

# Ovarian Renin–Angiotensin System-regulating Aminopeptidases Are Involved in Progesterone Overproduction in Rats with Mammary Tumours Induced by *N*-Methyl Nitrosourea

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**Abstract.** A local renin-angiotensin system (RAS) has been found in ovary. This ovarian RAS may regulate ovarian steroidogenesis. Ample studies show that the ovarian hormones estradiol (E2) and progesterone (P) are strongly implicated in the development of breast cancer. **Materials and Methods:** The aim of the present work was to elucidate if alterations in ovarian RAS, analyzed through their proteolytic regulatory enzymes aminopeptidase A (APA), B (APB) and N (APN), could be responsible for an altered steroidogenesis in rats with mammary tumours induced by *N*-methyl nitrosourea (NMU). **Results:** We describe here a highly significant increase of serum P levels in NMU-treated rats, concomitantly with an increase in ovarian aspartyl and glutamyl aminopeptidase activities (named together as APA activity). Moreover, we did not find changes in APB or APN activities, suggesting an increased metabolism from Ang II to Ang III and a decreased catabolism of Ang III. **Conclusion:** The relationship between ovarian RAS and P overproduction in a rat model of mammary carcinogenesis indicates ovarian RAS as a new potential target in breast cancer therapy.

Breast cancer, the most frequent spontaneous malignancy diagnosed in women in the Western world, is continuously increasing in incidence in industrialized nations. Although

breast cancer develops in women as the result of a combination of external and endogenous factors such as exposure to ionizing radiation, diet, socioeconomic status, and endocrine, familial, or genetic factors, no specific etiological agent(s) or the mechanism responsible for the initiation of the disease has been identified as yet. On the basis of experimental, epidemiological, and clinical studies, the ovarian hormones estradiol (E2) and progesterone (P) are strongly implicated in the development of this disease. Both hormones drive cellular proliferation in the breast; this increased proliferation provides greater opportunity for the accumulation of random errors, leading to breast tumorigenesis (1, 2).

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 (3). NMU-induced mammary tumors are estrogen-dependent, aggressive and locally invasive, to a similar degree to that observed in the human disease (4-8).

It is well known that the renin angiotensin system (RAS) plays important roles in mammals in general homeostasis, including the regulation of systemic blood pressure and of fluid and electrolyte balance (9). Besides its classical roles, a local RAS has been found in the female reproductive system in several mammals. Thus, binding sites for angiotensin II (Ang II) have been identified in rat ovarian follicles (10, 11) and corpora lutea (CL) (12, 13). Renin- and prorenin-like activities have been shown in bovine follicles (14) and angiotensin-converting enzyme (ACE) is also present in rat ovarian follicles and CL (15). The presence of angiotensin receptor subtypes (AT1 and AT2) have been documented and all studies have conclusively shown that by far the predominant receptor in the rat ovary is the AT2 (16). Furthermore, evidence has been accumulating that Ang II locally regulates ovulation, oocyte maturation (17, 18), and steroidogenesis (19). These findings

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suggest that ovarian RAS may function independently, or in concert with the systemic RAS, and regulate ovarian function through the paracrine/autocrine actions of Ang II. However, although classically AngII has been considered as the main bioactive peptide of the RAS, it has been postulated that is actually angiotensin III (AngIII) which plays this role (20, 21). In fact, Ang III has most of Ang II properties and shares the same receptors (22, 23). Ang III is obtained by deletion of the N-terminal aspartic residue by glutamyl-aminopeptidase (GluAP: EC 3.4.11.7) and aspartyl-aminopeptidase (AspAP: EC 3.4.11.21). Both enzymes are named together as aminopeptidase A (APA). Furthermore, AngIII is metabolized to angiotensin IV (Ang IV) by arginyl-aminopeptidase (aminopeptidase B, APB: EC 3.4.11.6) and alanyl-aminopeptidase (aminopeptidase N, APN: EC 3.4.11.14) (23-25).

In recent work, we determined that Ang III is the main peptide involved in ovarian P (but not E2) production in the rat (26). Therefore, the aim of the present work was to elucidate if alterations in ovarian RAS, analyzed through their proteolytic regulatory enzymes, could be responsible for an altered steroidogenesis in rats with mammary tumours induced by NMU.

## Materials and Methods

**Animal and treatments.** 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 of the University of Jaén, and maintained in a controlled environment 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 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 NMU 50 mg/kg body weight dissolved in distilled water (10 mg/ml) at 50, 80 and 110 days after birth, as described by Rivera *et al.* (27). All rats were at estrous at the first NMU injection, verified by daily vaginal smears. The control group received the vehicle only. For tumour detection, 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, with a value of 113.0±4.2 days (mean±SEM); tumour incidence (TI), as the percentage of the rats that developed at least one tumour, with a value of 60%; and mean tumour number per rat (n/t), as the number of tumours per rat in animals developing at least one tumour, with a value of 1.93±0.4 tumours (mean±SEM).

**Sample preparation.** After 122 days from 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 for ten minutes at 3000×g to obtain the serum. These samples were frozen and stored at -80°C, until use. The right ovary was quickly removed and also frozen until use. To obtain soluble fraction, tissue samples were homogenized in 10 volumes of 10 mM HCl-Tris buffer (pH 7.4) and ultracentrifuged at 100,000×g for 30 min (4°C). The supernatants were used to measure

soluble enzymatic activity and protein content in triplicate. To solubilize membrane proteins, the pellets were homogenized again in HCl-Tris buffer (pH 7.4) plus 1% Triton X-100. After centrifugation (100,000×g, for 30 min at 4°C), the supernatant was used to determinate solubilized membrane-bound activity and proteins in triplicate.

**RAS-regulating aminopeptidase assays.** AspAP was determined fluorimetrically using aspartyl-β-naphthylamide (AspNNap) as the substrate, according to the method previously described by Carrera *et al.* (28). Briefly, ten microliters of each sample was incubated in triplicate for 30 min at 37°C with 100 µl of the substrate solution: 100 µM AspNNap, 1.3 µM ethylenediaminetetraacetic acid (EDTA) and 2 mM MnCl<sub>2</sub> in 50 mM of phosphate buffer, pH 7.4.

GluAP activity was measured in the same way using glutamyl-β-naphthylamide (GluNNap) as the substrate, as previously described (29). Ten microliters of each sample was incubated in triplicate for 30 min at 37°C with 100 µl of the substrate solution: 100 µM GluNNap, 0.65 mM dithiothreitol (DTT) and 50 mM CaCl<sub>2</sub> in 50 mM of phosphate buffer, pH 7.4.

APN and APB were also measured fluorometrically using alanyl-β-naphthylamide (AlaNNap) or arginyl-β-naphthylamide (ArgNNap) as the substrate, as previously described by Garcia *et al.* (30). Ten microliters of each supernatant were incubated for 30 min at 37°C with 100 µM of the substrate solution: 100 µM AlaNNap or 100 µM ArgNNap and 0.65 mM DTT in 50 mM of phosphate buffer, pH 7.4.

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 activities was measured fluorimetrically at 412 nm emission wavelength with an excitation wavelength of 345 nm. Proteins were quantified also in triplicate by the method of Bradford, using bovine serum albumin (BSA) as standard. Specific soluble and membrane-bound APN, APB, AspAP and GluAP activities were expressed as nanomoles of Ala-, Arg-, Asp- and Glu-β-naphthylamide hydrolysed per min per mg of protein, by using a standard curve prepared with the latter compound under corresponding assay conditions.

**Assessment of sex hormones.** Serum samples were assayed by dissociation enhanced lanthanide fluorescence immunoassay (DELFA) for E2 and P (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland), according to the manufacturer's instructions. For E2, the lower limit of the assay detection was 0.05 nmol/l (13.6 pg/ml) and the interassay coefficient of variation was between 1.8-2.1%. For P, the lower limit of assay detection was 0.8 nmol/l (0.25 ng/ml) and the interassay coefficient of variation was between 1.9-4.5%.

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

## Results

Figure 1 shows ovarian soluble and membrane-bound RAS-regulating aminopeptidase activities. Neither soluble nor membrane-bound APN (Figure 1A) or APB (Figure 1B) activities were modified in NMU-treated animals. Instead, a significant increase was found in soluble AspAP (Figure 1C)

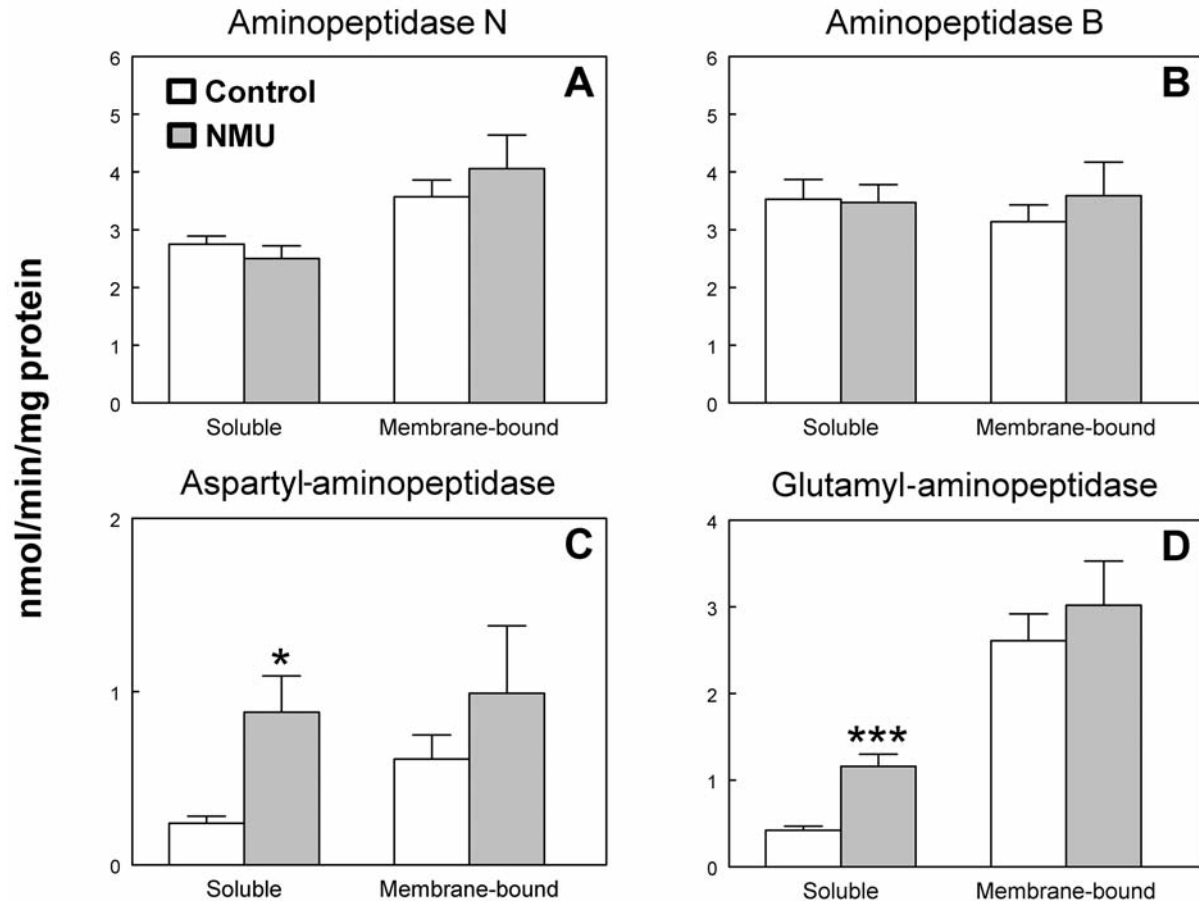


Figure 1. Specific soluble and membrane-bound renin-angiotensin system (RAS) regulating aminopeptidase N (A), aminopeptidase B (B), aspartyl aminopeptidase (C) and glutamyl aminopeptidase (D) activities in ovary of control and N-methyl nitrosourea (NMU)-treated rats. Results are expressed in nanomoles of the corresponding aminoacyl- $\beta$ -naphthylamide (see Material and Methods section) hydrolysed per min and per mg of protein (mean $\pm$ SEM;  $n=10$ ; \* $p<0.05$ , \*\*\* $p<0.001$ ).

( $p<0.05$ ) ( $0.24\pm0.04$  nmol/min/mg protein *versus*  $0.88\pm0.21$  nmol/min/mg protein) and GluAP (Figure 1D) ( $p<0.001$ ) ( $0.42\pm0.047$  nmol/min/mg protein *versus*  $1.16\pm0.14$  nmol/min/mg protein) activities in ovary from NMU-treated rats. However, no significant changes were observed in membrane-bound activities (Figure 1C and 1D).

Figure 2 shows serum circulating levels of E2 and P in control and NMU-treated animals. No significant differences were found in E2 levels, but a significant increase ( $p<0.01$ ) was observed in serum P levels in NMU-treated rats. The serum P level was increased by more than 50% ( $31.07\pm3.45$  ng/ml in the control group *versus*  $46.97\pm4.05$  ng/ml in the NMU-treated group).

## Discussion

Although the action of ovarian hormones on the breast does not appear to be genotoxic, they affect the rate of cell division (31, 32). Thus, E2 stimulates ductal growth and

increases cell proliferation rates, which can increase the likelihood of a random genetic error. P stimulates alveolar growth and also has proliferative effects (33). In the same way, sex steroid hormone receptors (ER and PgR, respectively) have been characterized in mammary tumours of NMU-treated rats in the cytosolic fraction (34). The total incidence of ER and PgR positivity was 100%, PgR presence being an expression of ER functionality (35). In the present study, we observed a highly significant increase of serum P levels in NMU-treated rats. Under physiological conditions, the major developmental role of P in the normal breast is the formation of lobular-alveolar structures during pregnancy (36), but in the case of breast cancer cells, a decrease in PgR associated with exposure to progestins (37-39) has been documented. Furthermore, many of the effects of P are thought to be due to its ability to oppose the action of E2, particularly in the uterus. P abrogates E2 induction of many of the known hormone-responsive genes. This effect is mediated by down-regulation of cytoplasmic and nuclear ER

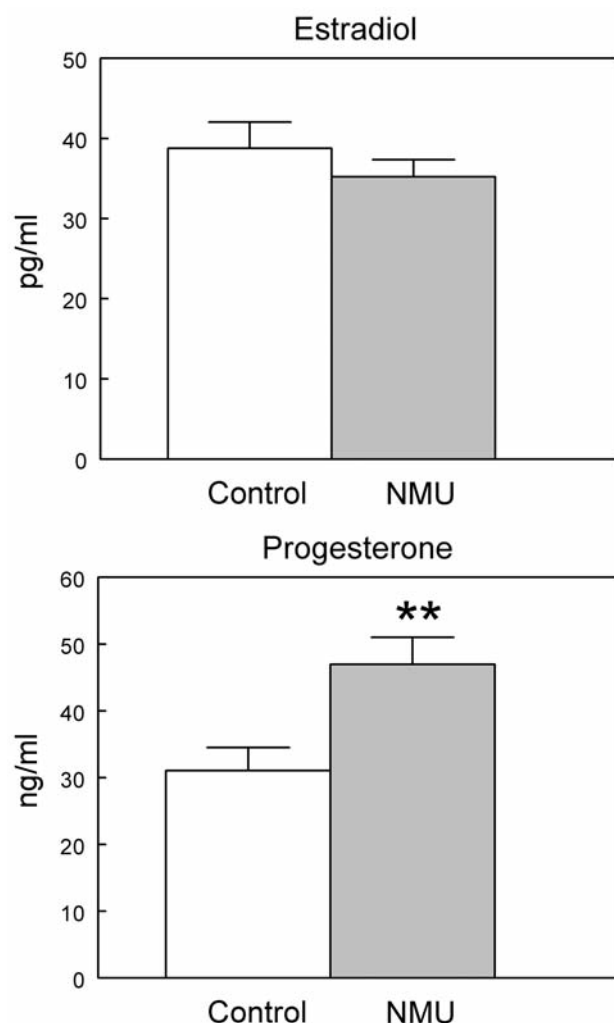


Figure 2. Serum circulating levels of estradiol (A) and progesterone (B) in control and *N*-methyl nitrosourea (NMU)-treated animals (mean±SEM; n= 10; \*\*p<0.01).

protein concentrations (40, 41). Thus, the rise in P levels in this breast cancer model could lead to a reduction in PgR levels in breast tumour, and therefore, reduces ER activity in mammary gland. In this manner, P will counteract the proliferative effect of estrogens.

In this way, it is well known that ovarian RAS is involved in steroidogenesis (10, 11, 42) Ang III being responsible, at least in the rat, for P production (26). In the present work, we also found an increase in ovarian AspAP and GluAP activities (named together as aminopeptidase A or APA activity) in NMU-treated animals, suggesting an increased metabolism from Ang II to Ang III. In the same way, we did not find changes in APN or APB activities, responsible for Ang III catabolism. Therefore, a rise in ovarian Ang III levels would be expected, leading to an increase in P production. To our

knowledge, the literature regarding the effects of endogenous progesterone on breast cancer risk is confusing and inconsistent, as the effects of this hormone can only be studied in ovulatory menstrual cycles of premenopausal women. Most studies that have evaluated progesterone levels do not report whether measurement was restricted to ovulatory cycles. During anovulatory menstrual cycles, the P level remains low throughout the cycle. Furthermore, it is impractical in most epidemiological studies to make certain that the progesterone measurement occurs on the day of the progesterone peak; this requires daily measurements throughout the luteal phase of the cycle. Finally, with recent studies showing a marked increase in breast cancer risk associated with addition of a progestin to estrogen replacement therapy regimens (43, 44), the seroepidemiology of progesterone is of great interest, and the present report shows for the first time the relationship between ovarian RAS and progesterone overproduction in a rat model of mammary carcinogenesis, pointing to ovarian RAS as a new potential target in breast cancer therapy.

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