Effect of GnRH Analogs on the Expression of TrkA and p75 Neurotrophin Receptors in Primary Cell Cultures From Human Prostate Adenocarcinoma

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BACKGROUND. GnRH analogs have antiproliferative and/or apoptotic effects on prostate cancer cells. Also, neurotrophin receptors TrkA and p75 have been reported in normal prostate suggesting a role in the gland growth control. In prostate cancer, TrkA receptors seem to be overexpressed and p75 receptors show a decreased expression. These changes in neurotrophin receptors may be related with unbalanced growth in malignant cells. In the present study we investigate the effects of GnRH analogs (leuprolide and cetrorelix) on the expression of TrkA and p75 neurotrophin receptors in primary cultures of human prostate cancer cells.

METHODS. Tissue was obtained from radical prostatectomies due to prostate adenocarcinoma. Cells were isolated after sequential enzyme digestion and cultured in defined media. Nerve growth factor (NGF) receptors in untreated cultures were estimated by immunofluorescence. Cultures were treated with leuprolide (agonist) or cetrorelix (antagonist) and expression of TrkA and p75 receptors were evaluated by semi quantitative RT-PCR (polymerase chain reaction) and western blot. Cell proliferation was estimated by MTT method and apoptosis through COMET assay.

RESULTS. Both leuprolide and cetrorelix induced a significant increase in p75 receptor gene and protein expression at a concentration that induce apoptosis and decrease proliferation. TrkA receptors showed no changes in presence of GnRH analogs.

CONCLUSIONS. GnRH analogs, leuprolide, and cetrorelix, change the ratio between neurotrophin receptors TrkA and p75 by increasing gene and protein expression of p75 receptor. Considering that TrkA receptor is related with proliferation and p75 with apoptosis, we suggest that our findings may explain, in part, the effect of GnRH analogs on prostate cancer growth.

KEY WORDS: **prostate cancer; GnRH analogs; neurotrophin receptors; primary cell culture**

INTRODUCTION

Prostate cancer is a leading cause of male cancer deaths worldwide and its incidence significantly increases in elder man. Late diagnosis and lack of efficient treatments for advanced stages are the major complications for the patients [1,2]. At the beginning, prostate carcinoma is androgen sensitive and most treatments are intended to low testosterone levels [3]. For that

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purpose, GnRH analogs are widely used to block hypothalamic-pituitary axis and decrease androgen plasma concentration in-patient with prostate adenocarcinoma [4]. In addition, GnRH receptors have been found in prostatic malignant tissue and carcinoma derived cell lines [5–7]. Several GnRH agonists such goserelin, buserelin, leuprolide, and antagonists (antide, cetrorelix) have been used in different experimental models to study their effects on cell survival. Most experimental studies have been carried out in established cell lines derived from prostate carcinoma [8–18]. GnRH agonists mainly induce arrest in proliferation rate and/or apoptosis, whereas antagonist effects seem to be less consistent. Some reports show no effect in proliferation activity in cell lines cultures treated with antagonist [18]. Other studies have shown similar effect on proliferation and apoptosis with both agonist and antagonist of GnRH [16,17]. Recently, we have found antiproliferative and apoptotic effects of leuprolide and cetrorelix using a primary culture system [submitted]. On the other hand, neurotrophins, as nerve growth factor (NGF), and their receptors have been found both in normal and malignant prostate [19,20]. Neurotrophins exert their actions through activation of high-affinity tyrosine kinase receptors, Trk (A, B, and C), and a low-affinity receptor p75 (TNF-family receptor) [21–23]. Both TrkA and p75 NGF receptors are expressed in epithelial, but not stromal, cells of normal prostate both in vivo and in vitro [24,25]. Interestingly, in benign and malignant prostate tumors, p75 expression is significantly reduced and absent in metastatic cell lines, suggesting a negative correlation between p75 expression and cancer progression [26,27]. Several studies have reported maintained or increased expression of TrkA receptor in malignant prostate cells [20,28]. In addition, TrkB and TrkC receptors have been found ectopically expressed in malignant prostate [20,28]. In most tissues, TrkA receptor activation increases proliferation rate while p75 receptor has been associated with apoptosis [29–31]. TrkA and p75 have been proposed as an associated receptor system in which the balance between these two kinds of receptors may have a role in normal cell survival [21,22]. Conversely, the unbalanced expression of TrkA and p75 receptors in cancer prostate may explain, at least in part, the increased proliferation rate and the low apoptosis level in tumor cells. In addition, p75-transfected LNCaP cells, which do not express endogenous p75 receptor, underwent apoptosis when deprived of NGF [32]. It has been suggested that p75 is a negative modulator of epithelial growth by inducing apoptosis. Then lost of p75 expression seems to contribute to malignancy by promoting proliferation and inhibiting the apoptotic pathway [26,27]. In the present study we investigate whether treatments with GnRH analogs influence the expression of NGF receptors in a primary cell culture from human prostate cancer.

MATERIALS AND METHODS

Reagents

All reagents, unless otherwise indicated, were obtained from Sigma Chemical Co. (St. Louis, MO).

Prostatic Tissue

The prostatic samples were obtained from patients undergoing prostatectomy due to benign hyperplasia or adenocarcinoma, from our Institutional Hospital. Prostate fragments were received in sterile culture medium containing RNAse inhibitors. Later on, tissue was brought to the laboratory and, if present, soft hyperplasic tissue was separated from harder malignant nodes. For control purpose, sections of each sample were processed by routine histological technique for Gleason classification [33]. In this study six prostate samples with a Gleason score range of 5–7 were included. This protocol was approved by Bioethics Committee of our Institution.

Cell Isolation and Culture

Prostatic cells were isolated, cultured, and characterized essentially as described previously [34]. Briefly, small tissue fragments (1 mm^3) were digested in collagenase (2.5 mg/ml), hyaluronidase (1 mg/ml), and deoxyribonuclease (0.01 mg/ml), for 2–3 hr at 37°C in a shaking water bath. The resulting large epithelial cell aggregates were washed and further digested in collagenase for another 8–12 hr in the same conditions. Resulting small aggregates of prostatic cancer cells were mechanically dispersed, washed, and plated in cell culture plates (Falcon, Becton Dickinson Co., NJ). During the first days of culture media were supplemented with 5% of fetal bovine serum (FBS) (Gibco, Invitrogen Co., CA). For immunofluorescence assays, cultures from benign hyperplasia were obtained as a reference of non-malignant cells.

GnRH Analogs Treatments

After first medium change, cells were washed and fresh medium without FBS and supplemented with human transferrin 5 mg/L, insulin 2 mg/L, epidermal growth factor (EGF) 10 μg/L, vitamin A, and E 200 μg/L, hydrocortisone 10 nM, sodium selenite 2 μg/L, dihydrotestosterone (DHT) 10 nM and, when indicated NGF 15 ng/ml, was added. Media were changed every 48 hr and cultures maintained for 1 week. GnRH

analogs treatments were carried out during day 4 and 6 of culture. At the end of the treatment, cells were treated for RT-PCR or western blot. In parallel control cultures, cells were evaluated for proliferation and apoptosis. According to our previous results, both leuprolide (Sigma) and cetrorelix (Serono, Novartis Chile) were used at a concentration of 20 ng/ml.

Immunocytochemistry of NGF Receptors

The epithelial prostatic cells were grown on coverslips, rinsed in PBS and fixed in paraformaldehyde 3% (Merck, Germany)-sucrose 2% in PBS for 30 min at room temperature. Then, the samples were blocked with PBS containing glycine 20 mM and bovine serum albumin 1% (BSA). NGF receptors were determined immunocytochemically using specific rabbit antibodies against TrkA and p75 proteins (Santa Cruz Biotechnology, Inc., CA). Then, antirabbit-FIT-C (Santa Cruz Biotechnology) was used to evidence specific antibody binding. The cell cultures were evaluated on a Zeiss Fluorescence Microscope.

Proliferative Activity and Apoptosis

To confirm that GnRH analogs treatments effectively induce changes in proliferative an apoptotic activity the cell cultures, cell number was evaluated by a spectrophotometric method based in mitochondrial dehydrogenases assay with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [35], and apoptosis was estimated by DNA fragmentation using COMET assay [36].

ReverseTranscription-Polymerase Chain Reaction of TrkA and p75 Receptors

Total RNA was isolated from prostate cells using Trizol Reagent (Gibco BRL, Rocksville, MD) according to the manufacturer's instructions. Aliquots of RNA (2 μg) from each culture were used in an RT reaction, to a final volume of 20 μl. Resulting cDNA was subjected to polymerase chain reaction (PCR). The specific primers used to amplify TrkA receptor DNA fragments were: 5′-CCA-TCG-TGA-AGA-GTG-GTC-TC-3′, sense, and 5′-GGT-GAC-ATT-GGC-CAG-GGT−CA-3′, antisense. Primers for p75 receptor were: 5′-AGC-CAC-CAG-ACC-GTG-TGT−G-3′, sense, and 5′-TTG-CAG-CTG-TTC-CAC-CTC−TT-3′, antisense. The amplification was carried out using a Thermal Cycler (PT-100, MJ Research, Inc.) in a reaction mix that included: 2 μl cDNA, 2.5 μl PCR Buffer 10X, 0.75 μl $MgCl_2$ 25 mM, 0.625 μl dNTPs 10 mM, 0.125 μl Taq polimerase 500 UI (Biotools), 0.5 μl of each primer and 19 μl of DEPC water. β-actin was used as an internal standard. Amplification was performed for 35 cycles for TrkA and p75 receptors and 28 cycles for β-actin. The PCR products were electrophoretically resolved on 1.2% agarose gel and stained with ethidium bromide. The bands were evaluated using an image analyzer UN-SCAN-IT gel version for Windows 4.1 and Kodak Digital Science 1D Software (Rochester, NY), and normalized relative to β-actin product. Data were expressed as the ratios between mRNAs of the NGF receptors and β-actin.

Western Blot Analysis

Cells were lysed in buffer containing 500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 μg/ml PMSF, and 3 μg/ml aprotinin. Homogenates were centrifuged at 13,000 rpm for 15 min at 4°C and protein concentration of the supernatant was determined by Bradford method [37]. One-fifty micrograms of protein was separated by 7.5% SDS–PAGE and transferred into nitrocellulose membranes (Bio-Rad, CA). The membranes were blocked in 5% fatless milk during 1 hr at room temperature, and then incubated with a rabbit polyclonal antibody against human TrkA or p75 receptors (Santa Cruz Biotechnology, CA) at 1:100 dilution, or with a mousse monoclonal antibody against human protein (MP Biomedicals, CA) at 1:15,000 dilution for 1 hr at room temperature. Later, membranes were incubated for 45 min at room temperature with secondary antibodies coupled to HRP. Detection of antibodies complex was carried out by a chemoluminescence kit (Amersham Biosciences). The films were evaluated using an image analyzer UN-SCAN-IT gel version for Windows 4.1 and Kodak Digital Science 1D Software (Rochester, NY), and normalized relative to β-actin product. Data were expressed as the protein ratios between the NGF receptors and β-actin.

Statistic Analysis

Statistic evaluation of data will be performed using ANOVA analysis and non-parametric test of Kruskal–Wallis followed by Dunn's post-test. Statistic significance will be considered for $P < 0.05$. All experiments were repeated al least three to four times and results are expressed as mean ± SD.

RESULTS

Effect of GnRH Analogs on Proliferative and Apoptotic Activity

To confirm the effect of GnRH analogs on proliferative and apoptotic activities, primary cell cultures from prostate cancer were treated for 48 hr with 20 ng/ml of leuprolide, or cetrorelix in absence or presence of

TABLE I. Effect of Leuprolide and Cetrorelix on the Proliferation of Epithelial Cells From Prostate Cancer Cultured in Absence or Presence of NGF

	Cells/cm^2 ($\times 10^3$)	
	+NGF	−NGF
Control	755.9 ± 62.0	466.6 ± 32.1
Leuprolide	441.0 ± 43.4*	348.3 ± 33.3*
Cetrorelix	401.1 ± 41.2*	307.2 ± 30.0*

Epithelial cells were isolated from human prostate carcinoma and cultured as described in Materials and Methods, in absence or presence of 15 ng/mL of NGF. At day 4 of culture cell were treated with leuprolide or cetrorelix (20 ng/mL) for 48 hrs. At the end of the experiments, final cell number was estimated by MTT method. Values are mean ± SD of 3 independent experiments.
*$P < 0.05$ compared with respective controls.

TABLE II. Effect of Leuprolide and Cetrorelix on the Apoptosis of Epithelial Cells From Prostate Cancer Cultured in Absence or Presence of NGF

	Percentage of Comet positive cells	
	+NGF	−NGF
Control	6.8 ± 0.7	8.4 ± 1.0
Leuprolide	38.9 ± 4.2*	20.2 ± 2.8*
Cetrorelix	44.7 ± 5.3*	22.2 ± 3.0*

Epithelial cells were isolated from human prostate carcinoma and cultured as described in Materials and Methods, in absence or presence of 15 ng/mL of NGF. At day 4 of culture cell were treated with leuprolide or cetrorelix (20 ng/mL) for 48 hrs. At the end of the experiments, DNA fragmentation was estimated by COMET assay. Values are mean ± SD of 3 independent experiments.
*$P < 0.05$ compared with respective controls.

NGF 15 ng/ml. As expected, both agonist and antagonist of GnRH effectively induced a decrease in cell number and an increase in apoptotic cells (Tables I and II). Cetrorelix showed a slightly higher effect than leuprolide. These GnRH effects were more pronounced in presence of NGF.

Expression of TrkA and p75 Receptors in Epithelial Primary Cell Cultures From Prostate Adenocarcinoma

We detected the protein expression of NGF receptors in our primary culture system using specific antibodies. We found a consistently high expression of TrkA receptor in all cultures of prostate carcinoma compared with cultures from benign hyperplasia (Fig. 1A,C); while p75 protein expression was almost undetectable with the antibody used in this study in cultures from adenocarcinoma (Fig. 1B).

Effect of Leuprolide and Cetrorelix on Gene Expression of TrkA and p75 Receptors

To evaluate the influence of GnRH analogs on expression of NGF receptors, primary cultures of prostate epithelial cells were treated with 20 ng/ml of

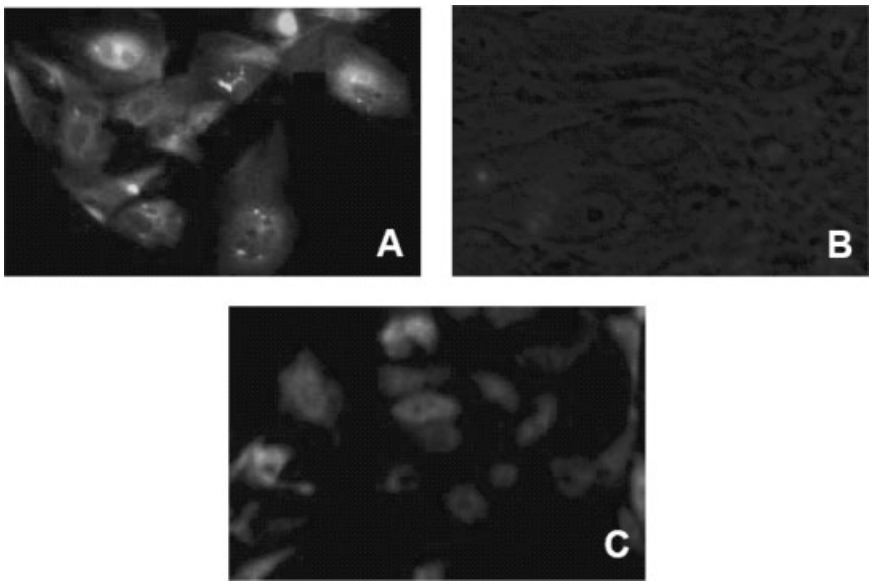

Fig. 1. Immunocytochemistry of cell cultures. The cells were treated with rabbit antibodies against TrkA and p75 nerve growth factor (NGF) receptors and then, detected with FITC-conjugated anti-rabbit IgGs. (400×). **A**: TrkA receptor expression in cultures from prostate cancer. **B**: p75 receptor expression in cultures from prostate cancer. **C**: TrkA receptor expression in cultures from benign hyperplasia as a reference of non-malignant cells. Representative cultures are showed.

leuprolide or cetrorelix and then, analyzed by RT-PCR for TrkA and p75 receptors. Expression of p75 receptor showed a significant increase in presence of both GnRH analogs (Fig. 2B), while TrkA receptor showed no changes at the concentrations used (Fig. 2A).

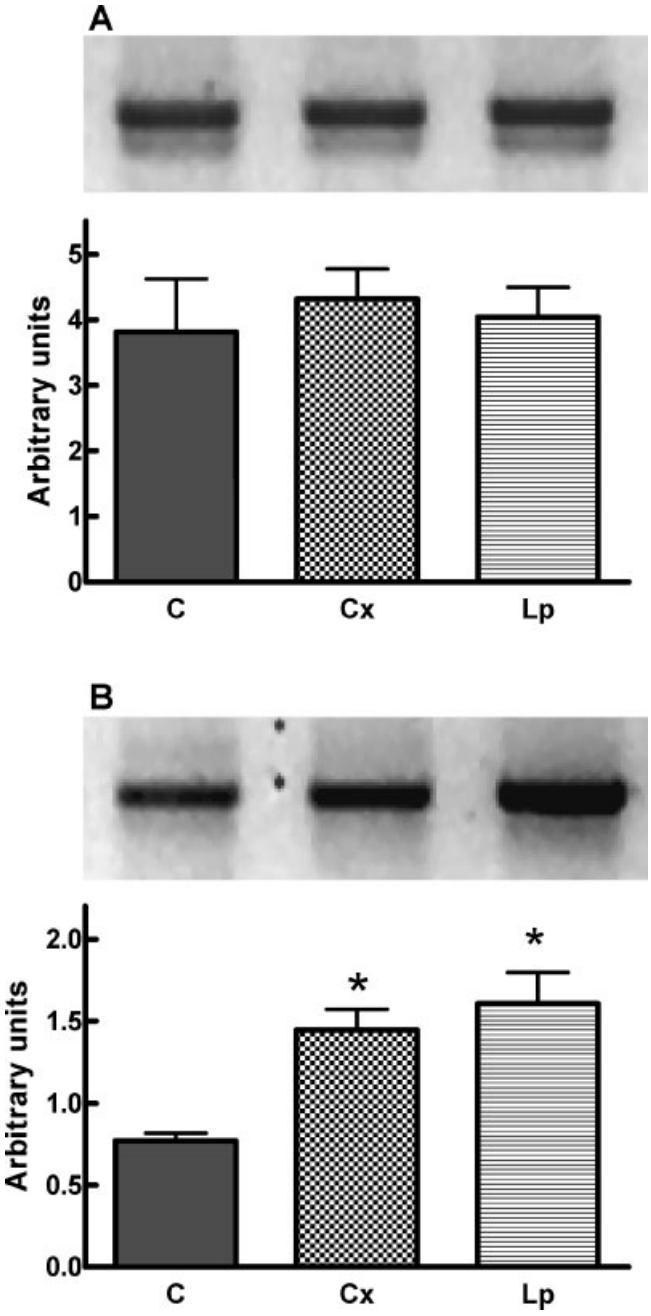

Fig. 2. Effect of leuprolide and cetrorelix on mRNA expression of TrkA and p75 receptors. RT-PCR (polymerase chain reaction) of NGF receptors from control and analog-treated epithelial culture cells from prostate carcinoma. Representative gels are shown. Graphs represent densitometric analysis of β-actin normalized bands. **A**: TrkA receptor. **B**: p75 receptor. Data are expressed as mean ± SD of three independent experiments. $^*P < 0.01$ compared with respective controls. C, Control; Cx, cetrorelix; Lp, leuprolide.

Effect of Leuprolide and Cetrorelix on Protein Expression of TrkA and p75 Receptors

To study the effect of GnRH analogs on protein expression of NGF receptors, leuprolide or cetrorelix-treated primary cultures of prostate epithelial cells were analyzed by western blot for TrkA and p75 receptors. In accordance with RT-PCR results, protein expression of p75 receptor showed a significant increase in presence of both GnRH analogs (Fig. 3B), while TrkA receptor showed no changes at the concentrations used (Fig. 3A).

DISCUSSION

GnRH-analog therapy has been extensively used to low testosterone plasma levels and therefore, control malignant prostate growth [4]. Also, GnRH receptors have been reported both in prostate cancer tissue and carcinoma derived cell lines, suggesting a local action for GnRH analogs [5–7]. On the other hand, several GnRH analogs have shown effect on proliferative and apoptotic activities in cell lines cultures. GnRH agonists have, mainly, antiproliferative and/or apoptotic effects. However, contradictory effects have been reported for antagonists [8–18]. Most of our knowledge on prostate cancer cells in vitro comes from cell lines experiments and most of these cell lines are derived from metastatic tumors, which may not represent the in situ carcinoma. Considering this limitation in established cell lines models, we have recently characterized a primary culture system from human prostate cancer cells [34]. In this system, we showed that GnRH agonists and antagonists have both antiproliferative and apoptotic effects [submitted]. Although many reports concerning GnRH analogs effects on prostate cancer cells have been published, little is known about the mechanisms underlying these effects. In normal and malignant prostate, paracrine, and autocrine mechanisms seem to play a key role in growth control [38,39]. Growth factors, like insulin-like growth factor I (IGF-I, EGF), fibroblast growth factor (FGF), NGF, and their receptors appear involved [40,41]. In the normal prostate, NGF is expressed mainly in the prostate stromal compartment while its receptors are expressed in epithelial cells [24,25]. However, neurotrophins are also expressed in epithelial cells of malignant tissue [42]. The high affinity TrkA receptor, but not TrkB or C, is present in normal prostate. Interestingly, TrkB and C receptors are ectopically expressed only in malignant prostate while TrkA is significantly overexpressed, or at least maintained, in carcinoma [20,28,42]. Trk receptors have been identified as members of tyrosine kinase family and their activation is mainly related with proliferation and differentiation processes [23,28,29]. Recent reports have suggested that inhibition of Trk

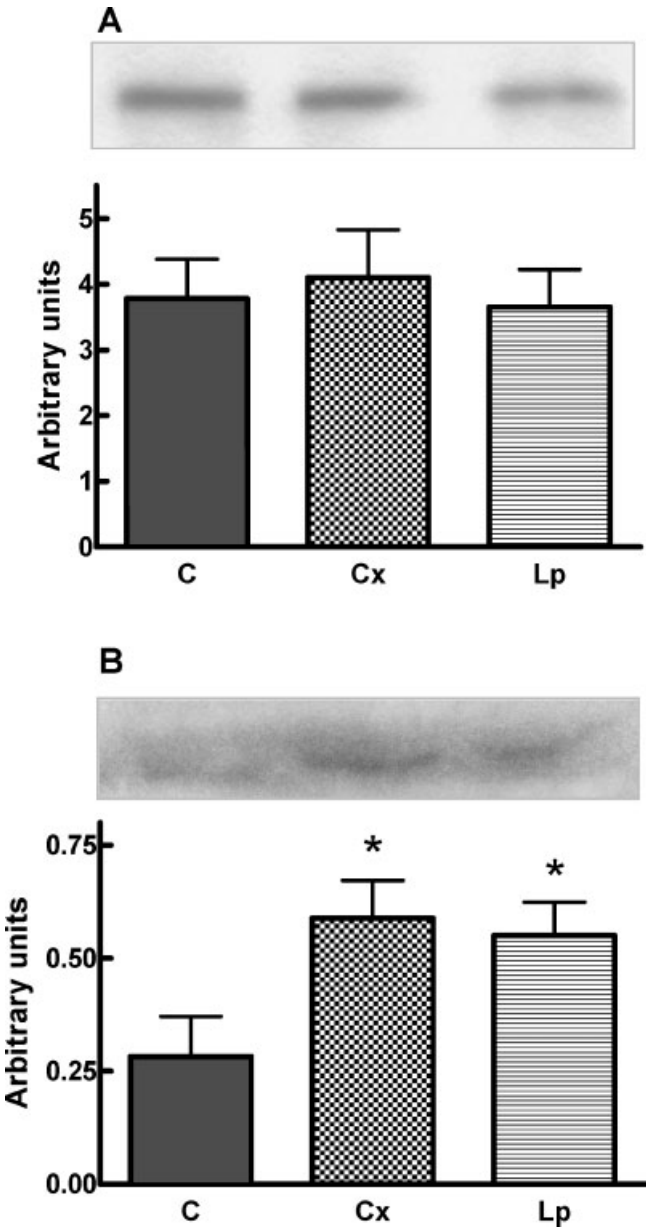

Fig. 3. Effect of leuprolide and cetrorelix on protein expression of TrkA and p75 receptors. Western blot of NGF receptors from control and analog-treated epithelial culture cells from prostate carcinoma. Representative membranes are shown. Graphs represent densitometric analysis of β-actin normalized bands. **A**: TrkA receptor. **B**: p75 receptor. Data are expressed as mean ± SD of three independent experiments. $^*P < 0.05$ compared with respective controls. C, Control; Cx, cetrorelix; Lp, leuprolide.

receptors may represent an interesting strategy for prostate cancer therapy [42]. On the other hand, the low affinity p75 receptor has been found widely expressed in normal prostate but its expression is reduced in adenocarcinoma and even absent in metastatic cell lines [23,26,27]. Although TrkA and p75 are co-expressed in most of NGF-responsive cells, independent expression of these receptors has also been reported. The TrkA/p75 ratio appears essential for the physiological response elicited by NGF [21]. Interestingly, p75 receptor has been consistently associated with apoptotic induction although specific mechanisms are not fully understood [30,31,43–46]. However, apoptotic effect of p75 receptor has been associated with changes in TrkA/p75 ratio [21,22]. In addition, p75 receptor expression also inhibits NGF-mediated metastasis in human prostate cancer cells [47]. Taken together, these data suggest that changes in the TrkA/p75 ratio occurring in prostate cancer may explain, in part, the enhanced proliferative activity and low apoptosis in malignant tissue. In addition, p75-transfected LNCaP cells, which do not express endogenous p75 receptor, underwent apoptosis when deprived of NGF [32]. We think that our primary culture system resembles more closely the in situ situation of prostate tumor than established cell lines. In this culture system, we have found a high TrkA and a very low p75 receptor expression at the level of protein both by immuncytochemistry and western blot. At the level of gene expression we were able to detect both TrkA and p75 transcripts. These data are consistent with other in vitro and in vivo studies. Indeed, the lost of expression of p75 receptor in prostate cancer is related with mRNA stability and translation rather than transcription activity [26]. Also, our data showed that treatment with leuprolide and cetrorelix, at doses that induce apoptosis and cell proliferation decrease, significantly increased gene and protein expression of p75 receptor. Although, no changes were found in TrkA expression, the ratio between these two types of receptors may resemble more closely the normal situation under GnRH analogs treatments. We suggest that the antiproliferative and apoptotic effects reported for GnRH analogs in prostate cancer cells may be mediated, at least in part, by changing the expression of NGF receptors. Although direct interactions between p75 and TrkB or C receptors have not been reported, so far, we cannot disregard a possible role for these types of Trk receptors in the apoptotic effect of GnRH analogs. We have demonstrated that p75 expression is increased with GnRH analogs treatments. Although this is not direct evidence that antiproliferative and apoptotic effects of these analogs are mediated by p75, it must be considered that p75 has been widely associated with survival inhibition in several tissues, including prostate [23,32]. Recently, proliferation arrest and apoptosis elicited by p75 was found associated with NF-kB/JNK pathway in prostate cancer derived PC-3 cell line [48] and gene therapy with this receptor has been proposed [49]. This point is very interestingly, considering that PC-3 cells are derived from androgen insensitive prostate cancer. This means that GnRH analogs may

have a positive effect in controlling malignant cell growth regardless of their androgen-suppressive effect. Considering all these data, our results reinforce the importance of GnRH-analog therapy for prostate cancer and allow us strongly suggest that antiproliferative and/or apoptotic effects of these analogs may be explain, at least in part, by the increased expression of p75 receptor.

CONCLUSION

We conclude that GnRH analogs, leuprolide, and cetrorelix, used at a concentration that induces proliferation arrest and apoptosis, caused a significant increase in p75 NGF receptor. This may be one of the survival inhibition mechanisms for these analogs.

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