

## Glutamyl- but Not Aspartyl- Aminopeptidase Activity is Modified in Serum of N-Methyl Nitrosourea-induced Rat Mammary Tumours

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**Abstract.** *Background: The rat model of breast cancer induced by the administration of N-methyl-nitrosourea (NMU) constitutes a useful tool for dissecting the initiation, promotion and progression process of carcinogenesis. Angiogenesis, the recruitment of new blood vessels, is an essential component of the metastatic pathway. Tumour vessels have an aberrant response to constrictor hormones, such as angiotensin II (Ang II). Ang II degradation to form angiotensin III (Ang III) begins with the action of glutamyl aminopeptidase (GluAP) and aspartyl aminopeptidase (AspAP), named together as aminopeptidase A activity (APA). The present work analyses GluAP and AspAP activities in serum of NMU-induced rat mammary tumours, to evaluate the putative value of these activities as biological markers of the initiation and promotion of the disease. Materials and Methods: Serum AspAP and GluAP activities were measured fluorimetrically using their corresponding aminoacyl- $\beta$ -naphthylamide. Results: The increase found in GluAP but not in AspAP suggests an increase in Ang III and a decrease in Ang II serum circulating levels. Conclusion: The decrease in Ang II may be responsible for the overexpression of AT<sub>1</sub> receptors described in breast cancer. However, increased levels of Ang III, which exhibit the same affinity for the AT<sub>1</sub> receptor, would favour the development of the disease.*

The rat model of breast cancer induced by the administration of N-methyl-nitrosourea (NMU) constitutes

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a useful tool for dissecting the initiation, promotion and progression process of carcinogenesis (1). NMU-induced mammary tumours are estrogen-dependent, aggressive and locally invasive, to a similar degree as that observed in the human disease (2-6).

Angiogenesis, the recruitment of new blood vessels, is an essential component of the metastatic pathway. These vessels provide the principal route by which tumour cells exit the primary tumour site and enter the circulation (7). Tumour vessels have an aberrant response to constrictor hormones, such as angiotensins and endothelins. Angiotensin peptides, besides regulating vascular tone and natriohydric balance through the renin-angiotensin system (RAS), are involved in the control of cell growth and vascular permeability (8). In fact, angiotensin II (Ang II) stimulates angiogenesis and tumour growth (9-13). Ang II degradation begins with the action of glutamyl aminopeptidase (GluAP; E.C: 3.4.11.7) and aspartyl aminopeptidase (AspAP; E.C: 3.4.11.21). These enzyme activities, classically named together as aminopeptidase A activity (APA), remove the N-terminal Asp to produce angiotensin III (Ang III), a less potent vasoconstrictor peptide than Ang II (14). However, GluAP and AspAP activities reflect two enzymes with different properties and roles (15-18).

The aim of the present work was to analyse GluAP and AspAP activities in the serum of rats with NMU-induced mammary tumours, and to evaluate the putative value of these activities as biological markers of the initiation and promotion of the disease.

### Materials and Methods

**Animals and treatment.** Forty female virgin Wistar rats (164.7 $\pm$ 4.7 g body weight) were used in this work. The animals were provided from the animal house-care of the University of Jaén, Spain, and maintained in an environment controlled under constant temperature (25°C) with a 12h-light / 12h-dark cycle. All animals were allowed access to water and food *ad libitum*. The experimental

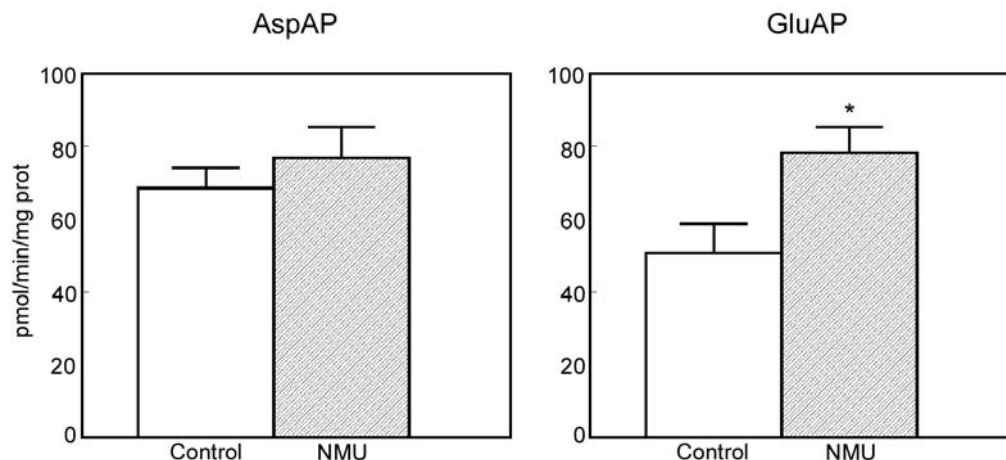
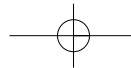


Figure 1. Aspartyl- and glutamyl- aminopeptidase activities in serum of control and NMU-treated rats. Results are expressed in picomoles of aspartyl- or glutamyl-  $\beta$ -naphthylamide hydrolysed per min and per mg of protein (Mean  $\pm$  SEM; n = 9; \*p < 0.05).

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 intraperitoneally injected 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.* (19). All rats were at estrus at the first NMU injection, verified by daily vaginal smears. The control group received the vehicle only. For tumour detection and growth control, rats were examined by palpation 2 days each week after the second NMU injection. The number of tumours were recorded and the major and minor diameters of each tumour was measured with a caliper. Body weight was determined every week. The following tumour growth parameters were also determined: latency period (LP), as the 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. After 122 days of first NMU injection, animals were sacrificed under equithesin anaesthesia (2 ml/kg body weight). Blood samples were obtained through the left cardiac ventricle and centrifuged for ten minutes at 3000g to obtain the serum. These samples were frozen and stored at  $-80^{\circ}\text{C}$ , until use.

**Aspartyl aminopeptidase activity assay.** Serum AspAP was determined fluorimetrically using aspartyl- $\beta$ -naphthylamide (AspNNap) as the substrate, according to the method previously described by us (20). Briefly, ten microlitres of each sample was incubated in triplicate for 30 min at  $37^{\circ}\text{C}$  with 100 microlitres of the substrate solution: 100  $\mu\text{M}$  AspNNap, 1.3  $\mu\text{M}$  ethylenediaminetetraacetic acid (EDTA) and 2 mM  $\text{MnCl}_2$  in 50 mM of phosphate buffer, pH 7.4. All the reactions were stopped by adding 100 microlitres of 0.1 M acetate buffer, pH 4.2.

**Glutamyl aminopeptidase activity assay.** Serum GluAP activity was measured in the same way using glutamyl- $\beta$ -naphthylamide (GluNNap) as the substrate, as previously described (21). Ten microlitres of each sample was incubated in triplicate for 30 min at  $37^{\circ}\text{C}$  with 100 microlitres of the substrate solution: 100  $\mu\text{M}$

GluNNap, 0.65 mM dithiothreitol (DTT) and 50 mM  $\text{CaCl}_2$  in 50 mM of phosphate buffer, pH 7.4. All the reactions were stopped by adding 100 microlitres of 0.1 M acetate buffer, pH 4.2.

The amount of  $\beta$ -naphthylamine released as the result of the enzymatic activities was measured fluorimetrically at 412 nm emission wavelength with an excitation wavelength of 345 nm. Proteins were also quantified in triplicate by the method of Bradford, using bovine serum albumin (BSA) as standard.

**Statistical analysis.** To analyse the differences between the control group and the animals with mammary tumours due to NMU injections, we used unpaired Student's *t*-test. 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 a LP (Mean  $\pm$  SEM) of  $113.0 \pm 4.2$  days between the first NMU injection and the appearance of the first tumour, with a 60% of TI. Mean tumour number per rat (Mean  $\pm$  SEM) was  $1.93 \pm 0.4$  tumours.

Specific AspAP and GluAP activities in serum of controls and NMU-treated rats are shown in Figure 1. Serum GluAP activity significantly increased ( $p < 0.05$ ) by 54 % in NMU-treated rats when compared with the control group. On the contrary, AspAP activity did not show significant differences between both groups.

## Discussion

To our knowledge, the present report describes, for the first time, changes in AspAP and GluAP activities in breast cancer at serum level in a rat model induced by NMU. Thus, serum GluAP activity increased whereas AspAP activity did not change. In the RAS, GluAP is the enzyme

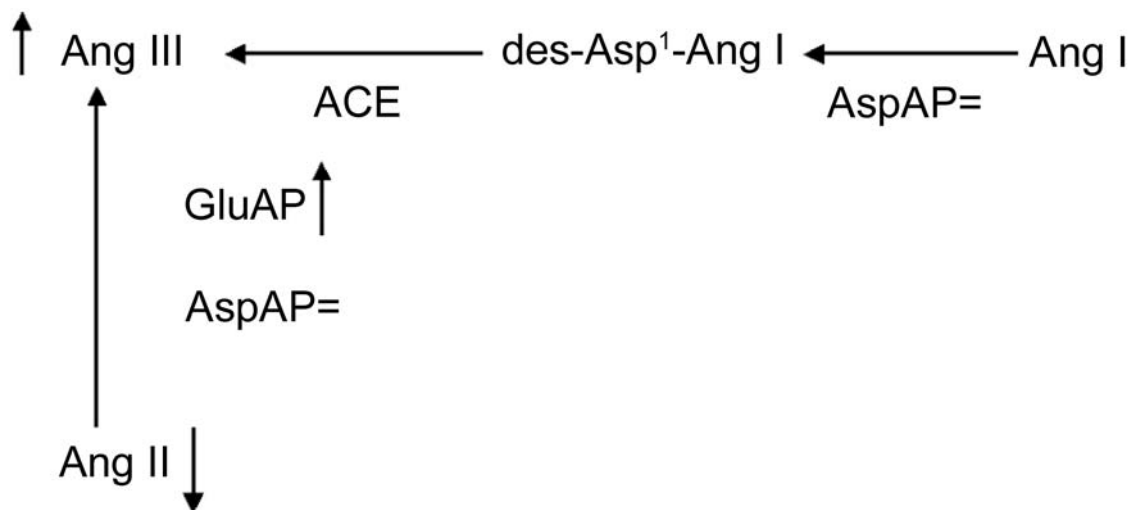
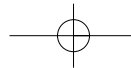


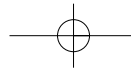
Figure 2. Hypothesized model of action of aspartyl aminopeptidase (AspAP) and glutamyl aminopeptidase (GluAP) on the metabolism of angiotensins in NMU-induced rat breast cancer. The increase of GluAP activity degrades angiotensin II (Ang II) to angiotensin III (Ang III) quickly, increasing circulating levels of Ang III and decreasing Ang II. Furthermore, Ang III is also produced from angiotensin I (Ang I) through the production of des-Asp<sup>1</sup>-Ang I by AspAP, which is further converted to Ang III by the action of the angiotensin-converting enzyme (ACE). Ang III could act through the overexpressed AT<sub>1</sub> receptors, favouring the promotion of the disease.

responsible for the degradation of Ang II to form Ang III. Ang III is also produced from Ang I through the production of des-Asp<sup>1</sup>-Ang I, which is further converted to Ang III by the action of the angiotensin-converting enzyme (ACE). AspAP activity degrades Ang I to des-Asp<sup>1</sup>-Ang I (22).

The RAS not only plays essential roles in the maintenance of vascular homeostasis (23), but also promotes angiogenesis directly or indirectly and growth of neoplastic cells (24). The two main bioactive peptides of the RAS, Ang II (25-32) and its direct metabolite Ang III (26-32), exhibit the same affinity for type 1 (AT<sub>1</sub>) and type 2 (AT<sub>2</sub>) angiotensin receptors (25, 26). Our results suggest an increase in Ang III and a decrease in Ang II serum circulating levels. The decrease in Ang II may be responsible for the overexpression of AT<sub>1</sub> receptors described in breast cancer (33). However, increased levels of Ang III, which exhibit the same affinity for the AT<sub>1</sub> receptor, would favour the development of the disease (Figure 2).

Although no references have been found in the literature about the role of Ang III in breast cancer, a number of *in vitro* studies in various cell types have implicated Ang II in cell hypertrophy and hyperplasia. Also, Ang II has been shown to correlate with increased fibrosis and mitotic activity. These proliferative effects are essentially mediated by AT<sub>1</sub>, as AT<sub>1</sub> blockers suppressed the Ang II action whereas AT<sub>2</sub> blockers were inactive (34). Abali *et al.* have demonstrated that AT<sub>1</sub> receptor is expressed on some neoplastic tissues like medroxyprogesterone-induced mouse

mammary adenocarcinoma and platelets. In most patients with cancer, some abnormalities like high platelet count have been noticed. Platelets may adhere to tumour vessels, form microthrombi and release their granules rich in potent pro-angiogenic factors like vascular endothelial growth factor (VEGF), one of the most important pro-angiogenic factors, a high serum level of which is associated with tumour progression (35). Angiogenesis refers to the formation of new blood vessels from the existing vasculature and occurs at extremely low levels in the adult organism. As a consequence, angiogenesis functions as a key factor in the development of metastasis. Ang II is itself an angiogenesis promoting factor and stimulates pro-angiogenic factors like tumour growth factor (TGF- $\beta$ ) or VEGF (24). Since the vascular density is linked to the availability of oxygen, poor density should be a potent marker of reduced blood perfusion and, therefore, hypoxia. On the other hand, the high vascular density and angiogenic ability of cancer is not synonymous with high blood flow since the geometry of the vascular/epithelial component distribution, vascular collapse due to increased interstitial blood pressure or non-functional vasculature due to immature structure of the vessels may not allow the establishment of an adequate blood flow, which results in tissue hypoxia (36). Sasaki *et al.* suggest that the AT<sub>1</sub> pathway plays an important role in ischemia-induced angiogenesis *in vivo* and would provide a unique strategy against angiogenic disorders, including malignant tumours (37).



Recent studies have demonstrated a specific overexpression of AT<sub>1</sub> receptor on the cytoplasmic membrane of cells of hyperplastic lesions with or without atypia and on DCIS of the breast, showing the strong positive correlation between membrane expression of AT<sub>1</sub> and hyperplastic lesions of the breast (33); and, on the other hand, binding of Ang II on AT<sub>1</sub> induces cell growth, while triggering *via* AT<sub>2</sub> causes an opposing effect of growth inhibition and apoptosis. Our results show changes in GluAP activity, but not in AspAP activity. GluAP is a membrane-bound homodimeric that is strongly expressed in peripheral tissues, such as in intestinal and renal epithelial cells and in the vascular endothelium (32,38). In addition to their role in regulation of responses to bioactive peptides, GluAP may be involved in cell activation, signal transduction and cell-matrix adhesion. By enzyme histochemistry, GluAP activity has also been localized in the microvessels of all organs in animal and human. Results obtained by Schlingemann *et al.* (39) showed that, in normal tissues, vascular staining with a monoclonal antibody that recognizes human GluAP was generally weak and often absent, while in tumours, marked microvascular staining was demonstrated.

On the other hand, the present results show different profiles for GluAP and AspAP. Previous evidence supports the existence of two enzymes with presumably different roles in the regulation of susceptible substrates (20,22,40).

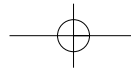
To conclude, we propose that alterations in the RAS through their regulating aminopeptidases could be responsible, at least in part, for the promotion and/or progression of breast cancer and the development of the angiogenic process.

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