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RESEARCH ARTICLE

The Transactivation of the Erbb Family of Receptor Tyrosine Kinases Is Regulated by Neurotensin Receptors in Cancer

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ABSTRACT

Neurotensin (NTS)-like peptides are autocrine growth factors for lung cancer. NTS is present in and secreted from lung cancer cells and binds to G protein-coupled receptors causing signal transduction and proliferation. The growth of non-small cell lung cancer (NSCLC) cells is stimulated by NTS and inhibited by SR48692, a small molecule NTSR1 antagonist. Adding NTS to NSCLC cells increases the tyrosine phosphorylation of ErbB receptor tyrosine kinases EGFR, HER2, and HER3 by transactivation. The NTSR1 regulation of EGFR, HER2, and HER3 transactivation is blocked by SR48692, specific tyrosine kinase inhibitors, and certain monoclonal antibodies. Additional agents which impair the transactivation process include PP2 (Src inhibitor), GM6001 (matrix metalloprotease inhibitor), and N-acetyl-cysteine (antioxidant). The results indicate growth stimulation caused by the adding NTS to NSCLC cells may be mediated by transactivation of ErbB RTKs.

Key words: neurotensin, SR48692, EGFR, HER2, HER3

INTRODUCTION

Receptor tyrosine kinase (RTK) phosphorylation can be mediated by peptide G protein-coupled receptors (GPCRs) through transactivation.[1] Activation of GPCRs for neurotensin (NTS) increases phosphatidyl inositol (PI) bisphosphate metabolism to inositol trisphosphate (IP_3) and diacylglycerol (DAG) which elevates cytosolic calcium (Ca^{2+}) and activates protein kinase C (PKC), respectively. [2,3] One minute after adding of NTS to non-small cell lung cancer (NSCLC) cells, Tyr^{1068} phosphorylation of the Epidermal growth factor receptor (EGFR) is increased 3-4 fold.[4] Two minutes after the addition of NTS to NSCLC cells ERK phosphorylation is increased 2-3 fold. The phosphorylated ERK can enter the nucleus and increase nuclear oncogene expression leading to cellular proliferation.[5] Adding of NTS to NSCLC stimulates their proliferation whereas adding of the NTSR1 antagonist SR48692 inhibits proliferation.[6] The NTSR1 mediated growth effects may be due to activating of receptor tyrosine kinases (RTKs) of the ErbB family.[7]

The EGFR or ErbB1 is activated by EGF or transforming growth factor α (TGFα).[8] The EGFR is mutated in approximately 15% of the NSCLC patients and they can be treated with tyrosine kinase inhibitors (TKIs) such as gefitinib or erlotinib.[9] ErbB2 or HER2 lacks an endogenous ligand but is activated when it forms heterodimers with the EGFR or HER3[8]. ErbB2 or HER2 is amplified in approximately 30% of the NSCLC patients.[10] Lapatinib is a TKI that blocks the ATP binding site of the EGFR and HER2. HER2 is blocked by monoclonal antibodies (mAbs) such as trastuzumab which is used to treat certain breast cancer patients.[11] ErbB3 or HER3 binds neuregulin (NRG) 1 or 2.[12] HER3 is biologically active when it forms heterodimers with HER2.[13] Certain mAbs block HER3 and their growth effects are being investigated in clinical trials.[14] While the EGFR, HER2, and HER3 increase cancer growth, ErbB4 or HER4 can stimulate or inhibit mammary gland development and carcinogenesis.[15] Because HER4 has 4 splice variants (SVs), the growth effects may differ depending on which SV is present.[16] This review will focus on how peptide GPCRs for NTS regulate transactivation of the EGFR, HER2, and HER3.

1. NEUROTENSIN

In the normal brain, NTS modulates neurotransmitter systems such as cholinergic, dopaminergic, serotonergic, GABAergic, and glutaminergic.[17] NTS plays a role in hypotension, hypothermia, analgesia, and appetite. NTSR1 is present on dopamine neurons in the ventral mesencephalon associated with the pathophysiology of Parkinson's disease.[18] In the normal periphery, NTS plays a role in fat storage, obesity, and metabolic disorders.[19] NTS is a 13 amino acid peptide that is derived from a 170 amino acid precursor protein.[20] NTS is present in and secreted from lung cancer cells.[21,22] Pro-NTS or long-fragment NTS (LF-NTS) is present in NSCLC patient sera and is neutralized by mAb LF-NTS.[23] Mab LF-NTS impairs the ability of LF-NTS to activate NTSR1 and reduces P-EGFR, P-HER2, P-HER3, P-ERK, P-AKT, P-Src, and P-c-jun in adenocarcinoma cells. It remains to be determined if mAb LF-NTS will be therapeutic for NSCLC patients.

The GPCRs NTSR1 and NTSR2 bind NTS with high affinity. The 418 amino acid NTSR1 is antagonized by SR48692.[24] NTSR1 has an extracellular N-terminal, 7 transmembrane (TM) domains, and an intracellular C-terminal of 42 amino acids. Met^{208} which is in TM4 and Arg ^{327} as well as Phe^{331} in TM6 are important in high affinity binding of NTS and SR48692 to NTSR1.[25] NTSR1 is present in numerous cancers including medullary thyroid cancer, lung cancer, pancreatic cancer, colon cancer, prostate cancer, and breast cancer. [26,27] The Wnt/APC/b-catenin pathway is important for overexpression of NTSR1 in colon cancer.[28] The 410 amino acid NTSR2 is antagonized by levocabastine. [29] NTSR2 is present in chronic lymphocytic leukemia and glioma.[30] Both NTSR1 and NTSR2 have 7 TM domains, whereas NTSR3 or sortolin has 1 TM domain.[31] NTSR3 is a coreceptor that participates in NTS/NTSR1 signaling in breast cancer. NSCLC cell lines are enriched in NTSR1 but not NTSR2.[32,33] NTS and NTSR1 are expressed in approximately 70% of the NSCLC lines examined.[34] NTS and NTSR1 are expressed in approximately 60% of the adenocarcinoma biopsy specimens examined and patients whose tumors had high NTSR1 had lower relapse-free survival. High NTSR1 expression in patients with ductal breast cancer is associated with poor prognosis.[35] The results indicate that NTS may function as an autocrine growth factor in lung and breast cancer.

2. EPIDERMAL GROWTH FACTOR RECEPTOR

The EGFR is an important RTK in lung cancer which causes 0.23 M and 2.1 M deaths in the U.S. and the world, respectively.[36] NSCLC, which is comprised of adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, is treated with chemotherapy but the 5-year survival rate is only 19%.[36] The EGFR is activated by EGF, TGFα, heparin binding-EGF, epigen, epiregulin, and betacellulin. The ligands are derived from precursor

proteins which are released from the plasma membrane by proteases derived from the matrix metalloprotease (MMP) family.[37] After shedding, the ligand binds to extracellular domains I and III of the EGFR. After binding, the EGFR changes from a tethered to an extended conformation to dimerize. Extracellular EGFR domain II is important in the forming of EGFR homodimers or EGFR/HER2 heterodimers.[8] The extended EGFR has increased TK activity and phosphorylates amino acids such as Tyr^{1068} of the EGFR which interacts with Grb2. The Grb2/SOS complex removes GDP from Ras. Activated Ras-GTP facilitates the phosphorylation of Raf, leading to the phosphorylation of MEK and ERK.[38] The EGFR regulates the MEK-ERK cascade leading to cellular proliferation. EGFR mutations occur in the kinase domain with L858R being the most common. These patients respond initially to the TKIs gefitinib or erlotinib. Drug resistance occurs after the secondary EGFR mutations such as T790M.[39] Cetuximab is a mAb that recognizes extracellular domain III of the EGFR, preventing ligand binding, and is used to treat patients with head and neck cancer or colon cancer.[9]

The EGFR has 1210 amino acids and the gene encodes for a 170 kDa glycoprotein.[8] Table I shows that 5 minutes after adding NTS to NSCLC cells, EGFR tyrosine phosphorylation increases significantly to 367%. The C-terminal of NTS is biologically active and NTS^{8-13} increases EGFR tyrosine phosphorylation to 282% whereas inactive NTS^{1-8} did not increase EGFR tyrosine phosphorylation. SR48692 did not affect basal EGFR tyrosine phosphorylation but inhibits the increase in EGFR tyrosine phosphorylation caused by adding NTS to NSCLC cells (Table I). Because the phosphorylated EGFR is metabolized by protein tyrosine phosphatases (PTP) the increase in P-EGFR is transient. Adding NTS to colon, gastrin, liver, neuroendocrine, or prostate cancer cells increases P-EGFR.[3,40,41,42,43] The increase in PY^{1068}EGFR caused by NTS addition to NSCLC cells is inhibited by gefitinib, SR48692 or NTSR1 siRNA.[4] Further, the EGFR transactivation mediated by NTSR1 is impaired by anti-TGFα or GM6001 which inhibits MMP. The results suggest that TGFα may be present in the plasma membrane as a precursor protein that is metabolized by MMP leading to the activation of the EGFR. The ability of NTS to increase EGFR phosphorylation is impaired by the Src inhibitor PP2.[4] Src can phosphorylate the EGFR leading to increased TK activity. EGFR transactivation mediated by NTSR1 is impaired by Tiron, which inhibits superoxide formation.[4] NTS increases reactive oxygen species (ROS) in NSCLC cells which is impaired by Tiron. The ROS may impair PTP increasing P-EGFR.[44] ROS are essential for NTS to increase P-EGFR in NSCLC cells. NTS stimulates but SR48692 or gefitinib inhibits NSCLC growth in vitro. Addition of siRNA to NTS plus NTSR1 to NSCLC cells impairs NSCLC tumor growth in vivo using mouse models.[34] The results indicate that the NTSR1 regulates EGFR transactivation in numerous cancers in a Src-, MMP- and ROS-dependent manner.

3. HER2

Breast cancer occurs in 0.26 M and 2.1 M women in the US and the world, respectively.[45] The 5-year survival rate is 90% and breast cancer patients are treated with surgery followed by chemotherapy, radiotherapy, and adjuvant hormonal therapy. Breast cancer patients can overexpress HER2, be hormone receptor-positive or triple negative which lacks HER2, estrogen receptors and progesterone receptors. HER2 is overexpressed in 20-30% of the breast cancers, and these patients are preferentially treated with trastuzumab, a mAb against the extracellular HER2 domain that causes HER2 internalization.[46] HER2-positive breast cancer patients can be treated with lapatinib. HER2 mutations are uncommon (less than 2% of the breast cancer patients) but L755S occurs in the RTK domain.[47]

HER2 has 1255 amino acids and is a 185 kDa glycoprotein that is in the extended conformation and hence does not need a ligand to dimerize.[8] NTS or NTS^{8-13} addition to NSCLC cells increases PY^{1248}HER2 to 292 or 267%, respectively (Table I). NTS^{1-8} or SR48692 does not alter PY^{1248}HER2 significantly, however, SR48692 decreases the transactivation of HER2 caused by adding NTS to NSCLC cells. PY^{1248}HER2 interacts with the Shc adapter protein.[8] Shc interacts with SOS activating the MEK/ERK signal cascade. Adding the NTS analog JMV449 to NSCLC cells increases EGFR, HER2 and HER3 expression after 48 hours.[7] Also, JMV449 increased the release of HB-EGF, which binds to the EGFR in NSCLC cells. This may facilitate the formation of EGFR/HER2 heterodimers.

Table I. NTS analogs and transactivation

Ligand	% EGFR transactivation	% HER2 transactivation	%HER3 transactivation
NTS, 0.1 μM	367 ± 21**	292 ± 18**	230 ± 23**
NTS^{8-13}, 0.1 μM	282 ± 24**	267 ± 15**	205 ± 12**
NTS^{1-8} 1 μM	105 ± 7	102 ± 6	98 ± 7
SR48692 5 μM	98 ± 5	99 ± 7	101 ± 6
NTS + SR	121 ± 10	112 ± 9	108 ± 8

NTS analogs were added to NCI-H441 cells for 5 min and the % PY^{1068}-EGFR, PY^{1248}-HER2, and PY^{1289}HER3 determined. The mean value ± S.D. of 5 determinations is indicated; $p < 0.01$, ** by ANOVA.

The phosphorylation of the EGFR and HER2 caused by adding of NTS to NSCLC cells is impaired by Tiron and diphenylene iodonium (DPI) an inhibitor of NADPH oxidase (NOX) and dual oxidase (DUOX) enzymes. NSCLC cells have enzymes for NOX1-5 and DUOX1-2, but DPI inhibits the production of ROS from these enzymes.[48] Adding NTS to NSCLC cells increases ROS 2-fold which is impaired by DPI.[33] Further, DPI inhibited the growth of NSCLC cells. The ability of NTS to cause increased tyrosine phosphorylation of the EGFR and HER2, is impaired by SR48692 and lapatinib.[33] Lapatinib and SR48692 were synergistic at inhibiting the NTSR1 mediated transactivation of the EGFR and HER2 as well as inhibiting proliferation. SR48692 increases the potency of TKIs so that lower drug doses are needed to inhibit the proliferation of NSCLC cells. It remains to be determined if GPCR antagonist can be combined with TKI to inhibit NSCLC proliferation in patients.

4. HER3

HER3 is a 180 kDa glycoprotein with 1342 amino acids which is activated by NRG1 or NRG2. NSCLC cells have NRG1 but not NRG2 immunoreactivity.[8] NRG1 has an extracellular EGF-like domain, a TM domain, and a cytosolic C-terminal.[49] Due to alternative processing, 6 different NRG1 proteins can be present as well as 31 isoforms. Proteolytic processing leads to the release of NRG1, which can activate HER3.[15] NRG1 is detected in the cytosol of NSCLC cells treated with NTS. Also, JMV449 increased but SR48692 decreased NRG1 release from NSCLC cells.[7] NRG1 gene fusions increase the proliferation of NSCLC cells.[50]

HER3 and HER4 bind NRG1 with high affinity. HER3 is overexpressed in breast cancer and HER3 can be mutated at E^{928} in the TK domain in some (< 1%) breast cancer patients.[51] Breast cancer patients who have overexpressed HER3 have lower survival rates.[52] While HER3 has a ligand binding site it has weak TK activity. Nonetheless, HER3 can form functional heterodimers with the EGFR or HER2. Table I shows that NTS or NTS^{8-13} addition to NSCLC cells increased HER3 tyrosine phosphorylation to 230 and 205%. NTS^{1-8} or SR48692 had little effect on P-Tyr^{1289}HER3, however, SR48692 antagonized the increase in P-HER3 caused by adding NTS to NSCLC cells. After NRG1 is released, it binds to HER3 and changes the conformation from tethered to extended form. As a result, HER3 phosphorylates p85, a subunit of PI3K, increasing enzymatic activity.

Figure 1 shows that PI3K phosphorylates phosphatidylinositol-4,5 bisphosphate $PI(4,5)P_2$ to $PI(3,4,5)P_3$ which is metabolized by PDK1 resulting in the phosphorylation of AKT at Ser^{473} and Thr^{308}. $PI(3,4,5)P_3$ can be degraded by the phosphatase PTEN. AKT phosphorylates BAD blocking apoptosis but increasing cellular survival.[53] In addition, P-HER3 can activate Grb2 or SHC which leads to the phosphorylation of ERK. The ability of NTS to increase phosphorylation of AKT but not HER3 or ERK is impaired by the PI3K inhibitor LY294002.[54] The ability of NTS to increase phosphorylation of ERK but not HER3 or AKT is impaired by PD98059, a MEK inhibitor.[54] The results indicate that HER3 is upstream from PI3K or ERK. The ability of NTSR1 to regulate HER3 transactivation is inhibited by SR48692 and mAb3481 (a HER3 blocker).

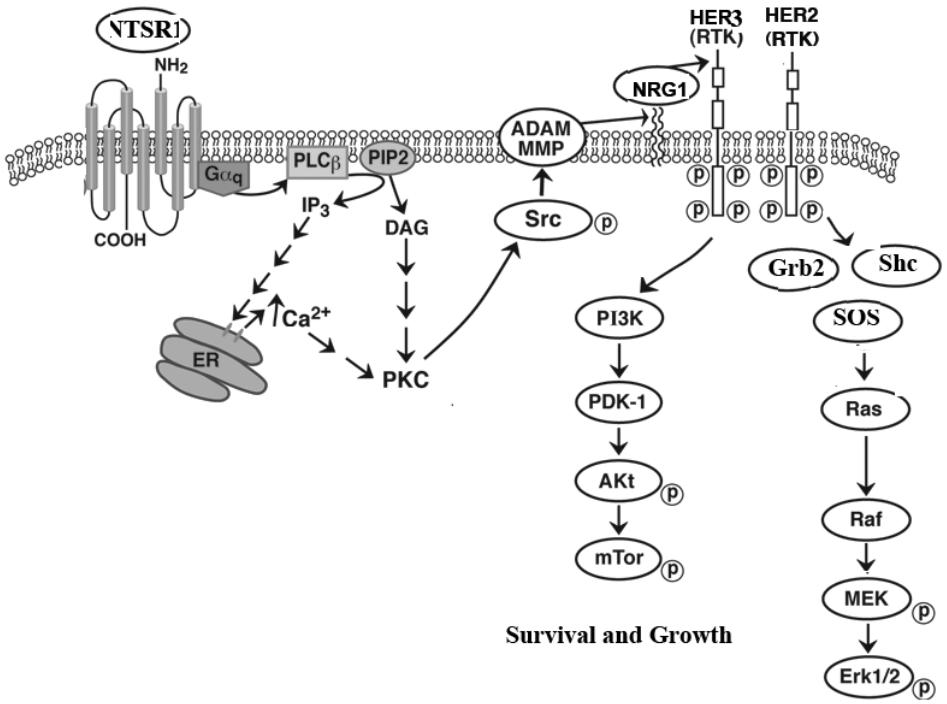

Figure 1. Effect of NTS on HER2 and HER3 transactivation.
NTS binds to NTSR1 and causes PI turnover leading to PKC activation and elevated Ca^{2+}. Subsequently, Src and MMP are activated leading to the shedding of NRG1. The NRG1 binds to HER3 causing the formation of HER2/HER3 heterodimers and increased RTK activity. Shc or Grb2 bind to SOS leading to the formation of Ras-GTP and phosphorylation of Raf, MEK, and ERK leading to increased cellular proliferation. PI3K is phosphorylated leading to the activation of PDK-1, Akt, and mTor increasing cellular survival.

Clinical trials are being conducted with HER3 mAbs. Patritumab inhibits HER3 ligand binding and dimerization and patritumab with erlotinib was well tolerated in patients with advanced NSCLC.[55] NSCLC patients were treated with erlotinib and seribantumab. Seribantumab prevents NRG1 binding to HER3.[56] It is being investigated if seribantumab and docetaxel inhibit tumor growth in patients who have high expression of NRG1.[14] Due to the reduced RTK activity of HER3, mAbs are used as blockers in preference to TKI.

5. CONCLUSIONS

This review focuses on how NTSR1 regulates phosphorylation of the EGFR, HER2 and HER3 in NSCLC cells. NTSR1 is a GPCR which causes ErbB transactivation in a ligand-dependent manner. Addition of NTS to NSCLC cells increases release of TGFα and NRG1 which results in tyrosine phosphorylation of the EGFR and HER3, respectively. HER2 is tyrosine phosphorylated when it forms heterodimers with the EGFR or HER3. When NTS is added to NSCLC cells the level of ErbB tyrosine phosphorylation is pEGFR > pHER2 ≥ pHER3. This may result because the EGFR can form homodimers and heterodimers whereas HER2 and HER3 can only form functional heterodimers. When the EGFR, HER2 and HER3 are phosphorylated the PI3K and ERK signal transduction pathways are activated leading to increased NSCLC proliferation. The transactivation of the ErbB RTK and proliferation of NSCLC are inhibited by SR48692 (NTSR1 antagonist) and lapatinib (TKI). SR48692 potentiates the ability of lapatinib to decrease transactivation and growth of NSCLC cells.

Using precision medicine techniques, it will be important to determine if the tumors of lung cancer patients have mutated EGFR, amplified HER2, overexpressed HER3, and/or overexpressed NTSR1. If the patient's tumor has mutated EGFR and overexpresses NTSR1, a combination of gefitinib and SR48692 may be a useful to inhibit RTK transactivation and tumor proliferation. It remains to be determined if peptide receptor antagonists combined with TKI will be a useful therapy for treatment of NSCLC.

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