Peptides 86 (2016) 153–161

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Growth hormone-releasing hormone induced transactivation of epidermal growth factor receptor in human triple-negative breast cancer cells

Eva Vacas[a], Laura Muñoz-Moreno[a], Pedro L. Valenzuela[b], Juan C. Prieto[a], Andrew V. Schally[c], María J. Carmena[a], Ana M. Bajo[a,*]

[a] Department of Systems of Biology, University of Alcala, Alcala de Henares, Spain
[b] Obstetrics and Gynaecology Department, Principe de Asturias Hospital, Alcalá de Henares University, Alcalá de Henares, Madrid, Spain
[c] Veterans Administration Medical Center and Departments of Pathology and Medicine, Division of Oncology and Hematology, Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine and South Florida Veterans Affairs Foundation for Research and Education, Miami, FL, USA

ARTICLE INFO

Article history:
Received 15 June 2016
Received in revised form 20 October 2016
Accepted 1 November 2016
Available online 2 November 2016

Keywords:
GHRH
Triple-negative breast cancer
EGFR
Transactivation
Cell migration
GHRH antagonist

ABSTRACT

Triple-negative breast cancer (TNBC) is a subset of breast cancers which is negative for expression of estrogen and progesterone receptors and human epidermal growth factor receptor-2 (HER2). Chemotherapy is currently the only form of treatment for women with TNBC. Growth hormone-releasing hormone (GHRH) and epidermal growth factor (EGF) are autocrine/paracrine growth factors in breast cancer and a substantial proportion of TNBC expresses receptors for GHRH and EGF. The aim of this study was to evaluate the interrelationship between both these signaling pathways in MDA-MB-468 human TNBC cells. We evaluated by Western blot assays the effect of GHRH on transactivation of EGF receptor (EGFR) as well as the elements implicated. We assessed the effect of GHRH on migration capability of MDA-MB-468 cells as well as the involvement of EGFR in this process by means of wound-healing assays. Our findings demonstrate that in MDA-MB-468 cells the stimulatory activity of GHRH on tyrosine phosphorylation of EGFR is exerted by two different molecular mechanisms: i) through GHRH receptors, GHRH stimulates a ligand-independent activation of EGFR involving at least cAMP/PKA and Src family signaling pathways; ii) GHRH also stimulates a ligand-dependent activation of EGFR implicating an extracellular pathway with an important role for metalloproteinases. The cross-talk between EGFR and GHRHR may be impeded by combining drugs acting upon GHRH receptors and EGFR family members. This combination of GHRH receptors antagonists with inhibitors of EGFR signalling could enhance the efficacy of both types of agents as well as reduce their doses increasing therapeutic benefits in management of human breast cancer.

1. Introduction

Breast cancer is the second leading cause of cancer deaths in western women [1]. Effective therapies have been developed for patients with hormone positive disease or HER-2 overexpression. However, in many cases as the development of metastasis, recurrence or the presence of a triple-negative carcinoma, chemotherapy is currently the only form of systemic therapy [2].

The definition of triple-negative breast cancer (TNBC) applies to a subset of breast tumors which do not express receptors for estrogen and progesterone and do not overexpress HER-2. This subgroup shows certain clinical features and represents 15–20% of breast carcinomas [3]. TNBC tends to affect most often young patients and is associated with pour prognosis [4–7]. Recurrence happens much earlier and most deaths occur in the first five years after diagnosis [6,8]. These clinical findings underscore the paramount importance of the development of new therapies aimed at TNBC.

Growth hormone-releasing hormone (GHRH) is a hypothalamic neuropeptide which regulates synthesis and release of growth hormone by the pituitary and is an autocrine/paracrine growth factor for multiple human cancers including breast cancer [9,10]. G-protein coupled receptors (GPCRs) such as receptors for pituitary type of growth hormone-releasing hormone (pGHRH-R) as well as four splicing variants (SV1-SV4) have been detected in various human cancers including TNBC cells [11–13]. GHRH antagonists

* Corresponding author at: Department of Systems of Biology, University of Alcalá, 28871 Alcalá de Henares, Spain.
E-mail address: ana.bajo@uah.es (A.M. Bajo).

http://dx.doi.org/10.1016/j.peptides.2016.11.004
0196-9781/

of MIA series designed recently such as MIA-602, MIA-606 and MIA-690 show a high binding affinity for tumoral GHRH receptors. In vitro and in vivo studies with GHRH antagonists demonstrate that they act directly on tumor cells and inhibit their proliferation [14].

Receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR/HER-1/ErbB1) have been identified as critical pathway elements in signaling from GPCRs, cytokines, other RTKs and integrins to a variety of cellular responses including MAPK activation, gene transcription and proliferation [15,16]. There is considerable evidence that agonists of some GPCRs, through a process of transactivation, can activate growth factor RTKs in the absence of added exogenous growth factors [17]. Two modes of transactivation of RTK by GPCRs have been identified. In the first one, GPCR stimulation induces activation of metalloproteinases of a disintegrin and metalloproteinase (ADAM) family which cause ectodomain shedding of a transmembrane RTK ligand precursor. This in turn activates its cognate receptor (ligand dependent transactivation). The second mode of RTK transactivation is independent of the cognate ligand [18]. Transactivation of EGFR and HER2 through several GPCRs has been reported in human prostate cancer [19,20]. On the other hand, recent findings indicate a bidirectional communication between both receptor types that involves the amplification of the malignant signals [18]. Functional crosstalk between GPCRs and EGFR contributes to the progression of colon, lung, prostatic, ovarian, head and neck, and breast tumors [17]. Thus, GPCR might be a suitable supplementary site for blocking tumorigenic signals. Consequently, GPCR-mediated functions could become promising therapeutic targets for development of drugs for treatment of cancer [17]. Src kinases, a family of non-receptor protein tyrosine kinases, have been involved in oncogenic processes including proliferation, survival, motility and angiogenesis. Furthermore, it has been reported that several GPCRs activate Src which, in turn, phosphorylates different RTKs [21,22]. Taken together, it appears that Src family kinases could be part of a complex of associated components of GPCRs and RTKs and participate in the two way communication between both receptor types. Therefore, we evaluated whether GHRH induces EGFR transactivation as well as the elements involved in such process in human triple-negative breast cancer MDA-MB-468 cells. We also report the participation of EGFR in the migration of triple-negative breast cancer cells. The findings suggest the merit of combining GHRH receptors antagonists with inhibitors of EGFR signalling in order to enhance the efficacy of both types of agents as well as reduce their doses increasing therapeutic benefits in management of human breast cancer.

2. Materials and methods

2.1. Cell lines

The MDA-MB-468 human triple-negative breast cancer cell line was purchased from the American Type Culture Collection ATCC (Rockville, MD, USA). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin/amphoterycin B. Cell culture supplies were purchased from Life Technologies (Alcobendas, Madrid, Spain). The culture was performed in a humidified 5% CO_2 environment, at 37 °C. After cells reached 70–80% confluence, they were washed with phosphate buffered saline (PBS), detached with 0.25% trypsin/0.2% EDTA, and plated at 30,000–40,000 cells/cm^2. The culture medium was changed every 2 days.

2.2. Reagents

hGHRH(1–29)NH_2 was purchased from NeoMPS (Strasbourg, France). GHRH antagonist MIA-690 was synthesized in the laboratories of one of us (A.V.S). The chemical structure of MIA-690 is [(PhAc-Ada)0-Tyr^1, D-Arg^2, Cpa^6, Ala^8, Har^9, Fpa5^{10}, His^{11}, Orn^{12}, Abu^{15}, His^{20}, Orn^{21}, Nle^{27}, D-Arg^{28}, Har^{29}]hGH-RH(1–29)NH_2. EGFR tyrosine kinase inhibitor (AG1478), 4-amino-5-8chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), n-[(2*R*)-2-(Hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (GM6001) and 2-aminoethyl amide (TAPI-1) were acquired from Calbiochem (Darmstadt, Germany). N-(2-(*p*-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide (H89) from Sigma-Aldrich (Alcobendas, Madrid, Spain).

2.3. Protein isolation

MDA-MB-468 cells coated into 6-well plates (2×10^5) were incubated with 0.1 μM GHRH and/or 1 μM MIA-690 for different time periods. The cells were washed twice with ice-cold PBS and then harvested, scraped into ice-cold PBS, and pelleted by centrifugation at 500 x *g* for 5 min at 4 °C. Cells were washed once with ice-cold PBS and then scraped into ice-cold PBS. Thereafter, cells were lysed with 25 mM triethanolamine buffer (pH 7.4) containing 150 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) Nonidet P-40 (NP-40), 30 mM NaF, 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% sodium dodecyl sulfate (SDS), 1 mM Na_3VO_4, 2 mM phenyl methylsulfonylfluoride (PMSF), 10 μg/μl aprotinin, 10 μg/μl leupeptin, and 10 μg/μl pepstatin. Protein content was measured by the Bradford assay using bovine serum albumin as standard.

2.4. Western blotting

Protein (20–40 μg) from cell lysates was solubilized in 50 mM Tris–HCl buffer (pH 6.8) containing 10% (v/v) glycerol, 3% (w/v) SDS, 0.01% bromophenol blue, and 0.7 M β-mercaptoethanol, and then heated for 5 min. Proteins were resolved on an 8% SDS-PAGE and then transferred to nitrocellulose sheets (BioTrace/NT, Pall Corporation, East Hills, NY, USA). Rabbit polyclonal antiEGFR and antiphospho-EGFR (Tyr^{1173}), (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:200 and 1:5000), antiMMP2 (Abcam, Cambridge, UK) (1:2000) and antiphospho-Src Family (Tyr^{416}) (Cell Signaling Technology, Danvers, MA, USA) (1:2000) antibodies was added followed by incubation for overnight. After treatment for 1 h at room temperature with the corresponding HRP-labelled secondary antiserum (BD Biosciences) (1:4000), signals were detected with enhanced chemiluminiscence reagent (Amersham, Uppsala, Sweden) using β-actin antibody (Sigma-Aldrich) (1:10,000) as a loading control.

2.5. Wound-healing assays

Human triple-negative breast cancer MDA-MB-468 cells were incubated in 24-well plates and a small wound area was performed in the confluent monolayer with a scraper. Then, cells were incubated in the absence or presence of 0.1 μM GHRH and/or 1 μM MIA-690. Three representative fields of each wound were photographed by means of a Nikon Diaphot 300 inverted microscopy at different times (0 and 24 h). Wound areas of untreated samples were averaged and assigned a value of 100%.

2.6. Data analysis

Quantification of band densities was performed using the Quantified One Program (Bio-Rad). Data shown in the figures are representative at least of three different experiments. The results