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Original article

Putative relationship between hormonal status and serum pyrrolidone carboxypeptidase activity in pre- and post- menopausal women with breast cancer

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ABSTRACT

In breast cancer, hormonal changes are rather constant in post-menopausal women since they tend to vary only over long time spans. However, in pre-menopausal women, the development of breast cancer is associated with hormonal physiological variations. The aim of the present work was to analyse the changes in circulating levels of gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in pre- and post-menopausal women that were healthy or with breast cancer, and their connection to serum pyrrolidone carboxypeptidase (Pcp) activity. We observed significant changes in the hormonal profile in post-menopausal women with breast cancer compared to the control group. In pre-menopausal women, we found significant changes in circulating GnRH levels with respect to the healthy group. Our present results support the existence of neuroendocrine misregulation that could be involved in tumour progression, with Pcp being a potentially new pharmacological target in breast cancer treatments.

Introduction

Menopause has historically been considered a primary ovarian event, associated with changes in pituitary gonadotropin secretion that occur secondarily to the decline in ovarian sex steroid.[1] Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels decrease progressively after menopause, as does gonadotropin-releasing hormone (GnRH) pulse frequency.[2] In breast cancer, a disease hormone-dependent and subject to hormonal influence, hormonal changes are rather constant in post-menopausal women since they tend to vary only over long time spans. In contrast, in pre-menopausal women, the progression of breast cancer is related to hormonal physiological variations.[3] Indeed, high FSH and LH levels have been associated with a significantly worse prognosis in pre-menopausal breast cancer patients.[4] FSH and LH levels are regulated by GnRH, which in turn is regulated by the proteolytic regulatory enzyme, pyrrolidone carboxypeptidase (Pcp) (E.C. 3.4.19.3). Pcp is an omega peptidase widely distributed in fluids and tissues and hydrolyses N-terminal pyroglutamic residues from biologically active peptides such as GnRH.[5,6] We previously reported a decrease in both rat[7] and human[8] Pcp activity in breast cancer, suggesting that GnRH may be an important local intracrine, autocrine and/or paracrine hormonal factor in the pathogenesis of breast cancer. We suggested that this decrease indicated the existence of high circulating levels of GnRH. In this sense, in human breast cancer, a significant decrease in Pcp activity in neoplastic and adjacent tissues have been found when compared to unaffected tissue, indicating that local factors may be selectively modified by tumoural processes in the affected tissue.[8] In this regard, GnRH receptors and GnRH mRNA have been found in breast tissue, raising the possibility of a local role for GnRH in the human mammary gland.[9] Here, we analysed fluorometrically serum Pcp activity in pre- and post-menopausal women diagnosed with infiltrating ductal carcinoma and corresponding healthy pre- and post-menopausal control women. We also determined circulating levels of GnRH, FSH and LH.

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Materials and methods

Experimental design

We analysed 22 pre-menopausal and 37 post-menopausal women with breast cancer and 22 pre-menopausal and 37 post-menopausal healthy volunteers (controls). The study was approved by the Hospital Ethics Committee and all patients signed an informed consent form. Sample collection was performed for six months and they were taken at the moment of the patient's diagnosis without any previous treatment. Furthermore, plasma levels of oestradiol and progesterone were analysed in women (control and patients with breast cancer) to confirm the existence of menopause. Table 1 shows the clinicopathological characteristics of the patients. Blood samples were obtained and centrifuged for 10 min at 3000 *g* to obtain serum. Samples were rapidly frozen in liquid nitrogen and stored at −80 °C, until use.

Pyrrolidone carboxypeptidase assay

Serum Pcp activity were measured fluorimetrically using pyroglutamyl-(3-naphthylamide (pGLUNNap) as the substrate according to the method previously described by us.[10] Briefly, 10 μl of each sample were incubated in triplicate for 30 min at 37 °C with 100 μl of the substrate solution: 100 mM of pGLUNNap, 0.65 mM dithiothreitol (DTT) and 1.3 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM of phosphate buffer at pH 7.4. All the reactions were stopped by adding 100 μl of 0.1 M acetate buffer at 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 excitation wavelength of 345 nm. Proteins were quantified also in triplicate using bovine serum albumin (BSA) as standard.

Determination of circulating levels of GnRH, FSH and LH

Hormone concentrations in all serum samples were assayed using a pool of several kits. Circulating LH and FSH were measured by enzyme immunoassays (Bioserv Diagnostics, Rostock, Germany). Circulating GnRH was dosed by a commercial RIA kit (Phoenix Pharmaceuticals, Inc, USA) according to the manufacturer's instructions. The lowest detection limit was 25.4 pg/ml. The detection range was 10–1280 pg/ml. The sensitivities of the enzyme immunoassays were 0.3 IU/l for LH and FSH. The values of 0.2 IU/l for LH and FSH were assigned to samples below the detection limit.

Statistical analysis

To analyse the differences between healthy pre- and post-menopausal women, and pre- and post-menopausal women diagnosed with infiltrating ductal carcinoma, we used unpaired Newman–Keuls test. All comparisons with *p*-values below 0.05 were considered significant.

Results

Fig. 1 illustrates the results obtained for specific serum pyrrolidone carboxypeptidase activity in healthy (control) pre- and post-menopausal women and in women diagnosed with infiltrating ductal breast cancer.

Healthy post-menopausal women showed higher values of Pcp activity than pre-menopausal women (98.69 ± 4.94. vs 79.06 ± 4.37 pmol/min/mg protein). On the contrary, no differences were found in serum Pcp activity between pre- and post- menopausal women with breast cancer (75.62 ± 4.42 vs 79.71 ± 4.63 pmol/min/mg protein). Only post-menopausal women with breast cancer showed lower values of Pcp activity than control women. This decrease occurred concomitantly with a decrease in circulating levels of GnRH and FSH, whereas circulating levels of LH was not modified among the groups.

Furthermore, a significant decrease was observed in pre- ($p < 0.001$) and post-menopausal ($p < 0.001$) women with breast cancer compared to corresponding healthy control groups. Fig. 2 shows the data for circulating GnRH levels in pre- and post-

Table 1
Clinicopathological description of the patients involved in this study.

	Premenopausal ($n = 22$)	Postmenopausal ($n = 37$)
Age at diagnosis (years ± SEM)	45.5 ± 0.85	62.2 ± 1.38
Median	45	61
Range	39–52	49–84
Body mass index	27.55 ± 0.88	31.57 ± 0.63
Median	26.7	32
Range	23.7–32.3	25.9–37.4
Histological type		
Ductal	22 (100%)	37 (100%)
Lobular	None	None
Mixed	None	None
Molecular receptor status		
ER+	86.95%	83.72%
PR+	73.92%	72.09%
HER-2/neu+	86.95%	53.65%
Scarf-Bloom-Richardson grade		
I	35.3%	15.6%
II	47%	65.7%
II	17.7%	18.7%
TNM classification		
I stage	14 (63.7%)	10 (27%)
II stage	8 (36.3%)	27 (73%)
III stage	None	None
IV stage	None	None

ER, estrogen receptors; PR, progesterone receptor; Her-2/neu, human epidermal growth factor receptor; TNM, tumor node metastasis classification.

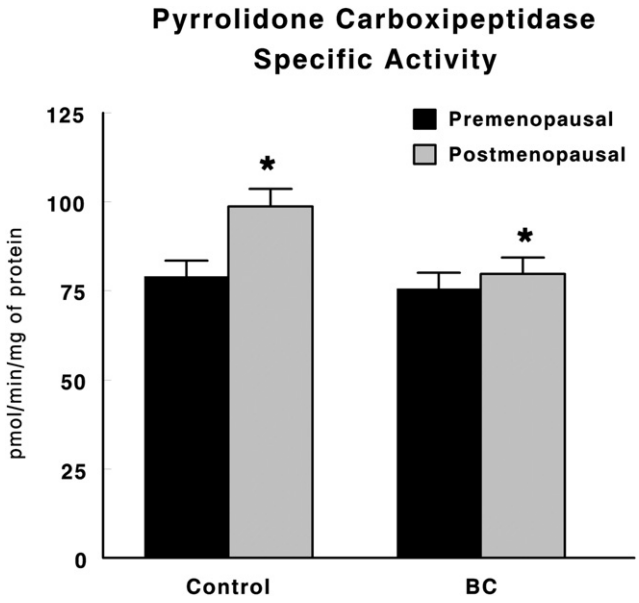

Fig. 1. Serum-specific pyrrolidone carboxypeptidase (Pcp) activities in healthy pre- and post-menopausal control women and pre- and post-menopausal women with breast cancer. Results are expressed as picomoles of pyroglutamyl-(β)-naphthylamide hydrolysed per min and per mg of protein (Mean ± SEM; $n = 22$ and 37; $^*p < 0.05$).

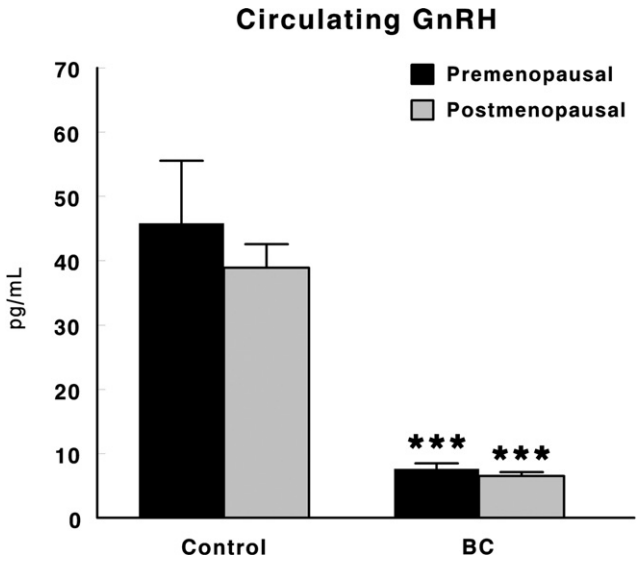

Fig. 2. Circulating levels of GnRH in healthy pre- and post-menopausal control women and pre- and post-menopausal women with breast cancer. Results are expressed as picograms per millilitre (Mean ± SEM; $n = 22$ and 37 $^{***}p < 0.001$).

menopausal women. We did not observe changes between healthy pre- and post-menopausal women, but we did find a significant decrease ($p < 0.01$) in circulating GnRH levels in both pre- and post-menopausal women with breast cancer compared to healthy controls.

On the other hand, post-menopausal women showed a significant increase ($p < 0.01$) in circulating FSH and LH levels compared to pre-menopausal women (Fig. 3). However, circulating levels of FSH significantly decreased in post-menopausal women with breast cancer, in relation to the control group, whereas no changes occurred in LH levels in pre-menopausal women. No significant differences were found in circulating FSH and LH levels in pre-menopausal women.

Discussion

Ovarian failure at menopause, associated with a reduction in oestrogen secretion, results in an increase in gonadotropic LH and FSH.[11] Progressive decreases in inhibin A and inhibin B elicit an early increase in FSH, which initially maintains folliculogenesis and oestradiol secretion. Changes in oestrogen feedback may contribute to cycle disruption. Studies in post-menopausal women show that oestradiol negative feedback is associated with a decrease in GnRH secretion, while oestradiol and progesterone are associated with a further decrease in the overall amount of GnRH secreted, with ageing not altering this inhibition on hypothalamic GnRH secretion in post-menopausal women.[12] In this sense, our results show an increase in circulating FSH and LH levels in post-menopausal women that lead to a rise in oestradiol and progesterone levels. However, in relation to circulating GnRH levels, we did not observe significant changes, although there was a decrease in post-menopausal women compared to pre-menopausal women. In contrast, the overall amount of GnRH increased with ageing, consistent with a significant degree of adaptability in the ageing brain in women.

The present work documented a change in Pcp activity in the serum of healthy post-menopausal women when compared to pre-menopausal women. This did not appear in post-menopausal women with breast cancer. Healthy post-menopausal women showed higher values of Pcp activity than pre-menopausal women.

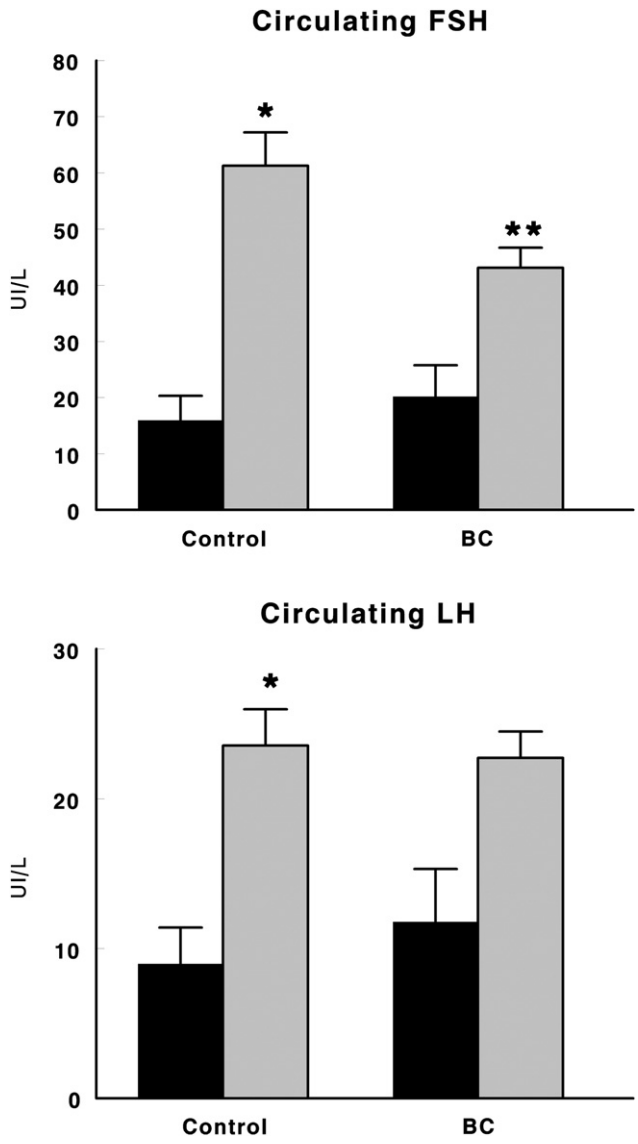

Fig. 3. Circulating levels of FSH and LH in healthy pre- and post-menopausal control women and pre- and post-menopausal women with breast cancer. Results are expressed in UI/l (Mean ± SEM; $n = 22$ and 37; $^{*}p < 0.01$; $^{***}p < 0.001$).

The increase observed in Pcp activity may reflect a decrease in circulating levels of GnRH that lead to a decrease in the gonadal steroid hormone production responsible, at least partly, for the initiation and promotion of the disease.

Breast cancer in menopausal women is associated with high plasma oestradiol concentrations.[13] Oestradiol exerts a negative feedback on FSH levels in post-menopausal women[3]; therefore, high oestradiol levels lead to low FSH levels in post-menopausal women. Our results agree with this affirmation since we observed a decrease in circulating levels of FSH in post-menopausal women with breast cancer. The negative feedback exerted by oestradiol could influence the hypothalamus-pituitary-ovary axis; therefore, GnRH release could be affected too. In this sense, we observed a decrease in circulating GnRH levels in post-menopausal patients with respect to healthy post-menopausal women. On the other hand, post-menopausal women with breast cancer showed lower values of Pcp activity than healthy post-menopausal women. In this regard, GnRH receptors and GnRH mRNA have been found in breast tissue, raising the possibility of a local role for GnRH in the human mammary gland.[9]

The decrease in serum Pcp activity in post-menopausal women with breast cancer occurred concomitantly with a decrease in the circulating levels of GnRH and FSH, whereas circulating levels of LH was not modified among the groups. Therefore, the results obtained for this activity could be a reflection of the functional status of the enzyme substrates, which can be selectively activated or inhibited as a result of specific conditions brought about by the tumour under different hormonal conditions.

Conclusion

During menopause, undoubtedly, the most notable changes in the neuroendocrine axis arise from the loss of ovarian function and thus, the loss of negative feedback on the hypothalamus and pituitary.[1] In this sense, although GnRH could have a role in the human mammary gland, our present results support the existence of neuroendocrine misregulation that could be involved in tumour progression, with Pcp being a potentially new pharmacological target in breast cancer treatments.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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