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The N-domain of angiotensin-converting enzyme specifically hydrolyzes the Arg-5-His-6 bond of Alzheimer's Aβ-(1-16) peptide and its isoAsp-7 analogue with different efficiency as evidenced by quantitative matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Chronic imbalance between production and degradation of the human amyloid-beta peptide (Aβ) is assumed to play an important role in pathogenesis of Alzheimer's disease (AD). Post-translational modifications of Aβ could influence its interactions with specifically cleaving proteases and, therefore, perturb the Aβ homeostasis. The angiotensin-converting enzyme (ACE) was previously shown to degrade non-modified Aβ *in vitro* and in cells. In the presented work, we investigated the effect of isomerization of Asp-7, a common non-enzymatic age-related modification found in AD-associated Aβ species, on hydrolysis of Aβ by ACE. Two synthetic peptides corresponding to the Aβ region 1-16 with either Asp or isoAsp residues in position 7 were examined as monomeric soluble substrates for the N- as well as for the C-domain of ACE. The use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) coupled with the ^{18}O-labeled internal standard approach has allowed us to show that (i) the N-domain of ACE (N-ACE), but not the C-domain, selectively cleaves the Arg-5-His-6 bond in both peptides, and that (ii) N-ACE hydrolyzes the isoAsp-7 analogue more efficiently than the non-modified one. Our results demonstrate a new endopeptidase activity of N-ACE as well as high preference of the domain to recognize and hydrolyze the isomerized Aβ species that were earlier suggested to promote AD pathogenesis. The results suggest the need for further analysis of biological effects of isomerized Aβ and its interaction with ACE in AD pathogenesis. Copyright © 2007 John Wiley & Sons, Ltd.

Alzheimer's disease (AD) is a progressive and fatal neurodegenerative disorder and is the most common cause of dementia in the elderly.[1] The disease is characterized by the extracellular accumulation of a 40-42 amino acid peptide, the human amyloid-β peptide (Aβ), in the AD brains as diffuse and dense neuritic amyloid plaques as well as cerebrovascular deposits.[2,3] *In vivo*, Aβ is normally present in brain and physiological fluids (cerebrospinal fluid, plasma and urine) at low nanomolar levels.[4] According to the amyloid cascade hypothesis of AD, the cerebral Aβ accumulation is the primary factor driving AD pathogenesis. Development of the disease results from a chronic imbalance between Aβ production and breakdown.[5]

The Aβ peptides isolated from AD brains have numerous endogenous post-translational modifications.[6,7] In particular, the L-isoAsp isoform is prevalent among the Asp-7 residues (approx 75%). This isoform has the same molecular mass as the common L-Asp, but differs from it by a one -CH_2- unit

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longer backbone and, accordingly, a one -CH_2- unit shorter side chain. It was suggested that the abundance of these structurally altered aspartyl residues might profoundly affect the conformation of the Aβ peptide within plaque cores and, therefore, significantly impact normal catabolic processes designed to limit deposition of AD plaques.[6] Indeed, it has been shown that substitution of the isoaspartic acid for the aspartic one in Aβ position 7 eliminates the Aβ activation effect on the classical complement pathway,[8] a process which would normally protect against the Aβ-induced neurotoxicity and might reduce accumulation or promote breakdown of degenerating neurons and Aβ plaques.[9] However, it is still unclear whether the isoaspartyl residues are the cause or the result of pathological accumulation of Aβ.[10]

Conformations of soluble Aβ-(1-40...42) species remain unknown because of their time-dependent aggregation under physiologically relevant conditions. However, the 3D structure of model fibrils formed by Aβ-(1-42) was resolved from solid-state nuclear magnetic resonance (NMR) studies *in vitro*.[11] In this structure two conformationally different regions have been observed, namely, residues 18-42 form a β-strand-turn-β-strand motif constituting the Aβ inner intermolecular core whereas residues 1-17 are disordered and located outside of the core thus forming a site that is not necessarily involved in amyloid stabilization and is quite accessible for interactions with partners other than Aβ. Indeed, the N-terminal region 1-16 was identified as the Zn/Cu-binding domain of Aβ.[12–16] Recently, the solution structure of a synthetic peptide corresponding to Aβ-(1-16) in Zn-free and Zn-bound state was resolved.[17] In the absence of Zn ions, the peptide conformation appeared poorly structured in the N-terminal region 1-6, whereas an irregular structure was observed in region 7-15. Binding of a Zn molecule resulted in a well-defined compact structure of the entire peptide Aβ-(1-16) wherein residues His-6, Glu-11, His-13, and His-14 formed the Zn-binding site. It is worth noting that the incorporation of the isomerized aspartate into Aβ-(1-16) position 7 appeared to alter the Zn coordination mode by introducing the residue as a chelator.[17]

The angiotensin-converting enzyme (ACE, dipeptidyl carboxypeptidase, EC 3.4.15.1), a zinc-metalloprotease, plays a key role in blood pressure regulation by cleaving angiotensin I to angiotensin II and inactivating bradykinin.[18,19] ACE is found as a membrane-bound ectoenzyme both in the vascular endothelial and various absorptive epithelial and neuroepithelial cells, as well as in soluble form in plasma and other body fluids. Somatic ACE is a single-chain protein containing two similar regions, termed the N- and C-domains, each possessing an active site. Despite their high homology, the domains are distinguished by their substrate specificities, which are presumed to be physiologically significant.[19]

Usually, ACE acts as an exopeptidase; however, endopeptidase activity of the enzyme can be rarely observed for some specific substrates. The most prominent case of such activity is the hydrolysis of Aβ *in vitro* and in a cellular context. It was shown that ACE as well as its N-domain, but not the C-domain, specifically cleaves the synthetic peptide Aβ-(1-40) into four fragments.[20,21] One of them was identified as Aβ-(8-40) while the rest corresponded to unidentified products of Aβ-(1-7) hydrolysis. Also, it was demonstrated that cellular expression of ACE promoted degradation of naturally secreted Aβ-(1-40) and Aβ-(1-42), leading to significant degradation of both species.[22]

In this study, we hypothesized that isomerization of Asp-7 might affect the ability of ACE to degrade Aβ. To test this suggestion, two monomeric soluble synthetic peptides corresponding to the Aβ region 1-16 and containing Asp or isoAsp residues in position 7 have been used as substrates for the N- or C-domains of ACE *in vitro*. To identify and quantify hydrolysis products, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) was performed in combination with the ^{18}O-labeled internal standard approach.[23]

EXPERIMENTAL

Amyloid-beta peptides

Synthetic peptides (purity >95% checked by reversed-phase high-performance liquid chromatography (RP-HPLC)), Aβ-(1-16/Asp-7) (CH_3CO-Asp-Ala-Glu-Phe-Arg^5-His-Asp-Ser-Gly-Tyr^{10}-Glu-Val-His-His-Gln^{15}-Lys-NH_2) and Aβ- (1-16/isoAsp-7) (CH_3CO-Asp-Ala-Glu-Phe-Arg^5-His-isoAsp-Ser-Gly-Tyr^{10}-Glu-Val-His-His-Gln^{15}-Lys-NH_2), acetylated at the N-terminus and amidated at the C-terminus, were purchased from Sigma-Genosys (The Woodlands, TX, USA). The amino acid sequence of each peptide was confirmed on a 7 T Apex Qe BRUKER ultrahigh-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) by using a *de novo* sequencing approach based on the collision-induced dissociation (CID) fragmentation technique. Analysis of the obtained mass spectra was performed using BioTools software (Bruker Daltonics, Billerica, MA, USA). The presence/absence of isoaspartic acid residues within Aβ-(1-16/isoAsp-7)/Aβ-(1-16/Asp-7) was determined by enzymatic methylation catalyzed by the protein isoaspartyl methyltransferase (PIMT) using the Isoquant isoaspartate detection kit (Promega, Madison, WI, USA) according to the manufacturer's protocols as described previously.[17] The peptide concentrations were determined by absorption spectroscopy using the extinction coefficient of 1450 M^{-1} cm^{-1} at 276 nm.[24]

Reagents

$H_2^{18}O$ with 95–98% ^{18}O content was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and α-cyano-4-hydroxycinnamic acid (HCCA) was from Bruker Daltonics (Bremen, Germany). All other reagents were of analytical grade or better and were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Trypsin was purchased from Promega (Madison, WI, USA). The N- and the C-domains of bovine ACE (N-ACE and C-ACE), homogenous according to sodium dodecyl sulfate polyscrylamide gel electrophoresis (SDS-PAGE), were provided by Dr P.V. Binevski (Moscow State University, Russia). Enzymatic activities of ACE domains were regularly measured by a fluorometric method using the Z-Phe-His-Leu substrate as described previously.[25] Briefly, the 2 mL reaction mixture contained barbital buffer (25 mM, pH 7.4), NaCl

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(50 mM for N-ACE assay or 200 mM for C-ACE assay), $ZnCl_2$ (1 μM), N-ACE (0.02 μM) or C-ACE (0.02 μM), Z-Phe-His-Leu (50 μM), which was added last to initiate the reaction. Incubation was performed at 37°C for 30 min. When assaying samples with lisinopril (10 μM), a 20 min pre-incubation with it was performed before substrate addition. Enzymatic reactions were terminated by the addition of 0.4 mL 2 N NaOH. Samples were developed by adding 1 mL bidistillate water, 0.1 mL of 1% *o*-phthaldialdehyde and after 6 min 0.2 mL 6 N HCl. Fluorescence was measured at an excitation wavelength of 370 nm and an emission wavelength of 500 nm. Fluorescence of a standard solution of His-Leu (10 nM) was measured in duplicate, simultaneously with that of samples and blanks. The N- and C-domain activities were 75 and 17.5 nM His-Leu/min/mg, respectively. Therefore, to keep similar enzymatic activity *in situ* for both ACE domains, the concentration of C-ACE was 5-fold higher than that of N-ACE.

Enzymatic digestions

The hydrolysis of Aβ-(1-16/Asp-7) or Aβ-(1-16/isoAsp-7) by the N- or C-ACE domains was performed during 10–130 min at 37°C in 20 μL of the reaction mixture containing 20 μM of the respective amyloid-beta peptide, 0.02 μM N-ACE or 0.1 μM C-ACE, 50–500 mM NaCl, 1 μM $ZnCl_2$, 50 mM sodium bicarbonate buffer (pH 7.8) or 25 mM barbital buffer (pH 7.4). For MS analysis, a 5 μL aliquot of the reaction mixture was put into 15 μL of 0.5% trifluoroacetic acid (TFA) to obtain an acidic solution (final pH ~3) thus terminating the digestion process; then 0.3 μL of this solution was used to prepare the MALDI probe as described in the next section.

Mass spectrometry

MALDI-TOFMS studies were performed on an Ultraflex instrument (Bruker Daltonics, Bremen, Germany). Mass spectra were acquired in a positive-ion reflector mode, 256–1500 laser shots were summed per spectrum. To prepare the matrix solution, HCCA was dissolved to a concentration of 20 μg/μL in acetonitrile /0.1% TFA (70:30 v/v). Usually, for the MALDI probe preparation, the dried-droplet method was used: 0.3 μL of 2% TFA was mixed with 0.3 μL of the sample (0.5–2 pmol per target) and 0.3 μL of the matrix solution, then loaded onto a MALDI sample plate, and measured by MS.

Quantitative determination of ACE digestion products

The quantitation of ACE digestion products (without previous fractionation of the reaction mixture under analysis) was performed by MALDI-TOFMS using ^{18}O-labeled internal standards as described earlier.[23] To prepare the ^{18}O-labeled internal standards, hydrolysis was performed at 37°C in 25 μL of ^{18}O-water solution containing 20 μM of the Aβ-(1-16/Asp-7) peptide, 50 mM of ammonium bicarbonate (pH 7.8), and 1 μg of trypsin. The reaction was terminated in 48 h by adding 0.25 μL TFA. To obtain the final standard solution, 5 μL of the terminated reaction mixture were added to 45 μL of the matrix solution (see previous section). For quantitation assay, 5 μL of the final standard solution were mixed with an equal volume of an ACE digestion mixture pre-incubated for 20 min, then, 1 μL of the resulted mixture was put directly on the MALDI target plate and subjected to MALDI-TOFMS analysis to produce an isotopic pattern of the corresponding analyte/internal standard mixture. Finally, the previously described algorithm[23] was used to calculate the absolute concentration of the peptide of interest on the basis of experimentally determined isotopic patterns of the analyte and the ^{18}O-labeled standard (of a known concentration) and of the analyte/internal standard mixture. The method error was estimated to be less than 10%.

RESULTS

The N- but not C-domain of ACE hydrolyzes human A*β*-(1-16) variants between Arg-5 and His-6

Oba *et al.*[21] reported that the N-domain of ACE degrades Aβ-(1-40) at the Asp-7-Ser-8 site, whereas, under the same experimental conditions, the C-domain did not show any proteolytic activity related to Aβ. In contrast, Hemming and Selkoe[22] reported that both domains of ACE contributed equally to Aβ degradation. In the present study, to determine which ACE domain was responsible for degradation of the Aβ fragment 1-16 and its isoAsp-7-containing analogue as well as to identify the cleavage site(s), each peptide (20 μM) was incubated in two different buffer systems (see Experimental) at 37°C with either N-ACE or C-ACE for 10, 20, 40, and 130 min. Additionally, these reactions were performed in the presence of lisinopril (10 μM) known as a specific inhibitor of ACE enzymatic activity. Samples from all of the reaction mixtures were subjected to direct MALDI-TOFMS analysis in order to identify the reaction contents.

Mass spectra of the peptide Aβ-(1-16/Asp-7) (Fig. 1(A)) as well as of its isomerized analogue, Aβ-(1-16/isoAsp-7) (Fig. 1(B)), which were prepared in 25 mM barbital buffer (pH 7.4), containing 150 mM NaCl and 1 μM $ZnCl_2$, show one major peak at *m/z* 1995.9, which corresponds to the singly protonated species, and one minor peak at *m/z* 2017.9, which is characteristic for the peptide sodium adduct ion. The same samples, incubated for 130 min at 37°C, demonstrate no changes in the corresponding mass spectra (data not shown) in comparison with the freshly prepared ones. These results were also reproduced in a 50 mM sodium bicarbonate buffer (pH 7.8). Data obtained in both buffer systems demonstrates that the peptides: (i) are homogenous; (ii) do not contain neither significant contaminants, nor degradation products; and (iii) do not undergo spontaneous degradation during 130 min ageing.

The mass spectra of samples obtained from the reaction mixture, wherein Aβ-(1-16/Asp-7) had been incubated for 10–130 min with N-ACE in the bicarbonate buffer system, are shown in Figs. 2(A)–2(D). Besides the peaks corresponding to the parent peptide molecular ion (*m/z* 1995.9) and its Na^+ adduct (*m/z* 2017.9), there are four other significant peaks with *m/z* values 679.3, 701.2, 1335.6, and 1357.6. The 679.3 and 701.2 signals correspond to the single protonated peptide Aβ-(1-5) and the peptide sodium adduct, respectively, whereas the 1335.6 and 1357.6 peaks are characteristic for the Aβ-(6-16/Asp-7) peptide.

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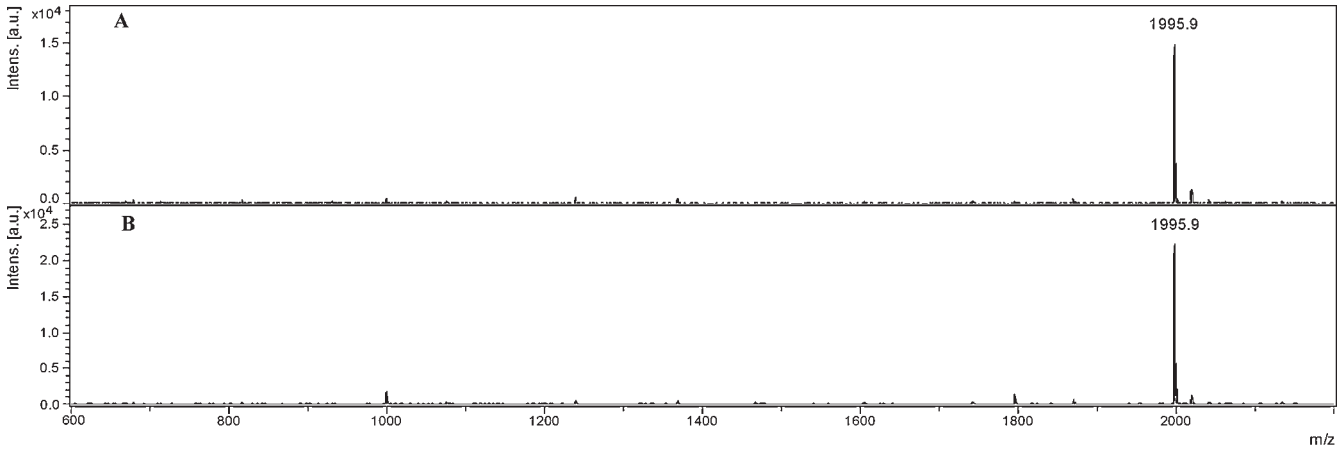

Figure 1. MALDI-TOF mass spectra of peptide samples freshly prepared in 25 mM barbital buffer (pH 7.4), containing 150 mM NaCl and 1 μM $ZnCl_2$: (A) Aβ-(1-16/Asp-7) and (B) Aβ-(1-16/isoAsp-7).

Our data demonstrates that N-ACE cleaves Aβ-(1-16/Asp-7) at the Arg-5-His-6 site. Since there is no apparent difference in the mass spectra obtained for various time points (from 10 to 130 min), one could tentatively suppose that hydrolysis has reached its steady state in 10 min or less; therefore, it appears that the reaction products inhibited Aβ-(1-16/Asp-7) cleavage by N-ACE. To verify that N-ACE preserves its enzymatic activity *in situ*, the following procedure was used: 3 μL were drawn from the 20 min incubated reaction mixture, then mixed with 1 μL of 100 μM angiotensin I (dissolved in the reaction buffer) and placed at 37°C. The prepared mixture was subjected to direct MALDI-TOF analysis immediately after mixing (Fig. 3(A)) and 20 min later (Fig. 3(B)). Besides the signals of Aβ-(1-5), Aβ-(6-16/Asp-7) and Aβ-(1-16/Asp-7), the peak at *m/z* 1296.7 corresponding to angiotensin I is observed in the first spectrum but disappears in the second one. Instead of it, a new peak emerges with *m/z* value of 1046.5, which corresponds to the normal proteolytic product of angiotensin I, namely, angiotensin II, thus confirming that N-ACE was not incactivated by products of Aβ-(1-16/Asp-7) proteolysis.

Peptide Aβ-(1-16/isoAsp-7) incubated with N-ACE under the same conditions which were used for Aβ-(1-16/Asp-7) proteolysis was characterized by the same pattern of peaks in

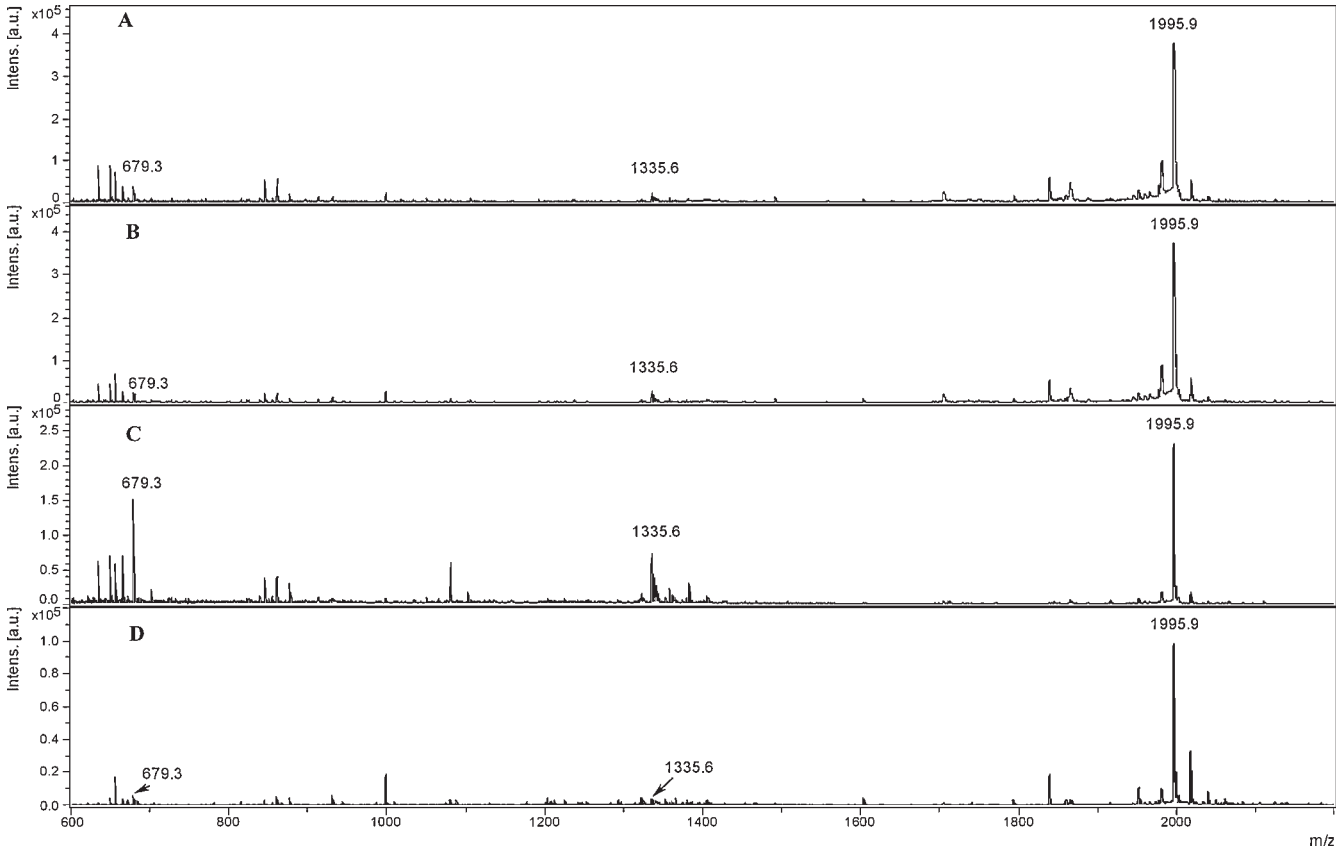

Figure 2. MALDI-TOF mass spectra of samples obtained from the reaction mixture, wherein Aβ-(1-16/Asp-7) was incubated with N-ACE during 10 (A), 20 (B), 40 (C), and 130 (D) min.

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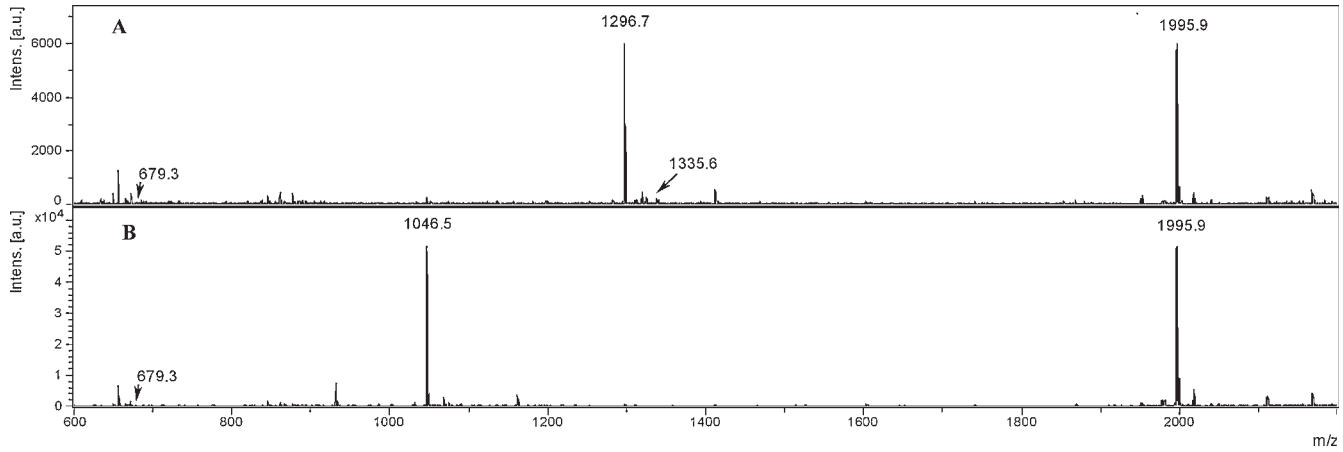

Figure 3. MALDI-TOF mass spectra of the reaction mixture (wherein the proteolysis of Aβ-(1-16/Asp-7) by N-ACE was performed for 20 min) immediately after adding angiotensin I (A) and after a 20 min incubation with angiotensin I (B).

mass spectra (Figs. 4(A)–4(D)) as that of peptide Aβ-(1-16/Asp-7). Based on these results, it appears that the Asp-7 isomerization does not influence the ability of N-ACE to hydrolyze the Arg-5-His-6 site within the Aβ region 1-16. It is worth noting that the specificity of N-ACE activity has been confirmed by complete inhibition of hydrolysis by the ACE inhibitor lisinopril (Figs. 5(A) and 5(B)).

In contrast to N-ACE, the C-domain of ACE under the same experimental conditions (150 mM NaCl, 50 mM sodium bicarbonate buffer, pH 7.8) hydrolyzes neither Aβ-(1-16/Asp-7) nor Aβ-(1-16/isoAsp-7) at any peptide bond as evidenced by MALDI-TOF mass spectra (Figs. 6(A) and 6(B)). Taking into account that sodium bicarbonate buffer would not be optimal for the C-ACE activity, we performed additional reactions between C-ACE and the Aβ-(1-16) peptides in 25 mM barbital buffer (pH7.4), in which the domain showed similar to N-ACE enzymatic activity on the model substrate (see Experimental). Moreover, since it was reported that the proteolytic activity of the C-domain of ACE is dependent on chloride concentration,[32]

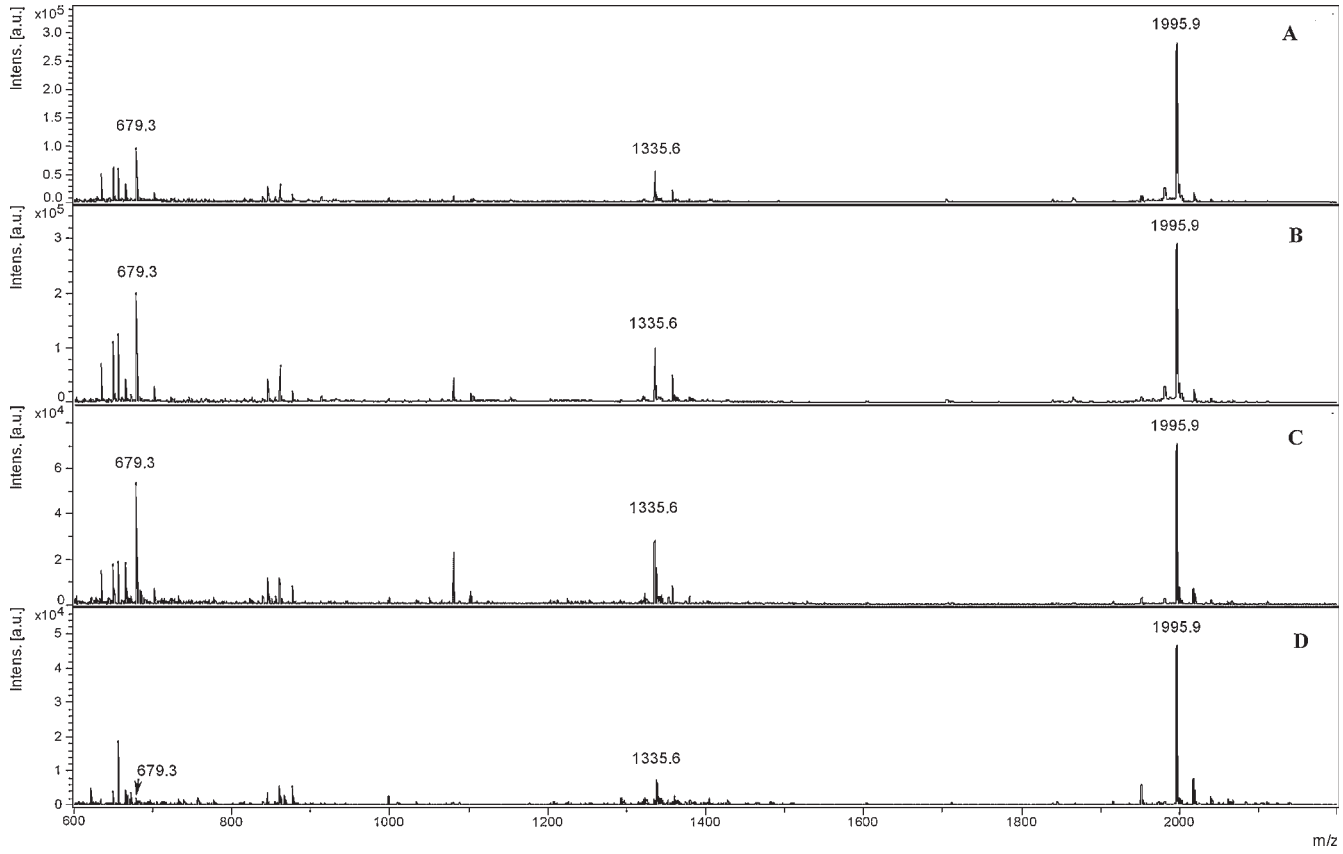

Figure 4. MALDI-TOF mass spectra of samples obtained from the reaction mixture, wherein Aβ-(1-16/isoAsp-7) was incubated with N-ACE for 10 (A), 20 (B), 40 (C), and 130 (D) min

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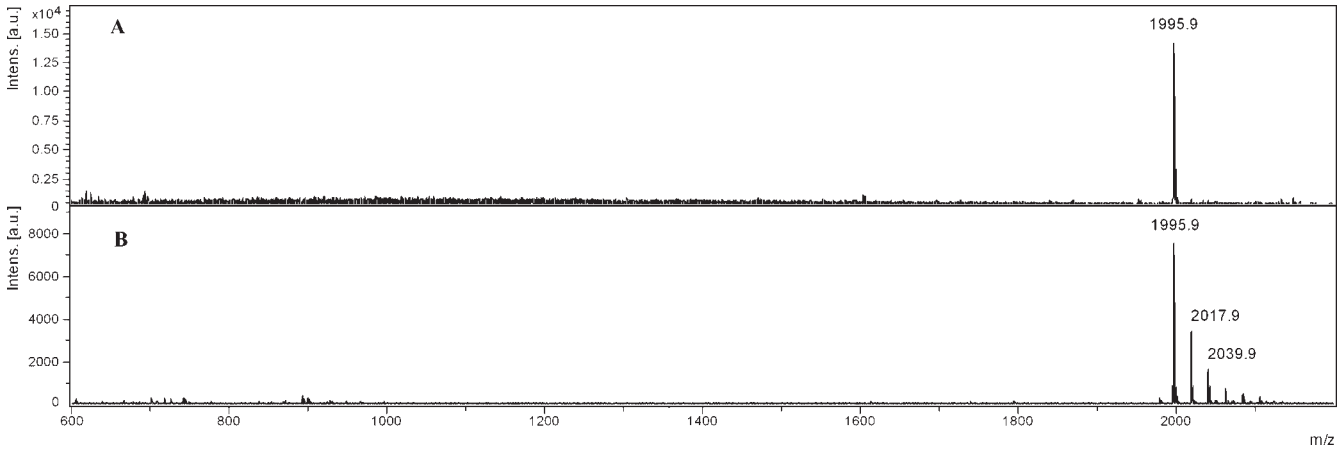

Figure 5. MALDI-TOF mass spectra of samples obtained from the reaction mixtures wherein Aβ-(1-16/Asp-7) (A) or Aβ-(1-16/isoAsp-7) (B) was incubated for 130 min with N-ACE in the presence of lisinopril. On both spectra only peaks corresponding to the respective peptides are observed.

we varied the NaCl concentrations from 50 to 500 mM (in 25 mM barbital buffer, pH7.4), but C-ACE showed no proteolytic activity at either of them (data not shown).

Quantitative MS analysis of ACE hydrolysis products using ^{18}O-labeled internal standards

To evaluate the effect of Asp-7 isomerization on the efficiency of the Arg-5-His-6 peptide bond cleavage of Aβ-(1-16) species by N-ACE, quantitation of ACE digestion products was performed by direct MALDI-TOFMS using ^{18}O-labeled internal standards as described earlier.[23,26] The absolute peptide concentrations were calculated by employing a linear correlation between the peak height ratio and the sample loading. However, this approach can only be used when a peptide similar to the analyzed one is used as an internal standard.[27] Since N-ACE cleaves the Arg-5-His-6 bond of Aβ-(1-16) thus producing Aβ-(1-5) and Aβ-(6-16), we used trypsin to prepare the appropriate ^{18}O-labeled standards. These standards were obtained by exhaustive trypsinolysis of a predefined amount of Aβ-(1-16/Asp-7) in ^{18}O-water. As a result of hydrolysis and C-terminal deamidation, accompanied by an isotopic exchange between oxygen atoms of the peptide C-terminus carboxy group and water, an equimolar mixture of two ^{18}O-labeled peptides, ^{18}O-Aβ-(1-5) and ^{18}O-Aβ-(6-16/Asp-7), was produced. Each labeled peptide contained one or two ^{18}O atoms, as confirmed by MALDI-TOF mass spectra (Fig. 7(B) and 7(F)) which show the substitution of monoisotopic peaks of the initial Aβ-(1-5) and Aβ-(6-16) peptides with *m/z* values 679.3 and 1335.6, respectively, by their singly (*m/z* values 681.3 and 1338.6) and doubly ^{18}O-substituted analogues (*m/z* values 683.3 and 1340.6). The relative abundances of the mono- and di-^{18}O-substituted species were 19% and 81% for Aβ-(1-5) and 18.5% and 81.5% for the Aβ-(6-16) peptide.

The isotopic patterns corresponding to the unlabeled compounds Aβ-(1-5) and Aβ-(6-16/Asp-7), labeled standards ^{18}O-Aβ-(1-5) and ^{18}O-Aβ-(6-16/Asp-7), and the analyte/standard mixtures of interest are shown in Fig. 7. Based on this data, absolute peptide concentrations of the analytes in respective reaction mixtures were calculated

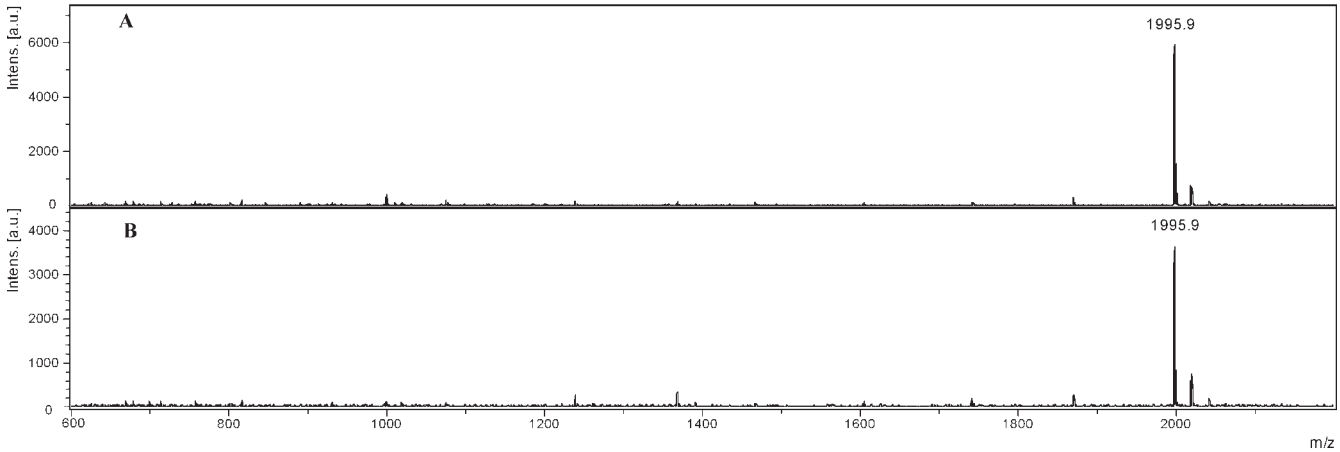

Figure 6. MALDI-TOF mass spectra of samples obtained from the reaction mixtures wherein Aβ-(1-16/Asp-7) (A) or Aβ-(1-16/isoAsp-7) (B) was incubated during 130 min with C-ACE. On both spectra only peaks corresponding to the respective peptides are observed.

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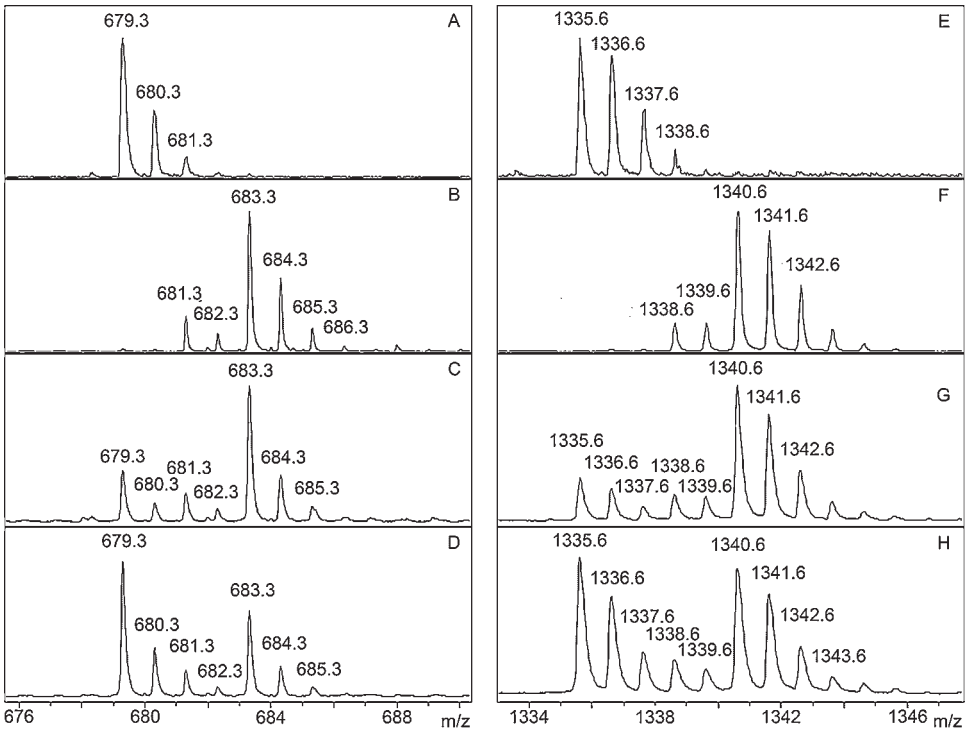

Figure 7. MALDI-TOF mass spectra presenting isotopic patterns of the major MS signals obtained from: unlabeled Aβ-(1-5) (A); ^{18}O-Aβ-(1-5) (B); ^{18}O-Aβ-(1-5) added to the reaction mixture, containing N-ACE and Aβ-(1-16/Asp-7) (C); ^{18}O-Aβ-(1-5) added to reaction mixture, containing N-ACE and Aβ-(1-16/isoAsp-7) (D); unlabeled Aβ-(6-16) (E); ^{18}O-Aβ-(6-16/Asp-7) (F); ^{18}O-Aβ-(6-16/Asp-7) added to the reaction mixture, containing N-ACE and Aβ-(1-16/Asp-7) (G); ^{18}O-Aβ-(6-16/Asp-7) added to the reaction mixture, containing N-ACE and Aβ-(1-16/iso Asp-7) (H).

(Fig. 8). It was found that the 20 μM peptide Aβ-(1-16/Asp-7) after hydrolysis by N-ACE produced 4 ± 0.4 μM Aβ-(1-5) and 4 ± 0.4 μM Aβ-(6-16/Asp-7), whereas, for the 20 μM peptide Aβ-(1-16/isoAsp-7), the efficiency was much higher, resulting in 13 ± 1.3 μM Aβ-(1-5) and 14 ± 1.4 μM Aβ-(6-16/isoAsp-7).

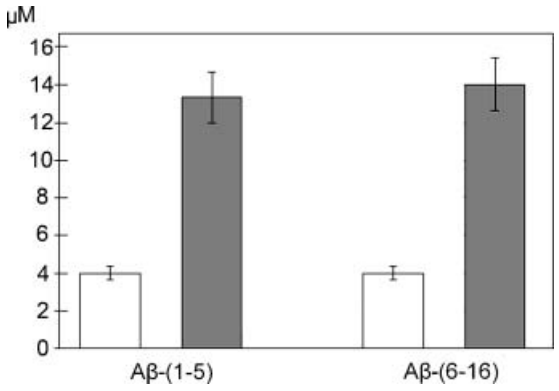

Figure 8. Concentrations of peptides Aβ-(1-5) and Aβ-(6-16) in the reaction mixtures wherein 20 μM of Aβ-(1-16/Asp-7) (white boxes) or Aβ-(1-16/isoAsp-7) (grey boxes) was incubated for 20 min with N-ACE.

DISCUSSION

In order to get a better understanding of the ACE/Aβ interaction, we have used two synthetic peptides, corresponding to the metal-binding domain of Aβ (residues 1-16), as model Aβ targets to study their hydrolysis by the N- or C-domain of ACE. Peptide solubility and monomeric state have allowed us to investigate hydrolysis by using direct MALDI-TOFMS of samples without previous fractionation of the respective reaction mixtures. Our results demonstrated that the Aβ region 1-16 was cleaved by N-ACE between residues Arg-5 and His-6, meanwhile, the C-domain of ACE failed to hydrolyze this region. This agrees with an earlier study[21] where the role of the N-domain of ACE, but not the C-domain, in specific hydrolysis of Aβ *in vitro* was reported. Although Oba *et al.*[21] claimed that N-ACE cleaved Aβ-(1-40) at the site Asp7-Ser8, in our opinion this statement was only one of possible interpretations of their experimental results because by using the HPLC technique the authors of that work observed a series of proteolytic products and were able to identify only one of them, Aβ-(8-40), whereas the three others were attributed to degradation products of Aβ-(1-7). Since this attribution was not proved by experimental results one could suggest that the cleavage occurs either at the Asp-7-Ser-8 site and/or at an unknown point to the left of that site. Our results indicating that N-ACE hydrolyzes the

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Arg-5-His-6 bond of Aβ-(1-16) strongly supports the second suggestion. It is worth noting that application of MALDI-TOFMS methodology successfully used in the present work for soluble and monomeric Aβ-(1-16) peptides[12,15,17] failed to be useful in similar studies of longer Aβ peptides which exist in an aqueous solution as a mixture of monomeric and oligomeric species,[28] and presumably aggregate in a time-dependent mode under our experimental conditions (unpublished data).

Cleavage of Aβ species at the Arg-5-His-6 bond is an unusual new property of ACE and is related to its N-domain only. The biological role of this ACE activity should be further investigated. Although it has been suggested on the basis of previous genetic studies that ACE might be associated with AD,[29,30] the molecular mechanism by which this linkage could be realized is still unclear. One common opinion is that ACE is implicated in AD via direct proteolysis of the Aβ peptides.[20–22] On the other hand, experiments on young transgenic mice have shown that ACE is not involved in the regulation of steady-state Aβ levels in the brain.[31] However, these studies on ACE/Aβ interactions, both *in vitro* and *in vivo*, were performed on unmodified Aβ species, whereas in AD brains the content of Aβ peptides containing various chemical modifications is very high, suggesting that such modifications could influence proteolysis. One of the most abundant naturally occurring modifications within Aβ is isomerization of Asp-7.[6] Here, to evaluate the effect of such modification on Aβ hydrolysis by ACE, we have examined *in vitro* interactions between enzyme domains and two different Aβ-(1-16) isoforms. The first one, Aβ-(1-16/Asp-7), is a model of Aβ species, which are normally present in biological fluids and tissues, whereas the second, Aβ-(1-16/isoAsp-7), corresponds to the AD-associated Aβ isoform containing an isoAsp residue in position 7. Quantitative MALDI-TOFMS based on the use of ^{18}O-labeled internal standards has allowed us to show that Aβ-(1-16/isoAsp-7) is cleaved by N-ACE much more efficiently than Aβ-(1-16/Asp-7). A possible explanation for this might be in the structure of the Aβ-(1-16).[17] Usually, incorporation of β-amino acids leads to the formation of a more rigid structure of the protein, thus limiting the accessibility for proteases to attack. However, this is true only if this incorporation is made into a structure at the surface of the protein.[33] In the case of Aβ-(1-16) the isoAsp is in position 7, which is part of an unstructured region of the peptide, thus it should not create a more rigid structure. Another important point to note is that the two amino acids to the right and left of the cleavage site play an important role in binding to the active site of ACE.[34] Since Asp7 is one of these amino acids its isomerization should affect binding efficiency. Introduction of isoAsp lengthens the backbone of the peptide, presumably making it more flexible and shortens the side chain, which might lead to a less bulky structure, so making it easier for the enzyme to attack.

Taking into account these results, one could suggest that N-ACE *in vivo* rather degrades the Aβ isoforms related to AD than participates in the breakdown of normal Aβ. Since the isoAsp-7-containing isoforms are associated with AD, they can potentially be the pathogenic ones. Earlier, the decreased activity of ACE in the aged human brain was hypothesized to promote Aβ accumulation and thus modulate the likelihood of development of AD.[22] Nevertheless, transgenic mice study results[31] appeared to contradict that hypothesis. In the context of our new findings, the contradiction may be eliminated by the assumption that ACE enzymatic deficiency primarily concerns the degradation of aged Aβ isoforms absent in the examined young mice[31] and does not affect the level of unmodified Aβ species. Our results, thus, suggest the need for further analysis of biological effects of the isomerized Aβ and its interaction with ACE on AD pathogenesis.

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