

ORIGINAL ARTICLE

Contributions of degradation and brain-to-blood elimination across the blood–brain barrier to cerebral clearance of human amyloid- β peptide(1-40) in mouse brainShingo Ito^{1,2,3}, Kohta Matsumiya¹, Sumio Ohtsuki^{1,2,3}, Junichi Kamiie⁴ and Tetsuya Terasaki^{1,2}

The purpose of the present study was to estimate the relative contributions of degradation and brain-to-blood elimination processes to the clearance of microinjected human amyloid- β peptide(1-40) (hA β (1-40)) from mouse cerebral cortex, using a solid-phase extraction method together with a newly developed ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) quantitation method for intact hA β (1-40). The clearance rate constant of hA β (1-40) in mouse cerebral cortex was determined to be 3.21×10^{-2} /min under conditions where the saturable brain-to-blood elimination process across the blood–brain barrier (BBB) was expected to be saturated. Thus, this clearance rate constant should mainly reflect degradation. The [¹²⁵I]hA β (1-40) elimination rate across the BBB under nonsaturating conditions was determined to be 1.48×10^{-2} /min. Inhibition studies suggested that processes sensitive to insulin and phosphoramidon, which inhibit neprilysin, insulin-degrading enzyme, and endothelin-converting enzyme, are involved not only in degradation, but also in elimination of hA β (1-40). In conclusion, our results suggest a dominant contribution of degradation to cerebral hA β (1-40) clearance, and also indicate that a sequential process of degradation and elimination of degradation products is involved in cerebral hA β (1-40) clearance.

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INTRODUCTION

The accumulation of amyloid- β peptide (A β) in extracellular space is thought to trigger A β aggregation and deposition, which contribute to development of Alzheimer's disease (AD).^{1,2} Amyloid- β peptide is normally produced and cleared at the same rate in human brain.³ In late-onset AD (99% of cases), the clearance rate of A β from brain is lower than that in non-AD brains, but there is no significant difference in A β production rate.⁴ Thus, impaired clearance of soluble A β could be the major cause of elevated levels of A β , ultimately leading to deposition of A β aggregates in the brain.⁵ Cerebral clearance of soluble A β involves both degradation in the brain^{6–8} and brain-to-blood elimination across the blood–brain barrier (BBB).^{9,10} The relative contributions of these processes are poorly understood, though this information would be important to understand in detail the pathogenesis of AD, and also to develop treatments.

Therefore, the aim of the present study was to evaluate the degradation and brain-to-blood elimination rate constants of human A β (hA β)(1-40) from mouse brain by using a newly developed ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) method for quantitation of intact hA β (1-40) after microinjection of hA β (1-40), and thus to estimate separately the contributions of the two processes to cerebral A β clearance in mouse. The elimination rate of hA β (1-40) from the

rodent cerebral cortex has been determined from the time course of ¹²⁵I radioactivity remaining in the ipsilateral cerebrum.^{10,11} However, intact and degraded hA β (1-40) cannot be distinguished by measuring radioactivity. The degradation of A β (1-40) has also been examined by using labeled hA β (1-40) or knockout mice.^{6–10} However, to separate the contributions of degradation or elimination to cerebral clearance of hA β (1-40), it is necessary to quantify both the radiolabel and intact hA β (1-40).

For quantification of intact hA β (1-40), enzyme-linked immunosorbent assay has been widely used. However, enzyme-linked immunosorbent assay results can be influenced by crossreactivity of A β antibody, the presence of A β binding protein, and/or nonspecific binding of compounds in brain extract.¹² However, quantification by means of liquid chromatography/tandem mass spectrometry (LC/MS/MS) with selected/multiple reaction monitoring (SRM/MRM) has the advantages of high selectivity and reliability.¹³ Therefore, for the present work we used a solid-phase extraction (SPE) procedure to concentrate A β from brain extract without anti-A β antibody treatment,¹⁴ followed by quantitation of intact hA β (1-40) with a newly developed UPLC/MS/MS method. The combination of this approach with radioactivity measurements allowed us to estimate separately the contributions of degradation and elimination to cerebral A β clearance in our experimental model.

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MATERIALS AND METHODS

Animals

Male C57BL/6J mice (8 to 10 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). The protocol was approved by the Institutional Animal Care and Use Committee at Tohoku University (Sendai, Japan). The investigation using animals described in this report conformed to the guidelines of the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University.

Reagents

Human amyloid- β peptide(1-40) was purchased from Bachem AG (Bubendorf, Switzerland) and Peptide Institute (Osaka, Japan). [^{15}N]-labeled hA β (1-40) ([^{15}N]hA β (1-40)) was obtained from rPeptide (Bogart, GA, USA). FITC-labeled inulin (FITC-inulin) was purchased from Sigma (St. Louis, MO, USA). Monoiodinated and nonoxidized [^{125}I]-labeled hA β (1-40) ([^{125}I]hA β (1-40)) was prepared from hA β (1-40) (Biosource, Camarillo, CA, USA) by Perkin-Elmer Life Sciences (Boston, MA, USA) (2,200 Ci/mmol). [^3H]Dextran (100 mCi/mg) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Insulin was purchased from Sigma. Thiorphan was obtained from Wako Pure Chemicals (Osaka, Japan). Phosphoramidon and human insulin were purchased from Peptide Institute. Xylazine hydrochloride was purchased from Sigma. Ketalar 50 (ketamine hydrochloride) was obtained from Sankyo Co. (Tokyo, Japan). All other chemicals were analytical-grade commercial products.

Preparation of Unlabeled Human Amyloid- β Peptide(1-40) and [^{15}N]-Labeled Human Amyloid- β Peptide(1-40)

Unlabeled hA β (1-40) and [^{15}N]hA β (1-40) were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Kanto Chemical, Tokyo, Japan) at a concentration of 1 mg/mL and stored at 4°C. The amounts of hA β (1-40) and [^{15}N]hA β (1-40) in HFIP solution were determined by amino-acid sequence analysis as described previously.¹⁵ Unlabeled hA β (1-40) peptide was dried and then dissolved in distilled water or 0.1% ammonium hydroxide containing 20% acetonitrile before use.

Mass Spectrometric Analysis of Unlabeled Human Amyloid- β Peptide(1-40) and [^{15}N]-Labeled Human Amyloid- β Peptide

Unlabeled hA β (1-40) and [^{15}N]hA β (1-40) were detected by following a specific *m/z* transition from a precursor peptide ion in Q1 to a product peptide ion in Q3 (SRM/MRM transition). A solution of hA β (1-40) in 0.1% ammonium hydroxide containing 20% acetonitrile (1 $\mu\text{mol/L}$) was injected into the MS/MS (API5000; Applied Biosystems, Foster City, CA, USA) at 5 $\mu\text{L}/\text{min}$ to optimize MS/MS SRM/MRM conditions in the negative ion mode (Table 1).

Preparation of Standard Solutions

For preparing the calibration curves, unlabeled hA β (1-40) was diluted in 1.5 mL tubes with 200 μL of 0.1% ammonium hydroxide containing 20% acetonitrile by serial dilution of hA β (1-40) (0, 20, 40, 200, 400, 2,000, and 4,000 fmol) spiked into 1 pmol of [^{15}N]hA β (1-40) as an internal standard. Aliquots of 50 μL of these solutions (0, 5, 10, 50, 100, 500, and 1,000 fmol standard/250 fmol internal standard) were injected into the column.

Quantification of Human Amyloid- β Peptide(1-40) by Ultraperformance Liquid Chromatography/Tandem Mass Spectrometry

Human amyloid- β peptide(1-40) and [^{15}N]hA β (1-40) were analyzed with a UPLC system (Waters ACQUITY UPLC system, Waters Co., Milford, MA, USA) connected to an ESI-triple quadrupole mass spectrometer (API5000; Applied Biosystems). hA β (1-40) solution was injected into a reversed-phase UPLC column (ACQUITY UPLC BEH C18 column 1.7 μm , 2.1 mm ID \times 50 mm, Waters) with the column oven set at 25°C. Mobile phase A consisted of 0.1% ammonium hydroxide and mobile phase B consisted of 0.1% ammonium hydroxide containing 20% acetonitrile. The peptides were separated and eluted from the column using the following gradient system: 0 to 2 minutes 15% B, 2 to 7 minutes 15–30% B, 7.0 to 7.01 minutes 30–60% B, 7.01 to 9 minutes 60% B, 9.0 to 9.01 minutes 60 to 15% B, 9.01 to 14 minutes 15% B at a flow rate of 0.5 mL/min. Samples dissolved in 0.1% ammonium hydroxide containing 20% acetonitrile were added to the sample tray held at 4°C, and 50 μL aliquots of the samples were injected into the column with an auto sampler. To prevent carry-over, 50 μL of 0.1% ammonium hydroxide containing 20% acetonitrile was injected between sample injections. The MS was set up to run an SRM/MRM experiment for detection of each hA β (1-40) (Table 1). The ion counts in the chromatograms were determined by using the quantitation procedures in Analyst software version 1.4.2 (Applied Biosystems). Human amyloid- β peptide was quantified by calculating the ratios of peak areas to those of isotope-labeled peptides, as described previously.¹⁵

Extraction of Human Amyloid- β Peptide(1-40) from Mouse Brain

[^{15}N]hA β (1-40) (1 pmol) was spiked into samples as an internal standard just before homogenization. Mouse brains were homogenized in nine volumes of 6 mol/L guanidinium hydrochloride (GuHCl) solution in a tissue homogenizer (Model PT1300D; Kinematica AG, Littau, Switzerland) and incubated for 3 hours on ice. Samples were ultracentrifuged at 100,000 *g* for 60 minutes at 4°C with a TLA-55 rotor using an Optima MAX-E ultracentrifuge (Beckman, Fullerton, CA, USA) and the supernatants were collected in new 1.5 mL tubes. Unlabeled hA β (1-40) and [^{15}N]hA β (1-40) were extracted from the supernatant of mouse brain homogenate by SPE as described previously, with minor modifications.¹² An Oasis HLB 1 mL (30 mg) extraction cartridge (Waters) was placed on a vacuum manifold and equilibrated using 1 mL of water followed by 1 mL of methanol. Samples were passed through the prepared SPE cartridges at 1 mL/min. To reduce sample loss, 200 μL of 6 M GuHCl solution was added to the sample tubes and these solutions were passed over the prepared SPE cartridges at 1 mL/min again. The SPE cartridges were then washed sequentially with 1 mL of 10% and 30% methanol, and then eluted with 1 mL of 2% NH_4OH in 90% MeOH. Eluted samples were dried in a centrifuge concentrator (Tomy Seiko Co., Ltd., Tokyo, Japan). Once samples had been dried completely, they were stored at –80°C until assay. The dried samples were resuspended in 200 μL of 0.1% ammonium hydroxide containing 20% acetonitrile, and these samples (50 μL) were analyzed by UPLC/MS/MS as described above.

Recovery of hA β (1-40) from mouse brain homogenates was determined according to equation 1;

$$\text{Recovery of hA}\beta(1-40) (\%) = \frac{\text{Peak area of } [^{15}\text{N}]\text{hA}\beta(1-40) \text{ spiked into brain homogenates before SPE}}{\text{Peak area } [^{15}\text{N}]\text{hA}\beta(1-40) \text{ spiked into brain homogenates after SPE}} \times 100 \quad (1)$$

Table 1. SRM/MRM channels and parameters of unlabeled and [^{15}N]-labeled hA β (1-40)

Target	Peptide sequence	Charge	SRM/MRM transition (<i>m/z</i>)					
			Q1	Q3	DP	CE	EP	CXP
hA β (1-40)	DAEFRHDSGYEVHHQKLVFF-	–5	864.7	861.3	–140	–10	–30	–31
	AEDVGSNKGAIIGLMVGGVV	–4	1,081.3	1,076.9	–190	–10	–40	–27
[^{15}N]hA β (1-40)	[^{15}N]DAEFRHDSGYEVHHQKLVFF-	–5	875.3	871.5	–140	–10	–30	–31
	AEDVGSNKGAIIGLMVGGVV	–4	1,094.4	1,090.0	–190	–10	–40	–27

CE, collision energy; CXP, collision cell exit potential; DP, declustering potential; EP, entrance potential; hA β (1-40), human amyloid- β peptide(1-40); SRM/MRM, selected/multiple reaction monitoring.

Measurement of FITC-Inulin in Mouse Brain

The supernatants of mouse brain homogenate were 10-fold diluted with 500 mmol/L HEPES (pH 7.4), because pH significantly affects the fluorescence of FITC.¹⁶ The duplicate samples were then loaded onto a 96-well plate (100 μ L sample/well). The standard solution was diluted with mouse brain homogenate without microinjection of FITC-inulin. Fluorescence was determined using a Fluoroscan Ascent FL (FIN-00811; Labsystems, Helsinki, Finland) with excitation at 485 nm and emission at 538 nm. Recovery of inulin in the supernatant of mouse brain homogenate after microinjection was $96.5 \pm 0.5\%$ ($n=4$, data not shown), as determined by ¹⁴C-radioactivity measurement of [¹⁴C]inulin in the supernatant and pellet of brain homogenate.

Brain Efflux Index Method

The *in vivo* brain elimination experiments were performed using the intracerebral microinjection technique previously reported.¹¹ Briefly, mice were anesthetized with an intramuscular injection of xylazine (1.22 mg/kg) and ketamine (125 mg/kg), and placed in a stereotaxic frame (SRS-6; Narishige, Tokyo, Japan) that determines the coordinates of the mouse brain coinciding with the secondary somatosensory cortex (S2) region. A small hole was made 3.8 mm lateral to the bregma, and a fine injection needle fitted on a 5.0 μ L microsyringe (Hamilton, Reno, NE, USA) was advanced to a depth of 2.5 mm. The applied solution (0.30 μ L) containing hA β (1-40) and FITC-inulin or [¹²⁵I]hA β (1-40) and [³H]dextran in an extracellular fluid (ECF) buffer (122 mmol/L NaCl, 25 mmol/L NaHCO₃, 3 mmol/L KCl, 1.4 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 0.4 mmol/L K₂HPO₄, 10 mmol/L D-glucose, and 10 mmol/L HEPES, pH 7.4) was administered into the S2 region over a period of 30 seconds. After microinjection, the microsyringe was left in place for 4 minutes to minimize any backflow. At designated times after microinjection, the ipsilateral (left) and contralateral (right) cerebrum and cerebellum were excised. For measurement of intact hA β (1-40) and FITC-inulin by UPLC/MS/MS analysis and fluorescence spectrometry, the brain and applied solution (0.30 μ L) were snap frozen in liquid nitrogen. The amounts of intact hA β (1-40) and FITC-inulin in mouse brain were measured as described above. For measurement of ¹²⁵I and ³H radioactivity, brains were dissolved in 2.0 mL 2 M NaOH at 60°C for 1 hour. The ¹²⁵I and ³H radioactivity of the samples were measured in a γ -counter (ART300, Aloka, Tokyo, Japan) for 3 minutes and a liquid scintillation counter (TRI-CARB2050CA, Packard Instruments, Meriden, CT, USA) for 5 minutes, respectively. We previously showed that no significant radioactivity was found in the contralateral cerebrum, cerebellum, or CSF at 60 minutes after microinjection of [¹²⁵I]hA β (1-40),¹¹ indicating that [¹²⁵I]hA β (1-40) did not diffuse to these regions from the injected hemisphere. In that study, the amount of microinjected [³H]dextran, a BBB-impermeable compound, was not significantly changed after microinjection,¹¹ indicating that the BBB remained intact during the Brain Efflux Index (BEI) study. The cerebral clearance was defined according to equation 2, and the percentage of intact hA β (1-40) remaining in the ipsilateral cerebrum was determined using equation 3:

$$\text{Cerebral clearance (\%)} = \frac{\text{Clearance of intact hA}\beta(1-40) \text{ from the ipsilateral cerebrum}}{\text{Intact hA}\beta(1-40) \text{ injected into the brain}} \times 100 \quad (2)$$

$$\text{Percentage remaining in the ipsilateral cerebrum (\%)} = \frac{\text{Amount of intact hA}\beta(1-40) \text{ in the brain/amount of FITC-inulin in the brain}}{\text{Concentration of intact hA}\beta(1-40) \text{ injected/concentration of FITC-inulin injected}} \times 100 \quad (3)$$

The clearance rate constant, reflecting both degradation in the brain and elimination across the BBB, was determined from the slope obtained by fitting a semilogarithmic plot of remaining percentage of intact hA β (1-40) in the ipsilateral cerebrum versus time, using the nonlinear least-squares regression analysis program MULTI.¹⁷

To characterize the clearance system in the brain, the clearance rate constant calculated from the slope of the profile of percentage remaining in the ipsilateral cerebrum at 5 and 30 minutes was determined in the presence or absence of several inhibitors.

The BEI was defined according to equation 4, and the percentage of substrate remaining in the ipsilateral cerebrum (100-BEI) was determined

using equation 5:

$$\text{BEI (\%)} = \frac{[^{125}\text{I}]\text{hA}\beta(1-40) \text{ undergoing efflux at the BBB}}{[^{125}\text{I}]\text{hA}\beta(1-40) \text{ injected into the brain}} \times 100 \quad (4)$$

$$100 \text{ BEI (\%)} = \frac{\text{Amount of } [^{125}\text{I}]\text{hA}\beta(1-40) \text{ in the brain/amount of } [^3\text{H}]\text{dextran in the brain}}{\text{Concentration of } [^{125}\text{I}]\text{hA}\beta(1-40) \text{ injected/concentration of } [^3\text{H}]\text{dextran injected}} \times 100 \quad (5)$$

The apparent brain-to-blood elimination rate constant across the BBB was determined from the slope obtained by fitting a semilogarithmic plot of (100-BEI) versus time, using the nonlinear least-squares regression analysis program MULTI.¹⁷

To characterize the efflux transport system at the BBB, the elimination rate constant across the BBB calculated from the slope of the profile of (100-BEI (%)) at 5 and 30 minutes was determined in the presence or absence of several inhibitors.

Data Analysis

Unless otherwise indicated, all data represent the mean \pm s.e.m. One-way analysis of variance followed by Dunnett's test was used to assess the statistical significance of differences among the means of more than two groups.

RESULTS

Quantification of Human Amyloid- β Peptide(1-40) by Liquid Chromatography/Tandem Mass Spectrometry with Selected/Multiple Reaction monitoring

Full scanning analyses were performed in the range of m/z 600 to 1,200 for hA β (1-40) and [¹⁵N]hA β (1-40) and two product ions were selected for SRM/MRM analysis (-5 ion transition and -4 ion transition). Mixtures of different amounts of hA β (1-40) (5, 10, 50, 100, 500, and 1,000 fmol) and 250 fmol of [¹⁵N]hA β (1-40), used as an internal standard, were measured under optimal conditions using the two SRM/MRM transitions. Calibration curves showed linear regression lines in the range of 5 to 1,000 fmol for hA β (1-40) ($R^2 = 0.999$ for the -5 ion transition and 0.996 for the -4 ion transition) (Figure 1A). There was no significant difference in peak area ratio determined using the two different SRM/MRM transitions ($R^2 = 0.999$; Figure 1B). The limit of detection of hA β (1-40) was 1.42 fmol for the -5 ion transition and 3.24 fmol for the -4 ion transition. To improve reliability, the amount of hA β (1-40) was determined from the values of both SRM/MRM transitions.

Before homogenizing brain tissue, a fixed amount of [¹⁵N]hA β (1-40) was spiked into each sample as an internal standard, and loss of hA β (1-40) during sample preparation, including SPE, was compensated by determining the peak area ratio of hA β (1-40) to [¹⁵N]hA β (1-40). Nevertheless, a low recovery of hA β (1-40) might still result in inaccuracy of quantification. Therefore, the recovery rate of hA β (1-40) was determined by comparing the amounts of [¹⁵N]hA β (1-40) in two samples: one in which [¹⁵N]hA β (1-40) was added before brain homogenization and one in which it was added after SPE. The recovery rate of [¹⁵N]hA β (1-40) determined from 116 measurements ($n=58$ each for -5 ion transition and -4 ion transition) was $74.2 \pm 1.2\%$. This suggested that recovery of hA β (1-40) from mouse brain in the procedure with SPE was sufficient.

Detection of Human Amyloid- β Peptide(1-40) in Mouse Brain After Microinjection

Human amyloid- β peptide(1-40) (5 μ mol/L) was microinjected into the S2 region of mouse brain, and the remaining amount was measured at 30 minutes after microinjection by means of UPLC/MS/MS analysis. The peaks of hA β (1-40) and [¹⁵N]hA β (1-40) were detected at same retention time using either the -5 or -4 ion transition (Figures 2A and 2B). In contrast, no significant peak of

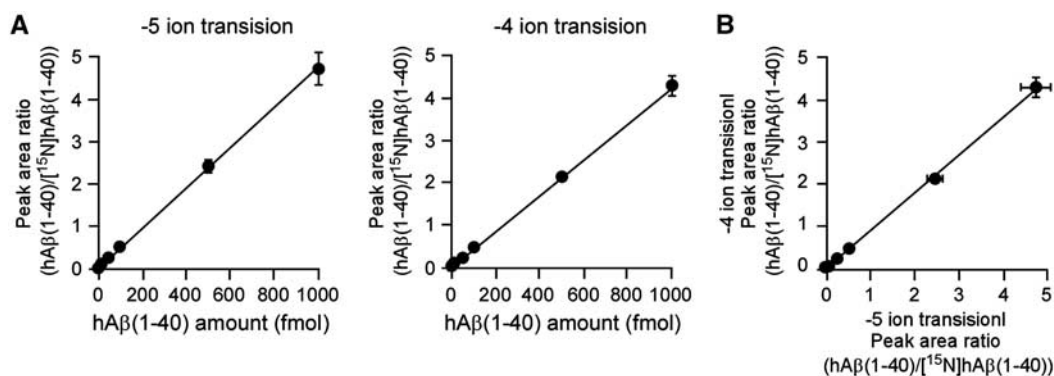


Figure 1. Linearity of calibration curves for human amyloid- β peptide(1-40) (hA β (1-40)) by ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS). **(A)** Calibration curve for hA β (1-40) by UPLC/MS/MS. Mixtures of hA β (1-40) (5, 10, 50, 100, 500, or 1,000 fmol) and [^{15}N]hA β (1-40) (250 fmol) dissolved in 50 μL of 0.1% ammonium hydroxide containing 20% acetonitrile were injected directly into the UPLC/MS/MS and two product ions of each hA β (1-40) were measured by selected/multiple reaction monitoring (SRM/MRM) analysis (– 5 ion transition and – 4 ion transition). Peak area ratios were calculated from the peak areas of hA β (1-40) and [^{15}N]hA β (1-40) (unlabeled/labeled). Each point represents the mean \pm s.e.m. ($n = 3$). **(B)** Comparison of calibration curves of the two product ions of each hA β (1-40) generated by SRM/MRM analysis. Each point represents the mean \pm s.e.m. ($n = 3$).

hA β (1-40) or [^{15}N]hA β (1-40) was observed in mouse brain in the absence of microinjection (Figures 2C and 2D). There was a high peak at the retention time of 7.5 minutes in all measured samples in either the – 5 or – 4 ion transition, and this appears to be a nonspecific peak derived from the mouse brain. The peak area ratio of the – 5 ion to – 4 ion of [^{15}N]hA β (1-40) from brain (0.953; Figures 2A and 2B) was decreased compared with that in the calibration curve sample (1.53; Figures 2E and 2F). This is presumably due to a greater ion suppression effect of the more highly charged ion, although the ratio of hA β (1-40) to [^{15}N]hA β (1-40) was not different in the two cases.

Clearance of Intact Human Amyloid- β Peptide(1-40) from Mouse Brain

Clearance of intact hA β (1-40) from mouse brain was investigated by quantifying intact hA β (1-40) remaining in the brain after microinjection. Figure 3 shows the time profile of the percentage of intact hA β (1-40) remaining in the ipsilateral cerebrum after microinjection of 5 $\mu\text{mol/L}$ hA β (1-40) into the S2 region of mouse brain. Remaining intact hA β (1-40) decreased in a time-dependent manner, and the clearance rate constant was determined to be $3.21 \times 10^{-2} \pm 0.42 \times 10^{-2}/\text{min}$ (mean \pm s.d.).

Inhibitory Effect of Amyloid- β Peptide-Degrading Enzyme Inhibitors on Clearance of Intact Human Amyloid- β Peptide(1-40) in Mouse Brain

To investigate the contribution of degradation processes to hA β (1-40) clearance, the effects of various enzyme inhibitors on the clearance of intact hA β (1-40) in mouse brain were examined (Table 2). A mixture of insulin (100 $\mu\text{g/mL}$) and phosphoramidon (10 mmol/L), which inhibit neprilysin (NEP), insulin-degrading enzyme (IDE) and endothelin-converting enzyme, markedly suppressed the clearance of intact hA β (1-40) by 94.6%. Thiorphan (1 mmol/L), a selective inhibitor of NEP, and insulin (100 $\mu\text{g/mL}$) significantly inhibited intact hA β (1-40) clearance by 85.7% and 54.8%, respectively.

Inhibitory Effect of Amyloid- β Peptide-Degrading Enzyme Inhibitors on Brain-to-Blood Elimination of [^{125}I] Radioactivity from Mouse Brain After [^{125}I]-Labeled Human Amyloid- β Peptide(1-40) Injection

The inhibitory effects of the same inhibitors were examined on elimination of [^{125}I] radioactivity from the mouse brain across the BBB after microinjection of [^{125}I]hA β (1-40). [^{125}I] radioactivity was

cleared from mouse brain with an elimination rate constant of $1.48 \times 10^{-2} \pm 0.17 \times 10^{-2}/\text{min}$ (mean \pm s.d., Table 3); this represents brain-to-blood elimination of intact and/or degraded [^{125}I]hA β (1-40) from the brain to blood across the BBB. Co-administration of a mixture of insulin (100 $\mu\text{g/mL}$) and phosphoramidon (10 mmol/L), thiorphan (1 mmol/L), and insulin (100 $\mu\text{g/mL}$) significantly inhibited the elimination of [^{125}I] radioactivity and the elimination rate constants were decreased by 97.8%, 35.7%, and 41.4%, respectively (Table 3).

DISCUSSION

In the present study, we developed a new quantitation method for intact hA β (1-40) in mouse brain by means of SPE followed by UPLC/MS/MS (Figures 1 and 2). Since hA β (1-40) is identified in terms of LC retention time and molecular mass, with stable isotope-labeled [^{15}N]hA β (1-40) as an internal standard, UPLC/MS/MS quantification affords higher specificity than antibody-based quantification. Although the sensitivity of the present UPLC/MS/MS quantification of hA β (1-40) is at least 10-fold lower than that of commercial enzyme-linked immunosorbent assay, the SPE step can concentrate hA β (1-40) from large volumes of biologic samples. Human amyloid- β peptide(1-42) was previously reported to be recovered by the same SPE method¹² and quantified by LC/MS/MS followed by immunoprecipitation.¹³ Therefore, it seems likely that various derivatives of hA β can be quantified by UPLC/MS/MS with high specificity, provided that they can be recovered by SPE.

Using the developed method, the clearance rate constant of intact hA β (1-40) (5 $\mu\text{mol/L}$) in mouse brain was determined to be $3.21 \times 10^{-2}/\text{min}$ (Figure 3). The clearance of intact hA β (1-40) reflects both degradation by proteases and brain-to-blood elimination by efflux transport at the BBB. We previously reported that the K_m value of [^{125}I]hA β (1-40) elimination across the rat BBB was 247 nmol/L (injectate concentration).¹⁰ The elimination rate constant in rat was $1.42 \times 10^{-2}/\text{min}$, and 28.5% of the elimination was estimated to be due to a nonsaturable (or low affinity) component.¹⁰ A self-inhibition study showed that 20 $\mu\text{mol/L}$ hA β (1-40) decreased the elimination rate constant across the BBB to 28.9%.¹⁰ Similarly, in the mouse study, the elimination rate constant across the BBB was $1.48 \times 10^{-2}/\text{min}$ (Table 3), and 20 $\mu\text{mol/L}$ hA β (1-40) decreased the elimination rate constant to 37.5%.¹¹ Therefore, it appears that the K_m value of [^{125}I]hA β (1-40) elimination across the BBB in mouse is similar to that in rat. If this is the case, then the concentration of microinjected hA β (1-40)

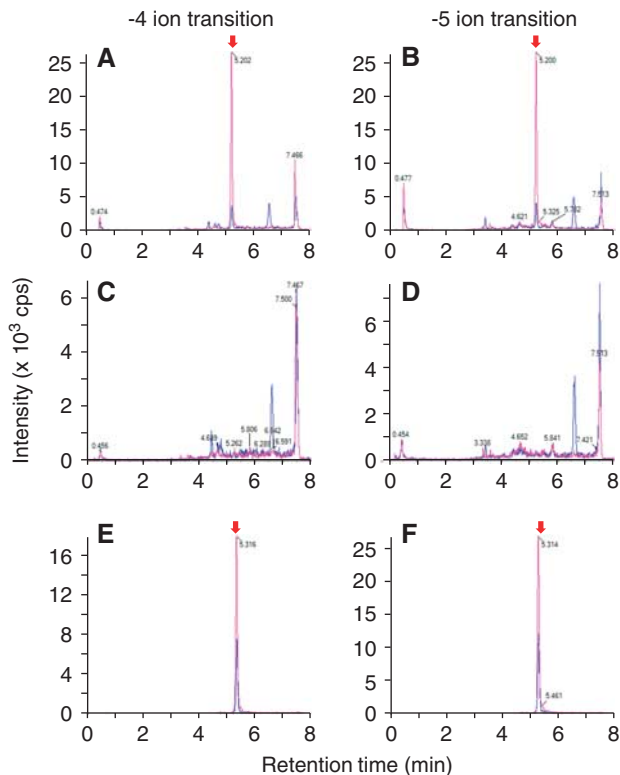


Figure 2. Detection of human amyloid- β peptide(1-40) (hA β (1-40)) in mouse brain after microinjection. In all figures, chromatograms of hA β (1-40) and [^{15}N]hA β (1-40) (internal standard) are indicated by blue and red lines, respectively. The peaks of hA β (1-40) and [^{15}N]hA β (1-40) are indicated by red arrows. (A, B) A mixture of hA β (1-40) (1.5 pmol) and FITC-inulin (9 μg) dissolved in 0.30 μL of extracellular fluid (ECF) buffer was microinjected into the S2 region of mouse brain. Five minutes after microinjection, whole mouse brain was removed and the microinjected region of the ipsilateral cerebrum was isolated. The brain tissue sample was homogenized in 6 mol/L guanidinium hydrochloride (GuHCl) solution after addition of [^{15}N]hA β (1-40) (1 pmol) and then ultracentrifuged. The supernatant was passed through an HLB column, dried, and resuspended in 0.1% ammonium hydroxide containing 20% acetonitrile. Two product ions of hA β (1-40) (blue line) and [^{15}N]hA β (1-40) (red line) were measured by selected/multiple reaction monitoring (SRM/MRM) analysis (-4 ion transition (A) and -5 ion transition (B)) for each hA β (1-40). (C, D) Mouse brain without microinjection of a mixture of hA β (1-40) and FITC-inulin in ECF buffer was homogenized in 6 mol/L GuHCl solution without addition of [^{15}N]hA β (1-40) and then ultracentrifuged. The supernatant was passed through an HLB column, dried, and resuspended in 0.1% ammonium hydroxide containing 20% acetonitrile. Two product ions of hA β (1-40) (blue line) and [^{15}N]hA β (1-40) (red line) were measured by SRM/MRM analysis (-4 ion transition (C) and -5 ion transition (D)). (E, F) The mixture of hA β (1-40) (100 fmol, blue line) and [^{15}N]hA β (1-40) (250 fmol, red line) was subjected to SRM/MRM analysis (-4 ion transition (E) and -5 ion transition (F)) for the calibration curve.

(5 $\mu\text{mol/L}$) was 20.2-fold greater than the K_m value. Therefore, we can assume that the saturable component of hA β (1-40) elimination from mouse brain across the BBB was saturated. Accordingly, the elimination rate constant across the BBB can be estimated to be $0.560 \times 10^{-2}/\text{min}$ when 5 $\mu\text{mol/L}$ hA β (1-40) was microinjected, as shown in Table 2 (see Supplementary Information for detailed calculations).

The K_m values of NEP, IDE, and endothelin-converting enzyme for hA β (1-40) have been reported to be in the micromolar range,^{18–20} being much greater than the estimated hA β (1-40) concentration in the injected area, and this suggests that the A β

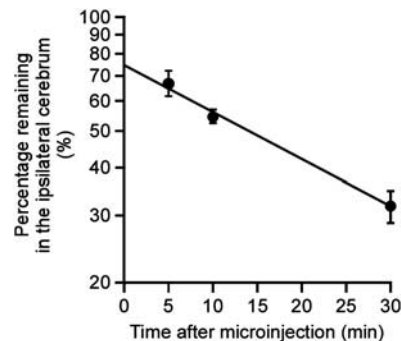


Figure 3. Time course of intact human amyloid- β peptide(1-40) (hA β (1-40)) remaining in the ipsilateral cerebrum after intracerebral microinjection. A mixture of hA β (1-40) (1.5 pmol) and FITC-inulin (9 μg) dissolved in 0.30 μL of extracellular fluid (ECF) buffer was injected into the S2 region of mouse brain. After the indicated times, the amounts of hA β (1-40) and FITC-inulin in mouse brain were measured as described in Materials and methods. The solid line was obtained with the nonlinear least-squares regression analysis program, MULTI. Each point represents the mean \pm s.e.m. ($n = 4$ to 11).

degradation process was not saturated. Taking these findings into consideration, the clearance and elimination rate constants across the BBB determined in this study are summarized in Figure 4. From the observation that A β -degrading enzyme inhibitors inhibited [^{125}I]hA β (1-40) elimination across the BBB (Table 3) and our previous finding that both intact and degraded [^{125}I]hA β (1-40) were detected in plasma at 5 minutes after microinjection into rat cerebral cortex,¹⁰ two different brain-to-blood elimination pathways appear to operate; one is the elimination of intact hA β (1-40), and the other is the elimination of degradation products of hA β (1-40). The elimination rate constant of intact hA β (1-40) across the BBB was estimated to be less than $0.560 \times 10^{-2}/\text{min}$ from the UPLC/MS/MS study (Figure 4A). The degradation rate constant of intact hA β (1-40) was estimated to be at least $2.69 \times 10^{-2}/\text{min}$ ($3.25 \times 10^{-2} - 0.560 \times 10^{-2}/\text{min}$), which is at least 1.8-fold greater than the apparent elimination rate constant of hA β (1-40) across the BBB (Figures 4A and 4B). On the basis of these calculations, it appears that degradation has a dominant role in cerebral clearance of hA β (1-40).

The clearance of hA β (1-40) was almost completely inhibited by insulin and phosphoramidon (Table 2), indicating that hA β (1-40) was degraded mainly via insulin- or phosphoramidon-sensitive pathways, which include the reactions catalyzed by IDE, NEP, and endothelin-converting enzyme. The inhibitory effects of insulin alone and thiorphan alone suggest that insulin- and thiorphan-sensitive degradation processes account for at least 54.8% and 84.6% of hA β (1-40) degradation in the brain (Table 2). It was reported that microinjection of thiorphan (1 mmol/L) and phosphoramidon (10 mmol/L) into rat hippocampus markedly inhibited degradation of radiolabeled hA β (1-42).²¹ The K_m value of insulin for IDE is 25 nmol/L,²² which is ~ 23 -fold lower than the estimated insulin concentration at the injection site (568 nmol/L). Thus, these inhibitors appear to be present at sufficiently high concentrations to inhibit A β degradation in the brain. The roles of NEP and IDE in A β accumulation in mouse brain have been investigated using the corresponding gene-deficient mice.^{7,8} In NEP-deficient mouse brain, endogenous A β (1-40) was increased by ~ 2 -fold compared with wild-type mice.⁸ In IDE-deficient mouse brain, endogenous A β (1-40) was increased by ~ 1.2 - to 1.6-fold compared with wild-type mice.^{7,23} The greater effect of NEP is consistent with the contribution of each protease suggested by the present study (Table 2). Furthermore, when the percentage contributions of IDE and NEP to the degradation were added, the total was greater than 100%, suggesting that some part of intact hA β (1-40) is degraded by both IDE and NEP.

Table 2. Effects of inhibitors of A β -degrading enzymes on hA β (1-40) clearance from mouse brain, obtained from measurements of intact hA β (1-40)

Inhibitor	Clearance rate constant (1/min)	% Of control
Control	$3.25 \times 10^{-2} \pm 0.46 \times 10^{-2}$	100
100 μ g/mL insulin + 10 mmol/L phosphoramidon	$0.180 \times 10^{-2} \pm 0.51 \times 10^{-2}$	5.42***
100 μ g/mL insulin	$1.47 \times 10^{-2} \pm 0.34 \times 10^{-2}$	45.2**
1 mmol/L thiorphan	$0.499 \times 10^{-2} \pm 0.467 \times 10^{-2}$	15.4***

BEI, Brain Efflux Index; ECF, extracellular fluid; hA β (1-40), human amyloid- β peptide(1-40).

A mixture of hA β (1-40) (1.5 pmol) and FITC-inulin (9 μ g) dissolved in 0.30 μ L of ECF buffer was injected into the S2 region of mouse brain in the presence or absence of inhibitors. The clearance rate constant was calculated from the slope of the profile of (100-BEI (%)) at 5 and 30 minutes (each point $n = 3$ to 4). Each value represents the mean \pm s.d.. ** $P < 0.01$, *** $P < 0.001$, significantly different from control.

Table 3. Effects of inhibitors for A β -degrading enzymes on the elimination of 125 I radioactivity from mouse brain after microinjection of [125 I]hA β (1-40)

Inhibitor	Elimination rate constant (1/min)	% of control
Control	$1.48 \times 10^{-2} \pm 0.17 \times 10^{-2}$	100
100 μ g/mL insulin + 10 mmol/L phosphoramidon	$0.03 \times 10^{-2} \pm 0.27 \times 10^{-2}$	2.17***
100 μ g/mL Insulin	$0.86 \times 10^{-2} \pm 0.23 \times 10^{-2}$	58.6**
1 mmol/L thiorphan	$0.95 \times 10^{-2} \pm 0.28 \times 10^{-2}$	64.3*

BEI, Brain Efflux Index; ECF, extracellular fluid; hA β (1-40), human amyloid- β peptide(1-40).

A mixture of [125 I]hA β (1-40) (0.012 μ Ci, 18.2 nmol/L) and [3 H]dextran (0.12 μ Ci) dissolved in 0.30 μ L of ECF buffer was injected into the S2 region of mouse brain in the presence or absence of inhibitors. The elimination rate constant was calculated from slope of the profile of (100-BEI (%)) at 5 and 30 minutes (each point $n = 4$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from control.

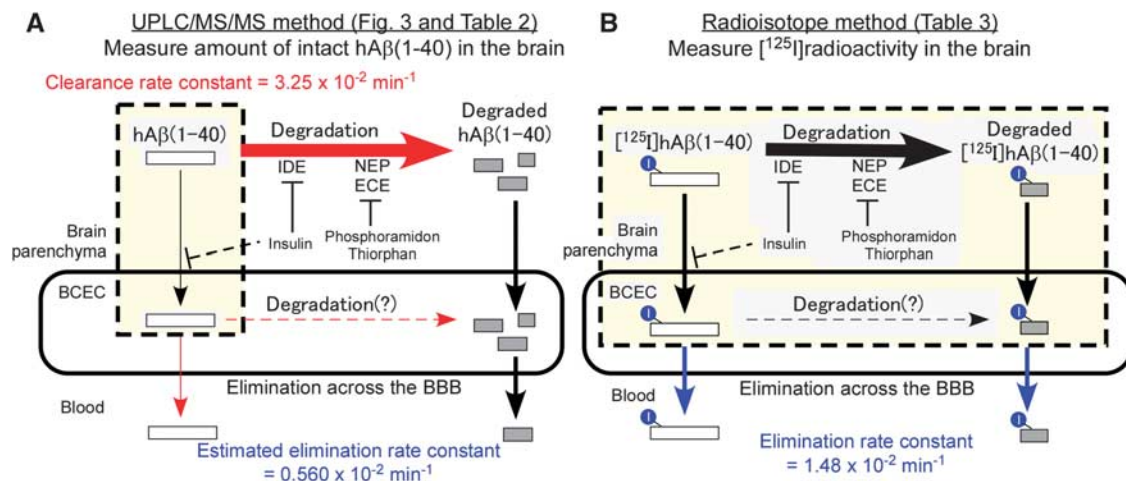


Figure 4. Schematic representations of analysis of cerebral human amyloid- β peptide(1-40) (hA β (1-40)) clearance by ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) and radioisotope methods. **(A)** UPLC/MS/MS-based analysis of cerebral hA β (1-40) clearance. UPLC/MS/MS measures the amount of intact hA β (1-40) in the brain (yellow box), which includes the brain parenchyma and brain capillary endothelial cells (BCEC). The determined clearance rate constant represents both degradation of intact hA β (1-40) and brain-to-blood elimination of intact hA β (1-40) across the blood-brain barrier (BBB) (red arrows). The elimination rate constants of intact and degraded hA β (1-40) were estimated as described in Discussion and Supplementary Information. **(B)** Radioisotope-based analysis of brain-to-blood [125 I]hA β (1-40) elimination across the BBB, which measures [125 I] radioactivity in the brain (yellow box), including the brain parenchyma and BCEC. The determined elimination rate constant represents brain-to-blood elimination of both intact and degraded hA β (1-40) across the BBB (blue arrows). Insulin inhibited [125 I]hA β (1-40) uptake into cultured immortalized mouse BCEC, and the possible inhibitory effect *in vivo* is indicated by dashed lines. NEP, neprilysin; IDE, insulin-degrading enzyme; ECE, endothelin-converting enzyme.

The elimination of [125 I]hA β (1-40) from the brain across the BBB was inhibited by thiorphan (Table 3). Thiorphan (10 μ mol/L) did not inhibit the internalization of [125 I]hA β (1-40) into conditionally immortalized mouse brain capillary endothelial cells (TM-BBB4).²⁴ This *in vitro* concentration was close to the estimated concentration of thiorphan at the injection site *in vivo* (33 μ mol/L = 1 mmol/L/30.3), since injectate was reported to be diluted at least 30.3-fold by diffusion in the brain.²⁵ This result suggests that

NEP-degraded [125 I]hA β (1-40) was partially eliminated from the brain by efflux transport at the BBB. The thiorphan-sensitive clearance rate constant was estimated to be 2.75×10^{-2} /min (3.25×10^{-2} (control) - 0.499×10^{-2} /min (+ thiorphan); Table 2), and the thiorphan-sensitive elimination rate constant across the BBB was estimated to be 0.53×10^{-2} /min (1.48×10^{-2} (control) - 0.95×10^{-2} /min (+ thiorphan); Table 3). On the basis of these rate constants, ~19% of the [125 I]hA β (1-40) degradation products

generated by NEP appears to be eliminated from the brain across the BBB on the assumption that thiorphan inhibited only the hA β (1-40) degradation process. Since the elimination study using [¹²⁵I]hA β (1-40) covered the elimination of only degraded peptides containing ¹²⁵I-labeled tyrosine, further study may be necessary to examine the elimination of degraded peptide(s) not containing labeled tyrosine and also to clarify the molecular mechanism of the elimination of hA β (1-40) from the brain across the BBB.

Insulin also inhibited the elimination of [¹²⁵I]hA β (1-40) from the brain across the BBB. One possible explanation is that degradation products of [¹²⁵I]hA β (1-40) generated by IDE were partially eliminated from the brain by efflux transport at the BBB. However, insulin is also a ligand of various receptors, and the possibility that insulin inhibited the internalization of [¹²⁵I]hA β (1-40) into capillary endothelial cells cannot be excluded. Our previous report showed that low-density lipoprotein receptor-related protein 1 does not contribute to the elimination of [¹²⁵I]hA β (1-40) from mouse cerebral cortex, but unknown molecules, whose function may be insulin sensitive, have a role in the elimination.^{10,11} Indeed, [¹²⁵I]hA β (1-40) internalization into TM-BBB4 cells was inhibited by insulin (100 μ g/mL), but not by thiorphan (10 μ mol/L) or phosphoramidon (100 μ mol/L), supporting the idea that an insulin-sensitive internalization process is involved in the elimination of A β from the brain across the BBB.²⁴

The present study examined the clearance of hA β (1-40) in mouse brain. Nprilysin and IDE are expressed in human brain, and their activities are reduced age and region specifically.^{26–28} Postmortem studies indicate that NEP protein expression and activity were inversely associated with AD pathology.^{26–33} Thus, it seems reasonable to assume that these proteins, especially NEP, are involved in A β (1-40) clearance in human brain. As for the brain-to-blood elimination process, our *in vitro* study showed that [¹²⁵I]hA β (1-40) internalization into mouse brain capillary endothelial cell line (TM-BBB4) was significantly inhibited by unlabeled mouse A β (1-40),²⁴ suggesting that hA β (1-40) and mouse A β (1-40) share the same molecular mechanism of internalization into mouse brain capillary endothelial cells. The molecule responsible for hA β (1-40) elimination across the BBB remains unknown even in rodents. The available evidence suggests that cerebral hA β (1-40) clearance in humans involves similar molecular mechanisms to those in rodents, at least in part. Although the *in vivo* experiments performed in the present study could not be performed in humans, several human BBB models have recently become available, such as D3 cells,³⁴ TY08 cells,³⁵ and human brain capillary endothelial cells derived from induced pluripotent stem cells.³⁶ Further experiments using these human cells should help to establish the relevance of our mouse data to humans, as well as providing more details of the mechanisms of cerebral hA β (1-40) degradation and elimination in human brain.

In conclusion, our present study suggests that both degradation and elimination are involved in the cerebral clearance of hA β (1-40). Furthermore, an insulin- or thiorphan-sensitive process was involved in both degradation and elimination. A part of hA β (1-40) was eliminated from the brain across the BBB via successive processes of degradation and elimination of the degradation product(s). It remains important to clarify the molecular mechanisms of this multistep clearance system to understand the pathophysiologic role of hA β (1-40), and to identify potential therapeutic targets for AD.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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