Insulin-regulated Aminopeptidase/Placental Leucil Aminopeptidase (IRAP/P-LAP) and Angiotensin IV-forming Activities are Modified in Serum of Rats with Breast Cancer Induced by *N*-methyl-nitrosourea

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Abstract. *Background: In previous reports, changes in oxytocinase activity in human breast cancer tissue and in the serum of N-methyl-nitrosourea (NMU)-induced rat mammary tumors were described. Insulin-regulated aminopeptidase (IRAP) has been identified with oxytocinase and has also been referred to as placental leucine aminopeptidase (P-LAP). Materials and Methods: The IRAP/P-LAP activity in rat serum was assayed to analyze the putative role that IRAP/P-LAP may play in regulating mammary gland carcinogenesis induced by NMU. Furthermore, as it has been recently described that IRAP/P-LAP is the angiotensin IV (Ang IV) receptor AT_4, the activities of Ang IV-forming aminopeptidase N (APN) and aminopeptidase B (APB) were also assayed. Results: Changes in serum IRAP/P-LAP and Ang IV-forming APB activities were found in rats with mammary tumors induced by NMU. Both activities were greatly increased, although the Ang IV-forming APN activity was not modified. Conclusion: These 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 tumor. Thus, these enzymatic activities may be involved in the promotion and progression of breast cancer through oxytocin (OT), vasopressin (AVP) and/or renin-angiotensin system (RAS) misregulation.*

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Little information is available regarding the role of aminopeptidases in 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 tumor. In a previous report, we described changes in oxytocinase activity in human breast cancer, where a highly significant increase occurred in tumoral tissue (1). The oxytocinase activity in the serum of *N*-methyl-nitrosourea (NMU)-induced rat mammary tumors was also analyzed. This enzyme activity correlated with the number and size of tumors and the body weight of the animals, confirming the involvement of oxytocin (OT) in carcinogenesis and suggesting a major role for oxytocinase activity in the development of breast cancer (2). In both reports, cystyl-β-naphthylamide was used as the artificial substrate to assay oxytocinase activity. However, insulin-regulated aminopeptidase (IRAP; E.C: 3.4.11.1), a cell surface aminopeptidase, was identified with oxytocinase (3) and has also been referred to as placental leucine aminopeptidase (P-LAP) (4). This enzyme activity is usually assayed using leucyl-β-naphthylamide as an artificial substrate. The aim of the present report was to assay IRAP/P-LAP activity in rat serum in order to analyze the putative role that IRAP/P-LAP

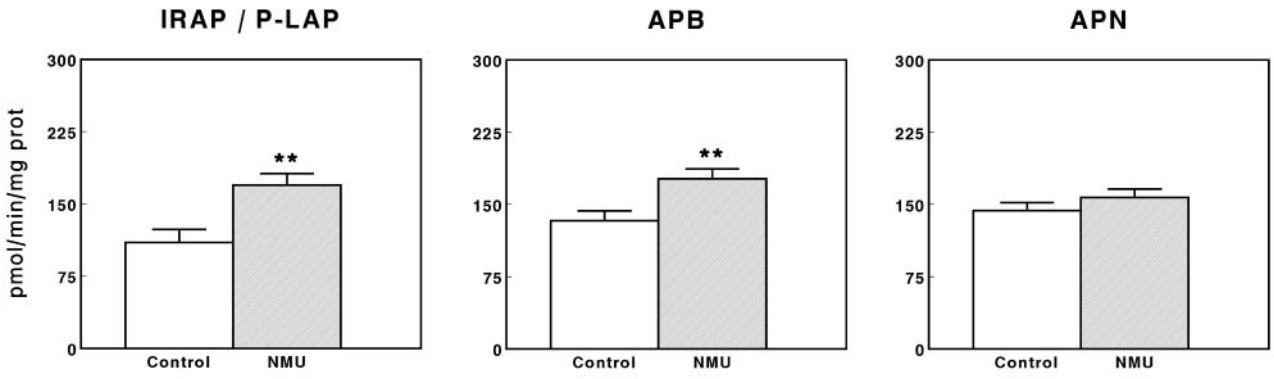

Figure 1. *Insulin-regulated aminopeptidase (IRAP/P-LAP), aminopeptidase B (APB), and aminopeptidase N (APN) activities in the sera of control and NMU- treated rats. The results are expressed in picomoles of leucyl-, arginyl- or alanyl- β-naphthylamide hydrolyzed per min and per mg of protein (mean±SEM; n= 9; **p<0.01).*

may play in regulating mammary gland carcinogenesis induced by NMU. Furthermore, as it has recently been described that IRAP/P-LAP is the angiotensin IV (Ang IV) receptor AT_4 (5), the activities of Ang IV-forming aminopeptidase N (APN; E.C: 3.4.11.2) and aminopeptidase B (APB; E.C: 3.4.11.6) were also assayed.

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 facility of the University of Jaén, Spain, and maintained in an environment controlled under constant temperature (25˚C) with a 12-h light / 12-h dark cycle. All animals were allowed 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 2 groups. One group was injected intraperitoneally with 3 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 previously described (2). Tumors induced by this method are estrogen-dependent (6). All rats were at estrus at the first NMU injection, as verified by daily vaginal smears. The control group received the vehicle only. For tumor detection and growth control, the rats were examined by palpation 2 days each week after the second NMU injection. The number of tumors was recorded and the major and minor diameters of each tumor were measured with a caliper. The body weight was measured 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 tumor, and mean tumor number per rat (n/t), defined as the number of tumors per rat in animals developing at least one tumor. One day 122 after the first NMU injection, the animals were sacrificed under equithensin anesthesia (2 ml/kg body weight). The blood samples were obtained through the left cardiac ventricle and centrifuged for 10 min at 3000 xg to obtain the serum. These samples were frozen and stored at –80˚C, until use.

Enzyme activity assay. Serum IRAP/P-LAP, aminopeptidase N (APN) and aminopeptidase B (APB) were measured fluorometrically using leucyl-β-naphthylamide (LeuNNap), alanyl-β-naphtylamide (AlaNNap) and arginyl-β-naphtylamide (ArgNNap) as substrates. Ten microliters of each sample were incubated in triplicate for 30 min at 37˚C with 100 μL of the substrate solution containing 100 μM of LeuNNap, AlaNNap or ArgNNap and 0.65 mM dithiothreitol (DTT) in 50 mM phosphate buffer, pH 7.4.

All the reactions were stopped by adding 100 μl of 0.1M 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 excitation wavelength of 345 nm. The proteins were also quantified in triplicate by the method of Bradford, using bovine serum albumin (BSA) as standard.

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

Results

The tumor growth parameters in the rats showed a latency period (mean±SEM) of 113.0±4.2 days between the first NMU injection and the appearance of the first tumor, with 60% tumor incidence. The mean tumor number per rat (mean±SEM) was 1.93±0.4 tumors. The specific IRAP/P-LAP, APN and APB activities in the sera of the controls and NMU-treated rats are shown in Figure 1. The serum IRAP/P-LAP and APB activities significantly increased ($p<0.01$) by 54.4% and 32.7%, respectively, in NMU-treated rats compared with the control group, whereas serum APN activity did not show significant differences between these 2 groups.

Discussion

The present work reports changes in serum IRAP/P-LAP and Ang IV-forming APB activities in rats with mammary tumors induced by NMU. Both activities were greatly increased, although the Ang IV-forming APN activity was not modified.

IRAP/P-LAP is a type II membrane-spanning protein. Upon the cloning of human oxytocinase/P-LAP, it became evident that IRAP and P-LAP were the rat and human homologs of the same protein (4). IRAP/P-LAP 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 is rarely or never detected in breast cancer (7). In addition, OT inhibits the proliferation of human breast cancer cell lines (8) and, thus, may play a role in preventing this disease (9). *In vivo*, OT significantly reduced the growth of mouse mammary carcinoma TS/A (10).

OT acts as growth regulator through the activation of specific G-couple receptors (OTR) distributed on the cell surface (10, 11). In contrast to other hormone receptor systems, in which biological responses are modulated by changing hormone concentrations, the OT/OTR system is largely regulated by changes in OTR expression, although, to date, the regulators of OTR levels in the mammary gland are not known (12). Using immunohistochemistry and RT-PCR, OTR and OTR mRNA were detected in normal and pathological breast tissue (10). In the human breast, OTR were detected in myoepithelial cells along normal lobules and in intraductal cells in benign hyperplasic lesions. OTR were also described in cases of primary and metastatic carcinomas of the breast (13), but it is not clear whether the receptors were functional. Copland and coworkers (12) demonstrated that OTR were functional in human breast Hs578T cells. We previously described a significant increase of oxytocinase activity in the serum of rats with mammary tumors induced by NMU (2). The levels of activity in this model correlated with the number and size of the tumors and, to a lesser extent, with the body weight of the animals, as demonstrated by the changes observed concomitantly with the appearance of tumors. The lower availability of OT suggested by our results may also be responsible for the increase in the OTR number described by others in breast cancer.

To our knowledge, there are no reports linking IRAP and breast cancer. However, altered P-LAP levels have been implicated in certain pathological conditions (14) and changes in P-LAP have been used for several clinical assays (15). Gupta and coworkers (16) studied serum P-LAP levels in patients with breast tumor and found an increase with clinical staging. Furthermore, serum P-LAP values were correlated with the histological subtypes of breast carcinoma, suggesting serum P-LAP as a sensitive prognostic indicator of invasiveness in breast carcinoma. IRAP/P-LAP is the only aminopeptidase known to cleave vasopressin (AVP) (17). It has been described that all breast cancers express the AVP gene, which leads to the production of both normal and abnormal forms of tumor AVP mRNA and proteins. Breast cancer cells also express normal genes for all AVP receptors. Through these receptors, AVP exercises multifaceted effects on tumor growth and metabolism. It has been proposed that biologically active peptide production by tumors is an important part of a special process of oncogenic transformation, rather that a pre-existing condition of progenitor cells; this concept has been called selective tumor gene expression of peptides essential for survival (STEPS) (18). The increase in serum IRAP/P-LAP activity in NMU-treated rats could also be an indicator of a misregulation of AVP functions.

On the other hand, our finding of an increased APB activity suggests that the metabolism of Ang III to Ang IV is rapid, indicating an increase in circulating levels of Ang IV. The action of Ang IV is mediated by the AT_4 receptor (5). Albiston and coworkers have recently identified this receptor as the IRAP/P-LAP and have also shown that the AT_4 receptor ligands dose-dependently inhibit the catalytic activity of IRAP/P-LAP (19). Although our results did not agree with an inhibitory effect of Ang IV on IRAP/P-LAP activity, we can conclude that both IRAP/P-LAP and Ang IV-forming activities are involved in this animal model of breast pathogenesis through unknown mechanisms. Although to our knowledge no authors have commented on the role of Ang IV in breast cancer, it has been described that Ang IV (and to a lesser extent angiotensin II, Ang II) stimulates the activity of tyrosine kinases and, therefore, cell proliferation in estrogen-induced rat pituitary tumors (20).

Our results indicated that APN activity in the sera from controls and NMU-treated rats did not differ. However, soluble APN activity was reported to be highly increased in neoplastic tissue (1). Furthermore, Severini and coworkers (21) also described an increase in serum APN activity in various disorders including breast cancer. It has been reported that the biological function of APN varied depending on the tissue microenvironment (22) and might be responsible for the differences observed. It has been suggested that APN plays an important role in the progression of tumor vasculogenesis and it has been identified as a critical regulator of angiogenesis (23).

To conclude, the differences found between control and NMU-treated rats regarding IRAP/P-LAP and APB activities suggest that these enzymatic activities may be involved in the promotion and progression of breast cancer through OT, AVP and/or renin-angiotensin system

misregulation. Furthermore, these activities could be useful serum markers for the rapid prediction of the sensitivity of a tumor to therapy, the maintenance or remission or an eventual occult disease, thereby permiting a better monitoring of cancer and a rapid selection of effective therapeutic/experimental means.

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