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Neuromedin B receptors regulate EGF receptor tyrosine phosphorylation in lung cancer cells

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Abstract

Neuromedin B (NMB), a member of the bombesin family of peptides, is an autocrine growth factor for many lung cancer cells. The present study investigated the ability of NMB to cause transactivation of the epidermal growth factor (EGF) receptor in lung cancer cells. By Western blot, addition of NMB or related peptides to NCI-H1299 human non-small cell lung cancer (NSCLC) cells, caused phosphorylation of Tyr¹⁰⁶⁸ of the EGF receptor. The signal was amplified using NCI-H1299 cells stably transfected with NMB receptors. The transactivation of the EGF receptor or the tyrosine phosphorylation of ERK caused by NMB-like peptides was inhibited by AG1478 or gefitinib (tyrosine kinase inhibitors) and NMB receptor antagonist PD168368 but not the GRP receptor antagonist, BW2258U89. The transactivation of the EGF receptor caused by NMB-like peptides was inhibited by GM6001 (matrix metalloprotease inhibitor), PP2 (Src inhibitor), or transforming growth factor (TGF) α antibody. The transactivation of the EGF receptor and the increase in reactive oxygen species caused by NMB-like peptides was inhibited by N-acetylcysteine (NAC) or Tiron. Gefitinib inhibited the proliferation of NCI-H1299 cells and its sensitivity was increased by the addition of PD168368. The results indicate that the NMB receptor regulates EGF receptor transactivation by a mechanism dependent on Src as well as metalloprotease activation and generation of reactive oxygen species.

Keywords

lung cancer; neuromedin B; epidermal growth factor receptor; transactivation; reactive oxygen species

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1. Introduction

Numerous studies demonstrate that members of the mammalian bombesin receptor family [gastrin-releasing peptide (GRP) receptor, neuromedin B (NMB) receptor, bombesin receptor subtype 3 (BRS-3)] can play an important role in the growth of a number of normal and neoplastic tissues (Jensen et al., 2008; Patel et al., 2006; Jensen and Moody, 2006, Lango et al., 2002). This occurs because these receptors are frequently over-expressed or ectopically expressed by many tumors [85–100% small cell lung cancer (SCLC) and non-SCLC (NSCLC), 40–75% breast cancer, 100% head/neck cancer, 65–100% prostate cancer, 100% ovarian cancer, 75–100% pancreatic cancer] (Jensen et al., 2008, Patel et al., 2006, Jensen and Moody 2006, Lango et al., 2002, Reubi et al., 2002, Siegfried et al., 1999). GRP and NMB are frequently synthesized and released by these tumors and both peptides can have an autocrine growth effect or a potent direct effect on tumor growth/differentiation (Cuttitta et al., 1985; Giaccone et al., 1992).

The growth mechanisms involved and the possible therapeutic potential have been well studied in the case of the GRP receptor, particularly in lung, prostate and head/neck cancer cells (Jensen et al., 2008; Jensen and Moody 2006, Liu et al., 2003; Zhang et al., 2007). Recent studies show activation of the GRP receptor not only leads to proliferation, it also results in the rapid tyrosine phosphorylation of the EGF receptor and ERK by stimulating matrix metalloproteases to cause TGF α and amphiregulin release, by a Src-dependent mechanism (Lui et al., 2003; Zhang et al., 2007; Thomas et al., 2005). Activation of c-Src and EGF receptor transactivation are essential for GRP receptors to stimulate proliferation in some tumor cells (Zhang et al., 2004). Furthermore, this cascade has important therapeutic implications because the combination of a GRP receptor antagonist and an EGF receptor tyrosine kinase inhibitor resulted in markedly increased anti-proliferative activity in head/neck squamous cell cancers (Xiao et al., 2003; Liu et al., 2007). Transactivation of the EGF receptor due to GRP receptor activation occurs in a number of head/neck, lung and prostate cancer cells (Zhang et al., 2004; Xiao et al., 2003, Liu et al., 2007), as well as a number of other GRP receptor-containing cells (Santiskulvong et al., 2003). These findings may have important implications for treatment of a number of cancer patients.

The NMB receptor is widely expressed in tumors, particularly lung cancer (Jensen et al., 2008; Jensen and Moody, 2006). Similar to the GRP receptor, activation of the NMB receptor is known to stimulate phospholipase C and D resulting in calcium mobilization, activation of the serine/threonine kinase protein kinase C (Fathi et al., 1996; Corjay et al., 1991; Moody et al., 1992; Lach et al., 1995) and activation of some tyrosine kinase cascades resulting in tyrosine phosphorylation of a number of proteins (p125^{FAK}, paxillin and ERK). The results suggest that the GRP and NMB receptors have similar signal transduction mechanisms. In this communication, the ability of the NMB receptors to regulate the tyrosine phosphorylation of the EGF receptor and ERK was investigated.

2. Materials and Methods

2.1 Cell culture

NSCLC NCI-H1299 cells, which are known to contain low levels of native NMB receptor and wild type EGF receptor (Corjay et al., 1991), were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% heat-inactivated fetal bovine serum (Invitrogen, Grand Island, NY). To study the effect of NMB receptor expression level on various parameters, NCI-H1299 cells with increased stable expression of the NMB receptor were used (Benya et al., 1992) and were grown in RPMI 1640 supplemented with 300 mg/l G418 sulfate (Sigma-Aldrich, St. Louis, MO). The cells were split weekly 1/20 with trypsin-

ethylenediaminetetraacetic acid (EDTA). The cells were mycoplasma free and were used when they were in exponential growth phase after incubation at 37°C in 5% CO₂/95% air.

2.2. Receptor binding

BA1, which binds with high affinity to all human bombesin receptors, was radiolabeled using iodogen and HPLC purified as reported previously (Mantey et al., 1997). The ability of NMB-like peptides and the NMB receptor selective antagonist, (S)-a-Methyl-a-[[[(4-nitrophenyl)amino]carbonyl]amino]-N-[[1-2-pyridinyl]cyclohexyl]methyl]-1H-indole-3-propanamide (PD168368; Ryan et al., 1999, Moody et al., 2000), to inhibit specific [¹²⁵I]BA1 binding to NCI-H1299 cells transfected with NMB receptor were investigated. PD168368 was dissolved in dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO) at a concentration of 10 mM. GRP, NMB, bombesin and BA1 were purchased from Phoenix Pharmaceuticals, Belmont, CA, whereas the GRP receptor antagonist ((4'-hydroxy)-3-phenylpropanoyl)-His-Trp-Ala-Val-D-Ala-His-D-Pro-psi(CH₂NH)-Phe-NH₂ (BW2258U89) was a gift from Dr. J. McDermid (Univ. North Carolina). The NCI-H1299 cells were washed 3 times in SIT medium (RPMI-1640 containing 3 × 10⁻⁸ M sodium selenite, 5 µg/ml bovine insulin and 10 µg/ml transferrin (Sigma-Aldrich, St. Louis, MO)). The cells were incubated in SIT buffer containing 0.25% bovine serum albumin and 250 µg/ml bacitracin (Sigma-Aldrich, St. Louis, MO) and [¹²⁵I]BA1 (100,000 cpm) added as well as various concentrations of unlabelled competitor. After incubation at 37°C for 30 min, free [¹²⁵I]BA1 was removed by washing 3 times in buffer and the cells which contained bound [¹²⁵I]BA1 dissolved in 0.2 N NaOH and counted in a gamma counter. The half maximal inhibitory concentration (IC₅₀) was calculated for each unlabeled competitor.

2.3. Western Blot

The ability of NMB to stimulate tyrosine phosphorylation of EGF receptor or ERK (p42/p44 MAP kinase) was investigated by Western blot. NCI-H1299 cells were cultured in 15 cm dishes. When a monolayer of cells formed they were placed in SIT media for 3 h. Routinely, NSCLC cells were treated with gefitinib, (N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinazolinamine (AG1478), PD168368, BW2258U89, (R)-N4-Hydroxy-N1-[(S)-2-(1H-indol-3-yl)-1-methylcarbamoyl-ethyl]-2-isobutyl-succinamide (GM6001), 4-(4'-Phenoxyanilino)-6,7-dimethoxyquinazoline, 6,7-Dimethoxy-N(4-phenoxyphenyl)-4-quinazolinamine (PP2), N-acetyl-cysteine (NAC), Tiron or TGFα antibodies for 30 min prior to stimulation with NMB-like peptides. Then cells were treated with 0.1 µM NMB or BA1 for 2 min, washed twice with PBS and lysed in buffer containing 50 mM Tris.HCl (pH 7.5), 150 mM sodium chloride, 1% Triton X-100, 1% deoxycholate, 1% sodium azide, 1 mM ethyleneglycoltetraacetic acid, 0.4 M EDTA, 1.5 µg/ml aprotinin, 1.5 µg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride and 0.2 mM sodium vanadate (Sigma-Aldrich, St. Louis, MO). The lysate was sonicated for 5 s at 4°C and centrifuged at 10000 × g for 15 min. Protein concentration was measured using the BCA reagent (Pierce Chemical Co., Rockford, IL), and 400 µg of protein was incubated with 4 µg of anti-phosphotyrosine (PY) monoclonal antibody, 4 µg of goat anti-mouse immunoglobulin IgG and 30 µl of immobilized protein G overnight at 4°C. The immunoprecipitates were washed 3 times with phosphate buffered saline and analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and Western blotting. Immunoprecipitates were fractionated using 4–20% polyacrylamide gels (Novex, San Diego, CA). Proteins were transferred to nitrocellulose membranes and the membranes were blocked overnight at 4°C using blotto (5% non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM CaCl₂, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide) and incubated for 16 h at 4°C with 1 µg/ml anti-EGF receptor antibody (Cell Signaling Technologies, Danvers, MA) followed by anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Upstate Biotechnologies, Lake Placid, NY). The membrane was washed for 10 min with blotto and twice for 10 min

with washing solution (50 mM Tris/HCl (pH 8.0), 2 mM CaCl₂, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide). The blot was incubated with enhanced chemiluminescence detection reagent for 5 min and exposed to Kodak XAR film. The intensity of the bands was determined using a densitometer.

Alternatively, 20 µg of cellular extract was loaded onto a 15 well 4–20% polyacrylamide gels. After transfer to nitrocellulose, the blot was probed with anti PY¹⁰⁶⁸-EGF receptor, anti-EGF receptor, anti-PY-ERK, anti-ERK or anti-tubulin (Cell Signaling Technologies, Danvers, MA).

2.4. Reactive oxygen species

NCI-H1299 cells were placed in 96 well plates (30,000 cells/well) and cultured overnight. The cells were treated with 10 µM dichlorofluoresceindiacetate (H₂DCF) for 1 h and washed 3 times with serum free SIT medium. Some of the cells were treated with 5 mM NAC, 10 µM PP2, 10 µM GM6001 or 5 mM Tiron for 30 min and then stimuli such as 0.1 µM BA1, 0.1 µM NMB or 10 µM H₂O₂ added. Fluorescence measurements were taken at the various times using an excitation wavelength of 485 nm and emission wavelength of 585 nm.

2.5 Proliferation

Growth studies *in vitro* were conducted using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and clonogenic assays (Moody et al., 2004; Moody et al., 1996). In the MTT assay, NCI-H1299 cells (10⁴/well) were placed in SIT medium and various concentrations of gefitinib or PD168368 added. After 4 days, 15 µl of 0.1 % MTT solution added. After 4 h, 150 µl of dimethylsulfoxide was added. After 16 h, the optical density at 570 nm was determined. In the clonogenic assay, the effects of gefitinib and PD168368 were investigated on NCI-H1299 cells. The bottom layer contained 0.5% agarose in SIT medium containing 5% FBS in 6 well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose, gefitinib, PD168368 and 5 × 10⁴ NCI-H1299 cells. Triplicate wells were plated and after 2 weeks, 1 ml of 0.1% p-iodonitrotetrazolium violet was added and after 16 hours at 37°C, the plates were screened for colony formation; the number of colonies larger than 50 µm in diameter were counted using an Omnicon image analysis system.

3. Results

The ability of NMB-like peptides to increase EGF receptor tyrosine phosphorylation in native NCI-H1299 cells or NMB receptor transfected H1299 cells was investigated (Fig. 1). Figure 1A shows that 0.1 µM NMB, BA1 and bombesin, but not GRP, caused tyrosine phosphorylation of the 170 kDa EGF receptor in native NCI-H1299 cells. NMB or BA1 caused a 1.9-fold increase in the EGF receptor tyrosine phosphorylation, whereas bombesin, but not GRP, caused a 1.4-fold increase in EGF receptor tyrosine phosphorylation (Fig. 1B). These differences were not due to unequal lane loading, because equal amounts of total EGF receptor were loaded onto the gel (Fig. 1A). Similar results were obtained using NCI-H345 lung cancer cells (data not shown). The ability of NMB to cause EGF receptor transactivation was increased if NCI-H1299 cells were stably transfected with NMB receptors (Fig.1). Fig. 1C shows that 100 nM BA1 increased EGF receptor tyrosine phosphorylation in NCI-H1299 cells transfected with NMB receptors 10.2-fold. The increase caused by BA1 was significantly reversed by 1 µM PD168368, a NMB receptor antagonist, but not 1 µM BW2258U89, a GRP receptor antagonist (Fig. 1D). Also, PD168368 antagonized the ability of NMB to cause EGF receptor transactivation (data not shown).

Table I shows that specific [125 I]BA1 binding to NCI-H1299 cells was inhibited with high affinity by NMB or BA1 (IC_{50} = 1 and 12 nM, respectively), but not bombesin or GRP (IC_{50} = 63 and 145 nM, respectively). Also, specific [125 I]BA1 binding was inhibited by the NMB receptor antagonist, PD168368 (IC_{50} = 47 nM), but not by the GRP receptor antagonist, BW2258U89 (IC_{50} > 1000 nM). These results suggest that the NMB receptor regulates the transactivation of EGF receptor in NCI-H1299 lung cancer cells.

The ability of NMB-like peptides to stimulate tyrosine phosphorylation of ERK was investigated (Fig. 2). Fig. 2A shows that the EGF receptor was strongly tyrosine phosphorylated at 0.5, 1 or 2 min after NMB addition to lung cancer cells. In contrast, the 42 and 44 kDa bands of ERK were strongly tyrosine phosphorylated 1 or 2, but not 0.5 min after addition of NMB to NCI-H1299 cells stably transfected with NMB receptors. As a control, equal amounts of ERK or EGF receptor were added to the gels. These results suggest that the NMB receptor regulates tyrosine phosphorylation of ERK and the EGF receptor in lung cancer cells.

The ability of the tyrosine kinase inhibitor, gefitinib to affect EGF receptor transactivation was investigated. Fig. 2B shows that the transactivation of the EGF receptor caused by NMB addition to NCI-H1299 cells was reversed by the addition of 1 or 10 μ g/ml, but not 0.1 μ g/ml of gefitinib. Similarly, 10 μ g/ml but not 1 μ g/ml of AG1478 inhibited the EGF receptor tyrosine phosphorylation caused by NMB (data not shown). These results indicate that tyrosine kinase inhibitors block the ability of NMB to transactivate the EGF receptor.

To investigate the mechanism of transactivation of EGF receptors caused by NMB, the release of EGF-like growth factors was investigated (Fig. 3). Fig. 3A shows the effect of various EGF-like antibodies on EGF receptor transactivation caused by NMB. The increase in EGF receptor tyrosine phosphorylation caused by NMB was decreased by pretreatment of NCI-H1299 cells with anti-TGF α , but not anti-amphiregulin, anti-EGF or anti-HB-EGF antibodies. NMB caused increased ERK tyrosine phosphorylation. The increase in ERK tyrosine phosphorylation caused by NMB addition to NCI-H1299 cells transfected with NMB receptor was inhibited by anti-TGF α , but not anti-amphiregulin (Amph), anti-EGF or anti-HB-EGF (Fig 3A). As controls, equal amounts of EGF receptor or ERK were loaded onto the gels. NMB caused a significant 1.7-fold increase of immunoreactive TGF α in the incubation media exposed to lung cancer cells (Table II). The increased secretion of TGF α from NCI-H1299 cells caused by NMB was reversed if the cells were pretreated with PP2 (Src inhibitor) or GM6001 (metalloprotease inhibitor) (Table II). These results suggest that NMB causes EGF tyrosine phosphorylation as a result of increased release of endogenous TGF α from NCI-H1299 cells.

Furthermore, PP2 and GM6001 markedly inhibited the EGF receptor transactivation caused by NMB (Fig. 3B). As a control, equal amounts of housekeeping protein tubulin were loaded onto the gel. The increase in EGF receptor tyrosine phosphorylation caused by NMB (11-fold) was reduced significantly if PP2 or GM6001 were added to NCI-H1299 cells transfected with NMB receptor (Fig. 3C). The results indicate that NMB receptor stimulation of Src and activation of a matrix metalloprotease are essential for release of endogenous TGF α and subsequent EGF receptor tyrosine phosphorylation.

Reactive oxygen species are important in tyrosine phosphorylation of the EGF receptors in human epidermoid carcinoma cells (Bae et al., 1997). Fig. 4A shows that the addition of NMB to NCI-H1299 cells enriched in NMB receptors, increased EGF receptor tyrosine phosphorylation 9.6-fold (Fig. 4B), which was inhibited if the cells were pretreated with 5 mM NAC, an antioxidant. Figure 4C shows that Tiron, a superoxide scavenger (Ushio-Fukai et al., 2001), inhibited in a dose-dependent manner the ability of NMB to cause EGF

receptor transactivation. Addition of 0.1 or 1 mM Tiron to NCI-H1299 cells transfected with NMB receptors had little effect, however, addition of 10 mM Tiron significantly inhibited the ability of NMB to cause transactivation of the EGF receptor (Fig. 4D). The results suggest that NMB receptors regulate EGF receptor transactivation by generation of reactive oxygen species.

To investigate this possibility directly we assessed reactive oxygen species by determining the relative fluorescence intensity of NCI-H1299 cells loaded with 2',7'-dichlorofluorescein diacetate (H₂DCF) relative to no additions. Table III shows that BA1, NMB or H₂O₂ significantly increased the generation of reactive oxygen species. Tiron had little effect on the NCI-H1299 basal reactive oxygen species. In contrast, the ability of NMB and H₂O₂ to increase the reactive oxygen species was greatly reduced in the presence of Tiron. Similarly, NAC reduced the ability of BA1 and H₂O₂ to increase reactive oxygen species (data not shown). Table III shows that PP2 and GM6001 had little effect on the basal reactive oxygen species but inhibited the increase in reactive oxygen species caused by BA1 addition to lung cancer cells. The results demonstrate directly that GM6001, NAC, PP2 or Tiron impair the ability of NMB receptors to regulate reactive oxygen species in lung cancer cells.

The effects of PD168368 and gefitinib were investigated on lung cancer cellular growth. Figure 5A shows that using the MTT assay, gefitinib inhibited weakly the proliferation of NCI-H1299 cells (IC₅₀ > 30 µg/ml) with concentrations of 1 and 3 µg/ml gefitinib having no effect. The NMB receptor selective antagonist, PD168368 alone inhibited the proliferation of NCI-H1299 cells in a dose-dependent manner. Increasing concentrations of PD168368 caused a progressive shift to the left of the gefitinib dose-response curve demonstrating increased sensitivity of gefitinib. The combination index (CI) was calculated from the MTT data (Chou and Talaly, 1984). The combination index ranged from 0.42–0.63 when varying concentrations of PD168368 were added with gefitinib to NCI-H1299 cells. Because the combination index was significantly less than 1, gefitinib and PD168368 were synergistic at inhibiting lung cancer cellular proliferation.

In the clonogenic assay large robust NCI-H1299 colonies formed (Fig. 5B1). The addition of 0.3 µM PD168368 had little effect on colony formation (Fig. 5B2). The addition of 1 µg/ml gefitinib slightly reduced colony number and size (Fig 5B3). The addition of the combination of 0.3 µM PD168368 and 1 µg/ml gefitinib resulted in a marked 77% decrease in colony number, supporting the synergistic effect of these two compounds together (Fig. 5B4).

4. Discussion

Lung cancer causes over 160,000 deaths in the United States annually and over 80% of the patients have NSCLC. Most patients initially have advanced disease and systemic treatment only modestly affects survival in patients with advanced NSCLC. Among the novel anticancer agents that have been approved for the treatment of advanced NSCLC are the EGF receptor tyrosine kinase inhibitors (erlotinib and gefitinib). Activating mutations of the ATP binding site of the EGF receptor have been associated with dramatic responses to the EGF receptor tyrosine kinase inhibitors and tumor reduction (Paez et al., 2004; Helfrich et al., 2006; Lynch et al., 2004). Unfortunately only approximately 10% of Caucasians have activating mutations. The vast majority of patients who have wild type EGF receptor have tumors that are resistant to the concentrations of gefitinib achieved *in vivo*. Strategies to improve the sensitivity in the majority of patients who have wild type EGF receptor would be beneficial.

GRP and NMB and their receptors are present in NSCLC cells and function as autocrine growth factors (Jensen et al., 2008; Patel et al., 2006; Jensen and Moody 2006; Lango et al., 2002). Recent studies show that activation of the GRP receptor causes rapid transactivation of the EGF receptors in head/neck cancer cells (Zhang et al., 2007). This has important clinical implications because the inhibition of the EGF receptor by a tyrosine kinase inhibitor is markedly augmented by the simultaneous addition of a GRP receptor antagonist (Zhang et al., 2007). Even though the other mammalian BN-like peptides and receptors, like the NMB receptor, are known to have growth effects on NSCLC cells, very little is known about its growth signaling cascade and if the EGF receptor is involved.

The present study provides important insights into the NMB receptor signaling cascade in NSCLC cells and a number of the results support the conclusions that transactivation of the EGF receptor is an important mechanism in this signaling cascade and for growth of these cells. First, the NMB receptor regulates the transactivation of the EGF receptor, was directly demonstrated by showing that NMB addition to lung cancer cells caused tyrosine phosphorylation of the EGF receptor. Second, the activation of ERK occurring after NMB receptor stimulation was largely dependent on the transactivation of EGF receptor because it was inhibited by EGF receptor tyrosine kinase inhibitors in a similar fashion to that seen with NMB receptor antagonists (T. Moody, unpublished). Previous studies demonstrate that NSCLC cell lines including NCI-H1299 have immunoreactive NMB and/or NMB mRNA (Giaccone et al., 1992), secrete NMB and that NMB has an autocrine growth effect in these cells. These observations coupled with the findings in our study demonstrate that this autocrine growth effect is mediated in large part by NMB receptor mediated transactivation of the EGF receptor. The EGF receptor can be transactivated by numerous G-protein coupled receptors such as adenosine, angiotensin II, bradykinin, cholecystokinin, endothelin, gastrin, GRP, lysophosphatidic acid, neurotensin, prostaglandins, vasoactive intestinal peptide and vasopressin (Schafer et al., 2004; Cheng-Hsien et al., 2006). This can lead to the activation of numerous signaling cascades including protein kinases (PKC, PKD), MAP kinases (ERK, JNK, p38) and PI3K/Akt/mTOR/p70S6Ks (Jensen and Moody, 2006).

The signaling cascades for these different G protein-coupled receptors to stimulate EGF receptor transactivation varies markedly in different cells and includes: activation of a metalloprotease with release of an EGF-like molecule; activation of second messengers including, PKCs, cellular calcium or reactive oxygen species; activation of intracellular non-receptor tyrosine kinases such as PYK-2 or Src family kinases and inactivation of protein tyrosine phosphatases (Cheng-Hsien et al., 2006). Our results support the conclusion that with NMB receptor activation in NSCLC cells the subsequent EGF receptor transactivation requires activation of Src, stimulation of a metalloprotease, release of TGF α and generation of reactive oxygen species. These results have similarities and differences with other G protein-coupled receptors stimulating EGF receptor transactivation in other tumors or cells. The requirement for activation of a Src kinase for NMB receptor stimulation of EGF receptor transactivation is similar to GRP receptor activation in head/neck squamous cell cancers and prostate cancer cells (Zhang et al., 2004) and neurotensin or endothelin receptor activation in prostate cancer cells, however, differs from other G protein-coupled receptors where Src activation is not required for EGF receptor transactivation (Jensen and Moody, 2006). Similar to NMB receptor activation, GRP receptor activation in head/neck squamous cancer cells increased TGF α release (Zhang et al., 2004). These results demonstrate that even closely related receptors such as the NMB receptor and GRP receptor can stimulate transactivation in both lung cancer cells and other tumor cells by stimulating metalloproteases to release EGF receptor growth factors. Lastly, the transactivation of EGF receptor caused by NMB receptor stimulation in lung cancer cells requires the generation of reactive oxygen species as an early event, because EGF receptor transactivation was inhibited by NAC or Tiron, which reduce reactive oxygen species (Ushio-Fukai et al., 2001).

This conclusion was demonstrated directly by showing NMB receptor activation increased reactive oxygen species in lung cancer cells, and that the increase was significantly inhibited by NAC or Tiron. Further PP2 and GM60001 inhibited the increase in reactive oxygen species caused by NMB receptor activation. These results indicate that matrix metalloprotease and src activation occur upstream of generation of reactive oxygen species.

This result has both similarities and differences from EGF receptor transactivation by G protein-coupled receptors in other cells. Reactive oxygen species are an important event in the EGF receptor phosphorylation resulting from endothelin ET1 receptor activation in rat renal tubular cells (Cheng-Hsien et al., 2006) or angiotensin AT1 receptor activation in rat aortic vascular smooth muscle cells (Touyz et al., 2003). Stimulation of reactive oxygen species was due to NADPH oxidase activation. This in turn leads to oxidation of cysteine in the catalytic Src homology 2-containing tyrosine phosphatase, which reduces phosphatase enzymatic activity (Cheng-Hsien et al., 2006). Whether a similar mechanism is involved with NMB receptor in NSCLC cells is not established at present.

Cell line NCI-H1299, like most NSCLC tumors, has wild-type EGF receptor (Das et al., 2006). The proliferation of NCI-H1299 cells is inhibited weakly by gefitinib ($IC_{50} = 8.6 \mu M$), whereas the growth of NCI-H3225 cells, which have mutant EGF receptors, is strongly inhibited by gefitinib ($IC_{50} = 0.015 \mu M$) (Witta et al., 2006). Figure 5 shows that that PD168368 potentiates the cytotoxicity of gefitinib using NCI-H1299 cells. Using $3 \mu M$ PD168368 in the MTT assay, the gefitinib dose response curve is shifted to the left relative to no additions. Similar results were obtained using NCI-H1299 cells transfected with NMB receptors (data not shown). Currently 10% of NSCLC patients respond to gefitinib and particularly from a subgroup with EGF receptor mutations. If the *in vivo* sensitivity to gefitinib could be increased, a much higher percentage of patients with NSCLC might respond to gefitinib. Over half of the NSCLC cancer cells overexpress EGF receptor (Ushio-Fukai et al., 2001) and lung cancer cells respond to gefitinib *in vitro* (Witta et al., 2006; Ono et al., 2004; Janmaat et al., 2003), even though their sensitivities vary up to 2700-fold.

Previous studies of the GRP receptor in NSCLC as well as other tumors (Lui et al., 2003; Thomas et al., 2005; Xiao et al., 2003), demonstrated that GRP receptor blockade potentiates the action of gefitinib by increasing the tumoral sensitivity to the drug, suggest a novel treatment combining gefitinib and antagonists to these autocrine growth factor receptors. This is particularly appealing because receptor antagonists e.g. PD176252 have been described which have high affinity for both GRP receptor and NMB receptor (Jensen et al., 2008; Ashwood et al., 1998) and thus could be used to treat NSCLC patients.

5. Conclusions

The NMB receptor regulates the tyrosine phosphorylation of the EGF receptor in NSCLC cells via signaling cascades involving Src, matrix metalloproteases and generation of reactive oxygen species. It remains to be determined if agents which disrupt the NMB-induced transactivation of the EGF receptor will be useful in inhibiting the proliferation of NSCLC tumors.

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Fig. 1. Specificity of EGF receptor transactivation. (A) The ability of bombesin (BN), GRP, BA1 or NMB (0.1 μ M) to cause tyrosine phosphorylation of the EGF receptor (R) was determined using native NCI-H1299 cells. As a control equal amounts of EGF receptor were loaded onto the gel. (B) Densitometry analysis of 4 experiments; $P < 0.05$, *; $P < 0.01$, ** using Student's t-test. (C) The ability of the NMB receptor antagonist, PD168368 (PD) or the GRP receptor antagonist, BW2258U89 (BW) to inhibit the tyrosine phosphorylation of the EGF receptor caused by BA1 addition to NCI-H1299 cells transfected with NMB receptor was investigated. (D) Densitometry analysis of 4 experiments; $P < 0.01$ relative to no additions, **, $P < 0.01$ relative to BA1 addition, ^a. These experiments are representative of 3 others.

Fig. 2.

ERK tyrosine phosphorylation and EGF receptor transactivation is caused by NMB. (A) The ability of NMB (0.1 μ M) to stimulate tyrosine phosphorylation of EGF receptor and ERK in NCI-H1299 cells transfected with NMB receptor was determined as a function of time. As controls, equal amounts of EGF receptor or ERK were loaded onto the gel. (B) The ability of NMB (0.1 μ M) to cause tyrosine phosphorylation of the EGF receptor was investigated as a function of gefitinib dose. These experiments are representative of 2 others.

Fig. 3.

EGF receptor transactivation is inhibited by TGF α antibodies, GM6001 or PP2. (A) The effects of amphiregulin, EGF, HB-EGF or TGF α antibodies (5 μ g/ml) on activation of the NMB receptor resulting in EGF receptor and ERK tyrosine phosphorylation was investigated using NCI-H1299 cells transfected with NMB receptor. As a control equal amounts of EGF receptor and ERK were loaded onto the gel. (B) The ability of a matrix metalloprotease inhibitor (GM6001, 10 μ M) or the Src inhibitor (PP2, 10 μ M), to affect stimulation of EGF receptor transactivation with NMB receptor activation. As a control, equal amounts of tubulin were loaded onto the gel. These experiments are representative of

3 others. (C) Densitometer analysis of 3 experiments; $P < 0.01$ relative to no additions, **; $P < 0.01$; *, $P < 0.5$ relative to NMB addition, ^a using Student's t-test.

Fig. 4.

EGF receptor transactivation is inhibited by NAC or Tiron. (A) The ability of NMB (0.1 μ M) to stimulate NMB receptor mediated-EGF receptor transactivation was inhibited by 5 mM NAC. As a control, equal amounts of EGF receptor were loaded onto the gel. (B) Densitometer analysis of 4 experiments, $P < 0.01$, ** using Student's t-test. (C) The ability of varying doses of Tiron (T) to affect the ability of NMB to cause EGF receptor transactivation. As a control, equal amounts of tubulin were loaded onto the gel. (D) Densitometer analysis of 4 experiments, $P < 0.01$, ** using Student's t-test.

Fig. 5.

Ability of the EGF receptor tyrosine kinase inhibitor, gefitinib, the NMB receptor antagonist, PD168368, either alone or in combination to inhibit NCI-H1299 cellular proliferation. (A) The ability of varying doses of gefitinib and PD168368 (PD) to inhibit NCI-H1299 proliferation was investigated as a function of dose using the MTT assay. The mean value $\pm$ S.E. of 8 determinations are indicated. (B) Results of the clonogenic assay evaluating these inhibitors. The ability of no additions (B1), 0.3 μ M PD168368 (B2), 1 μ g/ml gefitinib (B3), and 0.3 μ M PD168368 plus 1 μ g/ml gefitinib (B4) to inhibit NCI-H1299 colony formation is indicated. These experiments are representative of 4 others.

Table I

Binding to lung cancer cells

Ligand	IC ₅₀ , nM
BA1	12 ± 2
Bombesin	63 ± 13
BW2258U89	>1000
GRP	145 ± 26
NMB	1.1 ± 0.1
PD168368	47 ± 8

The IC₅₀ value to inhibit specific [¹²⁵I]BA1 binding to NCI-H1299 cells was investigated using PBS containing 0.25 % BSA and 0.25 mg/ml bacitracin at 37°C for 30 min. The mean value ± S.D. of 3 experiments each repeated in quadruplicate is indicated.

Table IITGF α release

Addition	Immunoreactive TGF α , pg
None	1020 $\pm$ 250 ^a
NMB, 100 nM	1720 $\pm$ 190
NMB + GM6001	1160 $\pm$ 150 ^a
NMB + PP2	1240 $\pm$ 110 ^a

GM6001 and PP2 were incubated with NCI-H1299 cells for 5 min at 37°C. Then 100 nM NMB was added and after 5 min the supernatant was removed and assayed in a TGF α immunoassay. The mean value $\pm$ S.D. of 4 determinations is indicated;

P < 0.05, ^a using Student's t-test relative to NMB.

Table III

Reactive oxygen species

Addition	% Fluorescence Intensity
None	100 ± 7
NMB, 0.1 µM	145 ± 10 ^b
H ₂ O ₂ , 10 µM	589 ± 60 ^b
Tiron, 5 mM	82 ± 11
Tiron + NMB	92 ± 9
Tiron + H ₂ O ₂	200 ± 14 ^b
BA1, 0.1 µM	139 ± 7 ^b
PP2 10 µM	93 ± 5
PP2 + BA1	109 ± 12
GM6001	94 ± 4
GM6001 + BA1	119 ± 10 ^a

The mean ± S.D. of 6 determinations is indicated;

P < 0.01, ^b; P < 0.05, ^a; by ANOVA.

The relative fluorescence intensity was determined 2 hour after the addition of NMB, BA1, or H₂O₂ to NCI-H1299 cells. Tiron, PP2 or GM6001 was added 30 min prior to the addition of NMB, BA1 or H₂O₂.