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Gastrointestinal hormones stimulate growth of Foregut Neuroendocrine Tumors by transactivating the EGF receptor

Alessia Di Florio[1], **Veronica Sancho**[1], **Paola Moreno**[1], **Gianfranco Delle Fave**[2], and **Robert T. Jensen**[1]

[1]Digestive Diseases Branch, NIDDK, National Institutes of Health, Bethesda, MD 20892-1804, USA

[2]Digestive and Liver Disease Unit, II Medical School, University La Sapienza, S. Andrea Hospital, Via Di Grottarossa 00189, Rome, Italy

Abstract

Foregut Neuroendocrine Tumors[NETs] usually pursuit a benign course, but some show aggressive behavior. The treatment of patients with advanced NETs is marginally effective and new approaches are needed. In other tumors, transactivation of the EGF receptor(EGFR) by growth factors, gastrointestinal(GI) hormones and lipids can stimulate growth, which has led to new treatments. Recent studies show a direct correlation between NET malignancy and EGFR expression, EGFR inhibition decreases basal NET growth and an autocrine growth effect exerted by GI hormones, for some NETs. To determine if GI hormones can stimulate NET growth by inducing transactivation of EGFR, we examined the ability of EGF, TGFα and various GI hormones to stimulate growth of the human foregut carcinoid, BON, the somatostatinoma QGP-1 and the rat islet tumor, Rin-14B-cell lines. The EGFR tyrosine-kinase inhibitor, AG1478 strongly inhibited EGF and the GI hormones stimulated cell growth, both in BON and QGP-1 cells. In all the three neuroendocrine cell lines studied, we found EGF, TGFα and the other growth-stimulating GI hormones increased Tyr^{1068} EGFR phosphorylation. In BON cells, both the GI hormones neurotensin and a bombesin analogue caused a time- and dose-dependent increase in EGFR phosphorylation, which was strongly inhibited by AG1478. Moreover, we found this stimulated phosphorylation was dependent on Src kinases, PKCs, matrix metalloproteinase activation and the generation of reactive oxygen species. These results raise the possibility that disruption of this signaling cascade by either EGFR inhibition alone or combined with receptor antagonists may be a novel therapeutic approach for treatment of foregut NETs/PETs.

Keywords

neuroendocrine tumors; transactivation; gastrointestinal hormones; cell growth; pancreatic endocrine tumors

Corresponding author: Dr. Robert T. Jensen, 10 Center Dr., Bldg. 10, room 9C103, Bethesda, MD-20814, Tel.:(301) 496-4201, Fax: (301)-402-0600, robertj@bdg10.niddk.nih.gov.

1. Introduction

Foregut Neuroendocrine tumors (NETs)/pancreatic endocrine tumors (PETs) originate from the neuroendocrine cells of the diffuse endocrine system of the stomach, lung, first part of duodenum and pancreas [1,2]. Although these tumors have been considered rare, their incidence is increasing [2]. They are classified either on a clinical basis, depending on the presence of hormone hypersecretion (functioning vs. non-functioning), their grade of differentiation (well vs. poorly differentiated) or location of the primary lesion [2]. Overall, foregut NETs/PETs display a common secretory system and physiological response (production and/or secretion of neural/hormonal regulators), with a heterogeneous clinical presentation at diagnosis [3,4]. Although in some cases they present with a hypersecretion syndrome and show a relative slow growth rate, a subset show more aggressive growth. At present 60–80 % of cases are diagnosed at advanced stages, with metastases [1,3]. The only effective approach is surgical resection, but it is possible in only 10–20% [3,4]. Other treatments for patients with advanced foregut NETs/PETs include the use of kinase inhibitors, peptide-radioligand therapies, biotherapy (interferon, somatostatin and chemotherapy [1,2,5,6], but in most cases the survival rates remain low [1]. For this reason there is a need for new therapeutic approaches [5–7].

Growth factors and their receptors play an important role in controlling cell growth, differentiation and migration and this represents a key component in tumor development and growth [7,8]. Epidermal growth factor (EGF) and its receptor (EGFR) are frequently overexpressed in by tumors [8]; including foregut NETs/PETs [9–12], especially in the most aggressive forms. *In vitro* studies on carcinoids and PET cell lines demonstrate that activation of the EGFR receptor can stimulate stimulate growth and that its inhibition can inhibit basal growth [11,13,14].

A frequent mechanism in tumors in controlling their growth is transactivation of EGFR by the activation of G protein-coupled receptors (GPCRs), generally by gastrointestinal (GI) hormones/neurotransmitters or other biologically active substances, such as prostaglandins and lipids [15–17]. The abnormal secretion, either autocrine or paracrine of these substances in tumors, leads to the activation of GPCRs, which, in turn, transactivate EGFR [15,16,18]. This occurs in a number of cancers [head/neck squamous cell carcinoma (HNSCC) [16], lung, prostate, breast, colon] as well various endocrine cancers and those of other tissues [15,17–21]. Among GI hormones/neurotransmitters, neurotensin, bombesin-related peptides, bradykinin, gastrin and PACAP are known to play an important role in controlling tumor growth by transactivation of EGFR in a number of cancers [17–23].

It is reported that a number of GI hormones/neurotransmitters can affect the growth of neuroendocrine tumors [7,24]. Even though it has been shown that activaton of EGFR can alter the basal growth of NETs [11,13,14], it is unknown whether EGFR-transactivation is involved in the stimulation by GI hormones/neurotransmitters of their growth or if so, the cellular mechanisms involved.

Therefore the aim of this study was to address these issues by investigating the ability of GI hormones/neurotransmitters to stimulate the growth of various foregut neuroendocrine tumor cell lines and determining whether transactivation of EGFR was important in mediating this growth effect and if so, define the celluar signaling cascades involved.

2. Materials and Methods

Materials

Dulbecco's minimum essential medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS), were from Invitrogen (Carlsbad, CA); Thymidine, [6-^{3}H], >97%, 1 mCi (37MBq) from Perkin Elmer (Boston, MA); Tris buffered saline (TBS) from Cellgro® (Mediatech Inc. Manassas, Va); Dimethyl sulfoxide (DMSO), trichloroacetic Acid (TCA), MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DL-dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), Tiron and N-Acetyl-cysteine were from Sigma–Aldrich (St. Louis, MO); Human EGF (Recombinant, E. coli), PACAP27, the tyrosine kinase inhibitor AG178, the Src family kinases inhibitor PP2 and PP3, GF 109203X and GM 6001 were from Calbiochem (San Diego, Ca). TGFα BioVision (Mountain View, Ca), neurotensin and bradykinin were from Bachem (Torrence, CA); the bombesin analogue, [DTyr^6, β Ala^{11}, Phe^{13}, Nle^{14}]Bn (6–14)] obtained from Dr. David H. Coy, Tulane University (New Orleans, La); protease inhibitor tablets were from Roche (Basel, Switzerland); α-tubulin and phospho-EGF receptor (Tyr^{1068}) antibodies and non-fat dry milk were from Cell Signaling Technology, Inc. (Beverly, MA) and horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit) and Supersignal Western Pico/Dura were from Thermo Scientific (Rockford, IL).

Methods

Cell culture—The metastatic human pancreatic carcinoid cell line, BON was obtained from Cancer Research UK Cell Services and grown in DMEM/F12K (1:1), with 10% FBS and supplemented with penicillin/streptomycin. The human somatostatinoma cell line, QGP-1, was obtained from Cancer Research UK Cell Services and the rat islet cell tumor, Rin-14B was obtained from ATCC (Manassas, Va). Both cell lines were grown in RPMI with 10% FBS and supplemented with penicillin/streptomycin. The cells were mycoplasma free and incubated at 37 °C in 5% CO_2/95% air.

Western blotting—Briefly, after adding lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium azide, 1 mM EGTA, 0.4 mM EDTA, 1 mM DTT, 0.4 mM sodium orthovanadate, 1 mM PMSF, and one protease inhibitor tablet per 10 ml], lysates were sonicated, centrifuged at 13,000 rpm for 20 min at 4 °C and protein concentration was measured using the Bio-Rad protein assay reagent (Hercules, CA). Equal amounts of protein from lysates were loaded on to SDS-PAGE using 4–20% Tris-Glycine gels Invitrogen (Carlsbad, Ca). After electrophoresis, proteins were transferred to nitrocellulose membranes overnight. Membranes were washed twice in washing buffer (TBS plus 0.1% Tween®20), at room temperature for 1 h, and then incubated with primary antibody at 1:1000 dilution in washing buffer + 5% BSA [Phospho-EGFR (Tyr^{1068}) or tubulin, as loading control] overnight at 4°C. Then, membranes were washed twice in blocking buffer (TBS, 0.1% Tween® 20, 5% non-fat dry milk) for 4 min and then incubated with HRP-conjugated secondary antibody (anti-rabbit) for 1 h at room temperature. Membranes were washed again twice in blocking buffer for 4 min, and then twice in washing buffer for 4 min followed by incubation with chemiluminescence detection reagents for 4 min and finally were exposed to Kodak Biomax film (MR, MS). The intensity of the protein bands was measured using Kodak ID Image Analysis.

[^{3}H]-Thymidine uptake—BON (1.5×10^5 cells/well) and QGP-1 (4×10^4 cells/well) cells were plated in 24-well plates in DMEM/F12K with 10% FBS or RPMI with 10% FBS, respectively. After 24 hours cells were washed twice with PBS and starved in DMEM/F12K or RPMI without FBS, for additional 24 hours. Then, peptides and hormones were added for 24 hours. Six hours before the end of the incubation 1 mCi/ml of [^{3}H]-Thymidine was added

to each well. Then cells were washed 3 times with 1 ml iced cold PBS and incubated with 1 ml of ice cold 5% TCA at 4 °C for 30 min. After that, TCA was aspirated and cells were washed twice with iced cold PBS. Finally, cells were resuspended in 0.5 ml of 0.5 N NaOH/ 0.5% SDS and placed in a scintillation vial and counted in a scintillation counter.

MTT assay—5×10^3 BON cells were seeded in 96-well plates and incubated for 24 hours in 100 ml of DMEM/F12K with 10% FBS. Then, cells were washed twice in PBS and the starvation media (DMEM/F12K) with peptides and hormones were added for 24 hours. Three hours before the end of the treatments 10 ml of MTT (1.2 mM, final concentration) was added to each well; after 3 hours, 50 ml of 0.04N HCl in isopropanol was added to each well and incubated at room temperature for 5 min. The plate was read at 595 nm using a microplate reader.

Statistical analysis—All results are expressed as mean±SEM from at least 3 experiments, and results were considered significant if, in a paired t-Test and in one-way ANOVA (Dunnett's multiple tests, as a post test), p was < 0.05. PRISM GraphPad software (GraphPad Software Inc., La Jolla, CA) was used for all statistical analysis.

3. Results

3.1. Ability of EGF, TGFα or various GI hormones to stimulate growth of BON and QGP-1 cells and effect of an EGFR inhibitor, AG1478

To assess the effect of GI hormones on BON cell line growth, we performed two different assays, the MTT assay (Fig. 1A) and an assessment of [^{3}H]-Thymidine uptake (Fig. 1B). EGF and TGFα stimulated BON cells growth by 30% and 40% in the MTT assay, respectively. With [^{3}H]-Thymidine assessment, growth stimulated by EGF or TGFα was 32% and 42%, and was 180% with the addition of 10% fetal bovine serum. With both experimental approaches, each GI hormone/neurotransmitter [a bombesin analogue (Bn-analogue), bradykinin, PACAP and neurotensin (NT)], stimulated cell growth by 20–30. In contrast, treatment with the hormones galanin and CCK/gastrin, did not exert any effect on cell growth (data not shown). To assess if the cell growth induced by the GI hormones required EGFR activation, we pre-treated BON cells with the EGFR tyrosine kinase inhibitor AG1478 (1 μM) (Fig. 1A, B). The stimulation of cell proliferation induced by each GI hormone was completely inhibited by AG1478 pre-treatment, as was the stimulation of growth by EGF or TGFα. In the somatostatinoma cell line QGP-1, the growth factor TGFα as well as the GI hormones NT and a Bn-Analogue, stimulated cell growth, in an EGFR-dependent way, determined by assessing [^{3}H]-Thymidine incorporation (Fig. 1C).

3.2. Ability of EGF, TGFα or the GI hormones to stimulate EGFR tyrosine phosphorylation in NET cell lines

To directly assess the ability of GI hormones to transactivate the EGFR in BON cells, we analyzed, by Western blotting, Y^{1068} phosphorylation of EGFR, which correlates with EGFR activation [25]. BON cells treated with EGF (16nM), TGFα (3.6nM), NT (1μM), BN-analogue (1 μM), PACAP (1 μM) or bradykinin (1 μM) for 30 minutes (Fig. 2A, B), as well as with EGF or TGFα, significantly stimulated Y^{1068} EGFR phosphorylation (Fig. 2A, B). To examine this in more detail, we studied the effect of the EGFR tyrosine kinase inhibitor, AG1478 on stimulation of Tyr^{1068} phosphorylation by EGF or by two GI hormones [NT or a Bn-analogue]. AG1478 (1 μM) (inhibited EGF stimulation by 88% and inhibited completely Y^{1068} EGFR phosphorylation, stimulated by the Bn-analogue or by NT in BON cells (Fig. 2C, D).

To assess if the effect of GI hormones to stimulate EGFR phosphorylation was seen in other foregut NET/PET cell lines, we performed the same experiment, on the human somatostatinoma QGP-1 cell line and on the rat islet cell tumor, Rin-14B. As showed in Figure 2, in QGP-1 (Fig. 2E, F) and in Rin-14B (Fig. 2G, H), the GI hormones, Bn-analogue (1 μM), NT (1 μM) and PACAP (1 μM) as well as the growth factor, EGF (16 nM) are able to stimulate the Y^{1068} EGFR phosphorylation. This stimulation is reversed, in each case, by AG1478 (1 μM) pre-treatment.

3.3. Time-dependent and dose-dependent EGFR phosphorylation induced by Bn-analogue and NT

Next, we investigated, in more detail, the ability of EGF, Bn-analogue and NT to stimulate Y^{1068} EGFR phosphorylation in BON cells. EGF treatment caused a rapid phosphorylation in Y^{1068} EGFR, with a $t_{1/2}$ at 3 min (Fig. 3A, B). Conversely, the Bn-analogue (Fig 3. C, D) and NT (data not shown) induced a slower time-dependent EGFR phosphorylation, reaching a 3.4-fold increase at 62 min with a $t_{1/2}$= 12 min, followed by a decrease in stimulation with the Bn-Analogue. Moreover, we evaluated the increase of EGFR phosphorylation in response to increasing doses of Bn-analogue (Fig. 3E, F) or NT (Fig. 3G, H). EGFR Y^{1068} phosphorylation increased with increasing GI hormone concentration reaching a 2.4-fold increase with 1 μM Bn-analogue (EC_{50}= 17±2.2 nM) (Fig. 3F) and a 2.6-fold increase with 1 μM NT (EC_{50}= 0.44±0.08 nM) (Fig. 3H).

3.4. Src kinases mediate the EGFR-transactivation induced by Bn-analogue and NT in BON cells

To further explore the cellular pathways that mediate EGFR-transactivation induced by Bn-analogue and NT, we studied the effect of the Src kinase inhibitor, PP2 on Y^{1068} EGFR phosphorylation induced either by Bn-analogue or NT. BON cells pre-treated with 10 μM PP2, but not PP3, an inactive PP2 analogue, strongly reduced Y^{1068} EGFR phosphorylation stimulated by Bn-analogue (Fig. 4A, B) or NT (Fig. 4A, C).

3.5. PKC and Matrix Metalloproteinases (MMPs) activation are involved in Bn-analogue and NT induced EGFR-transactivation

To determine whether EGFR-transactivation by Bn-analogue and NT is mediated by PKC activity, BON cells where pre-treatment with the PKC inhibitor, GF 109203X. EGFR Y^{1068} phosphorylation stimulated by either the Bn-analogue (Fig. 5A, B) or NT (Fig. 5C, D) is strongly inhibited (86 and 77%, respectively) by GF 109203X pre-treatment (compare in Fig. 5A, lanes 2,4 to Fig. 5C, lanes 5,6).

To investigate the ability of Bn-analogue and NT to possibly induce EGFR-transactivation through activation of MMPs leading to cleavage of an EGF-related peptide, as demonstrated in other systems [19], we pre-treated BON cells with the MMP inhibitor, GM 6001. Inhibition of MMPs activity, by GM 6001, reduced the EGFR Y^{1068} phosphorylation stimulated either by Bn-analogue (Fig. 5A, B) or NT (Fig. 5C, D) by 75 and 60%, respectively (compare in Fig. 5A, lanes 2,3 and in Fig. 5C, lanes 2,3).

3.6. Production of reactive oxygen species (ROS) is involved in Bn-analogue and NT induced EGFR-transactivation in BON cells

In some cells the generation of ROS is required for various stimulants to cause EGFR-transactivation [19,21,26]. To determine if the generation of ROS was involved in EGFR-transactivation by Bn-analogue and NT, we pre-treated BON cells with the antioxidant, N-acetylcysteine (NAC) or Tiron, a superoxide scavenger [19]. EGFR Y^{1068} phosphorylation stimulated by the Bn-analogue (Fig. 6A, B) or NT (Fig. 6C, D) were strongly impaired by

both antioxidants pre-treatment, by 85% for Bn-analogue (In Fig. 6A compares lanes 2,3,4) and by 37–45%, for NT (In Fig. 6C, compare lanes 2,3,4).

4. Discussion

The aim of this study was to examine the role of EGF receptor transactivation in mediating the ability of gastrointestinal hormone/neurotransmitters to stimulate growth of Foregut NET/PETs. This study was undertaken for a number of reasons.

Foregut NETs/PETs represent a heterogeneous group of tumors whose incidence has increased in the last 30 years [2,4]. Although generally slow growing, 60–80 % of cases are diagnosed with metastatic disease, and a proportion is rapidly growing causing death [1,2,5]. The only curative treatment is surgery, but this is frequently limited due to metastatic spread and in general, current treatment of most patients with advanced metastatic disease is unsatisfactory [3]. Current approaches include the use of tyrosine kinase and mTOR inhibitors, peptide radio-receptor therapies, chemotherapy, biotherapy (somatostatin, interferon) and local ablative treatments of the metastatic disease [1,2,5]. None of these, generally, results in a complete response and they generally show an incomplete response with a 5-year survival rates at 20%–40% [1]. Therefore, there is a need for new treatment approaches [1,3,4].

The importance of growth factors and their cognate receptors in tumor growth, differentiation and invasion is fully established in a number of tumors [8]. Several growth factors and/or their receptors are reported in various Foregut NETs/PETs, as well as frequently over- or ectopically-expressed and to show gene amplification [9–12]. They include insulin-growth factor I (IGFI) [12]; platelet derived growth factor (PDGF) [14] and EGF/TGFα [27]. In some Foregut NETs/PETs, EGF/EGFR appears to be particularly important in growth as an autocrine growth factor [9,10,12,27,28]. Furthermore, a direct correlation between EGFR expression and malignant behavior is reported and can result in activation of growth cascades, involving AKT and MAPK [29]. In the rat insulinoma cell lines, epiregulin, an EGFR ligand, stimulates cell growth [30] and in the enterochromaffin (EC) cell line KRJ-I, TGFα and TGFβ increase cell growth [13]. Moreover, in the ileal carcinoid cell line, HC45, and in the rectal carcinoid cell line HC49, EGFR and its activated/phosphorylated form are found, and treatment with an EGFR kinase inhibitor reduces cell proliferation [14].

One of the most frequently used mechanisms for growth involving EGFR, besides overexpression, mutation and amplification, is EGFR-transactivation [15,16,31]. EGFR-transactivation is most frequently due to activation of various G protein-coupled receptors (GPCRs)[16], frequently due to ectopic autocrine secretion, and the release from other sites, of GI hormones/neurotransmitters, or to the release of other biologically active substances (bioactive lipids, prostaglandins, etc.) [15]. This mechanism occurs in a number of common tumors, including prostate, lung, breast, colon and head and neck squamous cell carcinoma (HNSCC) [16,17,32]. In lung cancer, cell proliferation is increased by transactivating EGFR due to the activity of bombesin-related peptides [19–21] and pituitary adenylate cyclase activating polypeptide (PACAP) [17]. The cross-talk between these two pathways is complex and frequently involves second messengers, including changes in intracellular Ca^{+2}, activation of protein kinase C (PKC), adenylate cyclase, generation of reactive oxygen species (ROS), activation of non-receptor tyrosine kinases, such as Src, and the activity of matrix metalloproteinases (MMPs) [16].

GI hormones/neurotransmitter GPCRs are present in Foregut NETs/PETs and can effect cell proliferation [24]. Somatostatin receptors are highly expressed and homogenously distributed in these tumors [34–36], as well as receptors for VIP, gastrin/cholecystokinin

(CCK) [24], secretin [36], PACAP and mammalian bombesin receptors (GRPR, NMBR). Although numerous studies have been performed to assess the effect of GI hormones/neurotransmitters on growth of various cancers [15], few studies have focused on their effect in Foregut NETs/PETs proliferation [13,38–42]. Somatostatin receptor agonists reduce growth of a number of NETs [3,36,43,44], as well as antagonists of CCK2R inhibit gastric carcinoid tumor growth [42]. In the EC cell line, KRJ-I cell line and in the broncho-pulmonary NET cell line, NCH720, serotonin stimulates cell proliferation and the synthesis of several growth factors [41]. In no cases were the role and the mechanism of EGFR-transactivation in mediating these growth effects, explored.

To address this question, in this study, the role of EGFR-transactivation in growth control by various GI hormones/neurotransmitters, was evaluated in a number of Foregut NET/PET cell lines [BON, QGP-1, Rin-14B cells] and detailed mechanistic studies were performed on BON cells. BON cells, are derived from a lymph node metastasis of a pancreatic neuroendocrine carcinoid tumor and resemble both foregut and midgut characteristics [45]. Previous studies have shown that treatment of the BON cells with EGF, as well as with other growth factors and GI hormones/neurotransmitters, like PACAP, stimulates cell proliferation. Moreover, treatment with Gefitinib (Iressa, Astra Zeneca) or AG1478, inhibitors of EGFR [31], completely blocks BON cell basal proliferation and induces apoptosis [11]. Among the substances synthesized and secreted, BON cells produce serotonin and several peptides such as neurotensin (NT) and bombesin [45] and display high bombesin receptor (GRPR, BRS-3) and high VIP receptor (VPAC1) densities, as well as for somatostatin receptors (sst1, 2, 5) [43,46]. To confirm our results of the importance of EGFR trasactivation in BON cells and establish as it a common mechanism occurring in neuroendocrine tumors, we also evaluated the effect of GI hormones/neurotransmitters in other two Foregut NET/PET cell lines: the human somatostatinoma QGP-1 and the rat islet cell tumor Rin-14B.

A number of our findings support the conclusion that various GI hormones/neurotransmitters, acting through their GPCRs, are able to stimulate foregut NET/PET cell line's proliferation and that it is mediated in large part by transactivating EGFR. First, using two different experimental approaches assessing cell growth, an MTT assay and assessment of [^{3}H]-thymidine intake, we found that NT, a bombesin receptor agonist (Bn-analogue), PACAP, bradykinin, as well as TGFα and EGF, were all able to stimulate BON cell growth, as well as in QGP-1 cells. Second, pre-treatment with the EGFR tyrosine kinase inhibitor, AG1478, blocked the increase of cell growth induced by the GI hormones in both growth assays and in both the cell lines, supporting the conclusion that the activation of EGFR is an important step in mediating their growth effect. Third, we demonstrated directly that the GI hormones/neurotransmitters inducing growth activate the EGFR, inducing the activation of downstream signaling cascades [15], by determining, with Western blotting, the generation of the Y^{1068} phosphorylated form of EGFR, which has been shown to correlate with the activation of EGFR [25]. Fourth, the Y^{1068}EGFR phosphorylation induced by NT or Bn-analogue, as well as that induced by EGF, was prevented by pre-treatment with the EGFR tyrosine kinase inhibitor, AG1478, under similar conditions that it inhibited BON cells growth by these agents. Moreover, these results were confirmed in two other foregut NET/PET cell lines, QGP-1 and Rin-14B. Neurotensin has been previously demonstrated to stimulate cell growth in prostate cancer cells [23,47] and in pancreatic adenocarcinoma cells [48]; bombesin [21], as well as NMB [19], stimulate cell proliferation in non-small-cancer lung cells and HNSCC [37]. and bradykinin stimulates growth of HNSCC through its B2 receptor [32]. Our results are consistent with other studies, which demonstrate PACAP [38], can stimulate growth in BON cells and that EGF can stimulate growth of both KRJ-I cells and BON cells [13]. Similarly in various non-endocrine tumors, AG1478 or other EGFR inhibitors [PD153035 or gefitinib] are able to inhibit cell growth stimulated by various GI

hormones/neurotransmitters [19,21,23,32,47,48] suggesting their growth stimulation is at least partially mediated by transactivation of the EGF receptor. In contrast to results in neuroblastoma tumors and other non-endocrine tumors [49], we found no effect of galanin on growth of BON cells, nor did we find that CCK/gastrin receptors stimulate BON cells growth, as reported in other Foregut NETs/PETs [13].

Our studies demonstrated that the activation of EGFR by GI hormones/neurotransmitters has important similarities and differences from that of the growth factor, EGF in BON cells and in other tissues. The action of EGF and the GI hormones (neurotensin and the bombesin agonist analogue) differed in their kinetics of EGFR activation. Specifically, EGF-induced Y^{1068}EGFR tyrosine phosphorylation was very rapid (peak at 2 minutes) followed by a rapid decline whereas, Y^{1068}EGFR tyrosine phosphorylation induced by either the Bn-analogue or NT was slower with a peak at 60 min, followed by a slight decrease. These results differ from studies in non-endocrine tumors where bombesin receptor agonists in HNSCC [22] and in non-small cancer lung cells [19–21] cause rapid EGFR tyrosine phosphorylation. In contrast, the delayed time course for neurotensin stimulation of EGFR tyrosine phosphorylation, in BON cells, is similar to that reported in prostate cancer cells [23]. The rapid kinetics of EGFR tyrosine phosphorylation we observed in BON cells with EGF was also observed in non-transformed human fibroblasts cells [50]. However, it differed from that reported in HNSCC studies, in which EGF-stimulated EGFR tyrosine phosphorylation showed a slower kinetic[23,51]. Moreover, EGFR phosphorylation induced by a bombesin receptor agonist and neurotensin in BON cells was dose-dependent, with an EC_{50} in the nanomolar range, similar to the ability of NT to stimulate EGFR tyrosine phosphorylation in the prostate cancer cell line, PC3 [47] and of bombesin receptor agonists in non-small lung cancer cells [21]. These results demonstrated that the kinetics/stoichiometry of GI hormones/neurotransmitters and growth factors stimulation of EGFR-transactivation can vary in different cells, raising the possibility that different mechanisms of activation may be involved.

Two principal mechanisms play important roles in mediating EGFR-transactivation by GPCRs: a ligand-dependent mechanism, involving the generation through matrix metalloproteinases(MMPs) of an EGF-like ligand or a ligand-independent mechanism, that involves the activation of EGFR by the generation of intracellular signals from activation of the GPCR [16]. A number of our results support the conclusion that both mechanisms are important in the GPCR-mediated EGFR-transactivation in BON cells. This conclusion was supported by the finding that Y^{1068}EGFR phosphorylation induced by NT and Bn-analogue in BON cells is inhibited 60–70%, but not completely, by the pre-treatment with GM6001, a broad spectrum MMP inhibitor suggesting the ligand-dependent mechanism plays a particularly important role. This result is similar to those obtained for bombesin-related peptides, in non-small cancer lung cells and HNSCC [19,21,22], but it is different from the effect of endothelin-1 in non-transformed fibroblasts and in prostate cancer cells, in which the inhibition of MMPs completely block EGFR-transactivation [52].

In various non-endocrine cells, transactivation of the EGFR, in some cases by various GPCRs, requires activation of protein kinase C (PKC), activation of Src kinases and the generation of reactive oxygen species (ROS) [17,19–21,48,53]. Activation of receptors for PACAP, NT, bradykinin and bombesin are all known to activate PKCs in various cells [17,47,54]. Our results are consistent with the conclusion that the transactivation of EGFR in BON cells occurring with activation of NT or Bn receptors is mediated by both PKC-dependent and PKC-independent mechanisms. This conclusion is supported by our finding that after pre-incubation with the broad spectrum PKC inhibitor GF109203X, both NT and the bombesin receptor analog activation of stimulation of Y^{1068} EGFR tyrosine phosphorylation is markedly reduced (70–80 %), but not completely inhibited. Similar

results are found in some non-endocrine cancer cells, such as for NT- and bombesin-stimulated transactivation of EGFR in prostate cancer cells [47] and in non-small cancer lung cells [21] is through a PKC-mediated mechanism [16]. Our results also demonstrate that activation of Src kinases is important in mediating EGFR-transactivation in BON cells, because we found that EGFR activation induced by NT or the Bn-analogue, is completely inhibited by the specific Src kinase inhibitor, PP2, but not by a inactive related compound, PP3. This result is consistent with findings obtained for bombesin-related peptides in non-small cancer lung cells [19,21], NT in prostate cancer cells and endothelin and angiotensin in smooth muscle cells [23,26]. Reactive oxygen species (ROS) are products of normal metabolism and pathological processes that can have numerous intracellular signaling effects involved in different far ranging cellular processes including regulation of intracellular calcium, inhibition of protein function, growth effects and promoting apoptosis [16,19]. We have found that the generation of ROS is required for transactivation of EGFR induced by NT or the Bn-analogue in BON cells, since by treating BON cells either with the ROS scavenger Tiron or the antioxidant N-acetylcysteine, the phosphorylation of Y^{1068} of EGFR was inhibited. Findings in a number of non-endocrine tissues are consistent with these results with angiotensin II and endothelin EGFR-induced transactivation, in vascular smooth muscle cells, urotensin EGFR transactivation in renal tubular cells [53] also shown to be redox sensitive [26], as well as bombesin related peptides and PACAP induced EGFR-transactivation, in non-small cancer lung cells [17,19,21].

In conclusion our study demonstrates that a number of gastrointestinal homones/neurotransmitters have growth effects on Foregut NETs/PETs and for the first time demonstrates that these growth effects are highly dependent on transactivation of the EGF receptor. Mechanistic studies show the EGFR-transactivation is partially dependent on the activation of PKC, Src kinases, matrix melloproteinases and the generation of oxygen free radicals. Because NETs of the gastrointestinal system frequently possess EGF receptors as well as receptors for various GI hormones/neurotransmitters [9–14,24,24], these findings raise the possibility that the inhibition of this transactivation could be a novel method of treating these tumors. This conclusion is supported by clinical findings in a phase II trial of the EGFR inhibitor, Gefitinib in patients with progressive metastatic NETs, has shown stabilization of the tumors [3]. Furthermore, the combination of gefitinib combined with a receptor antagonist for the bombesin receptor which transactivates the EGFR, have a potentiated cytotoxic effect on lung cancer cells, head/neck squamous cancer cells and prostate cancer cells [19,20,31,55–57].

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Abbreviations

Bn	bombesin
EGFR	epidermal growth factor receptor
GI	gastrointestinal
GPCR	G protein coupled receptor
HNSCC	head and neck squamous cell cancers
MMP	matrix metalloproteinases
NETs	neuroendocrine tumors

NT	neurotensin
PETs	pancreatic endocrine tumors
PACAP	pituitary adenylate cyclase activating peptide
PKC	protein kinase C
ROS	reactive oxygen species
TGFα	Transforming growth factor alpha
VIP	vasoactive intestinal peptide

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Research highlights

- In the foregut NET cells BON and QGP-1, various GI hormones/ neurotransmitters stimulated growth (bradykinin, Bombesin, PACAP, Neurotensin).
- NET growth by the GI hormones neurotransmitters was inhibited by AG1478, an EGFR inhibitor.
- Each of the GI hormones/neurotransmitters stimulated EGFR-transactivation with Y1068 tyrosine phosphorylation of the EGFR.
- EGFR-transactivation required activation of Src kinases, PKC and matrix metalloproteinases.
- EGFR-transactivation required activation of reactive oxygen species.

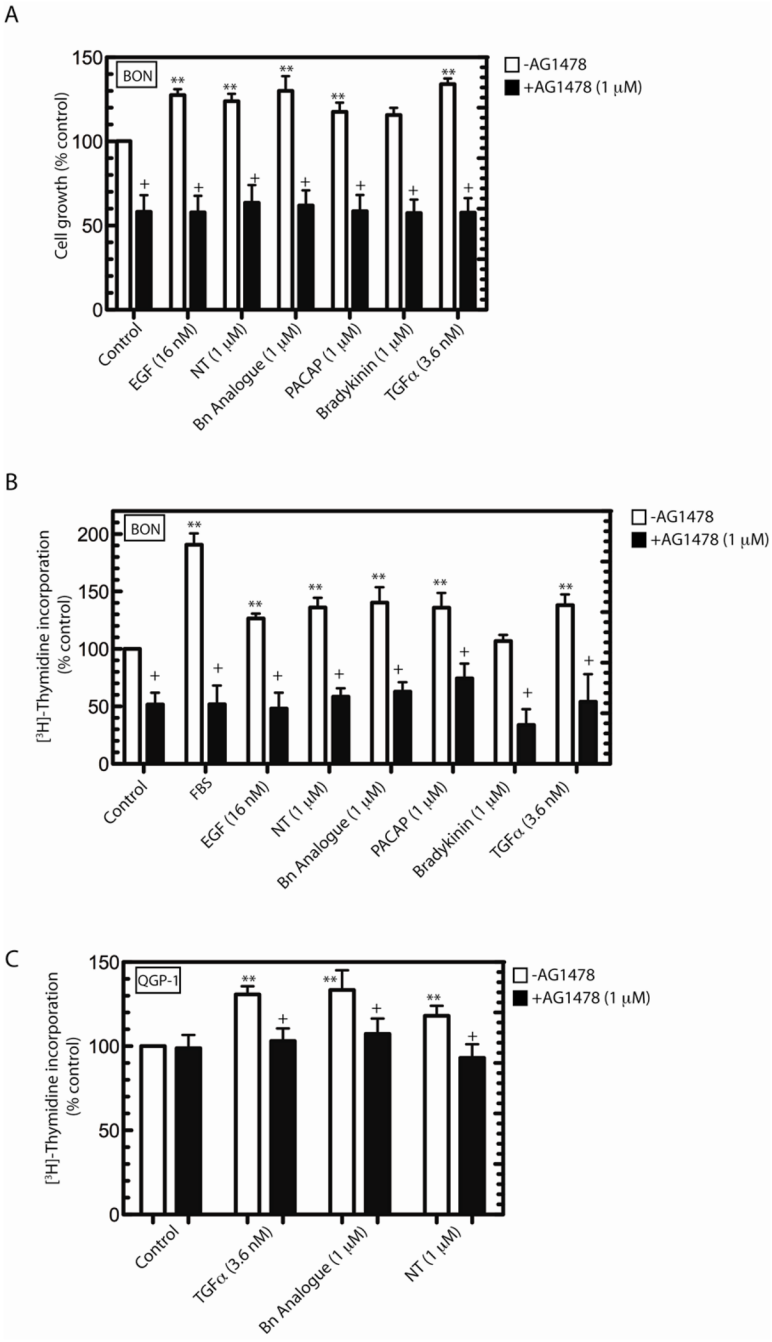

Figure 1. Effect of the EGFR inhibitor, AG1478 on growth of BON and QGP-1 neuroendocrine tumor cells stimulated by EGF, TGFα and various GI hormones

BON and QGP-1 cells were incubated for 24 hours, in serum-free medium, either with EGF, TGFα or the indicated GI hormones, at the indicated concentrations, in the presence or in the absence of the EGFR inhibitor, AG1478 (1 μM). Cell growth was evaluated by either an MTT assay (A) or measuring [^{3}H]-Thymidine uptake (B) for BON cells, and measuring [^{3}H]-Thymidine uptake for QGP-1 cells (C). **p< 0.01 stimulants *vs.* control; +p<0.01 stimulants *vs.* AG1478 co-treatment. Results are mean±SEM from 6 experiments.

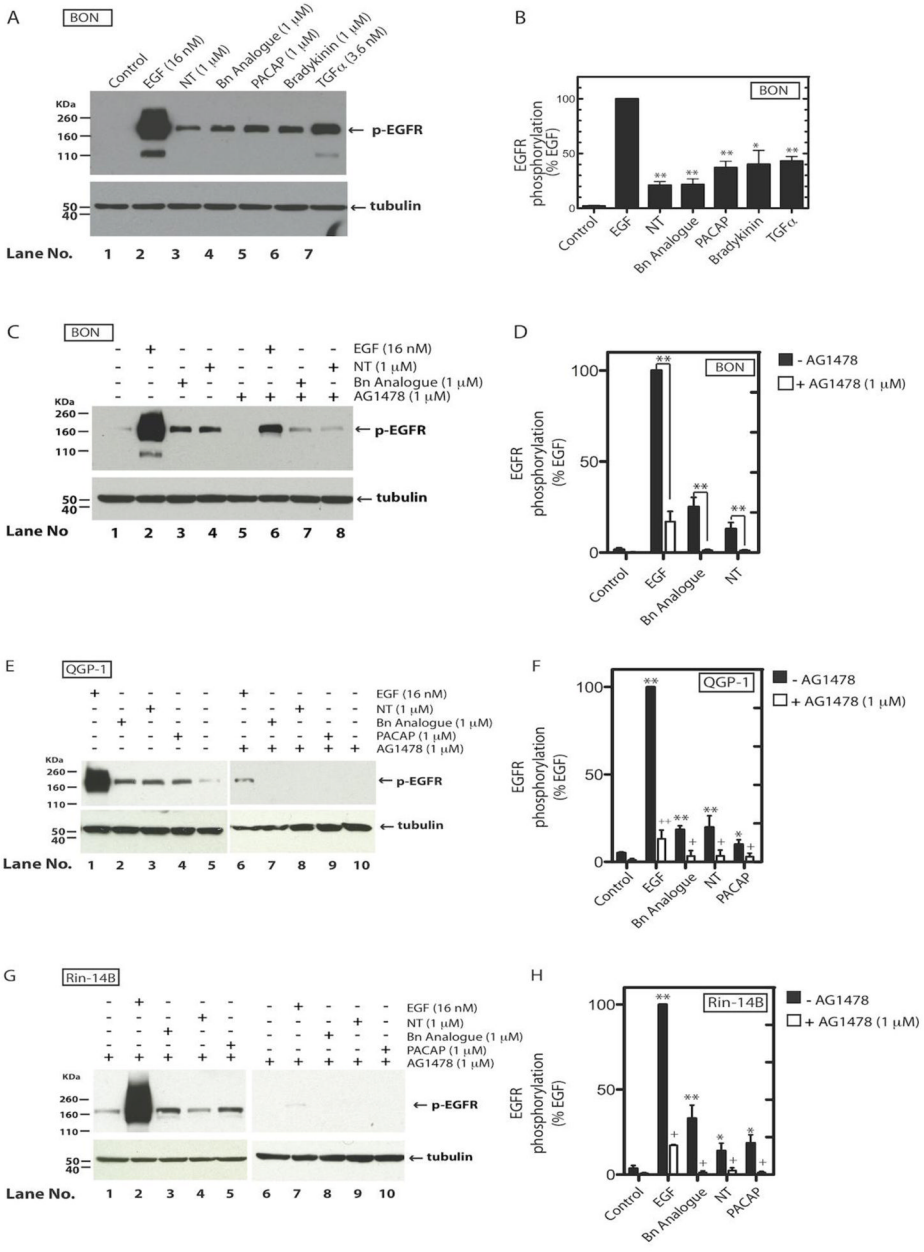

Figure 2. Ability of EGF, TGFα or various GI hormones to stimulate EGFR tyrosine 1068 phosphorylation and the effect of the EGFR inhibitor, AG1478 in BON cells (Panels A–D) and in QGP-1 and Rin-14B neuroendocrine tumor cells (Panels E–H)

Left panels (Panels A, C, E, G) show representative Western blot analysis of EGFR Y^{1068} phosphorylation of the indicated NET cells treated for 30 min with EGF, TGFα or the various GI hormones at the indicated concentrations (these experiments are representative of 4 others). Right panels (Panels B, D, F, H) show the results of the densitometric analysis of the stimulation of EGFR Y^{1068} phosphorylation shown in the corresponding left panel for the indicated NET cells. Shown are means± SEM from 4 separate experiments **p< 0.01; *p<0.05 *vs.* no AG1478.

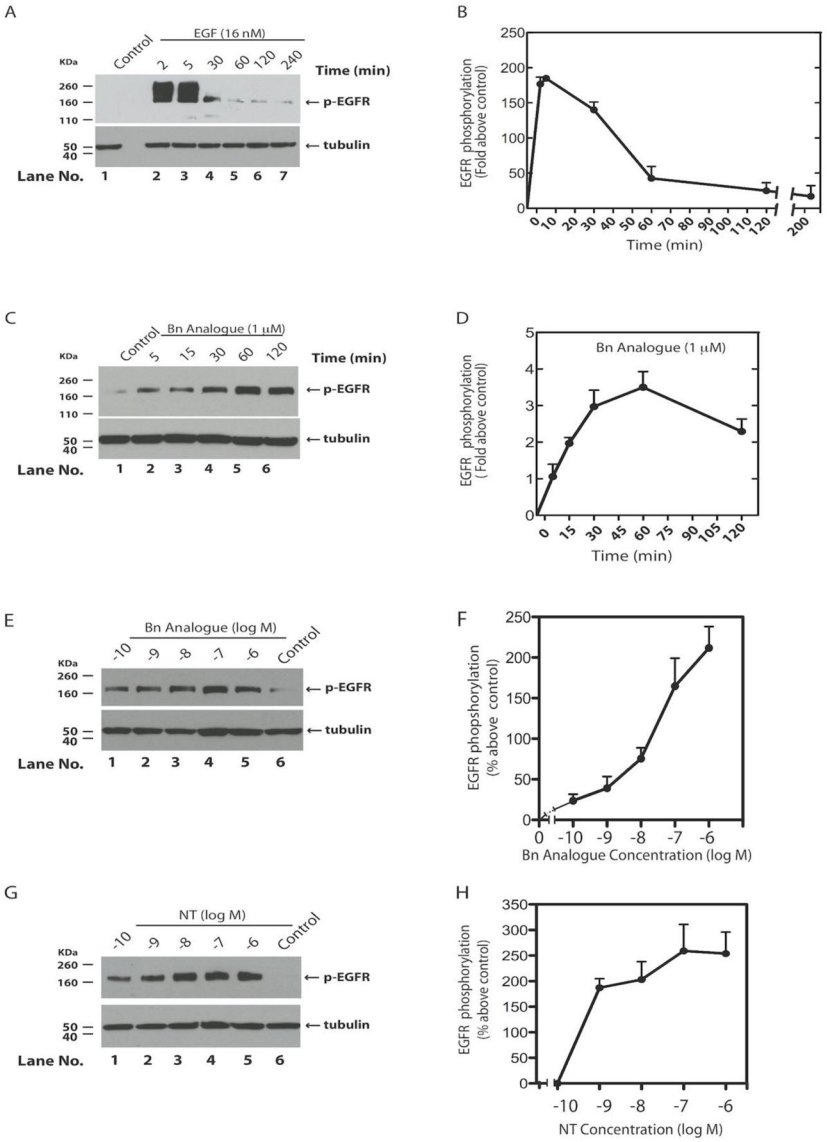

Figure 3. Time courses (Panels A–D) of EGFR Tyrosine 1068 phosphorylation stimulation (EGF, the Bn-analogue) and Dose-response stimulation (Panels E–H) (NT, the Bn-analogue) in BON cells

Left panels (Panels A, C, E, G) show a representative Western blot analysis of EGFR Y^{1068} phosphorylation in BON cells treated with EGF (16 nM) (Panel A), Bn-analogue (1 μM) (Panel C) or NT (1 μM) (Panel G), at the indicated times. This experiment is representative of 4 others. The right panels (Panels B, D, F, H) show the results of a densitometric analysis of results of experiments shown in the accompanying left panel. Shown are mean± SEM of EGFR Y^{1068} phosphorylation induced by EGF (Panel B), Bn-analogue (Panel D) or NT (Panel H) treatments. Results are the mean± SEM of 4 separate experiments.

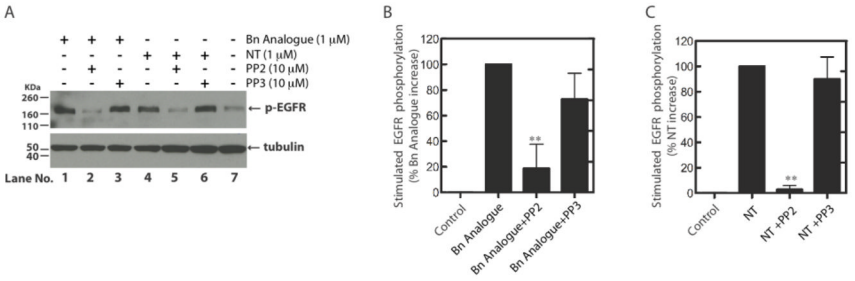

Figure 4. Effect of Src inhibition on EGFR-transactivation by Bn-analogue and NT in BON cells

(A) Representative Western blot analysis of EGFR Y^{1068} phosphorylation of BON cells treated with Bn-analogue (1 μM) or NT (1 μM) for 1 hour, with or without the Src inhibitor PP2, (10 μM) or its inactive related peptide, PP3 (10 μM). This result is representative of 4 others. (B, C) Densitometric analysis of the EGFR phosphorylation, induced by Bn-analogue (B) or NT (C), shown in panel A. Results are expressed as the mean± SEM of 4 experiments. **p<0.01 *vs.* stimulant alone.

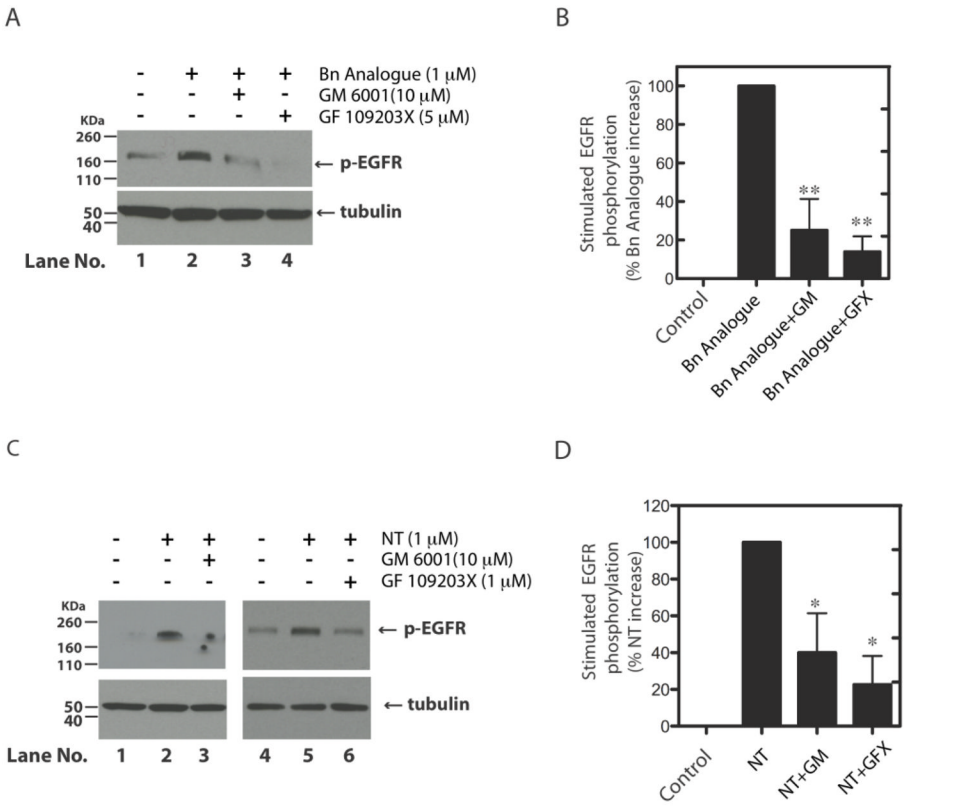

Figure 5. The effect of PKC inhibition or inhibition of metalloproteinases (MMPs) on EGFR-transactivation induced by Bn-analogue or NT, in BON cells

(A, C) Representative Western blot analysis of EGFR Y^{1068} phosphorylation of BON cells treated with Bn-analogue (1 μM) (A) or NT (1 μM) (C) for 1 hour, with or without the PKC inhibitor, GF 109203X or the MMP inhibitor, GM 6001. These experiments are representative of 3 different experiments. (B, D) Densitometric analysis of the effect of inhibition of PKC or MMP, on EGFR phosphorylation, induced by Bn-analogue (B) and NT (D), as shown in panels A and C. Results are the mean± SEM of 3 experiments. **p< 0.01; *p<0.05 *vs.* stimulant alone.

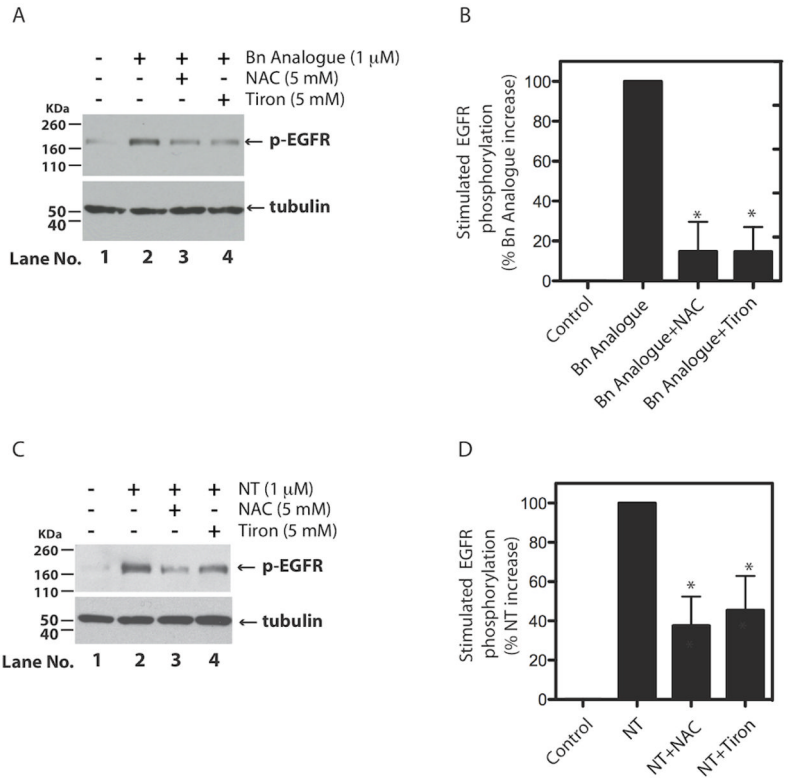

Figure 6. Effect of reactive oxygen species (ROS) inhibition on EGFR-transactivation induced by Bn-analogue or NT, in BON cells

(A, C) Representative Western blot analysis of EGFR Y^{1068} phosphorylation of BON cells treated with Bn-analogue (1 μM) (A) or NT (1 μM) (C) for 1 hour, with or without the ROS production inhibitors, N-acetylcysteine (NAC) or Tiron. These experiments are representative of 3 different experiments. (B, D) Densitometric analysis of the effect of inhibition of PKC or MMP, on EGFR phosphorylation, induced by Bn-analogue (B) and NT (D), as shown in panels A and C. Results are the mean± SEM of 3 experiments. *$p<0.05$ *vs.* stimulant alone.