Reduction of Brain β-Amyloid (Aβ) by Fluvastatin, a Hydroxymethylglutaryl-CoA Reductase Inhibitor, through Increase in Degradation of Amyloid Precursor Protein C-terminal Fragments (APP-CTFs) and Aβ Clearance

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Epidemiological studies suggest that statins (hydroxymethylglutaryl-CoA reductase inhibitors) could reduce the risk of Alzheimer disease. Although one possible explanation is through an effect on β-amyloid (Aβ) metabolism, its effect remains to be elucidated. Here, we explored the molecular mechanisms of how statins influence Aβ metabolism. Fluvastatin at clinical doses significantly reduced Aβ and amyloid precursor protein C-terminal fragment (APP-CTF) levels among APP metabolites in the brain of C57BL/6 mice. Chronic intracerebroventricular infusion of lysosomal inhibitors blocked these effects, indicating that up-regulation of the lysosomal degradation of endogenous APP-CTFs is involved in reduced Aβ production. Biochemical analysis suggested that this was mediated by enhanced trafficking of APP-CTFs from endosomes to lysosomes, associated with marked changes of Rab proteins, which regulate endosomal function. In primary neurons, fluvastatin enhanced the degradation of APP-CTFs through an isoprenoid-dependent mechanism. Because our previous study suggests additive effects of fluvastatin on Aβ metabolism, we examined Aβ clearance rates by using the brain efflux index method and found its increased rates at high Aβ levels from brain. As LRP1 in brain microvessels was increased, up-regulation of LRP1-mediated Aβ clearance at the blood-brain barrier might be involved. In cultured brain microvessel endothelial cells, fluvastatin increased LRP1 and the uptake of Aβ, which was blocked by LRP1 antagonists, through an isoprenoid-dependent mechanism. Overall, the present study demonstrated that fluvastatin reduced Aβ level by an isoprenoid-dependent mechanism. These results have important implications for the development of disease-modifying therapy for Alzheimer disease as well as understanding of Aβ metabolism.

Alzheimer disease (AD) is a progressive neurodegenerative disease, being the most prevalent disorder among dementia. The discoveries that the genes of familial AD-linked mutation up-regulate Aβ production and the increased rate of Aβ42 production is associated with the age of onset provide conclusive evidence for the amyloid hypothesis in the pathogenesis of AD (1). Several therapies based on the amyloid hypothesis are being examined, including γ-secretase inhibitors and Aβ vaccine therapy, as disease-modifying therapy. However, there are still many unresolved issues with their clinical application (2, 3). Furthermore, recent failures of clinical trials of these therapies raise questions on delayed timing of intervention and the efficacy of targeting only one pathway of Aβ metabolism (4, 5). More efficient treatment with higher safety is needed to treat AD.

On the other hand, from basic and clinical reports, statins (hydroxymethylglutaryl-CoA reductase inhibitors), which are widely used for the treatment of hypercholesterolemic patients, might be beneficial in AD. Clinically, many case control studies support the protective effect of statins (6–8), whereas the results of prospective studies and randomized clinical trials are still controversial (9–11). Larger randomized clinical trials considering intervention timing, dose, duration, kind of statins, and main end point (i.e. prevention or cognitive decline) are required to clarify the efficacy of statins (12).

Although statins affect Aβ metabolism, their proposed mechanism of action on Aβ production is quite diverse as follows: up-regulation of α-secretase processing, down-regulation of β-secretase processing, down-regulation of γ-secretase processing, modulation of APP trafficking, and up-regulation of APP-CTF degradation (13–24). However, it should be noted that these various mechanisms were demonstrated mostly by in vitro studies. In considering the in vivo effects on Aβ metabolism, several points should be clarified. Firstly, which of the two
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Effects (cholesterol-dependent effect and isoprenoid-dependent effect; Ref. 21) affects Aβ metabolism *in vivo* more strongly! Secondly, because the concentration of statin might be important (17), what are the physiological levels of statins at clinically relevant doses and how do statins affect Aβ metabolism at those levels? Thirdly, we previously demonstrated that the protective effect of fluvastatin in an Aβ-induced memory impairment mouse model was associated with reduced Aβ accumulation, suggesting additional effects on Aβ metabolism other than Aβ production (25). Here, the present study demonstrated that fluvastatin affected Aβ metabolism in the brain through a reduction of Aβ production and an increase in Aβ clearance via up-regulation of lysosomal degradation of Aβ.

**EXPERIMENTAL PROCEDURES**

*Animals*—C57BL/6 mice as well as APP23 transgenic mice were used in this study. APP23 transgenic mice overexpress human APP with Swedish double mutation (KM670/671NL) under the control of Thy-1 promoter (26). All hemizygous (+/−) transgenic animals were crossed with nontransgenic background strain animals (C57BL/6) to obtain transgenic (+/−) animals. Animals were housed in specific pathogen-free facilities under a standard 12:12-h light/dark cycle with free access to both food and water. All experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of Osaka University School of Medicine.

*Drug Administration to Animals*—Administration of fluvastatin was started at 8 weeks of age and continued for 4 weeks in all experiments, except for that of co-administration with lysosomal inhibitors. In experiments with lysosomal inhibitors, fluvastatin treatment was continued for 5 weeks. Mice received fluvastatin at 5 mg/kg/day added as a diet admixture (0.008%) or vehicle. This dose of fluvastatin is equivalent to the dose in clinical usage (20 mg/day) and did not affect plasma cholesterol level or markers of hepatic toxicity (data not shown).

One week of chronic administration of leupeptin or E64 (Peptide Institute Inc., Osaka Japan) into the cerebral ventricle was performed as described previously (27, 28). An osmotic minipump (model 2002; ALZET, Cupertino, CA) was loaded with ACSF buffer (148 mM NaCl, 3 mM KCl, 1.4 mM CaCl2, 0.75 mM MgCl2, 0.8 mM Na2HPO4, 0.2 mM NaH2PO4), leupeptin (20 mg/ml in ACSF), or E64 (20 mg/ml in ACSF) connected to the brain infusion assembly (brain infusion kit 3; ALZET) and incubated with sterile saline at 37 °C for 48 h. Anesthetized mice were placed in a stereotaxic apparatus (Narishige, Tokyo, Japan), and a midline incision was made to expose an area of the skull. A catheter was inserted into the lateral ventricle of the brain, and the connected osmotic minipump was implanted subcutaneously in the midscapular area of the back of each animal. The coordinates for cannula placement were: antero-posterior, 0.2 mm to bregma; mediolateral, 0.8 mm to bregma; and dorsoventral, 2.5 mm to cranium. A hole was drilled in the skull, the cannula was glued to the cleaned and scraped skull with Aron Alpha (jelly type; Toagosei, Tokyo, Japan), and the incision was closed over the assembly. During the intracerebroventricular administration of leupeptin or E64, there were no significant changes in body weight, food intake, or general appearance (data not shown).

*In Vitro Cell Culture*—Primary cultures of cortical neurons were prepared from day 16–18 C57BL/6 mouse embryos as described previously (29). In brief, cerebral cortices devoid of meninges were digested with trypsin/DNase I and dispersed by pipetting. Dissociated neurons were seeded onto polyethyleneimine (Sigma-Aldrich, Tokyo, Japan)-coated culture dishes at a density of 105 cells/cm2. Neurons were cultivated in Dulbecco’s modified Eagle’s medium with high glucose (Invitrogen, Tokyo, Japan) plus 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin. The medium was changed every 2 days. Before experiments, 5 μM cytosine β-D-arabinofuranoside (Sigma-Aldrich) was added from the 4th day for 2 days to block the increase in glia cells. Primary human brain microvascular endothelial cells (HBMEC; ScienCell, San Diego, CA) were plated on collagen type I-coated dishes (Iwaki, Tokyo, Japan) and cultured in endothelial cell basal medium-2 (EBM-2) supplemented with EGM®-2 containing 2% fetal bovine serum (Lonza, Walkersville, MD) and 100 μg/ml penicillin/streptomycin according to the manufacturer’s instructions.

*Sample Preparation*—Following drug administration, anesthetized mice were transcardially perfused with saline, and the brain was removed and cut sagittally into the left and right hemispheres. After removing the olfactory lobe and cerebellum, the hemispheres were snap-frozen in liquid nitrogen and stored at −80 °C. For biochemical analysis, these brains or the harvested cells were homogenized in radioimmuno precipitation lysis buffer (Millipore, Billerica, CA) containing a protease inhibitor mixture (PIM; Roche Diagnostics K.K., Tokyo, Japan) with a Teflon-glass homogenizer and then sonicated on ice. The resulting supernatants (total fraction) were used for Western blot analysis. In a separate study, the microsomal fraction was prepared as described previously (25). In brief, brain samples were homogenized in tissue homogenization buffer (THB; 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA) containing PIM. After centrifugation at 1600 × g for 5 min at 4 °C, the postnuclear supernatant was further centrifuged at 100,000 × g for 60 min at 4 °C to collect the pellet. After washing with ice-cold 100 mM Na2CO3 (pH 11.3), the precipitant was resuspended in THB containing PIM, solubilized by sonication, and used as the microsomal fraction, and the supernatant was also collected as the “soluble fraction.” Protein concentration was determined by the Lowry method (Bio-Rad, Tokyo, Japan) to load an equal quantity of sample in Western blot analysis, which was performed as described previously (25).

*Assessment of Aβ and APP Metabolites*—The microsomal fraction as described above was used to detect full-length APP (APP-FL) and APP C-terminal fragment (APP-CTFs: APP-CTFβ and APP-CTFα), and the soluble fraction was used to detect secreted APP (sAPPs: sAPPα and sAPPβ) in Western blot analysis and ELISA analysis (IBL, Gunma, Tokyo, Japan). For measurement of endogenous Aβ in the brain, the diethylamine extraction method was used (30). Briefly, the above described brain homogenate in THB was further homogenized with an equal volume of cold 0.4% diethylamine and 100 mM NaCl on ice. After centrifugation at 100,000 × g for 1 h at 4 °C, the supernatant was neutralized with a 1/10 volume of 0.5 M...
Tris base (pH 6.8) and applied to the Aβ ELISA system (Wako, Osaka, Japan).

To check our detection methods, APP metabolites in the brain of C57BL/6 mice (male, 8 weeks old) were measured after treatment with γ- and β-secretase inhibitors, using APP23 mice as a positive control (supplemental Fig. 1). Firstly, we measured their levels in the brain 4 h after subcutaneous administration of DAPT (Peptide Institute Inc.) at 100 mg/kg as reported previously (31). DAPT administration reduced the Aβ levels by ~60% (data not shown) and increased the APP-CTF levels (supplemental Fig. 1A). Secondly, we measured their levels in the brain at 3 h after intracerebroventricular administration of BACE inhibitor-IV (Calbiochem) according to previously described methods (32). BACE inhibitor-IV reduced the sAPPβ level and increased the sAPPα level (supplemental Fig. 1B). These preliminary studies confirmed that the changes in APP metabolites in the brain could be tracked by the methods employed in our study. The brain microsomal fraction was also subjected to dephosphorylation with protein phosphatase (Millipore) for 4 h in a supplied buffer containing PIM and 10 μM DAPT to detect dephosphorylated APP-CTFs as described previously (33).

Biochemical Fractionation—The hemispheres of the mouse forebrain were homogenized in THB buffer containing PIM. After collecting the postnuclear supernatant, the samples were centrifuged at 100,000 × g for 60 min at 4 °C and resuspended in suspension buffer (500 mM sucrose, 40 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA) followed by an additional 20 strokes in 1-ml syringes fitted with a 24-, 27-, and 30-gauge needle, sequentially. Then, the samples were mixed with 2 volumes of 60% iodixanol (Axis-Shield Plc, Dundee, Scotland, UK) and applied to the bottom of a preformed 2–30% iodixanol continuous gradient in THB containing PIM and DAPT, which was made by Gradient Master (BIOCOMP, Fredericton, NB, Canada). The gradient was centrifuged for 20 h at 100,000 × g in a rotor (model SW41; Beckman Coulter, Fullerton, CA) at 4 °C. Twenty fractions were collected from the top of the tubes by a piston gradient fractionator (BIOCOMP). The samples were trichloroacetic acid-precipitated and resolubilized in 2 volumes of sample buffer for Western blot analysis.

Brain Efflux Index (BEI) Study—The in vivo brain elimination experiments were performed using the intracerebral microinjection technique reported previously (34–36). Fluvastatin-treated or untreated C57BL/6 mice were anesthetized with intraperitoneal xylazine and ketamine (20 and 100 mg/kg, respectively) and placed in a stereotaxic apparatus. Core body temperature was maintained at 37 °C using a heating pad with a thermprobe (ATC-101B; Unique Medical, Tokyo, Japan). A 30-gauge needle (TOP, Tokyo, Japan) connected via Teflon tubing (JT-10; Eicom, Kyoto, Japan) to a 10-μl gas-tight Hamilton syringe (RN1701; Hamilton) was inserted into the somatosensory cortex, through a 0.3-mm hole at 0.5 mm anterior and 3.5 mm lateral to the bregma and at a depth of 1.3 mm. 125I-Aβ (1–40) (0.02 μCi; PerkinElmer Life Sciences) and [14C]inulin (0.01 μCi; PerkinElmer Life Sciences) dissolved in 0.3 μl of ECF buffer (122 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1.4 mM CaCl2, 1.2 mM MgSO4, 0.4 mM K2HPO4, 10 mM d-glucose, and 10 mM HEPES, pH 7.4; 125I-Aβ concentration = 20 nM) were administered at a speed of 0.1 μl/min using a micro-syringe pump (NE-1000; Neuroscience, Osaka, Japan). The intactness and quality of 125I-Aβ were confirmed by HPLC fractionation, SDS-PAGE fluorography, trichloroacetic acid precipitation, and ELISA analysis, as reported previously (34). Synthetic Aβ(1–40) peptides (Peptide Institute Inc.) were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (Kanto Chemical, Tokyo, Japan), dried, and resolubilized in ECF buffer. To minimize any backflow of injectate, the needle was left in place for 3 min after administration. The dose-dependent effect with or without fluvasatin treatment was determined at 60 min. At the designated time, the ipsilateral cerebrum was excised, snap-frozen in liquid nitrogen, and homogenized in THB with PIM. About half of this homogenate was solubilized in tissue solubilizer (Soluene®-350; PerkinElmer Life Sciences) at 65 °C and mixed with liquid scintillation mixture (LumaSafe Plus; PerkinElmer Life Sciences). The remains of the homogenate were trichloroacetic acid-precipitated to calculate the rate of intact Aβ (34). 125I radioactivity of the samples was measured with a γ-counter (ARC-2000; Aloka, Tokyo, Japan), and 14C radioactivity was measured with a liquid scintillation counter (WALLAC 1409; PerkinElmer Life Sciences) using a double-channel system.

BEI was defined by Equation 1, and the percentage of substrate remaining in the cerebrum (100 − BEI) was determined using Equation 2, as previously described (35).

\[
BEI(%) = \left( \frac{\text{Test substrate undergoing efflux at BBB}}{\text{Test substrate injected into brain}} \right) \times 100
\]

(Eq. 1)

\[
100 - \text{BEI}(\%) = \left( \frac{\text{Amount of intact [125I]Aβ in brain}}{\text{Amount of intact [125I]Aβ injected}} \right) \times 100
\]

(Eq. 2)

The apparent elimination rate constant, \( k_{\text{app,el}} \), was estimated by linear regression analysis of the semilogarithmic plot of (100 − BEI)% versus time. Kinetic parameters of Aβ clearance were calculated by using Equation 3.

\[
k_{\text{app,el}} = \frac{k_{\text{el,max}}}{(K_m + C)} + k_{\text{el,NS}}
\]

(Eq. 3)

\( k_{\text{el,max}}, K_m, \) and \( k_{\text{el,NS}} \) represent the maximum elimination rate, half-saturation concentration, and nonsaturable elimination rate constant, respectively. C is the Aβ concentration in the injectate. Regression analysis was performed to estimate these parameters using JMP software (version 7; SAS Institute Inc., Cary, NC).

In Vitro Uptake Assay of Aβ—The uptake of 125I-Aβ into HMVEC was examined as reported previously (37). Briefly, HMVEC cultured onto collagen I-coated 24-well dishes were grown to 90–100% confluence. Cells were washed three times with warmed ECF buffer and incubated at 37 °C with 125I-Aβ (0.1 nM) in ECF buffer for 20 min. Then, incubation medium was collected, and the cells were washed three times with ice-cold ECF buffer and an additional three times with acid wash.
buffer (28 mM CH₃COONa, 120 mM NaCl, 20 mM sodium barbital, pH 3.0). Cells were solubilized with radioimmune precipitation buffer, and the protein amount in the cells was measured. The cell/medium ratio (μl/mg of protein) is equal to 125I counts in the cells (cpm/mg of protein)/125I counts in the incubation medium (cpm/μl).

Isolation of Brain Microvessels—Brain microvessels were isolated by previously described methods (38) with some modifications in the cells (cpm/mg of protein)/125I counts in the incubation medium (cpm/μl). In brief, mouse brain devoid of leptomeninges was homogenized on ice in Dulbecco’s modified Eagle’s medium supplemented with 20 mM HEPES with a Teflon-glass homogenizer (0.25-mm clearance; Sansyo, Osaka, Japan) and resuspended with ice-cold 17% dextran (Sigma-Aldrich). Following centrifugation at 10,000 × g for 15 min at 4°C, the precipitate was suspended with ice-cold culture medium (10% fetal bovine serum in Dulbecco’s modified Eagle’s medium) and filtered through sterilized glass beads (425–600 μm, acid-washed; Sigma-Aldrich) on a 70-μm nylon cell strainer (BD Biosciences, Tokyo, Japan). These glass beads were washed with the culture medium and transferred onto a plastic dish. After gentle shaking in culture medium, dissociated vessels from glass beads were collected, washed with phosphate-buffered saline, and solubilized in radioimmune precipitation buffer containing PIM. Purification of the microvessels was assessed by microscopic observation and immunoblotting using anti-CD31 (an endothelial cell marker), anti-α-SMA (a smooth muscle cell marker), and anti-NeuN (a neuronal marker). In this evaluation, the rate of microvessels to brain parenchyma in this microvesSEL fraction was at least 20-fold higher when compared with that in the starting sample (data not shown).

Measurement of Fluvastatin Concentration—Fluvastatin level in each tissue was quantified using liquid chromatography-tandem mass spectrometry. In brief, animal serum or brain homogenate in saline was mixed with saturated sodium chloride and acetonitrile and centrifuged. Then, 0.1% acetic acid was added to the supernatant to prepare samples. Liquid chromatography was carried out on an HPLC apparatus (Agilent, Tokyo, Japan) equipped with an X Terra reverse phase column (RP18, 50 mm × 3.0 mm, inner diameter 3.5 μm; Waters, Tokyo, Japan) at a flow rate of 0.5 ml/min. The mobile phase consisted of 35% solvent A (0.1% acetic acid) and 65% solvent B (methanol). Electrospray data were acquired using a 4000 QTRAP triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The peak signals of transition from the parent ion to its major fragments (m/z 410–348) were measured. Quantification of fluvastatin was based on internal standardization using the peak area ratios of the analyte and the internal standard.

Antibodies and Other Reagents—The following antibodies were used: anti-APP N-terminal, anti-ADAM10/kuzbanian, anti-CD31 and anti-NeuN (Millipore), anti-APP C-terminal (IBL or Calbiochem), anti-sAPPβ wild type, anti-sAPPβ Swedish mutant type and anti-sAPPα (IBL), anti-amyloid precursor-like protein 1, anti-P-glycoprotein, anti-LRP1-heavy chain and anti-LRP1-light chain (Calbiochem), anti-N-cadherin, anti-Bip/GRP 78, and anti-Lamp1 (BD Biosciences), anti-calnexin (Assay Designs, Ann Arbor, MI), anti-Na⁺/K⁺ ATPase; (Abcam, Cambridge, MA), anti-cathepsin-B and anti-cathepsin-L (R&D Systems, Minneapolis, MN), anti-LRP1 N20, anti-Rab4, anti-Rab5a, anti-Rab5b, anti-Rab7, anti-ADAM17/TNF-α converting enzyme, and anti-β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin and anti-α-SMA (Sigma-Aldrich), anti-apolipoprotein E (Monosan, Uden, the Netherlands), anti-BACE1 (ABR, Rockford, IL), and anti-RhoA, anti-Rac1/2/3, and anti-Cdc42 (Cell Signaling, Danvers, MA). Anti-cathepsin-D antibody was developed as described previously (39). Chloroquine and mevalonate were purchased from Sigma-Aldrich. A human receptor-associated protein was purchased from Oxford Biomedical Research (Oxford, MI).

Statistics—All data were expressed as mean ± S.E. Comparison of two groups was performed by two-tailed t test. Comparison among three or more groups was performed by analysis of variance (ANOVA) or repeated measures ANOVA followed by two-tailed t test. p values of less than 0.05 were considered significant. All statistical analyses were performed using JMP software. In Western blot experiments, the density of each band was measured by ImageJ (version 1.37). The density of protein bands was standardized to the density of loading controls and compared with those for control mice, to which 100% was assigned.

RESULTS

Reduction of Aβ Production by Fluvastatin in Brain through the Lysosomal Degradation of Endogenous APP-CTFs via Enhanced Trafficking of APP-CTFs to Lysosomes—Initially, we investigated the in vivo effects of fluvastatin on Aβ production...
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in the brain of C57BL/6 mice. Fluvastatin treatment (5 mg/kg/day) significantly reduced both Aβ40 and Aβ42 levels in the brain (p < 0.01; Fig. 1A). Higher dose fluvastatin treatment (10 and 20 mg/kg/day) also reduced Aβ level in the brain to a similar degree (supplemental Fig. 2A), although neither 5 mg/kg/day nor 20 mg/kg/day fluvastatin treatment affect brain cholesterol level (supplemental Fig. 2B). To assess whether fluvastatin would affect α-, β-, or γ-secretase cleavage of APP, we measured the levels of other APP metabolites (APP-FL, APP-CTFα, APP-CTFβ, sAPPα, and sAPPβ) in the brain. As shown in Fig. 1, B and C, fluvastatin treatment (5 mg/kg/day) significantly reduced both APP-CTFα and APP-CTFβ (p < 0.01). Because these protein bands of APP-CTFs (APP-CTFα and APP-CTFβ) included some phosphorylated products (33), we quantified APP-CTF levels after dephosphorylation and found a reduction that fluvastatin reduces Aβ production in the brain through up-regulation of the lysosomal degradation of APP-CTFs. Meanwhile, fluvastatin did not affect the levels as well as the activity of lysosomal proteinases (cathepsin-B, -D, and -L; supplemental Fig. 4) and the level of Lamp1, a lysosomal receptor protein (supplemental Fig. 4A). This suggests that an increase in the lysosomal degradation of APP-CTFs is not due to up-regulation of lysosomal activity.

Next, we examined the hypothesis that fluvastatin enhances the trafficking of APP-CTFs to lysosomes. It is noteworthy that fluvastatin treatment shifted the subcellular distribution of APP-CTFs to heavier fractions (Fig. 3B), which showed considerable overlap with cathepsin-D, a lysosome marker (Fig. 3C), and partial overlap with Rab7, a late endosome marker (Fig. 3E), as assessed by iodixanol gra-
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Levels of small GTPases in microsomal and soluble fractions in brain with or without fluvastatin. Western blot shows levels of Rab small GTPases (Rab4, Rab5a, Rab5b, Rab7) in brain microsomal fraction (A) and soluble fraction (C). C = control; F = fluvastatin. B, quantification of Rab protein family levels in brain microsomal fraction. Calnexin was a loading control for the microsomal fraction. D, quantification of Rab protein family levels in brain soluble fraction. β-tubulin was a loading control for the soluble fraction. *, p < 0.05, N.S. = not significant; two-tailed Student’s t test. n = 6/group.

FIGURE 4. Levels of small GTPases in microsomal and soluble fractions in brain with or without fluvastatin. Western blot shows levels of Rab small GTPases (Rab4, Rab5a, Rab5b, Rab7) in brain microsomal fraction (A) and soluble fraction (C). C = control; F = fluvastatin. B, quantification of Rab protein family levels in brain microsomal fraction. Calnexin was a loading control for the microsomal fraction. D, quantification of Rab protein family levels in brain soluble fraction. β-tubulin was a loading control for the soluble fraction. *, p < 0.05, N.S. = not significant; two-tailed Student’s t test. n = 6/group.

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Enhancement of Brain Aβ Clearance through In vivo Experiments

As our previous study showed that fluvastatin reduced Aβ accumulation in the brain in an Aβ intracerebroventricular injection model (25), we next focused on Aβ clearance from the brain in C57BL/6 mice using the BEI method. The estimated half-life of intact 125I-Aβ and [14C]inulin in the brain was 43.6 and 187.4 min, respectively (Fig. 6A). Aβ clearance was composed of saturable and nonsaturable components (Fig. 6B), indicating an active process involving Aβ clearance. Indeed, fluvastatin significantly increased Aβ clearance rate at an injected concentration of 10 μM (Fig. 6C), whereas it failed to affect Aβ clearance rate at 20 nM and 1 μM. As the injected drug was diluted at least 30-fold by diffusion immediately after administration and spread farther (42) and the soluble Aβ level in the brain of AD patients is near or over 100 nM (43–45), our present study might be relevant in the pathological state of AD rather than in the normal physiological state (physiologi-
Because we previously showed that fluvastatin did not affect the activity and level of major Aβ-degrading enzymes such as neprilysin and insulin-degrading enzyme in the brain (25), we focused on the levels of apolipoprotein E, P-glycoprotein and the low density lipoprotein-related protein 1 (LRP1), which are reported to be involved in Aβ clearance from the brain (46). Levels of ApoE as well as P-glycoprotein in the soluble and membrane fractions of the brain and brain microvessels were not affected by fluvastatin (Fig. 6, D and E). However, LRP1 was significantly increased by fluvastatin in brain microvessels (Fig. 6, D and E), which might contribute to the increase in Aβ clearance.

To evaluate the effect of fluvastatin, we employed HBMEC. As shown in Fig. 7A, fluvastatin significantly increased the expression of LRP1 in HBMEC in a dose-dependent manner (p < 0.01), whereas co-treatment with mevalonate attenuated it (Fig. 7B), indicating that up-regulation of LRP1 by fluvastatin is due to an isoprenoid-dependent mechanism. To analyze the functional properties of LRP1, the initial internalization step of Aβ clearance at the BBB was examined using these cells as described previously (37). Expectedly, fluvastatin up-regulated Aβ uptake, whereas the addition of mevalonate attenuated it (Fig. 7C). Moreover, receptor-associated protein as well as anti-LRP1 antibody, which inhibit LRP1 function, blocked the effect of fluvastatin (Fig. 7, D and E), indicating that the increased uptake of Aβ by fluvastatin is mediated by LRP1. In this experiment, we selected the doses (10–100 nM) to achieve a serum concentration of fluvastatin close to that in treated mice (Table 1) because LRP1 located at the BBB is influenced by the serum concentration.

Finally, we examined the effect of fluvastatin in APP transgenic mice (APP23). Fluvastatin treatment reduced brain Aβ levels (Fig. 8A). Moreover, we confirmed that fluvastatin also increased brain microvessel LRP1 level (p < 0.01; Fig. 8B). These results suggest that up-regulation of LRP1-mediated clearance at the BBB could be involved in the regulation of Aβ metabolism in this AD mouse model.

**DISCUSSION**

It is well known that statins reduce the Aβ level in the brain (20, 23, 47–49). However, their molecular mechanisms are largely unknown. Chauhan et al. (23) reported that lova-
statin and pravastatin increased the sAPP\textalpha{} level in the brain of young TgCRND8 mice. They proposed up-regulation of \( \beta \)-secretase processing, resulting in reduced A\( \beta \) production, whereas Burns et al. (20) reported that simvastatin, lovastatin, and atorvastatin reduced the level of APP-CTF\( \beta \) among A\( \beta \) precursors (including sAPP\( \alpha \)) in the brain of wild-type mice and proposed a reduction of \( \beta \)-secretase processing. Several reasons for this inconsistency can be speculated. Variations in APP (wild type, Swedish, Indiana) or its level might shift the locus of APP processing and affect the effects of statins on APP metabolism (50), producing different results among wild-type mice and APP23 and TgCRND8 mice. In previous studies, which focused on the effect of statins on cholesterol synthesis, high doses of statins were administered mostly to reduce the serum cholesterol level (20, 47, 48, 51). In addition, as pleiotropic effects of statins are different, the results could be explained by the kind of statin used. Especially, fluvastatin has a potent antioxidant action and more potent pleiotropic effects on blood vessel walls and several tissues (52). Indeed, our previous study demonstrated a different preventive effect of fluvastatin from that of other statins in an A\( \beta \)-induced cognitive impairment model (25).

Our study demonstrated that fluvastatin up-regulated lysosomal degradation of endogenous APP-CTFs in brain. Previously, Ostrowski et al. (17) reported up-regulation of lysosomal degradation of APP-CTFs by statins via an isoprenoid-dependent mechanism in H4 neuroglioma cells expressing wild-type APP or APP-CTF\( \beta \). They proposed that Rho proteins might be involved in the degradation of APP-CTFs by statins. In this study, we failed to detect any change in the levels of Rho family proteins (RhoA, Rac1/2/3 and Cdc2) in the brain microsomal fraction and RhoA-GTP in the brain (supplemental Fig. 6). Further analyses are required to ascertain the involvement of other Rho members such as RhoB and RhoC.

On the other hand, we found a marked shift in the intracellular distribution of APP-CTFs to heavier fractions. This result suggests that the up-regulation of lysosomal degradation of APP-CTFs is mediated by enhanced trafficking of APP-CTFs from early endosomes to late endosomes and subsequently lysosomes (Fig. 9A). Furthermore, the present

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**FIGURE 6. Effects of fluvastatin on brain A\( \beta \) clearance and A\( \beta \) clearance-related proteins.** A, time course of \( ^{125} \text{I}-\text{A}\( \beta \) and \( ^{14} \text{C}\text{inulin} \) elimination rate from mouse brain by BEI method (\( n = 3/\)each point). Kinetic parameters of the concentration elimination rate constant curve were calculated as \( k_{\text{el,NS}} = 1.29 \times 10^{-3} \) (\( \mu \text{g/min} \)), \( k_{\text{el,max}} = 0.97 \) (\( \mu \text{g/min} \)), and \( k_{\text{el,NS}} = 6.7 \times 10^{-3} \) (min\(^{-1} \)). C, effects of fluvastatin treatment (5 mg/kg/day) on A\( \beta \) elimination rate with vehicle or with indicated concentrations of cold-A\( \beta \) (\( n = 5/\)group). D, Western blot analysis of ApoE in brain microsomal and soluble fractions, P-glycoprotein, and LRP1 in brain microvessels of C57BL/6 mice after fluvastatin treatment. Calnexin was a loading control for the brain microsomal fraction. \( \beta \)-Actin was a loading control for the brain soluble fraction and brain microvessels. C = control; F = fluvastatin; LRPI-LC = LRP1-light chain. E, quantification of level of apolipoprotein E in brain microsomal and soluble fractions and P-glycoprotein and LRP1 in brain microvessels of C57BL/6 mice (\( n = 5/\)group). APOE, apolipoprotein E; P-gp, P-glycoprotein. *, \( p < 0.05 \); **, \( p < 0.01 \); N.S. = not significant; two-tailed Student’s \( t \) test.
study documented a shifted subcellular distribution of Rab7 and reduced levels in Rab5. These changes are noteworthy because several groups propose that abnormal endosomal function is involved in the pathogenesis of AD (53). In the diseased state, APP is thought to be stacked in endosomes, where it is cleaved sequentially by β- and γ-secretase, leading to Aβ generation and deposition (50). The increase of lysosomal degradation of APP-CTFs by fluvastatin might be mediated via modification of the endosomal-lysosomal pathway. Indeed, we found that other proteins (i.e. APLP1) were similarly affected by fluvastatin. Complete suppression or strong activation of Rab5 function through overexpression of several Rab5 constructs causes a marked change in early endosome dynamics and APP metabolism (54–56). However, it remains to be determined how a partial reduction of Rab5 affects them. Recent studies showed that Rab5 and Rab7 act sequentially and cooperatively in the endosomal-lysosomal pathway (54, 57). Therefore, moderate change in Rab5 function through the inhibition of isoprenoid pathways by low levels of statin might be involved in shifting the subcel-

FIGURE 7. Effects of fluvastatin on LRP1 in human brain microvessel endothelial cells. A, Western blot analysis of LRP1 (LRP1-heavy chain (LRP1-HC) and LRP1-light chain (LRP1-LC)) after treatment with fluvastatin for 20 h. Graph represents quantification of LRP1-LC level (n = 9/group). B, effects of mevalonate (200 μM) on the reduction in LRP1 by fluvastatin (100 nM). Graph represents quantification of LRP1-LC level (n = 8/group). β-Actin was used as a loading control. C, the uptake assay of 125I-Aβ into HBMEC after treatment with or without fluvastatin (100 nM) in the presence or absence of mevalonate (200 μM) for 20 h (n = 6/group). D, the uptake assay of 125I-Aβ into HBMEC in the absence or presence of 400 nM receptor-associated protein (RAP) after treatment with fluvastatin (100 nM) for 20 h (n = 6/group). E, the uptake assay of 125I-Aβ into HBMEC in the presence of anti-LRP1 N20 antibody or control IgG (each 160 μg/ml) after treatment with fluvastatin (100 nM) for 20 h (n = 9/group). *, p < 0.05; **, p < 0.01, N.S. = not significant; ANOVA with two-tailed Student’s t test.

FIGURE 8. Effects of fluvastatin on Aβ levels and LRP1 levels in APP23 mice. A, Aβ40 and Aβ42 levels in the brain were determined by human/mouse Aβ ELISA after 4 weeks of treatment with or without 5 mg/kg/day fluvastatin (n = 8/group). B, Western blot analysis of LRP1 in brain microvessels of APP23 mice with or without 5 mg/kg/day fluvastatin. β-Actin was used as a loading control. Graph represents quantification of level of LRP1 (n = 5/group). C = control; F = fluvastatin; LRPI-LC = LRPI-light chain. *, p < 0.05; **, p < 0.01, two-tailed Student’s t test.
lular distribution of Rab7, resulting in the degradation of APP-CTFs (and APLP1-CTFs). Further studies are needed to clarify this hypothesis.

Our study also showed that fluvastatin enhanced Aβ clearance from the brain at high Aβ levels. In addition, LRP1 level, which is involved in Aβ clearance, was increased at the BBB of wild-type mice and also APP23 mice. Moreover, the in vitro model of Aβ clearance also supports that fluvastatin increases LRP1-mediated Aβ clearance at BBB. LRP1, a member of the low-density lipoprotein receptor family, is thought to be involved in Aβ clearance through an efflux process across the BBB (46) and/or a degradation process (58). The involvement of LRP1 in Aβ clearance was reported by several groups (37, 58–60). However, it remains to be resolved what proportions of LRP1-mediated clearance contribute to the total Aβ clearance in the normal brain and in the AD or the pre-AD brain. Up-regulation of LRP1 at the BBB was associated with an increase in Aβ clearance at high Aβ levels and also with the reduction of APP23 Aβ levels. These results imply the potential effect of fluvastatin in the pathological state of AD (Fig. 9B) rather than in the normal physiological state. Although the present study did not sufficiently address details of how fluvastatin increases LRP1-mediated Aβ clearance, as it is known that statins promote ligand uptake by the low density lipoprotein receptor through an increase in the receptor level (61), similar mechanisms might exist. In the present study, we believe that the local concentration of statins might be important in understanding the therapeutic value of statins as the levels of fluvastatin are quite different between brain and serum (1 nM in the brain versus 100 nM at the BBB; Table 1 and Fig. 9).

Overall, the present study demonstrated that fluvastatin reduced the Aβ level in vivo. This phenomenon might be due to up-regulation of the lysosomal degradation of endogenous wild-type APP-CTFs, a shift in the intracellular distribution of APP-CTFs to heavier fractions and changes of Rab proteins, leading to a reduction in Aβ production, and an increase in LRP1 in brain microvessels, leading to an increase in Aβ clearance from the brain at high Aβ levels. Our in vitro studies indicated that these effects are mediated by an isoprenoid-dependent mechanism. A recent study suggested that an increase in isoprenoid levels might be involved in the pathogenesis of AD (62). Additionally, other pleiotropic effects such as altered levels of phospho-Akt and endothelial nitric oxide synthase might be associated with improved learning and memory (49). The present results would have important implications in the development of disease-modifying therapy for AD as well as in basic understanding of Aβ metabolism, and further studies are needed to clarify the therapeutic mechanisms of statins in AD.

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REFERENCES
1. Masters, C. L., and Beyreuther, K. (2006) Brain 129, 2823–2839
2. Kong, G. T., Mantra, D., Poulet, F. M., Zhang, Q., Jossien, H., Bara, T., Engstrom, L., Pinzon-Ortiz, M., Fine, J. S., Lee, H. J., Zhang, L., Higgins, G. A., and Parker, E. M. (2004) J. Biol. Chem. 279, 12876–12882
3. Boche, D., Zotova, E., Weller, R. O., Love, S., Neal, J. W., Pickering, R. M., Wilkinson, D., Holmes, C., and Nicoll, J. A. (2008) Brain 131, 3299–3310
4. Burns, A. (2009) Lancet Neurol. 8, 4–5
5. Holmes, C., Boche, D., Wilkinson, D., Yadegaarfar, G., Hopkins, V., Bayer, A., Jones, R. W., Bullock, R., Love, S., Neal, J. W., Zotova, E., and Nicoll, J. A. (2008) Lancet 372, 216–223
6. Jick, H., Zornberg, G. L., Jick, S. S., Sheshadri, S., and Drachman, D. A. (2000) Lancet 356, 1627–1631
7. Wolozin, B., Kellman, W., Ruoos, P., Ceselias, G. G., and Siegel, G. (2000) Arch. Neurol. 57, 1439–1443
8. Wolozin, B., Wang, S. W., Li, N. C., Lee, A., Lee, T. A., and Kazis, L. E. (2007) BMC Med. 5, 20
9. Li, G., Higdon, R., Kukull, W. A., Peskind, E., Van Valen Moore, K., Tsu...
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ang, D., van Belle, G., McCormick, W., Bowen, J. D., Teri, L., Schellenberg, G. D., and Larson, E. B. (2004) *Neurology* **63**, 1624–1628

10. McGuinness, B., Craig, D., Bullock, R., and Passmore, P. (2009) *Cochrane Database Syst. Rev.* CD003160

11. Haag, M. D., Hofman, A., Koudstaal, P. J., Stricker, B. H., and Breteler, M. M. (2009) *J. Neurol. Neurosurg. Psychiatry* **80**, 13–17

12. Kandiah, N., and Feldman, H. H. (2009) *J. Neurol. Sci.* **283**, 230–234

13. Shibata, M., Yamada, S., Kumar, S. R., Calero, M., Bading, J., Frangione, B., Holtzman, D. M., Miller, C. A., Strickland, D. K., Ghiso, J., and Zlo Kov, B. V. (2000) *J. Clin. Invest.* **106**, 1489–1499

35. Shiki, T., Ohtsuki, S., Kurihara, A., Nagamura, H., Nishimura, K., Tachikawa, M., Hosoya, K., and Terasaki, T. (2004) *J. Neurosci.* **24**, 9632–9637

36. Akahama, S., Ohtsuki, S., Doi, Y., Tachikawa, M., Ito, S., Hori, S., Asashima, T., Hashimoto, T., Yamada, K., Ueda, K., Iwasubo, T., and Terasaki, T. (2008) *Neurochem. Int.* **52**, 956–961

37. Yamada, K., Hashimoto, T., Yakubi, C., Nagae, Y., Tachikawa, M., Strickland, D. K., Liu, Q., Bu, G., Basak, J. M., Holtzman, D. M., Ohtsuki, S., Terasaki, T., and Iwasubo, T. (2008) *J. Biol. Chem.* **283**, 34554–34562

38. Jung, S. S., and Levy, E. (2005) *Methods Mol. Biol.* **299**, 211–219

39. Koike, M., Nakanishi, H., Saitg, P., Ezaki, J., Ishara, K., Ohsawa, Y., Schulz-Schaeffer, W., Watanabe, T., Wagi, S., Kametaka, S., Shibata, M., Yamamoto, K., Komimani, E., Peters, C., von Figura, K., and Uchiyama, Y. (2000) *J. Neurosci.* **20**, 6988–6996

40. Steenmark, H. (2009) *Nat. Rev. Mol. Cell Biol.* **10**, 513–525

41. Liao, J. K. (2002) *J. Clin. Invest.* **110**, 285–288

42. Kakae, T., Terasaki, T., and Sugiyama, Y. (1996) *J. Pharmacol. Exp. Ther.* **277**, 1530–1559

43. McLean, C. A., Cherny, R. A., Fraser, F. W., Fuller, S. J., Smith, M. J., Beyreuther, K., Bush, A. I., and Masters, C. L. (1999) *Ann. Neurol.* **46**, 860–866

44. Tabaton, M., and Piccini, A. (2005) *Int. J. Exp. Pathol.* **86**, 139–145

45. Hellström-Lindahl, E., Vittanen, M., and Maratulle, A. (2009) *Neurochem. Int.* **55**, 243–252

46. Zlukovic, B. V. (2008) *Neuron* **57**, 178–201

47. Fassbender, K., Simons, M., Bergmann, C., Strohik, M., Lutjohnn, D., Keller, P., Runz, H., Kuhl, S., Bertsch, T., von Bergmann, K., Hennerici, M., Beyreuther, K., and Hartmann, T. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98**, 5856–5861

48. Petanceska, S., DeRosa, S., Oilm, V., Diaz, N., Sharma, A., Thomas-Bryant, T., Duff, K., Pappolla, M., and Refolo, L. M. (2002) *J. Mol. Neurosci.* **19**, 155–161

49. Li, L., Cao, D., Kim, H., Lestar, R., and Fukuchi, K. (2006) *Ann. Neurol.* **60**, 729–739

50. Small, S. A., and Gandy, S. (2006) *Neuron* **52**, 15–31

51. Park, I. H., Hwang, E. M., Hong, H. S., Boo, J. H., Oh, S. S., Lee, J., Jung, M. W., Bang, O. Y., Kim, S. U., and Mook-Jung, I. (2003) *Neurobiol. Aging* **24**, 637–643

52. Morishita, R., Tomita, N., and Ogiha, T. (2002) *Curr. Drug. Targets* **3**, 379–385

53. Nixon, R. A. (2005) *Neurobiol. Aging* **26**, 373–382

54. Rink, J., Chigo, E., Kalaidzidis, Y., and Zerial, M. (2005) *Cell* **122**, 735–749

55. Grbovic, O. M., Mathews, P. M., Jiang, Y., Schmidt, D. D., Dinarak, R., Summers-Terio, N. B., Ceresa, B. P., Nixon, R. A., and Cataldo, A. M. (2003) *J. Biol. Chem.* **278**, 31261–31268

56. Takai, Y., Sasaki, T., and Matozaki, T. (2001) *Physiol. Rev.* **81**, 153–208

57. Rojas, R., van Vlijmen, T., Mardones, G. A., Prabhu, Y., Rojas, A. L., Mohammed, S., Beck, A. J., Raposo, G., van der Sluijs, P., and Bonifacino, J. S. (2008) *J. Cell Biol.* **183**, 513–526

58. Nazer, B., Hong, S., and Selkoe, D. J. (2008) *Neurobiol. Dis.* **30**, 94–102

59. Deane, R., Wu, Z., Sagare, A., Davis, J., Dun, S., Samm, K., Xu, F., Parisi, M., LaRue, B., Hu, H. W., Spirek, P., Guo, H., Song, X., Lenting, P. J., Van Nostrand, W. E., and Zlokovic, B. V. (2004) *Neuron* **43**, 333–344

60. Ot, S., Ohtsuki, S., and Terasaki, T. (2006) *Neurosci. Res.* **56**, 246–252

61. Goldstein, J. L., and Brown, M. S. (2009) *Arterioscler. Thromb. Vasc. Biol.* **29**, 431–438

62. Eckert, G. P., Hooff, G. P., Strandjord, D. M., Igbavbova, U., Volmer, D. A., Müller, W. E., and Wood, W. G. (2009) *Neurobiol. Dis.* **35**, 251–257