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The inhibition of growth and down-regulation of gonadotropin releasing hormone (GnRH) receptor in αT3-1 cells by GnRH agonist

Sham S Kakar, Sati Nath, Jason Bunn and Lothar Jennes[1]
Department of Physiology and Biophysics, University of Alabama at Birmingham, AL 35294-0005. USA. Tel: (+1) 205 934-7034; Fax: (+1) 205 934-1445. [1]Department of Anatomy and Neurobiology, University of Kentucky, College of Medicine, Lexington, KY 40536-0084, USA.

Gonadotropin releasing hormone (GnRH) and its analogs inhibit the growth of hormone-dependent tumors *in vivo* and *in vitro*. The inhibition of growth and proliferation of tumor cells *in vitro* by GnRH and its analogs indicates that the tumor suppressing effect of the hormone is only partially due to suppression of pituitary gonadotropin release which reduces circulating steroid levels that are required for proliferation. Demonstration of GnRH-binding sites on some tumors suggests a direct inhibitory effect of GnRH and its analogs. However, the mechanism by which GnRH and its analogs inhibit tumor cell growth is not known. Our hypothesis is that the inhibition of growth and proliferation of tumor cells by GnRH and its analogs are mediated through down-regulation of its receptor expression. To test this hypothesis, mouse pituitary gonadotrope cell line (αT3-1) was selected as a model since this is the only cell line which expresses a sufficiently high level of GnRH receptors for precise measurements of the mRNA for the receptor. Addition of GnRH agonist (D-Lys^6)GnRH to the cell cultures caused a time-dependent decrease in both cell growth, as measured by cell number, and cell proliferation, as measured by [^{3}H]thymidine incorporation into DNA. After 1 h of treatment of αT3-1 cells with 1 μM of (D-Lys^6)GnRH, the cell number was reduced to 83.0 ± 13.4 compared to control, decreased to 75.1 ± 3.2 at 2 h, 63.2 ± 0.66 at 4 h and 52.2 ± 0.87 at 24 h. This decrease in cell number was accompanied by a parallel decrease in [^{3}H]thymidine incorporation into DNA. The inhibition of cell growth and [^{3}H]thymidine incorporation by treatment with 1 μM of (D-Lys^6)GnRH was sustained for at least 72 h. Inhibition of αT3-1 cell growth and [^{3}H]thymidine incorporation was dose-dependent; thus 10^{-9} M (D-Lys^6)GnRH resulted in about 30% inhibition within 4 h which was comparable to 10^{-6} M (D-Lys^6)GnRH, whereas 10^{-12} M (D-Lys^6)GnRH was ineffective. Measurement of mRNA for the GnRH receptor by Northern blot analysis showed a decrease in levels of mRNA by 5% within 2 h of treatment of αT3-1 cells with 1 μM (D-Lys^6)GnRH, by 30% at 4 h and by 50% at 24 h. In conclusion these data demonstrate that treatment of αT3-1 cells with (D-Lys^6)GnRH causes an inhibition of cell growth and proliferation, and down-regulates the GnRH receptor mRNA levels.

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Correspondence to SS Kakar

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Introduction

Hypothalamic gonadotropin releasing hormone (GnRH) plays a key role in the reproductive process in mammals. After its release from the hypothalamus, it binds to specific receptors on the pituitary gonadotropes. Activation of these receptors stimulates the secretion of gonadotropins, which in turn regulate the secretion of steroid hormones.[1] In addition to the pituitary gonadotropes, it is now known that GnRH receptors are also present in extra-pituitary tissues, hormone-responsive tumors and tumor-derived cell lines,[2,3] suggesting that GnRH receptors may serve additional functions.

GnRH agonists have been explored for the treatment of hormone-dependent tumors.[4–7] The major mechanism of action by which GnRH agonists inhibit tumor growth is thought to be through desensitization of the pituitary receptors which causes a decline in gonadotropin secretion.[5] However, a direct effect of GnRH on certain tumors is indicated by studies that showed a reduction in tumor growth in postmenopausal women whose gonadal steroid levels are minimal.[8–10] Inhibition of tumor cell growth *in vitro* and [^{3}H]thymidine incorporation in a number of tumor cell lines[11–18] support the concept of a direct antitumor effect of GnRH that is independent of the pituitary gonadal axis. However, the mechanism by which GnRH or GnRH/GnRH receptor interaction inhibits tumor growth remains unclear.

Recently, it has been shown that GnRH agonists and antagonists given continuously down-regulate GnRH receptor expression at the pituitary level and, consequently, inhibit luteinizing hormone secretion.[19–22] Based on these observations, it is hypothe-

sized that treatment of tumor cells with GnRH agonists and antagonists also results in down-regulation of GnRH receptor expression which leads to inhibition of tumor cell growth. Because of the low level of expression of GnRH receptor mRNA in human tumors which limits its measurements by using conventional techniques, we selected the αT3-1 cell line as our model to test our hypothesis. The αT3-1 cell line is a mouse pituitary gonadotrope cell line that expresses high levels of GnRH receptor mRNA.[23,24] This cell line was developed by targeted tumorigenesis in the mouse pituitary with SV40 large T antigen driven by human glycoprotein hormone α-subunit promoter.[25] In addition, this cell line has been used extensively to study many aspects of gonadotrope physiology, including GnRH receptor regulation. Using this cell line, our studies show that GnRH agonist (D-Lys^6)GnRH inhibits cell growth and proliferation in a time- and dose-dependent manner, and down-regulates the receptor mRNA levels.

Materials and methods

Cell culture

Mouse gonadotrope cell line αT3-1 was kindly provided by Dr Pamela Mellon (University of California San Diego, La Jolla, CA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco/BRL), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 μg/ml penicillin, 100 units/ml streptomycin and 0.25μg/ml amphotericin (Sigma, St Louis, MO). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell proliferation studies

αT3-1 cells in log phase were trypsinized and plated in six-well plates (approximately 5×10^5 cells/well). After 1 day, the medium was replaced with fresh medium containing 1% fetal bovine serum and various concentrations of GnRH agonist (D-Lys^6)GnRH or thyrotropin-releasing hormone (TRH) or vehicle (DMSO). Both (D-Lys^6)GnRH and TRH were solubilized in DMSO to a final concentration of DMSO of 0.01%. The medium was changed every 24 h supplemented with the appropriate concentrations of hormones. After incubating the cells for 1, 2, 4, 24, 48 or 72 h, cells were harvested and counted with a hemocytometer. The average cell mortality, determined by Trypan blue (Gibco/BRL) exclusion, was less than 5.0% and did not vary among treatment groups. Each experiment was performed in duplicate and reproduced at least three times in different passages of the cell line.

[^{3}H]Thymidine uptake

[^{3}H]Thymidine incorporation into DNA in αT3-1 cells was measured at various time intervals after treatment with (D-Lys^6)GnRH. One microcurie of [^{3}H]thymidine (Amersham, Arlington Heights, IL; TRK.120, 102 mCi/mg) was added to each well (containing 5×10^6 cells/well) and was incubated at 37°C for 4 h. The medium containing free [^{3}H]thymidine was carefully aspirated and the cells were washed twice with PBS. Then 600 μl of trypsin–EDTA media was added to the washed cells followed by incubation for 3–4 min at 37°C. The detached cells were collected in a microcentrifuge tube and centrifuged for 2 min. The cells were washed twice with ice-cold PBS and twice with ice-cold (10%) trichloroacetic acid. The pellet was dissolved in 0.3 ml of 0.1 N NaOH and counted for radioactivity in a liquid scintillation counter using 15 ml of liquid scintillation cocktail (Scinti Safe Econo 2; Fisher, Fair Lawn, NJ).

Preparation of RNA and Northern blot analysis

Effects of GnRH agonist (D-Lys^6)GnRH on steady-state levels of GnRH receptor mRNA were examined by Northern blot analysis after treatment of αT3-1 with 1 μM of (D-Lys^6)GnRH. αT3-1 cells in the log phase were plated in 60 mm Falcon culture dishes. After 24 h, the medium was replaced with fresh medium containing 1% fetal bovine serum and 1 μM of (D-Lys^6)GnRH or 1 μM of TRH. After 0, 1, 2, 3, 4 and 24 h of incubation the cells were washed with ice-cold PBS and stored at −80°C for RNA preparation. Total RNA from αT3-1 cells was prepared according to the procedure as described previously[26] using an RNA isolation system from Bioteex (Houston, TX). RNA was quantitated spectrophotometrically by measuring the absorption at OD_{260}. Purity of RNA was determined by the OD_{260}/OD_{280} ratio.

For Northern blot analysis, 10 μg of total RNA from each sample was electrophoresed on a denaturing 1% agarose gel containing formaldehyde. The gel was then blotted onto a Nylon membrane. After cross-linking the RNA by using a UV cross-linker

(BioRad, Hercules, CA), the hybridization was carried out at 65°C for 16–18 h in a hybridization buffer (0.5 M $NaHPO_4$, pH 7.2; 1 mM EDTA; 7% SDS; and 1% BSA) containing ^{32}P-labeled human GnRH receptor cDNA representing the open reading frame[27] prepared by random hexamer-primed synthesis. The blots were washed three times (20 min each) in 2 × SSC/0.1% SDS at room temperature and once at 55°C in 0.2 × SSC/0.1% SDS for 30 min.[26,28] The blots were rehybridized with ^{32}P-labeled β-actin to normalize the variation in loading and transfer of RNA. Levels of dominant 3.5 kb GnRH receptor mRNA and β-actin mRNA were determined by using BioRad video densitometer model 620 and quantitative analysis.

Results

Inhibition of αT3-1 cell growth by GnRH agonist (D-Lys^6)GnRH

The effects of (D-Lys^6)GnRH on αT3-1 cell growth were initially monitored over a period of 72 h using 1 μM (D-Lys^6)GnRH. The doubling time of the control cells was about 20 h. This high growth rate was significantly suppressed by exposing the cells to (D-Lys^6)GnRH. At 24 h after the addition of (D-Lys^6)GnRH to the cultures, the cell number was reduced to 62.2 ± 10.7% compared to controls (DMSO treated) (Figure 1). This level of inhibition of cell growth by (D-Lys^6)GnRH was sustained for up to 72 h, suggesting that the maximum level of inhibition of αT3-1 cells growth was achieved within 24 h. It should be noted that the inhibitory effects of (D-Lys^6)GnRH were not caused by cytotoxic activity, since cellular viability determined by Trypan blue exclusion (> 95%) did not change after treatments and TRH, which binds to GnRH receptor with very low affinity,[27] was ineffective (Figure 1).

To determine if inhibition of αT3-1 cell growth by (D-Lys^6)GnRH is time dependent, we incubated the αT3-1 cells with 1 μM (D-Lys^6)GnRH or TRH for 0, 1, 2, 4 and 24 h. As shown in Figure 2, (D-Lys^6)GnRH inhibited αT3-1 cell growth in a time-dependent fashion. Incubation of αT3-1 cells with (D-Lys^6)GnRH resulted in a reduction of cell number to 83.0 ± 13.4 at 1 h, 75.1 ± 3.2 at 2 h, 63.2 ± 0.66 at 4 h and 52.2 ± 0.87 at 24 h compared with controls. Again TRH was ineffective. Inhibition of αT3-1 cell growth by (D-Lys^6)GnRH was also found to be dose dependent. As shown in Figure 3, 10^{-9} M (D-Lys^6)GnRH reduced the cell number to 69.0% compared with control after 4 h of incubation and was comparable to 10^{-6} M of (D-Lys^6)GnRH, whereas a lower concentration of (D-Lys^6)GnRH (10^{-12} M) was ineffective.

Inhibition of [^{3}H]thymidine incorporation into DNA by GnRH agonist (D-Lys^6)GnRH

To determine if inhibition of αT3-1 cell growth by (D-Lys^6)GnRH was a result of inhibition of DNA synthesis, we studied [^{3}H]thymidine incorporation into DNA. As shown in Figures 1–3, (D-Lys^6)GnRH inhibited [^{3}H]thymidine incorporation into DNA in a time- and dose-dependent manner. About 30% inhibition was achieved within 24 h after addition of 1 μM (D-Lys^6)GnRH and this level of inhibition was maintained for up to 72 h. Incubation of αT3-1 cells with 10^{-9} M of (D-Lys^6)GnRH for 4 h caused 16.2% inhibition of [^{3}H]thymidine incorporation and 10^{-6} M (D-Lys^6)GnRH resulted 33.6% inhibition compared with controls (Figure 3). Addition of equal amounts of TRH to the cultures did not cause any changes in [^{3}H]thymidine incorporation (Figures 1–3).

Down-regulation of GnRH receptor mRNA by GnRH agonist (D-Lys^6)GnRH

To determine whether inhibition of αT3-1 cell growth and proliferation was associated with altered GnRH receptor gene expression, αT3-1 cells were treated with 1 μM of (D-Lys^6)GnRH for 0–24 h and GnRH receptor mRNA levels were measured by Northern blot analysis. As shown in Figure 4, Northern blot analysis revealed the existence of two transcripts with sizes 3.5 and 1.6 kb in αT3-1 cells. Treatment of cells with (D-Lys^6)GnRH resulted in decreased levels of both the transcripts in a time-dependent fashion. Densitometric analysis of the 3.5 kb transcript showed that GnRH receptor mRNA levels were decreased by 5% after 2 h of treatment of cells with 1 μM (D-Lys^6)GnRH which decreased to 30% at 4 h and to 50% at 24 h compared with controls or cells treated with TRH. Expression of the 1.6 kb mRNA changed in parallel with that of the 3.5 kb mRNA.

Discussion

The results of the present studies clearly demonstrate that the GnRH agonist (D-Lys^6)GnRH inhibits the growth and proliferation of mouse gonadotrope

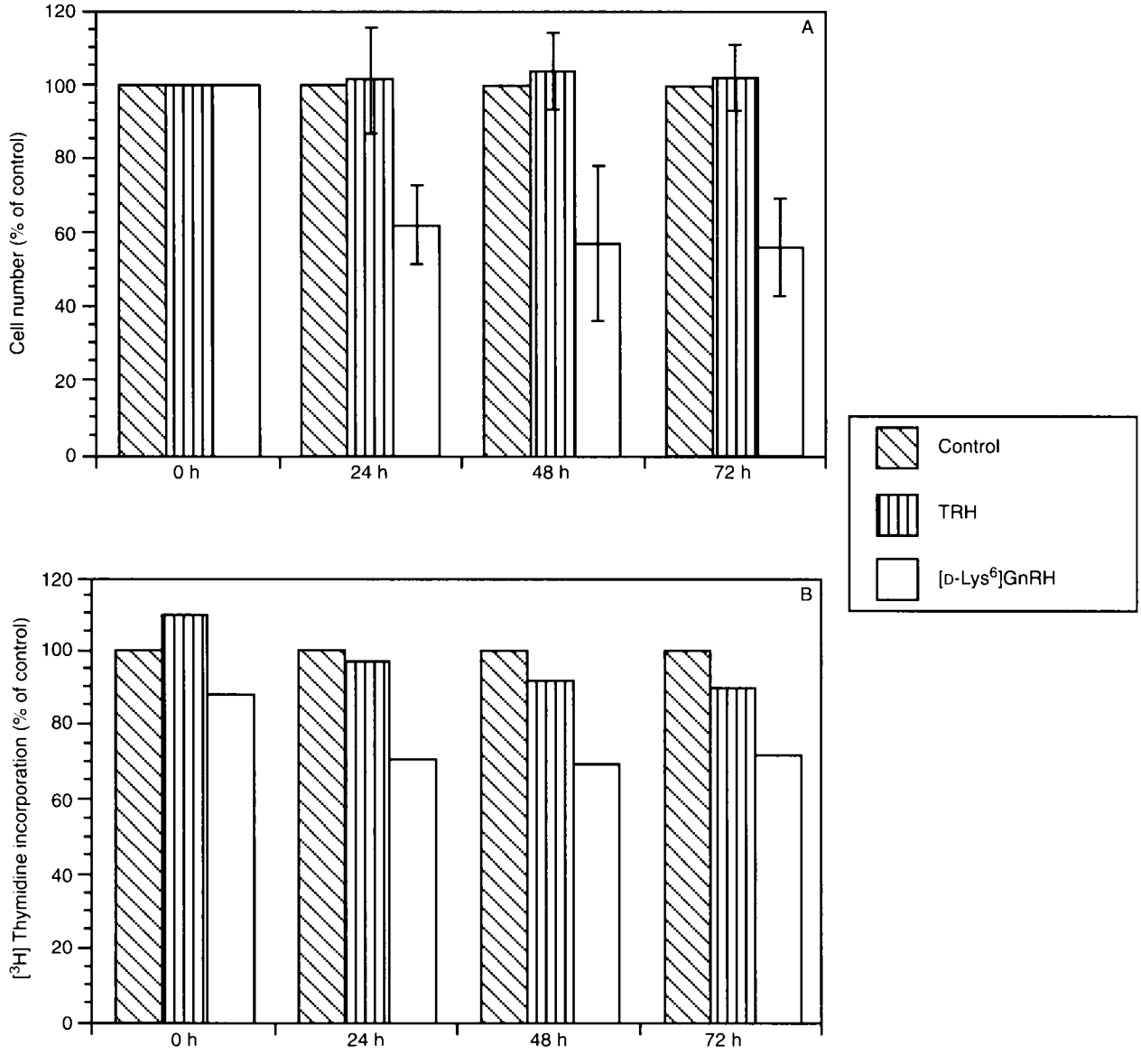

Figure 1. Inhibition of αT3-1 cell growth and [^{3}H]thymidine incorporation by GnRH agonist (D-Lys^6)GnRH. αT3-1 cells were incubated with 1 μM (D-Lys^6)GnRH or TRH for 0–72 h. (A) The cells were trypsinized and counted. Each value represents the mean of three independent experiments and is expressed as percent of cells compared with control (vehicle). (B) After incubation with (D-Lys^6)GnRH, 1 μCi of [^{3}H]thymidine was added to the cultures and incubated for an additional 4 h. [^{3}H]Thymidine incorporation into DNA was determined. Each value represents the mean of two independent experiments.

cell line (αT3-1) *in vitro* in a time- and dose-dependent manner (Figures 1–3). Inhibition of αT3-1 cell growth paralleled by an inhibition of [^{3}H]thymidine incorporation into DNA even after 1 h of incubation of cells with (D-Lys^6)GnRH suggests that DNA synthesis is reduced rapidly in αT3-1 cells by the GnRH agonist treatment. Inhibition of cell growth and [^{3}H]thymidine incorporation in various tumors cells by GnRH analogs have been documented.[11–18] However, a higher level of inhibition in a short incubation period is evident in αT3-1 cells than compared to most other tumor cells. This difference could be due to the high level of expression of GnRH receptors in αT3-1 cells compared with tumor cells.[23,24] The existence of GnRH and GnRH receptor in various normal human tissues[2,3,26,29] suggests that under normal physiological conditions, GnRH not only regulates the secretion of

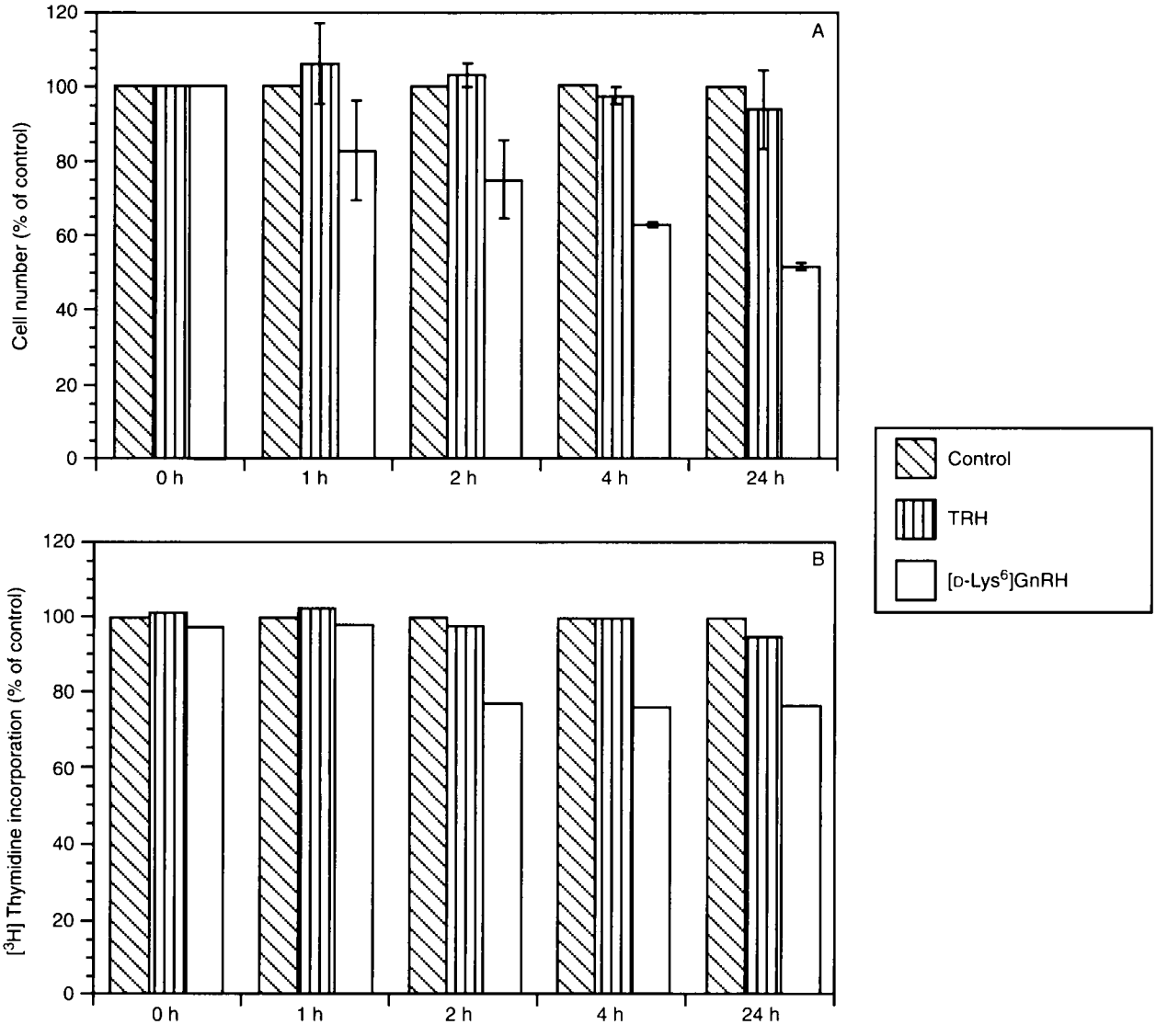

Figure 2. Inhibition of αT3-1 cell growth and [^{3}H]thymidine incorporation by GnRH agonist (D-Lys^6)GnRH. αT3-1 cells were incubated with 1 μM (D-Lys^6)GnRH or TRH for 0–24 h. (A) The cells were trypsinized and counted. Each value represents the mean of three independent experiments and is expressed as percent of cells compared to control (vehicle). (B) After incubation with (D-Lys^6)GnRH for the specified time, 1 μCi [^{3}H]thymidine was added to the cultures and incubated for an additional 4 h. [^{3}H]Thymidine incorporation into DNA was determined. Each value represents the mean of two independent experiments.

FSH and LH from the anterior pituitary but may also regulate cell growth and proliferation mediated through its high-affinity receptors in an autocrine or paracrine fashion.

Our results shown in Figure 4 demonstrate that treatment of αT3-1 cells causes down-regulation of GnRH receptor mRNA in a time-dependent manner. The parallelism in the inhibition of αT3-1 cell growth and proliferation, and down-regulation of receptor mRNA suggests a direct correlation between these parameters. Maximum inhibition of cell growth was achieved within 24 h of treatment of αT3-1 cells with 1 μM of (D-Lys^6)GnRH which was sustained at least for 72 h. Previous studies have shown that treatment of rats as well as rat pituitary cells in cultures with GnRH agonists and antagonists results in down-regulation of GnRH receptor number.[19–21,30] Changes in GnRH receptor number in αT3-1 cells in culture by treatment with GnRH agonists have also been reported.[31,32] Agonist-induced decrease in receptor number could occur at the post-translational level, translational level or gene

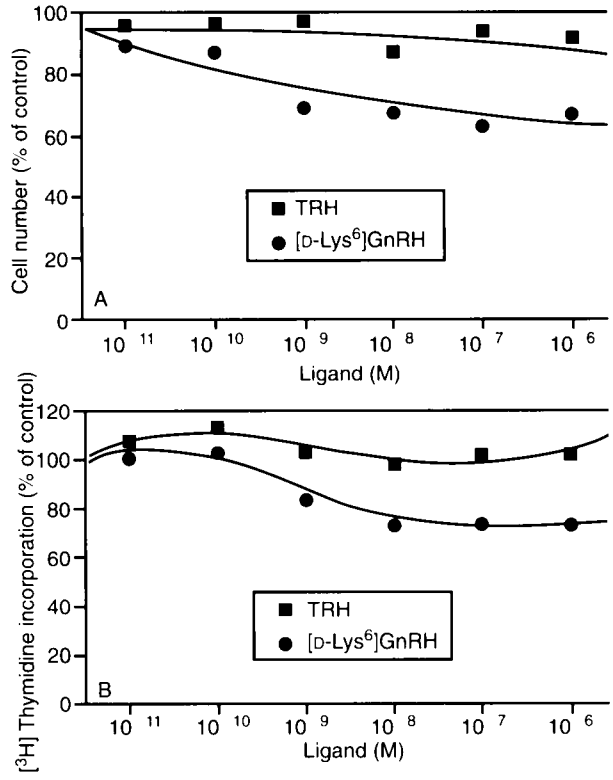

Figure 3. Inhibition of αT3-1 cell growth and [^{3}H]thymidine incorporation by GnRH agonist (D-Lys^6)GnRH. αT3-1 cells were incubated with increasing concentrations of (D-Lys^6)GnRH or TRH for 4 h. (A) Cells were trypsinized and counted. Each value represents the mean of two independent experiments and is expressed as percent of cells compared to control (vehicle). (B) After incubation with (D-Lys^6)GnRH, 1 μCi [^{3}H]thymidine was added to the cultures, incubated for an additional 4 h and [^{3}H]thymidine incorporation into DNA was determined. Each value represents the mean of two independent experiments.

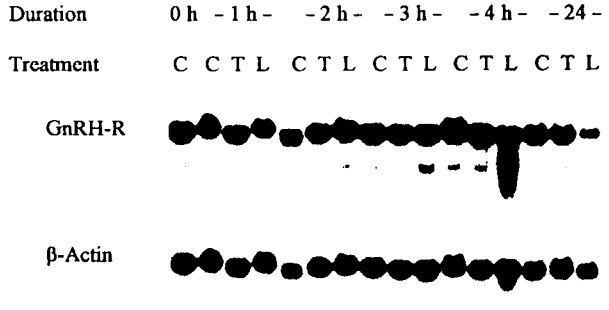

Figure 4. Northern blot analysis of the αT3-1 cell RNA. αT3-1 cells were treated with 1 μM of (D-Lys^6)GnRH for 0 24 h. Total RNA was prepared and 10 μg total RNA from each sample was used for Northern blot analysis.^{32}P-labeled human GnRH receptor cDNA representing the open reading frame or β-actin was used as probe.

transcriptional level. Down-regulation of GnRH receptor mRNA levels in αT3-1 cells by (D-Lys^6)GnRH in the present studies (Figure 4) suggests that loss of receptors can be at least, in part, be attributed to decreased receptor mRNA expression or mRNA stability. The decrease in mRNA levels in αT3-1 cells, and in rat and sheep pituitaries after treatment with GnRH or GnRH agonist (D-Ala^6)GnRH has also been shown.[19,22,31] Taken together these results suggest that inhibition of cell growth and proliferation of αT3-1 cells by GnRH agonists is accompanied by down-regulation of GnRH receptor mRNA levels.

In conclusion, our results demonstrate that GnRH can inhibit cell growth and [^{3}H]thymidine incorporation, and can decrease the levels of GnRH receptor mRNA levels in αT3-1 cells. Since αT3-1 cells express a high level of GnRH receptor, this cell line is a very useful model to study the mechanisms of regulation of tumor cell growth and proliferation by the GnRH/GnRH receptor system.

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