mice suggests that a putative neurogenic component in DSS-induced colitis includes Y1 receptor activation.

The cellular sites of Y1 receptor activation could involve the myenteric and submucosal nerve cell bodies and endothelial and endocrine-like cells in the colon, because all of these cell types have been described to express Y1 receptors (7, 11, 15, 31). In agreement with previous experimental data (1, 2), we observed increased NPY immunoreactivity in both Y1−/− and Y1+/+ mice receiving 5% DSS, and thus this increase occurs independent of the Y1 receptor. The increased immunoreactivity was observed in the myenteric and submucosal plexi and mucosa in nondamaged colonic sites but also in regions of crypt abnormalities. NPY-immunoreactive nerve fibers have been reported in the myenteric plexus, submucosal, and mucosal layers of the GI tract (10, 42). Abdominal sympathetic-


26. Sandhu H, Reiter R, and Heilig M. Diverse functions of neuropeptide Y revealed by 10.220.33.1 on November 7, 2017 http://ajpgi.physiology.org/ Downloaded from