Expression of neuropeptide Y receptors in human prostate cancer cells

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Summary

Background: Neuroendocrine molecules are now believed to play a significant role in the progression of human prostate cancer (CaP), especially in the androgen-independent stage.

Materials and methods: In the present study, we evaluated the presence and the function of the receptors for neuropeptide Y (NPY) in human CaP cell lines (the androgen-dependent LNCaP, and the androgen-independent DU 145 and PC-3).

Results: The presence of high-affinity binding sites for NPY was shown on PC-3 cells (radioreceptor assay). Reverse transcription-polymerase chain reaction analysis indicated that these sites correspond to the Y1 and Y2 receptor isoforms. A Y1 receptor protein (70 kDa) was also detected in PC-3 cell extracts by Western blot analysis. The activation of these receptors by NPY resulted in a reduction of forskolin-induced cAMP accumulation and an increase of [Ca2+]. Moreover, a prolonged treatment with NPY induced a dose-related proliferation of PC-3 cells.

Conclusions: By showing that NPY receptors are expressed in the androgen-independent cell line PC-3 and that their activation results in cell proliferation, the present data suggest that NPY-related mechanisms might be relevant in certain stages of CaP, such as the progression of the disease during the androgen-independent stage.

Key words: neuroendocrinology, neuropeptide Y, neuropeptide Y receptors, prostate cancer

Introduction

Prostate cancer (CaP) is initially often androgen dependent, and it may progress to androgen independence in later stages [1]. In this condition, hormonal therapy is no longer useful and the prognosis becomes worse. It is believed that the molecular basis underlying this transition includes a host of factors, some of which are now being identified as peptidic molecules, such as growth factors and neurohormones. Several studies suggest that neuroendocrine mechanisms play an important role in the control of the development and the function of the normal prostate, as well as in the progression of CaP [2, 3]. Few data, however, are presently available about one of these neuroendocrine modulators, neuropeptide Y (NPY), and on the related receptors in the normal as well as in the tumoral prostate. NPY, a peptide of 36 amino acids, is abundantly distributed through the nervous system, and activates specific membrane receptors that exist in at least five different isoforms [4]. NPY participates to the regulation of a variety of physiological functions, including regulation of neuroendocrine mechanisms, cognitive functions, eating behavior and cardiovascular activity, and has also been shown to stimulate cell proliferation [5]. In the context of the normal human prostate, NPY is mainly localized in the nerve fibers, and in the neuroendocrine (NE) cells [6]. In addition, NPY immunopositivity has also been shown in specimens from CaP patients (about 75% of a series of patients [7]), indicating a possible participation of this factor in CaP growth and progression.

With the aim of a better understanding of the role of this neuropeptide in CaP biology, the present study was designed to investigate the expression and the function of NPY and its receptors in three commonly utilized human CaP cell lines (LNCaP, androgen-dependent, and DU 145 and PC-3, androgen-independent).

Materials and methods

Cell cultures. The human prostate cancer cell lines LNCaP, DU 145, and PC-3 (American Type Culture Collection (ATCC), Rockville, Maryland) and the human neuroblastoma cell lines SK-N-MC and SK-N-BE (also from ATCC) were grown in monolayer at 37 °C. The culture medium was RPMI 1640 (Biochrom, Berlin, Germany), containing 5%-10% fetal calf serum (FCS) (Gibco, Grand Island, New York) for LNCaP, DU 145, and PC-3 cells, and MEM (Biochrom, Berlin, Germany) containing 10% FCS for SK-N-MC and SK-N-BE cells. Confluent cells were harvested with 0.05%-0.02% trypsin/EDTA (Biochrom, Berlin, Germany) and were seeded in Petri dishes. Experiments were performed using subconfluent cell cultures.

Receptor binding assay. The receptor binding assay was performed using [125I]NPY (SA, 2000 Ci/mmol) (Amersham Pharmacia Biotech Italia, Milan, Italy) as the ligand. The competition curves were performed by incubating crude membrane preparations (50–150 mg of protein per tube) from CaP cells and from SK-N-MC cells with 20 pM [125I]NPY in the absence or presence of human NPY (Bachem, Bubendorf, Switzerland) for 2 h and 30 min at room temperature. At the end, membrane preparations were pelleted down by centrifugation, and the pellets were counted in a g counter (Packard Instruments, Milan, Italy).
RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR). Cells were washed with cold PBS, collected, snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total cellular RNA was extracted by the guanidine/chloroform method. For RT-PCR analysis of the expression of the genes encoding for NPY and NPY receptor subtypes, 1 mg of total RNA was incubated with 50 ng of random hexamers at 70°C. The RT reaction using MuLV-RT was carried out using an RT-PCR kit (PE Italia, Milan, Italy). The PCR reaction was carried out in a DNA thermal cycler (PE Italia, Milan, Italy), and included an initial denaturing step at 95°C for 5 min and 35 cycles for all target genes. The sequence of the primers used for NPY, each NPY receptor subtype and β-actin (housekeeping gene), was obtained from previously published studies [8–12]. The amplified products were resolved in a 2% agarose/1X TBE gel and the DNA was visualized by ethidium bromide fluorescence on a UV transilluminator.

Cell proliferation studies. For proliferation studies, PC-3 cells were seeded in 60 mm Petri dishes (100,000 cells/dish). After 72 h, the culture medium was substituted by an experimental medium (RPMI 1640 + 0.1% BSA), containing different concentrations of human NPY. At the end of the incubation (96 h), cells were collected with 0.05%-0.02% trypsin/EDTA, and resuspended in RPMI 1640. Cell viability was assessed by the trypan-blue exclusion method and then cells were counted. Analysis of the data. Statistical analysis was performed by using the Systat statistical analysis package. Data are given as mean ± SEM; n = number of replicates within an experiment. Significance of differences between treatment groups was evaluated by ANOVA. Receptor binding experiments were analyzed by ANOVA. Receptor binding experiments were analyzed by the program Ligand.

Results

The NPY radioligand binding assay detected the presence of specific binding of [125I]NPY in only one of the three human CaP cell lines tested, the PC-3 cells (Figure 1a). The binding of [125I]NPY to PC-3 membranes was saturable and completely displaced by 10⁻⁶ M NPY concentrations, and showed a high affinity (Kd, 30 nM). The maximal binding capacity (Bmax) was 4870 fmol/mg protein, which corresponds to approximately 45,000 sites/cell. As expected, [125I]NPY bound to SK-N-MC membranes, a cell line expressing high amounts of Y1-Rs and thus used as positive control, with high affinity (Kd, 0.53 nM) and a Bmax of 1782 fmol/mg protein, corresponded to approximately 10,000 sites/cell (Figure 1a). The gene expression of two NPY receptor isoforms, the Y1-R and the Y2 receptor (Y2-R), in CaP cells was evaluated by RT-PCR analysis (Figure 1b). It was found that Y1-R and Y2-R mRNA are expressed only in PC-3 cells.

To evaluate the possible proliferative effects of NPY treatment, PC-3 cells were exposed for 96 h to different concentrations of this agent (10⁻⁹ to 10⁻⁷ M). Treatment with NPY did not modify the survival rate of these cells (viable cells, 95%, Trypan blue exclusion), but significantly stimulated cell proliferation, with a bell-shaped dose-response curve and a maximal effectiveness of NPY at 10⁻⁸ M (Figure 2).

Discussion

The present study shows that functional receptors for NPY, corresponding to the Y1 and the Y2 isoforms, are present in the androgen-independent human CaP cell line PC-3, and that their activation by NPY stimulates the proliferation of these cells.

Additional experiments conducted in our laboratory confirmed that these receptors correspond at least in

![Figure 1](attachment:figure1.png)

**Figure 1.** (a) Radioreceptor binding assay for NPY in PC-3 and SK-N-MC cells. NPY inhibits the binding of [125I]NPY to PC-3 and SK-N-MC cell membrane preparations. (b) RT-PCR analysis of the gene expression of NPY receptors in CaP cell lines. Total RNA extracts from the human CaP cell lines LNCaP, DU 145, and PC-3 and from the appropriate control cell lines (SK-N-MC for Y1-R, SK-N-BE for Y2-R) were subjected to RT-PCR analysis using oligonucleotides specific for the NPY receptor isoforms Y1 and Y2, and for β-actin. Ethidium bromide-stained gels for each RT-PCR reaction are shown.

![Figure 2](attachment:figure2.png)

**Figure 2.** Effect of NPY treatment on the proliferation of PC-3 cells. PC-3 cells were treated for 96 h with different concentrations of NPY. At the end of the incubation cells were collected and counted in a hemocytometer. Data are expressed as mean ± SEM, n = 5. *P < 0.001 vs. controls (-), (ANOVA).
part to the Y1-R, as suggested by an immunoblot study revealing the presence of a 70 kDa protein in PC-3 cell extracts; moreover, activation of the NPY receptors present on PC-3 cells by 10^{-8} M NPY resulted in a significant reduction of the cAMP increase induced by forskolin (~53%), and in an immediate increase of [Ca^{2+}i] (Magni and colleagues, personal observation). All these data are in agreement with previous studies conducted on neural tissue or cells [14, 15], suggesting that the NPY receptor system present in PC-3 cells is substantially identical to that expressed in the nervous system. The proliferative action induced by NPY treatment followed a bell-shaped curve with a maximal efficacy at the NPY concentration of 10^{-8} M, which is in the same range of the Kd calculated for the NPY binding sites present on these cells. It should be noted that this Kd value is only slightly lower than that found for SK-N-MC cells (used in this study as a positive control) by us and other authors [13]. Proliferative effects of NPY have been previously shown for vascular smooth muscle cells and seem to work via the Y1-R [16, 17]. This effect of NPY on proliferation seems to be quite specific, since this peptide has been reported to have no effect on the in vitro invasion and migration of PC-3 cells [18].

These findings might have a pathophysiological significance in the context of CaP progression; although according to our personal observations NPY is not produced in vitro by the human CaP cell lines tested, NPY expression has been shown in the context of the human normal [6] as well as tumoral [7] prostate. Therefore, in the prostate, NPY could be released by NE cells or by nerve terminals to reach NPY receptors present on nearby epithelial cells of normal or tumoral origin, and, in the latter case, act as a proliferative factor.

In conclusion, the present study, together with other data present in the literature [7], suggests that the prostatic NPY neuroendocrine system might participate in the modulation of the proliferation of CaP cells. Moreover, the presence and the activation of NPY receptors might represent a marker of CaP progression toward a stage sensitive to non-androgenic trophic and proliferative agents. Further studies in this field might also give indications about possible novel future lines for the treatment of CaP, especially when this disease has progressed to the androgen-independent stage.

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References


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