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ORIGINAL ARTICLE

GnRH receptor expression in human prostate cancer cells is affected by hormones and growth factors

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Abstract GnRH receptors (GnRH-R) have been found in various malignancies, including prostate cancer (PCa). They mediate the direct antitumor effects of GnRH analogs. Nevertheless, few reports concern drug-induced modulation of GnRH-R levels. In this study, we investigated GnRH-R expression in androgen-sensitive (LNCaP) and -insensitive (PC-3) PCa cells treated for 4 and 6 days with a GnRH agonist (Leuprorelin acetate, LA, 10^{-11} or 10^{-6} M), Dihydrotestosterone (DHT, 10^{-9} M), Cyproterone acetate (CA, 10^{-7} M), and Epidermal growth factor (EGF, 10 ng/ml), either alone or combined. The RT-PCR analysis showed no variation in GnRH-R mRNA levels of both treated LNCaP and PC-3 cells. On the contrary, immunoblotting indicated that in LNCaP and PC-3 cells, LA upregulated membrane GnRH-R expression (up to 92%). In androgen-sensitive cells, DHT induced a GnRH-R increase (up to 119%) always comparable to that occurring in the presence of CA. GnRH-R upregulation by LA/DHT or CA/DHT association was similar to that promoted by the single agents. In PC-3 cells, EGF upregulated GnRH-R (up to 110%). A prolonged treatment (for 12 days) determined a greater EGF-induced increase in GnRH-R levels (142%). Lower (or no) receptor enhancement occurred when LA and EGF were associated. Our findings indicate that LA post-transcriptionally upregulates its own membrane receptor in androgen-sensitive and -insensitive PCa cells, counteracting the receptor enhancement produced by DHT and EGF. The effects, obtained with a relatively long and continuous treatment, may have implications in the choice of therapy modality with GnRH analogs and may render the receptor a novel therapeutic target, particularly in hormone-refractory PCa.

Keywords GnRH receptor · Prostate cancer cells · Leuprorelin acetate · Dihydrotestosterone · Cyproterone acetate · Epidermal growth factor

Introduction

The binding of Gonadotropin Releasing Hormone (GnRH) to its hypophyseal type I receptors (type I GnRH-R) stimulates gonadotropin secretion. The existence of an autocrine/paracrine GnRH system has also been suggested by the detection of GnRH mRNA and protein [1–3] and, afterward, by the determination of GnRH-R in various normal and neoplastic extra-pituitary tissues or cell lines [1, 3–7]. In prostate cancer (PCa), the presence of GnRH membrane-binding sites has been demonstrated by a number of laboratories in human cancer specimens, cell lines, and animal experimental models. Nevertheless, controversial results have sometimes been reported concerning the receptor-binding affinity [3, 5, 8–11]. In biopsies from patients with PCa, Fekete et al. [8] described both high and low affinity-binding sites, while Qayum et al. [3]

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found only high affinity-binding sites. GnRH-R have been detected in both androgen-sensitive LNCaP [3, 9, 10] and androgen-insensitive DU-145, and PC-3 PCa cells [3, 10]. In LNCaP and in the two hormone-independent cell lines, high and low affinity-binding sites were found, respectively [3, 10]. On the other hand, a single class of low affinity-binding sites has been described by other authors in LNCaP cells [9].

Two classes of binding sites have also been reported in hormone-dependent Dunning tumors [8], while in the androgen-independent AT-1 rat PCa model, only high affinity-binding sites have been found [11].

The expression of the transcript representing the type I, pituitary GnRH-R has been demonstrated by Kakar et al. [4] in normal and neoplastic tissues and cell lines derived from them, including PC-3 and LNCaP cells. In this latter model and in DU-145 cells, the GnRH-R which have been identified correspond to the pituitary receptor, in terms of both mRNA expression and protein molecular size [12]. A transcript of the more recently discovered type II GnRH-R is also highly expressed in normal mammalian prostate [13]; in contrast, although indirect evidence suggests the presence of a functional receptor in TSU-Pr1 and DU-145 PCa cells [14, 15], the expression of a full-length functional protein in human PCa has not still been established [13].

The extra-pituitary GnRH-R account for the direct, mainly inhibitory, effect of GnRH analogs on the growth of various neoplastic cells, including PCa cell lines [3, 9, 16, 17]. Thus, it has been suggested that the effectiveness of GnRH analogs in the treatment of PCa may be not only based on the suppression of gonadal androgen secretion, but also based on their direct activity influencing tumor cell behavior at different levels.

In our experience, GnRH agonists are ineffective in regulating cell proliferation when used alone both in androgen-sensitive and -insensitive models, but they counteract or suppress growth stimulation induced by androgens in hormone-sensitive LNCaP cells and by EGF in hormone-insensitive PC-3 cells [18]. In a series of studies on these cell lines, we have also demonstrated the ability of the GnRH agonist Leuprorelin acetate (LA) to downregulate PSA gene expression and to induce variations in apoptosis-related gene expression [18–20]. More recently, the ability of LA to counteract the EGF-induced ERK1/2 activation in LNCaP cells has been demonstrated by our group [21].

The clinical relevance of GnRH-R in prostate tumors is not yet known. Moreover, there is little literature dealing with the modulation of these receptors by hormones or growth factors. In this article, we investigated whether the expression of type I GnRH-R in androgen-sensitive (LNCaP) and -insensitive (PC-3) PCa cells might be affected by a relatively long and continuous treatment with the GnRH agonist LA. This may have some relevance to the analog administration modalities in the management of PCa.

The effect on GnRH-R of DHT (in LNCaP cells) and EGF (in PC-3 cells), used either alone or in combination with LA, was also tested. In the androgen-sensitive model, the anti-androgen CA, which paradoxically exerts androgen-like effects, was also supplied either alone or associated with DHT. The effects of the treatments were investigated both at the mRNA (by RT-PCR) and at the protein level (by immunoblotting and immunocytochemistry). Immunoblot analysis was performed on the membrane fraction to determine extracellularly available receptor.

Results

Expression of GnRH-R mRNA in untreated and treated LNCaP and PC-3 cells

GnRH-R mRNA was detected in both LNCaP and PC-3 cells by RT-PCR analysis.

In the untreated PC-3 cells, the transcript level was slightly lower than in the untreated LNCaP cells. No significant variations in GnRH-R mRNA expression were detected in both LNCaP and PC-3 cells treated as above described for 4 and 6 days (Fig. 1). The same results were obtained by quantitative real time RT-PCR analysis (not shown).

Expression of GnRH-R protein in untreated and treated LNCaP and PC-3 cells

Western blot analysis

Western blot analysis of GnRH-R in LNCaP and PC-3 cells (Figs. 2, 3, and 4) revealed a signal corresponding to a protein of approximately 64 kDa, the molecular mass of the human pituitary receptor reported in the literature [22]. In addition, as for mRNA, a relatively stronger expression of GnRH-R was found in the more-differentiated, androgen-sensitive model (LNCaP) with respect to the less-differentiated, androgen-insensitive PC-3 cells (not shown).

Treatment of LNCaP cells for 4 days with LA (10^{-11} or 10^{-6} M) resulted in a mild upregulation of GnRH-R (20–30% compared with control, $P < 0.001$). A greater enhancement (66% compared with control, $P < 0.001$) was obtained with 10^{-9} M DHT, which did not differ significantly from that produced by 10^{-7} M CA ($P < 0.005$). The DHT/CA association led to the same effects as those observed with the two single hormones. Similarly, the LA/DHT combined administration produced an increase in receptor level quite comparable to that obtained in the presence of the single agents (Fig. 2a). The above effects underwent a time-dependent increase. In fact, as shown in

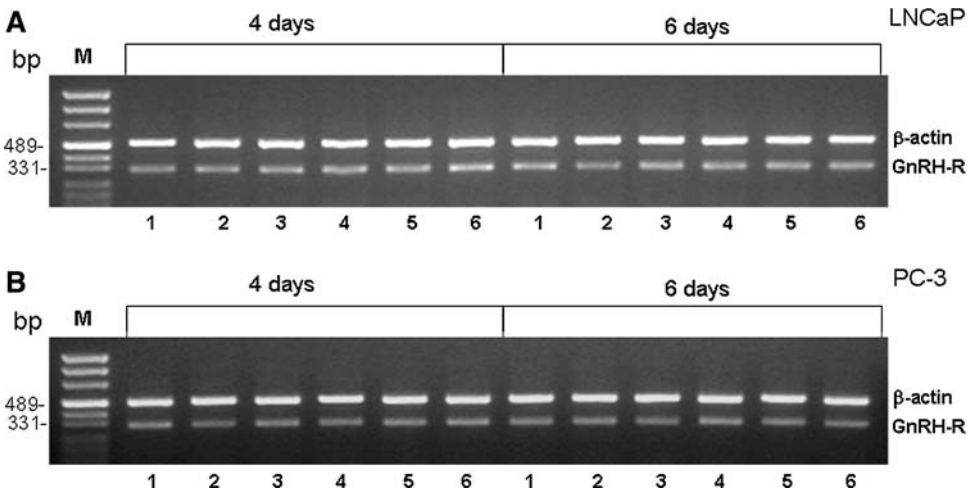

Fig. 1 RT-PCR analysis of GnRH-R (319-bp product size) and *β*-actin (496-bp product size) in LNCaP (**a**) and PC-3 (**b**) cells treated as above described (see Sect. "Materials and Methods") for 4 and 6 days. **a**: Control (1), 10^{-9} M DHT (2), 10^{-11} M LA (3), 10^{-6} M LA (4), 10^{-11} M LA + 10^{-9} M DHT (5), 10^{-6} M LA + 10^{-9} M DHT (6). **b:** Control (1), EGF, 10 ng/ml (2), 10^{-11} M LA (3), 10^{-6} M LA (4), 10^{-11} M LA + EGF, 10 ng/ml (5), 10^{-6} M LA + EGF, 10 ng/ml (6). PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Molecular weight markers are loaded on the first lane (M, pUC Mix Marker 8, Fermentas). The image shown is representative of three separate experiments yielding similar results

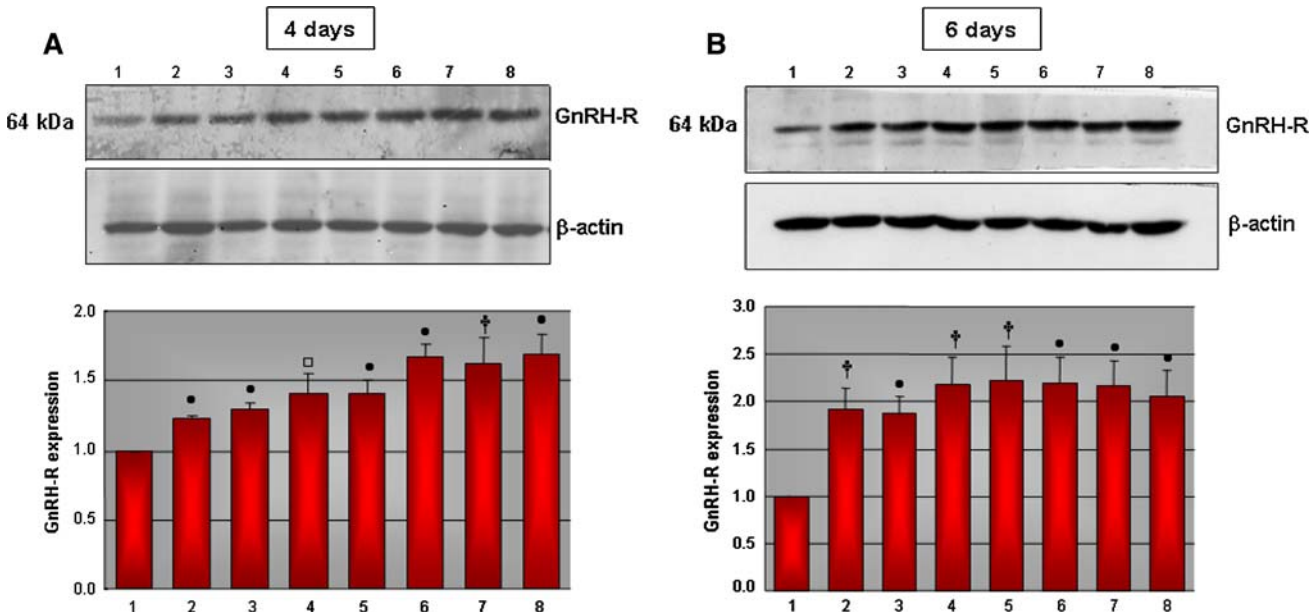

Fig. 2 Western blot analysis of GnRH-R expression in LNCaP cells treated with LA, DHT (either alone or associated to LA) and CA (either alone or associated to DHT) for 4 days (**a**), and 6 days (**b**): Control (1), 10^{-11} M LA (2), 10^{-6} M LA (3), 10^{-11} M LA + 10^{-9} M DHT (4), 10^{-6} M LA + 10^{-9} M DHT (5), 10^{-9} M DHT (6), 10^{-7} M CA (7), 10^{-9} M DHT + 10^{-7} M CA (8). The intensity of signals was quantified by densitometric scanning and normalized to that of *β*-actin. Data are the ratios between values of treated and untreated samples (control, set to 1), and they are shown as mean ± SE of three independent experiments. $^{\square}$ $P < 0.05$, † $P < 0.005$, $^{\bullet}$ $P < 0.001$ *vs* control (Student's *t*-test). A representative blot from three separate experiments yielding similar results is shown

Fig. 2b, a 6-day treatment with LA (10^{-11} or 10^{-6} M) or 10^{-9} M DHT in LNCaP cells resulted in a strong enhancement in GnRH-R expression (about 90% and 119%, respectively, $P < 0.005$–$P < 0.001$). Approximately the same increment produced by DHT occurred in the presence of CA ($P < 0.001$). When the anti-androgen was combined with DHT, the effects did not significantly change. Once again, the association LA/DHT displayed almost the same effects as those obtained when the single agents were administered to the cells.

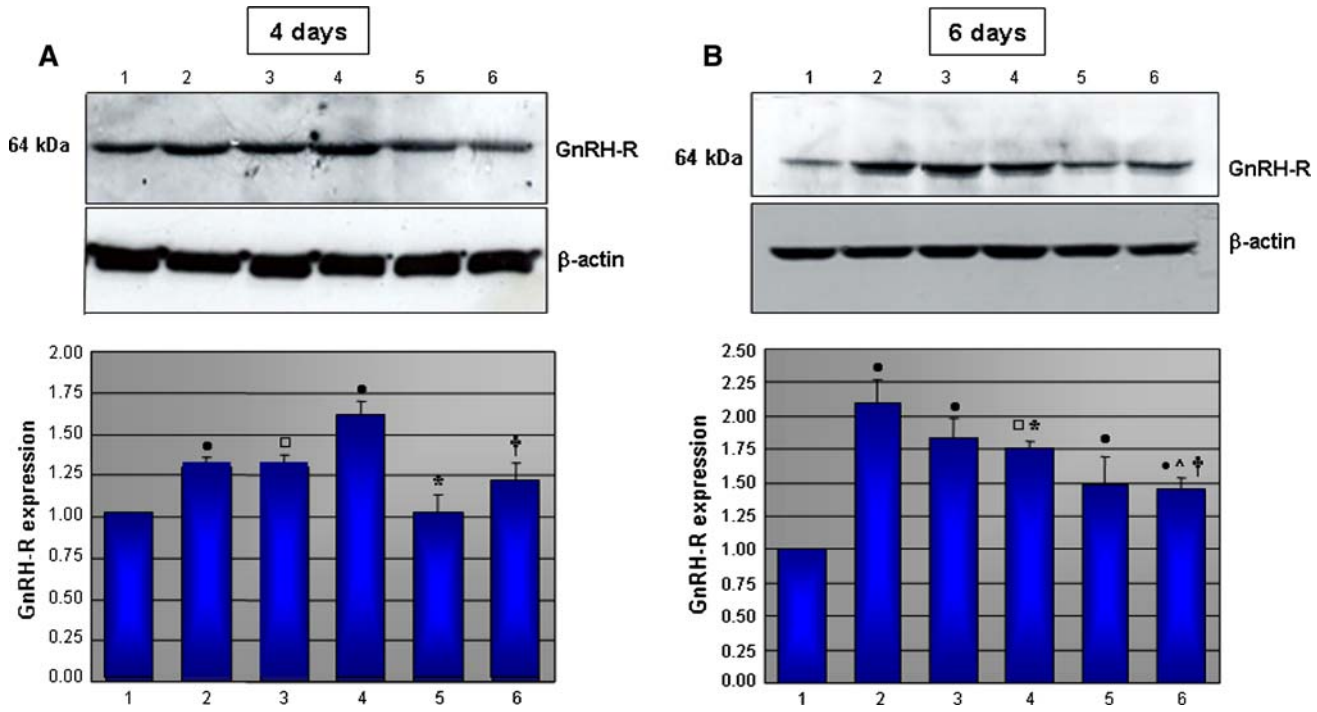

Fig. 3 Western blot analysis of GnRH-R expression in PC-3 cells treated for 4 days (**a**), and 6 days (**b**) with LA and EGF, supplied individually or in association to the cells: Control (1), EGF, 10 ng/ml (2), 10^{-11} M LA (3), 10^{-6} M LA (4), 10^{-11} M LA + EGF, 10 ng/ml (5), 10^{-6} M LA + EGF, 10 ng/ml (6). The intensity of signals was quantified by densitometric scanning and normalized to that of β-actin. Data are the ratios between values of treated and untreated samples (control, set to 1), and they are shown as mean ± SE of three independent experiments. $^{\square}$ $P < 0.05$, $^{\bullet}$ $P < 0.001$ vs control; * $P < 0.05$, ^$P < 0.01$ vs EGF, † $P < 0.05$ vs 10^{-6} M LA (Student's *t*-test). A representative blot from three separate experiments yielding similar results is shown

In the androgen-insensitive PC-3 cells, a 4-day treatment with a low LA concentration (10^{-11} M) produced a 30% increase ($P < 0.05$) in GnRH-R level, as also observed in the presence of 10 ng/ml of EGF ($P < 0.001$, Fig. 3a). A greater enhancement (60% with respect to control, $P < 0.001$) was obtained increasing the analog concentration (10^{-6} M). In this model, an antagonism between LA and EGF activity appears more clearly defined than that observed in LNCaP cells between the analog and DHT. In fact, the LA/EGF association determined a GnRH-R increase which was reduced (or completely absent) compared to those induced by the single agent treatments ($P < 0.05$). As observed in the androgen-sensitive cells, extending the treatment with LA or EGF to 6 days, the increase in GnRH-R signal was greater. The LA- or EGF-induced upregulation of the receptor reached the values of 75–85% and 110%, respectively ($P < 0.001$). A significant reduction in the stimulatory effect of both LA and EGF was observed when the two agents were supplied in combination to the cells ($P < 0.05$–$P < 0.01$) (Fig. 3b).

In PC-3 cells, a further extension of LA (10^{-11} or 10^{-6} M) or EGF (10 ng/ml) treatment to 12 days did not substantially modify the effect produced by the analog after 6 days ($P < 0.001$), while the EGF-induced upregulation reached the value of 142% ($P < 0.001$). The long-time treatment made more evident the interference occurring between LA and EGF, since their association not only abolished the induced increases in GnRH-R signal ($P < 0.001$), but even reduced it below the control value ($P < 0.02$, Fig. 4).

Immunocytochemical analysis

Immunocytochemical analysis of GnRH-R showed that PC-3 cell population was entirely immunopositive with moderate staining in 58% and weak reactivity in 33% of cells. LNCaP cells were moderately reactive in 74% and weakly stained in about 15% of elements, while a very small number (about 1%) of GnRH-R-negative cells were found. Few strong positive cells (9–10%) were detected in both the models. According to other authors [23], the staining was mainly localized in the cytoplasm, with a weak positivity at the plasma membrane (Figs. 5 and 6).

As shown in Fig. 5, in LNCaP cells, a 6-day treatment with 10^{-11} or 10^{-6} M LA did not induce any variations in the described cell categories. On the other hand, DHT and/or CA affected GnRH-R expression in each immunoreactivity group, and these effects were quantified using the H-score method. Considering the DHT-induced changes in each of the staining intensity categories, the overall enhancement in

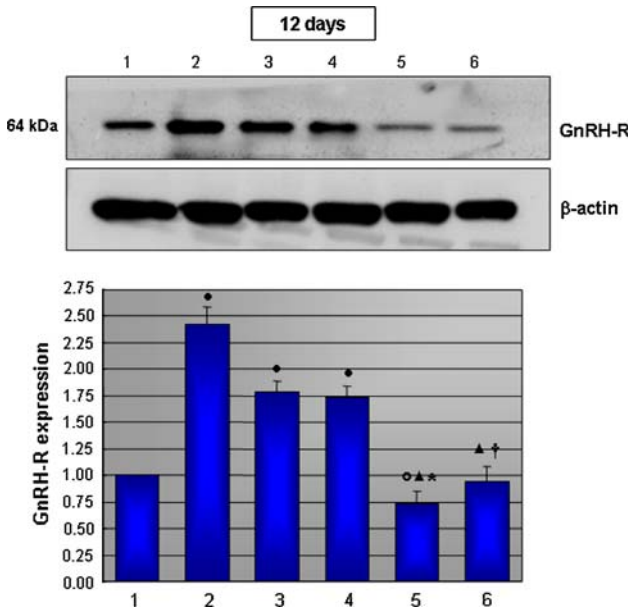

Fig. 4 Western blot analysis of GnRH-R expression in PC-3 cells treated for 12 days with LA and EGF, supplied individually or in association to the cells: Control (1), EGF, 10 ng/ml (2), 10^{-11} M LA (3), 10^{-6} M LA (4), 10^{-11} M LA + EGF, 10 ng/ml (5), 10^{-6} M LA + EGF, 10 ng/ml (6). The intensity of signals was quantified by densitometric scanning and normalized to that of β-actin. Data are the ratios between values of treated and untreated samples (control, set to 1), and they are shown as mean ± SE of three independent experiments. $^{\circ}$ $P < 0.02$, $^{\bullet}$ $P < 0.001$ vs control, $^{\blacktriangle}$ $P < 0.001$ vs EGF, * $P < 0.001$ vs 10^{-11} M LA, † $P < 0.001$ vs 10^{-6} M LA (Student's t-test). A representative blot from three separate experiments yielding similar results is shown

GnRH-R was about 19% ($P < 0.001$, Table 1). To the same extent as that induced by the androgen was the receptor upregulation elicited by DHT/LA, CA, or DHT/CA treatment ($P < 0.001$, Table 1).

In the androgen-insensitive PC-3 cells, a 6-day treatment with LA (10^{-11} or 10^{-6} M) did not lead to any appreciable variation in GnRH-R levels. On the contrary, EGF (10 ng/ml) influenced GnRH-R expression in all the cell categories. The effect of EGF, quantified by using the H-score system, resulted in an overall increase in GnRH-R levels of about 18% ($P < 0.001$, Table 2). After a 12-day treatment, LA did not still modify GnRH-R levels in any of the intensity categories, whereas the overall effect of EGF on GnRH-R level was scored as a 30% upregulation ($P < 0.001$, Table 2 and Fig. 6). The EGF effect remained essentially unchanged even if it was associated with LA.

Discussion

Hormone-responsive PCas are successfully treated with GnRH agonists owing to their ability to suppress gonadal testosterone secretion through a desensitization of the gonadotropes. However, increasing reports in the literature testify that the GnRH analog efficacy may also be attributed to a direct inhibitory effect of PCa cell growth, which is mainly expressed by inhibiting the mitogenic effect of androgens and peptide growth factors [18, 24]. These extrapituitary effects of GnRH analogs progressively assume the countenance of a multifaceted phenomenon, mostly dependent on the degree of expression and signaling pathway of GnRH-R which both differ in the different tissues [1, 25].

In this study, we investigated the effect of LA, a GnRH agonist, on GnRH-R mRNA and protein expression in the two phenotypically distinct PCa cells: the well-differentiated/weakly invasive, androgen-sensitive LNCaP cells and the poorly differentiated/highly invasive, androgen-insensitive PC-3 cells. In addition to the analog effect, the activity of DHT, CA, or EGF, either alone or variously combined with LA, on GnRH-R levels was studied.

GnRH-R are transmembrane G-protein-coupled receptors [26]. The literature reporting the presence of GnRH-R in benign and malignant prostate lesions is controversial. Straub et al. [27] described augmented GnRH-R levels in hormone-refractory PCas with respect to both benign prostate hyperplasia and primary PCas. On the other hand, Halmos et al. [5] demonstrated an inverse relationship between GnRH-R concentration and tumor grade in human PCa specimens. Similarly many but discordant are the data on the affinity-binding characteristics of GnRH-R found in PCa cell lines and specimens [3, 5, 8–10].

Little information is available regarding the variations induced by hormones and growth factors on GnRH-R expression, which may be crucial in view of the extensive GnRH analog treatment of the disease. In vitro studies on GnRH-R modulation by GnRH agonists mostly concern pituitary cell lines in which the hormone administration modality seems to play a key role in determining the effect on GnRH-R expression. A short or pulsatile exposure to the analogs leads to a receptor increase, whereas a continuous and/or prolonged treatment is ineffective in regulating GnRH-R levels [28]. Similar results were obtained in the in vivo studies performed on rats [29]. Regarding the effects of GnRH agonists on GnRH-R expression in PCa, very heterogeneous findings have been reported in the few published studies dealing with this topic. Bono et al. [7] investigated, by immunohistochemistry, the expression of GnRH-R in surgical specimens obtained from PCa patients who were treated for 3 months with neoadjuvant hormonal therapy with leuprolide and the antiandrogen bicalutamide (preceded by a 15 day-long antiandrogen therapy, maximal androgen blockade). The same authors compared their findings with GnRH-R expression found in the samples derived from PCa patients who did not receive any treatment and described a relative treatment-induced decrease

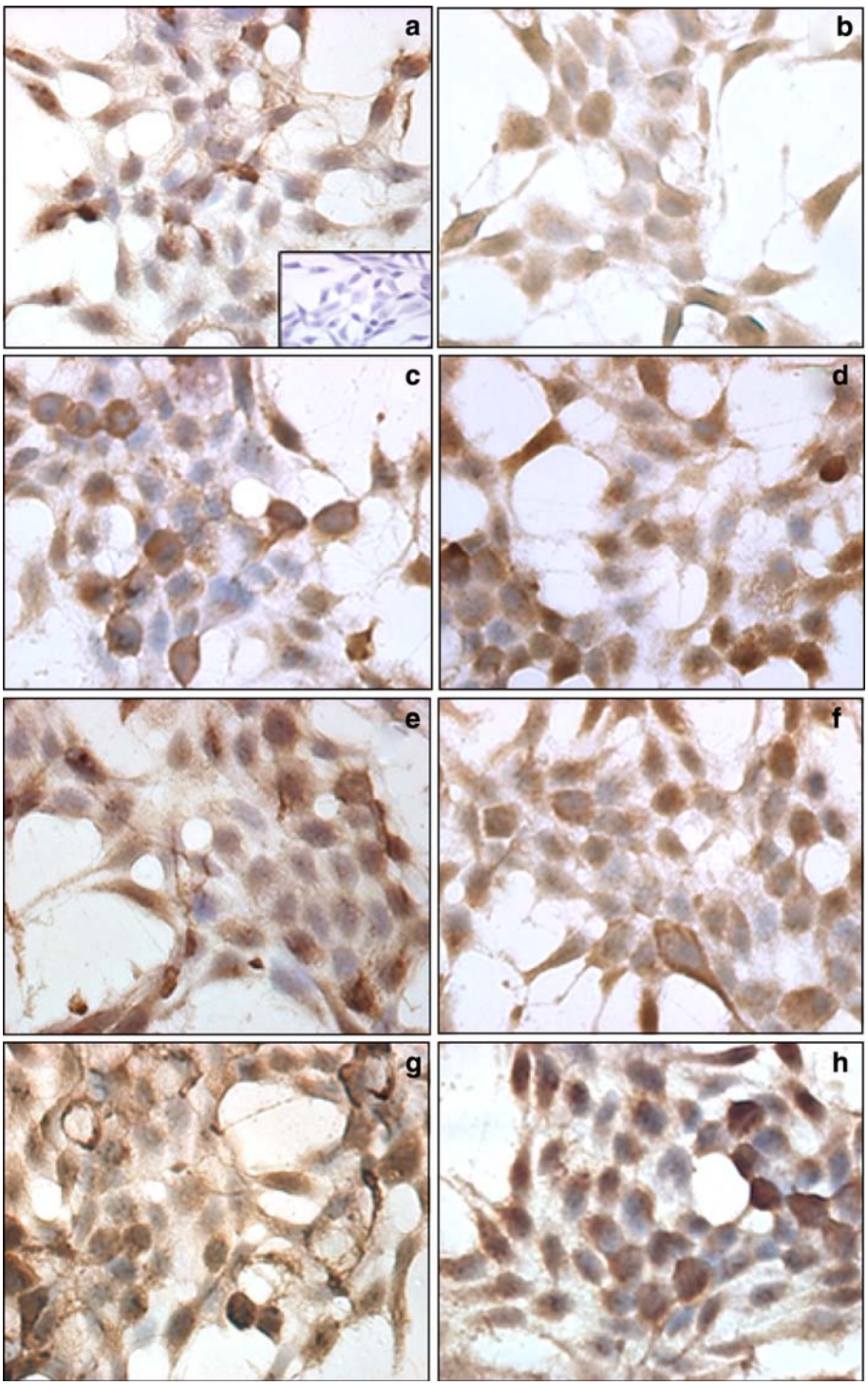

Fig. 5 Immunocytochemical analysis of GnRH-R expression in LNCaP cells exposed for 6 days to LA, DHT, and CA: **a** control cells (untreated cells); **b** 10^{-11} M LA; **c** 10^{-6} M LA; **d** 10^{-9} M DHT; **e** 10^{-11} M LA + 10^{-9} M DHT; **f** 10^{-6} M LA + 10^{-9} M DHT; **g** 10^{-7} M CA; **h** 10^{-9} M DHT + 10^{-7} M CA. The inset in the control cells (untreated cells) photomicrograph shows a negative control. The images are representative of three independent experiments yielding similar results. Original magnification 400×

in GnRH-R immunoreactivity which, in their opinion, may be due to the analog binding to PCa cells. In partial agreement with our data are those by Tieva et al. [30] who demonstrated the ability of the GnRH agonist Goserelin to induce a large increase in GnRH-R mRNA in the rat ventral prostate after a 28-day-long treatment. The opposite effect was observed by the same authors in pituitary samples. On the other hand, a slight decrease in GnRH-R concentration was described by Lamharzi et al. [31] in DU-145 xenografted PCas treated for 28 days with a GnRH agonist. No significant variations in GnRH-R mRNA were observed in this study. In a recent report, Castellon et al. [17] demonstrated no effect of a leuprolide treatment on GnRH-R mRNA and protein levels in PCa cells cocultured with fibroblasts, both isolated from human PCa specimens. The disagreement with our results may be assigned to the

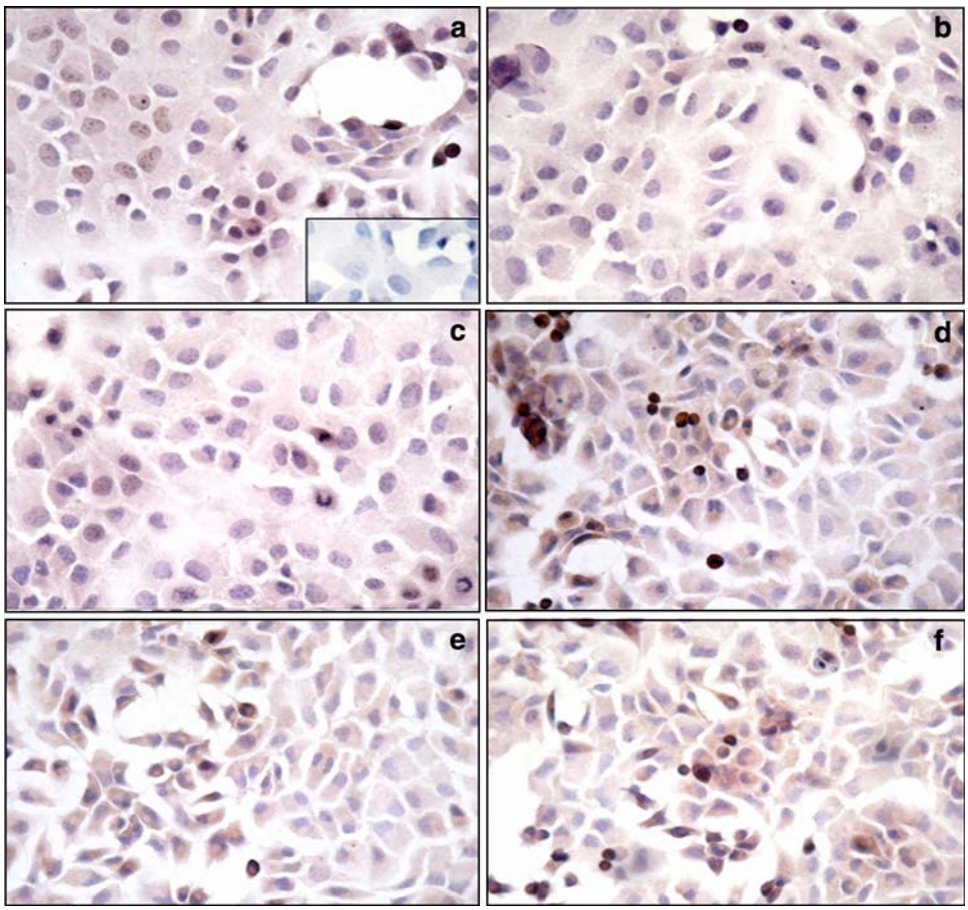

Fig. 6 Immunocytochemical analysis of GnRH-R expression in PC-3 cells exposed for 12 days to LA and EGF, supplied individually or variously combined to the cells: **a** control cells (untreated cells); **b** 10^{-11} M LA; **c** 10^{-6} M LA; **d** 10 ng/ml EGF; **e** 10^{-11} M LA + 10 ng/ml EGF; **f** 10^{-6} M LA + 10 ng/ml EGF. The inset in the control cells (untreated cells) photomicrograph shows a negative control. The images are representative of three independent experiments yielding similar results. Original magnification 400×

Table 1 Semiquantitative immunocytochemical GnRH-R scores in LNCaP cells

6 days of treatment	H-score ± SD
Untreated cells	193.46 ± 0.23
10^{-11} M LA	191.75 ± 1.32
10^{-6} M LA	195.54 ± 1.68
10^{-9} M DHT	229.67 ± 3.05°
10^{-11} M LA + 10^{-9} M DHT	223.02 ± 1.10°
10^{-6} M LA + 10^{-9} M DHT	220.21 ± 1.12°
10^{-7} M CA	226.42 ± 1.99°
10^{-9} M DHT + 10^{-7} M CA	225.71 ± 0.18°

Result are expressed as mean ± SD

° $P < 0.001$ vs untreated cells; ANOVA and Tukey's multiple comparison tests

different sources of the malignant cells, as the models used in our study are represented by established cell lines obtained from PCa metastatic lesions.

A substantial agreement exists on the in vivo effects of the androgens on pituitary GnRH-R expression, a rise in GnRH-R being observed after rat castration [28, 32], whereas a general mismatch emerges from the in vitro studies regarding pituitary cell cultures [33, 34]. As far as PCa is concerned, Tieva et al. [35] found an increase in GnRH-R mRNA levels in Dunning tumors following castration, while no variation in GnRH-R protein level was observed by Gnanapragasam et al. [36] after a 24-h treatment of LNCaP cells with the synthetic androgen Mibolerone. The discrepancy, arising between these latter results and the DHT-induced increase in GnRH-R protein that we observed in the same model, may be ascribed to the different hormones and/or times of treatment.

In our experience, the use of CA, a synthetic steroidal anti-androgen, produced substantially the same stimulatory effects on GnRH-R expression in LNCaP cells as those in DHT-treated cells. This paradoxical activity of CA is not surprising since LNCaP cells keep a mutated AR that illicitly binds progestins, oestrogens, and anti-androgens, which act as agonists [37].

A close interplay has been demonstrated in PCa cells among the actions of GnRH analogs and androgens. To date, no classical androgen response elements have been found in the human GnRH-R promoter. Nevertheless, Maudsley et al. [38] demonstrated a novel GnRH signaling pathway occurring in different cell types, including LNCaP and PC-3 cells, which induces nuclear translocation of AR and renders it transcriptionally inactive. We may

Table 2 Semiquantitative immunocytochemical GnRH-R scores in PC-3 cells

	6 days of treatment	12 days of treatment
	H-score ± SD	
Untreated cells	176.07 ± 2.20	175.26 ± 0.68
10^{-11} M LA	177.89 ± 1.24	173.45 ± 1.56
10^{-6} M LA	176.53 ± 1.46	179.60 ± 0.19
10 ng/ml EGF	208.23 ± 1.63°	228.27 ± 0.04°
10^{-11} M LA + 10 ng/ml EGF	201.20 ± 0.38°	217.67 ± 0.04°
10^{-6} M LA + 10 ng/ml EGF	199.99 ± 3.03°	211.90 ± 0.84°

Result are expressed as mean ± SD

° $P < 0.001$ vs untreated cells; ANOVA and Tukey's multiple comparison tests

hypothesize that part of the GnRH inhibition of the androgen growth stimulating activity in PCa cells as well as the agonist/DHT interference concerning the GnRH-R expression modulation could be ascribed to the agonist targeting the androgen signaling pathway.

The antitumor efficacy of GnRH agonists in peripheral tissues depends, amongst others, on the cross-talk with growth factor signaling cascades [21, 24, 39]. GnRH agonist direct inhibition of PCa growth acts by attenuating a well-documented autocrine proliferation signaling loop. EGF-R pathway has been shown to be required for the proliferation of PCa cells, whose inhibition by GnRH agonist resulted in a decrease of EGF-R levels or in a limitation of the receptor tyrosine kinase activity in vitro and/or in vivo [24, 31, 39]. In androgen-insensitive, highly metastatic PC-3 cells, we observed an EGF-induced increase in GnRH-R after 4, 6, and 12 days of treatment. This novel finding accords with data in human pancreatic cells and cultured rat pituitary cells [40, 41]. It is conceivable that the reduction or disappearance of GnRH-R increment, we observed when EGF was supplied to PC-3 cells in combination with LA, could be partly ascribed to the above EGF-limiting activity of GnRH agonists in PCa cells.

In both the androgen-sensitive and the androgen-insensitive cells, immunocytochemical analysis of GnRH-R expression revealed less dramatic effects of the treatments at both the membrane and cytoplasm level. The discrepancy with the immunoblotting results may be due, in part, to the observed prevalent cytoplasmic immunoreactivity that has made the estimation of variations in GnRH-R occurring at the cell surface difficult.

This study demonstrates that LA is able to increase the membrane expression of its own receptor in both androgen-sensitive and -insensitive PCa cells and that this event occurs at a post-transcriptional level. This effect, which appears after a relatively long-time and continuous exposure, may support the antimitogenic and/or proapoptotic activity of GnRH agonists, may suggest for these agents a differentiating action, and may also have clinical implications. In fact, the augmented availability of receptors at the membrane level may maintain in PCa cells the response to the GnRH-analog treatment and, in addition, render them as tools for targeted therapies with drugs such as anti-GnRH-R monoclonal antibodies conjugated to chemotherapeutic agents. In a recent report by Morgan et al. [42], who engineered cell lines expressing GnRH-R at different levels at the cell surface, the degree of GnRH-R expression has been shown to correlate with the extent of GnRH analog-induced tumor growth inhibition both in vitro and in vivo. The same author hypothesizes the potential usefulness of a patient screening and phenotyping in order to identify those expressing sufficiently high GnRH-R levels and thus those patients who may benefit from an additional response to GnRH agonist therapy based on their direct activity [43]. Our in vitro results have been obtained starting from very low doses of LA (10^{-11} M), consistent with the plasma concentrations of the analogs attainable through the subcutaneous administration of therapeutic doses of slow-release pharmaceutical preparation [44]. Nevertheless, the in vitro events may only partially resemble the more intricate network of environmental influences occurring in vivo.

A recently available polymeric matrix formulation of LA achieves a sustained drug delivery and a higher serum concentration of the analog, which renders the in vivo direct effects more probable. In addition, this formulation allows to attain high reliable and maintainable testosterone suppression to castration level ($\leq$20 ng/dl) [45]. This is quite important, if we consider that in our experience the antiproliferative effect of LA in androgen-sensitive models refers to the antagonization of androgen activity [18].

On the basis of the in vitro observations in this study, it is not easy to predict what exactly might happen in vivo both in androgen-responsive and -refractory tumors when LA faces with DHT or growth factors. Nevertheless, our findings seem to support the idea that, even in hormone-independent PCa, the administration of GnRH analogs may provide some benefits related to their described direct activity on cancer cells.

Materials and methods

Cell lines and culture conditions

The human PCa cell lines LNCaP (hormone-sensitive, passages 45–47) and PC-3 (hormone-insensitive, passages 88–90) were routinely cultured on 100-mm plastic dishes

in RPMI-1640 medium (Eurobio, Les Ulis Cedex B, France) and Dulbecco's modified Eagle's medium (DMEM, Eurobio), respectively. Media were supplemented with foetal bovine serum (FBS, Life Technologies, Paisley, Scotland) (10% for LNCaP and 5% for PC-3 cells), antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, Eurobio), and 2 mM glutamine (Eurobio).

The human mammary carcinoma cells, T47D, used as positive control for GnRH-R expression in Western blot analysis, were maintained in RPMI supplemented with 10% FBS, antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin), and 0.2 IU bovine insulin/ml.

All the cell lines were incubated in a humidified atmosphere of 5% CO_2-95% air at 37°C.

Hormones

The GnRH analog [*D*-Leu^6-(des-Gly^{10}-NH_2)]LH-RH ethylamide (Leuprorelin acetate, LA) was kindly donated by Takeda Italia Farmaceutici SpA, Rome, Italy. 5α-Dihydrotestosterone (DHT), the synthetic steroidal anti-androgen Cyproterone acetate (CA), and Epidermal growth factor (EGF) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antibodies

Mouse monoclonal antibody (mAb) to human GnRH-R (clone GNRH03; Lab Vision Corporation, Fremont, CA, USA) was used for Western blot analysis and immunocytochemistry. Mouse mAb to β-actin (clone AC-15) was from Sigma-Aldrich. A horseradish peroxidase (HRP)-labeled goat anti-mouse secondary Ab (Vector, Burlingame, CA, USA) was used in Western blot analysis. A biotinylated goat anti-mouse secondary Ab (Vector) was used for immunocytochemical analysis.

Cell treatments

On the basis of our previous results [18, 20], and with the aim of evaluating the effect of a relatively long-time treatment on the expression of GnRH-R, in this study, the cells were treated for 4 and 6 days with two LA concentrations: 10^{-6} M and 10^{-11} M. This latter dose is near to the concentration attainable in the serum of PCa patients treated with therapeutic doses of the analog [44].

DHT concentration (10^{-9} M) used in these experiments is close to the dissociation constant (Kd) value (9×10^{-10} M) of the androgen receptor in LNCaP cells, and it was proved to be effective in stimulating LNCaP cell proliferation [18, 46].

As far as CA is concerned, we are aware of the paradoxical effect induced by this anti-hormone on the proliferation of LNCaP cells which keep a mutated androgen receptor [37]. In this study, CA was used in combination with DHT, in order to verify its capability to compete with the androgen. A 100-fold higher concentration (10^{-7} M) than that of DHT was used, as is commonly used in such experiments assessing the anti-androgenic activity of these compounds [46].

For the evaluation of the effect of LA, DHT, CA, and EGF on GnRH-R (mRNA and protein) expression cells were treated as follows:

LNCaP cells were plated out at a density of 50,000 cells/ml of standard culture medium in 100-mm dishes (for Western blotting and RT-PCR) or on microscope circular slides (for immunocytochemistry). Cells were allowed to adhere and, 48 h after plating, medium was changed with fresh RPMI-1640 supplemented with 5% charcoal-treated FBS (CH-FBS) and containing LA (10^{-11} or 10^{-6} M), DHT (10^{-9} M) or their association. Cells were also exposed to 10^{-7} M CA, alone and associated to DHT.

PC-3 cells were seeded at a density of 25,000 cells/ml of standard culture medium in 100-mm dishes or on microscope circular slides. Once cells have adhered to the culture plates (24 h), medium was renewed with DMEM supplemented with 5% CH-FBS. LA (10^{-11} or 10^{-6} M) and EGF (10 ng/ml) were added separately or in combination to the culture medium.

For both cell lines, the medium was changed every 48 h, while LA was daily added to the cultures. Treatments were stopped after 4 or 6 days. The immunocytochemical analysis of receptor expression was performed only after a 6-day treatment in both cell lines, because on the 4th day, the number of adherent cells was too low.

In androgen-insensitive PC-3 cells, drug exposure was also extended until the 12th day. In this case, cells were treated for 6 days as above described; subsequently, they were trypsinized, seeded at the usual density and, as they adhered to the culture plates, they were exposed to the above agents for a further 6-day period. In androgen-sensitive LNCaP cells, the prolonged treatment in androgen-/growth factor-depleted medium did not allow the cells to survive.

In all the experiments, control cultures were run in parallel.

RT-PCR analysis

LNCaP and PC-3 cells were treated for 4 and 6 days as previously described, and total RNA was extracted by Trizol plus RNA purification kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After measuring RNA yield, an equal amount of total RNA (1 μg) from each sample was reverse transcribed in a 20 μl of a reaction mixture containing 20 U AMV RT and 3.2 μg random primer p(dN)$_6$ at 42°C for 1 h by the first strand

cDNA synthesis kit (Roche, Indianapolis, IN). For multiplex PCR, 2 μl of the cDNA were placed in a final volume of 50 μl of 1× reaction buffer containing 2 mM $MgCl_2$, 1 mM PCR grade nucleotide mix, 2 U Fast Start Taq DNA Polymerase (Roche). Primers for GnRH-R were: sense, 5′-GAC CTT GTC TGG AAA GAT CC-3′; and antisense, 5′-CAG GCT GAT CAC CAC CAT CA-3′; and for β-actin: sense, 5′-GGC ATC CTC ACC CTG AAG TA-3′; and antisense, 5′-CCA TCT CTT GCT CGA AGT CC-3′. The PCR temperature profiles were: 5 min of pretreatment at 95°C and 33 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 1 min, and extension at 72°C for 2 min. Amplified PCR products were resolved in 2% agarose gel, stained with ethidium bromide, and visualized under UV light. The intensity of the bands was analyzed by densitometric scanning (Chemi Doc Documentation System/Quantity One quantitation software, Bio-Rad, Hercules, CA, USA). The GnRH-R mRNA levels were normalized to the control gene, β-actin, and the data were obtained as a ratio of the normalized values of treated versus untreated (control) samples.

Quantitative real-time RT-PCR analysis

GnRH-R gene expression in LNCaP and PC-3 cells, treated for 4 and 6 days as previously described was also quantified by real-time quantitative RT-PCR using LC FS MasterPLUS SYBR Green I (Roche). DNA amplification was carried out using a LightCycler 2.0 System (Roche), according to the manufacturer's instructions. The relative quantities of GnRH-R gene mRNA against the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were evaluated by adopting a ΔC_T method [47]. Primers for GAPDH were: sense, 5′-TGA ACG GGA AGC TCA CTG G-3′; antisense, 5′-TCC ACC ACC CTG TTG CTG TA-3′.

Western blot analysis

At the end of each period of treatment described above, plasma membrane-enriched fractions from LNCaP and PC-3 cells were prepared according to the protocol reported by Limonta et al. [12]. Samples were homogenized in 10 mM Tris-HCl (pH 7.6) buffer containing 1 mM dithiothreitol on ice. The homogenates were centrifuged two times for 10 min each at 800×*g* to remove cellular debris, and the resulting supernatants were centrifuged at 18,000×*g* to pellet down the membrane fractions. The pellets were solubilized in RIPA buffer [50 mM Tris-HCl (pH 7.7), 150 mM NaCl, 0.8% Triton X-100, 0.8% sodium deoxycholate, 0.08% SDS, 10 mM EDTA, 100 μM Na_3VO_4, 50 mM NaF, 0.3 mM PMSF, and 5 mM iodoacetic acid]. The total protein concentration was determined by the modified Lowry method [48]. Samples were equally loaded at 100 μg/lane and electrophoresed on 10% polyacrylamide gel under reducing conditions. Proteins were then electroblotted onto a polyvinylidene difluoride membrane (Immobilon P, Millipore, Bedford, MA, USA) which was probed (overnight, 4°C) with the primary mAb (1:60 dilution) in TBS containing 0.02% Tween 20 (TBS-T) and 5% nonfat dried milk (blocking buffer). The blot was then overlaid with the HRP-labeled secondary Ab diluted 1:5,000 for 40 min, at room temperature (r.t.) in blocking buffer. The protein bands were detected using an enhanced chemiluminescence system (ECL, Amersham, Buckinghamshire, UK) and visualized on Hyperfilm ECL (Amersham). The membrane was stripped and rehybridized with an anti β-actin mAb (1:10,000) used as an internal control for protein loading. The signals were quantitated by densitometry (Chemi Doc Documentation System/Quantity One quantitation software, Bio-Rad). Densitometric units of the protein of interest were then corrected for the densitometric units of β-actin. The specific protein/β-actin ratio from each treated sample was divided by the value obtained under control conditions to obtain the fold enhancement or reduction of the protein.

Immunocytochemistry

At the end of each described treatment, cells were washed twice in PBS (without Ca^{++} and Mg^{++}) and fixed with 3% paraphormaldehyde for 20 min at r.t. After washings with 50 mM Tris-HCl (pH 7.4), endogenous peroxidase was inhibited with 2% hydrogen peroxide for 10 min. Cells were rinsed and then incubated overnight at 4°C with the anti-GnRH-R mAb (1:15 dilution). After rinsing, cells were incubated for 30 min with biotinylated secondary Ab solution (1:200 dilution) at r.t. The Abs were diluted in blocking solution (5% goat serum in Tris-HCl). The antigen–Ab complex was visualized by an avidin–biotin peroxidase complex solution by using an ABC kit (Vector) with 3,3′-diaminobenzidine (Vector) as chromogen. Harris' hematoxylin was used for nuclear counterstaining. Negative controls were performed by omitting the primary Ab. Tissue sections of human pituitary were used as positive control.

Cells were counted under a light microscope (Axioskop 2 plus, Zeiss), and the staining intensity was graded as absent (−), weak (+), moderate (++), and strong (+++). Two independent observers counted at least 500 cells in 4–10 random fields per slide, and the percentage of stained cells in each intensity category was determined.

The immunocytochemical analysis was scored in a semiquantitative fashion incorporating both the intensity and distribution of specific staining (H-score method).

Scoring was generated as follows: (3 × [% of strongly stained cells]) + (2 × [% of moderately stained cells]) + (1 × [% of weakly stained cells]) [49]. This gives a possible range of 0–300. Scoring was performed on high-power-fields (400x).

Statistical analysis

In the RT-PCR and Western blotting experiments, the significance of the difference between the two groups was determined by an unpaired two-tailed Student's *t*-test.

Immunocytochemical data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. A value of $P < 0.05$ was considered statistically significant.

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