Signal Transduction Pathways Mediating Neurotensin-stimulated Interleukin-8 Expression in Human Colonocytes*

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Neurotensin (NT), a neuropeptide released in the gastrointestinal tract in response to several stimuli, is involved in the pathophysiology of colonic inflammation. However, the molecular mechanism(s) mediating this proinflammatory response remains unclear. We found that NCM460, non-transformed human colonocytes, express a functional high affinity NT receptor that mediates NT-induced Erk activation. By using NCM460 cells stably transfected with NTR1, we show that NTR1 activation leads to interleukin (IL)-8 secretion that is mediated via both NF-κB and Erk-dependent pathways. In addition, NT-stimulated NF-κB activation is dependent on intracellular calcium release. NT-stimulated Erk activity requires Ras activation because overexpression of the dominant negative Ras mutant Ras-17N almost completely inhibits the Erk activation. Furthermore, NT directly stimulates Ras-GTP formation as shown by a Ras-GTP pull-down assay. By using reporter gene FBS, bictegravir-containing targeted substitutions in the IL-8 promoter, we show that the NF-κB, AP-1, and to a lesser degree the C/EBP sites in the IL-8 promoter region are required for IL-8 gene expression induced by NT. In summary, our results demonstrate that NT stimulates calcium-dependent NF-κB and Ras-dependent Erk pathways that mediate the release of IL-8 from non-transformed human colonocytes. We speculate that these NT-related proinflammatory pathways are important in the pathophysiology of colonic inflammation.

Neurotensin (NT), a 13-amino acid neuropeptide originally isolated by Carraway and Leeman (1), is highly expressed in the gastrointestinal tract (2). In the ileal mucosa NT is synthesized and secreted by specific endocrine cells (3), in response to diverse stimuli (4). NT increases small bowel, colonic, and gastric motility and stimulates ileal, pancreatic, and biliary secretion (4, 5) as well as Cl− secretion from human colonic mucosa (6), indicating that this peptide may contribute to the pathophysiology of human diarrhea. NT also stimulates growth of the intestinal mucosa under physiological and pathological conditions and causes proliferation of intestinal epithelial cells in vivo and in vitro (7–11). Two G-protein-coupled receptors (GPCRs) have been described for NT, a high affinity (NTR1) and a low affinity (NTR2) receptor (12). Administration of the specific NTR1 antagonist SR 48692 to rats inhibits colonic mucin and prostaglandin E2 secretion in response to immobilization stress (13), suggesting the importance of NTR1 in stress-mediated colonic responses. Our recent studies (14) demonstrate that NT is a proinflammatory peptide in the colon because blockage of the NT-NTR1 interaction with SR 48692 inhibited colonic secretion and inflammation mediated by Clostridium difficile toxin A. We also showed that, compared with normal colonic epithelial cells, there was a dramatic up-regulation of NTR1 during human colonic inflammation (15) as well as in Clostridium difficile toxin A-mediated colitis (14). NT exerts its proinflammatory effects by interacting with several cell types, including mast cells (13, 14, 16, 17), leukocytes (14, 18), endothelial cells (19), and macrophages (20).

The intracellular events stimulated by NT have been studied previously in human colon and pancreatic cell lines that express endogenous NTR1 (21, 22). NT stimulates the formation of inositol 1,4,5-trisphosphate, increases intracellular calcium (23, 24), and activates Erk, a member of the mitogen-activating protein kinase family (25), in colonic adenocarcinoma HT29 cells and in pancreatic MIA PaCa-2 cells (22). NT also stimulates Erk activation in Chinese hamster ovary cells overexpressing NTR1, which is partially inhibited by PTX and completely blocked by the protein kinase C inhibitor GF 109203X (25). Although NT triggers calcium release and Erk activation, their involvement in NT-induced release of proinflammatory cytokines has not been studied. In addition, the signaling pathways stimulated by NT that lead to Erk activation have not been elucidated.

Studies in human intestinal microvascular endothelial cells showed that NT stimulates translocation and DNA binding activity of NF-κB (15). NF-κB is a transcriptional factor critical for expression of genes involved in inflammation of the gastrointestinal tract (26–28). It consists of homo- and heterodimers of Rel family proteins, typically p65 and p50, sequestered in an inactive form in the cytoplasm by IκB inhibitory proteins. Upon stimulation, IκBs become phosphorylated, ubiquitinate, and subsequently degraded, resulting in the nuclear translocation of NF-κB and the activation of NF-κB-responsive genes. NF-κB

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¶ The abbreviations used are: NT, neurotensin; GPCR, guanine nucleotide-binding protein-coupled receptor; Erk, extracellular signal-regulated protein kinase; BAPTA/AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, acetoxymethyl ester; FMA, phorbol 12-myristate acetate; PTX, pertussis toxin; IL, interleukin; NTR, neurotensin receptor; EGF, epidermal growth factor; RT-PCR, reverse transcriptase-polymerase chain reaction; hp, base pair; PBS, fetal bovine serum; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay(s); MAP, mitogen-activated protein.
has been shown to be essential for expression of inflammatory genes such as IL-8. IL-8, a potent chemotactic factor for neutrophils, basophils, and T lymphocytes, has been implicated in the pathogenesis of several gastrointestinal inflammatory states (29–33). Three cis-regulatory elements (AP-1, C/EBP-like, and xB-like sites) in the IL-8 promoter region are involved in IL-8 gene expression (34, 35). However, the relative importance of the sites regulating IL-8 gene expression depends on the cell type and the particular stimulus.

In this study, we utilized NCM460, a non-transformed human colonic epithelial cell line, to investigate the molecular mechanisms by which NT exerts its proinflammatory effects. We found that NCM460 cells express a functional NTR1 receptor. Exposure of NCM460 cells transfected with the NTR1 to NT stimulates IL-8 secretion as well as gene transcription in a time- and dose-dependent manner. We also demonstrate that NF-xB and Erk activation are required for NT-induced IL-8 gene expression. Our results indicate that NT-stimulated NF-xB DNA binding activity is calcium-dependent. More importantly, we show for the first time that NT stimulates Ras-GTP formation and that NT-induced Erk activation is Ras-dependent.

**EXPERIMENTAL PROCEDURES**

**Reagents**

NT was purchased from Phoenix Pharmaceuticals (Belmont, CA). [3,11-tyrosyl-3,5,3H]NT was from PerkinElmer Life Sciences. BAPTA/AM and sulfasalazine were obtained from Calbiochem. Sulfipyrazone and collagenase were from Sigma, and Fura-2 AM and Pluronic F127 were obtained from INCELL Corporation (San Antonio, TX). BCA reagents and collagenase were from Sigma. [125I]-tyrosyl were purchased from PerkinElmer Life Sciences. BAPTA/AM and sulfasalazine were obtained from Calbiochem. Sulfinpyrazone and collagenase were from Sigma, and Fura-2 AM and Pluronic F127 were obtained from INCELL Corporation (San Antonio, TX). BCA reagents for measurement of protein concentration were purchased from Pierce.

Creation of a Stably Transfected Human Colonic NCM460 Cell Line Expressing NTR1 (NCM460-NTR1)

**Construction and Functionality of a Purinomycin-resistant Retroviral Vector**—To create stably transfected cell lines that selectively grow in medium containing puromycin, we constructed a highly efficient puromycin-resistant expression retroviral vector named pCMBP (kindly provided by Dr. Richard C. Mulligan, Children’s Hospital, Harvard Medical School) and pBabe-puro (kindly provided by Dr. Steve R. Farmer, Boston University School of Medicine). The retroviral promoter from pCMMP was removed with the restriction enzymes SbfI and Pmel (the fragment named Sbf-Pmel), and then ligated into the pBabe-puro vector which was first digested with BamHI, end-blunted with Klenow, and digested with PstI. To test the functionality of this vector, we also ligated a bacterial β-galactosidase gene (lacZ) into the pBabe vector, by a three-fragment ligation. To achieve that, pBabe-puro was first digested with PstI and EcoRI to remove the entire Moloney murine leukemia virus 5′-long terminal repeat and adjacent sequence. A LacZ fragment was isolated from pBlueScript-lacZ by first digesting this plasmid with BamHI, end-blunting with Klenow, and then digesting with EcoRI. The digested vector was then ligated with the fragment Sbf-Pmel and the LacZ fragment. The retroviruses expressing β-galactosidase were prepared following the procedure described below and used to infect NIH3T3 cells to measure the titers of produced virus.

Cloning of Full-length Human NTR1 cDNA from NCM460 Cells

**Construction of NTR1-expressing Retroviral Vectors**—Total cell RNA was isolated from NCM460 cells by the guanidinium isothiocyanate/phenol/chloroform extract procedure as described previously (36). Two micrograms of RNA were reverse-transcribed with Moloney murine leukemia virus-reverse transcriptase (Invitrogen, Grand Island, NY) according to the manufacturer’s instruction. Then 2 μl of RT mix was used for PCR amplification of full-length human NTR1 cDNA using Taq DNA polymerase (QIAGEN) under the following conditions: 94 °C, 4 min followed by 35 cycles of 94 °C, 1 min; 60 °C, 1 min and 72 °C, 1 min and then 72 °C, 5 min. The primers were designed based on the published sequence of human NTR1 (GenBank™ accession number X70070; Ref. 37). The forward primer containing an EcoRI site was 5′-aggtagatG-GACTCCAGGGCCACGAGG-3′; and the reverse primer containing a BamHI site was 5′-taaggatcaACACGGTCCGCGGACGACG-3′. A 1315-bp fragment was subcloned into pCR2.1 vector using the TA cloning kit (Invitrogen), and the identity of the DNA was confirmed by DNA sequencing. The NTR1 fragment was then removed from pCR2.1 by EcoRI digestion and ligated into the puromycin-expressing retroviral vector pCMBP (pCMBP-NTR1) described above. Retroviruses containing NTR1 were prepared using pCMBP-NTR1 following the procedure described below.

**Preparation of Retroviruses and Infection of NCM460 Cells—**293T cells (kindly provided by Dr. Richard A. Mulligan) were seeded at a density of 4–5 × 105 cells in 100-mm plates containing 10 ml of 10% FBS/Dulbecco’s modified Eagle’s Medium for 24 h before transfection. The plasmids were replaced with fresh medium 4 h before transfection. The plasmids pMD-gag-pol, pMD-VSVG (both kindly provided by Dr. Richard C. Mulligan), and pCMBP-NTR1 were combined in a ratio of 3:1:4 and used to prepare transfection mixtures using Effectene Transfection Reagent (Qiagen) according to the manufacturer’s instruction. Forty-eight hours after transfection, the media were collected and filtered through 0.45-μm disc filters, and the supernatants were either used immediately or stored at −80 °C. Infection of NCM460 cells was carried out as follows. Cells were seeded at 4 × 105 cells/cm² culture surface for 24 h and incubated with medium containing 2 volumes of filtered virus-containing supernatant and 1 volume of fresh growth media for 16–24 h in the presence of 10 μg/ml Polybrene (Sigma). The infected cells were then incubated in medium containing 10% FBS and 2 μg/ml puromycin for 6 days. Positively selected cells were pooled (named NCM460-NTR1 cells) and used for this study.

Ligand Binding Assay

NCM460 cells or NCM460-NTR1 cells in 24-well plates were incubated in M3D media containing 10% FBS until they reached ~80% confluence. The cells were then washed once with PBS and incubated with M3D media for 24 h. The cells were incubated with 0.3 ml of fresh M3D media containing increasing concentrations of [3,11-tyrosyl-3,5,3H]NT (0.05–20 nM) to determine nonspecific binding. 1 μM NT was added to the incubation mixture. After incubation for 3 h at 4 °C, the cells were placed on ice and washed three times with cold PBS. Cells were then lysed in 0.3 ml of 0.3 M NaOH for 30 min at 37 °C, and the radioactivity in the lysate was measured to determine the amount of ligand bound. All determinations were performed in triplicate. The number of receptors was determined from a saturation curve generated by SigmaPlot 3.0 software (Jandel Scientific, San Rafael, CA).

IL-8 Measurements

IL-8 protein levels in colonic epithelial cell-conditioned media were determined by a double-ligand enzyme-linked immunosorbent assay (ELISA) using goat anti-human IL-8 (R & D Systems Inc.) as described previously (30). Results were expressed as mean ± S.E. (ng/ml). At least three independent experiments were performed for each experimental condition, each with triplicate measurements.

Construction of Human IL-8 Promoter-Luciferase Constructs and Luciferase Assay

Construction of a reporter construct containing 1521 bp (nucleotides −1481 to +40) of the promoter region of human IL-8 gene has been described previously (31). IL-8 reporter constructs containing mutations in NF-κB, AP-1, or C/EBP sites were created by overlapping PCR-based site-directed mutagenesis. The substitution mutants were based on the sequences described by Wu et al. (35). The mutated NF-κB sequence was cggTAACTTTCGct; the mutated AP-1 sequence was gATATCTCAGag; and the mutated C/EBP sequence was cAGCTACGAGTgc. The sequences were confirmed by DNA sequencing using primers specific for the pGL2-basic luciferase expression vector (GL vector 1.8 P; Promega Corp., Madison, WI). Three IL-8 promoter activity in response to NT, cells were seeded in 12-well plates (0.2 × 105 cells/well) overnight and transiently transfected using Effectene Transfection Reagent (Qiagen) with IL-8 promoter-luciferase constructs or a control luciferase construct pRL-TK (Promega) or other DNA constructs as indicated. Transfected cells were serum-starved for 24 h followed by exposure to NT for 4 h. Firefly and Renilla luciferase activities were first determined by Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity was then calculated by normalizing IL-8 promoter-driven firefly luciferase activity to control Renilla luciferase activity. Data from all experiments are presented as the relative luciferase activity (mean ± S.E.) from at least two independent sets of experiments, each with triplicate measurements.
Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extracts were prepared for DNA binding assays as described previously (38). Cells growing in 100-mm plates were washed 2× with ice-cold PBS and then collected into 1 ml of TNE buffer (40 mM Tris (pH 7.4), 1 mM EDTA, 0.15 mM NaCl) and centrifuged at 5000 × g for 10 s. The cell pellets were resuspended with 800 μl of buffer A (10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride) for 10 min before addition of 46 μl of 10% Nonidet P-40 for an additional 2 min. Nuclei were pelleted by centrifugation at 5000 × g for 10 s, incubated with 120 μl of buffer B (20 mM Hepes (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride) for 45 min, and centrifuged at 13,000 × g for 10 min. Ten micrograms of nuclear extracts were incubated with 3 μg of poly(dI-dC) (Promega), 2 μl of bandshift buffer (50 mM MgCl2, 340 mM KCl), and 8 μl of 1× bandshift buffer (40 mM KCl, 25 mM Hepes (pH 7.6), 8 mM Ficoll 400, 1 mM dithiothreitol) at 4 °C for 15 min. 32P-Labeled double-stranded oligonucleotide probe (100,000 cpm) was then added to the reaction mixture and incubated for 30 min on ice. For supershift assays, the appropriate antibody was added to the nuclear extract and incubated at 4 °C for 30 min before addition of the probe. The anti-NF-κB p65 and anti-p50 (as well as normal rabbit IgG (control antibody) were from Santa Cruz Biotechnology (Santa Cruz, CA). Binding of specific nuclear protein to the probe was determined by fractionating the nuclear proteins through a non-denaturing 6% polyacrylamide gel at 200 V for 2 h at room temperature in TBE buffer (80 mM Tris borate, 2 mM EDTA (pH 8.0)). The gel was dried at 80 °C for 2 h under vacuum before exposure to x-ray autoradiography film. The NF-κB consensus oligonucleotide was purchased from Promega. The double-stranded oligonucleotide was end-labeled by T4 DNA polynucleotide kinase (New England Biolabs, Beverly, MA) and γ-32P]ATP (PerkinElmer Life Sciences).

Intracellular Ca2+ Release

Serum-starved cells grown in 100-mm plates were washed twice with PBS and incubated with 4 ml of collagenase solution (0.2 mg/ml collagenase, 0.2 mg/ml collagenase, 1 mg/ml bovine serum albumin, 2 ml EDTA in PBS) at 37 °C for 30 min. Cells were detached by gentle scraping and centrifuged at 1000 rpm for 3 min. Cell pellets were resuspended in 2 ml of Ca2+ buffer (5 mM KCl, 140 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 0.1% bovine serum albumin, 0.25 mM saponin, and 10 mM Hepes (pH 7.5)) and centrifuged again at the same speed. Cell pellets were resuspended in 2 ml of Ca2+ buffer containing 1 μg/ml Fura-2 and 0.02% pluronic F-127 and incubated at 37 °C for 30 min. Cells were then collected, centrifuged at 1100 rpm for 3 min, and resuspended in 2 ml of Ca2+ buffer. Half of the cell suspension was preincubated with Mn2+SO4 vehicle or BAPTA/AM (50 μM) for 5 min before stimulation with NT. Intracellular Ca2+ concentration was measured with the DeltaScan Illumination System plus Felix software (Photon Technology International, Lawernville, NJ).

Erk Phosphorylation Assay

Cells were washed twice with ice-cold PBS and then incubated in RIPA buffer containing a protease inhibitor mixture (Roche Molecular Biochemicals) for 10 min. Cell lysates were centrifuged at 1000 × g for 10 min. Equal amounts of cell extracts were separated by SDS-polyacrylamide gel electrophoresis (10%), and proteins were transferred onto nitrocellulose membranes (Bio-Rad) at 100 V for 1 h at 4 °C. Membranes were blocked in 5% nonfat, dried milk in TBST (50 mM Tris (pH 7.5), 0.15 mM NaCl, 0.05% Tween 20) and then incubated with phosphospecific antibodies (0.2 μg/ml) to Erk 1/2 (New England Biolabs, Beverly, MA). Horseradish peroxidase-labeled antibodies were detected by SuperSignal Chemiluminescent Substrate (Pierce).

Ras Activation Assay

Ras activity was measured using a Ras Activation Assay Kit (Upstate Biotechnology Inc., Lake Placid, NY) following the manufacturer's instructions. Briefly, quiescent cells were stimulated with NT (10−7 M), washed 2× with ice-cold PBS, and then incubated with 1× Mg2+ lysis/washing buffer containing a protease inhibitor mixture (Roche Molecular Biochemicals) for 10 min at 4 °C. Cell lysates were centrifuged at 1000 × g for 10 min. The supernatants were then treated with glutathione-Sepharose-4B beads (Amersham Pharmacia Biotech) for 30 min and then centrifuged at 1000 × g for 10 min. The supernatants were incubated with Ras-1-RBD-conjugated agarose beads for 30 min at 4 °C, and the beads were washed 5× with 1× Mg2+ lysis buffer. The beads were then boiled with 1× SDS sample buffer, and equal volumes of the samples were subjected to Western blot analysis using a monoclonal antibody against Ras. To normalize the amount of GTP-bound Ras to total amount of Ras, equal volumes of cell lysate were also subjected to Western blot analysis using the Ras monoclonal antibody.

Statistical Analyses

Results were expressed as means ± S.E. Data were analyzed using the SIGMASTAT™ professional statistics software program (Jandel Scientific Software, San Rafael, CA). Analyses of variance with protected f test were used for intergroup comparison.

RESULTS

NT Stimulates IL-8 Secretion and Gene Transcription in NCM460-NTR1 Cells—Our recent work (14) demonstrated that NTR1 mediates colonic inflammation. To examine whether NTR1 mediates release of the proinflammatory cytokine IL-8, we used the non-transformed human colonic epithelial cell line, NCM460 (39). We first determined whether NCM460 cells express NTR1 mRNA by RT-PCR, and we examined the functionality of this receptor. Our results show that the NCM460 cells express NTR1 as indicated by an expected 1315-bp PCR product that corresponds to the full-length coding region of human NTR1 cDNA (data not shown). Moreover, when quiescent NCM460 cells were exposed to NT (10−10 to 10−6 M) for 10 min, NT induced Erk phosphorylation even at a concentration of 10−10 M (Fig. 1a). Pretreatment with the specific NTR1 antagonist SR 48692 at a concentration of 10−6 M completely inhibited NT (10−9 M)-induced Erk activation, indicating that NTR1 mediates this response (Fig. 1b). NT (10−6 M, 4–24 h of exposure), however, failed to stimulate IL-8 secretion (data not shown), suggesting that the levels of the receptor may not be high enough to mediate this response. Therefore, we sought to overexpress NTR1 in NCM460 cells to examine whether increased expression of NTR1 results in IL-8 secretion in response to NT. To do that, the NTR1 cDNA fragment generated by RT-PCR from total mRNA derived from NCM460 cells was subcloned into the retroviral vector pCMVB. Retroviruses expressing NTR1 were produced and used to generate stably transfected NCM460 cell lines expressing NTR1 (NCM460-NTR1) as described under “Experimental Procedures.” To find out whether NCM460-NTR1 cells express increased number of NT receptors, we performed a ligand binding assay using [3H]NT. The results (data not shown) demonstrated that compared with the parental NCM460 cell that expressed 24,000 NT receptors per cell, NCM460-NTR1 cells had ~360,000 receptors per cell.

Exposure of NCM460-NTR1 cells to NT (10−7 M) strongly stimulated IL-8 secretion with a maximal induction 4 h after NT exposure (Fig. 1c). IL-8 secretion induced by NT was dose-dependent at concentrations ranging between 10−10 and 10−8 M, and a significant (1.6-fold, p < 0.01) induction was obtained at 10−9 M (Fig. 1d). However, NT (10−6 M, 4 h of exposure) could not stimulate IL-8 secretion in the parental NCM460 cells (Fig. 1d). We also determined whether NT stimulates IL-8 gene transcription in quiescent NCM460-NTR1 cells transiently transfected with a luciferase reporter construct containing the human IL-8 promoter. The results showed that NT (10−7 M) significantly stimulated IL-8 promoter-driven luciferase activity by 51-fold in NCM460-NTR1 cells but had no effect in the parental NCM460 cells (Fig. 1e, left). Taken together, these results demonstrate that NT stimulates IL-8 secretion as well as IL-8 gene transcription in NCM460-NTR1 cells.

NT-induced IL-8 Expression Requires NF-κB Activation—To confirm the requirement of NF-κB activation for NT-induced IL-8 secretion, we examined whether NT stimulated nuclear translocation and DNA binding activity of NF-κB in NCM460-NTR1 cells. NT (10−7 M) dramatically increased DNA binding of a nuclear protein complex that contained NF-κB p65 and p50 subunits as shown by supershift assays (Fig. 2a). The specific-
Fig. 1. NT stimulates IL-8 secretion and gene transcription in nontransformed human colonocytes overexpressing NTR1. a, NCM460 cells were incubated in M3D media for 24 h to render cells quiescent and then stimulated with NT (10⁻¹⁰ to 10⁻⁶ M) for 10 min. Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-phospho-Erk1/2 (upper panel) and anti-Erk2 (lower panel); the latter was used as a protein loading control. b, quiescent NCM460 cells were pretreated with NTR1-specific antagonist SR 48692 (1–50 nM) for 10 min and then treated with NT (10 nM) for 10 min. Erk phosphorylation was measured as in a. c, NCM460 cells stably transfected with NTR1 (NCM460-NTR1) were incubated with M3D media for 24 h and then treated with NT (10⁻⁷ M) for the indicated time points. The conditioned media were collected and IL-8 was measured by ELISA. Asterisk indicates p < 0.001, 2-h NT-stimulated cells versus non-stimulated cells. d, NCM460 cells or NCM460-NTR1 cells were rendered quiescent and treated for 4 h with NT at the indicated concentrations, and IL-8 was measured in the conditioned media by ELISA. Asterisk indicates p < 0.01, 10⁻⁹ M NT-stimulated cells versus non-stimulated cells. Results are expressed as mean ± S.E. (ng/ml). e, NCM460 cells or NCM460-NTR1 cells were transfected with IL-8 promoter construct with an internal control plasmid as described under “Experimental Procedures.” Cells were serum-starved and then treated with NT (10⁻⁷ M) for 4 h. Cell extracts were prepared to measure IL-8 promoter activity that was expressed as mean ± S.E. (relative luciferase activity, n = 3). Asterisk indicates p < 0.001, 10⁻⁷ M NT-stimulated cells versus non-stimulated cells. Results are from a single experiment each with triplicate determinations, representative of three separate experiments.

Fig. 2. NT-induced IL-8 expression requires NF-κB activation. a, quiescent NCM460-NTR1 cells were treated with NT (10⁻⁷ M) for 30 min, and nuclear extracts were prepared for EMSA using a 3²P-labeled NF-κB probe as described under “Experimental Procedures.” For supershift assays, 2 μg of anti-p65, anti-p50, or normal rabbit IgG were preincubated with nuclear extracts for 30 min on ice before EMSA reactions were performed. b, quiescent cells were pretreated with sulphasalazine (2 mM) for 30 min and then exposed to NT (10⁻⁷ M) for 30 min, and NF-κB binding activity was examined by EMSA. c, NCM460-NTR1 cells were infected with IκBα- or β-galactosidase (lacZ)-expressing retroviruses and incubated with M3D media for 24 h before treatment with NT (10⁻⁷ M) for 4 h for measurement of IL-8 secretion. Asterisk indicates p < 0.01, IκBα-expressing cells versus LacZ-expressing cells with NT stimulation. d, NCM460-NTR1 cells were transfected with IL-8 promoter construct with an internal control plasmid and pCMBP-lacZ or pCMBP-IκBα. Cells were incubated with M3D media for 24 h and then treated with NT (10⁻⁷ M) for 4 h. Cell extracts were prepared to measure IL-8 promoter activity which was expressed as mean ± S.E. (relative luciferase activity, n = 3). Asterisk indicates p < 0.01, IκBα-expressing cells versus LacZ-expressing cells with NT stimulation. e, cells were transiently transfected with wild-type or NF-κB mutant promoter constructs together with a control luciferase construct. The transfected cells were incubated with media for 24 h and then exposed to NT (10⁻⁷ M) for 4 h. Cell extracts were prepared to determine luciferase activity as described under “Experimental Procedures.” The results were representative of three separate experiments.
ity of the shifted band was confirmed by preincubating the nuclear extract with a control antibody that did not bind NF-κB as well as by addition of excess unlabeled (cold) probe into the binding mixture (Fig. 2a).

We next determined whether inhibition of NF-κB activation blocks NT-induced IL-8 secretion. Pretreatment of NCM460-NTR1 cells with the NF-κB inhibitor sulfasalazine significantly inhibited NT-induced NF-κB DNA binding activity (Fig. 2b) and IL-8 secretion (data not shown). To confirm the role of NF-κB in NT-induced IL-8 expression, we overexpressed IκBα, an endogenous NF-κB inhibitor. Our data show that NT-induced IL-8 secretion was reduced by 65% in cells overexpressing human IκBα, as compared with the control (Fig. 2c). To examine whether overexpression of IκBα inhibits NT-induced IL-8 gene transcription, NCM460-NTR1 cells were transiently transfected with the IL-8 luciferase reporter construct together with an IκBα plasmid or control vector. Our data clearly indicate that cotransfection with the IκBα plasmid significantly reduced NT-induced IL-8 promoter-driven gene transcription (Fig. 2d). To confirm the NF-κB requirement for NT-induced IL-8 transcription, we used an IL-8 promoter construct that contained targeted mutations in the NF-κB-binding site. The results showed that the NF-κB mutant construct did not respond to NT treatment, whereas the wild-type IL-8 promoter construct was strongly activated by NT (Fig. 2c). Thus, NF-κB activation is a major requirement for NT-induced IL-8 gene transcription and IL-8 protein release.

**Calcium Dependence of NT-induced NF-κB Activation and IL-8 Secretion**—NT (10⁻⁷ M) stimulated intracellular calcium release in NCM460-NTR1 cells within a few seconds, which returned to control values after ~3 min (Fig. 3a). Moreover, pretreatment of the cells with BAPTA/AM completely inhibited NT-induced Ca²⁺ release, even below the basal levels (Fig. 3a), and abolished NT-induced IL-8 secretion (Fig. 3b). To find out whether the effect of BAPTA/AM was mediated through NF-κB, quiescent NCM460-NTR1 cells were preincubated with BAPTA/AM and then treated with NT (10⁻⁷ M), and NF-κB DNA binding activity was measured by EMSA. Our results showed that pretreatment with BAPTA/AM significantly attenuated NT-stimulated NF-κB DNA binding activity, indicating that the effect of intracellular calcium on NT-induced IL-8 secretion involves NF-κB activation (Fig. 3c). We also found that preincubation of NCM460-NTR1 with 20 μM W7, a Ca²⁺-calmodulin antagonist, or 10 μM cyclosporin A, an inhibitor of the calcium-dependent phosphatase calcineurin, inhibited NT-induced IL-8 secretion by 61.3 and 60.7%, respectively (p < 0.05, n = 3) (data not shown). These results suggest that the Ca²⁺-dependent NF-κB activation in response to NT might be mediated through Ca²⁺-activated kinase(s) and phosphatase(s).

**Erk Activation Is Required for NT-induced IL-8 Expression**—We next determined whether an Erk-dependent pathway mediates NT-induced IL-8 expression. Quiescent cells were pretreated with PD98059, a specific MAP kinase/Erk kinase inhibitor, and treated with NT (10⁻⁷ M) for 4 h to measure IL-8 release and IL-8 promoter-dependent transcription. PD98059 significantly inhibited both NT-induced IL-8 release (Fig. 4a) and IL-8 promoter activity (Fig. 4b), suggesting that Erk activation is involved in NT-induced IL-8 expression.

**NT Stimulates Ras-GTP Formation**—To examine whether Ras is upstream of NT-induced Erk activation, we first examined whether NT directly activates Ras using a Ras-GTP pull-down assay (40). Quiescent NCM460-NTR1 cells were treated with NT (10⁻⁷ M) for various times or EGF for 3 min, and the levels of Ras-GTP in the cell extracts were then measured. We found that NT increased Ras-GTP loading from 3 to 5 min after NT exposure. As expected, EGF (100 ng/ml) strongly stimulated Ras-GTP loading (Fig. 5). These data demonstrate for the first time that binding of NT to NTR1 activates Ras.

**NT-induced Erk Activation Is Ras-dependent**—It is known that GPCR-stimulated Erk activation can be Ras-dependent or Ras-independent (41, 42). However, whether Ras is involved in the NT signaling pathway is not known. To explore this possibility, we used a dominant negative Ras mutant Ras-17N to block downstream events of Ras activation. NCM460-NTR1 cells were infected with the Ras-17N-expressing retroviruses and then rendered quiescent. Cells were then exposed to either NT (10⁻⁷ M) or PMA (2 μM), a phorbol ester that activates Erk (42), and nuclear extracts were prepared, and NF-κB DNA binding activity was determined by EMSA. Results are representative of three separate experiments.

**Calcium Mobilization Is Essential for NT-induced NF-κB Activation and IL-8 Secretion**—NT (10⁻⁷ M) stimulated intracellular calcium release in NCM460-NTR1 cells within a few seconds, which returned to control values after ~3 min (Fig. 3a). Moreover, pretreatment of the cells with BAPTA/AM completely inhibited NT-induced Ca²⁺ release, even below the basal levels (Fig. 3a), and abolished NT-induced IL-8 secretion (Fig. 3b). To find out whether the effect of BAPTA/AM was mediated through NF-κB, quiescent NCM460-NTR1 cells were preincubated with BAPTA/AM and then treated with NT (10⁻⁷ M), and NF-κB DNA binding activity was measured by EMSA. Our results showed that pretreatment with BAPTA/AM significantly attenuated NT-stimulated NF-κB DNA binding activity, indicating that the effect of intracellular calcium on NT-induced IL-8 secretion involves NF-κB activation (Fig. 3c). We also found that preincubation of NCM460-NTR1 with 20 μM W7, a Ca²⁺-calmodulin antagonist, or 10 μM cyclosporin A, an inhibitor of the calcium-dependent phosphatase calcineurin, inhibited NT-induced IL-8 secretion by 61.3 and 60.7%, respectively (p < 0.05, n = 3) (data not shown). These results suggest that the Ca²⁺-dependent NF-κB activation in response to NT might be mediated through Ca²⁺-activated kinase(s) and phosphatase(s).

**Erk Activation Is Required for NT-induced IL-8 Expression**—We next determined whether an Erk-dependent pathway mediates NT-induced IL-8 expression. Quiescent cells were pretreated with PD98059, a specific MAP kinase/Erk kinase inhibitor, and treated with NT (10⁻⁷ M) for 4 h to measure IL-8 release and IL-8 promoter-dependent transcription. PD98059 significantly inhibited both NT-induced IL-8 release (Fig. 4a) and IL-8 promoter activity (Fig. 4b), suggesting that Erk activation is involved in NT-induced IL-8 expression.

**NT Stimulates Ras-GTP Formation**—To examine whether Ras is upstream of NT-induced Erk activation, we first examined whether NT directly activates Ras using a Ras-GTP pull-down assay (40). Quiescent NCM460-NTR1 cells were treated with NT (10⁻⁷ M) for various times or EGF for 3 min, and the levels of Ras-GTP in the cell extracts were then measured. We found that NT increased Ras-GTP loading from 3 to 5 min after NT exposure. As expected, EGF (100 ng/ml) strongly stimulated Ras-GTP loading (Fig. 5). These data demonstrate for the first time that binding of NT to NTR1 activates Ras.

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ual experiments, each with triplicate determinations. The results were representative of three individual experiments, each with triplicate determinations.

Experimental Procedures.

Dominant Negative Ras Inhibits NT-induced IL-8 Expression—To determine whether Ras activation is required for NT-induced IL-8 secretion and IL-8 gene transcription, NCM460-NTR1 cells were infected with Ras-17N-expressing retroviral vectors and then exposed to NT (10^{-7} M) for 4 h. Expression of the dominant negative Ras significantly inhibited NT-induced IL-8 protein release (Fig. 7a). To explore whether the Ras-17N mutant also inhibited NT-induced IL-8 transcription, cells were transfected with the Ras-17N construct along with IL-8 promoter-luciferase construct and then exposed to NT (10^{-7} M) for 4 h. As shown in Fig. 7b, overexpression of Ras-17N significantly inhibited NT-stimulated IL-8 promoter activity.

DISCUSSION

We have reported previously (14, 15) that NTR1 are up-regulated in colonic inflammation and presented indirect evidence for increased expression of NTR1 receptors on colonic epithelial cells. This study shows that NCM460 human colonic epithelial cells express the high affinity NT receptor that mediates Erk activation in response to NT. Moreover, exposure of NCM460 cells overexpressing NTR1 to NT caused secretion of the potent chemoattractant IL-8 in a time- and dose-dependent manner. NT-induced IL-8 secretion and gene transcription were dependent on NF-κB and Erk activation. In addition, NT-mediated NF-κB activation was mediated by intracellular calcium release. We also report here for the first time that NT stimulates Ras activation which is required for NT-induced Erk activation and that both calcium and Ras activation are involved in IL-8 release in response to NT.

Our studies demonstrate that the mechanism of NF-κB activation and IL-8 secretion in response to NT in colonocytes is calcium-dependent. Interestingly, calcium-dependent NF-κB...
activation is required for IL-8 expression induced by the neuropeptide substance P in human monocytes (45). The mechanisms by which intracellular calcium release leads to activation of NF-κB activity following NT exposure have not been elucidated. Exposure of cells to various stimuli such as cytokines triggers a signaling cascade causing phosphorylation and subsequent degradation of IkB proteins leading to the release, activation, and nuclear translocation of NF-κB (46). The effect of increased intracellular calcium on IkB phosphorylation may be mediated by a calcium-calcmodulin-dependent pathway as W7, a calmodulin-antagonist, can inhibit IkB degradation (47, 48). In addition, the calcium-dependent protein phosphatase calcineurin may synergize with protein kinase C to activate SB203580, and the inhibitor of MAP kinase/Erk kinase, 

NF-κB-induced NF-κB translocation in a Gi/o protein and calcium-independent manner remains clear. Our results demonstrate that a dominant negative Ras 109203X (25), the pathway that leads to Erk activation is not Ras-dependent. The exact edge, this is the first report to show that NT activates Ras and that NT-induced Ras activation is via the calcium-dependent tyrosine kinase Pyk2, which is triggered by PTX-insensitive Gβ and phospholipase Cβ (58). However, the inability of PTX or BAPTA/AM to affect NT-induced Erk activation in our study indicates that these two pathways are unlikely to mediate NT-induced Ras-dependent Erk activation. In addition, transactivation of the EGF receptor has also been shown to mediate Erk activation induced by many GPCRs such as the receptors for lysophosphatidic acid, thrombin (59), and neuromodulin, and Ras-dependent Erk activation further our understanding of the molecular mechanism by which this neuropeptide mediates its pro-inflammatory effect in the gastrointestinal tract.

In summary, our findings that NT induces expression of pro-inflammatory cytokine IL-8 via Ca\textsuperscript{2+}-dependent NF-κB and Ras-dependent Erk activation further our understanding of the molecular mechanism by which this neuropeptide mediates its pro-inflammatory effect in the gastrointestinal tract.

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