Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation

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Brun, Paola, Cristina Mastrotto, Elisa Beggiao, Annalisa Stefani, Luisa Barzon, Giacomo C. Sturniolo, Giorgio Palù, and Ignazio Castagliuolo. Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 288: G621–G629, 2005; doi:10.1152/ajpgi.00140.2004.—Because neurotensin (NT) and its high-affinity receptor (NTR1) modulate immune responses, chloride secretion, and epithelial cell proliferation, we sought to investigate their role in the repair process that follows the development of mucosal injuries during a persistent inflammation. Colonic NT and NTR1, mRNA, and protein significantly increased only after dextran sodium sulfate (DSS)-induced inflammatory damage developed. Colitis-induced body weight loss, colonic myeloperoxidase activity, and histological damage were significantly enhanced by SR-48642 administration, a nonpeptide NTR1 antagonist, whereas continuous NT infusion ameliorated colitis outcome. To evaluate the NT and NTR1 role in tissue healing, mucosal inflammatory injury was established administering 3% DSS for 5 days. After DSS discontinuation, mice rapidly gained weight, ulcers were healed, and colonic NT, NTR1, and cyclooxygenase (COX)-2 mRNA levels were upregulated, whereas SR-48642 treatment caused a further body weight loss, ulcer enlargement, and a blunted colonic COX-2 mRNA upregulation. In a wound-healing model in vitro, NT-induced cell migration in the denuded area was inhibited by indomethacin but not by an antitransforming growth factor-β neutralizing antibody. Furthermore, NT significantly increased COX-2 mRNA levels by 2.4-fold and stimulated PGE₂ release in HT-29 cells. These findings suggest that NT and NTR1 are part of the network activated after mucosal injuries and that NT stimulates epithelial restitution at least, in part, through a COX-2 dependent pathway.

Neurotensin receptor type 1; healing; colitis; cyclooxygenase-2; inflammatory bowel disease

INFLAMMATORY BOWEL DISEASE (IBD) is a chronic intestinal inflammatory disorder that is considered the consequence of an aberrant response of the immune system to luminal antigens (13). Although the event(s) triggering the chronic inflammatory disorder has not been identified, a variety of cells and soluble factors mediate the extended mucosal damage (13). However, after the development of mucosal injuries, the intestinal epithelium rapidly tends to reestablish its integrity (26). To reestablish mucosal integrity, epithelial cells migrate into the wounded area (epithelial restitution), and they then proliferate to replace the decreased cell pool. Studies over the past several years have shown that a variety of soluble peptides, prostaglandins, growth factors, and cytokines are secreted in a coordinated fashion in the injured area to restore mucosal integrity (11, 26, 29).

Neuropeptides are small molecules produced by a variety of cells, including neurons and immune cells, that in the intestine modulate several biological processes including motility, electrolytes transport, mucosal blood flow, and cell growth (43). Neuropeptides released during inflammatory conditions are able to modify the activity of cells responsible either to trigger tissue damage and to promote healing (34). Furthermore, nature and amplitude of neuropeptide-mediated effects may be different in the normal tissue and in inflammatory disorders, because levels of receptors and cell populations expressing neuropeptide receptors may be strikingly different (18, 28). For example, substance P (SP) in physiological conditions stimulates intestinal motility and secretion mainly through its neuronal receptors (43), during acute enteritis enhances tissue damage activating macrophages and mast cells (7), and during chronic colitis stimulates mucosal healing through newly expressed receptors (5, 12). In addition, it should be taken into consideration that neuropeptide-mediated effects may be modulated by complex interactions with other mediators involved in tissue restitution, such as growth factors and cytokines (23).

Neurotensin (NT) is a tridecapeptide widely distributed in the nervous system and intestine (43) that binds to specific high and low affinity receptors (20, 41) expressed in the intestine (4) and at different in the normal tissue and in inflammatory disorders, nature and amplitude of neuropeptide-mediated effects may be different (18, 28). For example, substance P (SP) in physiological conditions stimulates intestinal motility and secretion mainly through its neuronal receptors (43), during acute enteritis enhances tissue damage activating macrophages and mast cells (7), and during chronic colitis stimulates mucosal healing through newly expressed receptors (5, 12). In addition, it should be taken into consideration that neuropeptide-mediated effects may be modulated by complex interactions with other mediators involved in tissue restitution, such as growth factors and cytokines (23).

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advantage of the DSS colitis model, we investigated expression and functional relevance of NT and NTR1 during both the development of mucosal damage and the healing process in the period subsequent to DSS discontinuation (44). Furthermore, we analyzed possible mechanisms involved in NT-mediated mucosal restitution using a well-established model of intestinal epithelial wound healing in vitro (17).

MATERIALS AND METHODS

Colonic biopsy procurement. Two colonic biopsies were collected from ulcerative colitis (UC) patients (n = 10) and healthy controls (n = 8) during endoscopic examination (20 cm from the anus) and were snap frozen in liquid nitrogen. The healthy controls did not have a history of intestinal inflammatory disorder or irritable bowel syndrome and underwent follow-up endoscopy examination for colonic polyps. They showed a macroscopically and histologically normal mucosa. Diagnosis of UC was based on standard clinical, endoscopic, and histological criteria, and all patients had moderately active disease (disease activity index ≥ 4) as determined by clinical and endoscopic parameters (35). The study was approved by the institutional review board and ethical committee of the University of Padua, and all patients gave written informed consent before the study.

Animals. Male Balb/c mice, 10 to 12 wk old purchased from Charles River Laboratories (Oderzo, Italy), were used in all the experiments. Mice were housed in groups of five per cage and kept under controlled temperature and humidity conditions. Animals received standard pelleted chow and tap water ad libitum, unless otherwise specified. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Padua.

Induction of colitis by DSS and assessment of colitis severity. Mice were randomly divided into groups receiving either regular tap water (control group) or drinking water containing 5% (wt/vol) DSS (TDB Consultancy, Uppsala, Sweden). To assess colitis severity, we evaluated daily animal body weight, stool consistency, and the presence of blood in the stools. A previously validated clinical score ranging from 0 to 4 was calculated by using the parameters of weight loss, stool consistency, and the presence or absence of fecal blood (9). Mice were then killed at day 5, and the colon was removed and divided for histology examination or snap frozen in liquid nitrogen for evaluation of MPO activity and IL-1β levels. For histology examination, colonic segments were fixed in 4% paraformaldehyde, paraffin embedded, and then longitudinal sections (10-μm thick) were collected, homogenized in 2 ml ice-cold 0.1 N HCl, the mucosa and underlying submucosa were collected, and the whole colons from control and DSS-treated mice were homogenized in ice-cold phosphate buffer saline (pH 7.4) containing a mixture of protease inhibitors (1 μM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Homogenates were then centrifuged 10,000 g (10 min at 4°C), the supernatant was collected, and MPO activity was measured by using a colorimetric assay method and expressed as units per milligram of tissue (7).

Administration of SR-48642, a nonpeptide NTR1 receptor antago-
nist. Mice were randomly divided into groups receiving either the specific NT receptor antagonist SR-48692 (Sanofi Recherche, Toulouse, France) or the same volume of vehicle. SR-48692 was dissolved in DMSO at a concentration of 5 mg/ml. Approximately 50 μl of the solution (17 μmol·kg body wt−1·24 h−1) were given daily by intraperitoneal injection (19). In one set of experiments, SR-48692 was given to mice receiving 5% DSS, starting 48 h after DSS addition to the drinking water. In a second series of experiments, the same dose of SR-48642 was administered daily to mice after the 5 days of 3% DSS regimen.

Administration of NT. For NT treatment, the peptide was dissolved at the concentration of 1.8 mg/ml in PBS containing 0.5% (wt/vol) BSA. Miniosmotic Alzet pumps (Alza, Palo Alto, CA) filled with the NT solution were implanted subcutaneously in the interscapular region of mice under light anesthesia. The pumps deliver 0.45 μl/h for up to 14 days corresponding to 0.54 μmol neurotensin·kg−1·24 h−1, known to stimulate proliferation of cells expressing NTR1 in vivo (19). Control mice received PBS containing 0.5% (wt/vol) BSA.

In situ hybridization for NT mRNA. A riboprobe complementary to exon 4 of the rat neurotransin gene (a generous gift from Paul Dobin, University of Massachusetts) was labeled with digoxigenin and quantified following the manufacturer’s instructions (6). Colonic sections (5 μm) obtained from control and inflamed colons were briefly fixed in 4% paraformaldehyde-PBS (pH 8.5) and acetylated for 15 min at room temperature in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine HCl-PBS buffer (pH 8.0). In situ hybridization was performed in a moisture chamber overnight at 53°C using a digoxi-
genin-NT cRNA probe at a concentration of 4 ng/ml in hybridization buffer (6). After hybridization, slides were digested with RNase (20 mg/ml) for 1 h at 37°C and washed with 2× SSC, 1× SSC, and 0.1× SSC for 1 h each at 50–58°C. Sections were then incubated with a fluorescein-labeled sheep antidigoxigenin conjugate (Bohringer-Manheim, Indianapolis, IN) to detect NT mRNA. Slides were then examined by using a Leica TCS-NT/SP2 confocal microscope using a ×40 objective, and images were digitally stored in a Leica software.

Measurement of colonic NT content. To determine colonic NT content, the whole colons from control and DSS-treated mice were collected, longitudinally opened, and washed in ice-cold saline. The muscle layer was then removed, and the mucosa and underlying submucosa were collected, homogenized in 2 ml ice-cold 0.1 N HCl, for 20 s, and then centrifuged (10,000 g for 15 min at 4°C). The supernatants were collected and prepurified on C18 cartridge columns (Water, Milford, MA). Samples were then freeze-dried in a SpeedVac concentrator and reconstituted in 0.5 ml of sample buffer. NT levels were determined by using a commercially available enzymatic immunoassay (EIA) kit (Peninsula laboratories) (6). NT levels were expressed as femtomole of peptide per microgram protein.

Immunohistochemical determination of NTR1. Colons from normal or DSS-treated mice were opened longitudinally and washed in 1× TBS buffer (0.05 M Tris base, 0.15 M NaCl), fixed in 4% buffered PFA and then paraffin embedded. In addition, full-thickness colonic sections were also obtained from archival specimens of patients who underwent colectomy for either carcinoma (controls, n = 5) or active UC (n = 5). Human and mouse sections (10 μm thick) were deparaaffinized after standard procedures and rehydrated in 1× TBS. After incubation for 30 min in blocking buffer (2% donkey serum, 0.3% Triton X-100 in TBS), the sections were incubated for 1 h at 22°C with a rabbit polyclonal anti-NTR1 antibody (6). After washing three times (10 min each) in 1× TBS, sections were incubated for 1 h at 22°C with a donkey anti-rabbit IgG fluorescein-labeled (Immunoresearch Laboratories, PA). After being washed in TBS (3 times), the slides were mounted in a drop of 1 mg/ml of n-propyl gallate (Sigma)
in 90% glycerol-PBS. Sections were then analyzed and photographed by using a Leica TCS-NT/SP2 confocal microscope using a ×40 objective, and images were digitally stored in a Leica software. For control, slides were incubated with nonimmune rabbit IgG.

**Cell culture and measurement of PGE2.** HT-29 cells (American Type Cell Cultures) were cultured in DMEM-high-glucose containing 10% FBS, 50 mg/ml streptomycin, and 50 U/ml penicillin (1). Confluent cells were dissociated by addition of trypsin/EDTA and then seeded at a density of 106 cells in six-well plates and grown to confluence. Culture medium was then changed, and cells were kept for 24 h in medium containing 0.1% FCS before running the experiments. On the day of the experiments, cells were washed twice with warm sterile DMEM and then incubated for 3–24 h in fresh medium containing 0.1% FBS in the presence or absence of various concentrations of NT (0–100 μM). Conditioned medium was collected, and PGE2 levels were determined by an immunoenzymatic assay (Cayman Chemical, Ann Arbor, MI), and results are expressed as picograms of PGE2 per 106 cells. Total RNA was then extracted from the cellular monolayers as described (7).

**RNA isolation and quantitative real-time RT-PCR analysis.** Total RNA was isolated from mouse colonic fragments, human colon biopsies, or cultured HT-29 cells following a standard single-step acid guanidinium phenol chloroform extraction procedure employing RNAzol (Biotech Laboratories, Huston, TX), and contaminating DNA was removed with RP-1 RNase-free DNase (Promega) (7). Random-primed cDNAs were generated from total RNA using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). The thermal cycling conditions and primer sequences employed to amplify murine (m)GAPDH, mNT, mNTR1, and cyclooxygenase (COX)-1 and -2, as well as human (h)GAPDH, hNTR1, and hCOX-2 are indicated in Table 1. When required, amplification products were separated on 1.5% agarose gels and visualized by ethidium bromide staining by using an UV transilluminator. Real-time quantitative RT-PCR analysis was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems). We quantified transcripts of mGAPDH and hGAPDH as the endogenous RNA control. For each sample, the amount of the target and endogenous reference were extrapolated from a standard curve prepared by using serially diluted correspondent cDNAs cloned into pGEM-T vector (Promega). Amplified fragments were sequenced to confirm the identity of the amplification products by automated sequencing.

**Migration (restitution) assays.** Wounding assays were carried out as described previously (17). HT-29 cells, known to express functional NTR1, were dissociated with trypsin/EDTA, seeded on microscope coverslips, and grown in DMEM-high-glucose (GIBCO-BRL, Grand Island, NY) supplemented with 10% FBS, 50 mg/ml streptomycin, and 50 U/ml penicillin. Confluent monolayers were incubated for 24 h with medium containing 0.1% FBS before making linear wounds using sterile razor blades. Cells were washed with serum-free medium, and the wounded monolayers were further incubated for 3–24 h in fresh medium containing 0.1% FBS in the presence or absence of various concentrations of NT (0–100 μM). In addition, the effects of NT on HT-29 cell migration were determined in the presence of the prostaglandin synthesis inhibitor indomethacin (10 μM) or a neutralizing antitumor growth factor (TGF)-β antibody (30 mg/ml) (R&D Systems, Minneapolis, MN). Migration was assessed in a blinded fashion by determining the number of HT-29 cells across the wound border in a defined wound area by taking photomicrographs at ×40 magnification with a Leica DM-LB inverted microscope connected to a digital Leica DC-100 camera. Experiments were performed in triplicate, and at least 10 wound areas were used to quantify migration.

**Statistical analysis.** Results are expressed as means ± SE. Statistical analysis was performed by using ANOVA and Bonferroni’s test. Statistical significance was considered for P values < 0.05.

**RESULTS**

**Increased NTR1 protein and mRNA level in UC patients.** Immunohistochemical analysis of normal colon revealed few cells in the mucosa stained for NTR1 (Fig. 1A), consistent with our previous results (31). In comparison, NTR1 immunoreactivity in sections obtained from UC patients was significantly increased for the number of positive cells and intensity of the signal (Fig. 1A). NTR1-positive cells were present both in the crypt epithelium and in the colonic lamina propria. No staining was observed when the anti-NTR1 antisera was previously incubated with the peptides used to obtain the antibody (Fig. 1A). As expected, analysis of the steady-state NTR1 mRNA levels by quantitative RT-PCR showed low levels of NTR1 mRNA in the normal colonic mucosa, whereas the abundance of NTR1 mRNA was significantly increased in biopsies from UC patients (3.6-fold increase, P < 0.01) (Fig. 1B).

**Increased mucosal levels of NT during DSS colitis.** Because in the acute inflammation triggered by C. difficile toxin A, NT upregulation in the colonic mucosa occurs within a few minutes of toxin exposure before any sign of tissue damage was evident (7), we decided to investigate the effect of the inflammatory reaction induced by DSS supplementation on NT mucosal levels. As shown in Fig. 2A, low levels of NT were measurable by EIA in the colonic mucosa of normal mice. Colonic mucosal levels of NT were not modified during the first 3 days of DSS administration, although clinical signs of colitis, such as moderate body weight loss (1.2 ± 0.3 g) and microscopic bleeding, were present. On the fifth day of 5% DSS administration, severe signs of colitis developed (loss of 2.1 ± 0.4 g body wt and presence of bloody diarrhea), and NT mucosal levels showed a 5.8-fold increase over normal mice (P < 0.01) (Fig. 2A).

**Table 1.** Thermal cycling conditions and primer sequences employed to amplify mGAPDH, mNT, mNTR1, and mCOX-1 and -2, as well as hGAPDH, hNTR1 and hCOX-2

<table>
<thead>
<tr>
<th>Primer Forward</th>
<th>Primer Reverse</th>
<th>Temp</th>
<th>BP</th>
</tr>
</thead>
<tbody>
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<td>177</td>
</tr>
<tr>
<td>mNT</td>
<td>gcagccctactaataacagctgacag</td>
<td>61°C</td>
<td>185</td>
</tr>
<tr>
<td>mNTR1</td>
<td>cgtgattacccctcccagttgtaagacag</td>
<td>61°C</td>
<td>194</td>
</tr>
<tr>
<td>mCOX-1</td>
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<td>61°C</td>
<td>389</td>
</tr>
<tr>
<td>mCOX-2</td>
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<td>53°C</td>
<td>276</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>atggcaccagagtagacaggttggta</td>
<td>60°C</td>
<td>168</td>
</tr>
<tr>
<td>hNTR1</td>
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<td>61°C</td>
<td>165</td>
</tr>
<tr>
<td>hCOX-2</td>
<td>gtaaaccagcacagcagtagctgctag</td>
<td>61°C</td>
<td>237</td>
</tr>
</tbody>
</table>

Temp, temperature; BP, base pairs; mGAPDH, murine GAPDH; mNT, murine neurotensin; mNTR1, mNT receptor-1; mCOX, murine cyclooxygenase; h, human.
To determine whether the increase in colonic NT peptide content was associated with increased NT mRNA, we performed a quantitative RT-PCR analysis of the steady-state NT mRNA levels in the colonic mucosa after DSS administration. As shown in Fig. 2B, NT mRNA levels significantly increased only after 5 days of DSS administration compared with normal mice. To localize the cells expressing NT mRNA during DSS colitis, we then performed in situ hybridization. As expected, very little hybridization was detectable in normal colonic tissue (Fig. 2C), according to the low levels of NT mRNA measured by quantitative RT-PCR. However, after 5 days of DSS administration, NT mRNA levels drastically increased as indi-
cated by the number of positive cells and the intensity of the hybridization signal (Fig. 2C). Hybridization for NT mRNA was localized in cells of the lamina propria but not in the epithelium. No hybridization was obtained by using a sense probe for NT mRNA (Fig. 2C).

**Increased mucosal NTR1 levels during DSS colitis.** As expected (Refs. 7 and 31 and Fig. 1), the minimal NTR1 immunoreactivity detectable in the normal mouse colon was mainly localized in cells of the lamina propria (Fig. 3A). However, NTR1 immunoreactivity drastically increased after 5 days of DSS administration and was mainly localized in the epithelial layer, often in the vicinity of the wounded mucosa. Indeed, incubation of the anti-NTR1 antiserum with an excess of the two NH₂-terminal peptides used to generate the antisem before the application to the sections led to signal disappearance. Furthermore, the steady-state NTR1 mRNA in the colonic mucosa assessed by quantitative RT-PCR was barely detectable in normal mice, whereas it significantly increased (P < 0.01) after 5 days exposure to DSS (Fig. 3B).

**Administration of SR-48642 enhances, whereas NT reduces the severity of DSS-induced colitis.** Mice receiving 5% DSS in their drinking water exhibited a wasting syndrome, associated with bloody diarrhea and superficial mucosal ulcers associated with an inflammatory infiltrate rich in neutrophils within 5 days of treatment (Table 2 and Fig. 4). As shown in Table 2 and Fig. 4, daily intraperitoneal administration of SR-48642 exacerbated the severity of colitis. Thus SR-48642-treated mice showed a greater body weight loss and larger mucosal ulcerations compared with vehicle-treated animals. Interestingly, colonic levels of IL-1β and MPO were not significantly affected by SR-48642 treatment (197.3 ± 8.2 vs. 154.5 ± 12 ng IL-1β/mg protein and 4.2 ± 0.6 vs. 4.7 ± 1.1 U MPO/mg tissue in vehicle and SR-48642 treated animals, respectively).

The enhanced severity of DSS colitis after administration of a specific NTR1 receptor antagonist prompted us to test whether NT administration had any protective effect on the development of the inflammation-induced mucosal damage. As shown in Table 2, continuous subcutaneous delivery of NT (0.54 μmol·kg⁻¹·24 h⁻¹) by miniosmotic Alzet pumps significantly improved DSS-induced colitis outcome. Thus NT infusion during the 5 days of the experiment reduced the body weight loss and the severity of the histological damage compared with animals receiving only vehicle (Table 2 and Fig. 4).

**Administration of SR-48642 prevents mucosal healing after DSS-discontinuation.** We then tested the involvement of NT and NTR1 in the process of mucosal repair that follows the resolution of colitis. To address this question, we used a well-established model of mucosal repair after an inflammatory damage. Mice received 3% DSS in their drinking water for 5 days, and then DSS was discontinued to arrest the inflammatory process and promote mucosal healing (44). We first determined, by quantitative RT-PCR, the level of NT and NTR1 mRNA in the colonic mucosa during the mucosal restitution process that follows DSS discontinuation. Indeed, NT mRNA and NTR1 mRNA levels remained significantly increased by 10.22 ± 0.33 on July 6, 2017 http://ajpgi.physiology.org/ Downloaded from

![Image of Figure 3](http://ajpgi.physiology.org/)

**Fig. 3.** Increased NTR1 expression during DSS colitis. A: mice received only drinking water or water containing 5% DSS for 5 days and were then killed. Colonos were removed, washed in ice-cold PBS, fixed in 4% PFA, and 10-μm thick colonic sections were were processed for immunohistochemical detection of NTR1 using a rabbit polyclonal antibody. Shown are representative sections from a normal colon (I) and a mouse with DSS colitis (II). Note the presence of faint signal for NTR1 in the mucosa of normal colon (I). The number of cells showing NTR1 immunoreactivity increases in animals with DSS colitis (II). Staining is present both in the epithelium and in lamina propria cells. Preabsorption of the NTR1 antiserum caused complete disappearance of staining (III). Results are representative of 3–5 animals per group. Original magnification, ×40. B: total RNA was isolated from normal mice or after 2–5 days exposure to 5% DSS, and quantitative RT-PCR was performed to quantify NTR1 mRNA. Expression of target gene was normalized to mGAPDH as the endogenous RNA control. Each experiment was performed at least 2 times with triplicate determinations (n = 6 each per condition). *P < 0.01 vs. control.

**Table 2. Effect of SR-48642 and NT administration on DSS colitis outcome in 2017 mice.**

<table>
<thead>
<tr>
<th>Body Weight Change, %</th>
<th>Clinical Score</th>
<th>Histologic Score</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>+4.8±0.2</td>
<td>0</td>
</tr>
<tr>
<td>DSS</td>
<td>-8.1±0.4*</td>
<td>2.3±0.2*</td>
</tr>
<tr>
<td>DSS + SR-48642</td>
<td>-15.3±0.8†</td>
<td>3.5±0.3*</td>
</tr>
<tr>
<td>DSS + NT</td>
<td>-2.6±0.4†</td>
<td>1.1±0.2*</td>
</tr>
</tbody>
</table>

Data represent the means ± SE of 8–10 animals per group. To induce colitis, mice received 5% DSS in their drinking water for 5 days. Animals were randomly divided to receive a specific NT receptor antagonist, SR-48692 (17 μmol·kg body wt⁻¹·24 h⁻¹) by daily intraperitoneal injection, NT (0.54 μmol·kg body wt⁻¹·24 h⁻¹) via a miniosmotic pump implanted subcutaneously or just vehicle. Severity of colitis was monitored by measuring of the body weight, and results are shown as %change over initial body weight. A clinical score (scale 0–4) was calculated taking into account daily body weight loss, stool consistency, and presence of fecal blood. A full thickness segment from the proximal colon of each animal was fixed in 4% PFA, paraffin embedded, and sections stained with hemotoxylin and eosin were analyzed by a pathologist in a blinded fashion. DSS, dextran sodium sulfate. *P < 0.01 vs. control, †P < 0.05 vs. DSS alone.
increased in the colonic mucosa of mice 5 days after DSS discontinuation compared with control animals, as the inflammatory infiltrate was roughly resolved and the mucosal restitution process was nearly completed (Fig. 5C).

We then inhibited the activity of NT during the mucosal healing process by administering SR-48642 to mice after DSS discontinuation. Thus after DSS discontinuation, the stool consistency returned to normality within a few days, and the animals rapidly gained weight, whereas as a consequence of SR-48642 administration, further body weight loss and persistence of diarrhea was observed (Fig. 5A). In addition, colonic levels of MPO and IL-1β remained elevated in the animals receiving SR-48642 (5.1 ± 0.9 U MPO/mg tissue and 188.1 ± 10.5 ng IL-1β/mg protein, respectively), whereas they significantly decreased and returned to values similar to control animals in the untreated mice (2.2 ± 1.1 U MPO/mg tissue and 25.6 ± 5.5 ng IL-1β/mg protein after DSS discontinuation vs. 1.8 ± 0.9 U MPO/mg tissue and 13.3 ± 6.6 ng IL-1β/mg protein observed in normal animals). Histological examination revealed a nearly complete resolution of the mucosal inflammatory process within 5 days after DSS discontinuation, whereas mucosal ulcers and neutrophil infiltrate further increased in animals treated with SR-48642 (Fig. 5B).

Neurotensin promotes HT-29 monolayer restitution. Because administration of SR-48642 inhibited the repair process that follows the mucosal inflammatory damage, we decided to...
investigate the effects of NT on migration of colonic epithelial cells in vitro. As shown in Fig. 6, addition of NT to serum-starved cell monolayers significantly enhanced migration of HT-29 cells into the denuded area of a model wound compared with migration of HT-29 cells cultured in serum-deprived medium alone. Enhancement of HT-29 cell migration was dose dependent, reaching the maximum at an NT concentration of \(10^{-8}\) M (data not shown). As shown in Fig. 6, NT-mediated HT-29 restitution was entirely blocked by addition of SR-48642 and indomethacin to the culture medium, whereas addition of anti-TGF-β1 neutralizing antibody had no effect on NT-mediated cell migration.

**NT increases expression of COX-2.** Because COX-2 upregulation and PG release are well-known mechanisms of mucosal protection and restitution (22), we then investigated whether NT had any effect on COX-2 gene expression and PGE2 release. As shown in Fig. 7, NT (\(10^{-8}\) M) significantly increased COX-2 mRNA steady-state levels in HT-29 cells within 3 h of exposure. Accordingly, PGE2 levels in the conditioned medium from HT-29 cells exposed for 16 h to NT (\(10^{-8}\) M) were significantly increased (8.97 ± 3.2 pg/ml vs. 20.82 ± 4.3 pg/ml, respectively \(P < 0.05\)). Furthermore, COX-2 but not COX-1 (data not shown) mRNAs steady-state level were also increased in the colonic mucosa of mice during the tissue repair phase that follows DSS discontinuation (Fig. 5C). Indeed, administration of SR-48642 after DSS discontinuation prevented tissue healing and blunted COX-2, but not NT, mRNA upregulation in the colonic mucosa (Fig. 5).

**DISCUSSION**

The main findings of this study are threefold. First, the expression of the high-affinity NT receptor type 1 is upregulated in patients with active UC. The NT system is upregulated after DSS-induced mucosal damage, and it is part of the network of factors contributing to mucosal healing. Thus inhibition of NT effects in vivo by administration of a nonpeptide NT receptor antagonist enhanced the severity of mucosal damage leading to a more severe clinical outcome. This view is further supported by the observation that NT in vitro stimulated intestinal epithelial cell migration in a model of restitution of wounded epithelial monolayers and augmented the production of prostaglandins through the upregulation of COX-2 gene expression.

Although in the gastrointestinal tract neuropeptides mediate a variety of activities, their role during acute and chronic inflammatory disorders is still debated. A large body of in vivo studies have provided direct evidence that in response to acute injuries, the synthesis and release of certain neuropeptides contributes to enhance vascular permeability, mast cells degranulation, and polymorphonuclear neutrophils recruitment (6, 7, 15). The effect of chronic inflammation on tissue neuropeptide content and the functional relevance of these changes are more controversial. For instance, in patients with chronic inflammatory disorders, neuropeptide levels have been reported (37) as increased, decreased, and unchanged, due to their extreme instability and fluctuating expression in the inflamed tissue. The variety of biological activities mediated by neuropeptides and the different experimental models tested may justify the apparent conflicting results reported during inflammatory disorders. For example, we recently reported that NT gene upregulation was detectable before fluid secretion and neutrophil recruitment triggered by *C. difficile* toxin A was measurable (6). Indeed, in this study using the DSS colitis model, characterized by the appearance of mucosal ulcers after 2–3 days of DSS administration, we observed that NT upregulation occurred only when the mucosal damage was already established after 5 days of DSS supplementation (Fig. 2). Thus NT may mediate different effects depending on the nature of the tissue insult and the kind of inflammatory response triggered. This is not completely unprecedented, because previous studies indicated that other neuropeptides released during acute inflammatory reactions enhance tissue damage, whereas during chronic inflammation exerts a protective role (5, 12). We did not fully characterize the cells producing NT in the inflamed colonic lamina propria (Fig. 2). However, recent studies (2, 3, 42) reported that activated immune cells can produce neuropeptides such as SP, calcitonin gene-related peptide, and...
corticotrophin-releasing hormone. Therefore, we suggest that in situ hybridization-positive cells in the colonic lamina propria are activated inflammatory cells, such as macrophages and/or lymphocytes infiltrating the colonic tissue during colitis.

Second, our immunohistochemical and quantitative RT-PCR studies demonstrate that colonic NTR1 expression is profoundly modified as a consequence of the persistent inflammatory reaction in IBD patients after DSS administration as well as during the healing process that follows DSS-discontinuation (Figs. 1 and 3). This observation supports recent evidence pointing out that during chronic inflammation, the expression of genes encoding receptors for certain cytokines, growth factors, and neuropeptides are upregulated to reset tissue sensitivity to specific mediators. Thus receptors for EGF, CXC cytokines, SP, and somatostatin are upregulated in the colonic mucosa of IBD patients (18, 30). Although we did not directly investigate the nature of the cells expressing NTR1, it is possible to appreciate the different distribution pattern of NTR1-bearing cells in normal and inflamed tissues (Refs. 4 and 31 and Fig. 2). NTR1 immunoreactive cells are mainly located in the lamina propria of the normal tissue as opposed to a prevalent distribution in the epithelial layer within the inflamed mucosa. In support of this view, recent studies in vitro indicate that in the normal human colon, NTR1 is expressed mainly on enteric neurons, because NT-mediated chloride secretion is completely abolished by tetrodotoxin-treatment (31). However, during colitis, newly synthesized NTR1 expressed by intestinal macrophages and microvascular endothelial and epithelial cells can enhance and redirect NT-mediated effects (Refs. 6 and 44 and Figs. 1 and 3).

Third, we report here that administration of a specific NT receptor antagonist to mice enhanced the severity of DSS colitis and hampered mucosal healing (Figs. 4 and 6, Table 2). After an injury, the mediators secreted in and around the wounded area stimulate healing modulating cell proliferation and migration (26). In view of NT-mediated effects, this peptide may be involved in different stages of the healing process. Thus NT stimulates either migration of connective and epithelial cells (Ref. 40 and Fig. 6) as well as endothelial and epithelial cell proliferation (neangiogenesis). Furthermore, NT activates proteases, such as the urokinase-type plasminogen activator involved in tissue repair (40). NT also enhances the expression of growth-related genes as well as the activity of kinases leading to proliferation of intestinal epithelial cells in vivo and in vitro (19, 27, 36). Recent studies in vivo and in vitro (23, 46) indicated that neuropeptides enhance growth factor-mediated epithelial as well as connective cell proliferation and attachment to the extracellular matrix and migration. Some of these effects involve complex form of cross-talk between growth factors receptors with intrinsic tyrosine kinase activity and G protein-coupled neuropeptide receptors (8). Thus the inhibition of NT-mediated effects after mucosal damage, such as cell migration and proliferation, might interfere with the mucosal reparative process leading to the development of larger mucosal ulcers and more severe tissue damage (Fig. 5).

NT upregulated COX-2 gene expression in vivo and in vitro, thereby stimulating the production of protective PGs that inhibit the onset of mucosal damage and support mucosal healing. Indeed, PGs production within the gastrointestinal mucosa plays a key role to preserve or reestablish mucosal integrity (39). Mucosal PG synthesis depends on constitutive COX-1 and inducible COX-2, which is upregulated through an NF-κB-dependent pathway (22). The expression of COX-2, in mucosal protection is illustrated by protective effects associated with PGs administration during DSS colitis and by the more aggressive DSS colitis observed in COX-2−/− mice (22, 38, 24). Here we report that NT induces the expression of COX-2 in HT-29, a human intestinal epithelial cell line expressing functional NTR1, and stimulates PGE2 production (Fig. 7). In addition, mucosal COX-2, but not NT, upregulation during the process of tissue repair that follows DSS-induced colitis was drastically reduced after administration of a specific NT receptor antagonist (Fig. 5). These results are consistent with the reported ability of NT to stimulate arachidonic acid metabolites from endothelial cells in culture and SR-48642 to inhibit NT-mediated PGE2 release and COX-2 mRNA upregulation in rat colonic mucosa during immobilization stress (4, 32). Furthermore, NT has been recently shown to directly induce NF-κB activation and nuclear translocation in human intestinal epithelial cells (45). Therefore, this peptide may contribute to activate NF-κB in the cells surrounding the damaged mucosa inducing the expression of COX-2 gene. Although our studies concentrate on COX-2 and PGE2, it is quite probable that NT may induce expression or release of additional protective mediators to diminish tissue injury by additive or synergistic interactions with PGE2. For example, we recently reported that NT causes adenosine release from colonic mucosa (31) and adenosine through adenosine receptors can attenuate an inflammatory process and reduce tissue damage in vivo (25). Additional protective effects could be provided by the ability of NT to enhance mucin release from goblet cells and to stimulate IGF-I gene expression in human colonic epithelial cells (Ref. 1 and unpublished observation).

In summary, our data support the view that NT and NTR1 upregulation in the colonic mucosa follows the development of mucosal damage, and it is part of the network of adaptive mechanisms activated to protect the mucosa and enhance tissue healing. Possible NT-mediated mechanisms of mucosal protection may include mucin and adenosine release, whereas NT-stimulated wound repair involves direct epithelial cell migration induced through the upregulating of COX-2 and PGE2 release. Therefore, considering the variety of the effects mediated by the NT system, this peptide may represent a potential therapeutic target in disorders associated with chronic mucosal ulcerations.

GRANTS

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