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Plasma renin–angiotensin system-regulating aminopeptidase activities are modified in early stage Alzheimer's disease and show gender differences but are not related to apolipoprotein E genotype

María del Carmen Puertas [a,1], José Manuel Martínez-Martos [a,1], Manuela Cobo [b], Pedro Lorite [c], Rosa María Sandalio [c], Teresa Palomeque [c], María Isabel Torres [d], María Pilar Carrera-González [a], María Dolores Mayas [a], María Jesús Ramírez-Expósito [a,*]

[a] Experimental and Clinical Physiopathology Research Group, Department of Health Sciences, School of Experimental Sciences, University of Jaén, Jaén, Spain
[b] Division of Neurology, University Hospital of Jaén, Jaén, Spain
[c] Unit of Genetics, Department of Experimental Biology, University of Jaén, Jaén, Spain
[d] Unit of Cell Biology, Department of Experimental Biology, University of Jaén, Jaén, Spain

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ABSTRACT

Alterations in blood pressure and components of the renin–angiotensin system (RAS) contribute to the development and progression of Alzheimer's disease (AD), resulting in changes that can lead or contribute to cognitive decline. Aspartyl aminopeptidase (ASAP), aminopeptidase A (APA), aminopeptidase N (APN) and aminopeptidase B (APB) catabolise circulating angiotensins, whereas insulin-regulated aminopeptidase (IRAP) has been described as the AT_4 receptor. We have found in AD patients a significant decrease of APA activity in men but not in women, and of APN, APB and IRAP in both genders, when compared with control subjects. No changes were found in ASAP activity. Also, APN, APB and IRAP but not APA correlated with the Mini–Mental test, but no relationship with APOE genotype was found. We conclude that several components of the RAS are modified in AD patients, with gender differences. Furthermore, ROC analysis indicates that APN, APB and IRAP activities could be useful non-invasive biomarkers of AD from the earliest stages.
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1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder worldwide (Wray and Noble, 2009). It is characterized neuropathologically by the formation of senile plaques of ß-amyloid (Aß) and neurofibrillary tangles, and clinically by the progressive deterioration of memory and other cognitive functions (Frykman et al., 2010).

The accumulation and deposition of Aß in selective brain regions are major causes of neurotoxicity and are assumed to be a culprit for inducing the pathologic processes of AD (Puertas et al., 2012; Roychaudhuri et al., 2009; Yang and Teplow, 2008). However, several population-based studies demonstrated that elevated blood pressure in mid-life was associated with elevated risk for later development of AD (de la Torre, 2012; Skoog and Gustafson, 2006).

Hypertension, a risk factor for cerebrovascular and coronary heart diseases (Skoog and Gustafson, 2006), is often treated by administration of angiotensin-converting enzyme (ACE) inhibitors (ACEIs) or angiotensin receptor blockers (ARBs) which act to inhibit the production and action of angiotensin II (AngII), respectively, that occur through the renin–angiotensin system (RAS). However, these medications, in addition to lessening cerebrovascular and coronary morbidities and mortalities also seem to preserve cognitive function (Papademetriou, 2005).

The RAS has classically been identified in reno-cardiovascular organs including the kidney and the heart and vessel walls, where its enzymatic actions and released peptides lead to blood pressure

* Corresponding author at: Experimental and Clinical Physiopathology Research Group, Department of Health Sciences, School of Experimental Sciences, University of Jaén, Campus Universitario Las Lagunillas, E-23071, Jaén, Spain. Tel.: +34 953 21 20 10; fax: +34 953 21 29 43.
E-mail address: mramirez@ujaen.es (M.J. Ramírez-Expósito).
[1] Both authors contributed equally to this work.

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regulation and electrolyte/fluid homeostasis. However, current evidence points to the existence of a paracrine, locally acting RAS within almost every organ in the body. Thus, a complete and functional RAS is also present in the brain and is independent from the RAS of renal origin (Ganten et al., 1971; Grobe et al., 2008). Angiotensinogen (AGT), synthesized by neurons and glial cells (Kumar et al., 1988), is converted into the inactive decapeptide angiotensin I (AngI) by the action of renin. ACE, the principal effector of RAS, further converts AngI to AngII that acts on angiotensin II type 1 and 2 receptors (AT_1R and AT_2R). Angiotensin II degradation begins with the action of aspartyl aminopeptidase (ASAP) and aminopeptidase A (APA), which remove the N-terminal Asp to produce angiotensin III (AngIII), a less potent vasoconstrictor peptide than AngII (Le Noble et al., 1991; Marc and Llorens-Cortes, 2011). AngIII is also produced from angiotensin I (AngI) through the production of des-Asp1-AngI, which is further converted to AngIII by the action of ACE. AngIII is further converted to angiotensin IV (AngIV) by aminopeptidase B or aminopeptidase N (Ardaillou and Chansel, 1997). Whereas AngI is considered inactive, AngII and AngIII are full agonists at the AT_1 and AT_2 receptors subtypes (AT_1R and AT_2R). Also, AngIV binds with low affinity at the AT_1R and AT_2R, but with high affinity and specificity at the AT_4 receptor subtype (AT_4R). Furthermore, there is evidence that the AT_4R may be the insulin-regulated aminopeptidase (IRAP) (Albiston et al., 2001; Chai et al., 2008) (Fig. 1).

In addition to the classical functions on salt and water homeostasis and regulation of blood pressure, the RAS that is present in the central nervous system (CNS) appears to participate in the regulation of multiple brain functions including processing of sensory information, learning, memory, and emotional responses (von Bohlen and Albrecht, 2006).

Several pathological processes such as stroke, depression, and emotional stress, as well as AD, have been suggested to be associated with dysregulation of the brain RAS (Phillips and de Oliveira, 2008). Increased ACE activity has been demonstrated in different brain regions as well as in CSF of patients with AD (He et al., 2006; Miners et al., 2008; Miners et al., 2009), whereas a decrease was found in plasma (Vardy et al., 2009). Moreover, AT_1R (Savaskan et al., 2001) and AT_2R (Ge and Barnes, 1996) have also been found to be increased in AD cortex suggesting an augmented brain RAS activity during the disease process (Mogi and Horiuchi, 2013).

To further support the importance of RAS in AD, AT_1R blockers and ACE inhibitors commonly used in the treatment of hypertension have been associated with lower incidence of clinically diagnosed AD and improved cognitive function (Davies et al., 2011; Kehoe and Passmore, 2012; Kehoe et al., 2013; Khachaturian et al., 2006; Li et al., 2010). On the contrary, little is known about the role of AngIII and AngIV, about the proteolytic enzymes involved in their production and about the role of AT_4R/IRAP in AD.

In the present work, we analyze RAS-regulating aminopeptidase and IRAP activities in men and women patients diagnosed of AD at early stage to 1) better understand the relationship between the proteolytic regulatory enzymes of the RAS and AD; 2) to know the putative steps of RAS cascade modified as a consequence of the illness; and 3) to take into consideration the value of RAS-related aminopeptidase activities as biomarkers of AD. Furthermore, as the ε4 allele of apolipoprotein E (APO-ε4) has consistently shown to be related to non-familial onset of AD, we also investigate the association of these enzyme activities with the APOE polymorphism in AD patients. Finally, we have used receptor-operated-characteristic (ROC) curve

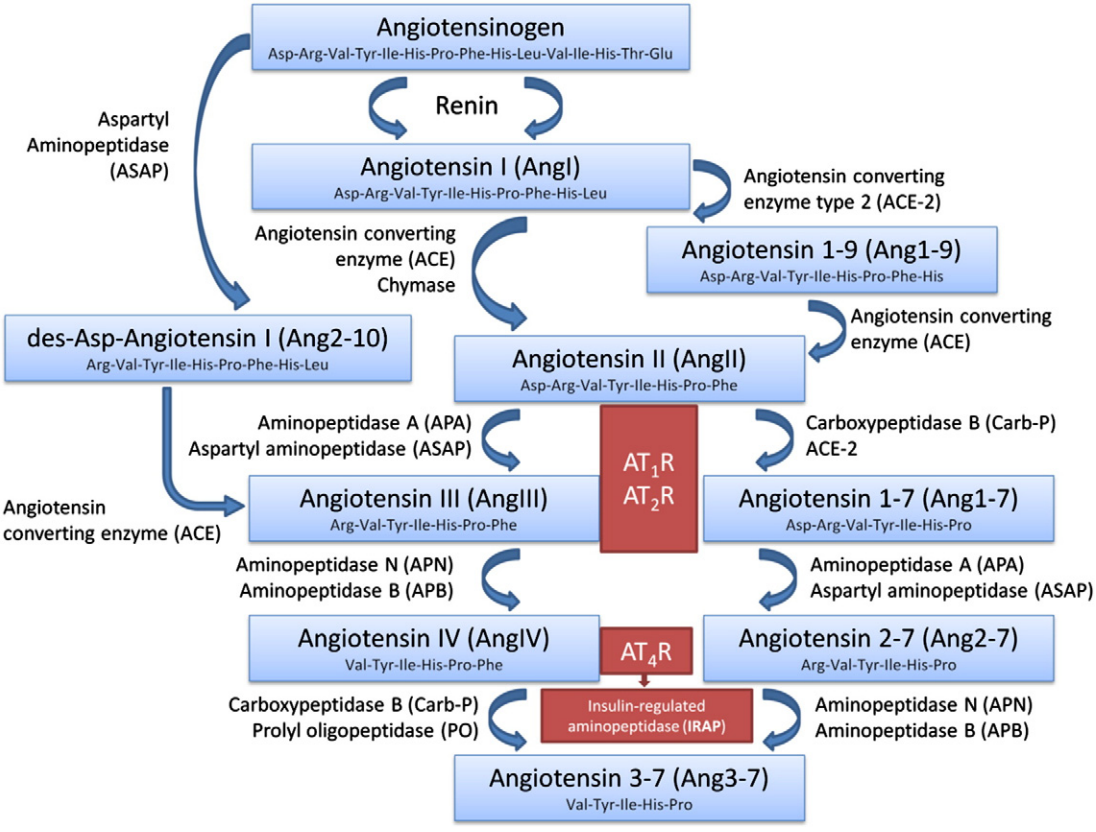

Fig. 1. Angiotensin peptides are derived from the precursor protein angiotensinogen through several enzymatic conversion pathways. The decapeptide angiotensin I (AngI) is formed by renin acting upon the amino terminal of angiotensinogen. AngI serves as a substrate for angiotensin converting enzyme (ACE), a zinc metalloprotease that hydrolyzes the carboxyl terminal dipeptide His-Leu to form the octapeptide angiotensin II (AngII). AngII is converted to the heptapeptide angiotensin III (AngIII) by aminopeptidase A (APA) that cleaves the Asp residue at the N-terminal. Aminopeptidase N (APN) cleaves Arg at the N-terminal of AngIII to form the hexapeptide angiotensin IV (AngIV). AngIV can be further converted to inactive peptide fragments and amino acid constituents. Angiotensins exert their actions through the different angiotensin receptor subtypes AT_1, AT_2 and AT_4. The AT_4 receptor has been proposed to be the insulin-regulated aminopeptidase (IRAP). Other conversion steps are also showed.

analysis to analyze how accurate RAS-related aminopeptidase activities are in identifying diseased cases.

2. Material and methods

2.1. Subjects

The subjects of this study were 46 individuals with AD (20 males; age 76.0 years ± 1.64; 26 females; age 73.96 years ± 1.49) and 46 healthy age-comparable controls (16 males; age 73.25 years ± 1.56; 30 females; age 73.83 years ± 1.24). Subjects were recruited from the Unit of Neurology of the University Hospital "Ciudad de Jaén", and had not histories of hypertension nor were taking antihypertensive drugs. Patients received diagnosis of AD if they met DSM-IV clinical criteria for dementia, and received a diagnosis of probable or possible AD according to NINCDS/ADRDA (National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association) criteria. People with AD who had comorbidity with other clinical major neurological illness were excluded.

Cognitive and functional status of AD patients was assayed by Mini–Mental State Examination (Men 23.7 ± 0.92; women 20.7 ± 0.66); Blessed Scale (men 6.50 ± 0.74; women 7.28 ± 0.69) and Brief Cognitive Rating Scale (BCRS) adapted as Functional Assessment Stage (FAST) (men 3.71 ± 0.22; women 3.78 ± 0.16).

Study participants' fasting blood samples were collected in the morning in tubes with heparin and centrifuged immediately. Plasma samples were stored at −80 °C until measurement.

The research protocol was approved by the local Clinical Research Ethical Committee at University Hospital of Jaén. All patients and their legal's guardians provided written informed consent.

2.1.1. Genomic extraction and APOE genotyping

Genomic DNA was extracted from blood using commercial kits according to the manufacturer's procedure. APOE genotyping was performed by a direct PCR-based RFLP (restriction fragment length polymorphism) method, as described by Hixson and Vernier (1990). A 244-bp APOE fragment was amplified by PCR using the primers APOE-F, 5′-TAAGCTTGGCACGGCTGTCCAAGGA and APOE-R, 5′-ACAG AATTCGCCCCGGCCTGGTACACTGCC. PCR reactions were set up in a 25 μl mixture containing 75 ng of genomic DNA, 0.4 mM dNTPs, 1.5 mM $MgCl_2$, 10% DMSO, 10 pmol of each primer and 1 U of Taq polymerase, using the following cycling profile: initial denaturation at 94 °C (2 min), 30 cycles at 94 °C (30 s), 56 °C (30 s), 72 °C (1 min), with a final elongation step of 72 °C for 4 min. PCR products were directly digested with 1 U of *Hha*I and the products visualized on a 4% Tris-buffered EDTA agarose gels. Each genotype gives a specific combination of *Hha*I fragment sizes (Hixson and Vernier, 1990).

2.2. Renin–angiotensin system-regulating aminopeptidase activities assay

2.2.1. Aspartyl aminopeptidase activity assay

Plasma ASAP was determinated fluorometrically using aspartyl-ß-naphthylamide (AspNNap) as the substrate. Briefly, 10 μl 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 $MnCl_2$ 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.

2.2.2. Aminopeptidase A activity assay

Plasma APA activity was measured in the same way using glutamyl-ß-naphthylamide (GluNNap) as the substrate. Ten microlitres 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 $CaCl_2$ 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.

2.2.3. Aminopeptidase N activity assay

Plasma APN was measured fluorometrically using alanyl-ß-naphthylamide (AlaNNap) as substrate. Ten microlitres of each sample was incubated by triplicate for 30 min at 37 °C with 100 μL of the substrate solution containing 100 μM of AlaNNap and 0.65 mM dithiothreitol (DTT) in 50 mM phosphate buffer, pH 7.4.

2.2.4. Aminopeptidase B activity assay

Plasma APB was measured fluorometrically using arginyl-ß-naphthylamide (ArgNNap) as substrate. Ten microlitres of each sample was incubated by triplicate for 30 min at 37 °C with 100 μL of the substrate solution containing 100 μM of ArgNNap and 0.65 mM dithiothreitol (DTT) in 50 mM phosphate buffer, pH 7.4.

2.2.5. Insulin-regulated aminopeptidase activity assay

Plasma IRAP was measured fluorometrically using leucyl-ß-naphthylamide (LeuNNap) as substrate. Ten microlitres of each sample was incubated by triplicate for 30 min at 37 °C with 100 μL of the substrate solution containing 100 μM of LeuNNap and 0.65 mM dithiothreitol (DTT) in 50 mM phosphate buffer, pH 7.4.

All the reactions were stopped by adding 100 ml of 0.1 M acetate buffer, pH 4.2. The amount of ß-naphthylamine released as the result of the enzymatic activities was measured fluorometrically at 412 nm emission wavelength and excitation wavelength of 345 nm. Proteins were quantified also in triplicate by the method of Bradford, using bovine serum albumin (BSA) as standard. Specific enzyme activities were expressed as picomoles of the corresponding aminoacyl-β-naphthylamide hydrolyzed per min per mg of protein, by using a standard curve prepared with the latter compound under corresponding assay conditions.

2.3. Statistical analysis

All values represent the mean of the individual determination ± standard error of the mean (SEM). Data were analyzed by MANOVA plus Newman–Keuls test, using IBM Pass V.19 software. ROC curves were analyzed and the area under the curve (AUC), sensitivity and specificity was calculated using MedCalc 12.2.1 package. Values of $P < 0.05$ were considered significant.

3. Results

Specific RAS-regulating ASAP, APA, APN and APB activities in plasma of AD patients and control group are shown in Figs. 2–6.

Whereas ASAP activity did not show differences between control and AD groups (Fig. 2A) or gender differences (Fig. 2B), the analysis of APA activity showed a significant decrease ($p < 0.01$) in AD patients when compared to control groups (Fig. 3A). By gender, APA activity was significantly lower ($p < 0.001$) only in men with AD, whereas no significant changes were observed in women with AD when compared to their respective control group (Fig. 3B). However, control women showed significant lower levels ($p < 0.05$) of APA specific activity than control men. Regarding APN (Fig. 4A), APB (Fig. 5A) and IRAP (Fig. 6A) specific activities, we found significantly decreased plasma levels in all activities ($p < 0.001$) in AD patients. These significant decreases ($p < 0.001$) were observed in both men and women AD patients for APN (Fig. 4B), APB (Fig. 5B) and IRAP (Fig. 6B). However, none of these changes were associated with −ε4 or +ε4 carriers in AD patients (Table 1). Fig. 7 shows the ROC curves obtained for APA, APN, APB and IRAP activities. For APA activity, the optimal balance between sensitivity and specificity was found at a cut-off of 5.974. The overall diagnostic accuracy showed an AUC = 0.665 ± 0.061, a sensitivity of 90.9 and a specificity of 38.1. For APN activity, the optimal

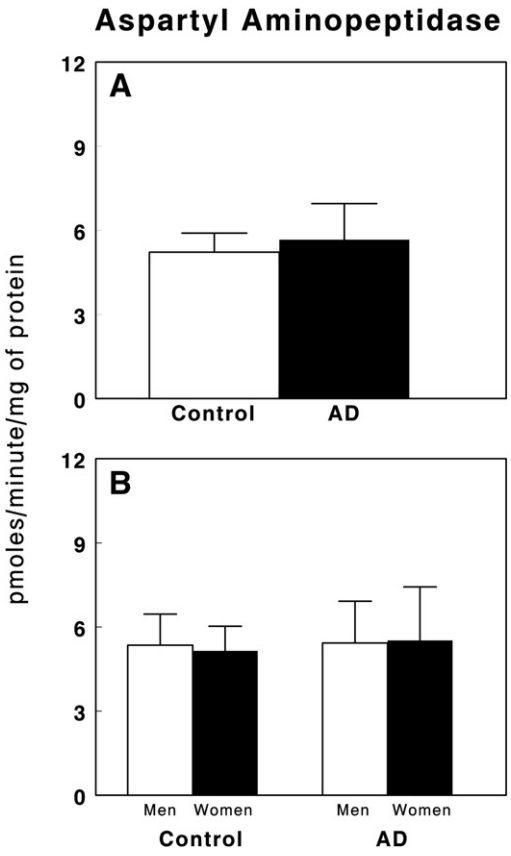

Fig. 2. Specific aspartyl aminopeptidase activity in plasma of patients with AD and their respective controls (A) and their gender differences (B). Results are expressed in pmol/min/mg of protein (mean ± SEM; n = 46 (16 control males, 30 control females, 20 AD males and 26 AD females)).

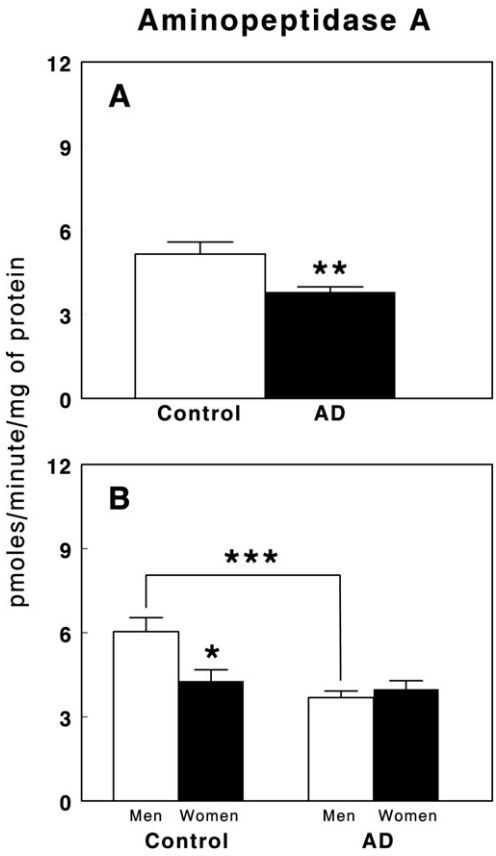

Fig. 3. Specific aminopeptidase A activity in plasma of patients with AD and their respective controls (A) and their gender differences (B). Results are expressed in pmol/min/mg of protein (mean ± SEM; n = 46 (16 control males, 30 control females, 20 AD males and 26 AD females); *p < 0.05; **p < 0.01; ***p < 0.001).

balance between sensitivity and specificity was found at a cut-off of 164.84. The overall diagnostic accuracy showed an AUC = 0.752 ± 0.056, a sensitivity of 62.8 and a specificity of 82.1. For APB activity, the optimal balance between sensitivity and specificity was found at a cut-off of 74.87. The overall diagnostic accuracy showed an AUC = 0.768 ± 0.053, a sensitivity of 74.4 and a specificity of 71.1. Finally, for IRAP activity, the optimal balance between sensitivity and specificity was found at a cut-off of 85.04. The overall diagnostic accuracy showed an AUC = 0.813 ± 0.048, a sensitivity of 90.9 and a specificity of 61.5. Finally, APN, APB, and IRAP but not APA specific activities correlated significantly with the Mini–Mental State Examination test (Pearson's correlation coefficient of 0.331 for APN (p = 0.025); 0.337 for APB (p = 0.022); and 0.375 (p = 0.010) for IRAP). Scatter diagrams are presented in Fig. 8.

4. Discussion

In this study we have found that overall, AD patients have significantly lower plasma RAS-related APA, APN, APB and IRAP specific activities compared to the healthy individuals, although APA activity decrease is found in men but not in women, indicating gender differences for this activity. Furthermore, these changes are not associated with the presence of APOE-ε4 allele, indicating that APOE-ε4 seems not to be related to the role of RAS in AD pathogenesis.

To date, ACE activity has been the component of the RAS most studied in AD. Since it was reported that ACE activity was increased in homogenates of post-mortem brain tissue from patients with AD and that the activity correlated with Aß load, several reports have showed that ACE correlates with AD severity (Barnes et al., 1991; Miners et al., 2008, 2009; Savaskan et al., 2001). However, studies of other RAS components in AD are keenly awaited. Initial investigations demonstrated increased AngII and AngII receptor binding and immunolabeling in AD brain (Abdalla et al., 2009; Savaskan et al., 1991, 2001). The decrease in APA activity observed in our AD group would support the fact that AngII levels were increased because the metabolism of AngII to AngIII was decreased and therefore the effect of AngII was predominant. However, this occurs in men, but not in women. In addition, our results also showed significant decreases in APN and APB activities, both involved in the metabolism of AngIII to AngIV. These data may also indicate that the conversion of AngIII to AngIV is also diminished and, therefore, AngIV functions. As a result, the effects of AngIII are potentiated (in a similar way to those of AngII), which could be extremely important in women with AD, who have not been seen modified their APA activity.

It is of great interest to notice that it has been evidenciated the possible involvement of AngII (and possibly AngIII as well) and AngIV in disorders of cognition. AngII inhibits potassium-mediated release of acetylcholine (Barnes et al., 1992). AngII also influences TNFα and TGFß signaling (Hamdi and Castellon, 2004; Suzuki et al., 2003), blood–brain barrier maintenance (Wosik et al., 2007) and cell survival (via AT_1R and AT_2R receptors) and through modulation of the activity of plasmin, another Aß degrading enzyme, all of potential relevance to AD (Hamdi and Castellon, 2004). Hippocampal AngII-immunopositive neurons with distorted processes were described in senile plaques (Savaskan et al., 1991). There is also evidence that pharmacological inhibition of AngII can reverse scopolamine-induced cognitive impairment (Gard, 2004). Intracerebroventricular administration of AngII also causes spatial memory impairment, reduces cerebral blood flow and acetylcholine levels, and induces oxidative stress (Tota et al., 2013). In contrast, AngIV, a metabolite of AngII, was found to facilitate

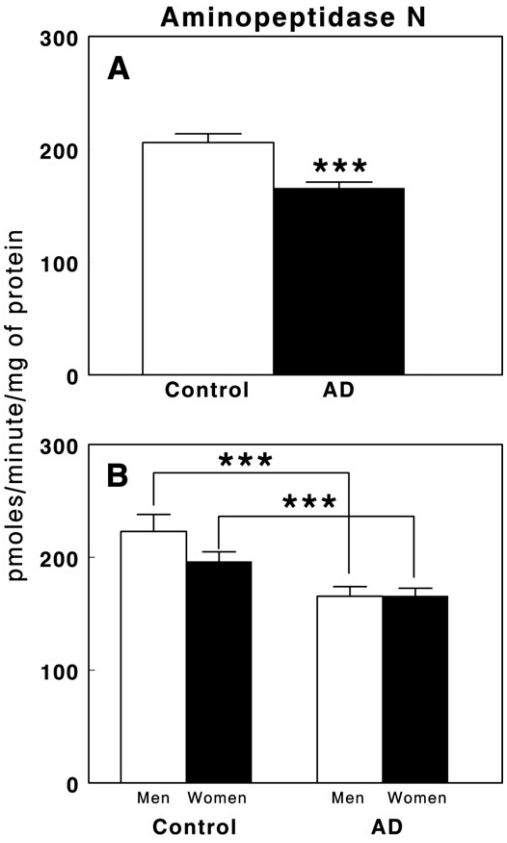

Fig. 4. Specific aminopeptidase N activity in plasma of patients with AD and their respective controls (A) and their gender differences (B). Results are expressed in pmol/min/mg of protein (mean ± SEM; n = 46 (16 control males, 30 control females, 20 AD males and 26 AD females); ***p < 0.001).

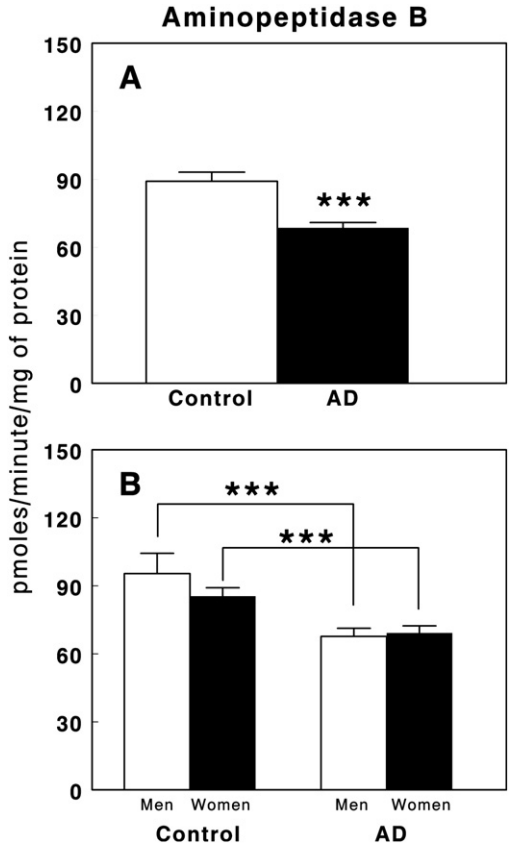

Fig. 5. Specific aminopeptidase B activity in plasma of patients with AD and their respective controls (A) and their gender differences (B). Results are expressed in pmol/min/mg of protein (mean ± SEM; n = 46 (16 control males, 30 control females, 20 AD males and 26 AD females); ***p < 0.001).

memory in various in vivo paradigms and promote long-term potentiation (Chai et al., 2008). AngIV is thought to mediate these effects through binding of IRAP, its putative 'receptor', which is abundantly expressed in regions of the brain involved in memory and which also facilitates the uptake of glucose by the insulin-responsive glucose transporter GLUT4 (Chai et al., 2008).

Research is underway to identify compounds that mimic the effects of AngIV on IRAP and that could serve as cognitive enhancers. At least in theory, increased availability of unbound AngII (e.g. through the action of ARBs) could increase its conversion to AngIV, contributing to some of the cognitive benefits of ARBs. Furthermore, Yang et al. (2011) have described a protective effect of AngIV via AT_4 receptors against AngII effects mediated by AT_1 receptors, being these routes of protection also diminished in AD patients. At present it is clear that AngIV is effective at facilitating spatial learning as measured using the Morris water maze (Wright et al., 2008; Wright and Harding, 2013), or Barnes circular maze, tasks of spatial learning (Lee et al., 2004). However, the identity and signaling mechanism of the AT_4 receptor has yet to be determined. There is the recent suggestion that AngIV may facilitate cognitive processing by promoting glucose uptake into hippocampal neurons (De Bundel et al., 2009). It is likely that many of the memory facilitating effects initially attributed to AngII are due to the conversion of AngII to AngIII and then to AngIV, which then acts at the AT_4 receptor subtype (Chai et al., 2004; Wright and Harding, 2010, 2013; Wright et al., 2008). This hypothesis is consistent with the finding that ARBs have cognitive enhancing effects (Basso et al., 2005; Davies et al., 2011; Ellul et al., 2007; Hajjar et al., 2005; Khachaturian et al., 2006; Li et al., 2010) presumably due to blockade of the AT_1 receptor subtype thus permitting conversion of endogenous AngII to AngIII to AngIV and activation of the AT_4 receptor subtype. This notion was recently supported by Braszko et al. (2006). It is also consistent with the majority finding that ACE inhibitors facilitate cognitive processing in that the increased AngI levels are likely converted to Ang(1–9) and then to AngII, III, IV and Ang(3–7) (Wright and Harding, 2010, 2013).

Taken together these findings support a new approach to understanding the memory enhancing effects of brain angiotensins. Several observations and research findings are relevant to the interpretation of this model (Wright and Harding, 2010). Firstly, the heaviest brain distributions of the AT_4 receptor subtype are in neocortex, hippocampus, amygdala, and nucleus basalis of Meynert, consistent with expectations concerning central locations for a mediator of cognitive processing (Chai et al., 2000; Gard, 2002; Harding et al., 1992; Wright et al., 2008). Secondly, this receptor subtype's ability to facilitate LTP, separate from N-methyl-D-aspartate (NMDA)-dependent LTP, suggests a nonglutamatergic signaling pathway (Davis et al., 2006). Thirdly, the finding that facilitation of the AT_4 receptor subtype results in increased internalization of calcium via at least three different calcium channels suggests a rapid and salient cell signaling event (Davis et al., 2006). And finally, the coupling of increased neural intracellular calcium with matrix metalloproteinase release into the extracellular space suggests a neural plasticity function (Meighan et al., 2006, 2007). Conversion of AngII to AngIII to AngIV appears to be necessary for AngII-induced dopamine release in the striatum (Stragier et al., 2004) and Ach release in the hippocampus (Lee et al., 2004).

Our investigation opens also the door to the treatment of AD trough and enzyme replacement therapy approach, administrating the properly functional proteolytic regulatory enzyme (APA, APN, APB and/or IRAP). A succeeding procedure requires that a different set of barriers must be surmounted. Thus, injection of foreign proteins into a patient will cause an immune response, the half-life of injected

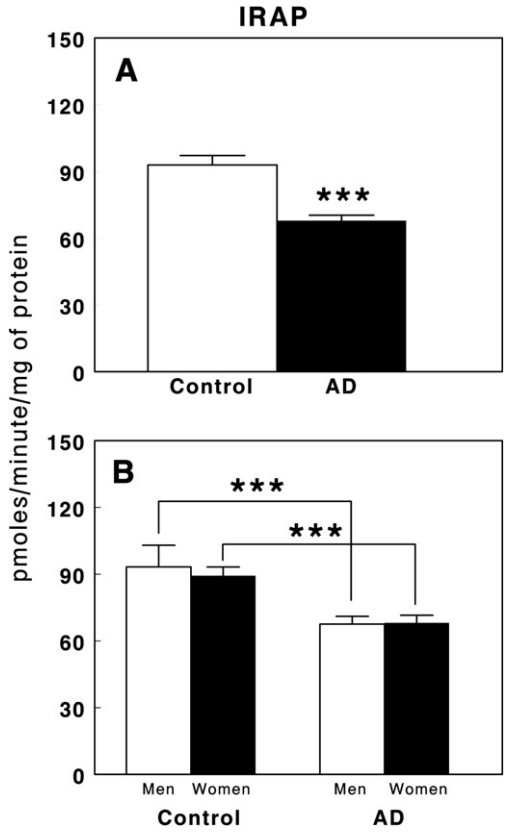

Fig. 6. Specific insulin-regulated aminopeptidase activity in plasma of patients with AD and their respective controls (A) and their gender differences (B). Results are expressed in pmol/min/mg of protein (mean ± SEM; n = 46 (16 control males, 30 control females, 20 AD males and 26 AD females); ***p < 0.001).

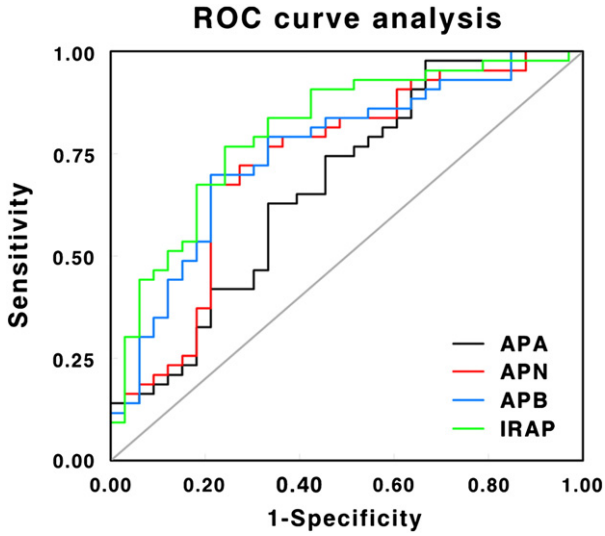

Fig. 7. Analysis using area under the curve (AUC) from receiver operator characteristic (ROC) curves showing differentiation between AD patients and healthy controls based on aminopeptidase A (APA), aminopeptidase N (APN), aminopeptidase B (APB) and insulin-regulated aminopeptidase (IRAP) specific activities (AUC = 0.66, 0.75, 0.76 and 0.81 respectively).

proteins can be quite short, and to be most effective the protein must become localized in the cells where the deficiency is manifest. An additional potential complication in the use of enzyme replacement therapy for the treatment of neurological disorders is the presence of a blood–brain barrier which serves as the gatekeeper controlling access to a neurological system. This structure provides a physical barrier, by means of tight junctions composed of membrane proteins and lipids that seal the gaps between endothelial cells, a chemical barrier that regulates the transport of material through these cells and pumps foreign substances away from the brain, and a metabolic barrier that hydrolyzes and inactivates toxic compounds. Thus, the blood–brain barrier effectively protects that brain against foreign substances, but also limits access to many therapeutic agents designed to treat neurological disorders. However, successful results have been described in both animal models of phenylketonuria (Gamez et al., 2004) and Canavan disease (Zano et al., 2011), and in children with severe combined immune deficiency caused by a defect in purine metabolism (Booth and Gaspar, 2009). To that, surface modifications of proteins can decrease the immunogenicity of foreign proteins. In this way, the introduction of polyethylene glycol polymers through covalent modifications of exposed protein functional groups has been shown to decrease the immune response to these modified proteins, increase their circulation half-life (Zano et al., 2011) and is able to traverse the blood–brain barrier. Therefore, further research must decide if enzyme replacement therapy is a viable approach for the treatment of AD and its putative adverse effects.

Finally, we have also evaluated the potential value of RAS-related aminopeptidase activities as diagnostic biomarkers of AD in its earliest stages, using ROC curve analyses. It is important because there is a

Table 1
Comparison of plasma RAS-regulating aminopeptidase activities ASAP, APA, APN, APB and IRAP between control and AD subjects with +ε4 and −ε4 carriers and gender differences.

n (%)	Control			AD patients						P value
	Men	Women	Whole population	Men		Women		Whole population		
				+ε4 allele	−ε4 allele	+ε4 allele	−ε4 allele	+ε4 allele	−ε4 allele	
Specific activity[1]				5 (25%)	15 (75%)	16 (61.5%)	10 (38.5%)	21 (45.7%)	25 (54.3%)	
ASAP	5.45 ± 1.1	3.78 ± 0.8	4.62 ± 0.7	3.71 ± 2.05	4.68 ± 1.1	3.92 ± 1.1	3.57 ± 1.6	3.81 ± 2.1	4.12 ± 1.3	n.s.
APA	6.13 ± 0.5	4.80 ± 0.4[a]	5.47 ± 0.3[b]	3.82 ± 2.6	3.65 ± 1.5	3.81 ± 0.3	4.05 ± 0.4	3.82 ± 0.2	3.7 ± 0.2	[a]P < 0.05 [b]P < 0.01
APN	213.3 ± 10.9	190.4 ± 8.2	201.9 ± 6.8[c]	165.5 ± 14.6	165.4 ± 8.4	174.6 ± 8.2	152.0 ± 10.3	172.4 ± 8.3	160.1 ± 5.3	[c]P < 0.001
APB	85.29 ± 4.9	83.04 ± 3.7	84.1 ± 5.1[d]	67.9 ± 6.6	57.5 ± 3.8	73.34 ± 3.7	62.5 ± 4.7	72.05 ± 3.7	65.5 ± 2.4	[d]P < 0.001
IRAP	87.84 ± 4.9	85.9 ± 3.7	86.9 ± 3.1[e]	68.4 ± 7.1	67.2 ± 4.1	71.6 ± 3.9	62.43 ± 5.03	70.9 ± 4.02	65.3 ± 2.5	[e]P < 0.001

n.s. = not significant.
[1] Data are expressed in pmol/min/mg of protein (mean ± SEM).
[a] vs. control men.
[b] Control vs. AD patients.
[c] Control vs. AD patients.
[d] Control vs. AD patients.
[e] Control vs. AD patients.

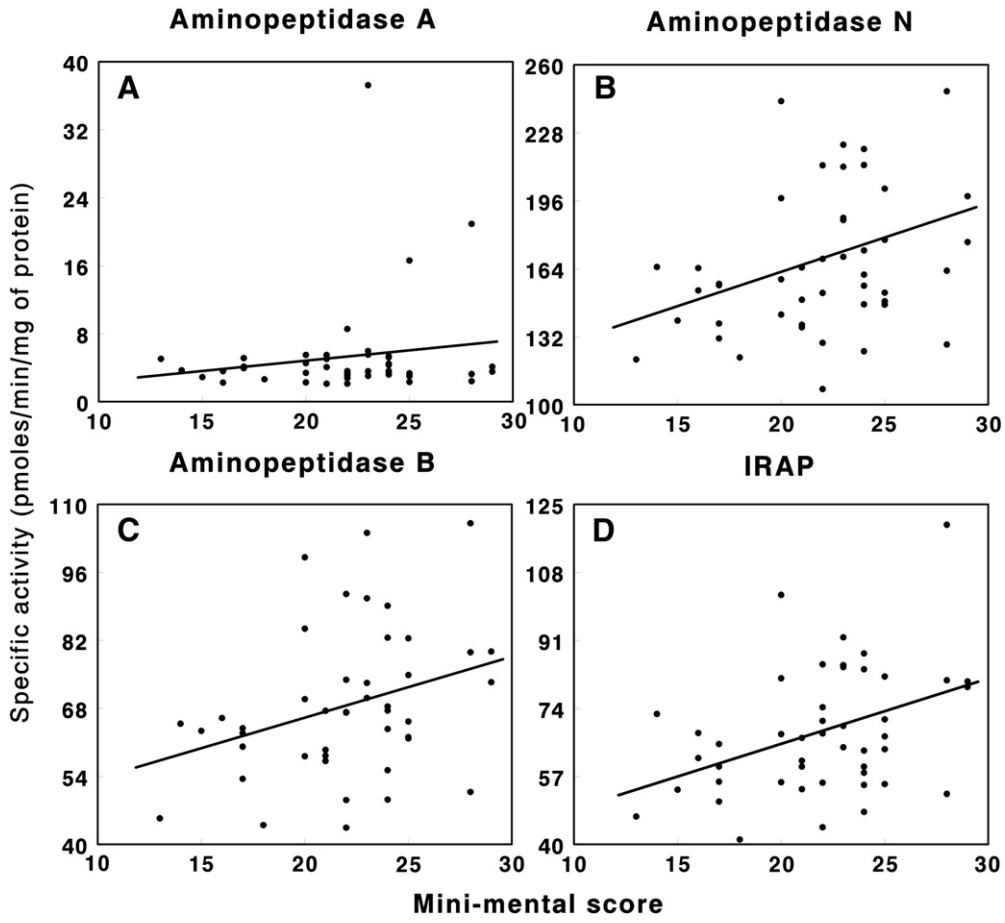

Fig. 8. Scatter plot diagram showing the relationship between plasma aminopeptidase A (A), aminopeptidase N (B), aminopeptidase B (C) and insulin-regulated aminopeptidase (D) specific activities and the Mini–Mental State Examination test in AD patients.

significant need for fast and cost-effective means of screening the rapidly growing elderly segment of the population, and that the ideal AD biomarker would come from blood (Graff-Radford et al., 2007). In our hands, IRAP activity showed the best diagnostic accuracy in AD, although APN and APB activities also show enough good values. In fact, pairwise comparison of ROC curves showed significant differences between IRAP and APB ($p = 0.027$) but not between IRAP and APN. In any case, plasma APN, APB and IRAP activities can be proposed alone or in combination, as rapid, readily accessible and cost-effective for providing routine screening of adults age 65 years and above for Alzheimer's disease. These activities can be also used as an optimal initial screening tool that could be followed up by advanced clinical, neuroimaging and/or CSF analysis.

Although with the limitations of the sample size, the risk of false positives in statistical analyses and the possible overlapping substrate specificity of the different proteolytic regulatory enzymes analyzed, which could contribute to the degradation of the substrates tested in the present report, we can conclude that 1) several components of the RAS are modified in AD patients; 2) some of these modifications are gender-related and 3) their consequences could be the potentiation of AngII and AngIII effects and the block of the protective effects of AngIV. Also, the precise mechanisms through which the different components of this system act on the central nervous system and mediate their various effects, including those on cognition, do not seem to be related to APOE genotype. Therefore, RAS-related aminopeptidases could be used to further investigate the relationship between renin–angiotensin system and AD, to be considered as alternative targets for the treatment of AD including enzyme replacement therapy, and/or to be used as adequate screening tools for the illness.

Conflict of interests

The authors declare no conflict of interest.

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