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# Effects of Dietary Fat on Mammary Gland Pyroglutamyl Aminopeptidase on Experimental Breast Cancer

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## Abstract

**Aim**: To analyze the relationship between dietary fat and breast cancer mediated by the local functions of gonadotropin-releasing hormone (GnRH), analyzed by its regulating peptidase. The presence of GnRH and its receptors has been demonstrated in tumor tissue of the breast, although the functions of this peptide remain unclear. Pyroglutamyl aminopeptidase (PAP) is the enzyme involved in the local regulation of GnRH, and changes in its soluble and membrane-bound activities have been described in both women with breast cancer and animals with mammary tumors induced by N-methyl nitrosourea (NMU).

**Methods**: We analyze here the effects of different normolipidic (4%) dietary fats (soybean oil (commercial), extra virgin olive oil (EVOO), refined sunflower oil (SO) and refined sunflower oil enriched with 50% oleic acid (OAESO)) on soluble and membrane-bound PAP specific activities in breast tissue of rats with mammary tumors induced by N-methyl-nitrosourea (NMU) administration.

**Results:** We found that animals with breast cancer showed higher levels of soluble PAP than control healthy animals, but in a different degree depending on the type of dietary fat. On the contrary, membrane-bound mammary PAP specific activity was modified only in animals fed on EVOO.

**Conclusion**: Dietary fat is involved in the regulation of GnRH functions mediated by PAP at mammary tumor tissue in different degree depending on its localization and the type of fat administrated.

**Keywords:** N-methyl nitrosourea; Breast cancer; Extra virgin olive oil; Sunflower oil; Oleic acid; GnRH; Pyroglutamyl aminopeptidase

## Introduction

Gonadotropin releasing hormone (GnRH) plays an important role in the control of mammalian reproduction. In addition to this classic hypophysiotropic action, GnRH might have a role as a modulator of cell growth and metastasis in a number of human malignant tumors including breast cancer [1]. Thus, GnRH receptors and GnRH mRNA have been found in breast tissue [2], raising the possibility of a local role for GnRH in the human mammary gland [3,4]. GnRH is regulated by the proteolytic regulatory enzyme pyroglutamyl aminopeptidase (PAP) [5,6]. PAP is an omega peptidase widely distributed in fluids and tissues that hydrolyses N-terminal pyroglutamic residues from biologically active peptides [7]. We have previously reported a decrease in both rat [8] and human [6] PAP activity in breast cancer, suggesting that GnRH may be an important local intracrine, autocrine and/or paracrine hormonal factor in the pathogenesis of breast cancer.

Several studies have been carried out to clarify which elements in the diet could play a protective or determining role in the development and/or progression of breast cancer, as well as the mechanisms through which the different nutrients can affect breast cancer progression, recurrence and/or mortality [9,10]. In this regard, dietary fat has been directly related with breast cancer incidence. Thus, high dietary fat is associated with increased breast cancer risk [11], whereas reduced fat intake is associated with lower recurrence rates and longer survival after breast cancer diagnosis [9]. Furthermore, the intake of lipids increases with a marked rise in saturated and polyunsaturated fatty acids (PUFA) versus monounsaturated fatty acids (MUFA). Different studies in animal models and humans have provided evidence that high n-6 PUFA intake stimulates several stages in the development of mammary and colon cancer [12-14]. Several mechanisms have been proposed to explain the promoting effects of these fatty acids on mammary carcinogenesis. They may be related to effects exerted by these fatty acids on the endocrine system, the immune system, eicosanoid metabolism, cell membrane fluidity, cell–cell interactions, or lipid peroxidation processes [11,15,16]. Rodents fed diets enriched with n-6 PUFA had a shortened latency period for tumor appearance, promoted tumor growth, and an increased incidence of mammary tumors compared to diets with high saturated fatty acid content. In contrast, a diet enriched with n-3 PUFA seems to prevent cancer by influencing the activity of enzymes and proteins related to intracellular signaling and cell proliferation [16-18]. Also, Different epidemiological studies [19] from Spain, Italy, and Greece have postulated that increased dietary intake of extra virgin olive oil is associated with a slight decreased risk, or at least not an increased risk, of breast cancer [20]. In any case, most of the experiments performed to analyze the relationship between dietary fat and breast cancer in animal models have used high amounts of fat (between 15-20%) [21] compared to the physiological levels of dietary fat adequate for these animals (4%). Therefore, in the present report we analyze the effects of 4% dietary fat in the form of soybean oil, extra virgin olive oil, refined sunflower oil, and refined sunflower oil enriched with 50% oleic acid on soluble and solubilized membrane-bound PAP specific activities in breast tissue

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in rats with experimentally induced mammary tumors to analyze the influence of a normolipidic dietary fat on PAP regulatory functions at breast level in this animal model.

## Material and Methods

### Animals and treatment

The animal protocol was designed to minimize pain or discomfort to the animals. Seventy three female virgin Wistar rats (149.4 ± 2.7 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 12-hr-light/12-hr-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 (2010/63/EU) and approved by the ethical committee of the University of Jaén. The rats were randomly divided into five groups. Four groups of 16 animals were injected intraperitoneally with three doses of 50 mg/kg body weight of NMU at 50, 80 and 110 days after birth. All rats were on estrus cycle during the first injection, verified by daily vaginal smears. Animals were fed with AIN-93M diets containing 4% of fat (Musal and Chemical, Spain), constituted by extra virgin olive oil (EVOO dietary group), refined sunflower oil (SO dietary group) or refined sunflower oil enriched with 50% oleic acid (OAESO dietary group). The fourth dietary group received a commercial standard diet with 4% soybean oil. Finally, a fifth group of 9 animals was considered as control and rats were injected intraperitoneally with the vehicle only and fed with the commercial diet. The parameters of carcinogenesis and the histopathological characteristics of the different dietary groups have been previously described [22,23].

### Sample preparation

After 17 weeks of the first NMU injection, animals were sacrificed under equithensin anesthesia (2 ml/kg body weight). Normal and tumor mammary gland tissues were quickly removed and frozen in liquid nitrogen and stored 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 × 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 × 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.

### Pyroglutamyl aminopeptidase (PAP) activity assay

PAP activity was measured fluorometrically using pyroglutamyl-ß-naphthylamide (pglunnap) as a substrate, as previously described [5]. Briefly, ten microliters of each supernatant were incubated during 30 min at 37°C with 100 µl of the substrate solution containing 100 µm pglunnap, 1.5 mm bovine serum albumin (BSA), 0.65 mm dithiothreitol and 1.3 mm EDTA in 50 mm phosphate buffer, ph 7.4. The reactions were stopped by adding 100 µl of 0.1 M acetate buffer, ph 4.2. The amount of ß-naphthylamine released as the result of the enzymatic activity was measured fluorometrically at 412 nm emission wavelength with an excitation wavelength of 345 nm. Proteins were quantified in triplicate by the method of Bradford, using BSA as a standard. Specific serum, soluble and membrane-bound PAP specific activities were expressed as picomoles of pglunnap hydrolyzed per minute and per milligram of protein, by using a standard curve prepared with the latter compound under corresponding assay conditions. The fluorogenic assay was linear with respect to time of hydrolysis and protein content.

### Statistical analysis

All values represent the mean of the individual determination ± standard error of the mean (SEM). Data was analyzed by multiple analyses of variance (MANOVA) plus Newman-Keul's post-hoc test, using IBM Pass V.19 software. Values of $P<0.05$ were considered significant.

## Results

Figure 1 shows histopathological sections of carcinomas of the rat mammary gland displaying papillary and cribiform patterns, extensive solid areas and tumoral necrosis. Well-circumscribed carcinomas with nodular appearance and zones with infiltrative pattern with intense desmoplastic reaction were also seen.

Figure 2A shows soluble PAP activity in breast tissue of control animals and animals with mammary tumors induced by NMU. Animals with mammary tumors show significantly higher values of mammary soluble PAP activity than control animals ($p<0.001$), independently of the dietary manipulation. Thus, an increase by 833% was found in animals fed on commercial diet and by 854% in animals fed on SO diet. However, animals fed on EVOO or OAESO diets show significant lower soluble PAP activities than animals fed on commercial or SO diets, with increases by 440% and 327% respectively. Figure 2B shows membrane-bound PAP activity in breast tissue of control animals and animals with mammary tumors induced by NMU. A significant increase by 94% ($p<0.001$) was found in this activity in mammary tissue of animals with breast cancer fed on EVOO diet when compared with control animals. No changes were also found on animals fed on commercial, SO or OAESO diets.

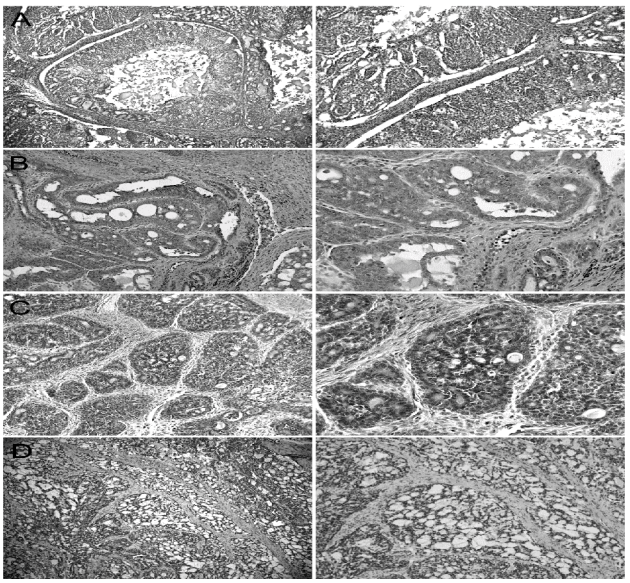


**Figure 1:** Histopathology of mammary tissue from animals with breast cancer induced by N-methyl-nitrosourea (NMU) fed with an AIN-93 commercial diet (A), or with AIN-93 diets with 4% of fat constituted by extra virgin olive oil (EVOO) (B), refined sunflower oil (SO) (C) and refined sunflower oil enriched with 50% oleic acid (OAESO) (D) (Left column, 20x magnification; right column, 40x magnification).

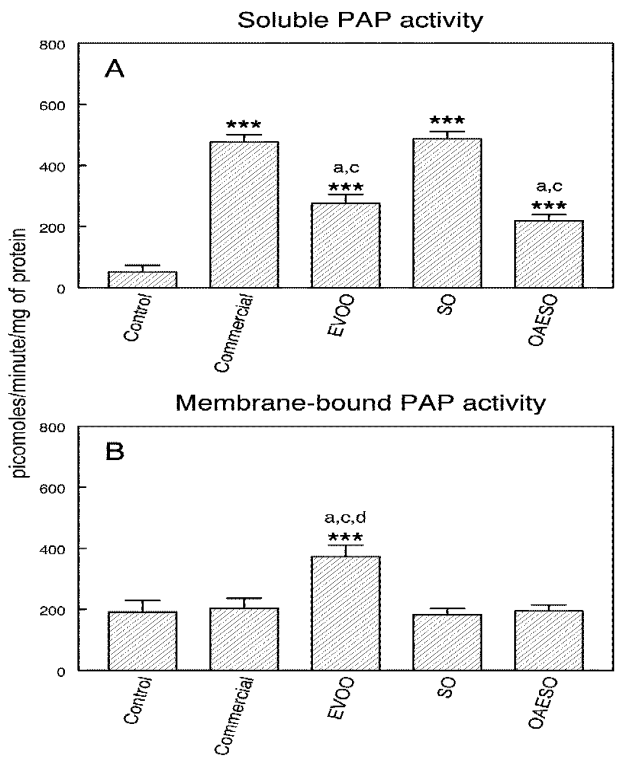


**Figure 2:** Mammary soluble (A) and membrane-bound (B) pyroglutamyl aminopeptidase specific activities in non-tumor healthy control animals and animals with breast cancer induced by N-methyl-nitrosourea (NMU) fed with an AIN-93 commercial diet, or with AIN-93 diets with 4 % of fat constituted respectively by extra virgin olive oil (EVOO), refined sunflower oil (SO) and refined sunflower oil enriched to 50% oleic acid (OAESO). Results are expressed in picomoles of pyroglutamyl-ß-naphthylamide hydrolyzed per min and per mg of protein (mean ± SEM; ***p<0.001; [a]p<0.01 vs. commercial diet; [c]p<0.01, vs. SO diet; [d]p<0.01 vs. OAESO diet).

## Discussion

Different studies in animal models as well as in humans [24-27] have shown that a high intake of n-6 pufas stimulates different steps of the development of breast and other types of cancer. To explain these promoter effects in mammary carcinogenesis, several mechanisms involving the endocrine and immune systems, eicosanoids metabolism, membrane fluidity, cell-cell interactions and the tendency of n-6 PUFAS to promote lipid peroxidation have been proposed [28]. In rodents, a diet rich in n-6 PUFAS shortens the latency period, promotes tumor growth and increases the incidence of mammary tumors when compared with other diets rich in saturated fatty acids [29]. On the contrary, n-3 pufas-enriched diet appears to prevent cancer by acting on enzymes and proteins associated with intracellular communication and cell proliferation [30,31]. Similarly, different experimental studies conducted in animals with chemically-induced mammary tumors have revealed that EVOO also has a protective effect in the prevention of these tumors [32] and that this effect has been related to its high content in oleic acid [20]. Previous studies in mice analyzed the influence of a high fat diet (20% fat supplemented with EVOO) on PAP activity in serum and in different tissues [33]. We did not observed changes in serum PAP activity in animals of the high fat group. However, PAP activity was modified in the soluble but not in the membrane bound fraction of the tissues affected. In the present work, we have observed the influence of several dietary fats on PAP activity in animals with mammary tumors, being soluble PAP activity differently modified depending on the type of dietary fat, whereas membrane-bound PAP activity was modified only by EVOO. In this regard, changes in PAP activities may lead to alterations in local GnRH levels in breast tissue. GnRH is a hypothalamic neuronal secretory decapeptide that plays a pivotal role in mammalian reproduction. Although hypothalamus and pituitary are the main source and target sites for GnRH, several reports have described extrahypothalamic GnRH and GnRH receptors (GnRH-R) in various reproductive tissues such as ovaries, prostate and mammary glands, although their functions in these tissues remain unknown [34]. The presence of GnRH and GnRH-R in normal and tumoral mammary tissue suggests an important local intracrine, autocrine and/or paracrine role for GnRH in the pathogenesis of breast cancer. Therefore, the level of GnRH in mammary tissue may be modified by alterations in PAP activities due to the intake of different dietary fats.

In this regard, both native GnRH and certain GnRH agonists and antagonists inhibited the proliferation of cancer cells in a dose and time dependent manner [1,3,4]. Thus, the continuous administration and in high doses of GnRH agonists suppress the pituitary gonadal axis through the down-regulation and desensitization of its own receptors. The observation that GnRH receptors are expressed in steroid-dependent tumors and that their activation reduces cell proliferation and metastatic behavior of cancer cell lines, both *in vitro* and *in vivo* (when inoculated into nude mice), indicates a possible additional and more direct antitumor activity for these compounds [35]. This statement may explain the result obtained for soluble PAP activity, which is significantly reduced in animals that were fed on EVOO. Therefore, this type of dietary fat, by increasing the local levels of GnRH, would inhibit mammary tumor development. In fact, we have previously reported that the type of dietary fat does not influence the parameters of the carcinogenesis, because no significant differences were found between groups either in the latency period, the incidence of animals with tumors and the incidence of mortality in animals with tumors or the tumor yield per rat [23], but histopathological analysis showed important morphological changes in breast tissue related to the type of dietary fat [22]. Thus, we found a decreased percentage of tubules in tumors of animals fed on SO and OAESO when compared to animals fed on commercial diet and those fed on EVOO. A decreased percentage of mitosis was also observed in the tumors of animal fed on EVOO and OAESO. Finally, differences were found in the final grading score between groups, with animals fed on a commercial diet showing 50% of type I and 50% of type II tumors; animals fed on EVOO showing 81% of type I and 19% of type II tumors; animals fed on SO showing 84% of type I and 16% of type II tumors and animals fed on OAESO showing 100% of type I tumors.

On the other hand, it is also well known that the type and amount of fat in the diet not only modify blood lipid concentration, including cholesterol levels [36], but also modifies the lipid composition of the cell membrane [37]. This can affect membrane-bound activities and carriers [38]. This also could explain the differences found between soluble and membrane-bound PAP activities in this experimental model, where membrane-bound PAP activity shows an inverse response to that obtained for soluble PAP activity. Regarding OAESO diet, in this experimental group the tumors appears earlier and the mortality is the highest [22]. In this sense, it has been difficult to establish associations between the total consumption of dietary fat and the risk of breast cancer, and therefore it has been raised the possibility that specific types of fat might have different effects.

It has been also studied in chemically-induced breast cancer animal models if oleic acid levels were the key to the protective effect of olive oil [20]. In this study, the inhibitory effects of three types of olive oil with different percentages of oleic and linoleic acid were compared. The results showed that oil with a greater percentage of oleic acid (80%) and less linoleic acid (5%) showed the highest levels of benign adenocarcinoma. On the other hand, it has also been demonstrated that diets enriched in polyunsaturated fatty acids of the n-6 series, especially linoleic acid, mainly present in seed oils such as corn, soybeans and sunflower, accelerate mammary carcinogenesis and tumor progression, whereas the polyunsaturated fatty acids of the n-3 series [39] and monounsaturated fatty acids [40] showed inhibitory effects. Therefore, the type of dietary fat clearly influences several aspects in various degrees of the development, promotion and/or progression of breast cancer.

## Conclusion

The type of dietary fat is related to several different mammary tumor characteristics in the NMU-induced rat breast cancer model even when normolipidic amounts of fat are used. Among the multiple factors that can be affected by this dietary manipulation, changes in PAP activity at this tissue level could be responsible of the changes in the processes mediated by the local regulation of GnRH. Further studies are necessary to clearly determine the exact role of GnRH and its receptors in cancer development and their relationship with dietary fat components.

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