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Neurotensin-induced Erk1/2 phosphorylation and growth of human colonic cancer cells are independent from growth factors receptors activation

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ABSTRACT

Neurotensin (NT) promotes the proliferation of human colonic cancer cells by undefined mechanisms. We already demonstrated that, in the human colon adenocarcinoma cell line HT29, the effects of NT were mediated by a complex formed between the NT receptor-1 (NTSR1) and-3 (NTSR3). Here we examined cellular mechanisms that led to NT-induced MAP kinase phosphorylation and growth factors receptors transactivation in colonic cancer cells and proliferation in HT29 cells. With the aim to identify upstream signaling involved in NT-elicited MAP kinase activation, we found that the stimulatory effects of the peptide were totally independent from the activation of the epidermal growth factor receptor (EGFR) both in the HT29 and the HCT116 cells. NT was unable to promote phosphorylation of EGFR and to compete with EGF for its binding to the receptor. Pharmacological approaches allowed us to differentiate EGF and NT signaling in HT29 cells since only NT activation of Erk1/2 was shown to be sensitive to PKC inhibitors and since only NT increased the intracellular level of calcium. We also observed that NT was not able to transactivate Insulin-like growth factor receptor.

Our findings indicate that, in the HT29 and HCT116 cell lines, NT stimulates MAP kinase phosphorylation and cell growth by a pathway which does not involve EGF system but rather NT receptors which transduce their own intracellular effectors. These results indicate that depending on the cell line used, blocking EGFR is not the general rule to inhibit NT-induced cancer cell proliferation.

1. Introduction

Colon cancer is an important cause of cancer death over the world. Although advances have been made in the treatment of colon cancer, the intracellular mechanisms responsible for growth of this type of cancer have not been clearly established. The proliferation of colonic cancer cells depends on multiple mechanisms in particular the involvement of the Epidermal Growth Factor (EGF) system through the EGF receptor (EGFR) [1,2]. EGFR can be activated directly by its ligands (EGF, Hb-EGF, Amphiregulin, TGFα) and indirectly by several neuropeptides through the activation of their G-protein coupled receptors (GPCR) [2]. Substance P, cholecystokinin (CCK), vasoactive intestinal peptide (VIP) and neurotensin (NT) are neuropeptides known to transactivate EGFR in gastrointestinal cancers (for reviews see [3] and [2]). NT belongs to the family of endogenous factors that regulate the development of cancers from various origins including pancreatic, prostatic and colonic cancers [2]. The neurotensinergic system is composed by three different identified NT receptors (NTSRs) [4]. NTSR1 and NTSR2 are both seven trans-membrane (TM) domain G protein-coupled receptors (GPCR) whereas NTSR3 is a single TM domain type 1 receptor which shows 100% homology with the sorting protein, sortilin [5,6].

NT treatment of the human colon cancer cell line KM20, which expresses high levels of NTSR1, results in calcium mobilization as well as activation of the ERK pathway [7]. These findings suggest that the proliferative effect of NT may be mediated through the ERK stimulatory pathway and that NT may be a growth factor in certain colon cancers. In the non-transformed human colonic epithelial NCM460 cells which over-express NTSR1, NT treatment activates MAP kinase pathway by a mechanism involving EGFR activation [8]. More recently, it was shown that Insulin-like growth factor-1 receptor transactivation modulated the inflammatory and proliferative responses of NT in the human colonic epithelial cells NCM460 [9]. In the human colonic epithelial cell line HT29, we previously found that the NT signaling pathways mediated by NTSR1 (ie; MAP kinase activation and inositol phosphate formation) were modulated by the presence of NTSR3 which can form a complex with NTSR1 [10]. We also shown that both MAP kinase activation and the growth effect of NT were dependent on the internalization process of NT receptors in these cells [11]. In the present study, we have challenged the hypothesis of EGFR transactivation by NT in the colonic adenocarcinoma cell lines HT29 and HCT116 with the

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goal to demonstrate that this mechanism of action of NT could be a general rule leading to cancer cell proliferation. The fact that intraluminal lipids are the most potent stimuli for NT release suggests an association between the known stimulatory effects of fats on colon carcinogenesis and peptides, such as NT, which increases colon cancer proliferation. This observation is of importance in order to develop tools to block receptor systems (ie; EGFR and/or NTSRs) responsible for cancer growth. We observed that although both NT and EGF enhance HT29 cells growth, the intracellular pathways involved in these effects are quite independent and NT is unable to transactivate EGFR in two cell lines, HT29 and HCT116.

2. Materials and methods

2.1. Materials

Neurotensin (NT) was from Peninsula Laboratories and Epidermal Growth Factor (EGF) was from Upstate Millipore. Dulbecco's modified Eagle's medium (DMEM) was from Life Technologies Inc. and fetal calf serum from BioWest. ZD1839 was a generous gift from Dr Jean-Louis Fischel (Nice) and SR48692 was a gift from Dr Danièle Gully (Sanofi-Avantis). U-73122 was from Calbiochem. PD98059, pertussis toxin (PTX), phorbol-12 myristate 13 acetate (PMA), lysophosphatydic acid (LPA), gentamycin, GF 109203X (GFX), thapsigargin, mammalian protease and phosphatase inhibitor cocktails were from Sigma France. Mouse anti-phospho-ERK1/2, rabbit total anti-Erk1/2, and antibodies against EGFR and IGF-1R were from Santa Cruz Laboratories. Cetuximab was from Merck. Insulin was from Novo Nordisk. HRP conjugated goat anti-rabbit and anti-mouse were from Cell Signaling. Fura-2AM was from Invitrogen.

2.2. Cell culture and proliferation measurement

The human cell lines HT29 and HCT116 were maintained in DMEM supplemented with 10% FBS and 50 μg/ml gentamicin at 37 °C under 5% CO_2. Cells were seeded into a flat-bottom 96-well plate (Corning, Ny, USA) at a density of 5×10^3 cells per well, in the presence of 10% FBS. After 24 h, cells were serum-starved and treated in the absence or in the presence of Cetuximab (2 μg/ml) with NT (10 nM) or EGF (10 nM) or 10% FBS. After 96 h of treatment, cells number was estimated using the fluorometric cell proliferation Assay Kit (CellTiter-Blue® from Promega). Each experiment was performed in triplicate.

2.3. Binding of ^{125}I-EGF on whole HT29 cells

^{125}I-EGF (600 Ci/mmol) was prepared and purified as previously described [12]. Binding experiments were carried out on whole cells as previously described [13]. Briefly, cells, preincubated in Earle's-Tris-HEPES buffer (pH 7.4) and incubated for 30 min at 37 °C with 0.4 nM ^{125}I-EGF (600 Ci/mmol) in the absence or in the presence of increasing concentrations of EGF or NT. Incubation was terminated by washing cells twice, cells were harvested with 1 ml of 0.1 M NaOH and counted in a Packard γ-counter.

2.4. Detection of phosphorylated MAP Kinase Erk1/2 and EGFR

Cells were incubated overnight in serum free DMEM and then stimulated with NT or EGF (1×10^{-8} M) for indicated times at 37 °C and then lysed in 50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.5% Na-deoxycholate, 1% NP40, 0.1% SDS in the presence of phosphatase and protease inhibitors (1/100).

In some experiments, cells were preincubated for 15 min with GFX (10 μM), U73122 (10 μM) and PD98059 (24 μM) or for 30 min with SR48692 (1 μM), ZD 1839 (10 μM), or overnight with PTX (10 ng/ml). When tested, cetuximab (2 μg/ml) was preincubated for 30 min before stimulation with NT.

Forty micro grams of proteins were analyzed on SDS-PAGE, electrotransferred onto nitrocellulose and subjected to immunoblotting using antibodies directed against phospho-EGFR (Tyr 1173) or against phospho-IGF-1R or the phosphorylated forms of Erk1/2. Results were standardized within the same blot using total EGFR or total Erk1/2 antibodies. The acquisition and quantifications were performed as previously described [10].

2.5. Measurement of cytosolic calcium variation

Cells, plated on coverslips, were loaded with Fura-2AM and the variations of fluorescence emission were measured as initially described [14]. Fluorescence images were acquired every 10 s on an EMCCD camera (Cascade 512, Roper Scientific, Evry, France).

2.6. Statistical analysis

Statistical analysis was made from the number of different independent assays by using the Student's *t*-test.

3. Results

3.1. Effects of NT and EGF on cell proliferation and Erk1/2 phosphorylation in HT29 cells

We first verified that both NT and EGF were able to induce cell growth. As shown in Fig. 1A, both NT and EGF (10 nM) significantly induced proliferation (by a factor of 1.7 and 2.6, respectively) of HT29 cells after 96 h in the absence of serum. We repeated the experiment in the presence of 2 μg/ml Cetuximab. As shown in Fig. 1A, we first confirmed that although the effect of NT on cell proliferation was not affected, the EGF-induced HT29 cell growth was efficiently inhibited by Cetuximab indicating that the proliferative action of NT is independent from the EGFR activation.

The ability of NT and EGF to activate Erk1/2 phosphorylation was measured in HT29 cells in the presence of specific blockers of EGFR, Cetuximab and the tyrosine kinase inhibitor ZD1839. As shown in Fig. 1B, EGF (0.1 μM) rapidly and transiently increased the phosphorylation state of Erk1/2 with a maximal stimulation after 10 min, this stimulation being totally inhibited both by ZD1839 and Cetuximab. In the same conditions, 0.1 μM NT increased the amount of activated Erk1/2 with a maximal effect between 5 and 10 min (Fig. 1C) but in this case, neither ZD 1939 nor Cetuximab were able to block this effect.

3.2. Study of NT and EGF crosstalk

To verify that EGFR activation is not involved in the response to NT on cell growth, we observed that EGF induced phosphorylation of EGFR between 2 and 15 min whereas NT was unable to stimulate phosphorylation of EGFR (Fig. 2A) even after 60 min of incubation (not shown) indicating that the action of NT on the HT29 cell growth is not mediated by EGFR activation.

Previous data reported that NT (1 μM) inhibited 20% of the binding of EGF to HT29 cells [15], we tested the ability of EGF and NT to inhibit the binding of ^{125}I-EGF (Fig. 2B). As expected, EGF displayed an IC50 value of 0.3 nM whereas NT was unable to displace the binding of ^{125}I-EGF even at 1 μM.

To verify that the distinct effects of NT and EGF on Erk1/2 and EGFR phosphorylation were not specific of the HT29 cell line, we repeated signaling and EGFR phosphorylation experiments in another colonic cancer cell line, HCT116 cells. Measurement of the

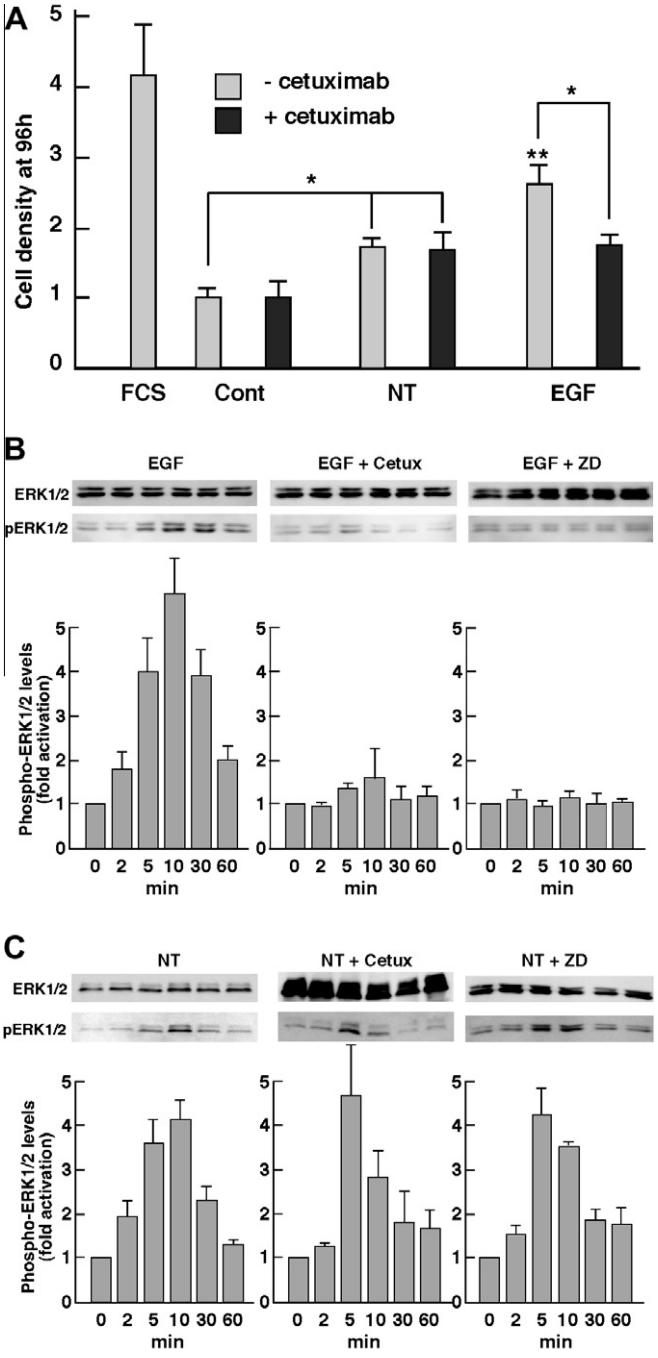

Fig. 1. Effects of NT and EGF on cell growth and Erk1/2 phosphorylation in HT29 cells. A – Cells were serum-starved, pre-incubated or not with Cetuximab (2 μg/ml) and incubated in the absence (Cont) or in the presence of NT (10^{-8} M) or EGF (10^{-8} M) or 10% serum (FCS) for 96 h at 37 °C. Cells number was determined using the fluorometric cell proliferation Assay Kit. Data are expressed as percent of cell number obtained in control conditions and are mean ± SEM from at least five independent experiments performed in triplicate. $^{*}p < 0.05$; $^{**}p < 0.01$. B – Cells were stimulated with EGF (10^{-8} M) for indicated times in the absence (EGF) or in the presence of ZD1839 (10 μM) (EGF + ZD) or Cetuximab (2 μg/ml) (EGF + Cetux). C – Cells were stimulated with NT (10^{-8} M) for indicated times in the absence (NT) or in the presence of ZD1839 (10 μM) (NT + ZD) or Cetuximab (2 μg/ml) (NT + Cetux). Cell lysates were analyzed by Western blotting using antibodies directed against phospho-Erk1/2. Results were standardized using anti-Erk1/2 total and are expressed as mean ± SEM from at least three independent experiments.

Tyr 1173 phosphorylation of EGFR revealed that only EGF efficiently activated this modification at 5 and 10 min whereas NT was without effect (Fig. 2C). In HCT116 cells, both NT and

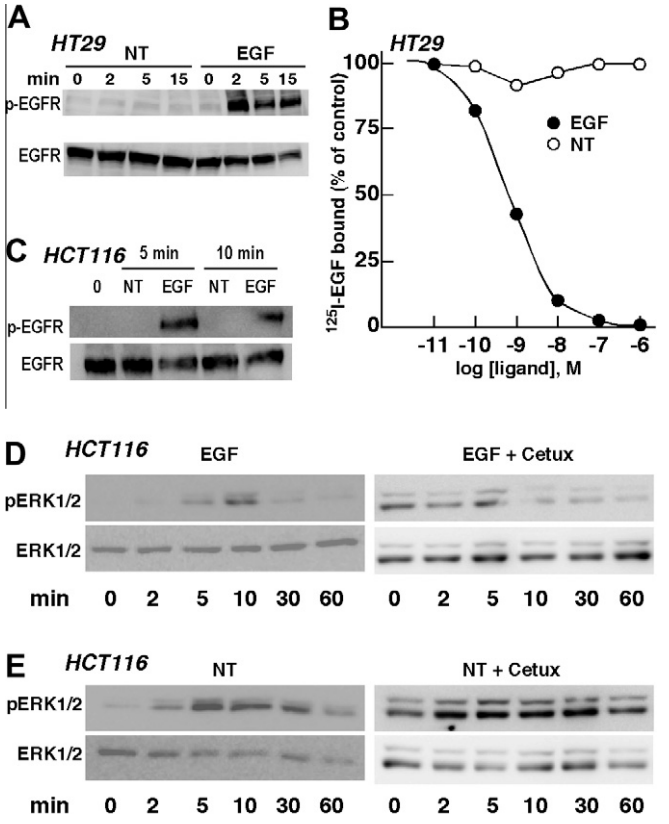

Fig. 2. Absence of NT and EGFR relationships in HT29 and HCT116 cells. A – HT29 cells were treated with NT (10^{-8} M) or EGF (10^{-8} M) for indicated times. Equal amount of cell proteins were separated on SDS-PAGE and immunoblotted with antibodies directed against phospho-EGFR (Tyr 1173) or total EGFR. The results are representative of three independent experiments. B – Cell monolayers were incubated in Earle's-Tris-HEPES buffer for 30 min at 37 °C with 0.4 nM ^{125}I-EGF in the absence or in the presence of increasing concentrations of EGF (closed circles) or NT (open circles). Binding experiments were terminated as described in Section 2. Data are expressed as the percent of binding obtained in the absence of unlabeled ligands and are mean from two independent experiments performed in triplicate. C – HCT116 cells were treated with NT (10^{-8} M) or EGF (10^{-8} M) for indicated times. Equal amount of cell proteins were separated on SDS-PAGE and immnoblotted with antibodies directed against phospho-EGFR (Tyr 1173) or total EGFR. The results are representative of three independent experiments. D – HCT116 cells were stimulated with EGF (10^{-8} M) for indicated times in the absence (EGF) or in the presence of Cetuximab (2 μg/ml) (EGF + Cetux). E – HCT116 cells were stimulated with NT (10^{-8} M) for indicated times in the absence (NT) or in the presence of Cetuximab (2 μg/ml) (NT + Cetux). Cell lysates were subjected to Western blotting using antibodies directed against phospho-Erk1/2 or total Erk1/2. Results were representative from at least three independent experiments.

EGF were able to rapidly and transiently stimulated the phosphorylation of Erk1/2 (Fig. 2D and E). Here again, the use of Cetuximab prevented the effect of EGF but not of NT.

3.3. Investigation on cellular pathways involved in the NT- and EGF-induced phosphorylation of Erk1/2

To investigate which effector pathways might be responsible for NT and EGF induced activation of Erk1/2, we examined the effect of several specific inhibitors. As shown in Fig. 3A, NT and EGF increased the phosphorylation of Erk1/2. Both effects were inhibited by the MAP kinase inhibitor PD98059. As expected, the NTSR1-selective antagonist SR48692 inhibited only the response of NT (Fig. 3A). PKC is known to represent an upstream regulator of MAP kinase cascade, we then explored this pathway by using the non-toxic PKC-specific inhibitor, GFX [16]. Fig. 3A shows that treatment of HT29

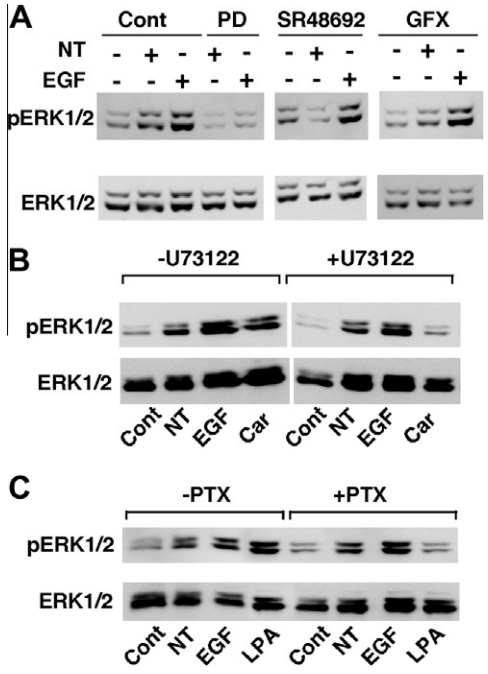

Fig. 3. Effects of pharmacological agents on NT and EGF-induced activation of Erk1/2 in HT29 cells. Cells were preincubated for 15 min with GFX (10 μM), U73122 (10 μM) and PD98059 (24 μM) or for 30 min with SR48692 (1 μM) or overnight with PTX (10 ng/ml) and then stimulated with NT (10^{-8} M) or EGF (10^{-8} M) for 10 min. Identical amounts of cell proteins were analysed by Western blotting to determine Erk phosphorylation. The results are representative from three independent experiments.

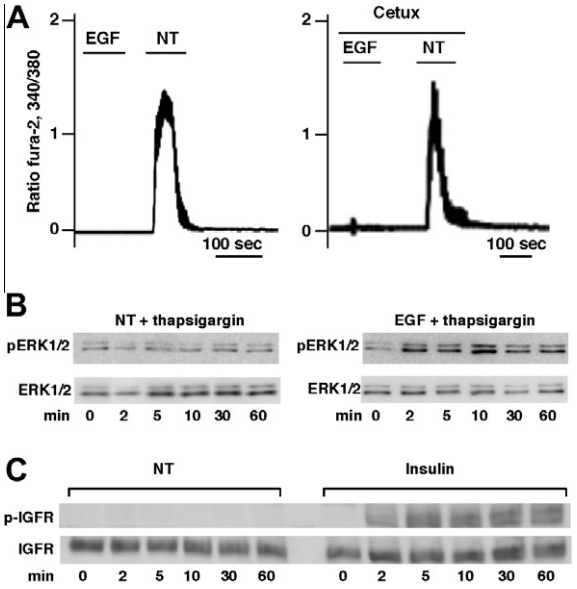

Fig. 4. Effects of NT and EGF on intracellular calcium concentration in HT29 cells. A – HT29 cells were incubated with NT (10^{-8} M) and EGF (10^{-8} M) in the absence or in the presence of Cetuximab (2 μg/ml). The intracellular calcium amount was determined as described in Section 2. The results are the mean of 20 recorded cells from two independent experiments. B – HT29 cells were preincubated 15 min with Thapsigargin (1 μM) and stimulated with NT (10^{-8} M) or EGF (10^{-8} M) for indicated times. Identical amounts of cell proteins were analysed by Western blotting to determine Erk phosphorylation. The results are representative from three independent experiments. C – Transactivation of the IGF receptor in HT29 cells. HT29 cells were treated with NT (10^{-8} M) or Insulin (10^{-8} M) for indicated times. Equal amount of cell proteins were separated on SDS-PAGE and immunoblotted with antibodies directed against phospho-IGFR (Tyr 1131) or total IGFR. The results are representative from three independent experiments.

cells with GFX markedly decreased NT-stimulated MAP kinase activity without affecting EGF-induced Erk1/2 phosphorylation.

To determine the involvement of phospholipase C (PLC) on the effects of NT and EGF on Erk activation, cells were treated with the PLC inhibitor U73122. Both NT and EGF responses were not attenuated by U73122 (Fig. 3B) whereas the response of carbachol, known to be inhibited by the drug, was efficiently reduced. We also tested the involvement of Gi by using pertussis toxin (PTX). PTX treatment was without effect both on NT and EGF Erk1/2 activation (Fig. 3C) whereas the drug strongly inhibited LPA-induced Erk activation.

3.4. Effect of NT and EGF on intracellular calcium

We investigated the effects of NT and EGF on HT29 cells intracellular calcium concentration by using Fura-2 fluorescence microscopy. As shown in Figure 4A, NT (0.1 μM), but not EGF (0.1 μM), induced a rapid and significant increase of intracellular calcium in HT29 cells ($n = 3$). Cetuximab was unable to block the NT-induced increase of intracellular calcium (Fig. 4A). This result suggests that the activation of Erk1/2 by NT but not by EGF could be the consequence of an effect on intracellular calcium concentration. Then, we tested the effect of depletion of intracellular calcium store on Erk1/2 phosphorylation. As shown in Fig. 4B, the use of thapsigargin (500 nM) to empty the endoplasmic reticulum (ER) calcium stores [17] revealed that only the response of NT was affected by the drug, the EGF response being unaffected (Fig. 4B). This result confirms that intracellular pools of calcium are involved in the activation of the MAP kinase pathway by NT.

3.5. Study on the possible involvement of the Insulin-like Growth Factor Receptor in the effects of NT

A recent study demonstrated that the Insulin-like Growth Factor-1 (IGF) receptor (IGF-1R) transactivation modulates the proliferation response of NT in human colonic epithelial cells [9]. For this reason, we tested the ability of NT and Insulin to phosphorylate the IGF-1R in HT29 cells. Fig. 4C clearly showed that only Insulin (10 nM) was able to phosphorylate IGF-1R between 2 and 60 min whereas NT was uneffective. Both treatments did not affect the amount of total IGF-1R (Fig. 4C).

4. Discussion

NT and EGF regulate the growth of several cell types, including human colonic epithelial cells [8,18], as well as prostate cancer epithelial cells [19]. Activation of both NT and EGF receptors by their ligands leads to the stimulation of MAP kinase phosphorylation. In several cell lines, the biological proliferation action of NT has been shown to be the consequence of EGFR transactivation [8,19]. In this work to analyze this hypothesis in two human colonic adenocarcinoma cell lines HT29 and HCT116, we characterized molecular mechanisms by which NT activates MAP kinases which are somewhat different from those induced by EGF.

Since the proliferative action of NT has been documented to be mediated by transactivation of EGFR in NCM460 colonic cells [8] and in PC3 cells [19], we first tested this hypothesis in HT29 cells. We observed that both NT and EGF stimulate the growth of HT29 cells via phosphorylation of Erk1/2 by a distinct mechanism since Cetuximab blocks the EGF-mediated cell growth and Erk1/2 phosphorylation but not the effects of NT. We obtained similar results in HCT116 cells. Moreover, NT was unable to induce the transactivation of EGFR in both cell lines. This statement indicates that in contrast to results obtained in other colonic and prostatic cancer cell lines, the cell growth effect of NT is independent from EGFR transactivation in HT29 and HCT116 cells [8,19]. These distinct pathways could be the consequence of the co-expression of NTSR1 and NTSR3 that were shown to be involved in complex for the ignaling of NT

[10]. However, a previous work reported that on intact HT29 cells, the binding of ^{125}I-EGF was slightly inhibited by 20% in the presence of 1 μM NT [15]. We measured the binding of ^{125}I-EGF in competition experiments on whole HT29 cells at 37 °C and observed that NT was unable to inhibit ^{125}I-EGF binding even at 1 μM concentration (Fig. 2B). The differences observed between our results and those obtained by Bozou et al. are likely due to the fact that they performed experiments by preincubation of NT before binding experiments. In the latter case, preincubation for 30 min with 1 μM NT likely induces endocytosis of a large part of NT receptors that could modify membrane expression of other receptors in HT29 cells.

To further investigate the pathways mediating growth response to NT and EGF, we tested cetuximab, the specific EGF binding blocker to EGFR and the specific tyrosine-kinase inhibitor ZD1839 for their ability to block NT-induced activation of Erk1/2 in HT29 cells. As expected, EGF-induced phosphorylation of Erk1/2 was totally inhibited by both drugs whereas these compounds were unable to reverse NT-induced MAP kinases phosphorylation. Here again, these results differ from those obtained in NCM460 cells and in PC3 cells in which both cetuximab and tyrosine kinase inhibitors blocked NT-induced MAP kinase activation [8,19]. This observation indicates that the cellular pathways leading to Erk1/2 activation and then to cell proliferation by NT and EGF are more complex in some cancer cell lines like HT29 and HCT116 and that the ways to develop tools to decrease tumor growth remain more complicated than expected from previous studies.

By using pharmacological tools, we attempted to delineate specific cellular pathways leading to NT and EGF-induced MAP kinases activation. Phosphorylation of Erk1/2 by NT and EGF is sensitive to PD98059 indicating the specific involvement of MAP kinases. The use of SR48692, a selective NTSR1 antagonist, efficiently inhibited the NT-induced Erk activation without any effect on EGF action, indicating a NTSR1-dependent effect of NT. However, previous studies have shown that, in CHO cells stably transfected with NTSR1, the activation of Erk1/2 was fully dependent on the activation of PKC [18]. We confirmed a partial involvement of PKC in the effects of NT in HT29 cells although SR48692 totally blocks the effect of NT. It is important to remember that HT29 cells also express the NTSR3, a non-G protein coupled NT receptor, which modulates the response to NT [10]. The results observed in this cell line could suggest that either SR48692 also recognizes NTSR3 or that the expression of NTSR3 changes the cellular signaling pathway as already hypothesized [10]. The weak involvement of PKC in the effects of NT, a process necessary to activate shedding of EGFR ligands, may explain the absence of EGFR transactivation upon peptide stimulation.

Finally, both NT and EGF effects on MAP kinase activation were insensitive to the Phospholipase C inhibitor U73122 and to the Gi inhibitor Pertussis toxin indicating that both responses are independent from inositol formation and adenylate cyclase. Although previous studies suggested that NT effects mediated by NTSR1 may be coupled to the heterotrimeric G proteins Gq and Gi and induce PKC activation via the Gq subfamily [4,20], there is no involvement of Gi or Gq in Erk1/2 phosphorylation induced by NT in HT29 cells. In this cell line, NT activated PLC leading to Inositol phosphate formation [10] but this activation is not responsible for MAP kinase activation. We already shown that activation by NT of Erk1/2 and phospholipase C were dissociated in this cell line [11]. In NCM460 colonic epithelial cell line, NT was shown to transactivate IGF-1R leading to cell growth [9], we also tested this hypothesis in HT29 cells and observed that NT was unable to phosphorylate IGF-1R confirming that the action of the peptide was strictly dependent on its own receptors and did not involve growth factor receptors.

In conclusion, NT and EGF activate cellular pathways to increase Erk1/2 phosphorylation but neither EGFR nor IGF-1R transactivation is responsible for NT-induced MAP kinase activation in the HT29 cell line. Some other parameters allow to distinguish between NT and EGF signaling such as the partial involvement of PKC for NT but not for EGF. The most important result that differentiate NT and EGF pathways is the observation that NT but not EGF rapidly increases intracellular calcium concentration that is necessary for NT-induced MAP kinase activation (Fig. 4B).

Acknowledgments

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