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Carboxy-terminal truncation of long-tailed amyloid β-peptide is inhibited by serine protease inhibitor and peptide aldehyde

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Abstract The 42/43-residue amyloid β-peptide (Aβ) is widely believed to play a major role in Alzheimer's disease. The present study shows that the rat brain contains a carboxypeptidase that efficiently deletes three amino acids from Aβ1–43. The carboxypeptidase activity in the brain was completely inhibited by 1 mM phenylmethylsulfonyl fluoride, suggesting the protease is a serine carboxypeptidase. The carboxy-terminal truncation of Aβ1–43 was moderately inhibited by carbobenzoxy-Leu-leucinal, carbobenzoxy-Leu-Leu-leucinal, and carbobenzoxy-Leu-Leu-norvalinal, and weakly by antipain. The present data suggest that the serine carboxypeptidase contributes to the generation of short-tailed Aβ peptides and is important in the intracellular clearance of Aβ1–42/43 in brains.

Key words: Alzheimer's disease; β-Amyloid; Lysosomal carboxypeptidase; Peptide aldehyde; Proteasome inhibitor; Calpain inhibitor

1. Introduction

Amyloid β-peptide (Aβ) is a 4-kDa peptide deposited as amyloid in the brains of all patients with Alzheimer's disease (AD) [1–3]. Aβ is a 39–43-residue heterogeneous peptide derived from proteolytic processing of the β-amyloid precursor protein (APP) by β-secretase, the protease that cleaves at the amino-terminus, and γ-secretase, the protease that cleaves at the carboxy-terminus [3]. Aβ1–40 is the major species found in cerebrospinal fluid [4] and approximately 90% of Aβ peptides secreted from cultured cells terminate at residue 40 [5,6]. However, all three early-onset familial Alzheimer's disease (FAD) genes identified to date have been shown to cause an enhanced production of Aβ ending at residues 42 or 43 [7–9]. Furthermore, the long-tailed Aβ is the major and early component within senile plaques of patients with sporadic AD, Down's syndrome, as well as FAD [10–13]. Therefore, Aβ1–42/43 appears to play a major role in FAD and AD. Even though the mechanism by which neurons are selectively killed by Aβ1–42 is not determined, the intracellular accumulation of Aβ1–42 is suspected to be very dangerous for neurons, because ERAB, a newly found protein locating the endoplasmic reticulum (ER) of neural cells, relocates to the cell membranes after binding to Aβ1–42 and causes apoptosis [14]. Recently, it was demonstrated that Aβ1–42 was generated within the ER or the Golgi and Aβ1–42 but less Aβ1–40 occurred in cultured cells transfected with APP [15]. In order to understand the mechanism to produce the carboxy-terminal heterogeneity of Aβ peptides several kinds of protease inhibitors have been examined and it was found that the secretion of Aβ1–40 into the medium from several cultured cells was reduced accompanying a slight increase of Aβ1–42 after treatment with peptide aldehydes such as MDL 28170, calpain inhibitor I, and carbobenzoxy-Leu-Leu-leucinal [16–18]. Based on these findings two types of γ-secretases (endopeptidases) have been postulated; one cleaves after amino acid 40 of Aβ and is sensitive to peptide aldehyde, and the other cleaves after amino acid 42 and is resistant to the inhibitor [17,18].

Since the binding of Aβ1–42 to ERAB protein in neural cells leads to apoptosis [14], the degradation of Aβ1–42/43 is supposed to be important to prevent neural dysfunction. In the previous paper I have reported that cathepsin D is the major endopeptidase that hydrolyzes Aβ in the middle of the sequence to eliminate the Aβ peptides [19]. However, to decrease intracellular levels of Aβ1–42/43, there must be a carboxypeptidase that is able to produce short-tailed Aβ from Aβ1–42/43, because cathepsin D hydrolyzes the long-tailed Aβ less efficiently than Aβ1–40 [20]. The present work shows that the rat brain contains the carboxypeptidase which successively removes three amino acids from Aβ1–43 producing Aβ1–40. In addition it was found that the carboxypeptidase activity was inhibited by peptide aldehyde protease inhibitors. The results suggest that the carboxypeptidase contributes to the generation of Aβ1–40 from Aβ1–42/43 and that the peptide aldehyde protease inhibitors may decrease the secretion of Aβ1–40 by their inhibition against the carboxypeptidase.

2. Materials and methods

Aβ1–40 and Aβ1–43 were obtained from Bachem. Phenylmethylsulfonyl fluoride (PMSF) and *p*-chloromercuribenzoic acid (PCMB) was purchased from Sigma. Carbobenzoxy-L-Leu-L-leucinal (Z-Leu-LeuH), carbobenzoxy-L-Leu-L-Leu-L-leucinal (Z-Leu-Leu-LeuH), carbobenzoxy-L-Leu-L-Leu-L-norvalinal (Z-Leu-Leu-NvaH), biotinyl-L-Asp-L-Glu-L-Val-L-aspart-1-al (biotin-Asp-Glu-Val-AspH), leupeptin, antipain, and pepstatin A were obtained from Peptide Institute, Inc., Osaka, Japan. Rat brain was homogenized with 5 volumes of 0.2 M acetate buffer, pH 5.2, containing 1 μM pepstatin A using a Polytron homogenizer at 0°C and centrifuged at 25 000×*g* for 60 min at 4°C. The supernatant fraction was dialyzed against acetate buffer (0.05 M, pH 5.2) and centrifuged to remove debris before use for enzyme assay. The carboxypeptidase activity was assayed by analyzing released amino acids after incubating the mixture of 20 μl of brain extract in acetate buffer (0.05 M, pH 5.2) and 20 μl of Aβ peptide (50 μM) at 37°C. Peptide aldehyde protease inhibitors were dissolved as stock solution of 10 mM just before use. Z-Leu-Leu-H, Z-Leu-Leu-Leu-H, Z-Leu-Leu-NvaH, and biotin-Asp-Glu-Val-AspH were dissolved

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Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; APP, β-amyloid precursor protein; FAD, familial Alzheimer's disease; PMSF, phenylmethylsulfonyl fluoride; PCMB, *p*-chloromercuribenzoic acid; Z-, carbobenzoxy-; LeuH, leucinal; NvaH, norvalinal; AspH, aspart-1-al

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in dimethyl sulfoxide and other peptide aldehyde inhibitors were in distilled water. In order to assay the effects of protease inhibitors 0.4 μl of inhibitors were added to 40 μl of the incubation mixture. Enzyme assay was performed three times independently. 10-μl aliquots of incubation mixture were used to analyze released amino acids using a method reported by Chang et al. [21].

3. Results and discussion

In the previous work it was shown that cathepsin D is the major protease in the rat brain that is able to hydrolyze Aβ peptides; inhibitor assay has shown that other lysosomal proteases did not degrade the peptides [19]. However, there is no reason to rule out the possibility that carboxy-terminus of Aβ peptides is deleted by lysosomal or other exopeptidase(s), since it has been shown that Aβ1–42 in media is internalized by cultured cells and truncated at the amino-terminus [22]. In order to test this possibility, the cleavage of carboxy-terminal amino acids from Aβ1–43 were assayed using rat brain extract. Since in the preliminary experiments it has been shown that carboxypeptidase activity of the rat brain was highest at pH 5.0–5.5, the enzyme activity was assayed in 0.05 M acetate buffer (pH 5.2) in the following experiments. When 1 nmol of Aβ1–43 was incubated with the rat brain extract, 0.29 nmol of threonine, 0.29 nmol of alanine, 0.26 nmol of isoleucine, and 0.13 nmol of valine were liberated in 2 h under the experimental conditions (Fig. 1). Release of other amino acids was negligible. The release of threonine from Aβ1–43 was two times faster than that of valine (Val-40 plus Val-39) from Aβ1–40. Since the release of Thr-43, Ala-42 and Ile-41 is faster than that of Val-40 plus Val-39, it would be reasonable to suppose that Aβ1–40 and Aβ1–39 are produced from Aβ1–42/43 by the action of carboxypeptidase and partly secreted from cells before further degradation by the carboxypeptidase and lysosomal endopeptidases. Fig. 2 shows that 1 mM PMSF completely inhibits the carboxypeptidase, while the inhibition by 2 mM EDTA or 1 mM PCMB was incomplete, suggesting the enzyme belongs to serine carboxypeptidase.

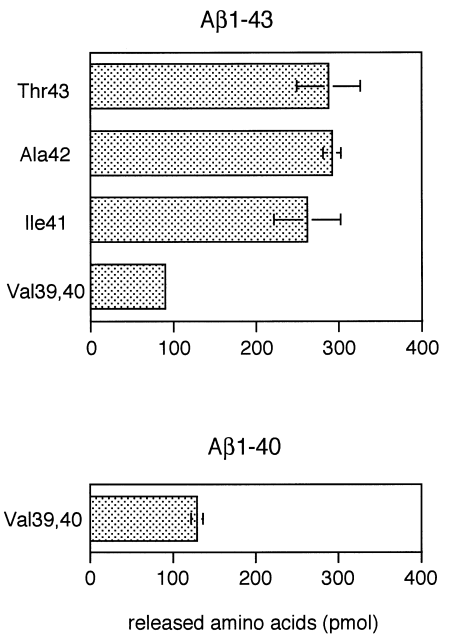

Fig. 1. Carboxy-terminal truncation of Aβ1–43 and Aβ1–40 by rat brain extract. Released amino acids were assayed after Aβ peptides (1 nmol) were incubated with rat brain extract for 2 h under the conditions described in Section 2. Data are means ± S.E. of three independent experiments.

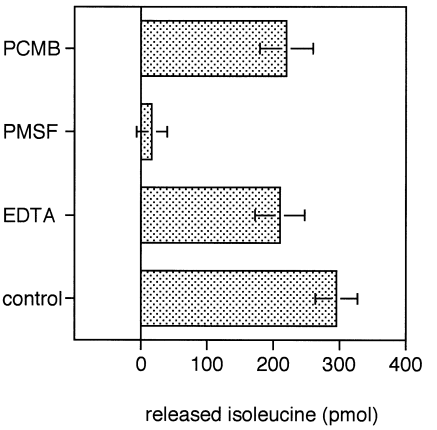

Fig. 2. Inhibition of carboxy-terminal truncation by PMSF. Aβ1–43 (1 nmol) was incubated with rat brain extract for 2 h in the presence or absence (control) of protease inhibitors and subsequently released Ile-41 was assayed. Data are means ± S.E. of three independent experiments.

Recently, peptide aldehyde protease inhibitors have been shown to affect differently on the generation of Aβ1–42 and Aβ1–40. Klafki et al. examined the secretion of Aβ1–40 and Aβ1–42 by cultured cells expressing APP with the Swedish double mutation and found the reduction of Aβ1–40 after treatment with calpain inhibitor I (*N*-acetyl-Leu-Leu-norleucinal) or Z-Leu-Leu-LeuH accompanying a slight increase of Aβ1–42 released into the medium [18]. Citron et al. also observed a peptide aldehyde with calpain inhibitor activity, MDL 28170 (carbobenzoxy-Val-phenylalaninal), reduces the amount of secreted Aβ40 peptide into media from cultured cells [17]. Both observations may suggest that two kinds of γ-secretases are present and peptide aldehydes reduce the secretion of Aβ1–40 by its inhibition against γ-secretase which is specific for the cleavage after residue 40, even though γ-secretase with such substrate specificity is yet to been identified. If the carboxypeptidase is engaged in the generation of Aβ1–40, it is expected that the enzyme is inhibited by peptide aldehyde protease inhibitors. Therefore, effects of peptide aldehydes on the carboxy-terminal cleavage of Aβ1–43 by rat brain extract were investigated (Fig. 3A). When 100 μM inhibitors were used, Z-Leu-LeuH, Z-Leu-Leu-LeuH, Z-Leu-Leu-NvaH, biotinyl-Asp-Glu-Val-AspH, and antipain inhibited the carboxypeptidase activity by 66%, 76%, 50%, 17%, and 7%, respectively. IC_{50} of Z-Leu-Leu-LeuH for the carboxypeptidase was approximately 60 μM (Fig. 3B). The data suggest that the carboxypeptidase is an important factor that plays a role in controlling the levels of Aβ1–40 and Aβ1–42/43 and that the inhibition of the enzyme by peptide aldehydes would lead to reduced secretion of Aβ1–40 from cultured cells accompanying increased production of Aβ1–42/43.

The brain carboxypeptidase is similar to cathepsin A (lysosomal serine carboxypeptidase, EC 3.4.16.1) in the following characteristics: cathepsin A has optimal activity at pH 4.5–6.0, has wide substrate specificity, and has been found in human brain [23]. However, sensitivity to antipain is different; IC_{50} of antipain for cathepsin A is about 2 μM [24], while only 7% of the brain carboxypeptidase activity was inhibited by the inhibitor at 100 μM. The purification of the carboxy-

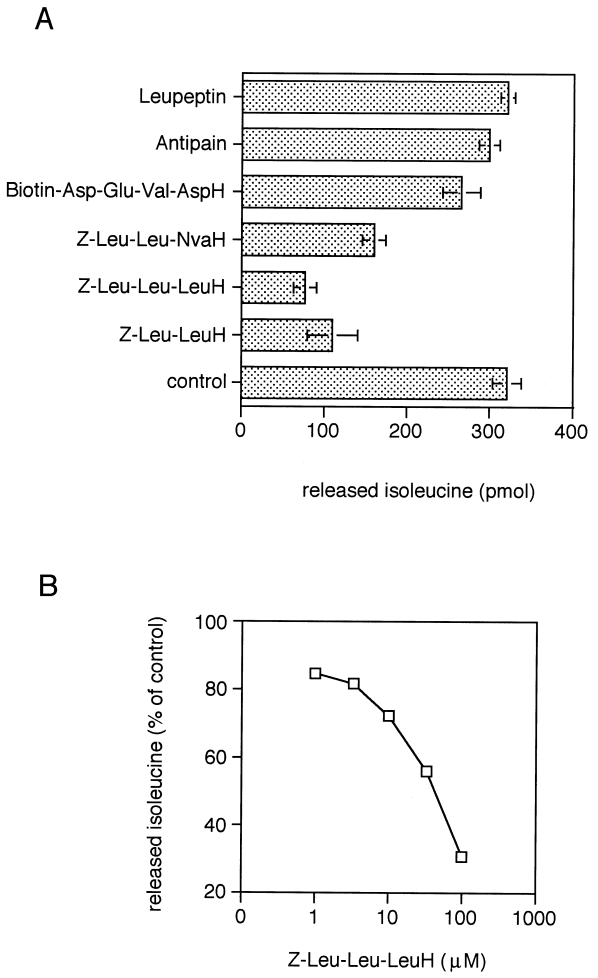

Fig. 3. Inhibition by peptide aldehydes. Release of Ile-41 from Aβ1–43 after 2-h incubation of Aβ1–43 with brain extract in the presence or absence (control) of peptide aldehyde. A: Inhibition by 100 μM peptide aldehyde. Data are means ± S.E. of three independent experiments. B: Dose dependence of inhibition by Z-Leu-Leu-Leu-H. Data are means of three independent experiments.

peptidase is under way. It is surprising that Aβ1–42/43 is more sensitive to the brain carboxypeptidase than Aβ1–40, since Aβ1–42/43 is more aggregatable and more resistant to endosomal/lysosomal endopeptidases than Aβ1–40 [20]. Since the long-tailed Aβ peptides are very cytotoxic for neural cells [14] and the intracellular clearance of Aβ peptides is supposed to be carried out mostly in endosomes/lysosomes, the carboxy-terminal truncation of Aβ1–42/43 by the serine carboxypeptidase would be essential to prevent neural dysfunction.

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