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Sex differences exist in brain renin-angiotensin system-regulating aminopeptidase activities in transplacental ethyl-nitrosourea-induced gliomas

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ABSTRACT

Introduction: The renin angiotensin system (RAS) is emerging as an important target for the treatment of glioma. We had described that the local RAS is involved *in vivo* in tumor growth in the rat model of experimental C6 glioma implanted at the subcutaneous region, through the modification of several proteolytic regulatory enzymes of aminopeptidase type.
Methods: We analyze RAS-regulating aminopeptidase activities in plasma and brain tissue of control male and female rats and rats with transplacental ethylnitrosourea-induced gliomas.
Results: No differences were found either the mean total number of tumors per animal or the tumor volume between male and female animals. However, we have found increased levels in aspartyl aminopeptidase in both males and females and of aminopeptidase B only in males. On the contrary, decreased levels were found in aminopeptidase N and insulin-regulated aminopeptidase activities in both males and females, whereas aminopeptidase A only decreased in females. Decreased levels of aminopeptidase N, aminopeptidase B and insulin-regulated aminopeptidase were also shown in plasma of only female rats.
Conclusions: Under the complexity of RAS cascade, the changes found suggest the predominant actions of angiotensin III against a decreased action of angiotensin II and angiotensin IV. We conclude that angiotensin peptides are involved in tumor growth in this rat model of glioma and that their role in tumor growth can be analyzed through their corresponding proteolytic regulatory enzymes, which make them new and attractive therapeutic targets beyond the use or angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs).

1. Introduction

Gliomas are the most common primary intraparenchymal tumors of the central nervous system (CNS). These tumors have a very poor prognosis. They represent approximately 26 % of all primary brain and other CNS tumors and 81 % of the malignant tumors. Glioblastoma accounts for the highest amount of gliomas (56.6 %). Relative survival estimates for glioblastoma are very low, with only 5.6 % of patients who survived five years after diagnosis (Ostrom et al., 2018).

The renin angiotensin system (RAS) has been revealed as an important target for the treatment of glioma, once its participation in various of the events that take place for a normal cell to become carcinogenic (Hanahan and Weinberg, 2011), as well as in the processes that take place later, such as angiogenesis, have been demonstrated for tumor development and spread. Within the RAS, a wide variety of components have been described, among which the most studied for their involvement in glioma are angiotensin II (AngII) and type 1 and type 2 angiotensin receptors (AT_1 and AT_2). Secondly, the new signaling systems mediated by angiotensin 1–7 ($Ang_{1–7}$) and its receptor (Mas receptor), are also currently being investigated (Perdomo-Pantoja et al., 2018). However, one of the regulatory elements of this system, the proteolytic enzymes of the aminopeptidase type, have received little attention, probably due to the belief that they have a ubiquitous location and very limited specificity. Several works of our group have shown on multiple

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occasions, both *in vitro* and *in vivo*, that the regulation carried out by these enzymes and the function associated with them are finely controlled at the local level of the tissue or cell that is involved (Martinez-Martos et al., 2019; Puertas Mdel et al., 2013; Ramirez-Exposito et al., 2019a; Ramirez-Exposito and Martinez-Martos, 2018, 2019; Ramirez-Exposito et al., 2020). Therefore and taking into account the specific location where brain tumors develop, proteolytic enzymes of aminopeptidase type are important objectives to be considered.

In this sense, we have used a model of heterotypic brain glioma performed by the implantation of C6 glioma cells in the subcutaneous region of the abdomen of the Wistar rat. We have shown significant alterations in the proteolytic enzymes of the RAS at the level of the tumor tissue, while there were no significant changes at the level of circulating activity (Mayas et al., 2012). Among these changes, a direct relationship of aspartyl aminopeptidase (ASAP) activity with tumor growth over time stood out, while an inverse relationship occurred with aminopeptidase N (APN) and aminopeptidase B (APB) activities. The changes found in these enzymes allowed us to infer that tumor development was fundamentally participated by angiotensin III (AngIII), which would exert its action through the AT_1 and AT_2 receptors.

Nevertheless, the use of a heterotopic experimental model has some limitations. In fact, the tissue environment in which the tumor develops is not exactly the same, as it would have under normal conditions. For this reason, in the present study we analyze the activity of the proteolytic regulatory enzymes of the renin-angiotensin system in glioma tumor tissue from tumors obtained by transplacental administration of ethyl-nitrosourea (ENU). In this way, we will better understand the role of the regulatory proteolytic enzymes of the renin-angiotensin system in the exact location at the brain level. It also will allow us to know the role these enzymes play in tumor development and their possible potential as therapeutic targets against the disease. It will provide us with additional treatment options beyond the ones limited to the administration of angiotensin converting enzyme inhibitors and angiotensin receptor blockers, and thus expanding the therapeutic possibilities against this terrible disease.

2. Material and methods

2.1. Animals, treatments and sample preparation

We have used six female and three male Wistar rats that were obtained from Harlan laboratories (Spain). The animals were maintained in a controlled environment under constant temperature (25 °C) with a 12 h-light/12 h-dark cycle. Rats were given free access to standard laboratory rat food and water. The experimental procedures for animal use and care were in accordance with the European Community Council directive (2010/63/EU). Protocols were approved by the Bioethical Committee of the University of Jaen (Reference number CVI09−4957 M). Female Wistar rats weighing 200−250 *g* were caged overnight with males, and the day when the sperm was confirmed in vaginal smears was designated as day 1 of gestation. On day 18 of gestation, a group of pregnant rats was injected i.v. with a single dose of N-ethyl-N-nitrosourea (ENU), 75 mg/kg body weight dissolved in saline solution. Other group of pregnant rats were injected with saline alone. Female and male offspring from both vehicle and ENU-treated rats were used in these experiments. The offspring were naturally delivered and weaned at 22 days old. At this time, males and females were housed separated into two groups: control healthy group, with 30 animals, 15 females and 15 males, and ENU group, with 27 animals, 13 males and 14 females. All animals were kept under weekly observation for any sign of neurological or health problems. At 30 weeks of age, surviving rats were sacrificed and tumor samples obtained and prepared for histopathological examination or frozen at −80 °C until use. Equivalent brain regions were obtained from control animals based in Paxinos and Watson (Paxinos and Watson, 2013). Tissue samples were homogenized in 10 mM HCl-Tris buffer (pH 7.4) and ultracentrifuged at 100,000 *g* for 30 min (4 °C). The resulting supernatants were used to measure soluble enzymatic activity and protein content in triplicate.

Blood samples were obtained from the left cardiac ventricle, drawn into tubes with heparin, allowed to clot and then centrifuged for 10 min at 3000xg to obtain the plasma, which was frozen and stored until −80 °C until use.

2.2. Magnetic resonance imaging (MRI) analysis

Magnetic resonance images were acquired at 9.4 T (Bruker Biospec, Ettlingen, Germany) at the Fundación IDICHUS (Santiago de Compostela, Spain) using procedures previously described (Ramirez-Exposito et al., 2019b).

2.3. Aminopeptidase activity assays

Aminopeptidase activities were measured fluorometrically using the corresponding aminoacyl-ß-naphthylamide (aminoacyl-NNap) as the substrate (AspNNap for ASAP, GluNNap for APA, AlaNNap for APN, ArgNNap for APB and LeuNNap for insulin-regulated aminopeptidase (IRAP)), as previously described (Martinez-Martos et al., 2011; Martinez et al., 1998; Mayas et al., 2012).

2.4. Statistical analysis

All values represent the mean ± standard error of the mean (SEM). The data was analyzed by two-way analysis of variance (ANOVA) using GraphPad Prism V.8 software. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Parameters of the carcinogenesis

Fig. 1 shows examples of ENU-induced glioblastomas by MRI in both male (Fig. 1A) and female (Fig. 1B) rats as well as histopathological sections of tumors from animals of the two sexes (Fig. 1C for males and Fig. 1D for females). ENU treated animals showed a tumor incidence, defined as the percentage of rats bearing at least one malignant tumor at sacrifice, of 100 % in both male and female rats. In addition, no differences were found between males and females in the mean tumor number per animal (Fig. 1E). Regarding tumor volume, no significant difference was found between male and female rats.

3.2. Plasma and tissue aminopeptidase activities

In Fig. 2 we present data related to the activities of ASAP and aminopeptidase A (APA) in plasma and tumor tissue of control male and female animals and animals with gliomas induced by ENU administration. It can be shown that there are no significant differences in the plasma levels of either of the two enzymatic activities, neither in male animals nor in female animals (Fig. 2A and B). On the contrary, in tumor tissue, we did find significantly increased levels of ASAP activity in both male and female animals (Fig. 2C). Even more striking are the results obtained for APA activity, where there are no significant differences in the tumor tissue of male animals compared to their respective controls. However, there is a very significant decrease in female animals (Fig. 2D), thus demonstrating the existence of sex differences in the behavior of this enzymatic activity because of the tumor process.

Likewise, in Fig. 3, the data relating to the enzymatic activities of APN, APB and IRAP can be observed in plasma and tumor tissue of male and female animals with ENU-induced tumors. It is worth noting the important sex differences observed in these enzymatic activities at the plasma level, where no significant differences appear for any of them in male animals, while female animals showed significantly lower enzyme activities for all of them when compared to their corresponding control

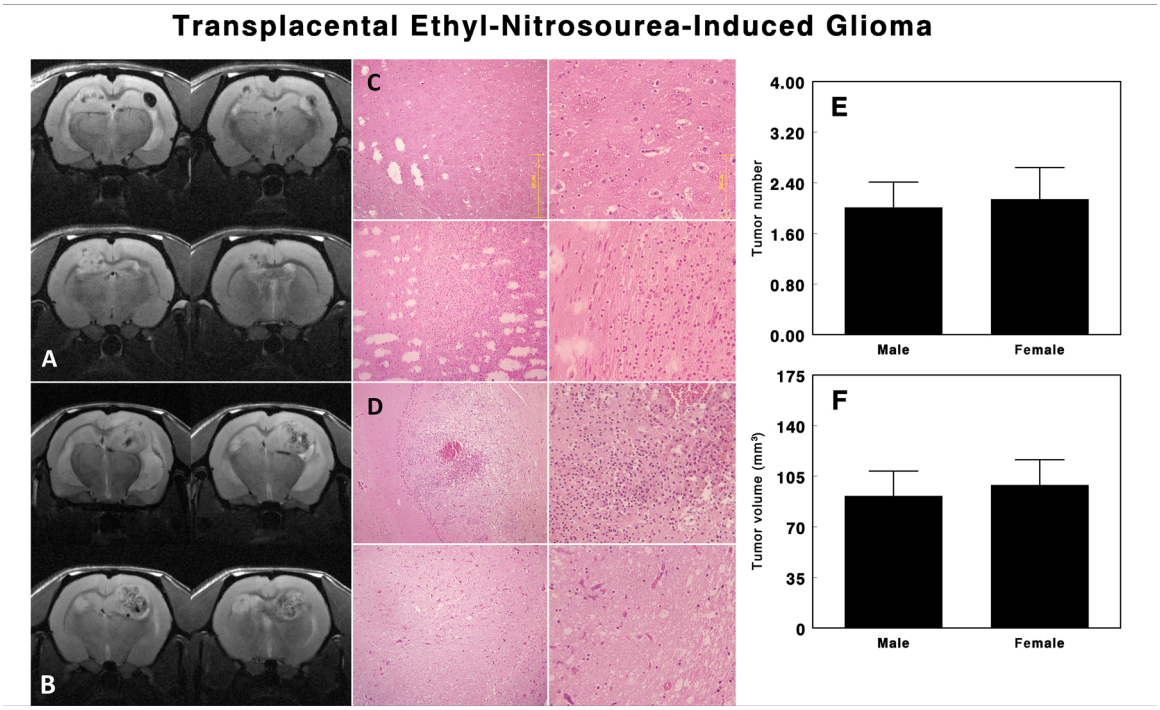

Fig. 1. Typical T2-weighted MRI images of transplacental N-ethyl-N-nitrosourea (ENU)-induced tumors on male (A) and female (B) animals. Histopathological studies showing representative specimens of the ENU-induced brain tumors in male (C) and female (D) rats (microscopic view with H&E staining using ×10 magnification (left pane) or 40x magnification (right pane) are included. Mean tumor number per animal (E) and tumor volume (F) are presented for male and female animals. Volume values represent the mean expressed in mm^3 ±SEM (n = 6).

animals (Fig. 3A-C). Regarding tumor tissue, both male and female animals showed significantly lower values of APN (Fig. 3D) and IRAP activity (Fig. 3F), while APB activity again showed sex differences, being significantly increased in tumor tissue of male animals compared to their healthy controls, while there were no significant differences in female animals that developed ENU-induced tumors (Fig. 3E).

4. Discussion

Alteration of renin-angiotensin system components plays a significant role in the proliferation, angiogenesis and invasion of glioma tumors. In this way, it is well described that AngII stimulates tumor neovascularization (Egami et al., 2003) and overexpression of ACE and AT1 is associated with tumor growth metastasis and progression (Arrieta et al., 2005, 2008, Arrieta et al., 2015; Rocken et al., 2007). For this reason, in recent years the renin-angiotensin system is emerging as an important pharmacological target against glioma, once it has been shown that the administration of either ACE inhibitors or angiotensin receptor blockers have positive effects on the disease. Thus, ACE inhibitors seem to reduce cancer incidence or at least, protects against it by reducing cell proliferation and migration, inflammation and angiogenesis (Perdomo-Pantoja et al., 2018; Ruiter et al., 2011). Furthermore, these compounds inhibit the cellular matrix metalloproteases activity and reduce the expression of vascular endothelial growth factor (VEGF) (Lindberg et al., 2004; Ruiter et al., 2011). Regarding angiotensin receptor blockers, it has been also demonstrated their ability to decrease tumoral volume, mitotic index, cell proliferation and the number of capillary vessels (Rivera et al., 2001).

However, a number of regulatory proteolytic enzymes also participate in the RAS, which have received much less attention as potential therapeutic targets against glioma. Interestingly, they are considered to be cell-surface peptidases involved in the control of cell proliferation and differentiation by modulating the access of peptides to their membrane receptors, and their alteration may contribute to neoplastic transformation or cell progression (Kenny et al., 1989; King et al., 1993; Shipp et al., 1991).

In the present work, we use the animal model of glioma induced by transplacental administration of ENU and show the changes found in the activity of various proteolytic enzymes of aminopeptidase type that regulates the RAS. Our laboratory had already described some alterations of these proteolytic enzymes in a heterotopic model of subcutaneous C6 glioma, but to avoid the limitations of this model, the data obtained from the model induced by ENU are presented here.

In the first place, it should be noted that the alterations in the proteolytic activity at the circulating level are limited to the activities of APN, APB and IRAP, but with an important sex differences, since they only appear altered in females. However, the circulating ASAP and APA activities show no variation in either male or female animals. We had already described the existence of sex differences in the development of gliomas in the ENU-induced animal model, where the alterations in males and females in the redox systems are also different (Ramirez-Exposito et al., 2019b). It is therefore obvious that the hormonal status decisively influences the progress of events that take place during the development of cerebral gliomas, and the circulating aminopeptidase activities could reflect the changes that occurs at brain level to a certain degree and depending on the sex.

Regarding the enzymatic activity in the tissue, significant differences do appear in most of the enzymatic activities analyzed, and there are also sex differences in some of them. Thus, for example, ASAP, APN and IRAP show the same behavior in male and female animals. In the case of ASAP, there is a significant increase in enzymatic activity, while APN and IRAP show a significant decrease. However, the activities of APA and APB do show sex differences, so that APA activity is only altered in female animals, where a significant decrease appears, while APB is only altered in males, where a significant increase occurs. In fact, the biological properties of angiotensins are associated with the hormonal status and invasive potential of cancer cells (Dominska et al., 2012). Therefore, hormonal influence on aminopeptidase activities is also plausible. In fact, we have described several sex differences in aminopeptidase activities (Martinez et al., 1998).

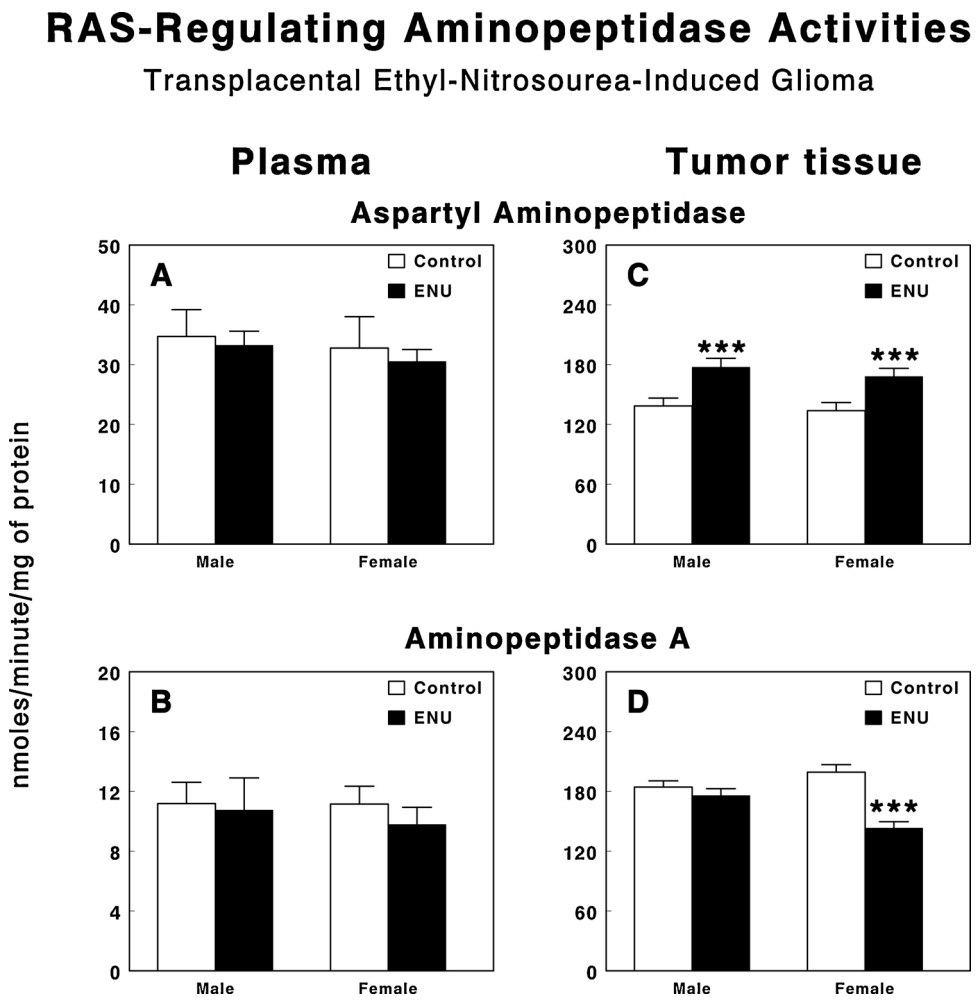

Fig. 2. Plasma (A, B) and tumor tissue (C, D) aspartyl aminopeptidase and aminopeptidase A specific activities in male and female healthy rats vs. rats with N-ethyl-N-nitrosourea (ENU)-induced brain tumors. Results are expressed in nanomoles of their corresponding aminoacyl-β-naphthylamide hydrolyzed per min and per mg of protein (mean ± SEM; n = 6; ***p < 0.001).

Taken as a whole, and considering the general scheme of the RAS cascade (Fig. 4), the data obtained here allow us to infer the alteration of three possible routes in a slightly different way in males and females, but with a common final destination. First, the activation of ASAP would promote, in both males and females, the formation of angiotensin 2–10 from angiotensinogen, providing the appropriate precursor for the formation of AngIII by ACE. Second, the cleavage of angiotensin II by ASAP is enhanced to form angiotensin III again. It has been reported a main role for AngIII in carcinogenesis, as previously proposed (Martinez-Martos et al., 2011; Mayas et al., 2012) or described for other authors (Teranishi et al., 2008). It has been described that similarly to AngII, AngIII activates the cell growth. The proliferative signal transduction of AngIII seems to be via MAPK phosphorylation, as for AngII (Uemura et al., 2003). AngIII appears to bind to the AT1 receptor in a concentration-dependent manner, resulting in cell proliferation (Teranishi et al., 2008). Interestingly, AngIII was shown to be several times more effective in the brain than AngII (Harding and Felix, 1987). A conflictive aspect at this point is the decrease in APA activity found in females, which would prevent the formation of AngIII from AngII. It has been described that APA was up-regulated and enzymatically active in blood vessels of human tumors, but was not detected in normal blood vessels (Marchio et al., 2004). It is possible that APA is implicated in neovascularization of tumor development. However, it has been described in tumoral cells; therefore, this enzyme may play a regulatory role in both neoplastic transformation and disease progression.

Third and last, ASAP and APA could also participate in the inactivation of angiotensin 1–7 in favor of its metabolite angiotensin 2−7. It has been demonstrated that Ang1–7 opposes to AT1 activation by AngII/AngIII acting through the Mas receptor. This Ang1–7/Mas axis plays an antitumorigenesis role, decreasing growth and invasiveness in some kinds of cancer. *in vitro*, Ang1–7/Mas signaling inhibition increased cell invasion and proliferation in glioblastoma multiforme (Liu et al., 2015; Ni et al., 2012; Wegman-Ostrosky et al., 2015). However, the conflict that arose in females between ASAP and APA activity would once again be present at this level of the cascade, since as we have commented, the first activity tends to increase while the second is inhibited. In this regard, an additional role for APA not related to neovascularization but related to the permeabilization of the blood brain barrier have been proposed (Juillerat-Jeanneret et al., 1997, 2000).

In the same way, the decrease found in APN and IRAP activities in both male and female animals also allow us to infer a decreased catabolism of angiotensin III towards angiotensin IV, which would again enhance AngIII effects. Special mention should be made of APB activity, which is significantly increased in male animals but unchanged in female animals, which could indicate a hormone-dependent alternative function to the conversion of AngIII to AngIV as explained above and that should be further investigated. However, a limited action of APB on AngIII has been described (Ohnishi et al., 2020), also supporting the idea that APB is involved in generating additional active peptides by cleaving extended amino-terminal arginine and lysine residues.

An additional fact is to consider that IRAP also acts as a receptor for angiotensin IV (AT4), so that the alteration of the enzymatic activity,

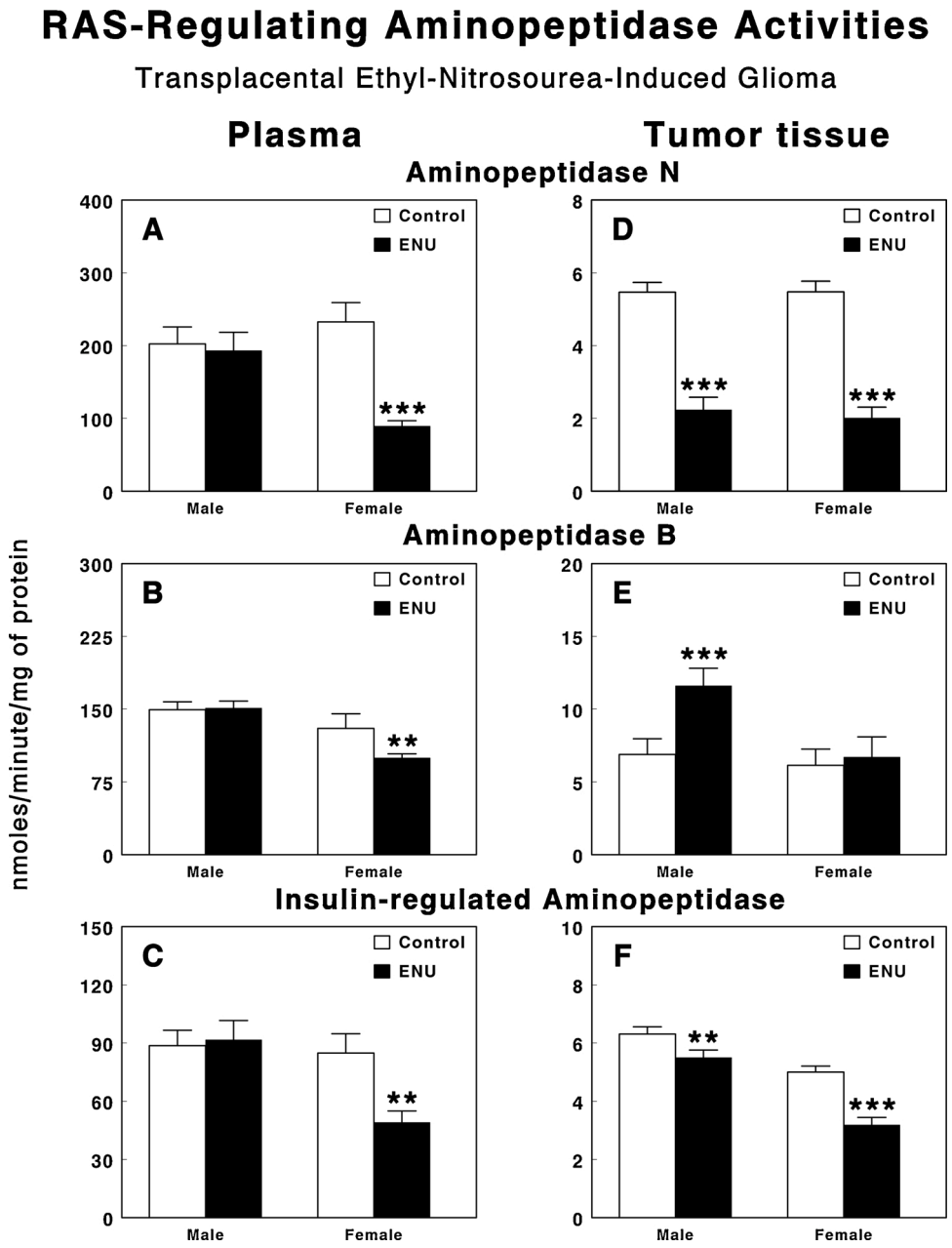

Fig. 3. Plasma (A, B, C) and tumor tissue (D, E, F) aminopeptidase N, aminopeptidase B and insulin-regulated aminopeptidase specific activities in male and female healthy rats vs. rats with N-ethyl-N-nitrosourea (ENU)-induced brain tumors. Results are expressed in nanomoles of their corresponding aminoacyl-β-naphthylamide hydrolyzed per min and per mg of protein (mean ± SEM; n = 6; **p < 0.01; ***p < 0.001).

which would promote a lower amount of angiotensin IV, could also reflect a lower activation of the AT4 receptor. Additionally, the changes that we have found in the circulating activity of aminopeptidases N, B and IRAP in female animals exclusively would also be in favor of suggesting an increased activity of angiotensin III, through regulatory mechanisms that would be different in males and females, thus suggesting a hormonal dependence. We should not lose sight of the fact that our group has already described the existence of several sex differences in tumor development in glioma as stated above.

Finally, IRAP is also related to the glucose transporter (GLUT) type 4 (GLUT4). IRAP is located together to the insulin-dependent glucose transporter GLUT4 in specialized vesicles. IRAP participates in the tethering and/or trafficking of these vesicles (Fernando et al., 2008; Keller, 2003; Keller et al., 2002), whereas the transporter facilitates glucose uptake into cells. Angiotensin IV enhanced this activity-dependent glucose uptake in some brain regions, but not others, supporting that the modulation of glucose uptake by angiotensin IV is region-specific and is critically dependent on a high degree of colocalization between IRAP and GLUT4. Several isoforms of GLUT have been analyzed in biopsies of glioblastoma, demonstrating the expression of GLUT1, GLUT3, and GLUT4 genes. However, only the transcripts of GLUT1 and GLUT3 were detected by northern blot, suggesting that the expression of GLUT4 is relatively low. Therefore, the facilitative glucose transport may be altered in tumor cells and thus promotes significant changes in glucose metabolism (Nagamatsu et al., 1993), which is in line with our results. In addition, the use of GLUT inhibitors enhance the action of BCNU and temozolomide against high-grade glioma (Azzalin et al., 2017), also supporting an unexplored role of IRAP in glioma treatment.

5. Conclusions

We can therefore conclude that gliomagenesis induced by transplacental administration of ENU shows an important participation of the local RAS in both male and female animals. Even in female animals, some of these alterations are reflected at the level of the circulating RAS, which demonstrates the existence of sex differences in the processes related to the development of this type of tumor. Likewise, RAS regulatory proteolytic enzymes are also a potential target for the treatment of this type of tumors. Their pharmacological manipulation can be as interesting as that of other RAS elements more analyzed, such as receptor blockers or ACE inhibitors, either alone or in combination with other drugs, which represents an innovative field of study due to proteolytic enzymes of RAS have not been given sufficient importance in the treatment of tumors.

CRediT authorship contribution statement

MJ Ramírez-Expósito: Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Project administration, Funding acquisition. **MP Carrera-González:** Conceptualization, Methodology, Investigation, Writing - review & editing. **JM Martínez-Martos:** Conceptualization, Methodology, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

None to declare.

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