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Pyrrolidon Carboxypeptidase Activities in the Hypothalamus-pituitary-thyroid and Hypothalamus-pituitary-ovary Axes of Rats with Mammary Gland Cancer Induced by N-methyl Nitrosourea

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Abstract

Pyrrolidon carboxypeptidase is an omega-peptidase that hydrolyses N-terminal pyroglutamyl residues from biologically active peptides such as gonadotropin-releasing and thyrotrophin-releasing hormones. We previously described a decrease in both rat and human pyrrolidon carboxypeptidase activity with breast cancer, suggesting that gonadotropin-releasing hormone may be an important local intracrine, autocrine and/or paracrine hormonal factor in the pathogenesis of breast cancer while playing a role in the tumoral process. However, the other susceptible substrate of pyrrolidon carboxypeptidase, thyrotrophin-releasing hormone, may also be modified with breast cancer, supporting an association between breast cancer and thyroid disorders. The present work analyses soluble and membrane-bound pyrrolidon carboxypeptidase activities in the hypothalamus-pituitary-thyroid and hypothalamus-pituitary-ovary axes in N-methyl nitrosourea-induced breast cancer in rats. Our aim was to determine

the possible relationship between gonadotropin-releasing hormone and thyrotrophin-releasing hormone regulation through pyrrolidon carboxypeptidase activity. We propose that pyrrolidon carboxypeptidase activity dysregulation at various local and systemic levels may participate in the initiation, promotion and progression of breast cancer induced in rat by N-methyl nitrosourea through the increase in gonadotropin-releasing hormone. Since pyrrolidon carboxypeptidase activity also acts on thyrotrophin-releasing hormone, the dysregulation of this enzyme's activity could indirectly affect hypothalamus-pituitary-thyroid axis function, and thus potentially represent a link between the diseases of thyroid and breast cancer.

Key words

Pyroglutamyl aminopeptidase · Breast cancer · GnRH · TRH · Hypothalamus-pituitary-thyroid axis · Hypothalamus-pituitary-ovary axis

Introduction

Pyrrolidon carboxypeptidase (Pcp) is an omega-peptidase that hydrolyses N-terminal pyroglutamyl (pGlu) residues from biologically active peptides such as gonadotropin-releasing hormone (GnRH), thyrotrophin-releasing hormone (TRH) and arylamide derivatives in a highly selective manner [1,2]. To date, three

distinct forms of pyrrolidon carboxypeptidase have been observed in mammalian tissues. The first, Pcp type I (PcpI; E. C. 3. 4. 19. 3), is typically localized in the cytosolic compartment [3], and has a broad pyroglutamyl-substrate specificity. This enzyme has been shown to cleave the N-terminal pGlu residue from a range of biologically active peptides, which include TRH, Acid TRH, GnRH, neurotensin, bombesin and anorexigenic peptide in

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vitro [3]. Pcp type II (PcpII; E. C. 3. 4. 19. 6) has been shown to be a membrane-bound metalloenzyme which, in contrast to PcpI, displays a narrow substrate specificity restricted to the pGlu-His bond of TRH or very closely related peptides [4]. A third manifestation of Pcp activity known as thyroliberinase (E. C. 3. 4. 19.), has also been observed in the serum of various mammalian species, which displays biochemical characteristics remarkably similar to those of tissue PcpII with a narrow substrate specificity restricted to TRH or closely related peptides. Serum thyroliberinase has been established as the soluble form of PcpII [5].

Mammary tumours induced in rats by administration of chemical carcinogens such as N-methyl-nitrosourea (NMU) constitute useful tools for dissecting the multi step process of carcinogenesis, which involves initiation, promotion and progression [6].

In a previous report, we have described that serum Pcp activity is decreased in a well-established model on NMU-induced rat mammary breast cancer [7]. We have suggested that this decrease indicates the existence of high circulating levels of the peptide hormone GnRH. We have also described in human breast cancer 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 tumoral process in the affected tissue [8]. In this connection, 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]. These indicate that GnRH may be an important local intracrine, autocrine and/or paracrine hormonal factor in the pathogenesis of breast cancer, suggesting a role in the tumoral process.

However, the other susceptible substrate of Pcp, TRH, could be also modified in breast cancer. In fact, several studies have reported an association between breast cancer and thyroid disorders [10–15]. Silva et al. [16] speculated on the association between breast cancer and thyroid disorders, affirming that this might be related to oncogenes affecting these organs or to hormonal effects. Smyth et al. [17] affirmed that although they did not yet have a definitive explanation for the association between both diseases, their results still provide strong evidence for a biological link between breast carcinoma and thyroid disorders.

The aim of the present work was to analyse soluble (PcpI) and membrane-bound (PcpII) activities in the hypothalamus-pituitary-thyroid (HPT) and hypothalamus-pituitary-ovary (HPO) axes in NMU-induced rat breast cancer to determine the possible relationship between GnRH and TRH regulation through Pcp activity.

Materials and Methods

Animals and treatment

Forty female virgin Wistar rats (164.7 ± 4.7 g body weight) were used in this study. The animals were provided from the animal house-care of the University of Jaén and maintained in an environment controlled under constant temperature (25°C) with a 12 h light-dark cycle. All animals were allowed access to water and food *ad libitum*. The experimental procedures for animals use and care were in accordance with the 86/609/EEC European

Community Council directive. The rats were randomly divided into two groups. One group was injected intraperitoneally with three doses of 50 mg/kg body weight of NMU dissolved in distilled water (10 mg/ml) at 50, 80 and 110 days after birth. Tumours induced by this method are oestrogen-dependent as described by Rivera et al. [18]. All rats were in oestrus at the first NMU injection as verified by daily vaginal smears. The control group received vehicle only. For tumour detection and growth control, rats were examined by palpation 2 days each week after the second NMU injection. The following tumour growth parameters were determined: latency period (LP), as the number of days between the first NMU injection and the appearance of the first tumour; tumour incidence (TI), as the percentage of the rats that developed at least one tumour; and mean tumour number per rat (n/t), as the number of tumours per rat in animals developing at least one tumour.

Samples

Animals were sacrificed under equithesin anaesthesia (2 ml/kg body weight) 122 days after the first NMU injection. Samples from hypothalamus, anterior and posterior pituitary, thyroid, and ovary were quickly removed and frozen at -80°C until use. To obtain the soluble fraction, tissue samples were homogenized in 10 volumes of 10 mM HCl-Tris buffer (pH; 7.4) and ultracentrifuged at $100,000 \times g$ for 30 min at 4°C . The resulting supernatants were used to measure soluble enzymatic activity and protein content, assayed in triplicate. To solubilize membrane-bound proteins, the pellets were rehomogenized in HCl-Tris buffer (pH; 7.4) plus 1% Triton X-100. After centrifugation ($100,000 \times g$, 30 min, 4°C), the supernatants were used to measure solubilized membrane-bound activity and proteins, also in triplicate. To ensure complete recovery of activity, the detergent was removed from medium by adding to the samples adsorbent polymeric Biobeads SM-2 (100 mg/ml) (Bio-Rad, Richmond, CA) and shaking for 2 h at 4°C . Proteins were quantified using BSA as standard.

Pyrrolidone carboxypeptidase assay

Pcp activity was measured fluorometrically using pyroglutamyl- β -naphthylamide (pGLUNNap) as the substrate according to the method previously described by us [19]. Briefly, ten microlitres of each sample were incubated in triplicate for 30 minutes at 37°C with 100 μl of the substrate solution: 100 μM of pGLUNNap, 0.65 μM 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 fluorometrically at 412 nm emission wavelength at an excitation wavelength of 345 nm. Specific soluble and membrane-bound Pcp activities were expressed as nmol or pmol of pGluNNap hydrolysed per min per mg protein by using a standard curve of β -naphthylamine under corresponding assay conditions. The fluorogenic assay was linear with respect to time of hydrolysis and protein content.

Statistical analysis

We used unpaired Student's *t*-test to analyse the differences between control group and the animals with mammary tumours due to NMU injections. All comparisons with *p*-values below 0.05 were considered significant.

Results

Tumour growth parameters in rats after 122 days of the first NMU injection showed LP values at 113.0 ± 4.2 days (mean \pm SEM) between the first NMU injection and the appearance of the first tumour at 60% TI. Mean tumour number per rat was 1.93 ± 0.4 (mean \pm SEM).

Specific soluble and membrane-bound Pcp activities in hypothalamus, anterior and posterior pituitary, ovary and thyroid of control rats and rats with breast cancer induced by NMU are shown in Fig. 1. In the hypothalamus (Fig. 1a), soluble Pcp activity decreased significantly ($p < 0.001$) in rats with breast cancer, whereas no change was found in membrane-bound Pcp activity. In the same way, neither soluble nor membrane-bound Pcp activity was modified in pituitary from animals with breast cancer when compared with control animals (Fig. 1b and 1c). In the ovary, a significant increase ($p < 0.001$) was found in soluble Pcp activity in rats with breast cancer, although membrane-bound Pcp activity was not modified (Fig. 1d). Also, no changes were shown either in soluble or membrane-bound Pcp activities in the thyroid (Fig. 1e).

Discussion

Recent reports have refocused attention on the long debated question of possible link between diseases of the thyroid and breast. Smyth et al. [17,20], demonstrated a direct relationship between thyroid enlargement and breast carcinoma. An associa-

tion between autoimmune thyroid disease and breast carcinoma has also reported [21], but not confirmed [22].

Breast cancer is a hormone- and sex-dependent malignancy whose development is influenced by a myriad of hormones and growth factors [23–26]. Ovarian oestrogen has been demonstrated as essential in this disease [27]. GnRH is secreted from hypothalamus in a pulsatile fashion, and pulsatile GnRH stimulates LH and FSH biosynthesis and secretion from pituitary to act in the ovary [28]. On the other hand, TRH is secreted from hypothalamus to stimulate the release of TSH from pituitary, which acts in the thyroid by regulating thyroid hormone production [29]. Both hypothalamic releasing factors TRH and GnRH are susceptible substrates for Pcp [30]. The present work shows a decrease in hypothalamic soluble but not membrane-bound Pcp activity in rats with breast cancer induced by NMU. In the same way, no changes have been found either in soluble or membrane-bound Pcp activities in the pituitary. These results suggest the existence of increased levels of GnRH and/or TRH in hypothalamus without any changes at pituitary level. Furthermore, soluble but not membrane-bound Pcp activity is highly increased in the ovary, while no changes have been observed in the thyroid either soluble or membrane-bound fractions. Therefore, the increase in ovarian Pcp activity could be a response to high systemic circulating levels of GnRH as a mechanism of regulation to avoid GnRH overfunctioning at this local level. As thyroid Pcp activity did not change, GnRH rather than TRH may be the main substrate of Pcp in hypothalamus and ovary, or, at least, Pcp may mainly be involved in the local regulation of GnRH levels in rats with breast cancer induced by NMU. In this way, we have recently described

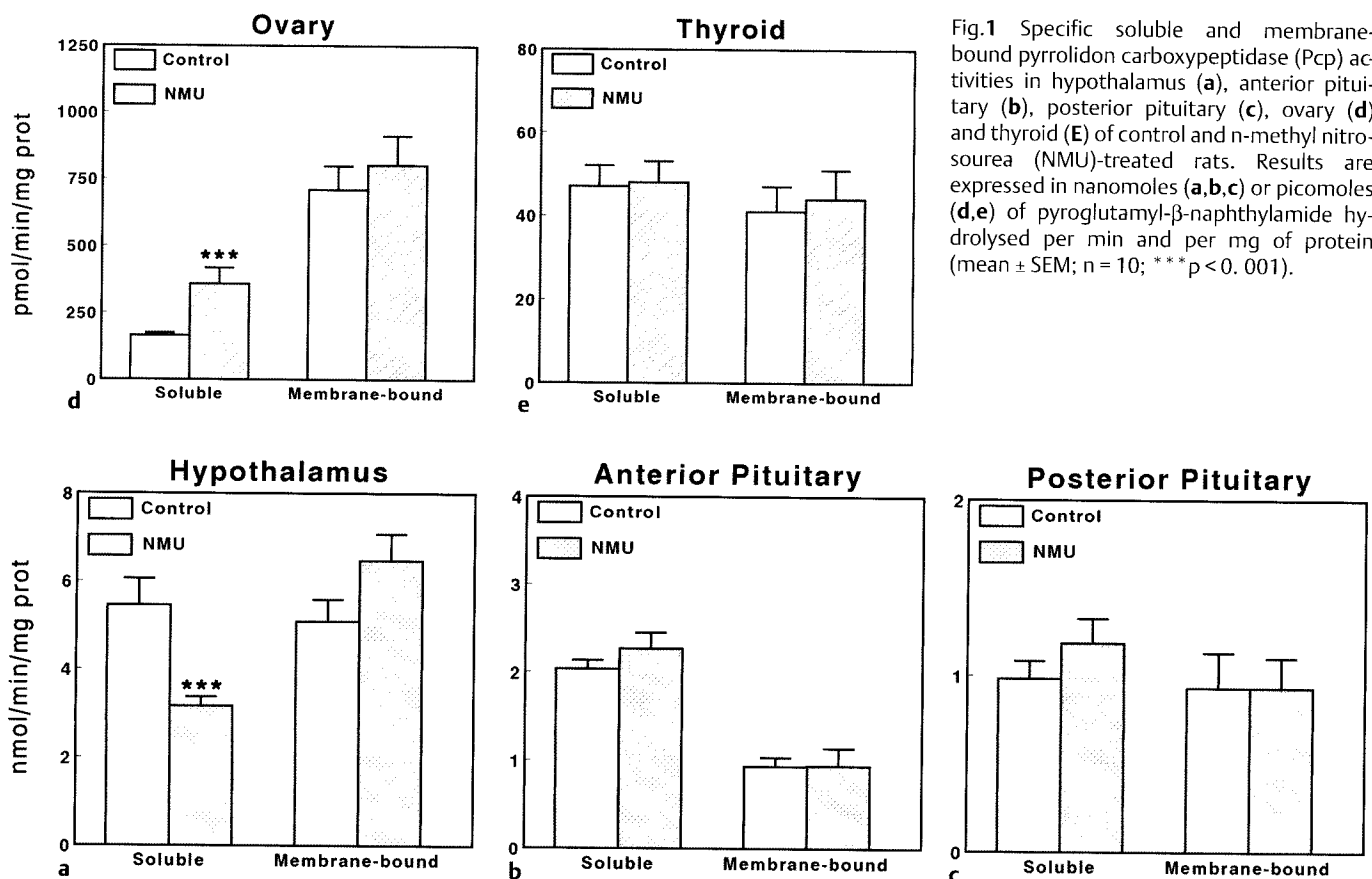


Fig.1 Specific soluble and membrane-bound pyrrolidon carboxypeptidase (Pcp) activities in hypothalamus (a), anterior pituitary (b), posterior pituitary (c) and thyroid (E) of control and n-methyl nitrosourea (NMU)-treated rats. Results are expressed in nanomoles (a,b,c) or picomoles (d,e) of pyroglutamyl- β -naphthylamide hydrolysed per min and per mg of protein (mean \pm SEM; n = 10; *** p < 0.001).

a decrease in serum Pcp activity in rats with NMU-induced breast cancer suggesting the existence of high circulating – systemic, not local – levels of GnRH [7]. However, we have also described a significant decrease in Pcp activity in neoplastic and adjacent tissues in human breast cancer compared to unaffected tissue, indicating again that local factors such as GnRH may be selectively modified by tumoral process in the affected tissue [8]. Therefore, GnRH may be an important local intracrine, autocrine and/or paracrine hormonal factor in the pathogenesis of breast cancer with an important role in the tumoral process. Moreover, increased GnRH levels also lead to increased gonadal steroid hormone levels [31], and NMU-induced carcinomas are mainly oestrogen-dependent. In any case, we must also take into account that the reduction in serum Pcp activity may lead to increase in TRH and, therefore, TSH levels. Higher serum TSH values may indicate susceptibility to future hypothyroidism [32]. The tendency towards thyroid hypofunction as evident in the skewed distribution of serum TSH values in breast carcinoma patients is consistent with previous reports showing an association between thyroid disease and breast carcinoma, in which hypothyroidism was the most frequently observed finding [33,34]. In fact, we have observed an important increase in body weight of rats with breast cancer induced by NMU concomitantly with the appearance of the tumours, although the body weight did return to control values after a few weeks, a possible consequence of “transient” hypothyroidism (data not shown). Therefore, the relationship between both releasing factor hormones and therefore the possible connection between thyroid disorders and breast cancer could be an indirect effect of local and systemic Pcp activities. Furthermore, it has been described as representing an important regulatory element controlling the stimulation of TRH target cells [35]. The TRH-R and GnRH-R are both members of the family of G-protein-coupled receptors, and both are coupled to G-proteins of the $G_{q/11}$ family [36,37]. Thus, the effects of TRH and GnRH appear to be mediated through the same or similar intracellular signal transduction pathways [38]. If TRH and GnRH share an identical signal transduction pathway for the stimulation of gene expression, the induction of gene expression by maximally effective concentrations of one hormone would be expected to prevent any further stimulation by the other.

To conclude, we propose that Pcp activity dysregulation at different local and systemic levels may participate in the initiation, promotion and progression of breast cancer induced in rat by NMU through the increase of GnRH. Since Pcp activity also acts on TRH, dysregulation of this enzyme's activity may indirectly affect HPT axis function, possibly representing a link between thyroid disease and breast cancer.

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