Type I Gonadotropin-Releasing Hormone Receptor Mediates the Antiproliferative Effects of GnRH-II on Prostate Cancer Cells

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Background: GnRH-II has been shown to exert a strong antiproliferative action on tumors of the female reproductive system. The data so far reported on the effects of GnRH-II on prostate cancer growth are controversial. Moreover, it is still unclear through which receptor [type I or type II GnRH-receptor (GnRH-R)] GnRH-II might modulate cancer cell proliferation.

Objective: The objective of this work was to investigate whether GnRH-II might affect the proliferation of prostate cancer cells and to identify the GnRH-R through which the peptide might exert its activity.

Design: We investigated the effects of GnRH-II on prostate cancer cell proliferation. We then transfected PC3 cells with a small interfering RNA targeted to type I GnRH-R. After receptor silencing we evaluated the effects of GnRH-II on cell proliferation and on forskolin-induced intracellular cAMP accumulation. Similar experiments were performed by silencing type II GnRH-R.

Results: GnRH-II exerted an antiproliferative activity on prostate cancer cells. Transfection of PC3 cells with a type I GnRH-R small interfering RNA resulted in a significant decrease of the expression of this receptor. After type I GnRH-R silencing: 1) the antiproliferative effect of GnRH-II was completely abrogated; and 2) GnRH-II lost its capacity to counteract the forskolin-induced cAMP accumulation. On the contrary, type II GnRH-R silencing did not counteract the antiproliferative effect of GnRH-II.

Conclusions: GnRH-II exerts a specific and significant antiproliferative action on prostate cancer cells. This antitumor effect is mediated by the activation of type I (but not of type II) GnRH-R and by its coupled cAMP intracellular signaling pathway. (J Clin Endocrinol Metab 94: 1761–1767, 2009)

GnRH was first identified as the hypothalamic key regulator of the reproductive functions. By binding to receptors (GnRH-receptor (GnRH-R)) on pituitary gonadotropes, GnRH stimulates gonadotropin synthesis/secretion and, therefore, steroid production from the gonads (1). GnRH agonists, when given continuously, suppress gonadal steroid secretion through the desensitization of pituitary GnRH-Rs; on the basis of this activity, these compounds are successfully used for the treatment of hormone-dependent pathologies (i.e. polycystic ovarian disease, precocious puberty, endometriosis) (2, 3), and particularly of steroid-dependent tumors (prostate, breast, and endometrial cancer) (4–6).

Later, it was shown that GnRH and GnRH-R are expressed in cancer tissues to act as a local negative regulator system of tumor growth (7–11). We have reported that activation of tumor GnRH-Rs reduces the proliferation as well as the migratory/invasive behavior of prostate cancer cells, either androgen dependent or androgen independent (12–16). These observations suggest that, when used for the treatment of cancers related to the reproductive system, GnRH agonists might exert an additional,
and more direct, antitumor activity; therefore, GnRH agonists might represent a targeted therapeutic approach also for the most aggressive prostate carcinoma. Clinical studies strongly support this hypothesis (17).

More recently, a second form of GnRH (GnRH-II) has been identified in humans (18). This form, which is encoded by a different gene, is a decapeptide showing three amino acid differences from the classical GnRH (now called GnRH-I). GnRH-II is widely distributed in the central nervous system, as well as in peripheral tissues, both normal and tumoral, suggesting that it might be involved in the control of several physiological and pathological functions (18). In particular, GnRH-II has been more potent than GnRH-I in inhibiting the proliferation of endometrial, ovarian, and breast cancers (19–23). In this context, efforts have been made to identify the receptor that might specifically mediate the actions of GnRH-II (type II GnRH-R). However, no transcripts that could be translated into a conventional, full-length, functional receptor have been found in humans (24, 25). Thus, it is still unclear through which receptor (type I or II) the peptide might exert its antitumor activity.

The data so far reported on the possible effects of GnRH-II on prostate cancer growth are controversial (26–28). Here, we demonstrate that GnRH-II exerts a strong antiproliferative activity on prostate cancer cells; this effect is mediated by activation of type I GnRH-R and of its coupled cAMP intracellular signaling pathway.

Materials and Methods

GnRH analogs

The GnRH-I agonist Zoladex [Goserelin acetate, t-Ser(Bu)6-Aza-Gly10-GnRH-I] was from Sigma Chemical Co. (St. Louis, MO). Human GnRH-II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2) was from Bachem (Heidelberg, Germany).

Cell cultures

The human androgen-dependent LNCaP and androgen-independent DU 145 and PC3 prostate cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium (Seromed Biochrom KG, Berlin, Germany) supplemented with fetal bovine serum (Life Technologies, Inc., Paisley, Scotland, UK) (5% for LNCaP and DU 145 cells; 10% for PC3 cells), glutamine (1 mM), and antibiotics (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate) in a humidified atmosphere of 5% CO2/95% air.

RT-PCR analysis

LNCaP, DU 145, and PC3 cells (2 × 105 cells per dish) were plated in 60-mm dishes. After 3 d, cells were treated daily with GnRH-II (10–2 to 10–5 μM) or Zoladex (10–3 μM), used as a control. After 7 d treatment, cells were harvested and counted by a hemocytometer. Each experimental group consisted of six replicates, and each experiment was repeated three times.

Type I GnRH-R silencing experiments

Type I GnRH-R small interfering RNAs (siRNAs) were from Invitrogen (Burlington, Ontario, Canada). Their sequences were: sense sequence 5′-UUGCAGAGAGUACUCUCAGCAUAACC-3′ and antisense sequence 5′-GGUAUGCUUAGAGAGUGUCUCCGAA-3′. A non-specific scrambled siRNA was used as a control (Medium GC Duplex; Invitrogen). Transfections were performed using Lipofectamine RNAi Max (Invitrogen) as the transfectant (according to the manufacturer’s instructions). Efficiency of transfection was assessed using the block-it Alexa fluor red fluorescence oligo. Transfection efficiency was usually higher than 70% of total cells. The effective knockdown of type I GnRH-R after siRNA transfections was monitored by RT-PCR, Western blot, and immunofluorescence analysis.

Cell proliferation assays

LNCaP, DU 145, and PC3 cells (1 × 104 cells per dish) were plated in 60-mm dishes. After 3 d, cells were transfected with type I GnRH-R siRNA or with scrambled siRNA (70 nM, final concentration) for 24 h. Reverse transcription was performed on total RNA, as described previously. PCR was performed for 33 cycles (94°C for 60 sec, 59°C for 60 sec, 72°C for 120 sec), in the presence of the primers: upstream primer, 5′-GACCTTGCTCTGAAAGAATGCCC-3′ (50 pmol) and downstream primer 5′-CAGGCTGATCCACCAATCATA-3′ (50 pmol) (30). The amplified cDNA products were separated on 1.5% agarose gels stained with ethidium bromide and visualized under UV light. The experiments were repeated three times.

Western blot analysis

PC3 cells were seeded in 10-cm dishes (1.5 × 106 cells per dish) and transfected, 2 d later, as described previously. After either 24 or 48 h, cells were lysed in radioimmunoprecipitation assay buffer [0.05 M Tris-HCl (pH 7.7), 0.15 M NaCl, 0.8% sodium dodecyl sulfate, 10 mM EDTA, 100 μM NaVO4, 50 mM NaF, 0.3 mM phenylmethylsulfonyl fluoride, and 5 mM iodoacetic acid] containing leupeptin (50 μg/ml), aprotinin (5 μg/ml), and pepstatin (50 μg/ml). Whole cell extracts (30 μg) were resuspended in sample buffer [0.5 M Tris-HCl (pH 6.8), 20% glycerol, 10% sodium dodecyl sulfate, and 0.05% blue bromophenol] and heated at 95°C for 5 min. After electrophoretic separation by 10% SDS-PAGE, proteins were transferred onto nitrocellulose membranes. Membranes were blocked in 3% BSA before incubation at room temperature, for 2 h, with either the mouse monoclonal type I GnRH-R antibody (1.5 μg/ml) (Clone FG14; Lab Vision Corp., Fremont, CA) or the mouse monoclonal type II GnRH-R antibody (1:500) (67-Rsc-100301; Santa Cruz Bio-


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technology, Inc., Santa Cruz, CA). Detection was done using a horse-radish peroxidase-conjugated antimouse secondary antibody and enhanced chemiluminescence reagents (SuperSignal Chemiluminescence Detection System; Pierce, Rockford, IL). Actin expression was detected with mouse antihuman actin (1:10,000) as the primary antibody (clone Ab-1; Oncogene, San Diego, CA). The experiments were repeated three times.

**Immunofluorescence analysis**

PC3 cells were seeded on 13-mm-diameter coverslips. After 2 d, cells were transfected, as described previously. After transfection (48 h), cells were fixed with 3% paraformaldehyde in 2% sucrose-PBS for 10 min and incubated with the unlabeled monoclonal antibody type I GnRH-R primary antibody (1:100) (Clone A9E4; Lab Vision Corp.), followed by fluorescein isothiocyanate-conjugated goat antimouse secondary antibody (Al- exa Fluor 488; Molecular Probes, Inc., Eugene, OR). Labeled cells were examined under a Zeiss Axiosvert 200 microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) with a 63 × 1.4 objective lens linked to a CoolSNAP ES charge-coupled device camera (Roper Scientific-Crystal Instruments, Rome, Italy). Images were acquired using the MetaVue program (Molecular Devices, Sunnyvale, CA) and analyzed using Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA). Each staining was repeated three times for three different prepreparations for each group.

**Cell proliferation studies after type I GnRH-R silencing**

PC3 cells were seeded (3 × 10^6 cells per well) in 24-multiwell plates and transfected with either type I GnRH-R siRNA or scrambled siRNA. After 2 d, cells were treated with GnRH-II (10^-6 m) or Zoladex (10^-6 m) for 48 h. Cells were harvested and counted by a hemocytometer. Each experimental condition was replicated six times, and each experiment was repeated three times.

**cAMP determination studies after type I GnRH-R silencing**

PC3 cells (3 × 10^6 cells per well) were transfected with either type I GnRH-R siRNA or scrambled siRNA. After 2 d, cells were washed with serum-free medium and pretreated for 1 h with GnRH-II (10^-6 m) or Zoladex (10^-6 m). Cells were treated with 3-isobutyl-1-methylxantine (0.5 mM) (Sigma Chemical) for 15 min at 37 °C before the addition of forskolin (5 mM, 15 min). Cells were extracted with ethanol 65%, at 4°C for 5 min and centrifuged. The supernatants were dried and stored at -20 °C. cAMP content was determined by the [3H]-cAMP assay system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Each experimental condition was replicated six times, and each experiment was repeated three times.

**Type II GnRH-R silencing experiments**

Type II GnRH-R siRNAs were from Invitrogen. Their sequences were as follows: type II GnRH-R-S, 5′-ACAAACUUAGUUUCAGCAGUCAAU-3′; and type II GnRH-R-AS, 5′-UUAGUAUGCCGAACAGUAAAGUUUGU-3′. A nonspecific scrambled siRNA was used as a control (Low GC Duplex; Invitrogen). Transfections were performed as described for type I GnRH-R silencing. The efficiency of type II GnRH-R knockdown was monitored by RT-PCR, as described for type II GnRH-R mRNA amplification. The experiments were repeated three times.

**Cell proliferation studies after type II GnRH-R silencing**

PC3 cells (3 × 10^6 cells per well) were transfected with either type II GnRH-R siRNA or scrambled siRNA. After 2 d, cells were treated with either GnRH-II (10^-6 m) or Zoladex (10^-6 m) for 48 h. Cells were then harvested and counted by a hemocytometer. Each experimental condition was replicated six times, and each experiment was repeated three times.

**Expression of GnRH-II and type II GnRH-R in prostate cancer cells**

The expression of GnRH-II mRNA in prostate cancer cells was analyzed by RT-PCR using a specific set of oligonucleotide primers (29). Figure 1A shows that the predicted 199-bp cDNA was detected in human immortalized PNT1A prostate epithelial cells (nontumoral control), and in prostate cancer cells, androgen dependent (LNCaP) or androgen independent (DU 145 and PC3).

The expression of type II GnRH-R was investigated as previously described (19). After RT-PCR, an amplified cDNA band of the expected length (338 bp) was obtained in PNT1A prostate cells, as well as in LNCaP, DU 145, and PC3 prostate cancer cells (Fig. 1B). The expression of the receptor was particularly high in PC3 cells. In these cells, analysis of the nucleotide sequence of the obtained cDNA revealed identity with the reported sequence of the human type II GnRH-R cDNA (31, 32).

**Effects of GnRH-II on prostate cancer cell proliferation**

LNCaP, DU 145, and PC3 prostate cancer cells were treated, daily, for 7 d with GnRH-II (10^-9-10^-6 m). The GnRH-I agonist Zoladex (10^-6 m) was used as a control. Figure 2 shows that GnRH-II reduces the proliferation of the three cell lines in a dose-dependent way. The antiproliferative effect of GnRH-II was significant at the doses of 10^-8 -10^-6 M. As expected, Zoladex (10^-6 m) significantly decreased the proliferation of the three cell lines (Fig. 2). The inhibitory effect of both GnRH-II (10^-6 m) and Zoladex (10^-6 m) was completely abrogated when prostate cancer cells (PC3) were cotreated with the GnRH-I antagonist Antide (10^-6 M; data not shown).

**Efficiency of type I GnRH-R silencing**

It is still unclear through which GnRH-R (type I or II) GnRH-II might exert its effects on cancer cells. The siRNA...
Mean analyzed by ANOVA followed by Bonferroni's test. Values are represented as the SE. *P < 0.05 vs. untreated controls (C).

FIG. 2. Effects of GnRH-II on the proliferation of prostate cancer cells, either androgen dependent (A, LNCaP), or androgen independent (B, DU 145; C, PC3). Cells were treated daily, for 7 d, with either GnRH-II (10^{-9} - 10^{-10} M) or Zoladex (10^{-6} M), used as the control. After the treatment, cells were harvested and counted by a hemocytometer. Each experimental group consisted of six replicates, and each experiment was repeated three times. The data were analyzed by ANOVA followed by Bonferroni's test. Values are represented as the mean ± se. *P < 0.05 vs. untreated controls (C).

Effects of type I GnRH-R silencing on the antiproliferative activity of GnRH-II in prostate cancer cells. These experiments were performed in PC3 cells, which showed a high level of expression of type II GnRH-R (Fig. 1B).

Transfection of siRNA resulted in a significant decrease of type I GnRH-R mRNA levels at 24 h (Fig. 3A). Silencing of type I GnRH-R was also followed by a significantly reduced expression of the receptor protein (Western blot analysis, Fig. 3B). The efficiency of the transfection was particularly clear at 48 h after receptor knockout. Immunofluorescence analysis of type I GnRH-R revealed a strong positive reaction in PC3 cells transfected with scrambled siRNA (Fig. 3C). Conversely, immunofluorescence staining was significantly reduced in type I GnRH-R siRNA transfected PC3 cells. These observations demonstrate that type I GnRH-R knockout efficiently reduces the expression of the receptor. Silencing of type I GnRH-R did not affect the expression of type II GnRH-R (Fig. 3D).

Effects of type I GnRH-R silencing on the cAMP accumulation

FIG. 3. Effects of type I GnRH-R silencing on the expression of type I GnRH-R, both at the mRNA (A) and protein (B and C) level, and on the expression of type II GnRH-R, at the protein level (D). A, PC3 cells were transfected with either the scrambled siRNA or type I GnRH-R siRNA. After transfection (24 h), RT-PCR was performed in the presence of specific oligonucleotide primers. β-Actin mRNA is reported as a loading control. One representative of three different experiments is shown. 1, untransfected controls; 2, PC3 cells transfected with the scrambled siRNA; and 3, PC3 cells transfected with type I GnRH-R siRNA. B, PC3 cells were transfected with either a scrambled siRNA or type I siRNA. After either 24 or 48 h, Western blotting was performed on whole cell extracts, using a mouse monoclonal type I GnRH-R antibody. Actin expression was analyzed as a loading control. One representative of three different experiments is shown. 1, untransfected controls; 2, PC3 cells transfected with the scrambled siRNA; and 3, PC3 cells transfected with type I GnRH-R siRNA.

Effects of type I GnRH-R silencing on the antiproliferative activity of GnRH-II

PC3 cells were transfected with either a scrambled siRNA or type I GnRH-R siRNA and treated with either GnRH-II (10^{-9} M) or Zoladex (10^{-6} M) for 48 h. As expected, in untransfected control cells, as well as in cells transfected with the scrambled siRNA, both GnRH-II and Zoladex significantly reduced cell number (Fig. 4, A and B). The antiproliferative effect of both compounds was completely abrogated in PC3 cells transfected with type I GnRH-R siRNA (Fig. 4C).

Effects of type I GnRH-R silencing on cAMP accumulation

We have previously showed that, in prostate cancer cells, type I GnRH-R is coupled to the Gs-cAMP signaling pathway (33). To confirm the role of this receptor in mediating the antiproliferative activity of GnRH-II, we investigated the effects of GnRH-II on FSK-induced cAMP accumulation in PC3 cells, after type I GnRH-R knockout. PC3 cells were transfected with either a scrambled siRNA or type I GnRH-R siRNA, and treated with FSK in the presence of GnRH-II (10^{-9} M) or Zoladex (10^{-6} M). Figure 5A shows that both GnRH-II and Zoladex significantly counteract the cAMP accumulation induced by FSK in untransfected PC3 cells. Similar results were obtained in cells transfected with the scrambled siRNA (Fig. 5B). On the other hand, both GnRH-II and Zoladex failed to antagonize FSK-induced cAMP accumulation after knockout of type I GnRH-R (Fig. 5C).
Effects of type II GnRH-R silencing on the antiproliferative activity of GnRH-II

Our results indicate that GnRH-II reduces the proliferation of prostate cancer cells through the activation of type I GnRH-R. However, according to Enomoto and Park (26), the two receptors (types I and II) might interact in mediating the activity of GnRH-II. To clarify this issue, we studied whether type II GnRH-R silencing might affect the antiproliferative activity of GnRH-II. PC3 cells were transfected with a type II GnRH-R siRNA; the efficiency of transfection was monitored by RT-PCR. Treatment with siRNA resulted in a significant decrease of type II GnRH-R mRNA levels at 24 h after transfection (Fig. 6, upper panel). To clarify the effects of type II GnRH-R silencing on cell proliferation, PC3 cells were transfected with either a scrambled siRNA or type II GnRH-R siRNA. Cells were treated with either GnRH-II (10^{-6} M) or Zoladex (10^{-6} M) for 48 h. Both compounds exerted a significant antiproliferative effect, not only on control cells or on cells transfected with the scrambled siRNA, but also on cells in which type II GnRH-R had been silenced (Fig. 6, A–C, lower panel). This demonstrates that the antimitogenic activity of GnRH-II on prostate cancer cells is not mediated by type II GnRH-R.

Discussion

The direct antiproliferative effects of GnRH-I and its analogs on different tumors, including prostate cancer, are well documented (7–11). On the other hand, clear evidence about the possible action of the second form of GnRH on prostate cancer growth is still lacking.

In this study we analyzed the effects of GnRH-II on prostate cancer cell proliferation and unraveled its mechanism of action. First, we demonstrated that GnRH-II is expressed in human prostate epithelial cells, both nontumoral (PNT1A) and tumoral (LNCaP, DU 145, and PC3). No association was found between the level of expression of the peptide and the grade of malignancy; this observation is consistent with previous reports (28). In all the cell lines tested, we demonstrated the presence of a mRNA specific for type II GnRH-R. The amplified cDNA spanned from nucleotide 319 (exon 1) to nucleotide 656 (exon 2). Sequence analysis of this 338-bp fragment revealed a complete identity with the reported sequence of the human type II GnRH-R (31, 32). This indicates that a transcript specific for type II GnRH-R is expressed in prostate cancer cells. These observations are in agreement with those previously reported by Enomoto and Park (26). The expression of type II GnRH-R has also been reported for other tumors, such as breast (22), endometrial, and ovarian (19, 34) cancer. However, it has been proposed that a full-length functional receptor protein does not exist.
in humans because of the presence of a frameshift and a premature stop codon in the receptor transcript (24, 25). Thus, the effects of GnRH-II on cancer growth might be mediated by type I, rather than type II, GnRH-R.

To clarify this issue, we investigated the effects of GnRH-II on prostate cancer cell proliferation and the receptor (type I or II) that might mediate these effects. We demonstrated that GnRH-II exerts a dose-dependent antiproliferative action on prostate cancer cells, either androgen dependent (LNCaP) or androgen independent (DU 145, PC3). To identify the receptor that might mediate the antitumor activity of GnRH-II, we used the siRNA technique. PC3 cells were transfected with a siRNA targeted against the type I GnRH-R gene or with a siRNA targeted against type II GnRH-R. Type I GnRH-R silencing completely antagonized the antiproliferative effects of GnRH-II, as well as that of the GnRH-I agonist Zoladex, as expected. On the other hand, type II GnRH-R silencing did not affect the antiproliferative action of GnRH-II. These results strongly indicate that the antiproliferative effect of GnRH-II on prostate cancer cells is mediated by type I, but not type II, GnRH-R.

The data so far reported in the literature regarding the receptor that specifically mediates the activity of GnRH-II in tumor cells are controversial. Our results are in agreement with those published by Kim et al. (34). These authors demonstrated that, in ovarian cancer cells, GnRH-II exerts an antiangiogenic action through the activation of type I GnRH-R. However, contrasting results were obtained by other authors. Enomoto and Park (26) reported that, in DU 145 cells, type II GnRH-R mediates the antiproliferative action of GnRH-II, through a direct interaction with type I GnRH-R. Grundker et al. (20) showed that GnRH-II significantly reduces the growth of endometrial and ovarian cancer cells; this effect persists after knockout of type I GnRH-R, suggesting the existence, in these cell lines, of a functional type II GnRH-R. Gunthert et al. (22) demonstrated that GnRH-II analogs inhibit the proliferation of breast cancer cells, expressing type II GnRH-R; however, whether this receptor might be crucial for the antimitogenic activity of GnRH-II was not investigated by these authors. The reasons for these discrepancies are unclear; however, as suggested by Kim et al. (34), they might be accounted for by the different experimental conditions adopted (cell lines, methods for type I GnRH-R silencing, etc.).

In the present paper, we also show that GnRH-II, as well as GnRH-I agonists, counteract the FSK-induced intracellular cAMP accumulation; these data confirm our previous reports showing that type I GnRH-R is coupled to the Gai-cAMP signaling pathway in prostate cancer cells (33). In ovarian cancer cells, Kim et al. (34) demonstrated that GnRH-II, by binding to type I GnRH-R, activates ERK1/2 through a protein kinase C-dependent pathway. The intracellular events that follow type I GnRH-R activation in cancer cells have been extensively investigated. In addition to the Gai-cAMP (7, 33, 35) and protein kinase C-ERK signaling pathways (34), GnRH-I analogs have acted through activation of c-Jun N-terminal kinase, p38 stress-activated kinases, phosphotyrosine phosphatases, and inhibition of the phosphatidylinositol 3-kinase/Akt (11, 36, 37). These signals are distinct from those reported to mediate the stimulatory activity of GnRH-I at the pituitary level. On gonadotrope cells, type I GnRH-R is known to be coupled to the Goq-phosphoinositide-phospholipase C pathway (24, 25, 38, 39). To explain this complex scenario, Millar et al. (40) have proposed the “ligand-induced selective-signaling” phenomenon. According to this concept, type I GnRH-R might assume various conformations, endowed with different selectivities for GnRH-I analogs and with different intracellular signaling pathways. Thus, each GnRH-I analog can recruit particular pathways, while bypassing others (40). As underlined by the authors, this might be crucial for opening the way to the development of new GnRH-I analogs that might improve anticancer therapeutic interventions.

In summary, in the present paper, we demonstrate that both GnRH-II and a type II GnRH-R transcript are expressed in prostate cancer cells. GnRH-II exerts a significant antiproliferative action on prostate cancer cells; this effect is mediated by type I GnRH-R and by its coupled cAMP intracellular signaling pathway.

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