Insulin-like Growth Factor-1 Receptor Transactivation Modulates the Inflammatory and Proliferative Responses of Neurotensin in Human Colonic Epithelial Cells*

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Dezheng Zhao‡1,2, Kyriaki Bakirtzi‡1, Yanai Zhan‡, Huiyan Zeng‡, Hon Wai Koon‡,§ and Charalabos Pothoulakis*2,3

From the ‡Division of Gastroenterology and Center for Vascular Biology and *Division of Vascular Medicine, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215 and the §Inflammatory Bowel Disease Center, Division of Digestive Diseases, David Geffen School of Medicine, UCLA, Los Angeles, California 90095-7019

Neurotensin (NT) is a gastrointestinal neuropeptide that modulates intestinal inflammation and healing by binding to its high-affinity receptor NTR1. The dual role of NT in inflammation and healing is demonstrated in models of colitis induced by Clostridium difficile toxin A and dextran sulfate sodium, respectively, and involves NF-κB-dependent IL-8 expression and EGF receptor-mediated MAPK activation in human colonocytes. However, the detailed signaling pathways involved in these responses remain to be elucidated. We report here that NT/NTR1 coupling in human colonic epithelial NCM460 cells activates tyrosine phosphorylation of the insulin-like growth factor-1 receptor (IGF-1R) in a time- and dose-dependent manner. NT also rapidly induces Src tyrosine phosphorylation, whereas pretreatment of cells with the Src inhibitor PP2 before NT exposure decreases NT-induced IGF-1R phosphorylation. In addition, inhibition of IGF-1R activation by either its specific antagonist AG1024 or siRNA against IGF-1 significantly reduces NT-induced IL-8 expression and NF-κB-dependent reporter gene expression. Pretreatment with AG1024 also inhibits Akt activation and apoptosis induced by NT. Silencing of Akt expression by siRNA also substantially attenuates NT-induced IL-8 promoter activity and NF-κB-dependent reporter gene expression. This is the first report to indicate that NT transactivates IGF-1R and that this response is linked to Akt phosphorylation and NF-κB activation, contributing to both pro-inflammatory and tissue repair signaling pathways in response to NT in colonic epithelial cells. We propose that IGF-1R activation represents a previously unrecognized key pathway involved in the mechanisms by which NT and NTR1 modulate colonic inflammation and inflammatory bowel disease.

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² Both authors contributed equally to this work.
³ To whom correspondence may be addressed: Center for Vascular Biology and Div. of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, 99 Brookline Ave., RN2701, Boston, MA 02215. Tel.: 617-667-0466; Fax: 617-667-3591; E-mail: dzhao@bidmc.harvard.edu.
4 To whom correspondence may be addressed: Inflammatory Bowel Disease Center, Div. of Digestive Diseases, David Geffen School of Medicine, UCLA, Los Angeles, CA 90095. Tel.: 310-825-9104; Fax: 310-825-3542; E-mail: cpothoulakis@mednet.ucla.edu.

Neurotensin (NT) is a 13-amino acid neuropeptide initially isolated from the bovine hypothalamus by Carraway and Leeman (1) and is highly expressed in the gastrointestinal tract (2). NT modulates motility in the stomach, small bowel, and colon (3–5) and stimulates secretion in the dog jejunum (6) and human colonic mucosa (7, 8). NT also stimulates growth and regeneration of the intestinal mucosa (9, 10) and has been implicated in the pathophysiology of colon cancer (11, 12).

Two G-protein-coupled receptors (GPCRs) have been described for NT: a high-affinity (NTR1) and a low-affinity (NTR2) receptor (13). A third, non-GPCR has also been identified (14). Several studies underlined the importance of NTR1 in several intestinal pathologic conditions. Administration of the specific NTR1 antagonist SR 48692 to rats inhibits colonic responses to stress (15, 16) and reduces colonic secretion and inflammation mediated by Clostridium difficile toxin A (17). Increased NTR1 expression is evident in the colonic mucosa during the course of acute colitis mediated by toxin A (17), in the colons of mice with experimental colitis (18), and in humans with inflammatory bowel disease (18). In a recent study, we also showed that involvement of NT in colonic inflammation may include direct stimulation of pro-inflammatory chemoattractant IL-8 transcription in colonic epithelial cells (19).

NT stimulates several intracellular signaling events, as shown in human colonic and pancreatic cell lines expressing endogenous NTR1 (20, 21). These include increased intracellular calcium release (22, 23) and activation of the MAPK family member ERK1/2 in pancreatic MIA PaCa-2 cells (24), transformed colonic adenocarcinoma HT29 cells (24), and non-transformed human colonic epithelial NCM460 cells (19). Our recent studies indicate that NT also activates the NF-κB pathway (19) and the Rho family of small GTPases (25, 26). Two previous studies showed that pretreatment with PKC inhibitors reduces NT-induced ERK1/2 activation in CHO cells overexpressing NTR1 (24) and in pancreatic carcinoma PANC-1 cells expressing endogenous NTR1 (27). In contrast, our laboratory and Hassan et al. have found that EGF receptor (EGFR) transactivation is involved in NT—in-
duced ERK activation in NCM460 cells (28) and PC3 (prostate epithelial cancer) cells (29). In addition, NT can also activate the PI3K/Akt pathway in PC13 cells, which is linked to cell proliferation (29).

In addition to EGFR, the insulin-like growth factor-1 receptor (IGF-1R) can be activated by its cognate ligand IGF-1 and transactivated in response to GPCR ligands such as thrombin (30) and angiotensin II (31). IGF-1R is expressed in human intestinal smooth muscle cells (32), cells of the muscularis propria, and the intestinal mucosa (33). IGF-1 regulates gastrointestinal tract growth and tissue repair as well as tumorigenesis through binding to its high-affinity receptor IGF-1R, which activates a number of signaling transduction pathways, including PI3K and downstream kinase Akt (34). IGF-1R is also altered in the intestines of Crohn disease patients (35), suggesting a possible role for IGF-1R in colitis. The PI3K/Akt pathway is involved not only in cell proliferation, differentiation, and survival but also in the NF-κB-mediated pro-inflammatory effects of TNFα (36, 37), IL-1β (38), and the GPCR B2-type bradykinin receptor (39). Although previous studies indicate that NT/NTR1 coupling activates the NF-κB pathway (19) and phosphorylates Akt (29), it is not known whether NT, or any other neuropeptide to our knowledge, stimulates phosphorylation of IGF-1R in human colonic epithelial cells and whether this response is involved in the pro-inflammatory and proliferative responses to NT.

In this study, we first show that NT rapidly induces tyrosine phosphorylation of IGF-1R and activates Akt in non-transformed human colonic epithelial NCM460 cells. Pretreatment with a specific IGF-1R inhibitor significantly attenuates NT-induced Akt phosphorylation, whereas transfection of colonocytes with siRNA specifically targeting either IGF-1R or Akt reduces NT-induced NF-κB-driven reporter expression and IL-8 promoter activity. In addition, inhibition of PI3K activation by its specific inhibitor LY 294002 significantly decreases NT-induced IkBα phosphorylation and IL-8 gene expression. In summary, our results demonstrate that transactivation of the IGF-1R/PI3K/Akt pathway is a novel mechanism in the pro-inflammatory responses of NT.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—Non-transformed human colonic epithelial NCM460 cells and NTR1-overexpressing NCM460 cells (NCM460-NTR1) have been described previously (19). NT was purchased from Phoenix Pharmaceuticals (Belmont, CA). Cell culture medium M3D for NCM460 and NCM460-NTR1 cells was obtained from INCELL Corp. (San Antonio, CA). Cell culture medium M3D for NCM460 and NCM460-NTR1 cells was treated with NT (10 μM), LY 294002 (25 μM), or vehicle for 10 min. Equal amounts of cell extracts were separated by SDS-PAGE (10%), and proteins were transferred onto nitrocellulose membranes (Bio-Rad) at 400 mA for 2 h at 4 °C. Membranes were blocked in 5% nonfat dried milk in Tris-buffered saline/Tween (50 mM Tris (pH 7.5), 0.15 M NaCl, 0.05% Tween 20) and then incubated with each primary antibody overnight at 4 °C. Membranes were washed with Tris-buffered saline/Tween and incubated with horseradish peroxidase-labeled secondary antibodies for 1 h. Peroxidase activity was detected by SuperSignal chemiluminescent substrate (Pierce). Western blot bands were quantified by densitometry using Scion Image analysis software.

** Luciferase Reporter Assay**—A reporter construct containing 1521 bp (nucleotides −1481 to +40) of the promoter region of the human IL-8 gene was used to measure transcription of the IL-8 gene as described previously (19). The NF-κB-luciferase reporter construct was purchased from Clontech and used to determine NF-κB transcriptional activity as described previously (19). To determine the IL-8 promoter activity or NF-κB transcriptional activity in response to NT, cells were seeded in 12-well plates (0.2 × 10⁶ cells/well) overnight and transiently transfected using Effectene transfection reagent with IL-8 promoter-luciferase constructs or NF-κB-luciferase reporter constructs together with a control luciferase construct, pRL-TK (Promega, Madison, WI), or siRNAs (control, IGF-1R, or Akt1/2 siRNA) as indicated. Transfected cells were serum-starved for 24 h and then treated with the indicated pharmacological inhibitors, followed by exposure to NT for 4 h. Firefly and Renilla luciferase activities in cell extracts were measured using the 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.

**Cell Proliferation Assay**—Serum-starved NCM460 cells, seeded on 96-well plates (1 million cells/plate) were pretreated with IGF-1R inhibitor (0.2 μM), LY 294002 (25 μM), or vehicle for 30 min and then exposed to NT (10 nm) or EGF (10 ng/ml). After 24 h, 20 μl of CellTiter AQ grieving One solution (Promega), which contains the tetrazolium compound MTS, was added to each well, and the plates were incubated at 37 °C for 1 h. Absorbance at 490 nm (indicating cell viability) was measured by using a 96-well plate reader as described previously (40, 41).

**Statistical Analyses**—Results were expressed as means ± S.E. Data were analyzed using the SIGMA-STAT™ professional statistics software program (Jandel Scientific Software, San Rafael, CA). Analyses of variance with protected t tests were used for intergroup comparison.

**RESULTS**

**NT Induces Tyrosine Phosphorylation of IGF-1R**—To determine whether NT can activate IGF-1R, NCM460 and NCM460-NTR1 cells were treated with NT (10⁻⁷ m) for various times. Equal amounts of cell lysates were subjected to Western blot analysis using the polyclonal antibodies against
phosphorylated IGF-1R and total IGF-1R. The results show that NT induced phosphorylation of IGF-1R with a maximal level at 1–2 min after stimulation (Fig. 1A). To examine whether NT induces phosphorylation of IGF-1R in a dose-dependent manner, quiescent NCM460 cells were treated with NT (10⁻⁷ M) for 1 min. Equal amounts of cell proteins were used to measure IGF-1R phosphorylation as described above. NT activated IGF-1R phosphorylation in a concentration-dependent manner, with a maximal stimulation at a concentration of 10⁻⁷ M (Fig. 1B). However, concentrations of 10⁻⁸ and 10⁻⁹ M were also able to induce this response (Fig. 1B).

NT is known to bind to three receptors, including the high-affinity GPCR NTR1 and two low-affinity receptors (one GPCR and another intracellular sortilin) (13). We have previously shown that NCM460 cells express the high-affinity receptor NTR1, which mediates NT-induced ERK1/2 activation (19). To further confirm that NT-induced IGF-1R phosphorylation is mediated by NTR1, NCM460 cells were pretreated with a specific NTR1 antagonist, SB48692 (1 μM), and then treated with NT (10⁻⁸ M) for 2 min. NTR1 antagonism almost completely reduced NT-induced IGF-1R phosphorylation, indicating that this receptor mediates NT-induced IGF-1R activation in these cells (Fig. 1C).

Involvement of Src Kinase in NT-induced IGF-1R Phosphorylation—We and others have previously shown that transactivation of the cell-surface EGFR by NT is via metalloproteinase-dependent EGFR ligand shedding (28, 29). To examine whether NT-induced IGF-1R phosphorylation might involve an autocrine mechanism involving release of IGF-1 and subsequent interaction with its receptor from colonocytes, NCM460 cells were pretreated with an IGF-1-neutralizing antibody or control antibody and treated with NT for 1 or 2 min. We found that the IGF-1-neutralizing antibody had no significant effect on NT-induced IGF-1R phosphorylation (Fig. 2A). We also assessed the levels of IGF-1 in the conditioned media collected from NCM460 cells treated with NT at 1, 2, 5, and 15 min. The proteins in the conditioned media were precipitated with 15% trichloroacetic acid, and the precipitated proteins plus positive control recombinant human IGF-1 were subjected to Western blotting using anti-IGF-1 antibody. The secreted IGF-1 was barely detectable and was not affected by NT stimulation (data not shown). The results together suggest that NT-induced IGF-1R transactivation is not mediated through an autocrine pathway in colonocytes. We next examined whether NT-induced IGF-1R phosphorylation involves intracellular calcium release. Cells were pretreated with the intracellular calcium chelator 1,2-bis-(o-
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Aminophenoxy)-ethane-N,N,N,N-tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM) at different concentrations and treated with NT for 1 and 5 min, and IGF-1R phosphorylation was evaluated. The data show that inhibition of intracellular calcium had no significant effect on the NT response (Fig. 2B).

NT induces tyrosine phosphorylation of Src tyrosine kinase in prostate cancer PC3 cell lines with a maximal 2-fold increase 60 min after treatment (42). We therefore sought to determine whether NT affects Src phosphorylation in human colonic epithelial cells. NCM460 cells were pretreated with different doses of the intracellular calcium blocker BAPTA/AM and then treated with NT (10^{-8} M) for 1 and 2 min. C, quiescent NCM460 cells were treated with NT (10^{-8} M) for the indicated times. Equal amounts of cell protein were subjected to Western blot analysis using anti-phosphotyrosine Src antibody (phospho-Y-Src) or anti-total Src antibody to ensure equal protein loading. D, NCM460 cells were pretreated with the Src inhibitor PP2 (0.5 or 10 μM) and then treated with NT (10^{-8} M) for 1 or 5 min. For all above treatments, equal amounts of total protein were used to determine IGF-1R phosphorylation as described above. The results are representative of three individual experiments.

**FIGURE 2. NT-induced IGF-1R phosphorylation involves Src activity independent of IGF-1 release and intracellular calcium mobilization.**

A, quiescent NCM460 cells were treated with NT (10^{-8} M) for 1 and 2 min in the absence or presence of IGF-1-neutralizing antibody. B, quiescent NCM460 cells were pretreated with different doses of the intracellular calcium blocker BAPTA/AM and then treated with NT (10^{-8} M) for 1 and 2 min. C, quiescent NCM460 cells were treated with NT (10^{-8} M) for the indicated times. Equal amounts of cell protein were subjected to Western blot analysis using anti-phosphotyrosine Src antibody (phospho-Y-Src) or anti-total Src antibody to ensure equal protein loading. D, NCM460 cells were pretreated with the Src inhibitor PP2 (0.5 or 10 μM) and then treated with NT (10^{-8} M) for 1 or 5 min. For all above experiments, equal amounts of cell protein were used to determine IGF-1R phosphorylation as described above. The results are representative of three individual experiments.

IGF-1R Activation Mediates the NT-induced PI3K/Akt Pathway—Hassan et al. (29) have shown that NT activates the PI3K/Akt pathway in prostate cancer PC3 cells. Because IGF-1R receptor stimulation is a major pathway for Akt activation and of anti-apoptotic effects in response to many stimuli (43), we examined whether NT stimulates Akt activation in human colonic NCM460 cells and determined whether Akt activation in response to NT involves IGF-1R activity.

NCM460 cells were treated with NT for various time intervals, and Akt phosphorylation was determined by Western blotting. The results show that NT significantly stimulated Akt phosphorylation in NCM460 cells, with a maximal activation level 5 min after NT treatment (Fig. 3A). Moreover, Akt phosphorylation is largely dependent on PI3K activity because the specific PI3K inhibitor LY 294002 blocked NT-induced Akt activation (Fig. 3A). Next, we examined whether NT-induced Akt activation is mediated through IGF-1R. For this purpose, cells were pretreated with different concentrations of the specific IGF-1R inhibitor AG1024 and stimulated with NT for 5 min. As shown in Fig. 3B, AG1024 completely inhibited NT-induced Akt phosphorylation at a concentration of as low as 0.2 μM (Fig. 3B). Because our data above show that NT-induced IGF-1R tyrosine phosphorylation is mediated by Src activation, we further examined the effect of the Src inhibitor PP2 on NT-induced Akt activation. We found that pretreatment with the Src inhibitor PP2 (10 μM), but not its inactive analog PP3 (10 μM), blocked NT-induced Akt activation (Fig. 3C). Together, our results indicate that NT activates Src, which subsequently triggers the IGF-1R-mediated PI3K/Akt pathway.

**FIGURE 3. Src-dependent IGF-1R phosphorylation mediates NT-induced Akt activation.** A, quiescent NCM460 cells were pretreated with the PI3K inhibitor LY 294002 at the indicated concentrations and then treated with NT (10^{-8} M) for 5 or 15 min. NS, non-specific. B, quiescent NCM460 cells were pretreated for 30 min with the IGF-1R inhibitor AG1024 at the indicated concentrations and then treated with NT (10^{-8} M) for 5 min. C, quiescent NCM460 cells were pretreated for 30 min with the Src inhibitor PP2 (10 μM) or its negative control PP3 (10 μM) and then treated with NT (10^{-8} M) for 2 or 5 min. For all above experiments, equal amounts of cell protein were used to determine Akt phosphorylation as described above. The results are representative of three individual experiments.

**FIGURE 4. IGF-1R and Akt are involved in NT-dependent anti-apoptotic effects.** A, quiescent NCM460 cells were treated with NT (10^{-8} M) for various time intervals and then treated with NT (10^{-8} M) for 5 min. B, quiescent NCM460 cells were pretreated with different concentrations of the PI3K inhibitor LY 294002 or the IGF-1R inhibitor AG1024 and then treated with NT (10^{-8} M) for 5 min. C, quiescent NCM460 cells were pretreated with different concentrations of the Src inhibitor PP2 or its negative control PP3 and then treated with NT (10^{-8} M) for 2 or 5 min. For all above experiments, equal amounts of cell protein were used to determine Akt phosphorylation as described above. The results are representative of three individual experiments.
mixed siRNA oligonucleotides that target both isoforms or a control siRNA. To confirm the efficiency of IGF-1R siRNA knockdown, 293T cells were transiently transfected with the IGF-1R siRNA mixture or control siRNA by Effectene transfection reagent, and total cell lysates were prepared 72 h after transfection and used for Western blotting using anti-IGF-1F antibody against both IGF-1R isoforms or control anti-actin antibody. IGF-1R siRNA almost completely knocked down the endogenous IGF-1R protein expression, whereas the control siRNA had little effect (Fig. 4B). We then examined the effect of the IGF-1R siRNA on NT-stimulated IL-8 gene transcription. NCM460-NTR1 cells were transiently transfected with IGF-1R siRNA or control siRNA along with an IL-8 promoter construct and an internal control plasmid, followed by incubation with NT. Compared with the control siRNA, IGF-1R-targeted siRNA significantly attenuated NT-induced IL-8 promoter activity (Fig. 4C). Next, we examined the effect of these IGF-1R siRNAs on NF-κB-driven gene expression. Cells were transiently transfected with a control siRNA or the IGF-1R siRNAs along with the NF-κB promoter construct and an internal control plasmid before 4 h of incubation with NT. Our results demonstrate that inhibition of IGF-1R expression significantly reduced NT-induced NF-κB-dependent reporter gene expression (Fig. 4D).

Inhibition of the PI3K/Akt Pathway Attenuates NT-induced IL-8 Gene Expression and NF-κB Activation—Because the PI3K/Akt pathway is one of the major downstream events after IGF-1R stimulation, we sought to determine whether inhibition of the PI3K/Akt pathway could have a similar effect as IGF-1R blockage on NT-induced IL-8 gene expression. Cells were pretreated with the PI3K inhibitor LY 294002 and treated with NT for 4 h, and IL-8 secretion in the conditioned medium was measured. The data show that inhibition of PI3K by LY 294002 significantly reduced NT-induced IL-8 secretion (Fig. 5A). To further determine the role of this pathway in the pro-inflammatory effect of NT, we used mixed siRNA oligonucleotides that target ubiquitously expressed Akt1 and Akt2 isoforms or a control siRNA. To confirm the efficiency of Akt1/2 siRNA knockdown, 293T cells were transiently transfected with the Akt1/2 siRNA or control siRNA by Effectene transfection reagent, and total cell lysates were prepared 72 h after transfection and used for Western blotting using anti-Akt antibody against both Akt1/2 isoforms or control anti-actin antibody. The data show that the Akt1/2 siRNA almost completely knocked down the endogenous Akt protein expression, whereas the control siRNA had little effect (Fig. 5B). To examine the effect of the Akt1/2 siRNA on NT-stimulated IL-8 gene transcription, NCM460-NTR1 cells were transiently transfected with Akt1/2 siRNA or control siRNA along with an IL-8 promoter construct and an internal control plasmid, followed by incubation with NT. As shown in Fig. 5C, Akt-targeted siRNA significantly inhibited NT-induced IL-8 promoter activity compared with the control siRNA. Next, we examined the effect of these Akt siRNAs on the NF-κB-driven gene expression. Cells were transiently transfected with a control siRNA or siRNA targeting two isoforms of Akt (Akt1 and Akt2) along with a NF-κB promoter con-
Inhibition of the IGF-1R/PI3K/Akt Pathway Attenuates NT-induced Colonic Epithelial Cell Growth—One of the major cellular outcomes of IGF-1R-induced activation of the PI3K/Akt pathway is increased cell proliferation and survival. To determine the pathway involved in NT-mediated colonic epithelial cell growth and survival, NCM460 cells were pretreated with the IGF-1R inhibitor AG1024 (0.2 μM), the PI3K inhibitor LY 294002 (20 μM), or a vehicle control EGF (20 ng/ml) for 24 h. Cell proliferation was measured by MTS assay. #, p < 0.01 versus vehicle-treated, non-stimulated cells; *, p < 0.01 versus NT-treated cells; **, p < 0.01 versus NT-treated cells. The results are representative of three individual experiments.

DISCUSSION

We report here for the first time that the neuropeptide NT transactivates IGF-1R in colonic epithelial cells by a mechanism that involves tyrosine phosphorylation of Src tyrosine kinase. We also present evidence that NT-associated phosphorylation of IGF-1R involves the high-affinity NT receptor NTR1 and is linked to Akt phosphorylation, cell proliferation, and NF-κB-driven IL-8 transcription. Because previous results from our laboratory and others have demonstrated an important role for NT and NTR1 in the pathophysiology of acute ileitis (17), acute colitis (44), and mucosal healing following colitis (18), IGF-1R transactivation in response to NT/NTR1 coupling may play an important role in the pathophysiology of intestinal inflammation.

We have previously reported a dramatic increase in NTR1 expression on various intestinal cell types, including colonic epithelial cells, within toxin A-associated enteritis and showed that antagonism of NTR1 significantly inhibits colonic inflammation and damage (17). Consistent with this response, NT stimulates NF-κB-dependent IL-8 production in NTR1-expressing human colonic epithelial cells (19, 25), supporting an important pro-inflammatory role for NT-NTR1 interactions during the course of colitis. However, NT-NTR1 interactions also play a crucial role in the healing process during intestinal inflammation. In this regard, Brun et al. (18) showed that NT and NTR1 expression is also dramatically up-regulated in mucosal epithelial cells after dextran
sulfate sodium-induced colitis and that antagonism of NTR1 aggravates dextran sulfate sodium-induced tissue damage, whereas a continuous NT infusion ameliorates colitis. This protective effect of NT was supported by studies of ours and others in which NT was shown to stimulate the EGFR-mediated MAPK pathway in human colonic epithelial cells (28) and in androgen-independent PC3 cells (29) or PKC-mediated MAPK activation in human pancreatic carcinoma PANC-1 cells (45). Although NT is able to transactivate EGFR in non-transformed colonic epithelial NCM460 cells, the levels of its activation are modest and transient, and NT-induced MAPK activation is only partially mediated by EGFR transactivation (28), suggesting that other receptor tyrosine kinases, such as IGF-1R, could also be transactivated by NT in human colonocytes. Our present results showing that NT induces a profound IGF-1R transactivation in parental and NTR1-transfected NCM460 cells clearly support this notion. This finding indicates that IGF-1R transactivation by NT could play an important role in pathological conditions associated with either low or high levels of NTR expression. Our data showing that inhibition of IGF-1R activation decreases NT-induced IL-8 expression as well as human colonic epithelial cell proliferation in colonocytes are also consistent with a dual role of NT in the pathophysiology of intestinal inflammation.

The possible involvement of IGF-1R in the pathogenesis of inflammatory bowel disease has been suggested for some time, but direct evidence is lacking because of the fact that IGF-1R homologous knockout mice die at birth of respiratory failure (46). Emerging evidence indicates that administration of IGF-1 partially reduces colonic damage induced by oral dextran sulfate sodium (47). In addition, the level of IGF-1R protein was shown to be significantly up-regulated in the colons of patients with ulcerative colitis (48). However, serum IGF-1 levels appear to be down-regulated in both ulcerative colitis and Crohn disease (49, 50), as well as in the 2,4,6-trinitrobenzenesulfonic acid (TNBS) models of colitis (51). It was shown recently that IGF-1 mRNA and colonic protein levels are highly increased only after 35 days of TNBS treatment, and administration of either anti-IGF-1 or anti-IGF-1R antibody inhibits TNBS-induced colonic fibrosis (52). The opposite expression patterns of IGF-1 and IGF-1R during intestinal inflammation suggest that an IGF-1R-initiated signaling event in vivo might not necessarily depend on its extracellular ligand, IGF-1, IGF-2, or insulin. Instead, intracellular signaling-mediated IGF-1R transactivation by other cell-surface receptors such as GPCRs or other receptor tyrosine kinases may also mediate the protective effect of IGF-1R in inflammatory responses. Recent studies have indicated that, in addition to IGF-1 and insulin, IGF-1R can also be transactivated by several GPCRs, including thrombin (30, 53), endothelin-1 and bombesin (54), and angiotensin II (31). Similar to NTR1 (17, 18), other GPCRs, including the receptors for thrombin (55, 56) and bombesin (57), are also shown to play dual roles in intestinal inflammation. Interestingly, these GPCRs also transactivate the EGFRs that stimulate MAPK activation, leading to cell proliferation (28, 58). Although the receptor tyrosine kinases, including EGFR and IGF-1R, primarily initiate both mitogenic and anti-apoptotic effects in response to different extracellular stimuli, the intracellular signaling activated by the receptor such as Akt could also influence the key pro-inflammatory and anti-apoptotic mediator NF-κB.

Our data further show that IGF-1R-mediated Akt activation indeed modulates NT-stimulated IkBa phosphorylation and NF-κB activation, as inhibition of the PI3K/Akt pathway by its specific inhibitor LY 294002 or Akt1/2 siRNA significantly decreased NT-induced IkBa phosphorylation and NF-κB-dependent gene expression, respectively. This is consistent with our previous study showing that other pathways, particularly protein kinase C, are also involved in the NT-induced IkBa/NF-κB cascade (59). Dual phosphorylation of the NF-κB inhibitory protein IkBa by its upstream kinase IKKa/β plays a key role in its degradation and the subsequent NF-κB nuclear translocation and targeting of gene transcription. The role of Akt in IKK-mediated IkBa phosphorylation and degradation has been shown in several culture systems, including TNFα (36, 37), IL-1β (38), and the G-protein-coupled B2-type bradykinin receptor (39). However, this Akt-mediated NF-κB activation appears to depend on different cell types and experimental conditions. For example, inhibition of the PI3K/Akt pathway has no effect on TNFα- or IL-1β-induced NF-κB activation in endothelial cells (60).

In addition to the major role of the canonical NF-κB pathway activated by the upstream kinases, including Akt (shown here) and protein kinase C (59), in NT-stimulated IL-8 gene transcription, our previous studies also showed that inhibition of MAPK activation partially inhibits NT-induced IL-8 expression (19). The involvement of MAPK in the IL-8 response is mediated through increasing induction of the two AP-1 subunits c-jun and c-Fos, thereby the transcriptional activity of AP-1. Consistent with this, our previous results showed that mutation of the proximal AP-1 site in the IL-8 promoter significantly reduces NT-induced IL-8 promoter activity (19). In contrast to Akt, inhibition of MAPK has no effect on NT-induced IkBa phosphorylation, NF-κB-dependent gene transcription, and Akt phosphorylation (data not shown). These results indicate that the MAPK and PI3K/Akt pathways regulate the pro-inflammatory response of the neuropeptide through two different signaling mechanisms.

In summary, we have demonstrated a novel pathway for NT-mediated cell proliferation and inflammatory mediator IL-8 expression in which NT activates its high-affinity receptor NTR1, leading to activation of a signaling cascade from Src to IGF-1R to PI3K/Akt and to IkBa and NF-κB nuclear translocation and gene transcription. The ability of neuropeptides such as NT to activate signaling pathways such as IGF-1R involved in both colitis and healing identifies this peptide as a target for future therapeutic approaches in intestinal inflammatory processes, in particular, inflammatory bowel disease.

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