Differential effects of estradiol and tamoxifen on pyrrolidon carboxypeptidase activity in the human estrogen-dependent and estrogen-independent breast cancer cell lines MCF-7 and EVSA-T

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Differential effects of estradiol and tamoxifen on pyrrolidon carboxypeptidase activity in the human estrogen-dependent and estrogen-independent breast cancer cell lines MCF-7 and EVSA-T

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**Abstract**

Pyrrolidon carboxypeptidase (Pcp) (E.C 3.4.19.3) is an omega peptidase widely distributed in animal fluids and tissues, which hydrolyzes N-terminal pyroglutamyl residues from biologically active peptides such as gonadotropin releasing hormone (GnRH). Previous results obtained in our laboratory showed a decrease of Pcp activity in human and rat breast cancer, suggesting that this enzymatic activity or its putative substrates may play a major role in breast cancer pathogenesis. In the present work, we analyze the effects of estradiol and tamoxifen on both Pcp activity and cell proliferation, in the estrogen-dependent and estrogen-independent breast cancer cell lines MCF-7 and EVSA-T. We found a stimulant effect of estradiol on MCF-7 but not on EVSA-T cells growth, whereas tamoxifen inhibited cell growth in both cell types. In the same way, we found a decrease on Pcp activity on MCF-7 cells treated with estradiol, whereas no effect was observed with tamoxifen. Contrarily, Pcp activity also decreased on EVSA-T cells when treated with tamoxifen, whereas no changes were observed in this cell line when treated with estradiol. We propose that Pcp activity and, therefore, its peptide substrate/s (mainly GnRH, but also TRH, neurotensin and bombesin) participates differentially in the pathogenesis of estrogen-dependent and estrogen-independent tumors, and could be considered a target for drug design and development against mammary tumors.

**Introduction**

Breast cancer is the most common cause of tumor-related death among women in the Western world [1]. Hormonal regulation mechanisms of tumor growth have been described for human mammary tumors [2], cell lines [3] and experimentally induced tumors [4].

Pyrrolidon carboxypeptidase (Pcp), also called pyroglutamyl aminopeptidase, (E.C 3.4.19.3), is an omega peptidase, widely distributed in animal fluids and tissues [5], which hydrolyzes N-terminal pyroglutamyl residues from biologically active peptides such as gonadotropin releasing hormone (GnRH), and ary lamide derivatives in a highly selective manner [6]. In previous reports, we have described changes in Pcp activity in human and N-methyl nitrosourea (NMU)-induced rat breast cancer. In humans, Pcp activity was decreased in tumor and surrounding tissue [7]. In rats, a decrease was also found in serum [8]. Therefore, the changes in this enzyme activity and/or its putative substrates may play a major role in breast cancer pathogenesis. Previous reports of our laboratory also showed the influence of sex hormones on Pcp activity in serum and different tissues of both humans and rodents [9-12]. Therefore, the aim of the present work was to analyze the effects of estradiol and tamoxifen on Pcp activity, using human breast cancer cell lines MCF-7, which depend on estrogen for proliferation [13,14] and EVSA-T, which is estrogen-independent [15,16].

**Methods**

**Cultures.**

Two human tumor cell lines (human MCF-7 and EVSA-T breast cancer cell lines) have been studied in this work, described previously [17] and derived from human breast cancer [18]. Cell lines were grown in 5% fetal bovine serum (FBS)-supplemented Dubelcco’s modified Eagle’s medium (DMEM) with penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Cells were incubated at 37°C in a modified atmosphere of 5% CO2/95% air. Freedom from mycoplasma contamination was checked regularly by testing with Hoescht 33528.

**Colorimetric cell proliferation assay.**

To set up the colorimetric cell proliferation assay (CCPA) [19,20], cells were trypsinized from monolayer and diluted to 4 x 104 cells/ml. Cells were in exponential phase of growth during the whole.
experiment. Aliquots of 1 ml of cells (MCF-7 or EVSA-T cells) were pipetted into wells of 24-well tissue culture plates (Nunc) and the plates were incubated for 24 h. Estradiol or tamoxifen (Sigma) was then added to the wells in a volume of 1 ml per well at a range of concentrations (0.01, 0.1, 1 and 10 M), each dose being used in at least four replicate wells. After 3 days incubation, estradiol and tamoxifen solutions were removed and the cultures, were washed with PBS prior to fixation with 10% trichloroacetic acid (TCA) (4°C) for 30 minutes and then washed with tap water to remove TCA. Plates were air dried and then stored until use. TCA-fixed cells were stained for 20 minutes with 0.4% (w/v) sulforhodamine B (SRB) (Sigma) dissolved in 1% acetic acid. At the end of staining period, SRB was removed and cultures were rinsed with 1% acetic acid to remove unbound dye. The cultures were air dried and bound dye was solubilized with 10mM Tris base (pH 10.5). Optical density (OD) was read in ThermoLabsystems multiscan Ascent plate reader at 592 nm. The photometer response was linear with dye concentration and it was proportional to cell numbers counted in parallel with a hemocytometer [21].

Pcp activity assay.

Pcp activity was measured fluorimetrically using pyroglutamyl-β-naphthylamide (pGluNNap) as substrate, in accordance with the method previously described [11] with modifications. Briefly, cells were trypsinized from monolayer and diluted to 4 × 104 cells/ml. Cells were in exponential phase of growth during the whole experiment. Aliquots of 1 ml of cells (MCF-7 or EVSA-T cells) were pipetted into wells of 24-well tissue culture plates (Nunc) and the plates were incubated for 24 h. Estradiol or tamoxifen (Sigma) was then added to the wells in a volume of 1 ml per well at a range of concentrations (0.01, 0.1, 1 and 10 M), each dose being used in at least four replicate wells. After 3 days incubation, estradiol and tamoxifen solutions were removed and the cultures, were washed with PBS. One mL of the substrate solution (100 µM pGluNNap, 0.65 mM dithiothreitol (DTT) and 1.3 mM ethylenediaminetetraacetic acid (EDTA) in DMEM without FBS) were pipetted into each well, and incubated for 120 minutes at 37°C. All the reactions were stopped by adding 1 mL of 0.1 M acetate buffer, pH 4.2. The amount of β-naphthylamine released as the result of the enzymatic activity was measured fluorimetrically at 412 nm emission wavelength with and excitation wavelength of 345 nm. Specific Pcp activity was expressed as pmol of pGluNNap hydrolysed per min per 105 cells, by using a standard curve of β-naphthylamine under corresponding assay conditions. The fluorimetric assay was linear with respect to time of hydrolysis and cell number.

Statistical Analysis

To analyse the differences between groups, we used One Way Analysis of Variance (ANOVA) and Newman-Keuls post-hoc test. All comparisons with P values below 0.05 were considered significant.

Results

Cell proliferation curves for MCF-7 and EVSA-T cells measured by the CCPA after estradiol or tamoxifen treatment are shown in figure 1. Estradiol 0.1-10 µM increases significantly (pµM) decreased significantly (p

Regarding MCF-7 cells, estradiol treatment decreased Pcp activity significantly (pµM estradiol and pµM estradiol. However, when MCF-7 cells were treated with tamoxifen, Pcp activity did not change at any of the concentrations considered (figure 2A).

On the contrary, estradiol treatment of EVSA-T cells did not change Pcp activity at any of the concentrations used, whereas the treatment with tamoxifen 0.01-10 µM decreased significantly (pµM tamoxifen and pµM tamoxifen) Pcp activity (figure 2B).

Discussion

In human breast cancer, we had previously described a significant decrease in Pcp activity in neoplastic and adjacent tissues when compared with unaffected tissue, indicating that local factors may be selectively modified by the tumoral process in the affected tissue [7]. Also, in NMU-induced rat breast cancer, we had reported a decrease in serum Pcp activity [8], supporting the role for this enzyme activity in cancer pathogenesis. Since one of the susceptible substrates of Pcp is GNRH, our results indicate that the regulation of the levels of this peptide hormone through its degrading peptidase could be a key factor in the development of the disease. In this connection, GNRH receptors and GNRH mRNA have been found in breast tissue, raising the possibility of a local and physiological role for GNRH in the mammary gland [22]. In fact, GNRH receptor (GnRH-R) had been immunolocalized in the cytoplasm of carcinoma cells and was also detected focally in the cytoplasm of morphologically normal glandular epithelial adjacent to the carcinoma, also supporting that GnRH-R is widely distributed in breast carcinoma cells and regulates.
GnRH actions locally [23,24]. Taken together, these results indicate that GnRH may be an important local intracrine, autocrine and/or paracrine hormonal factor in the pathogenesis of breast cancer and suggest that play a role in the tumoral process. Moreover, increased levels of GnRH are associated with increased levels of gonadal steroid hormones [25]. Transformation of normal breast cells, initiation, and maintenance of breast cancer growth all depend upon the interplay of a number of inhibitory and stimulant factors, including estrogens [26]. Estrogens stimulate the growth of breast cancer in about one-third of the patients, whereas estrogen deprivation induces tumor regression [27]. In the present work we show an important stimulant effect of estradiol on the growth of the estrogen-dependent MCF-7 cells from concentrations ranging between 0.1 and 10 µM. However, no effects of estradiol on cell growth were observed in the estrogen-independent EVSA-T cell line. Thus, the main goal of the endocrine treatment of breast cancer is the inhibition of estrogen activity achieved either by blocking the estrogen receptors (ER) or estrogen production [28-31]. Therefore, hormonal manipulation plays an important role in the treatment of ER-positive breast cancer because it is not only effective but also associated with minimal toxicity and excellent quality of life [32]. Compounds called "selective ER modulators" (SERMs) directly compete with estrogen binding to the ER, preventing downstream signaling. The original SERM tamoxifen is a synthetic antiestrogenic compound widely used to treat ER-positive breast cancer [33-36]. Tamoxifen is also used as an adjuvant treatment of several other types of tumors including malignant gliomas [37-39]. High concentrations of tamoxifen have been shown to inhibit cell proliferation and to induce apoptosis [38]. Our results also support this data, and both MCF-7 and EVSA-T cell lines growth are inhibited by tamoxifen. In the present work, we have found a decrease in Pcp activity in the human estrogen-dependent breast cancer cell line MCF-7 when it was treated with estradiol but not when it was treated with tamoxifen. Therefore, the administration of estrogen to this estrogen-dependent cells, decreases Pcp activity, enhancing the functions of GnRH. On the contrary, in the human estrogen-independent breast cancer cell line EVSA-T, Pcp activity did not change after estradiol treatment, although surprisingly, Pcp activity also decreased after the treatment with tamoxifen. Although the major biochemical effect of tamoxifen on breast cancer cells is the competitive binding to ER and a dissociated effects on gene expression [40], several other effects have been described such as the G1 blockade, the increase in the production of TGF-β, the inhibition of IGF1 production, the decrease in the levels of IGF1 and the increase in the levels of sex hormone-binding globulin [41], which could be responsible, at least in part, of the results obtained on EVSA-T cells after tamoxifen treatment. Therefore, further investigation is needed to understand the precise role of Pcp and its peptide substrate/s (mainly GnRH, but also TRH, neurotensin and bombesin) in the pathogenesis of estrogen-dependent and -independent tumors, but we can propose Pcp as a key enzyme to be used not only as a marker which might permit the monitoring of each type of breast cancer, but also a target for drug design and development against mammary tumors.

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Cell proliferation curves for the estrogen-dependent and estrogen-independent MCF-7 and EVSA-T cells measured by the colorimetric cell proliferation assay (CCPA) after estradiol (●) or tamoxifen (○) treatment. Results are expressed in percent of growth (Mean ± SEM; **p<0.01).
Illustration 2

Figure 2.

Specific Pyrrolidon Carboxypeptidase Activity

Specific pyrrolidon carboxypeptidase (Pcp) activity in the estrogen-dependent and estrogen-independent MCF-7 and EVSA-T cells after estradiol or tamoxifen treatment. Results are expressed in picomoles of pyroglutamyl-β-naphthylamide hydrolysed per min and per 10^5 cells (Mean ± SEM; *p<0.05; **p<0.01).
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