Curcumin Inhibits Neurotensin-Mediated Interleukin-8 Production and Migration of HCT116 Human Colon Cancer Cells

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Abstract

Purpose: Neurotensin, a gut tridecapeptide, acts as a potent cellular mitogen for various colorectal and pancreatic cancers that possess high-affinity neurotensin receptors. Cytokine/chemokine proteins are increasingly recognized as important local factors that play a role in the metastasis and invasion of multiple cancers. The purpose of this study was to (*a*) determine the effect of neurotensin on cytokine/chemokine gene expression and cell migration in human cancer cells and (*b*) assess the effect of curcumin, a natural dietary product, on neurotensin-mediated processes.

Experimental Design: The human colorectal cancer, HCT116, was treated with neurotensin, with or without curcumin, and interleukin (IL)-8 expression and protein secretion was measured. Signaling pathways, which contribute to the effects of neurotensin, were assessed. Finally, the effect of curcumin on neurotensin-mediated HCT116 cell migration was analyzed.

Results: We show that neurotensin, acting through the native high-affinity neurotensin receptor, induced IL-8 expression in human colorectal cancer cells in a time- and dose-dependent fashion. This stimulation involves Ca^{2+}-dependent protein kinase C, extracellular signal-regulated kinase–dependent activator protein-1, and extracellular signal-regulated kinase–independent nuclear factor-κB pathways. Curcumin inhibited neurotensin-mediated activator protein-1 and nuclear factor-κB activation and Ca^{2+} mobilization. Moreover, curcumin blocked neurotensin-stimulated IL-8 gene induction and protein secretion and, at a low concentration (i.e., 10 μmol/L), blocked neurotensin-stimulated colon cancer cell migration.

Conclusions: Neurotensin-mediated induction of tumor cell IL-8 expression and secretion may contribute to the procarcinogenic effects of neurotensin on gastrointestinal cancers. Furthermore, a potential mechanism for the chemopreventive and chemotherapeutic effects of curcumin on colon cancers may be through the inhibition of gastrointestinal hormone (e.g., neurotensin)–induced chemokine expression and cell migration.

The endocrine control of cancer growth is well established predominantly in breast and prostate cancers, where hormonal or antihormonal therapy represents a mainstay of treatment (1). In a manner analogous to breast and prostate cancers, certain gastrointestinal (GI) and pancreatic cancers possess native receptors for GI hormones; growth of these receptor-positive cancers can be altered by the specific peptide analogue or the receptor antagonist (2, 3). GI hormones, which are released from specialized endocrine cells in the gut and pancreas by ingestion of nutrients, can affect tumor growth in an endocrine, paracrine, and/or autocrine fashion (2, 3).

Our laboratory is focused on the mechanisms and downstream effector proteins that regulate proliferation of colorectal and pancreatic cancers by the GI hormone neurotensin. Neurotensin, a tridecapeptide predominantly found in the distal small bowel (4), is potently released by ingestion of fats (5). Physiologic functions of neurotensin include stimulation of GI motility and secretion (4) and stimulation of normal intestinal cell growth (2, 6). In addition, neurotensin is known to stimulate proliferation of human colorectal and pancreatic cancers that possess high-affinity neurotensin receptors (NTR; refs. 7, 8). The high-affinity NTR (designated NTR1), a member of the G-protein-coupled receptor family, is present in most human pancreatic and colorectal cancers (7, 9, 10), suggesting that neurotensin may act in an endocrine fashion to affect tumor growth. Acting through the NTR1, neurotensin is known to stimulate various signal transduction pathways, including intracellular calcium ($[Ca^{2+}]_i$), mitogen-activated protein kinase, extracellular signal-regulated kinase (ERK), and c-Jun NH_2-terminal kinase and various protein kinase C (PKC) isoforms (9, 11, 12). Ultimately, this stimulation results in the activation of various transcription factors, which can alter the expression of several tumor-promoting genes (9, 12–14).

A plethora of growth factors, secreted either locally or by the cancer cells, may affect various aspects of tumor progression.

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Increasingly, an important role for cytokines/chemokines in tumor growth and metastasis is being recognized (15). Chemokines and their receptors have recently been shown to act at all stages of tumor development and progression, including the neoplastic transformation of cells, promotion of aberrant angiogenesis, and clonal expansion and growth (16). In particular, interleukin (IL)-8 (or CXCL8), an inflammatory component originally identified as a chemotactic factor for leukocytes (17), has been shown to affect cancer progression through mitogenic, angiogenic, and motogenic effects (18). The expression of IL-8 and its receptors has been noted in a variety of human cancers, including colorectal and pancreatic cancers (19). In fact, some studies suggest that IL-8 may act as an autocrine and/or paracrine growth factor in human colorectal and pancreatic cancers (20) and that expression correlates with the aggressiveness and metastatic potential of these cancers (21). It has been reported that neurotensin induces the expression of macrophage inflammatory protein-2, monocyte chemoattractant protein-1, IL-1β, and tumor necrosis factor-α in murine microglial cells and stimulates IL-8 secretion in a nontransformed colon epithelial cell line stably transfected with the NTR (13); however, to our knowledge, the effect of neurotensin on cytokine/chemokine gene expression has not been analyzed in human cancer cells with native NTR.

Curcumin, a naturally occurring polyphenolic pigment isolated from the rhizomes of the plant *Curcuma longa*, is commonly used as a coloring and flavoring agent in food products. Current studies are assessing the role of curcumin as a chemopreventive and/or chemotherapeutic agent for certain cancers (22). Curcumin has been shown to improve cholecystokinin-induced pancreatitis and inhibit cholecystokinin-induced IL-8, tumor necrosis factor-α, and chemokine KC expression in a rat pancreatitis model (23); the effect of curcumin on cytokine/chemokine gene regulation by neurotensin and other GI hormones has not been investigated in GI cancer cells. In this study, we tested the effects of the GI hormone neurotensin, which is released by dietary fat, and curcumin, a natural product of dietary origin, on cytokine/chemokine gene regulation in human colon cancer cells. Importantly, we found that neurotensin selectively stimulated IL-8 gene expression and protein secretion in human colon cancer cells with native high-affinity NTR; curcumin inhibited neurotensin-induced IL-8 production and migration of HCT116 human colon cancer cells.

Materials and Methods

Materials. Neurotensin and curcumin were purchased from Sigma Chemical Co. (St. Louis, MO). A23187 and ionomycin were from Alexis Biochemicals (San Diego, CA). [α-^{32}P]UTP (3,000 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NY). U0126 was from Promega (Madison, WI). Adenovirus vector encoding hemagglutinin-tagged IκB-α superrepressor (Ad5IκB-AA) and its control vector (Ad5GFP) were gifts from Dr. Christian Jobin (University of North Carolina, Chapel Hill, NC). The NTR antagonists, SR48692 and SR142948A, were generous gifts from Dr. Danielle Gully (Sanofi Recherche, Toulouse, France). All other reagents were from Calbiochem (San Diego, CA).

Cell culture. Human colon cancer cell line (HCT116 and HT29) and the human pancreatic cancer cell line (MIA PaCa-2) were from American Type Culture Collection (Manassas, VA). MIA PaCa-2 cells were incubated in DMEM with 10% fetal bovine serum (FBS); HCT116 and HT29 were maintained in McCoy's 5A medium supplemented with 10% FBS. The human colon cancer cell line KM20 was obtained from Dr. Isaiah Fidler (M.D. Anderson Cancer Center, Houston, TX) and grown in MEM supplemented with 1% sodium pyruvate and 10% FBS.

RNA isolation and RNase protection assay. RNA was isolated from cultured cells using Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX) according to the manufacturer's protocol. A [α-^{32}P]UTP-labeled antisense RNA probe was prepared using the hCK-5 multiprobe template set (BD PharMingen, San Diego, CA) and MAXI-Script SP6/T7 *in vitro* transcription kit (Ambion, Austin, TX). RNase protection assays (RPA) were done using the RPA III RNase Protection kit (Ambion) according to the manufacturer's recommendations and as we have described previously (24). Finally, samples were analyzed by electrophoresis on 5% denaturing polyacrylamide gel and detected by autoradiography.

IL-8 measurement. The concentration of IL-8 from conditioned medium was determined using a human IL-8 ELISA kit (Pierce Biotechnology, Rockford, IL). Results were expressed as mean ± SD (pg/mL). At least three independent experiments were done for each experimental condition, each with triplicate measurements.

Ca^{2+} ratio imaging. Real-time recording of $[Ca^{2+}]_i$ was done in single cells as we have described previously (9). In brief, cells grown on glass coverslips (Carolina Biological, Burlington, NC) were washed with a physiologic medium (KRH) and then loaded with 2 μmol/L fura-2-AM for 50 minutes at 25°C to minimize dye compartmentalization. Loaded cells were washed thrice with KRH and incubated for 60 minutes at 25°C in the dark with KRH with 0.1% bovine serum albumin. Loaded cells attached to coverslips were mounted on a Leiden coverslip dish and placed in an Open Perfusion Microincubator (Medical Systems Corp., Greenvale, NY) covered with 3 mL KRH with 0.1% bovine serum albumin. The Ca^{2+} variations at the single-cell level were monitored using a Nikon Diaphot inverted microscope (Nikon, Garden City, NY), equipped with a Nikon ×40 (1.3 numerical aperture) oil immersion objective, coupled to a dual monochrometer system via a fiber optic cable (Photon Technology International, South Brunswick, NJ). Fura-2-AM intracellular fluorescence was measured at an emission wavelength of 510 nm by alternating the excitation wavelength between 340 and 380 nm. Full ratio images were obtained at 1 image per 1.5 seconds. Images were processed using ImageMaster software (Photon Technology International).

Preparation of nuclear extracts and electrophoretic mobility shift assays. The nuclear extracts were prepared from HCT116 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) according to the manufacturer's protocol. Electrophoretic mobility shift assays (EMSA) were done as described previously (25) with minor modifications. Nuclear extracts (10 μg) were incubated with a ^{32}P-labeled oligonucleotide (4×10^4 counts/min) containing consensus activator protein-1 (AP-1)–binding site or nuclear factor-κB (NF-κB)–binding site (Promega) and 2 μg poly(deoxyadenylate-deoxythymidylic acid) in a buffer containing 10% glycerol, 100 mmol/L KCl, 5 mmol/L MgCl, 12.5 mmol/L HEPES (pH 7.9), 1 mmol/L EDTA, and 1 mmol/L DTT in a final volume of 20 μL for 15 minutes at room temperature. For supershift studies, 2 μL antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture 1 hour before the addition of labeled probe. The reaction mixture was fractionated on 6% nondenaturing polyacrylamide gels.

Cell migration assay. The Costar Transwell System (8-μm pore size polycarbonate membrane, 6.5-mm diameter, Corning, Inc., Corning, NY) was used to evaluate cell migration. Both sides of each Transwell membrane were coated with 15 μg/mL collagen (Cohesion Technology, Palo Alto, CA) by immersion for 30 minutes at 37°C. Cells (50,000 in 100 μL serum-free medium) were added to the upper well, and 500 μL serum-free medium was added to the lower chamber. Neurotensin, curcumin, and vehicle were added to the lower chamber. At the end of the 16-hour incubation at 37°C, 5% CO_2, cells on the top of the membrane were removed by swiping with a damp cotton swab, and cells that had migrated to the lower surface were fixed in methanol for

15 minutes at room temperature and stained with 1% crystal violet. The migration activity was quantified by counting the migrated cells on the lower surface of the membrane of at least seven fields per chamber using a ×10 objective.

Statistical analysis. Data that were from experiments with only two treatment groups (e.g., neurotensin only and the control) were analyzed using the two-sample *t* test. Due to heterogeneous variability among treatment groups, data from experiments with more than two treatments were transformed using logarithm to the base 10 for the data analysis purpose. Then, the logarithm transformed data were analyzed using one-way classification ANOVA. All tests were assessed at the 0.05 level of significance. The Fisher's least significant difference procedure was used for multiple comparisons with Bonferroni adjustment for the number of comparisons. All statistical computations were conducted using the SAS system release 9.1.

Results

Neurotensin stimulates IL-8 gene expression and protein secretion in HCT116 colon cancer cells. To determine the potential effect of neurotensin on cytokine/chemokine expression in cancer cells, HCT116 human colon cancer cells, which possess the high-affinity NTR (NTR1), were treated with neurotensin (100 nmol/L) over a time course. RNA was extracted for RPA using a multiprobe (hCK-5) containing cDNAs for several cytokine/chemokine genes (Ltn, RANTES, IP-10, macrophage inflammatory protein-1β, macrophage inflammatory protein-1α, monocyte chemoattractant protein-1, IL-8, and I-309); the constitutively expressed genes, L32 and glyceraldehyde-3-phosphate dehydrogenase, are included as controls for RNA loading equality (Fig. 1). Neurotensin increased IL-8 gene expression by 1 hour with maximal stimulation occurring at 2 hours after treatment; IL-8 expression returned to control levels by 24 hours (Fig. 1A, *left*). We next determined the range of concentrations of neurotensin, which affect IL-8 expression at 2 hours after treatment. Induction of IL-8 was noted with 12.5 nmol/L neurotensin; maximal induction occurred at dosages of 25 to 200 nmol/L (Fig. 1B, *right*). The induction of IL-8 mRNA expression was

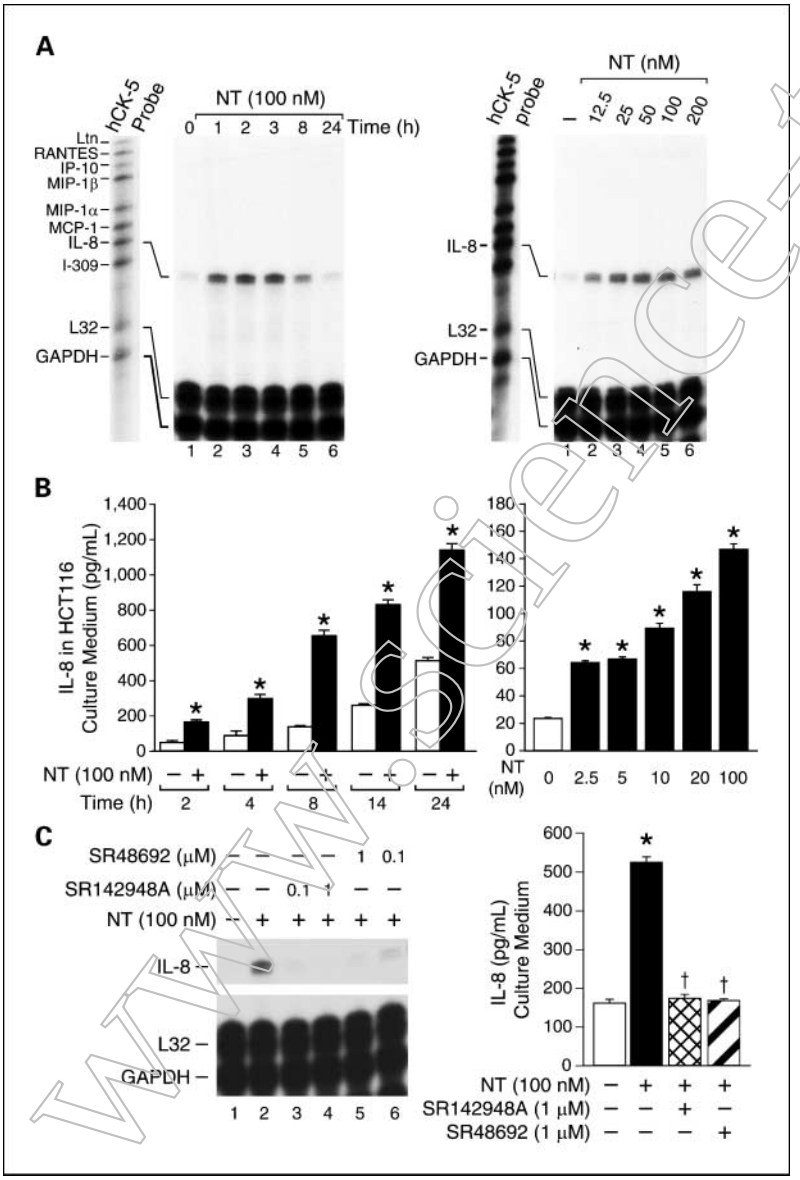

Fig. 1. Neurotensin stimulates IL-8 mRNA and protein in HCT116. *A,* HCT116 human colon cancer cells were incubated in McCoy's 5A medium without FBS for 24 hours and then treated with neurotensin (*NT*; 100 nmol/L) or vehicle (PBS) over a time course; total RNA was isolated and analyzed by RPA using a ^{32}P-labeled multiprobe template set (hCK-5; *left*). HCT116 cells were treated with various concentrations of neurotensin or vehicle for 2 hours; RNA was isolated and analyzed by RPA using the hCK-5 multiprobe (*right*). *B,* HCT116 cells were seeded in plates with McCoy's 5A medium and FBS. One day later, medium was changed to McCoy's 5A medium without FBS for 24 hours and then treated with neurotensin (100 nmol/L) or vehicle for the indicated time points (*left*) or various concentrations of neurotensin for 8 hours (*right*). *, $P < 0.05$ versus control at each time point. IL-8 protein (pg/mL) released from HCT116 cells into the culture medium was assessed by ELISA. *, $P < 0.05$ versus control. *C,* HCT116 cells were pretreated with the NTR inhibitors SR48692 or SR142948A at the indicated concentrations for 45 minutes and then treated with neurotensin (100 nmol/L) for 2 hours; RNA was isolated and analyzed by RPA using the multiprobe hCK-5 (*left*). HCT116 cells were seeded in plates using McCoy's 5A medium with FBS. One day later, the medium was changed to McCoy's 5A medium without FBS for 24 hours, pretreated with of SR48692 (1 μmol/L) or SR142948A (1 μmol/L) for 45 minutes, and then treated with neurotensin (100 nmol/L) for 8 hours; IL-8 secretion was measured in the medium (*right*). Representative of three separate experiments. Columns, mean; bars, SD. *, $P < 0.05$ versus control; †, $P < 0.05$ versus neurotensin only.

also noted in the colon cancer cells HT29 and KM20 and the pancreatic cancer cell line MIA PaCa-2, all of which possess the high-affinity NTR, thus indicating that neurotensin-mediated IL-8 induction is not limited to HCT116 cells (data not shown).

IL-8 functions as a paracrine and/or autocrine growth factor in several cancer cells; the secretion of IL-8 protein from cancer cells is a key step for these effects (20). To determine whether the neurotensin-stimulated induction of IL-8 mRNA expression was accompanied by an increase in secretion of IL-8 protein into the culture medium, IL-8 protein levels were measured by ELISA after neurotensin treatment. As shown in Fig. 1B (*left*), neurotensin (100 nmol/L) significantly increased IL-8 protein levels in HCT116 cell medium over a time course with maximal increases noted at 8 hours after treatment compared with vehicle treatment (control). Next, HCT116 cells were treated with various concentrations of neurotensin and IL-8 protein levels were quantitated in the medium. A concentration of 2.5 nmol/L produced a 2-fold increase in IL-8 secretion; a concentration of 100 nmol/L resulted in a 6-fold increase in IL-8 protein secretion into the medium (Fig. 1B, *right*).

To determine the specific effect of neurotensin, we tested whether the effects of neurotensin on IL-8 expression are mediated through the native high-affinity NTR. HCT116 cells were pretreated with SR48692, a selective nonpeptide NTR1 antagonist that binds with high affinity to NTR1 and has been proven to be extremely useful for delineating the functions of NTR1 (26), or SR142948A, another selective nonpeptide NTR1 antagonist, and then treated with neurotensin (100 nmol/L). Both NTR antagonists blocked neurotensin-mediated induction of IL-8 mRNA (Fig. 1C, *left*). Furthermore, both SR48692 and SR142948A blocked neurotensin-mediated stimulation of IL-8 protein secretion into the culture medium (Fig. 1C, *right*). These findings suggest that the cellular effects of neurotensin on IL-8 induction are mediated through the native high-affinity NTR.

Curcumin inhibits IL-8 stimulation by neurotensin. Curcumin, a therapeutic phytochemical of dietary and medicinal origin, has been shown to possess anti-inflammatory and cancer chemotherapy effects. Therefore, we postulated that curcumin may inhibit the effects of neurotensin on IL-8 expression and secretion. We found that the pretreatment of curcumin inhibited neurotensin-induced IL-8 mRNA expression in a dose-dependent fashion (Fig. 2A). In contrast, neither caffeic acid phenethyl ester (CAPE; another naturally occurring product), MG132, PDTC, nor sulfasalazine affected neurotensin-mediated IL-8 gene expression (data not shown). Furthermore, curcumin inhibited neurotensin-induced IL-8 protein secretion in a dose-dependent fashion (Fig. 2B). The inhibitory effects of curcumin on IL-8 protein secretion were more pronounced than for IL-8 mRNA expression, thus suggesting the possibility that additional mechanisms are involved in the inhibition of IL-8 secretion by curcumin.

Effect of neurotensin is dependent on $[Ca^{2+}]_i$ stimulation and PKC activation. Neurotensin can activate the $[Ca^{2+}]_i$ and PKC signaling pathways in cells with native NTR (11, 12); these pathways are likewise implicated in IL-8 gene regulation (27). To better delineate the signaling pathways acting downstream of NTR to induce IL-8 gene expression, we first determined the role of $[Ca^{2+}]_i$ on the effects of neurotensin. Pretreatment of HCT116 cells with the cell-permeable Ca^{2+} chelator, BAPTA/AM (30 μmol/L), completely blocked neurotensin-induced IL-8 expression (Fig. 3A) and protein secretion (Fig. 3B). In addition, treatment with Ca^{2+} ionophores, ionomycin and A23187, which increase $[Ca^{2+}]_i$, significantly enhanced IL-8 gene induction in combination with neurotensin compared with neurotensin treatment alone (Fig. 3C). In addition, either ionomycin or A23187 alone increased IL-8 mRNA expression compared with vehicle treatment.

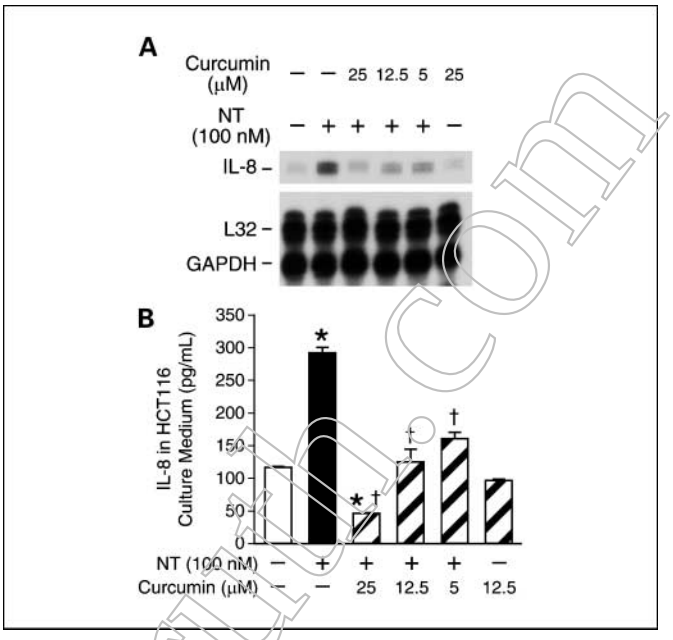

Fig. 2. Curcumin inhibits neurotensin-mediated IL-8 mRNA and secretion. *A,* HCT116 cells were pretreated with curcumin at the indicated concentrations for 25 minutes and then treated with neurotensin (100 nmol/L) for 2 hours. RNA was isolated and analyzed by RPA using the hCK-5 multiprobe. *B,* HCT116 cells were seeded in plates in McCoy's 5A medium with FBS. One day later, the medium was changed to serum free for 24 hours, pretreated with curcumin at the indicated concentrations for 30 minutes, and then treated with neurotensin (100 nmol/L) for 8 hours. IL-8 secretion was measured in the conditioned medium by ELISA. Representative of three separate experiments. Columns, mean; bars, SD. *, $P < 0.05$ versus control; †, $P < 0.05$ versus neurotensin only.

The conventional PKCs, which include α, βI, βII, and γ, are dependent on Ca^{2+} signaling. To examine the PKC isoforms contributing to IL-8 induction by neurotensin, HCT116 cells were pretreated with PKC isoform-selective inhibitors. GFx (which inhibits PKCα, βI, βII, γ, δ, and ζ), Ro-31-8220 (which inhibits PKCα, βI, βII, γ, and ε), and Gö6983 (which inhibits PKCα, β, γ, δ, and ζ) blocked neurotensin-induced IL-8 mRNA expression (Fig. 3D). Similar to the inhibition of IL-8 expression, pretreatment with GFx and Gö6983 completely blocked neurotensin-mediated IL-8 secretion; however, rottlerin, which is considered a relatively selective PKCδ inhibitor, exhibited no inhibitory effects on neurotensin-mediated IL-8 stimulation (data not shown). Collectively, these results suggest that Ca^{2+}-dependent PKC isoforms contribute to IL-8 regulation by neurotensin.

Involvement of NF-κB and the mitogen-activated protein kinase/ERK kinase/AP-1 pathway on neurotensin-mediated IL-8 stimulation. A well-described mechanism contributing to IL-8 gene induction is the activation of NF-κB, which binds the IL-8 promoter to stimulate IL-8. Moreover, neurotensin can increase NF-κB activation in certain cells (13). As shown in

Fig. 4A (*left*), we found that neurotensin (100 nmol/L) increased NF-κB-binding activity; in contrast, neurotensin had no effect on the DNA-binding activity of NFAT, a transcription factor that also binds the proximal IL-8 promoter (data not shown). The proteasome inhibitor MG132 inhibited NF-κB activation, whereas gliotoxin, PDTC, or the mitogen-activated protein kinase/ERK kinase (MEK) inhibitor, U0126, had no effect on neurotensin-induced NF-κB-binding activity, indicating that neurotensin-mediated NF-κB induction is specific and ERK independent. Supershift analysis showed that the upper of the two retarded DNA-protein complexes was supershifted with either p65 or p50 antibodies and the lower band was shifted with the p50 antibody (Fig. 4A, *right*); addition of IgG (control) did not affect neurotensin-mediated NF-κB activation.

To determine whether neurotensin-mediated NF-κB induction is important for IL-8 gene regulation, HCT116 cells were infected with adenovirus encoding the superrepressor of IκBα (IκB-AA) and the adenoviral control vector encoding green fluorescent protein (28). HCT116 cells were then treated with neurotensin (100 nmol/L) or vehicle, and RNA was extracted for RPA. Overexpression of IκB-AA significantly, but not completely, reduced neurotensin-mediated IL-8 mRNA expression compared with the control vector (Fig. 4B, *left*). EMSA analysis confirmed that overexpression of IκB-AA blocked neurotensin-induced NF-κB activity and strongly inhibited basal NF-κB activity compared with the control vector (Fig. 4B, *right*). These data suggest that NF-κB-dependent and NF-κB-independent pathways are involved in the regulation of IL-8 gene expression by neurotensin.

Activation of MEK/ERK by neurotensin and downstream induction of AP-1 transcription factors contributes to the proliferative effects of neurotensin (9, 12). In addition, the MEK/AP-1 pathway has been shown to contribute to IL-8 gene regulation (29). To determine the role of ERK1/2 on neurotensin-mediated IL-8 stimulation, HCT116 cells were pretreated with the MEK inhibitors PD98059 (30 μmol/L) or U0126 (10 μmol/L) and then treated with neurotensin (100 nmol/L). Treatment with both U0126 and PD98059, at concentrations that block ERK activation in HCT116 cells (30), inhibited neurotensin-induced IL-8 mRNA expression (Fig. 4C, *left*) and protein secretion (Fig. 4C, *right*), indicating that the MEK/ERK pathway is involved in IL-8 regulation by neurotensin. It is interesting to note that neither U0126 nor PD98059 completely blocked neurotensin-induced IL-8 mRNA, indicating ERK-dependent and ERK-independent regulation (such as NF-κB) of the IL-8 gene by neurotensin in HCT116 cells. In contrast, neither the p38 inhibitor SB203580 nor the c-Jun NH_2-terminal kinase inhibitor SP600125 affected neurotensin-mediated IL-8 gene induction (data not shown).

The AP-1 family of transcription factors can be activated by the upstream MEK/ERK pathway. c-Fos is a direct substrate of ERK *in vitro* and *in vivo* (31–33). Therefore, we speculated that one mechanism for neurotensin-mediated MEK/ERK pathway regulation of IL-8 gene expression is through AP-1 transcription factor activation. We treated HCT116 cells with neurotensin and determined AP-1 DNA-binding activity by EMSA. Neurotensin treatment for 30 minutes increased AP-1 DNA-binding activity; this increase was prevented by the ERK inhibitor U0126, suggesting that neurotensin-mediated AP-1 activation is regulated by the MEK/ERK pathway (Fig. 4D, *left*). In contrast, gliotoxin had no effect on AP-1-binding activity. Supershift analyses were done to delineate the AP-1 proteins in the binding complex. Preincubation with antibodies against JunB

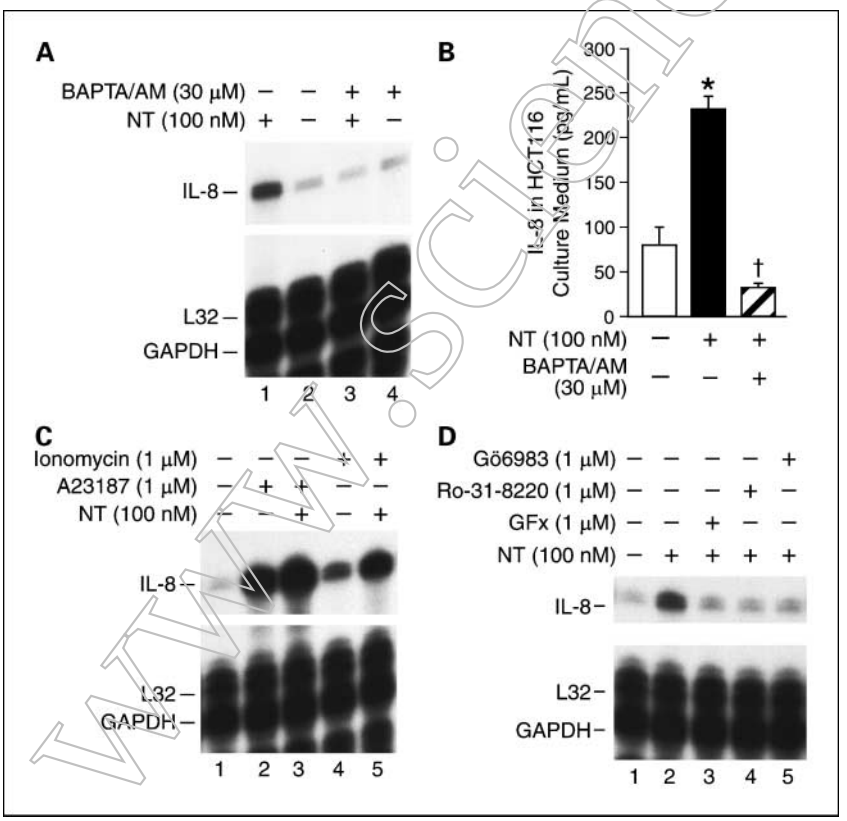

Fig. 3. Effect of neurotensin is dependent on $[Ca^{2+}]_i$ stimulation and PKC activation. *A,* HCT116 cells were pretreated with BAPTA/AM (30 μmol/L) for 20 minutes and then treated with of neurotensin (100 nmol/L) for 2 hours; RNA was extracted and analyzed by RPA using the hCK-5 multiprobe. *B,* HCT116 cells were seeded in plates in McCoy's 5A medium with FBS. Two days later, the medium was changed to serum free for 24 hours, pretreated with BAPTA/AM (30 μmol/L) for 20 minutes, and then treated with neurotensin (100 nmol/L) for 6 hours. The conditioned medium was collected and IL-8 secretion was measured by ELISA. Representative of three separate experiments. Columns, mean; bars, SD. *, $P < 0.05$ versus control; †, $P < 0.05$ versus neurotensin only. *C* and *D,* HCT116 cells were pretreated with 1 μmol/L ionomycin, A23187, Gö6983, Ro-31-8220, or GFx for 20 minutes and then treated with neurotensin (100 nmol/L) for 2 hours. RNA was extracted and analyzed by RPA using the hCK-5 multiprobe.

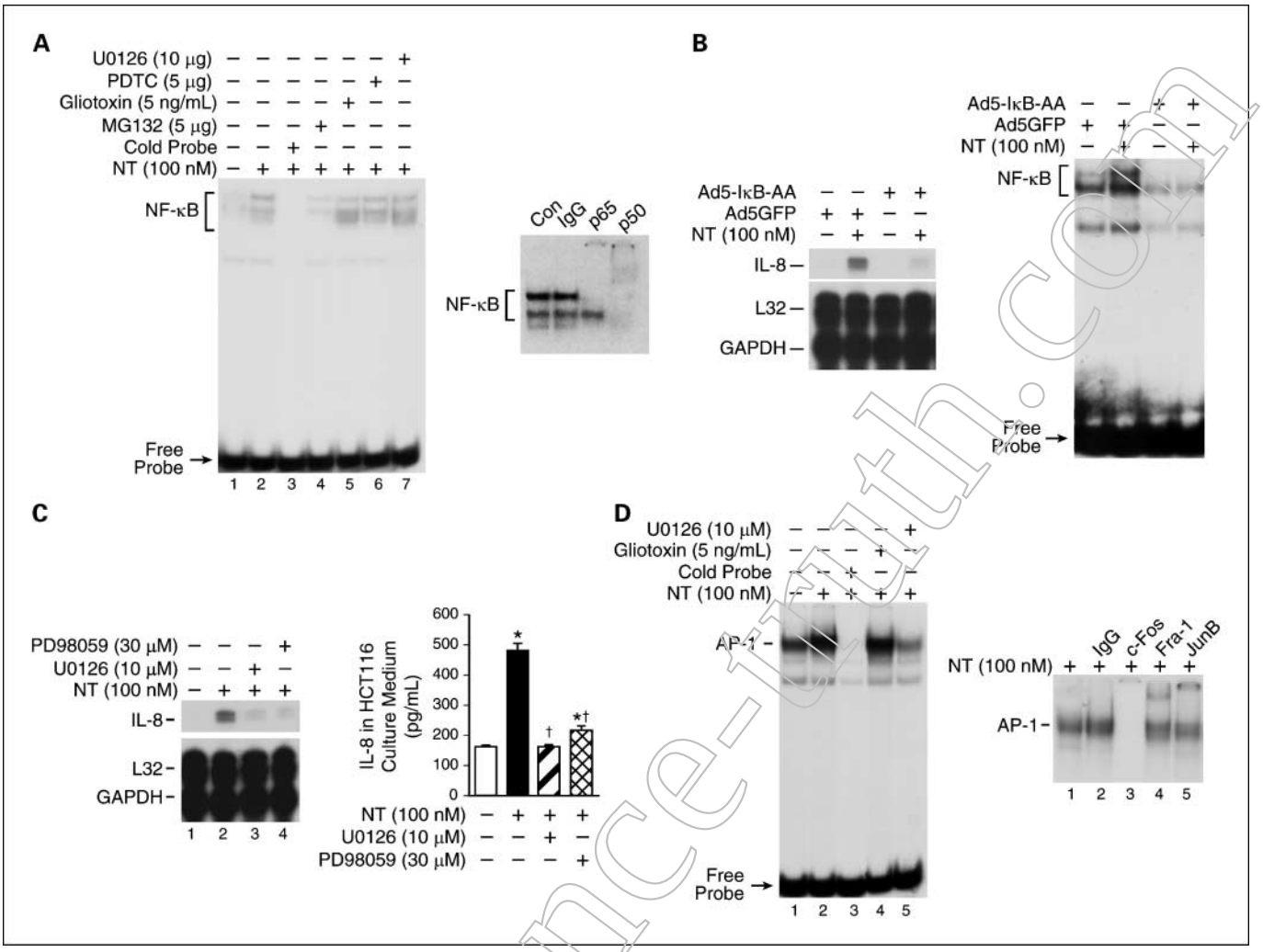

Fig. 4. NF-κB activation and the MEK/AP-1 pathway play a role in IL-8 regulation by neurotensin. *A,* HCT116 cells were pretreated with vehicle or U0126 (10 μmol/L), PDTC (5 μmol/L), gliotoxin (5 ng/mL), or MG132 (5 μmol/L) for 25 minutes and then treated with neurotensin (100 nmol/L) for 30 minutes. Cells were extracted for nuclear protein and analyzed by EMSA using a ^{32}P-labeled NF-κB probe as described in Materials and Methods (*left*). Nuclear protein (10 μg/lane) from HCT116 treated with neurotensin (100 nmol/L) was preincubated with specific antibodies (p65 and p50) or IgG before the addition of ^{32}P-labeled NF-κB probe, and DNA-binding activity was assessed by EMSA (*right*). *B,* HCT116 cells were infected with adenovirus encoding the superrepressor of IκBα (Ad5IκB-AA) or the adenovirus control vector encoding green fluorescent protein (Ad5GFP) for 1 hour, washed, and replated in McCoy's 5A medium without FBS for 24 hours and then treated with neurotensin (100 nmol/L) for 2 hours. RNA was isolated and analyzed by RPA using the hCK-5 multiprobe (*left*). To confirm inhibition of NF-κB activation, cells were extracted for nuclear protein and analyzed by EMSA using a ^{32}P-labeled NF-κB probe as described in Materials and Methods (*right*). *C,* HCT116 cells were pretreated with the MEK/ERK inhibitors PD98059 (30 μmol/L) or U0126 (10 μmol/L) for 20 minutes and then treated with neurotensin (100 nmol/L) for 2 hours; RNA was extracted and analyzed by RPA using the hCK-5 multiprobe (*left*). HCT116 cells were seeded in plates in McCoy's 5A medium with FBS. Twenty-four hours later, medium was changed to serum free and cells were pretreated with PD98059 (30 μmol/L) or U0126 (10 μmol/L) at the indicated concentrations for 25 minutes and then treated with neurotensin (100 nmol/L) for 8 hours. IL-8 secretion was measured in the conditioned medium by ELISA (*right*). Representative of three separate experiments. Columns, mean; bars, SD. *, $P < 0.05$ versus control; †, $P < 0.05$ versus neurotensin only. *D,* HCT116 cells were pretreated with U0126 (1 μmol/L) or gliotoxin (5 ng/mL) for 25 minutes and then treated with neurotensin (100 nmol/L) for 30 minutes. Cells were extracted for nuclear protein and analyzed by EMSA using a ^{32}P-labeled AP-1 probe as described in Materials and Methods (*left*). Nuclear extracts (10 μg/lane) from HCT116 cells were preincubated with specific antibodies (c-Fos, Fra-1, or JunB) before the addition of ^{32}P-labeled AP-1 probe, and DNA-binding activity was assessed by EMSA (*right*).

and Fra-1 resulted in supershifts of the DNA-protein band, whereas c-Fos antibody completely blocked the retarded band, indicating interaction of these proteins with the labeled AP-1 probe; in contrast, antibodies to FosB, c-Jun, and Fra-2 did not result in either a supershifted band or diminution of the AP-1 complex (Fig. 4D, *right*). These results suggest that ERK-dependent AP-1 activation by neurotensin may play a role in neurotensin-mediated IL-8 regulation.

Curcumin inhibits neurotensin-stimulated NF-κB and AP-1 activity and neurotensin-activated Ca^{2+} mobilization. Curcumin inhibits both NF-κB and AP-1 activation in different cells types. We next determined the effect of curcumin on neurotensin-stimulated AP-1 and NF-κB activation. Curcumin, at dosages of 5 and 25 μmol/L, blocked neurotensin-mediated NF-κB induction (Fig. 5A); however, CAPE, which has been reported to suppress NF-κB activation in certain cell lines, had no inhibitory effect on neurotensin-mediated NF-κB induction in HCT116 cells. Neurotensin increases AP-1 DNA-binding activity, which involves c-Fos, JunB, and Fra-1 proteins. Hahm et al. (34) reported that curcumin can inhibit the formation of the Fos-Jun DNA complex, thus suppressing AP-1-regulated gene expression. As shown in Fig. 5B, neurotensin-mediated AP-1 activation was blocked by curcumin, but not CAPE, in HCT116 cells, which suggests that the inhibition of AP-1

activation by curcumin is involved in IL-8 regulation. Taken together, neurotensin-mediated ERK-dependent AP-1 activation and ERK-independent NF-κB activation were blocked by curcumin, but not by CAPE, gliotoxin, MG132, or PDTC, in HCT116 cells. These data may explain the findings that neurotensin-mediated IL-8 expression was inhibited by curcumin but not by other agents.

The inhibitory effects of curcumin on IL-8 protein secretion were more pronounced than noted for IL-8 mRNA expression. We showed that IL-8 protein secretion was more sensitive to Ca^{2+} than IL-8 mRNA induction (Fig. 3A and B). Several studies have shown that curcumin suppresses Ca^{2+} activation by a variety of stimuli (35, 36); therefore, we examined the effects of curcumin on Ca^{2+} signaling changes in HCT116 cells (Fig. 5C and D). Curcumin strongly inhibited neurotensin-induced Ca^{2+} mobilization; a dosage of 10 μmol/L almost completely blocked neurotensin-induced Ca^{2+} activation and 25 μmol/L curcumin decreased basal levels of Ca^{2+} activity. Collectively, our findings indicate that curcumin inhibited neurotensin-induced IL-8 production likely through the inhibition of AP-1 and NF-κB activity as well as neurotensin-induced $[Ca^{2+}]_i$ mobilization.

Curcumin blocks neurotensin-dependent migration of HCT116 cells. IL-8 belongs the CXC chemokine family, and its action is mediated by membrane receptors, CXCR-1 and CXCR-2. Expression of these receptors and an autocrine effect of IL-8 in HCT116 cells was reported by Brew et al. (20). To address whether IL-8 plays a role in HCT116 cell migration, we treated HCT116 cells with recombinant IL-8 and migration assays were done using collagen-coated Costar Transwell membranes. Treatment with IL-8 (2.5 ng/mL) resulted in an ~2-fold increase in HCT116 cell migration, indicating that IL-8 can increase the migration of HCT116 colon cancer cells (Fig. 6A). Our previous results showed that curcumin blocks neurotensin-induced IL-8 expression and secretion (Fig. 2A and B). The inhibition of colon cancer cell growth by curcumin has been well described (37). Therefore, we next determined whether neurotensin stimulates HCT116 cell migration and if curcumin could block this effect. Treatment of HCT116 cells with neurotensin (100 nmol/L) significantly increased HCT116 cell migration (~3-fold) compared with vehicle treatment; pretreatment with curcumin (10 μmol/L) blocked the stimulatory effect of neurotensin on HCT116 cell migration (Fig. 6B). These results suggest that curcumin suppresses neurotensin-induced HCT116 cell migration at least partially through the inhibition of IL-8 expression.

Discussion

Neurotensin, an important intestinal hormone for multiple physiologic functions in the GI tract, stimulates proliferation of normal intestine and NTR-positive colorectal and pancreatic cancers through mechanisms that are not entirely understood (2). In our present study, we show that neurotensin selectively increases the expression and secretion of IL-8, a chemokine increasingly recognized as contributing to increased tumor

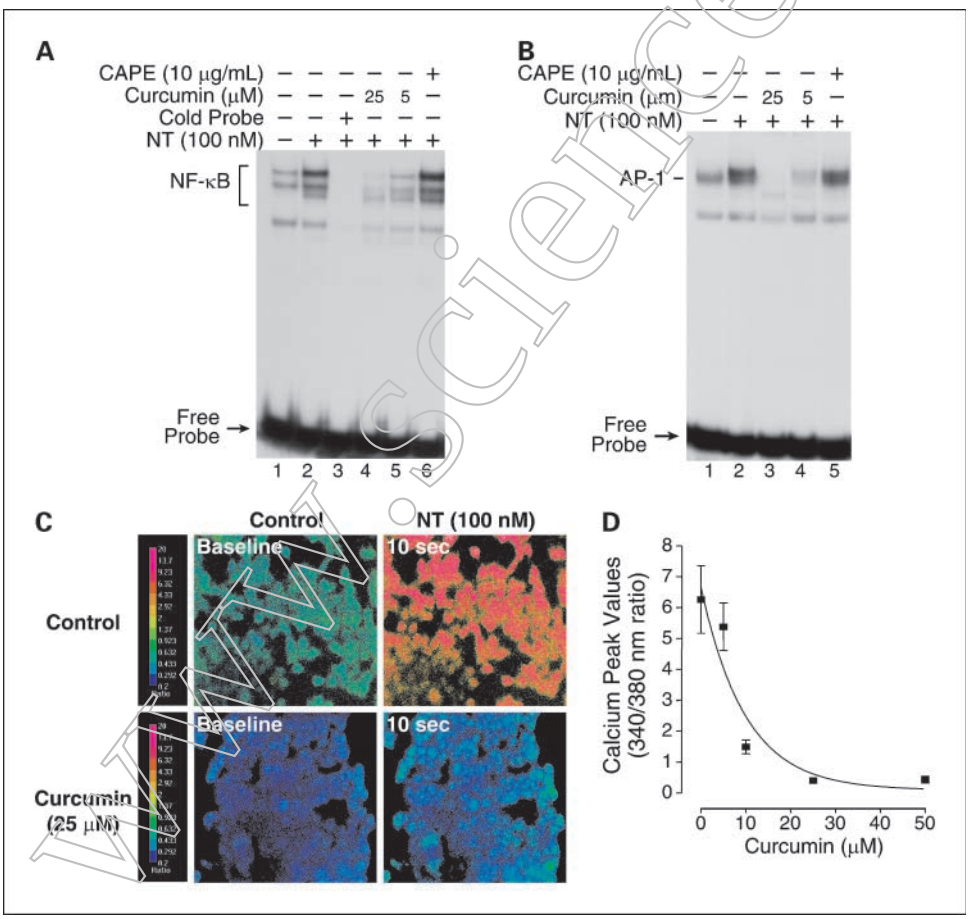

Fig. 5. Curcumin inhibits neurotensin-mediated IL-8 stimulation. *A,* HCT116 cells were pretreated with CAPE (10 μg/mL) or curcumin (either 5 or 25 μmol/L) for 25 minutes and then treated with neurotensin (100 nmol/L) for 30 minutes. Cells were extracted for nuclear protein and analyzed by EMSA using a ^{32}P-labeled NF-κB probe as described in Materials and Methods. *B,* HCT116 cells were pretreated with curcumin (either 5 or 25 μmol/L) or CAPE (10 μg/mL) for 25 minutes and then treated with neurotensin (100 nmol/L) for 30 minutes. Cells were extracted for nuclear protein and analyzed by EMSA using a ^{32}P-labeled AP-1 probe as described in Materials and Methods. *C,* pseudocolor images of HCT116 cells loaded with the Ca^{2+}-sensitive dye, fura-2-AM. The cells were pretreated with vehicle or curcumin (25 mmol/L) for 2 minutes and then treated with neurotensin (100 nmol/L). Red, highest level of $[Ca^{2+}]_i$; blue green, baseline levels. *D,* pretreatment with increasing concentrations of curcumin decreases the peak $[Ca^{2+}]_i$ response to neurotensin (100 nmol/L). Points, average 340/380 nm ratio from 40 individual cells; bars, SD.

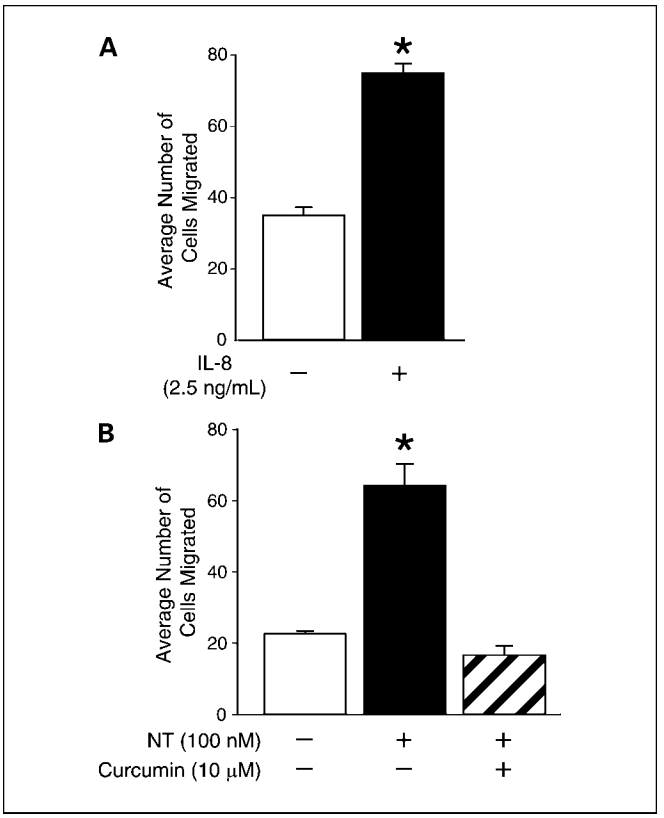

Fig. 6. IL-8 and neurotensin increase HCT116 cell migration. HCT116 cells (5×10^4) were seeded in the upper well of the Costar Transwell System as described in Materials and Methods; vehicle, IL-8 (2.5 ng/mL; *A*), neurotensin (100 nmol/L), or neurotensin + curcumin (10 μmol/L; *B*) was added to the lower well. After 16-hour incubation, the cells on the lower surface of the well were fixed in methanol and stained with 1% crystal violet. The average number of migrated cells ($n = 3$) was quantified by counting under a microscope in at least seven different viewing fields at ×10 magnification. Representative of separate experiments. Columns, mean; bars, SD. *, $P < 0.05$ versus control.

invasion, progression, and angiogenesis (16). These findings identify a potentially important mechanism contributing to enhanced tumor growth by neurotensin through the stimulation of multiple signaling pathways leading ultimately to the downstream regulation of tumor-secreted proteins, such as IL-8. In addition, we find that the natural product curcumin, which has been evaluated as an anti-inflammatory and antitumor agent, blocked neurotensin-mediated IL-8 gene induction, protein secretion, and cell migration.

The predominant proliferative effects of neurotensin seem to be mediated through the high-affinity NTR1, which is present in several colorectal, pancreatic, and prostate cancers. (38). We have used two nonpeptide NTR antagonists to further show that the effects of neurotensin are acting directly through the NTR1. Consistent with our findings, Zhao et al. (13) showed increased IL-8 promoter activity and protein secretion by neurotensin in nontransformed human colonocytes stably transfected with NTR1. Therefore, our study using cancer cells with native NTR1 and the previous studies using an artificially derived cell line (13) show that neurotensin selectively regulates IL-8 activity through the NTR1. Because we (8) and others (7, 39) have shown that SR48692 is nontoxic and effective *in vivo* to block neurotensin-mediated tumor growth, our findings have important implications in the possible development of novel therapeutic strategies for the treatment of human cancers based on NTR1 receptor blockade.

The signaling pathways and transcription factors regulating IL-8 expression have been described in different cell types in response to various stimuli. Similar to other physiologic agents (e.g., histamine, endothelin-1, and bradykinin), neurotensin acts through Ca^{2+}/PKC, ERK activation, and the ubiquitous AP-1 and NF-κB transcription factors to induce IL-8 gene expression in HCT116 cells. Most studies have shown that NF-κB plays the predominant role in IL-8 regulation. We found that both ERK-dependent AP-1 and ERK-independent NF-κB activation was necessary for neurotensin-mediated IL-8 mRNA and secretion in HCT116 cells. Consistent with our findings, AP-1 and NF-κB cooperatively regulate IL-8 expression in other cell types. For example, the activation of both AP-1 and NF-κB was essential for IL-8 induction in human breast cancer cells in response to glutamine deprivation (40), NF-κB-dependent AP-1 activity regulates vascular endothelial growth factor expression (41). Furthermore, ERK-dependent AP-1 activation synergizes with p65/NF-κB to stimulate IL-8 gene transcription in human epidermal carcinoma cells. Our findings suggest that either receptor blockade or, alternatively, an agent that inhibits both AP-1 and NF-κB activation would be required to block the effects of neurotensin on IL-8 gene induction and protein secretion.

Curcumin, a component of curry spice turmeric, possesses anti-inflammatory and anticancer effects through the inhibition of NF-κB and AP-1 activation. We found that curcumin effectively inhibited neurotensin-induced AP-1 and NF-κB induction and subsequent IL-8 gene expression and protein secretion in HCT116 cells. Similar to our findings, Nakamura et al. (42) found that curcumin decreased both AP-1 and NF-κB activation in prostate cancer cell lines. Moreover, curcumin markedly inhibited the activation of AP-1 and NF-κB DNA binding induced by lipopolysaccharide, H_2O_2, or tumor necrosis factor-α (43, 44). The inhibition of AP-1 and NF-κB by curcumin effectively suppressed IL-8 release in alveolar epithelial cells (43), which corroborates our findings in HCT116 cells. IL-8, regulated by AP-1 and NF-κB transcription factors in various cancer cells, is becoming increasingly recognized as an important local factor in tumorigenesis and metastasis. The results from our study and those of others (22, 45) suggest that an important mechanism for the antitumor effects of curcumin may be through the suppression of chemokines induced by GI hormone or other stimuli. Moreover, Zhao et al. (13) found that neurotensin stimulates IL-8 secretion through NF-κB activation and plays a role in colonic inflammation, whereas numerous other studies showed that curcumin possesses anti-inflammatory effects through inhibition of NF-κB and that colonic inflammation increases the risk for developing colorectal cancer (46). Our findings further suggest that curcumin may be useful for colon cancer treatment as well as potential colon cancer prevention. In fact, curcumin is currently being evaluated in clinical trials as a cancer chemotherapeutic and chemopreventive agent for colorectal cancers (47). Our findings provide additional evidence that curcumin may be beneficial in the suppression of certain chemokines (e.g., IL-8), which play a role in tumor progression.

Whereas several groups have confirmed that neurotensin stimulates GI cancer cell growth and that curcumin suppresses

the growth of various cancer types, few studies have analyzed the effect of neurotensin or curcumin on colon cancer cell migration and/or metastasis. In our current study, we found that neurotensin significantly increased HCT116 cells migration by ~3-fold and that curcumin blocked neurotensin-induced HCT116 cell migration at a concentration (10 μmol/L) that does not cause cell death as shown in our current experiments and by Kang et al. (44). The increase in HCT116 migration by IL-8 treatment suggested that the effect of curcumin on neurotensin-mediated HCT116 migration may be through the inhibition of IL-8. Numerous studies have shown that IL-8 can function as a motility factor for tumor cells, which is relevant to tumor invasion and metastasis. This concept was first shown in human melanoma cells (48) and subsequently shown in human colon carcinoma cells (49). HCT116 migration was increased ~2-fold by IL-8 and ~3-fold by neurotensin. We speculate that IL-8 is one of the motility factors in HCT116 cells, which is stimulated by neurotensin. In addition, neurotensin has been shown to stimulate other protumor factors, such as cyclooxygenase-2 (50), c-*myc*, and matrix metalloproteinases,[3] which may also contribute to the enhanced migration. Taken together, our current study has identified additional effects of neurotensin, which may enhance GI carcinogenesis. In addition to stimulating proliferation, neurotensin can promote the induction and secretion of IL-8, a proinvasive factor, and can increase tumor cell migration. The fact that curcumin, a natural product, can block these effects is appealing for future treatment strategies.

In summary, we show that neurotensin, an intestinal hormone that is potently released by fat ingestion (4, 5), acts through its native NTR to stimulate Ca^{2+}/PKC, ERK/AP-1, and NF-κB pathways and ultimately increases expression and secretion of IL-8 and enhances colon cancer cell migration. These effects were blocked by either NTR1 antagonists or curcumin, a diet-derived chemopreventive and/or chemotherapeutic agent that blocks AP-1 and NF-κB induction. In addition to neurotensin, other GI hormones (e.g., bombesin, gastrin, GRP, and substance-P) have been reported to stimulate expression of various cytokines/chemokines, such as IL-1β, IL-4, IL-6, IL-8, IL-12, and vascular endothelial growth factor. Therefore, it is intriguing to speculate that GI hormones, released in response to dietary components, may enhance tumor growth and promote invasion through the increased expression and secretion of cytokines/chemokines and that these effects may be suppressed by curcumin. Our findings have important clinical ramifications because most colorectal and pancreatic cancers possess receptors for various GI hormones, including neurotensin.

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[3] Q.D. Wang, preliminary results.

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