

Specific enkephalin-degrading aminopeptidase activity in the HPT and HPO axes of rats with breast cancer induced by *N*-methyl nitrosourea

María del Pilar Carrera^a, María Jesús Ramírez-Expósito^a, María Teresa Valenzuela^b,
María Jesús García^a, María Dolores Mayas^a, José Manuel Arias de Saavedra^c,
Rafael Sánchez^c, María del Carmen Pérez^d, José Manuel Martínez-Martos^{a,*}

^a*Facultad de Ciencias Experimentales y de la Salud, Departamento de Ciencias de la Salud, Área de Fisiología, Universidad de Jaén, Campus Universitario 'Las Lagunillas', E-23071, Jaén, Spain*

^b*Departamento de Radiología y Medicina Física, Unidad Mixta de Investigaciones Médicas, Universidad de Granada, Hospital Universitario San Cecilio, Granada, Spain*

^c*Sección de Bioquímica, Servicio de Análisis Clínicos, Hospital Universitario Médico Quirúrgico, Jaén, Spain*

^d*Sección de Inmunología, Servicio de Análisis Clínicos, Hospital Universitario Médico Quirúrgico, Jaén, Spain*

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Abstract

State and function of breast depend on an endocrinological balance, the upsetting of which can be a factor favorable to the development of cancer. Enkephalins (ENK) have been considered as a particular form of adaptation to defense to the organism against neoplastic processes. However, ENK may modify the endocrine functions of glands such as the ovary or the thyroid through the hypothalamus–pituitary axis, acting direct or indirectly as endocrine, paracrine or autocrine stimulatory growth factors. The present work analyses enkephalin-degrading tyrosyl aminopeptidase (EDA) activity in the hypothalamus–pituitary–thyroid (HPT) and hypothalamus–pituitary–ovary (HPO) axes in a rat model of breast cancer induced by *N*-methyl-nitrosourea (NMU) to state the relationship between ENK levels modification through EDA activity at different neuroendocrine levels and breast cancer. Results obtained show a decrease in EDA activity in hypothalamus, anterior and posterior pituitary, thyroid and ovary, suggesting increased levels of ENK in all these locations. These ENK may induce breast cancer cell growth and progression not only at breast level, but also acting at several neuroendocrine levels such as the HPT and HPO axes, inducing an unbalance of several other hormones, which could also facilitate the progression of cancer as an undesirable concomitant effect.

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1. Introduction

The breast is an organ subjected to different hormonal influences. State and function of breast depend, precisely, on an endocrinological balance, the upsetting of which can be a factor favorable to development of disease. It is well known that in the course of a neoplastic disease, many hormones are

released as a consequence of the “cellular stress”. Between them, enkephalins (ENK) have been considered humoral neurotransmitters, which act as antineoplastic defense mechanisms, as a particular form of adaptation to defense to the organism [1]. However, ENK themselves can influence breast cancer cell growth and progression, with the formation of metastases. Furthermore, ENK may modify the endocrine functions of glands such as the ovary, which induced steroid secretion [2], or the thyroid, which inhibits thyroid-hormones production [3]; and both gonads and thyroid hormones, under the control of the hypothalamus–

* Corresponding author. Tel: +34-953-212-600; fax: +34-953-212-141.

E-mail address: jmmartos@ujaen.es (J. M. Martínez-Martos).

pituitary axis, also influence breast cancer progression. Thus, ENK may therefore act, directly or indirectly, as endocrine, paracrine or autocrine stimulatory growth factors.

It has been assumed that ENK are hydrolyzed by specific enzymes, leading to their inactivation. To date, most information about ENK degradation has been described in brain tissue. Two enzymatic pathways are considered to be of great importance for the degradation of ENK [4]. These are the hydrolysis of the Gly-Phe bond by the membrane-bound enzyme neprilysin [5] and the breakdown of the Tyr-Gly bond by the enkephalin-degrading tyrosyl aminopeptidase (EDA) [6].

Mammary tumors induced in rats by administration of chemical carcinogens such as *N*-methyl-nitrosourea (NMU) constitute useful tools for dissecting the multistep process of carcinogenesis, which involves initiation, promotion and progression [7]. The aim of the present work is to analyse soluble and membrane-bound EDA activities in the hypothalamic–pituitary–thyroid (HPT) and hypothalamic–pituitary–ovary (HPO) axes to state the possible relationship between ENK levels modification through EDA activity at different neuroendocrine levels and breast cancer, using a rat model induced by NMU.

2. Materials and methods

2.1. 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/12 h-dark cycle. All animals were allowed to access to water and food ad libitum. The experimental procedures for animal use and care were in accordance with the European Community Council directive (86/609/EEC). The rats were randomly divided into two groups. One group were 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. Tumors induced by this method are oestrogen-dependent [8]. All rats were in estrus at the first NMU injection, verified by daily vaginal smears. Control group received the vehicle only. For tumor detection and growth control, rats were examined by palpation 2 days each week after the second NMU injection. The following tumor growth parameters were determined: latency period (LP), as the number of days between the first NMU injection and the appearance of the first tumor, with a value of 113.0 ± 4.2 days (Mean \pm S.E.M.); tumor incidence (TI), as the percentage of the rats that developed at least one tumor, with a value of 60%; and mean tumor number per rat (*n/t*), as the number of tumors per rat in animals developing at least one tumor, with a value of 1.93 ± 0.4 tumors (Mean \pm S.E.M.).

2.2. Samples

After 122 days of first NMU injection, animals were sacrificed under equithesin anaesthesia (2 ml/kg body weight). 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 the 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.

2.3. Enkephalin-degrading tyrosyl-aminopeptidase (EDA) activity assay

Specific EDA activity was measured fluorimetrically using Tyr- β -naphthylamide (TyrNNap) as the substrate. Ten microlitres of each sample were incubated in triplicate for 30 min at 37 °C with 100 microlitres of the substrate solution containing 100 μ M TyrNNap and 0.65 μ M dithiothreitol (DTT) in 50 mM of phosphate buffer, pH 7.4. All the reactions were stopped by adding 100 μ l 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 a 345 nm excitation wavelength. Specific soluble and membrane-bound EDA activities were expressed as nmol of TyrNNap hydrolyzed per minute and per mg of 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.

2.4. Statistical analysis

To analyse the differences between control group and the animals with mammary tumors induced by NMU injections, we used unpaired Student's *t*-test. All comparisons with *P* values below 0.05 were considered significant.

3. Results

Specific soluble and membrane-bound EDA activities in hypothalamus, anterior and posterior pituitary, thyroid and

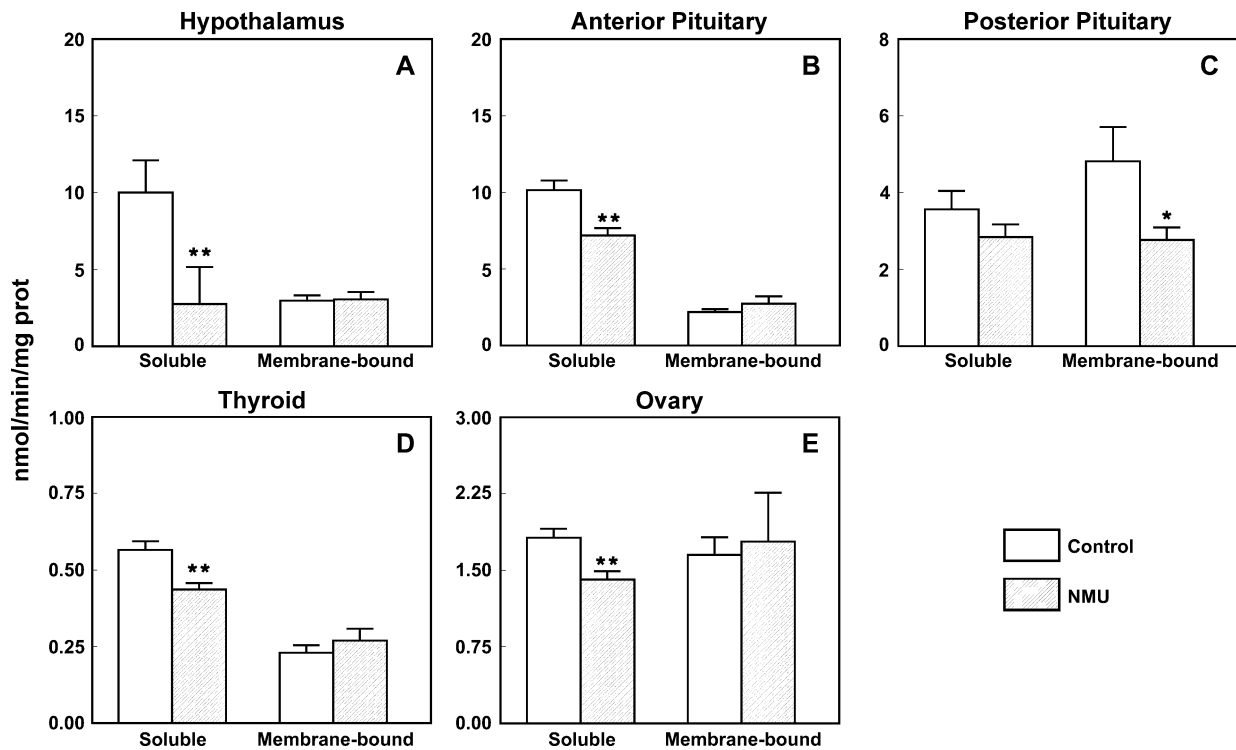


Fig. 1. Specific soluble and membrane-bound enkephalin-degrading tyrosyl aminopeptidase (EDA) activities in hypothalamus (A), anterior pituitary (B), posterior pituitary (C), thyroid (D) and ovary (E) of control and *N*-methyl-nitrosourea (NMU)-treated rats. Results are expressed in nanomoles of tyrosyl- β -naphthylamide hydrolyzed per min and per mg of protein (Mean \pm S.E.M.; $n=10$; * $P<0.05$; ** $P<0.01$).

ovary of control rats and rats with breast cancer induced by NMU are shown in Fig. 1. In the hypothalamus (Fig. 1A), soluble EDA activity decreased significantly ($P<0.01$) in rats with breast cancer, whereas no change was found in membrane-bound EDA activity. In the same way, soluble EDA activity decreased significantly ($P<0.01$) in the anterior pituitary of rats with breast cancer but membrane-bound EDA did not change. On the contrary, soluble EDA activity did not change in posterior pituitary (Fig. 1B) but membrane-bound EDA decreased significantly ($P<0.05$; Fig. 1C). In the thyroid and the ovary, a significant decrease ($P<0.01$) were found in soluble EDA activity in rats with breast cancer, although membrane-bound EDA activity were not modified (Fig. 1D and E).

4. Discussion

ENK are ubiquitous peptide hormones located with varying densities throughout the central, peripheral, and autonomic nervous systems as well as in several endocrine tissues and target organs. This widespread distribution is consistent with their involvement in a broad range of functions and behaviors, including regulation of pain, reinforcement and reward, release of neurotransmitters and autonomic and neuroendocrine modulation at different levels [9–16].

The results presented here show a decrease in EDA activity in hypothalamus, anterior and posterior pituitary,

thyroid and ovary, suggesting increased levels of ENK in all these locations. These results agree with previous reports indicating that ENK are released as a mechanism of defense of the organism against the neoplastic processes [1,17]. In this way, it has been demonstrated that steroid hormones increase the expression of the ENK precursor preproenkephalin mRNA in the posterodorsal part of the ventromedial hypothalamus, the bed nucleus of the stria terminalis and the medial preoptic nucleus [18]. We have recently described a decrease in serum pyrrolidone carboxypeptidase activity in rats with NMU-induced breast cancer, suggesting the existence of high circulating levels of GnRH which lead to increased levels of gonadal steroid hormones [19]. These high levels of gonadal steroid hormones could lead to increased expression of ENK precursor not only in hypothalamus, but also in other target organs such as the pituitary, the thyroid or the ovary. In any case, the increase of ENK through the inhibition of their degrading enzyme could be induced by the high levels of gonadal steroid hormones. However, little is known about the influence of steroid hormones on peptidase activities. We had evaluated the effect of gonadectomy and the *in vitro* response to the presence in the medium of steroid hormones on several peptide-regulating peptidases [20,21]. Aminopeptidase N and aminopeptidase B activities were measured in sera from male, female, orchidectomized and ovariectomized mice. Our results demonstrated highly significant sex differences, and an influence of steroid hormones on peptidase activity. Depending on the nature of the peptidase, these enzymes

responded in different ways to the presence of these substances.

Furthermore, in other experiments we have observed a significant increase in mouse hypothalamus and pituitary EDA activity after ovariectomy, which return to control levels after estradiol and/or progesterone administration (data not shown), supporting again an influence of steroid hormones on EDA activity at different locations. The results presented here also suggest an increase in ENK levels in the ovary, which could also be responsible of an overproduction of gonadal steroid hormones [2]. 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 [22]. The tendency towards thyroid hypofunction, 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 [23]. 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 tumors, although the body weight return to control values after a few weeks, which could be the consequence of a “transient” hypothyroidism [24]. Our results also suggest an increase in ENK in thyroid, and it has been demonstrated that ENK induce thyroid hypofunction, supporting also the former observations.

To conclude, although the increase in ENK levels found in breast cancer could be considered as a particular form of adaptation of the organism, creating defense mechanisms against breast cancer, the induction and growth of breast tumors appear to be related to the endocrine status of the host, not only at breast level, where ENK may act influencing breast cancer cell growth and progression, but also at several neuroendocrine levels such as the HPT and HPO axes, inducing an unbalance of several hormones which could also facilitate the progression of cancer as an undesirable concomitant effect.

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