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Serum oxytocinase activity is related to tumor growth parameters in N-methyl nitrosourea induced rat breast cancer

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Abstract

Oxytocinase has been reported to hydrolyse the peptide hormone oxytocin (OT). We have previously described changes in oxytocinase activity in human breast cancer, where a highly significant increase occurred in tumoral tissue. In the present work, we analysed oxytocinase activity in serum of rats with breast cancer induced by N-methyl-nitrosourea (NMU). We also correlated these data with the number and size of tumors and the body weight of the animals to evaluate the putative value of this activity as a biological marker of the disease. Our results confirm the involvement of OT in carcinogenesis and suggest a mayor role for oxytocinase activity in the development of breast cancer.
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Keywords: Oxytocin; N-methyl-nitrosourea; Mammary gland; Tumor growth parameters; Oxytocinase

Introduction

Tumors induced in rats by administration of N-methyl-nitrosourea (NMU) constitute a useful tool for dissecting the initiation, promotion and progression processes of carcinogenesis (Russo and Russo, 2000). NMU-induced mammary tumors are estrogen-dependent, aggressive and

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locally invasive, in a similar degree to that observed in the human disease (Gullino et al., 1975; Mc Cormick et al., 1981; Welsch, 1985; Russo et al., 1990; Thompson and Adlaka, 1991).

Little information is available about the role of aminopeptidases on breast cancer, although these enzymes have been implicated in the metabolism of several peptide hormones with important autocrine/paracrine functions. Thus, changes in aminopeptidase activities may reflect the local functional status of their substrates, which can be selectively activated or inhibited in the affected tissue as a result of specific conditions brought about by the tumour. Oxytocinase activity, also called cystyl aminopeptidase or placental leucyl aminopeptidase (EC 3.4.11.3) has been reported to hydrolyse the peptide hormone oxytocin (OT) (Tsujimoto et al., 1992; Mizutani et al., 1995). In a previous work, we have described changes in oxytocinase activity in human breast cancer, where a highly significant increase occurred in tumoral tissue (Martínez et al., 1999). In the present work, we analyse oxytocinase activity in serum of NMU-induced rat mammary tumors and correlate these data with the number and size of tumors and the body weight of the animals to evaluate the putative value of this activity as a biological marker of the disease. Our results confirm the involvement of OT in carcinogenesis and suggest a mayor role for oxytocinase activity in the development of breast cancer.

Materials and methods

Animals and treatment

Forty female virgin Wistar rats (164.7 ± 4.7 g body weight) were used in this work. 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 12hr-light / 12hr-dark cycle. All animals were allowed to access to water and food ad libitum. The experimental procedures for animals 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, as described by Rivera et al. (1994). Tumors induced by this method are oestrogen-dependent. All rats were at 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 number of tumors were recorded, and the major and minor diameters of each tumor was measured with a caliper. Body weight was determinated periodically every week. Other tumor growth parameters were also determined: latency period, defined as the days between the first NMU injection and the appearance of the first tumor; tumor incidence, defined as the percentage of the rats that developed at least one tumour, and mean tumor number per rat (n/t), defined as the number of tumor per rat in animals developing at least one tumour. After 122 days of the first NMU injection, animals were sacrificed under equithensin anaesthesia (2 ml/kg body weight). Blood samples were obtained through the left cardiac ventricle and centrifuged ten minutes at 3000g to obtain the serum. These samples were frozen and stored at −80°C, until use.

Table 1
Values of experimental and calculated oxytocinase activity (pmol/min/mg protein) from the multiple regression analysis

Rat #	Oxytocinase (experimental values)	Oxytocinase (calculated values)	Variation (%)[a]
1	112.23	106.685	5.19
2	148.26	133.514	11.04
3	131.58	129.46	1.63
4	163.76	157.723	3.82
5	123.018	129.813	− 5.23
6	105.56	103.918	1.58
7	108.102	115.787	− 6.63
8	136.37	145.769	− 6.44
9	125.083	131.319	− 4.74

[a] Variation (%) = (Oxytocinase experimental − Oxytocinase calculated)/(Oxytocinase calculated) × 100.

Oxytocinase activity assay

Serum oxytocinase activity was measured fluorimetrically using Cys-β-naphthylamide (CysNNap) as the substrate, according to the method previously described by us (Martínez et al., 1999). Briefly, ten microlitres of each sample were incubated by triplicate 30min at 37°C with 100 microlitres of the substrate solution: 100 μM CysNNap and 0.65μM dithiothreitol (DTT) in 50 mM of phosphate buffer, pH 6.0. All the reactions were stopped by adding 100 μl 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 an 345 nm excitation wavelength. Triplicated protein samples were quantified also by the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

Statistical Analysis

To analyse the differences between control group and the animals with mammary tumors induced by NMU injections, we used unpaired Student-t-Test. All comparisons with *P* values below 0.05 were considered significant.

The tumor number, the size of each tumor (major diameter x minor diameter) and the body weight were used to explain the changes of the dependent variable oxytocinase activity. A stepwise regression was carried out to select the best independent variables, following as criterion a minimum value for the

Table 2
Multiple regression model for oxytocinase activity[a]

Variable	Regression coefficient	Standard error	Contribution to R-SQ
Constant	221.392	45.65	
Tumor size	2.618	0.902	0.144
Tumors number	− 8.246	1.93	0.491
Body weight	− 0.321	0.16	0.125

[a] n = 9; Mallows'C*p* = 4.0; Mean absolute error: 6.69; R = 0.9068; R^2 = 0.8224; *F* (3,5)= 7.72; *P* = 0.0253.

Table 3
Correlation matrix for the variables used

Constant	1			
Tumor size	0.2559	1		
Tumor number	− 0.3381	− 0.1684	1	
Body weight	− 0.9844	− 0.3732	0.2511	1

Mallows'C*p*. Then, with the 9R program in the BMDP statistical package, the best regression was selected. The data and statistics obtained are given in Tables 1–3.

Results

Tumor growth parameters in rats showed a latency period (Mean ± SEM) of 113.0 ± 4.2 days between the first NMU injection and the appearance of the first tumour, with a 60% of tumor incidence. Fig. 1 shows body weight of control and NMU-treated rats during the whole experiment. A significant increase ($P < 0.01$) was found in NMU-treated rats concomitantly with the appearance of tumours. Body weight decreased after this time when compared with control rats. Fig. 2 shows mean tumor number per rat, major and minor diameters of tumors (Mean ± SEM) from the appearance of the first tumor to the end of the experiment. Specific oxytocinase activity in serum of controls and NMU-treated rats is shown in Fig. 3. Serum oxytocinase activity was significantly increased ($P < 0.05$) by 45% in NMU-treated rats when compared with control group.

Multiple regression analysis statistics are given in Tables 1–3. Table 1 shows the observed values versus the predicted values for the dependent variable oxytocinase activity. It can be used to detect cases

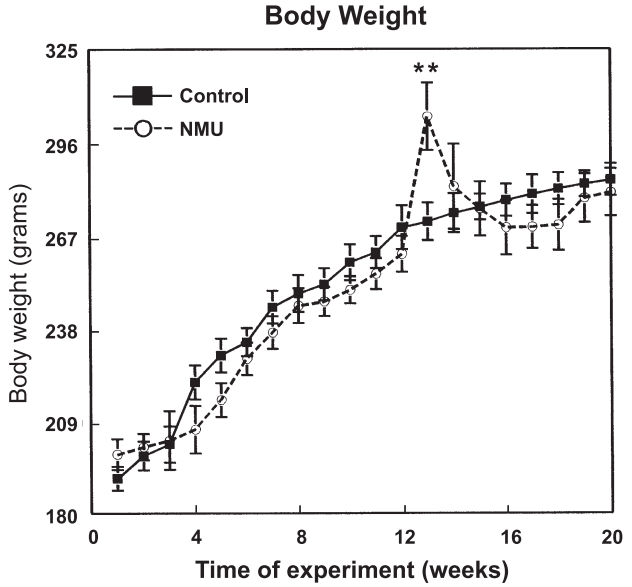

Fig. 1. Body weight of control (black square) and NMU-treated (white dot) rats during the time of the experiment (in weeks). Values are expressed in grams (Mean ± SEM; n = 9; $^{**}P < 0.01$).

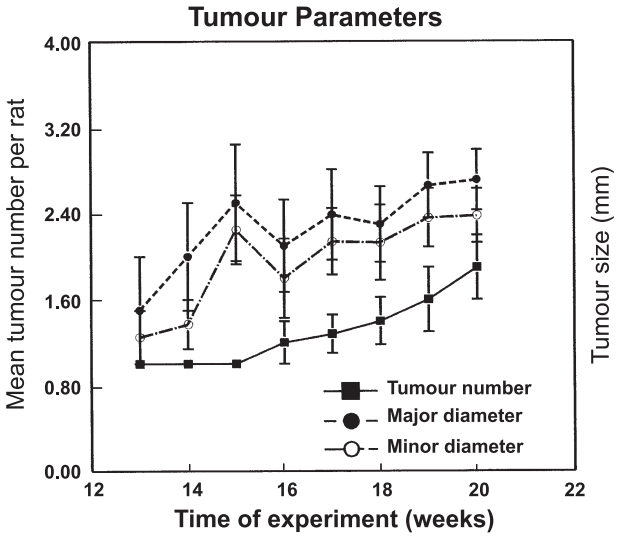

Fig. 2. Tumor growth parameters (mean tumor number per rat (black square), major diameter (black dot) and minor diameter (white dot) of NMU-treated rats from the appearance of the first tumor to the end of the experiment (in weeks).

in which the variance is not constant. Table 2 shows the results of fitting a multiple linear regression model to describe the relationship between oxytocinase activity and three independent variables. The equation of the fitted model is oxytocinase activity = 2.61805 × Tumor size − 8.24617 × Tumor number − 0.321669 × Body weight + 221.392. Since the *P* value in the ANOVA table is less than 0.05, there is a statistically significant relationship between the variables at the 95% confidence level. The R-Squared statistic indicates that this model explains 82.24% of the variability in oxytocinase activity. The mean absolute error of 6.69 is the average value of the residuals. Table 3 shows estimated

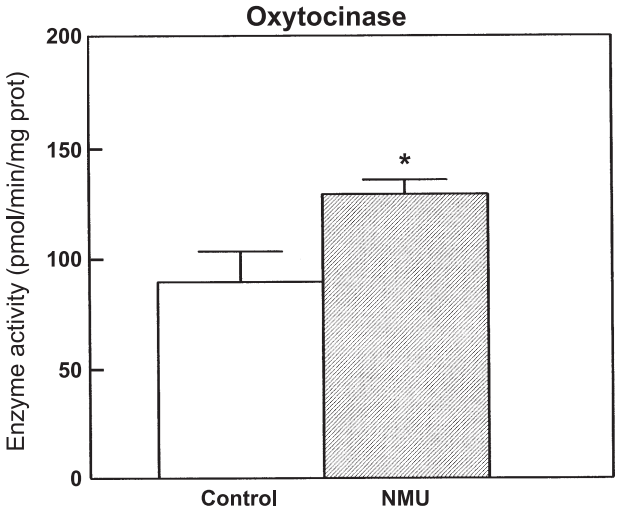

Fig. 3. Oxytocinase activity in serum of control and NMU-treated rats. Result are expressed in picomoles of cystyl-β-naphthylamide hydrolysed per min and per mg of protein (Mean ± SEM; n = 9; **P* < 0.05).

correlations between the coefficients in the fitted model. These correlations can be used to detect the presence of serious multicollinearity, i.e., correlation amongst the predictor variables. Fig. 4A shows the relationship between the experimental and expected values got using the regression equation. This plot allows to detect cases in which the variance is not constant. Fig. 4B shows the graph between the

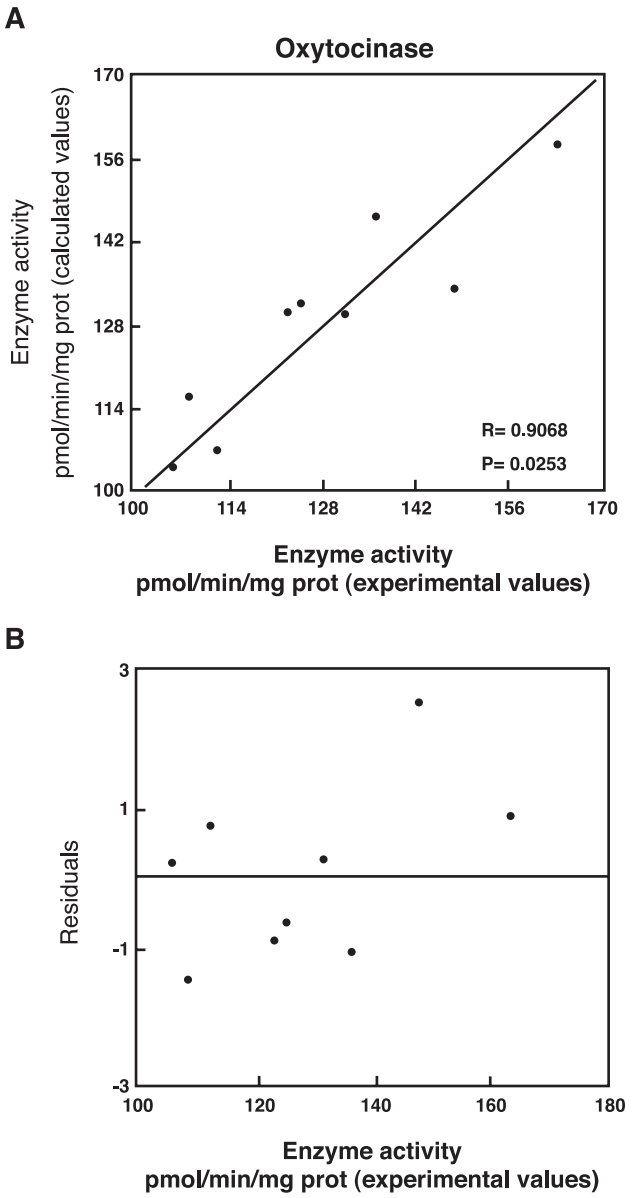

Fig. 4. A) Plot of the experimental vs. expected values of oxytocinase activity, showing the linear model that describes the relationship between the variables; B) Plot of residuals vs. experimental values of oxytocinase activity, showing that the model adequately describe the observed data.

residuals vs. experimental oxytocinase activity values. This plot helps to identify sequential correlations among the residuals.

Discussion

Although oxytocinase activity was firstly described in human placenta, several immunohistochemical studies have shown a wide distribution of this enzyme in a variety of sites other than placental, including vascular endothelium, gastrointestinal mucosa, pancreas, bile duct, bronchial epithelium, renal tubules, sweat glands, adipocytes and skeletal muscle (Nagasaka et al., 1997). These studies have suggested possible involvement of oxytocinase not only in pregnancy, but also in many other physiological processes. Oxytocinase activity is particularly interesting in breast cancer due to its role in the hydrolysis of OT. OT has been reported to be common in cells of healthy breast tissue but are rarely or never detected in breast cancer (North et al., 1995). In addition, OT inhibits proliferation of human breast cancer cell lines (Cassoni et al., 1994) and thus may play a role in preventing this disease (Murrell, 1995). In vivo, OT significantly reduced the growth of mouse mammary carcinoma TS/A (Sapino et al., 1998).

OT acts as growth regulator through the activation of specific G-couple receptors (OTR) distributed on cell surface (Sapino et al., 1998; Cassoni et al., 2001). In contrast to other hormone receptors systems, in which biological responses are modulated by changing hormone concentrations, the OT/OTR system is largely regulated by changes in OTR expression, although up to date, the regulators of OTR levels in the mammary gland are not known (Copland et al., 1998). Using immunohistochemistry and RT-PCR, OTR and OTR mRNA have been detected in normal and pathological breast tissue (Sapino et al., 1998). In the human breast, OTR have been detected in myoepithelial cells along normal lobules and in intraductal cells in benign hyperplasic lesions. OTR have also been described in cases of primary and metastatic carcinomas of the breast (Bussolati et al., 1996), but it is not clear whether the receptors are functional. Copland and coworkers (Copland et al, 1998) have demonstrated that OTR are functional in human breast Hs578T cells.

In the present report, our results show a significant increase of oxytocinase activity in the serum of rats with mammary tumors induced by NMU. The levels of activity in this model correlate with the number and size of the tumors and, in a lesser extend, with body weight of the animals, as demonstrated by the changes observed concomitantly with the appearance of tumours. It has been described that oxytocin may both increase and decrease body weight depending on the way and time after its administration. This influence in body weight depends on factors such as the endocrine profile and oxytocin sensitivity. Our results suggest a decreased level of oxytocin which could be responsible of the sudden increase of body weight concomitantly with the appearance of tumours. Probably, compensatory mechanisms led to an unchanged final body weight when compared with control rats. However, it is not known by which mechanisms oxytocin modifies body weight (Argiolas and Gessa, 1990). The lower availability of OT suggested by our results can be also responsible of the increase in the OTR number in breast cancer.

We have also recently described the decrease of pyrrolidon carboxypeptidase activity in serum of rats with mammary tumors induced by NMU (Carrera et al., 2003) which point out high circulating levels of its substrate gonadotropin releasing hormone (GnRH). Increased levels of GnRH lead to increased levels of gonadal steroid hormones (Huirne and Lambalk, 2001). However, Sapino and coworkers (Sapino et al., 1998) have reported the lack of correlation between OTR presence in human breast carcinomas and

the estrogen receptor status, however, progesterone and OTR expression correlated. Recent studies have also showed that both the human and the rat OTR interact with progesterone. The interactions steroid/receptor are modulated by GTPγS, indicating that there is a direct steroid / OTR interaction (Grazzini et al., 1998).

To conclude, although the regulation of OT-OTR-oxytocinase system is not well known yet, our results suggest that changes in oxytocinase activity might play an important role in the origin and evolution of breast cancer. Furthermore, serum oxytocinase activity could be a useful serum marker to rapidly predict the sensitivity of a tumor to a therapy, the maintenance or remission or an eventual occult disease, which might permit a better monitoring of cancer and a rapid selection of more effective therapeutic/experimental means.

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