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

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Abstract

Triple-negative breast cancer (TNBC) is a subset of breast cancers which is negative for expression of estro- gen 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.

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

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 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 fac- tor 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 methyl-sulfonylfluoride (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 13-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 13-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 are expressed as the mean ± SEM and were statistically treated following the Bonferroni's test for multiple comparisons after one or two-way analysis of variance (ANOVA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Effect of GHRH on phosphorylation of EGFR

The effect of GHRH on EGFR phosphorylation was analysed by Western blot assays in MDA-MB-468 human triple-negative breast cancer cell line. Treatment with 0.1 μM

GHRH at different times (0.5–120 min) significantly stimulated tyrosine phosphorylation of EGFR from 15 to 60 min with a highest response (80%) at 45 min of exposure to the peptide (Fig. 1A). No significant changes were observed on total EGFR levels after GHRH treatment. Moreover, when we tested at 45 min the specificity of the stimulatory effect using a GHRH antagonist, MIA-690, this antagonist totally abolished the effect of GHRH on the expression of phosphorylated EGFR (Fig. 1B). MIA-690 antagonist treatment showed no effect on the total EGFR levels.

3.2. Intracellular elements implicated in transactivation of EGFR induced by GHRH

Once we assessed the effect of GHRH on EGFR transactivation, we decided to explore intracellular signalling pathways elements implicated. For this purpose, MDA-MD-468 human triple-negative breast cancer cells were cultured in the absence or presence of 0.1 μM GHRH and/or several protein kinase inhibitors: PKA inhibitor (H89) and Src inhibitor (PP2). Treatment with 10 μM H89 and 10 μM PP2 fully abolished the stimulatory effect of GHRH on EGFR transactivation in MDA-MB-468 cells (Fig. 2A). The treatment with both inhibitors showed no effect on the total EGFR levels. These results point to PKA and Src as mediators of the effect of GHRH.

We performed a series of experiments focused on evaluating the sequence of elements in the signalling pathway involved in the EGFR transactivation by GHRH. TNBC MDA-MB-68 cells were cultured in the absence or presence of 0.1 μM GHRH and PKA inhibitor (H89) and then, *p*-Src expression was analyzed by Western blotting. Results revealed that H89 modified *p*-Src levels after GHRH treatment (Fig. 2B). This implies that EGFR transactivation induced by GHRH is performed through the PKA and subsequent activation of Src.

3.3. Involvement of matrix metalloproteinases in transactivation of EGFR induced by GHRH

EGFR transactivation occurs either by binding of EGF-like growth factors or by an intracellular mechanism. To determine whether EGF-like growth factors are involved in EGFR transactivation induced by GHRH, MDA-MB-468 cells were pretreated with a broad spectrum metalloproteinase inhibitor (GM6001) before an incubation for 45 min incubation with GHRH. GM6001 completely inhibited GHRH-induced EGFR phosphorylation (Fig. 3). Similarly, preincubation of breast cancer cells in the presence of TAPI-1, a metalloproteinase inhibitor with some specificity for TNFa converting enzyme/ADAM17, also blocked GHRH-induced EGFR phosphorylation (Fig. 3). No effect on total EGFR levels was observed after treatment with both inhibitors. Thus, metalloproteinase activity involved in the cleavage of membrane EGF-like growth factor is required for EGFR activation in MDA-MB-468 human triple-negative breast cancer cells.

3.4. Effect of GHRH on migration of MDA-MB-468 cells

To investigate the role of GHRH on breast cancer cell migration, wound-healing assays were performed. Firstly, a small wound area was made to the plate with confluent monolayer of cells. Under control conditions, the wound width was maintained for 24 h with administration of 0.1 μM GHRH. Images show that GHRH increased cell migration, provoking the closure of the wound 24 h (Fig. 4A). Moreover, we analyzed the effect of 1 μM GHRH antagonist MIA- 690. The results suggest that 10 min of preincubation with MIA-690 was satisfactory to block the elevated ability to migrate that GHRH exerts in MDA-MB-468 breast cancer cells. GHRH antagonist, MIA-690, alone also significantly decreased the mobility of TNBC cells (Fig. 4B). GHRH treatment considerably increased the MMP2

levels. However, no changes were observed after the treatment with MIA- 690 (Fig. 4C).

3.5. Involvement of EGFR on migration of MDA-MB-468 cells

Once established that GHRH may participate in the transactivation of EGFR and was also able to increase the migratory capacity of tumor cells, we decided to check whether both processes are interrelated in triple-negative breast cancer MDA-MB-468 cells. For this purpose, wound-healing assays were carried out with a specific inhibitor for EGFR tyrosine kinase activity (AG1478). Pretreatment with 10 μM AG1478 and subsequent incubation with 0.1 μM GHRH revealed that EGFR inhibitor strongly blocked the effect of GHRH on motility of MDA-MB-468 cells (Fig. 5). These findings could indicate that EGFR activation is implicated in effects of GHRH on cell migration in human triple-negative breast cancer cells.

4. Discussion

In the present study, we report that GHRH modulates the phosphorylation of one of the tyrosine kinase receptor family members, EGFR, in MDA-MB-468 human triple-negative breast cancer cells. Activation of EGFR may be achieved through two different molecular mechanisms, depending on the presence or the absence of ligand for the receptor tyrosine kinase [23].

HER family members are crucial participants in breast carcinogenesis [24]. In fact, EGFR has been previously characterized in the cell line studied, which exhibits features of advanced breast cancer [25,26]. Our results show that after treatment with GHRH, the levels of phosphorylated EGFR protein were increased in a significant manner in triple-negative breast cancer cells. On the same line, it has been described that GHRH stimulates mRNA for HER2 and EGFR and protein levels in human prostate cancer cells [27]. Moreover, vasoactive intestinal peptide (VIP), a neuropeptide structurally related to

GHRH, enhanced EGFR protein levels in human T47D and MDA-MB-468 breast cancer cells [25]. Thus, present results support the connection between GHRH receptors and EGFR. In this regard, tyrosine kinase receptor transactivation mediated by GHRH developed rapidly being maximal at 45 min after addition of GHRH. A similarly response has been observed for VIP/EGFR in colonic epithelial T84 [28] and breast cancer T47D cells [25] as well as for GHRH/EGFR in prostate cancer PC3 cells [27]. Our results reveal that the transactivation of pre-existing EGFR molecules appears to take place in this first response to GHRH (15 min). In addition, increased p-EGFR levels at longer time periods (60 min) may conceivably be the result of a slower induction of synthesis of both tyrosine kinase receptors. Such receptors can also be phosphorylated by a GHRH-dependent mechanism. This fact has been also observed for GHRH/EGFR in breast cancer MDA-MB-468 cells and prostate PC3 tumors [25,27]. Therefore, our results show that GHRH is linked to the EGFR signaling at least in two ways depending on how GHRH can stimulate EGFR tyrosine phosphorylation.

GHRH receptors are coupled to G protein and their stimulation leads to generation of cAMP and subsequent PKA activation in various tissues including breast [29,30]. Multiple cellular pathways jointly contribute to the development of breast cancers. Intracellular players such cAMP/PKA may be essential for breast cancer cells to adapt to invasive phenotypes in the absence of estrogen [31]. Present results demonstrate that GHRH induces the activation of EGFR through PKA. In this regard, the involvement of this kinase in EGFR transactivation induced by VIP and GHRH in breast and prostate cancer cells has been described [19,25,27]. Similarly, the signaling cascade involving cAMP and PKA is activated after a rapid transactivation of the EGFR by Gs-coupled receptors in colonic epithelial T84 cells [28].

Src is highly expressed in breast cancer cell lines as well as in the majority of advanced breast cancer specimens [32,33]. In addition, Src-family tyrosine kinases are known mediators of EGFR in GPCR-induced transactivation [34]. In our study, the transactivation of EGFR provoked by GHRH was Src-dependent since the Src inhibitor PP2 blocked the response to the neuropeptide. This fact would support previous reports about transactivations by GPCRs involving Src kinases [27,35]. On the other hand, GHRH increased phosphorylation of Tyr^{416} sited on the activation loop of the kinase domain of Src protein. Furthermore, our findings suggest that protein kinase A may be able to activate Src-kinases and subsequently lead the phosphorylation of pre-existing EGFR molecules in TNBC cells. Similar results were reported in human breast cancer cells with VIP and in human prostate cancer cells with GHRH [25,27]. Our results support the view that Src plays a key role in transactivation of RTK by GPCR [18,35].

Transactivation of EGFR induced by GHRH may involve EGFR-ligand-dependent pathway. In this case, GPCR activates metalloproteinase to induce EGF shedding from pro-HB-EGF present on the cell surface. Subsequent release of the mature growth factor activates EGFR and its downstream signaling cascades [36]. In our study, using inhibitor of matrix metalloproteinases, transactivation of EGFR provoked by GHRH suggests the involvement of an extracellular pathway in TNBC cells. On the other hand, it has been reported that the tumor necrosis factor-a-converting enzyme (TACE/ADAM-17) contributes to malignant phenotype of breast cancer cell by shedding of EGFR ligand TGF-α [37,38]. Similarly, it has been reported that Src interacts with the cytoplasmic tail of ADAM17, and this is accompanied by phosphorylation of Src and ADAM17 and their translocation to the cell membrane [39,40]. In our study, protein levels of

TACE/ADAM17 increased after treatment with GHRH supporting that such a metalloproteinase can be activated by other cellular signaling pathways including GPCRs [15,41]. Further experiments should be performed in order to clarify the elements involved in this extracellular pathway.

Growth hormone–releasing hormone (GHRH) and its receptors are involved in a variety of important cellular processes like proliferation, migration, angiogenesis and neoplastic transformation in various non-pituitary tissues including breast [42,43]. EGFR family is also involved in these processes which enhance tumor progression. In our study, transactivation of EGFR induced by GHRH appears to be involved in cell migration in TNBC cells provoked by GHRH. Moreover, our results show that the GHRH antagonists, MIA-690, blocked cell mobility. Thus continued development of GHRH analogues, which inhibit receptors for EGF expressed in more aggressive breast cancers may lead to a therapy for TNBC.

5. Conclusion

In conclusion, present findings demonstrate the stimulatory effect of GHRH on tyrosine phosphorylation of EGFR in human triple-negative breast cancer cells. Through its receptors, GHRH stimulates a ligand-independent activation of EGFR involving at least cAMP/PKA and Src family signaling pathways. GHRH also stimulates a ligand-dependent activation of EGFR involving an extracellular pathway with an important role for metalloproteinases. Consequently, the cross-talk between EGFR and GHRH-R may be impeded by combining drugs acting upon GHRH receptors and EGFR family members. This combination appears to potentiate the efficacy as well as reduces the doses of both types of agents thus increasing therapeutic effects in management of human breast cancer.

Author's contributions

P.L.V. and A.M.B. designed the transactivation experiments; J.C.P. and M.J.C. designed cell migration assays; A.V.S. provided the analogs; E.V. and L.M.-M. performed in vitro studies with cultured cells and Western blotting; E.V., L.M.-M., P.L.V., J.C.P., M.J.C, A.V.S. and A.M.B. wrote and edited the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1. Effect of GHRH on total EGFR levels and the tyrosine phosphorylation of EGFR protein in human MDA-MB 468 breast cancer cells. Cells were incubated in the absence or presence of 0.1 μM GHRH at different times (A) and previously treated with 1 μM MIA-690 and/or 0.1 μM GHRH at 45 min (B). Immunodetection using antibodies against EGFR, p-EGFR and β-actin was performed followed by densitometry of corresponding bands. Results are representative of three independent experiments and they are the mean ± S.E.M. *, $P < 0.05$; ***, $P < 0.001$ compared with untreated control; $^{###}$, $P < 0.001$compared to cells treated with GHRH.

Fig. 2. Influence of different inhibitors of protein kinases on the effect of GHRH on total EGFR levels and the transactivation of EGFR in MDA-MB 468 cell line. Cells were pretreated with specific inhibitors at different times: H89 (15 min) and PP2 (30 min). Subsequently, 45 min incubated with GHRH. Cell lysates were used to perform western blot assays using antibodies against EGFR, p-EGFR and 13- actin (A). The cells were pretreated with specific inhibitor H89 (15 min) and subsequently, 45 min incubated with GHRH, and next, cell lysates were used to perform western blot assays using antibodies against p-Src and β-actin (B). The results are representative of five independent experiments and correspond to the mean ± S.E.M. *, P < 0.05; ***, P < 0.001 versus control; ##, P < 0.01 compared to cells treated with GHRH.

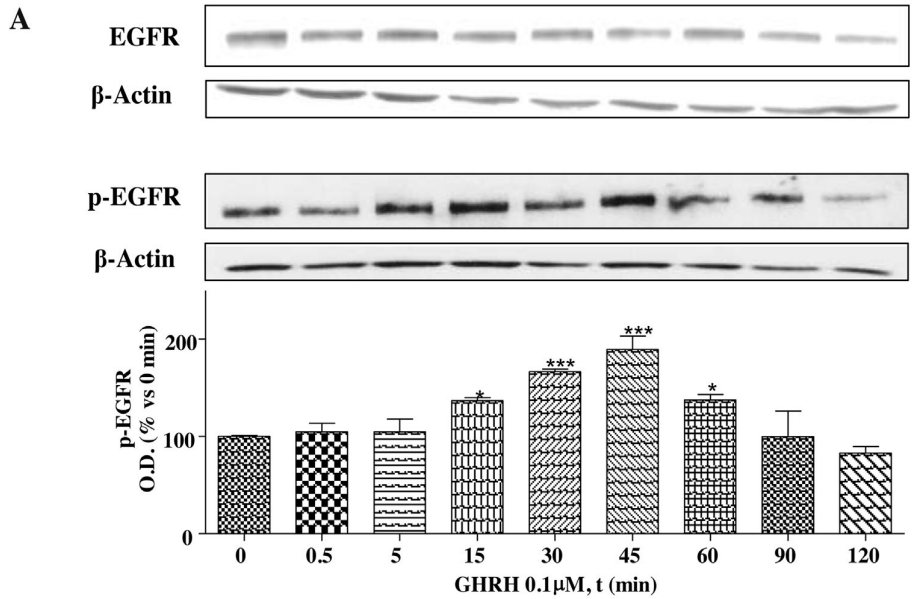
Fig. 3. Involvement of metalloproteinases on GHRH-induced phosphorylation of EGFR in MDA-MB-468 cell line. Breast cancer cells were pretreated for 60 min with specific inhibitors of metalloproteinases: GM6001 (for matrix metalloproteinases) and TAPI-1 (for ADAMs). Subsequently, cells were incubated in absence or presence of

with GHRH for 45 min. After that, cell lysates were used to perform western blot assays using antibodies against EGFR, p-EGFR and β-actin. The results are representative of five independent experiments and correspond to the mean ± S.E.M. ***, $P < 0.001$ *versus* control; ###, $P < 0.001$, compared to cells treated with GHRH.

Fig. 4. Effect of GHRH on MDA-MB-468 cell migration. Cells were preincubated with 1 μM MIA-690 (10 min) and/or 0.1 μM GHRH at 45 min. (A) Microscopic analysis of the cell−free area was carried out at 0 h and 24 h; (B) the area invaded by breast cancer cells was calculated; (C) MMP2 protein level by Western blotting. Data are the mean ± S.E.M. of five experiments. **, $P < 0.01$; ***, $P < 0.001$ *versus* control; ###, $P < 0.001$, compared to cells treated with GHRH.

Fig. 5. Effect of GHRH on cell migration in MDA-MB-468 cells, through activation of EGFR. Cells were pretreated for 30 min with 10 μM AG1478, a specific inhibitor for EGFR tyrosine kinase activity, and next, with or without of 0.1 μM GHRH for 24 h. (A) Microscopic analysis of the cell−free area was carried out at 0 h and 24 h; (B) the area invaded by breast cancer cells was calculated. Data are the mean ± S.E.M. of three experiments. **, $P < 0.01$ *versus* control; #, $P < 0.05$, compared to cells treated with GHRH.

Figure 1


A
EGFR
β-Actin
p-EGFR
β-Actin
p-EGFR
O.D. (% vs 0 min)
200
100
0
*
***
***
*
0
0.5
5
15
30
45
60
90
120
GHRH 0.1μM, t (min)

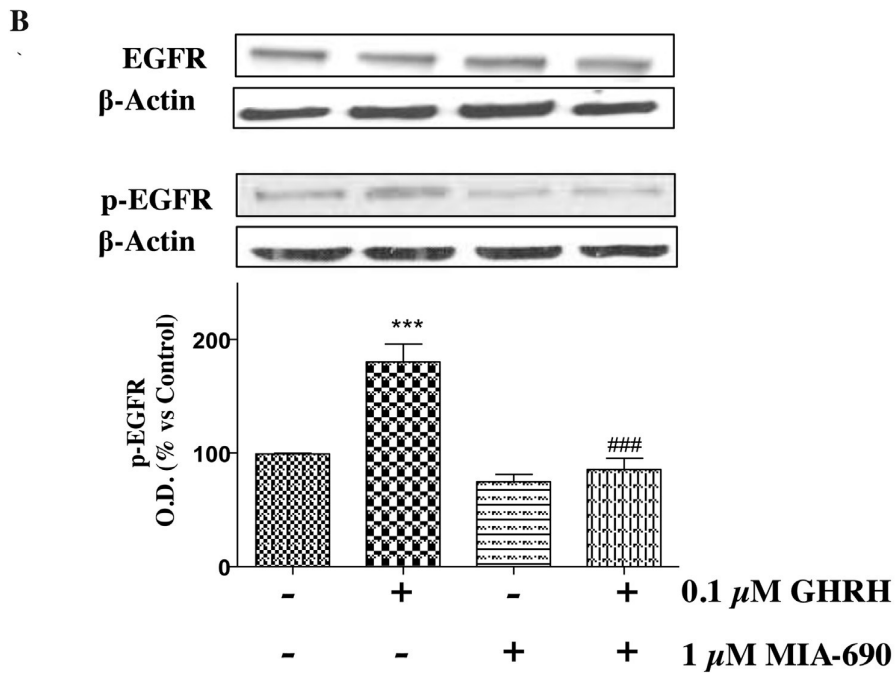
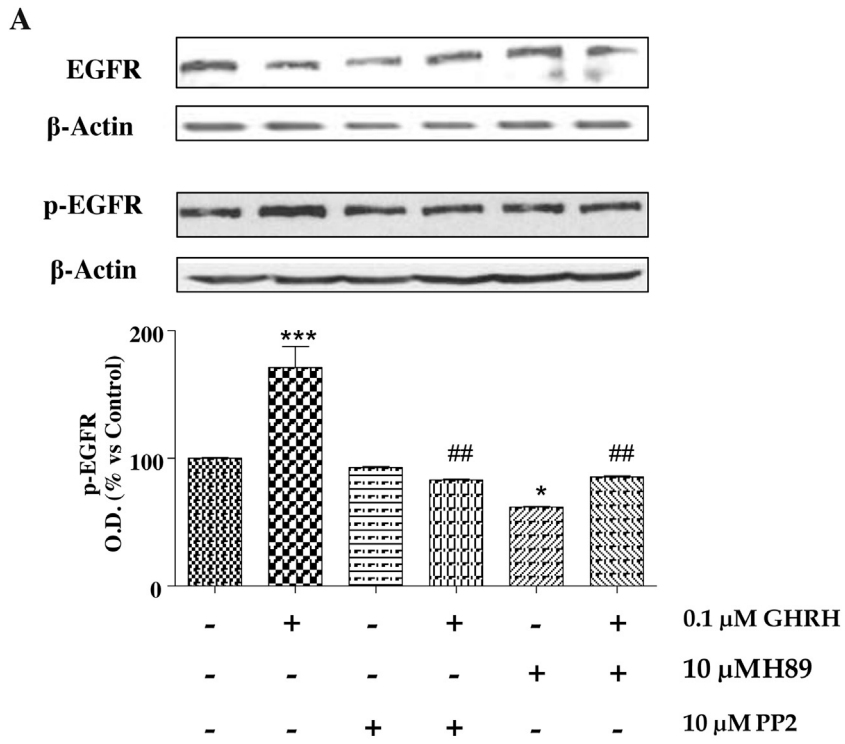

B
EGFR
β-Actin
p-EGFR
β-Actin
p-EGFR
O.D. (% vs Control)
200
100
0
***
###
-
+
-
+
0.1 μM GHRH
-
-
+
+
1 μM MIA-690

Figure 2


A
EGFR
β-Actin
p-EGFR
β-Actin
p-EGFR
O.D. (% vs Control)
200
100
0
***
##
*
##
- + - + - +
0.1 μM GHRH
- - - - + +
10 μMH89
- - + + - -
10 μM PP2

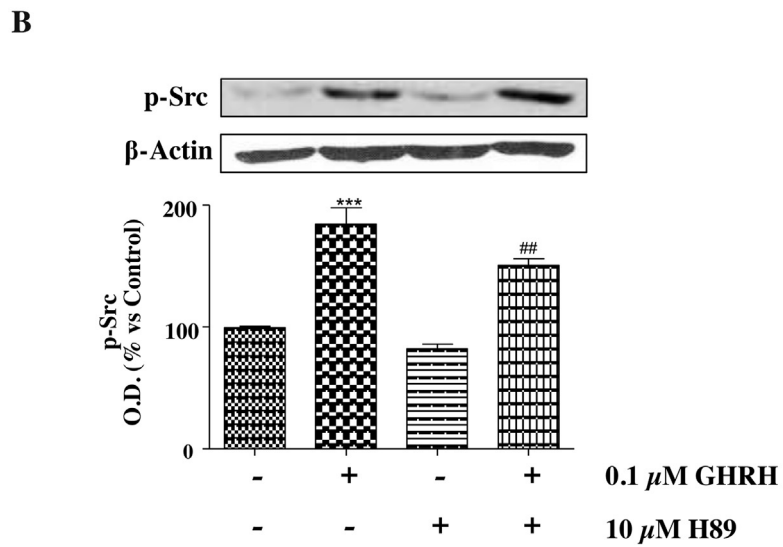

B
p-Src
β-Actin
p-Src
O.D. (% vs Control)
200
100
0
***
##
- + - +
0.1 μM GHRH
- - + +
10 μM H89

Figure 3

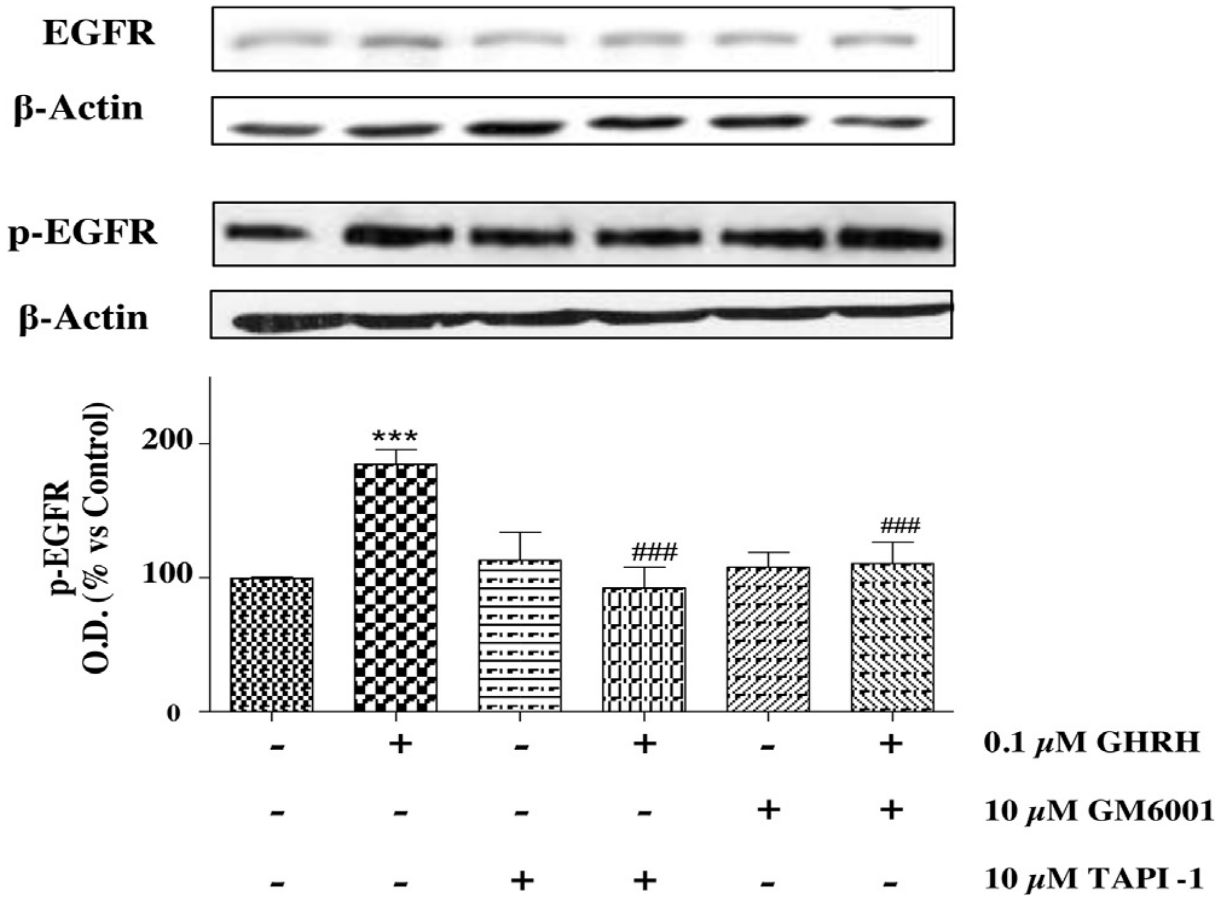

EGFR
β-Actin
p-EGFR
β-Actin
p-EGFR
O.D. (% vs Control)
200
100
0
***
###
###
- + - + - +
0.1 μM GHRH
- - - - + +
10 μM GM6001
- - + + - -
10 μM TAPI -1

Figure 5

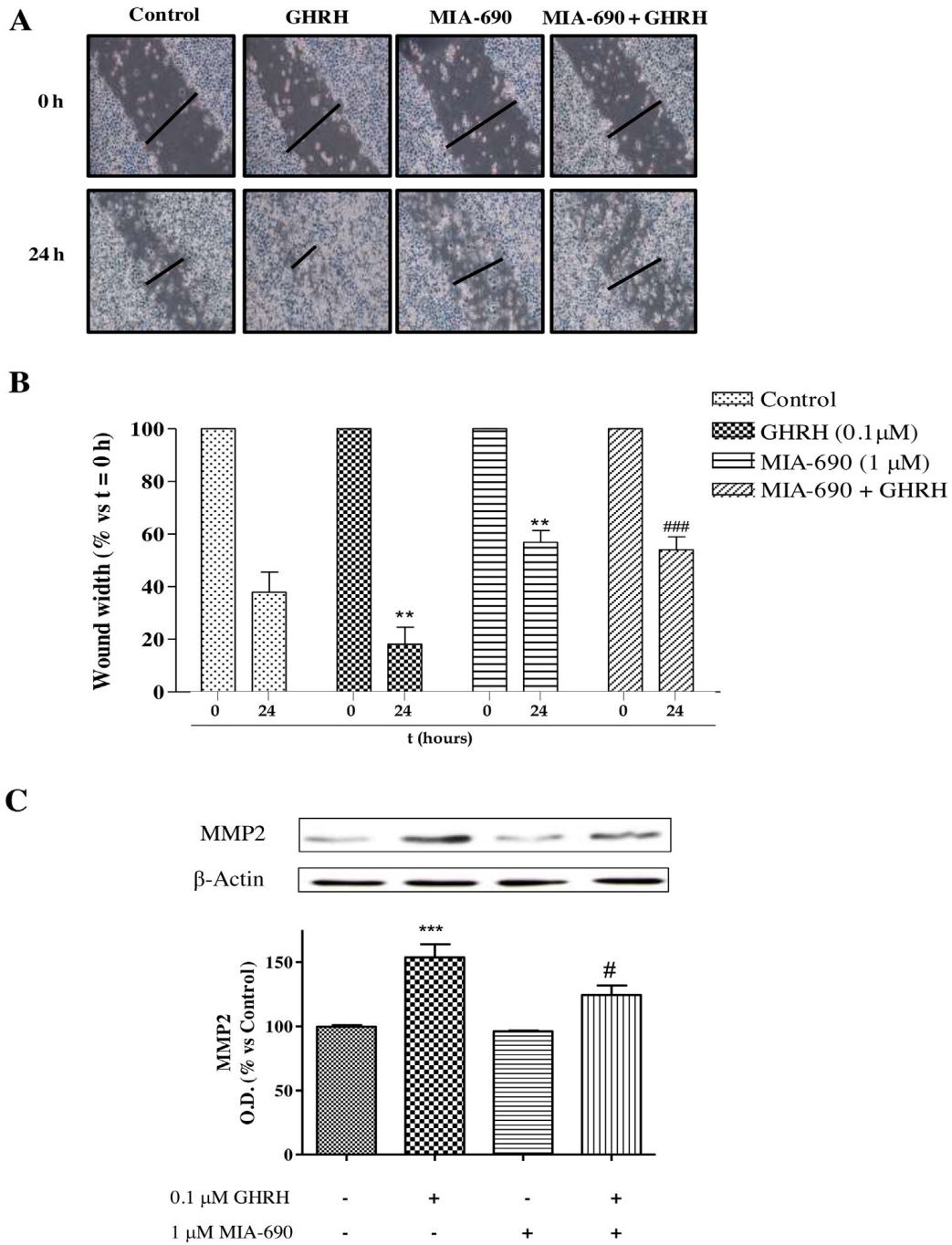
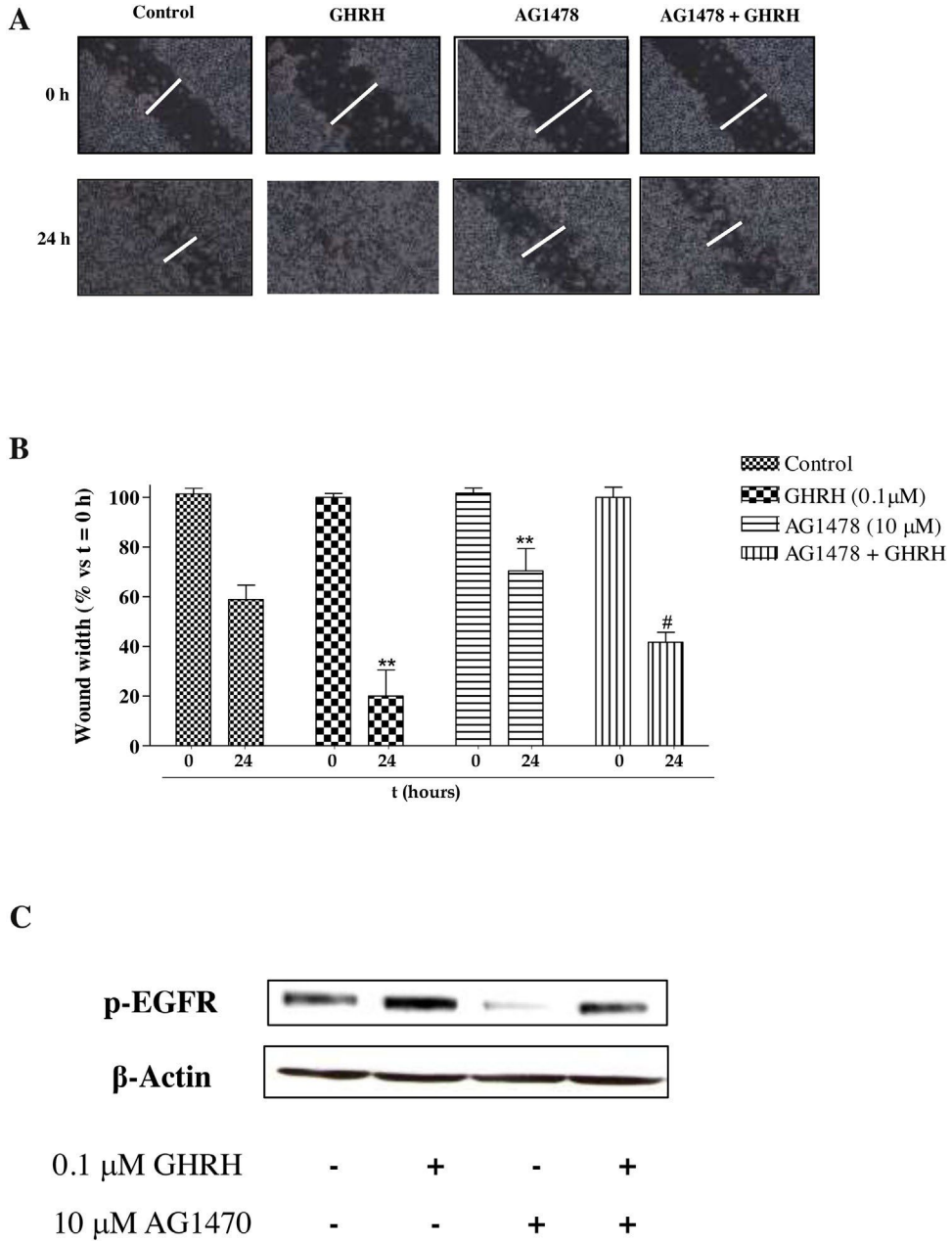

A
Control
GHRH
MIA-690
MIA-690 + GHRH
0 h
24 h
B
Control
GHRH (0.1μM)
MIA-690 (1 μM)
MIA-690 + GHRH
Wound width (% vs t = 0 h)
100
80
60
40
20
0
**
**
###
0
24
0
24
0
24
0
24
t (hours)
C
MMP2
β-Actin
MMP2
O.D. (% vs Control)
150
100
50
0
***
#
0.1 μM GHRH - + - +
1 μM MIA-690 - - + +

Figure 1


A
Control
GHRH
AG1478
AG1478 + GHRH
0 h
24 h
B
Wound width (% vs t = 0 h)
100
80
60
40
20
0
**
**
#
0
24
0
24
0
24
0
24
t (hours)
Control
GHRH (0.1μM)
AG1478 (10 μM)
AG1478 + GHRH
C
p-EGFR
β-Actin
0.1 μM GHRH - + - +
10 μM AG1470 - - + +