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PRECLINICAL STUDIES

Growth hormone-releasing hormone antagonists abolish the transactivation of human epidermal growth factor receptors in advanced prostate cancer models

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Summary Growth hormone-releasing hormone (GHRH) and its receptors have been implicated in a variety of cellular phenotypes related with tumorigenesis process. Human epidermal growth factor receptor family members (HER) such as EGFR and HER2 are involved in mitogenic signaling pathways implicated in the progression of prostate cancer. We analyzed the cross-talk between GHRH and EGF receptors in prostate cancer. The effects of GHRH in HER signaling were evaluated on human androgen-independent PC3 prostate cancer cells in vitro and GHRH antagonist in vitro and in nude mice xenografts of PC3 prostate cancer. Time-course studies indicated that GHRH had a stimulatory activity on both the expression of EGFR and HER2. GHRH analogues, JMR-132 and JV-1–38, endowed with antagonistic activity for GHRH receptors, abrogated the response to GHRH in PC3 cells. GHRH stimulated a rapid ligand-independent activation of EGFR and HER2 involving at least cAMP/PKA and Src family signaling pathways. GHRH also stimulated a slow ligand-dependent activation of EGFR and HER2 involving an extracellular pathway with an important role for ADAM. Preliminary results also revealed an increase of mRNA for GHRH and GHRH receptor induced by EGF. The inhibition of tumor growth, in vivo, was associated with a substantial reduction in the expression of mRNA and protein levels of EGFR and HER2 in the tumors. GHRH antagonist JV-1–38, significantly decreased the phosphorylated Src levels. The cross-talk between HER and GHRH-R may be impeded by combining drugs acting upon GHRH receptors and HER family members in human advanced prostate cancer.

Keywords GHRH · GHRH antagonists · HER · Cross-talk · Transactivation

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Introduction

Prostate cancer accounts for 29 % of all new cancer cases and is the second leading cause of cancer-related deaths among men in the Western world [1]. Progression of prostate carcinoma to a stage refractory to hormone therapy renders the disease resistant to currently available treatment options. Thus, the understanding of molecular events leading to such a stage is essential for the development of improved therapies for patients with advanced prostate cancer [2].

A large body of evidence indicates that G protein-coupled receptors (GPCRs) play a crucial role in tumorigenesis, and are involved in important steps of cancer progression from transformation, growth and survival to metastasis [3]. Pituitary-type growth hormone-releasing hormone (GHRH) receptors (pGHRH-R) as well as their four truncated splice variants (SV1-SV4) have been detected in different human cancers including prostate cancer [4, 5]. In the pituitary, the

binding of hypothalamic GHRH to pGHRH-R leads to the stimulation of the synthesis and release of GH. In turn, GH induces the production of hepatic insulin-like growth factor I (IGF-I), which is a known mitogen in many cell types, associated with malignant transformation, tumor progression and metastasis in various cancers [4]. SV1 has been described as the predominant type of GHRH receptor in several cancers including prostate cancer [6]. In vitro and in vivo studies with GHRH antagonists, demonstrates that they also act directly on tumor cells and inhibit their proliferation [7]. Intracellular second messengers such as cAMP and Ca^{2+}, which activate specific isoforms of protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and the oncogenes *c-fos* and *c-jun*, are involved in signaling pathways of GHRH analogs through tumoral GHRH-R [8,9]. Potent GHRH antagonists, such as JV-1–38 and JMR-132, have been shown to prevent cell proliferation in androgen-independent prostate cancer [10].

Receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR/HER-1/ErbB1) and human epidermal growth factor receptor-2 (HER-2/ErbB2) 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 [11, 12]. In addition, formation of EGFR/HER-2 heterodimers through ligand binding is involved in mitogenic signaling pathways implicated in prostate cancer progression towards androgen independence [13, 14].

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 [3]. This important pathway contributes to the growth-promoting activity of many GPCR ligands. Two modes of transactivation of RTK by GPCRs have been identified. In the first, 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 [15]. Transactivation of EGFR and HER2 through several GPCRs has been reported in human prostate cancer [16, 17]. On the other hand, recent findings indicate a bidirectional communication between both receptor types that involves the amplification of the malignant signals [15]. Functional crosstalk between GPCRs and EGFR contributes to the progression of colon, lung, breast, ovarian, head and neck, and prostate tumors [3]. 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 [3].

Src kinases, a family of non-receptor protein tyrosine kinases, have been involved in oncogenic processes including proliferation, survival motility, angiogenesis and androgen therapy resistance that lead to the hormone-refractory stage in prostate cancer [18, 19]. In addition, Src inhibition has been demonstrated to lead to a major decrease in cell invasion and growth in androgen-independent prostate carcinoma C4-2 cells [20]. 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.

Recently, we demonstrated that the treatment of PC3 human androgen-independent prostate cancers with GHRH antagonists JMR-132 and JV-1–38 decreased the expression of the main angiogenic and metastatic factors [10]. Therefore, we evaluated in PC3 tumors whether these antagonists can also affect the expression of EGFR, HER2 and Src, which play an important role in the mechanisms of angiogenesis and metastasis. In addition, we investigated the connection between GHRH receptors and EGFR/HER2 in PC3 prostate cancer cells. The effect of GHRH on EGFR and HER2 protein levels as well as the transactivation of both tyrosine kinase receptors was evaluated. We also report the participation of intracellular elements such as PKA, Src kinase, and matrix metalloproteinases in the molecular mechanism of transactivation. The findings suggest the merit of combining GHRH antagonists with inhibitors of EGFR and HER2 signaling for the therapy of different advanced stages of prostate cancer.

Material and methods

Peptides and chemicals

Synthetic GHRH (1–29) NH_2 was purchased from PolyPeptide (Strasbourg, France). GHRH antagonists JV-1–38 and JMR-132 were synthesized in the laboratories of one of us (AVS). JV-1–38 and JMR-132 structures are [PhAc-Tyr^1, D-Arg^2, Phe(4-Cl)^6, Har^9, Tyr(Me)^{10}, Abu^{15}, Nle^{27}, Har^{29}] human $GHRH_{1\text{-}29}NH_2$ and [$PhAc^0$-Tyr^1, D-Arg^2, Phe(4-Cl)^6, Ala^8, Har^9, Tyr(Me)^{10}, His^{11}, Abu^{15}, His^{20}, Nle^{27}, D-Arg^{28}, Har^{29}] human $GHRH_{1\text{-}29}NH_2$, respectively. Abu is α-aminobutyric acid, Har is homoarginine, Nle is norleucine, PhAc is phenylacetyl and Tyr(Me) is o-methyltyrosine. EGFR tyrosine kinase inhibitor (AG1478), 4-amino-5-(chlorophenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine (PP2), N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (GM6001) and 2-aminoethyl amide (TAPI-1) were acquired from Calbiochem (Darmstadt, Germany). HER-2 tyrosine kinase inhibitor (AG825) was from Tocris Bioscience (Bristol, United Kingdom), and N-(2-(p-bromocinnamylamino) ethyl)-5-isoquinolinesulfonamide (H89) was from Alexis (San Diego, CA).

Cell culture

PC3 cells, obtained from the American Type Culture Collection, were grown and maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin/amphoterycin B (Life Technologies, Carlsbad, CA). Cells were cultured in a humidified 5 % CO_2 environment at 37 °C. After the cells reached 70–80 % confluence, they were washed with PBS, detached with 0.25 % trypsin/0.2 % EDTA, and seeded at 30,000–40,000 cells/cm^2. The culture medium was changed every 3 days.

Animals, xenografts and processing of tumors

Athymic male nude mice (nu/nu) 5–6 weeks old were obtained from Harlan (Oxon, UK) and maintained in microisolator units on a standard sterilizable diet. Mice were housed under humidity-and temperature-controlled conditions, and the light/dark cycle was set at 12 h intervals. Experimental procedures are carried out according to Spanish Law 32/2007, Spanish Royal Decree 1201/2005, European Directive 609/86/CEE and European Convention of Council of Europe ETS 123. Tumors were initiated by subcutaneous injection into the right flank, as described previously [10]. The experiment was started when the tumors had grown to ~75 mm^3. Animals were randomly divided into three treatment groups: group 1 (ten mice), control, vehicle solution; group 2 (ten mice), GHRH antagonist JMR-132 subcutaneously injected once a day at a dose of 10 μg/animal, and group 3 (ten mice), GHRH antagonist JV-1–38, subcutaneously injected every day at a dose of 20 μg/animal. The experiment was ended on day 41. After mice were anaesthetized with halothane, tumors were dissected, cleaned, and weighed. Tumor specimens were divided into three approximately equal portions: one portion was processed for immunohistochemistry (10 % formalin fixed and paraffin embedded), and the other portions were frozen in liquid nitrogen and maintained at −80 °C for further experiments.

Isolation of cell and tissue lysates

Cell lysates were obtained as described previously [23]. Briefly, PC3 cells ($1.5–3\times10^6$) were washed and harvested in ice-cold PBS. Cells were then pelleted by centrifugation at 500×g for 5 min at 4ºC, lysed on ice for 30 min in a solution containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. Cell debris was removed by centrifugation at 4,000 × g for 5 min at 4ºC. Tumor specimens were homogenized in 1 M Tris–HCl (pH 7.6) containing 1 % Nonidet P40, 150 mM NaCl, 2 mM ortovanadate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin and then rotated for 30 min in a cold room. The extract was cleared by centrifugation at 15,000 × g for 30 min at 4 °C.

Western blot assays

Cell and tissue lysates (30 μg) were denatured by heating, separated by 10 % SDS-PAGE and blotted onto a BioTraceTM nitrocellulose membrane (Pall Corp., Madrid, Spain) overnight in 50 mM Tris–HCl, 380 mM glycine, 0.1 % SDS, and 20 % methanol. The antisera specific for EGFR (sc-3), HER-2 (sc-284) and TACE (C-15) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphospho-erbB/HER-2 (Tyr^{1248}) and antiphospho-EGFR (Tyr^{1173}) antibodies were acquired from Upstate (Lake Placid, NY). Phospho-Tyr^{416} Src was purchased from Calbiochem (Darmstadt, Germany). Blots were incubated with the primary and secondary antibodies at dilutions recommended for 1 h at room temperature according to manufacturer's instructions. Signals were detected with enhanced chemiluminescence reagent (Thermo Scientific, Rockford, IL) using β-actin (Sigma, St. Louis, MO) antibody as loading control.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells by using Tri® Reagent (Sigma) according to the manufacturer's instruction. Two micrograms of total RNA were reverse-transcribed into cDNA by Moloney murine leukemia virus reverse transcriptase according to the manufacturer's guidelines (Life Technologies). Reverse transcriptions were also performed without enzyme to rule out genomic DNA contamination. Primers were designed with the assistance of the computer program Primer3 v.0.4.0 [24]. Gene specific primers for human GHRH and human β-actin were as follows: GHRH: 5'-AATTGGAG AGCTCCTGGTG-3' (sense), 5'-CCAGTTGCATTTTGGC TACA-3' (antisense); GHRH-R: 5'-CCTGATCCCACTCT TTGGAA-3' (sense), 5'-CCTCTTGGTTGAGGAAGCAG-3' (antisense); EGFR: 5'-GGAGAACTGCCAGAAACTGA C-3' (sense), 5'-GCCTGCAGCACACTGGTTG-3' (antisense); HER-2: 5'-CTCACCTACCTGCCCACCAAT-3' (sense), 5'-GGGACCTGCCTCACTTGGTT-3' (antisense) and β-actin: 5'-AGAAGGATTCCTATGTGGGCG-3' (sense), 5'-CATGTCGTCCCAGTTGGTGAC-3' (antisense). The number of cycles was determined in preliminary experiments to be within the exponential range of PCR amplification. Negative controls containing water instead of cDNA were run in parallel to exclude contamination. PCR-conditions were: denaturation at 94ºC for 5 min, followed by 94ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min by 35 cycles for GHRH and GHRH-R and 25 cycles for β-actin and then a final cycle of 10 min at 72ºC. PCR products were subjected to electrophoresis on a 2 % agarose gel, stained with GelRed™ nucleic acid gel stain (Biotium, Hayward, CA) and visualized under ultraviolet light.

Immunohistochemistry

Serial sections, 5-μm-thick, were deparaffinised in xylene and rehydrated using graded ethanol concentrations. To retrieve the antigen, the sections were hydrated and placed in a glass jar containing 10 mM sodium citrate buffer, pH 6.0, and heated in a pressure cooker for 2 min. The endogenous peroxidase activity was inhibited by incubation with 3 % hydrogen peroxide for 20 min at room temperature. After rinsing in Tris-buffered saline (TBS), the slides were incubated with blocking solution (3 % normal donkey serum plus 0.05 % Triton in TBS) for 45 min to prevent non-specific binding of the first antibody. Afterwards, the sections were incubated overnight at 4ºC with the primary antibodies: pEGFR (1:100) and pHER-2 (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) in the blocking solution diluted 1:9. Then, the sections were washed in TBS and incubated for 20 min with biotinylated link universal antibody (Dako, Barcelona, Spain). After an extensive wash in TBS, detection was made by the conventional labeled-streptavidin-biotin method (LSAB-kit, Dako). The peroxidase activity was detected using the glucose oxidase-DAB-nickel intensification method. Sections were dehydrated, cleared in xylene, and mounted in DePex (Probus, Badalona, Spain). To assess the specificity of immunoreaction, negative and positive controls were used. Sections of samples identically processed, but not incubated with the primary antibodies, were used as negative controls. As positive controls, sections of skin, rat adrenal gland and kidney were processed with the same antibody.

Data analysis

Densitometry was performed using Quantitative One Program (Bio-Rad, Alcobendas, Spain). Data were subjected to one-way ANOVA and differences were determined by Bonferroni's multiple comparison test. Each experiment was repeated at least three times. Data are shown as the means of individual experiments and presented as the mean ± SEM. P values <0.05 were considered statistically significant.

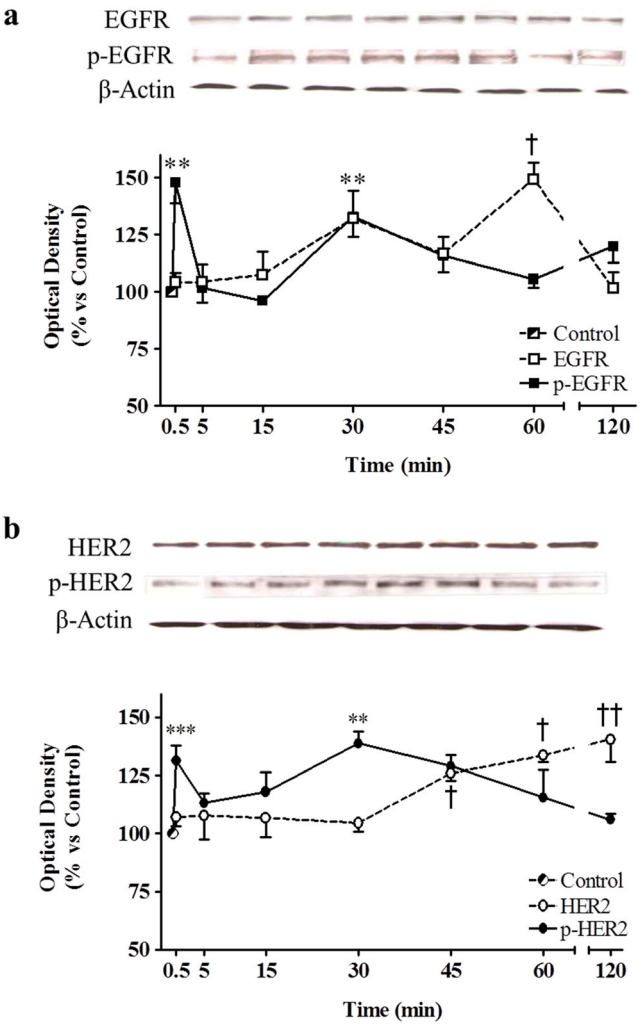

Fig. 1 Effect of GHRH on the expression and tyrosine phosphorylation of EGFR (**a**) and HER2 (**b**) proteins in human PC3 androgen-independent prostate cancer cells. Cells were incubated in the presence or absence of GHRH (0.1 μM) for the indicated times. Immunodetection using antibodies against EGFR, HER2, p-EGFR, p-HER2, and β-actin was performed followed by densitometry of the corresponding bands. A representative experiment of at least three performed is shown in the upper panels. Data are the mean ± SEM, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ versus each non-phosphorylated tyrosine kinase receptor; $^{**}P<0.01$, $^{***}P<0.001$ versus each phosphorylated tyrosine kinase receptor.

Results

Effect of GHRH on expression of EGFR and HER2 and their phosphorylation

GHRH enhanced protein levels of EGFR (Fig. 1a) and HER2 (Fig. 1b) in PC3 cells in a time-dependent pattern. The highest responses occurred at 30–60 min and at 45–120 min of incubation with 0.1 μM GHRH for EGFR and HER2, respectively. Furthermore, 0.1 μM GHRH stimulated tyrosine phosphorylation of EGFR (Fig. 1a) and HER2 (Fig. 1b). The onset of this effect was rapid, with maximal phosphorylation of EGFR (48 %) and HER-2 (33.5 %) within 30 s after the addition of the neuropeptide. A similar elevation was also observed at 30 min (37 % for p-EGFR and 38 % for p-HER2).

Effect of GHRH antagonists JMR-132 and JV-1–38 on the phosphorylation of EGFR and HER2

In order to evaluate whether transactivations of EGFR and HER2 observed at 0.5 and 30 min were mediated through GHRH receptors, we studied the effects of GHRH antagonists on the phosphorylation of EGFR (Fig. 2a) and HER2 (Fig. 2b) in PC3 cells. The response to GHRH was mediated by the

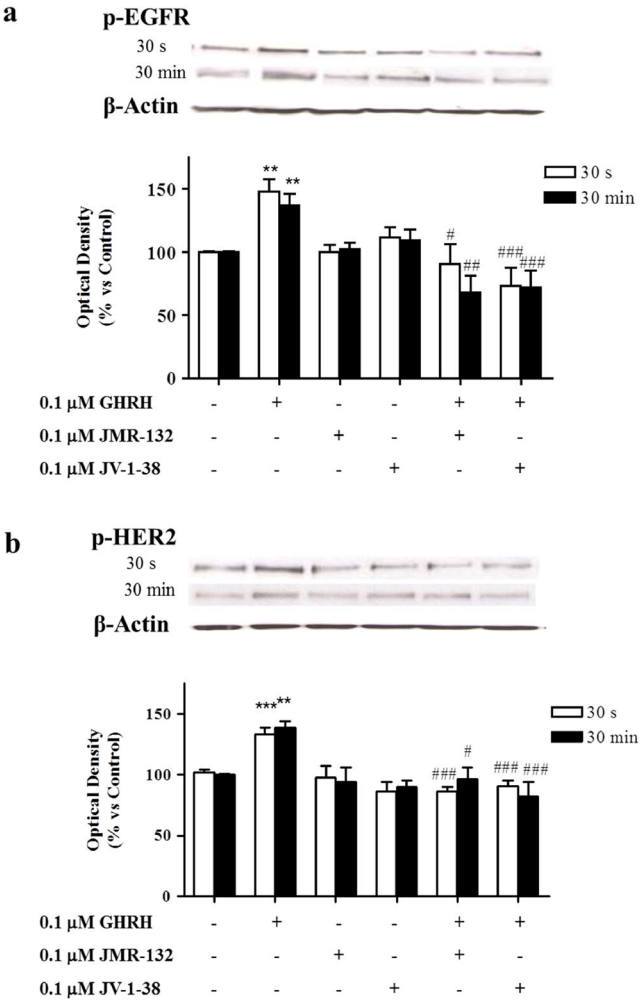

Fig. 2 Antagonistic effect of JMR-132 and JV-1–38 on the phosphorylation of EGFR and HER2 in PC3 androgen-independent human prostate cancer cells. Cells were preincubated for 30 min with the specific antagonists JMR-132 (0.1 μM) and JV-1–38 (0.1 μM). And then incubated in the presence or absence of 0.1 μM GHRH for 30 s or 30 min. After processing of cells, Western blot assay was carried out using antibodies against p-EGFR, p-HER2, and β-actin followed by densitometry of the bands. A representative experiment of at least three performed is shown (upper panels). Data in each bar are the mean ± SEM, $^{**}P<0.01$, $^{***}P<0.001$ versus the corresponding control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus GHRH.

binding of this neuropeptide to GHRH receptors since preincubation of cells for 30 min with specific antagonists 0.1 μM JMR-132 and 0.1 μM JV-1–38 completely blocked GHRH-induced EGFR and HER2 tyrosine phosphorylation. The antagonists alone showed no effect on the activation of both receptor tyrosine kinases.

Intracellular mediators of GHRH-mediated transactivation of EGFR and HER2

In order to determine whether PKA and Src signaling pathways mediate the stimulatory effect of GHRH on the phosphorylation of EGFR (Fig. 3a) and HER2 (Fig. 3b) in human PC3 prostate cancer cells, the protein kinase inhibitors H89 (for PKA) and PP2 (for Src kinase) were used. Both agents were able to block the 30 s-stimulatory effect of GHRH on the phosphorylation of EGFR (with decreases of 64 % for H89 and 74 % for PP2) and HER2 (with a reduction of 56 % for H89 and PP2) levels. However, neither inhibitor showed any effect on GHRH-mediated tyrosine kinase receptor activation at 30 min. Incubation of the cells with inhibitors alone had no effect on the transactivation (data not shown).

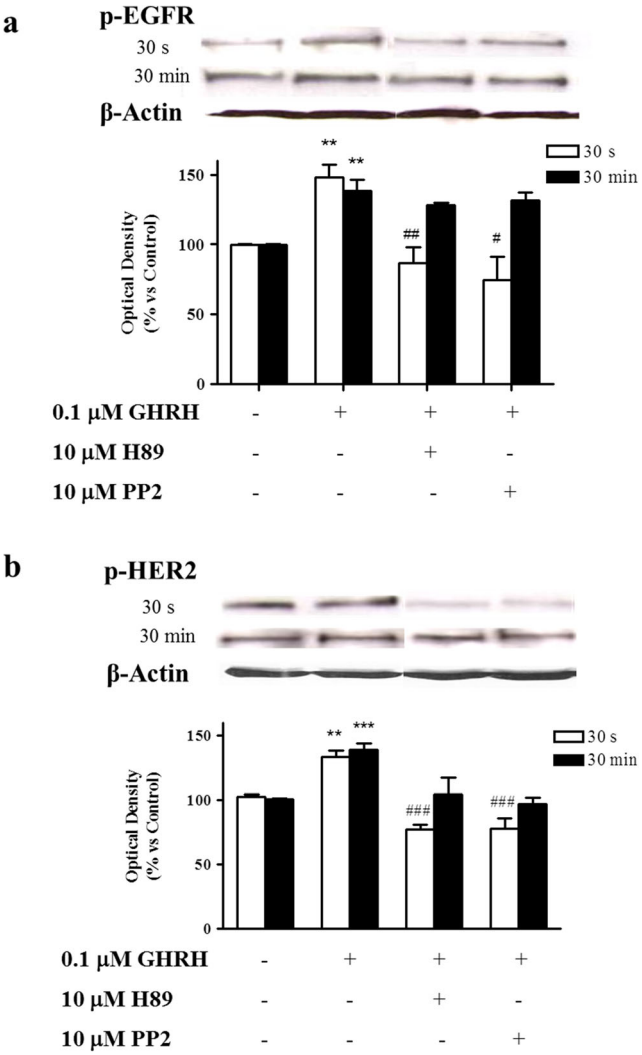

Fig. 3 Involvement of PKA and Src pathways in GHRH-induced phosphorylation of EGFR (**a**) and HER2 (**b**) in human PC3 androgen-independent prostate cancer cells. Cells were incubated with specific inhibitors of protein kinase, H89 (for PKA) or PP2 (for Src family) for 15 and 30 min, respectively. Afterwards, an incubation in the absence or presence of GHRH (0.1 μM) for 30 s or 30 min was carried out. Cell lysates were used to perform Western blot assays using antibodies against p-EGFR, p-HER2, and β-actin, followed by densitometry of the bands. A representative experiment of at least three performed is shown (upper panels). Data in each bar are the mean ± SEM, $^{**}P<0.01$, $^{***}P<0.001$ versus the corresponding control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus GHRH

Effect of GHRH on the phosphorylated Src

Src is involved in dependent and independent-ligand transactivations of HER family members by several GPCRs. Therefore, we assessed the phosphorylation of Src after the incubation with GHRH(1–29) at different times. GHRH elicited the activation of Src protein following a profile with two peaks with maximal stimulation at 30 min and 2 h (Fig. 4a). Then, we assessed the regulation of Src by GHRH receptors. GHRH-induced increases in phosphorylation of Src at 0.5 and 30 min were completely abolished by 0.1 μM antagonists JMR-132 and 0.1 μM JV-1–38 indicating that both stimulatory effects were due to specific binding of GHRH to its receptors.

Involvement of matrix metalloproteinases in the GHRH-induced transactivation of EGFR and HER2

The involvement of matrix metalloproteinases (MMPs) was assessed using specific inhibitors, GM6001 (for MMPs) or TAPI-1 (for ADAMs). The role of MMPs on the GHRH-mediated phosphorylation of EGFR and HER2 was demonstrated by the ablation of the effect of this neuropeptide by GM6001 and TAPI-1 (Fig. 5a). Both agents were able to block the 30 min stimulatory effect of GHRH on the phosphorylation of EGFR (with decreases of 52 % for GM6001 and 42 % for TAPI-1) and HER2 (with decreases of 76 % for GM6001 and 80 % for TAPI-1) levels. However, neither inhibitor showed an effect on the GHRH-mediated tyrosine kinase receptor activation at 30 s. The outcome to the cell incubation with inhibitors alone was ineffective on the transactivation (data not shown).

In turn we assessed the expression of the tumor necrosis factor-α-converting enzyme (TACE/ADAM-17) after cell incubation with GHRH at different times (Fig. 5b). The neuropeptide led to a significant increase in the levels of TACE protein levels in a time-dependent manner, with two peaks of maximal stimulation at 15 min and 8 h.

Heterodimerization of HER family members

In order to evaluate whether heterodimerization between EGFR and HER2 was produced after GHRH activation in PC3 cells, specific inhibitors of tyrosine kinase activity (AG1478 for EGFR and AG825 for HER2) were used (Fig. 6a). EGFR inhibitor, AG1478, alone did not show any effect on HER2 activation at the time intervals studied. HER2 inhibitor, AG825, alone led to a significant decrease (25–30 %) in p-HER2 protein levels at 0.5 and 30 min. Preincubation (30 min) with both specific tyrosine kinase inhibitors completely abolished the stimulatory effects of GHRH on p-HER2 levels. Importantly, AG1478 provoked a decrease of 60 and 56 % in p-HER2 protein levels at 0.5 or 30 min of exposure to GHRH, respectively.

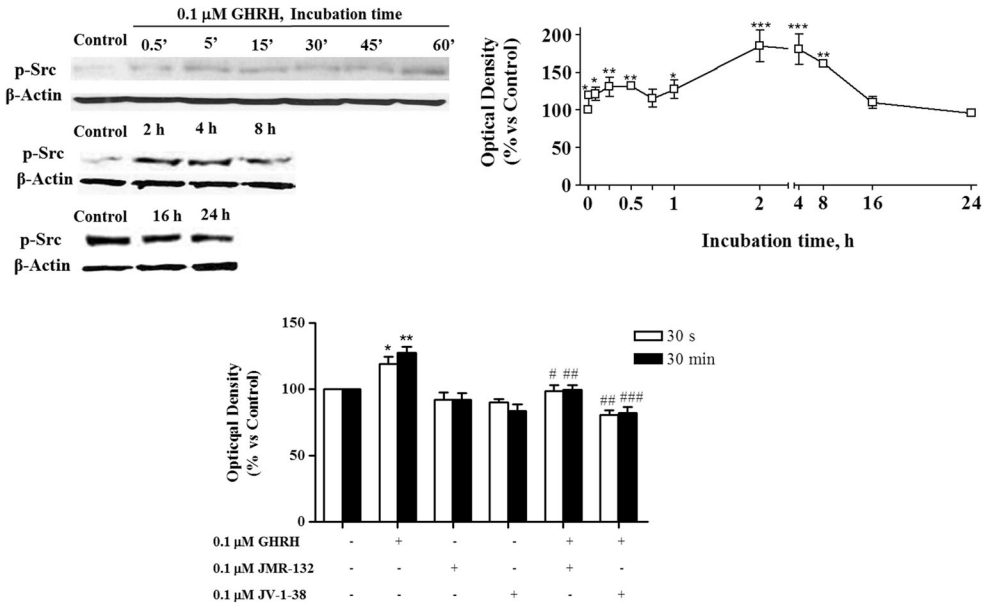

Fig. 4 Effect of GHRH on the expression of phosphorylated Src (p-Src) protein (**a**) and antagonistic effect of JMR-132 and JV-1–38 on p-Src (**b**) in human PC3 androgen-independent prostate cancer cells. A, Cells were incubated in the presence or absence of GHRH (0.1 μM) for the indicated times. B, Cells were preincubated for 30 min with the specific antagonists JMR-132 (0.1 μM) and JV-1–38 (0.1 μM). And then, incubated in the absence or presence of 0.1 μM GHRH for 30 s or 30 min. Immunodetection using antibodies against p-Src and β-actin was performed, followed by densitometry of the corresponding bands. A representative experiment of at least three performed is shown in the left panels. Data are the mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus GHRH

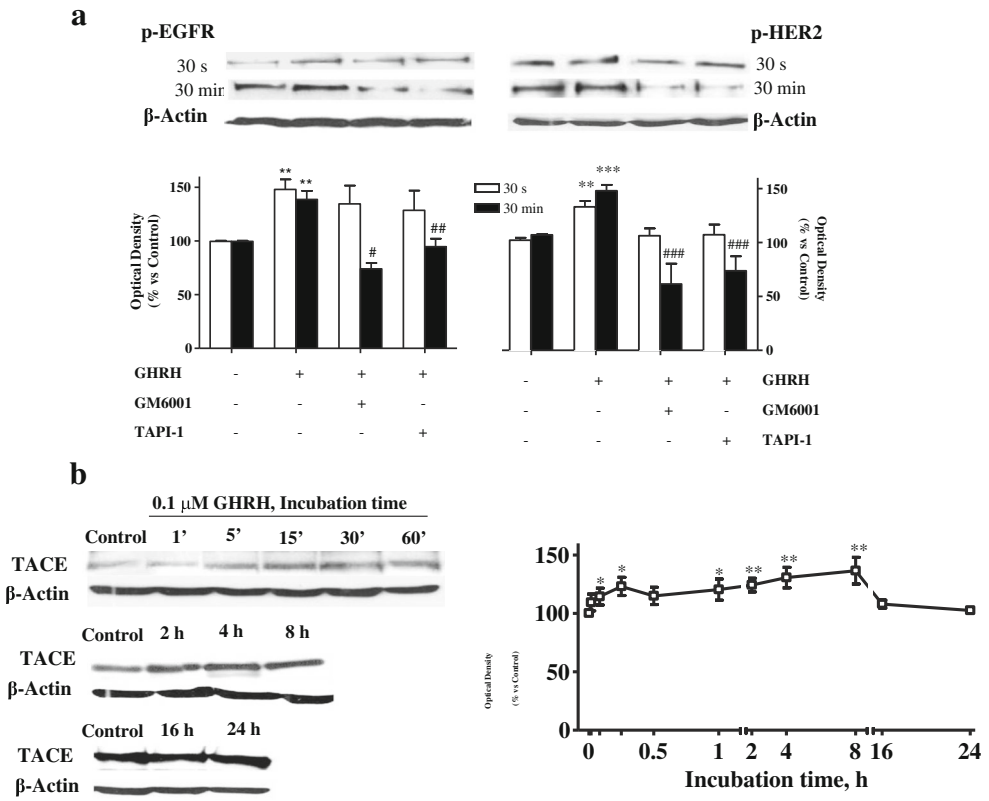

Fig. 5 Association of a disintegrin and metalloprotease (ADAM) on GHRH-induced phosphorylation of EGFR and HER2 in human PC3 androgen-independent prostate cancer cells. **a**, Cells were exposed to specific inhibitors of metalloproteinases, 10 μM GM6001 (for matrix metalloproteinases) or 10 μM TAPI-1 (for ADAMs) for 60 min. Afterwards, cells were incubated in the presence or absence of GHRH (0.1 μM) for 30 s or 30 min was performed. **b**, Immunodetection using antibodies against the tumour necrosis factor-α-converting enzyme (TACE/ADAM-17) and β-actin was performed followed by densitometry of the corresponding bands. A representative experiment of at least three is shown. Data are the mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus the corresponding control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus GHRH

Transactivation of GHRH signaling pathway by EGF

With the purpose of making a first preliminary approach to assess whether RTK ligands can transactivate the GHRH signaling pathway, we analyzed the effect of EGFR ligand on the expression of both GHRH and its receptor (Fig. 6b). The incubation of cells with 100 ng/ml EGF resulted in a significant increase (20–45 %) in mRNA levels for GHRH from 60 to 480 min. In addition, a significant rise in mRNA for GHRH receptors (36 %) was observed at 120 min after the incubation with the growth factor.

Effect of GHRH antagonists, JMR-132 and JV-1–38, on the activated forms of EGFR, HER2 and Src in PC3 prostate tumors

We evaluated the expression of activated EGFR and HER2 after treatment with GHRH antagonists in PC3 xenografts. The expression of phosphorylated EGFR and HER2 was analyzed by immunohistochemistry (Fig. 7a). Tumor cells from control groups showed an intense immunoreactivity to phosphorylated EGF receptor (Fig. 7aa). Xenografts from mice treated with JV-1–38 presented a lower number of positive cells to EGFR (Fig. 7ac) and the most of tumors from mice treated with JMR-132 were negative to this receptor (Fig. 7ab). Moreover, phosphorylated HER2 was detected in all experimental groups (Fig. 7a). In control group, the immunolabeling was observed in the plasma membrane of tumor cells (Fig. 7ad); however, in the xenografts from mice treated with the antagonists, some cells showed the immunoreaction grouped beneath the plasma membrane with a patchy pattern (Fig. 7ae-f).

In order to evaluate whether a less immunoreactivity was due to a less expression of both molecules, we checked the status of EGFR and HER2 mRNA and protein levels from tumor tissues by RT-PCR and Western blot assays (Fig. 7b). Protein and mRNA levels for both tyrosine kinase receptors were significantly decreased after treatment with GHRH antagonists (Table 1). Tyrosine kinase Src has been assessed by Western blot analysis (Fig. 7c). The p-Src levels were significantly diminished in the groups treated with JMR-132 (28 %) and JV-1-38 (44 %).

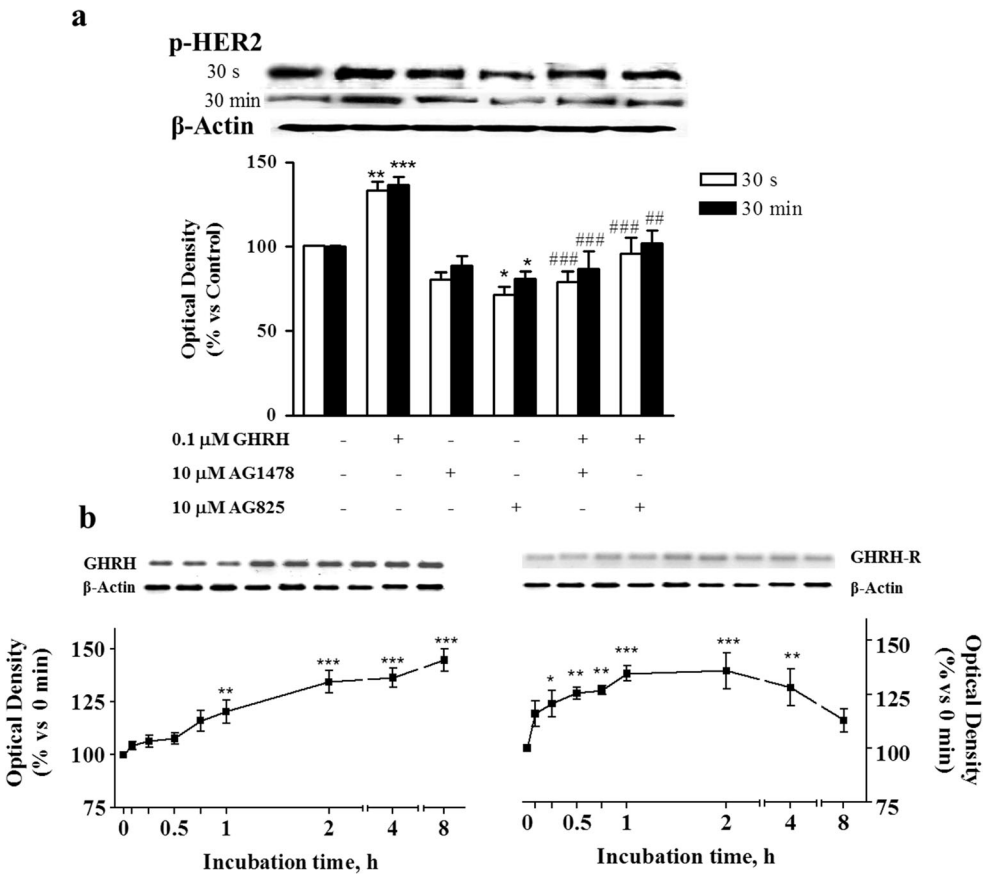

Fig. 6 Effect of GHRH on the heterodimerization of tyrosine kinase receptors such as EGFR and HER2 in human PC3 androgen-independent prostate cancer cells (**a**). Cells were incubated with specific inhibitors of tyrosine kinase activity, AG1478 (for EGFR) or AG825 (for HER2) for 30 min, respectively. Afterwards, an incubation in the presence or absence of GHRH (0.1 μM) for 30 s or 30 min was carried out. Cell lysates were used to perform Western blot assays using antibodies against p-HER2 and β-actin followed by densitometry of the bands. Time-dependent effect of EGF on the expression of mRNA for GHRH and GHRH receptors in human PC3 androgen-independent prostate cancer cells (**b**). Cells were incubated in the presence or absence of 100 ng/ml EGF for the indicated times. Semiquantitative RT-PCR analysis for GHRH, GHRH-R and β-actin mRNAs was performed. A representative experiment of at least three is shown (upper panels). Data in each bar are the mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus the corresponding control; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus GHRH

Discussion

In our study GHRH modulates the signaling of tyrosine kinase receptor family members in human PC3 androgen-independent prostate cancer cells in at least two ways. Such a modulation involves an increase in expression levels of EGFR and HER2 as well as in tyrosine phosphorylation of EGFR and HER2. The activation of HERs by GHRH is achieved through two mechanisms depending on the presence or the absence of their corresponding ligand. The treatment with GHRH antagonists, JMR-132 and JV-1–38, results in a significant reduction in the growth of PC3 human prostate cancers in nude mice, as reflected by tumor volumes [10].

There are tendencies for upregulation of HER2, increased co-expression of EGFR and HER2 and downregulation of HER3 in the prostate cancer lymph node metastases in comparison to the primary tumors [25]. Protein expression levels of EGFR and HER2 were evaluated in PC3 cells, which exhibit features of advanced prostate cancer [16, 26]. It has been described that EGFR and HER2 are involved in prostate cancer progression towards androgen independence [14, 27]. After treatment with GHRH, both EGFR and HER2 protein levels were increased in a significant manner in androgen-independent cells. Similarly, other members structurally related to GHRH enhanced HERs protein levels in enteric neurons, glial cells and human breast cancer cells [28, 29].

GHRH also induces tyrosine phosphorylation of EGFR and HER2 following a bimodal pattern in an advanced stage of prostate cancer. The first transactivation of HERs mediated by GHRH developed rapidly within 30 s after addition of GHRH. This fact could be due to the fast stimulation of pre-existing EGFR and HER2 molecules. The second transactivation of HERs induced by GHRH passed to longer time periods (30 min). In this case, increased p-EGFR and p-HER2 levels may be conceivably the result of a slower induction of synthesis of both tyrosine kinase receptors. In the same way, similar responses have been observed for other member structurally related to GHRH in colonic epithelial, breast

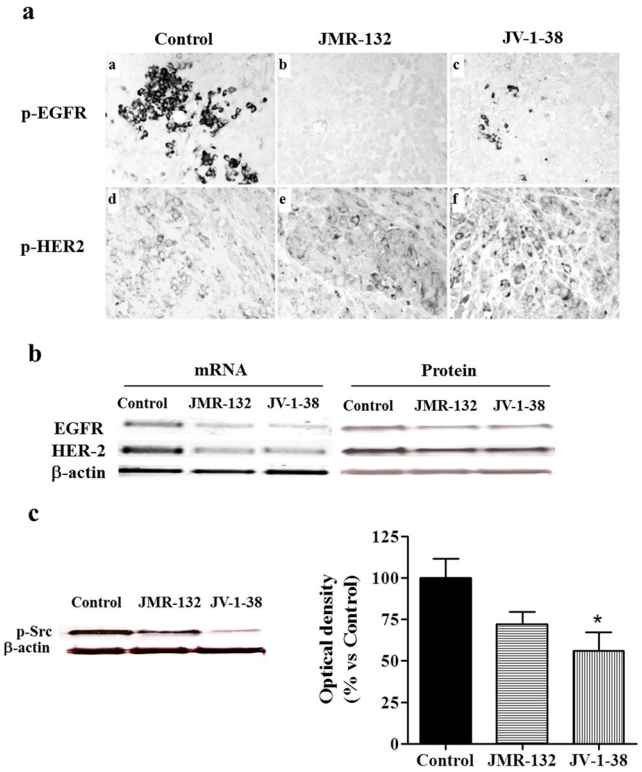

Fig. 7 pEGFR and pHER2 expression. Control samples showed an intense immunoreactivity to pEGFR (**a**) (**aa**). Tumors from mice treated with JMR-132 were negative to this receptor (**ab**) and JV-1–38 treated xenografts presented a low positive cell number (**ac**). Tumor cells from control samples showing pHER-2 in the plasma membrane (**ad**). In treatments with GHRH antagonists, the immunolabeling was observed beneath the plasma membrane with a dotted pattern (ae, af). Original magnification ×300. Effects of the GHRH antagonists JMR-132 (10 μg/day) and JV-1–38 (20 μg/day) on EGFR and HER2 expression in PC3 tumors (**b**) HER mRNA and protein levels were assessed by RT-PCR and Western blot assays, respectively. Expression levels were normalized with those for β-actin. Effect of the GHRH antagonists JMR-132 (10 μg/day) and JV-1–38 (20 μg/day) on activated Src expression in PC3 tumors (**c**). Immunodetection using antibodies against phosphorylated Src and β-actin was performed followed by densitometry of the corresponding bands. A representative experiment of at least three is shown. Data in each bar are the means ± SEM. *$P<0.05$ vs. control

cancer, and prostate cancer cells [16, 28, 30]. Therefore, our results show that GHRH is linked to the HER system by at least two ways. GHRH may induce the expression of specific genes for EGFR/HER2 and stimulate tyrosine phosphorylation of both receptors.

GHRH and its receptors have been involved in a variety of important cellular processes like cell survival, proliferation, apoptosis, angiogenesis and neoplastic transformation in various non-pituitary tissues including prostate [10, 31–33]. Previous studies detected the expression of splice variants (SV) of GHRH-R in the cancer models examined [6, 34]. Transactivation of EGFR and HER2 in a late stage of prostate tumor progression is initiated by GHRH binding to its receptors. Tyrosine phosphorylation of EGFR and HER2 was abolished by both GHRH antagonists, JMR-132 and JV-1–38.

GHRH receptors are coupled to Gs protein and their stimulation leads to the generation of cAMP and subsequent PKA activation in various tissues including prostate [35]. Intracellular cAMP/PKA signaling may be essential for prostate cancer cells to adapt to invasive phenotypes in the absence of androgen [35]. Present results demonstrate that GHRH induces the activation of EGFR and HER2 through PKA stimulation. Likewise, this enzyme participates in the transactivation of HERs provoked by other GPCRs in neurons and colonic epithelial, breast and prostate cancer cells [16, 28, 30, 36].

Src is highly expressed in prostate cancer cell lines as well as in the majority of prostate cancer specimens [37]. Furthermore, Src signaling is involved in androgen-induced proliferation of prostate cancer cells and may also participate in the transition to androgen-independent growth [37]. In addition, Src-family tyrosine kinases are known mediators of the transactivation of EGFR by GPCR [15, 36, 38, 39]. In our study, the rapid transactivation of HERs by GHRH is Src dependent because a Src inhibitor, PP2, blocked the response to the neuropeptide. On the other hand, GHRH increased Src activity at two different times. The faster effect may be due to the phosphorylation of the total content of Src. This first increase in p-Src levels could be involved in phosphorylation of pre-existing EGFR and HER2 molecules by GHRH in prostate cancer cells. The slow effect of GHRH on p-Src levels could be due to *the novo* synthesis of the non-receptor kinase as well as to its differential regulation by several growth factors. Again, the augmented Src activity induced by GHRH

Table. 1 Effect of treatment with GHRH antagonists JMR-132 (10 μg/day) and JV-1–38 (20 μg/day) on mRNA expression and protein levels of EGFR and HER-2 in PC3 tumors

	EGFR		HER-2	
Treatment	mRNA (% of control)	Protein (% of control)	mRNA (% of control)	Protein(% of control)
Control	100±6.7	100±5.6	100±8.1	100±6.0
JMR-132	69.3±12.1*	72.9±4.5*	71.5±6.7*	78.6±3.3*
JV-1–38	62.1±5.0*	77.9±6.9*	66.0±7.2*	70.5±8.3**

Values are mean ± SEM; mRNA and protein levels were quantified by densitometric analysis, and the data were normalized to actin values.*$P<0.05$ vs. control; **$P<0.01$ vs. control

was blocked with GHRH antagonists. In our system, PKA might activate Src-kinases which in turn could phosphorylate EGFR and HER2.

Transactivation of HER by GPCR may also involve EGFR-ligand-dependent pathway. This is mediated by a metalloproteinase which in turn induces the shedding of pro-HB-EGF present on the cell surface leading to the generation of EGF. Subsequent release of the mature growth factor activates EGFR and its downstream signaling cascades [40]. Furthermore, it has been reported that the tumor necrosis factor-α-converting enzyme (TACE/ADAM-17) contributes to the invasion of androgen-independent prostate cancer cell. Such a metalloproteinase stimulates the detaching of TGF-α which subsequently activates the EGFR-MEK-ERK signaling pathway [41]. In our study, an inhibitor of matrix metalloproteinases suppressed the slower transactivation of both HERs induced by GHRH. This fact suggests the involvement of an extracellular pathway of HER activation in advanced prostate carcinoma. In our study, protein levels of TACE/ADAM17 were increased after treatment with the neuropeptide GHRH supporting that such a metalloproteinase can be activated by GPCRs [42, 43]. On the other hand, Src non-receptor protein interacts with the cytoplasmic tail of ADAM17, which promotes its phosphorylation and translocation to the cell membrane [44, 45]. In our study, the slow transactivation of HER induced by GHRH is Src independent in human androgen-independent prostate cancer cells. Further experiments should be performed in order to clarify the elements involved in this extracellular pathway of HER activation.

HER2 is the preferred receptor partner for heterodimerization of EGFR and HER3 giving potent mitogenic signals [46]; it activates androgen receptor and has been linked to the clinical progression of castration-resistant human prostate cancer [14, 47]. After the exposure of PC3 androgen-independent prostate cancer cells to the neuropeptide GHRH, HER2 seems to form dimers with others members of HER family. GHRH induces the transactivation of HER2. However, this activation could involve the phosphorylation of EGFR since that effect was abolished by an inhibitor of EGFR tyrosine kinase activity. In addition, EGFR phosphorylation could activate other HER family members which in turn would activate HER2.

On the other hand, RTK activation induced by growth factors triggers extracellular release of GPCR ligands, which bind and activate the corresponding GPCR in an autocrine/paracrine manner [15, 48]. In this regard, in a first preliminary approach, we observed an increase of mRNA levels for both GHRH-R and GHRH by EGF ligand in androgen-independent prostate cancer cells.

Our in vivo study reveals that both GHRH antagonists caused either a lack or a decrease in the number of positive cells for activated EGFR in PC3 androgen-independent prostate tumors. In addition, Src tyrosine kinase expression levels were decreased after treatment with GHRH antagonists. Previous studies with the GHRH antagonist JMR-132 in human breast cancer xenografts have demonstrated that this antagonist caused a very substantial down-regulation of EGF binding sites [49]. Interestingly, the low levels of GHRH observed in tumors of treated mice [10] could diminish the activation of the receptor tyrosine kinase. In regard to phosphorylated HER-2, the groups treated with GHRH antagonists had a higher number of cells positive for this activated receptor. However, the distribution of HER-2 was different from the control group. The location of phosphorylated HER-2 beneath plasma membrane after treatment with GHRH antagonists could suggest a rapid down-regulation of the tyrosine kinase receptor. In this regard, one class of therapeutics is being developed to treat HER-2-positive cancers including geldanamycin. Such an antibiotic binds to and inactive an essential chaperone of membrane-bound HER-2, heat shock protein 90, inducing endocytosis and receptor down-regulation by proteasomal and lysosomal degradation [50,51]. Therefore, altering EGFR concentrations could provide an effective mechanism for the regulation of EGFR/HER-2 heterodimerization [52]. It is required the development of agents as GHRH analogues, that inhibit receptors for EGF and HER-2 both expressed in a more aggressive stage of the prostatic carcinoma [49].

The present findings shed more light in the activation mechanisms of HER by GHRH in human androgen-independent prostate cancer. Consequently, the cross-talk between HER and GHRH-R might be impeded by combining drugs acting upon GHRH receptors and HER family members. This combination could enhance the efficacy; reduce the doses of both types of these drugs and increase therapeutic effects in management of human advanced prostate cancer.

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Conflict of interest The authors declare that they have no conflict of interest.

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