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ApoE-isoform-dependent cellular uptake of amyloid-β is mediated by lipoprotein receptor LR11/SorLA

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ABSTRACT

The formation of senile plaques composed of β-amyloid (Aβ) in the brain is likely the initial event in Alzheimer's disease (AD). Possession of the *APOE* ε4 allele, the strong genetic factor for AD, facilitates the Aβ deposition from the presymptomatic stage of AD in a gene-dosage-dependent manner. However, the precise mechanism by which apoE isoforms differentially induce the AD pathology is largely unknown. LR11/SorLA is a type I membrane protein that functions as the neuronal lipoprotein endocytic receptor of apoE and the sorting receptor of the amyloid precursor protein (APP) to regulate amyloidogenesis. Recently, LR11/SorLA has been reported to be involved in the lysosomal targeting of extracellular amyloid-β (Aβ) through the binding of Aβ to the vacuolar protein sorting 10 (VPS10) protein domain of LR11/SorLA. Here, we attempted to examine the human-apoE-isoform-dependent effect on the cellular uptake of Aβ through the formation of a complex between an apoE isoform and LR11/SorLA. Cell culture experiments using Neuro2a cells revealed that the cellular uptake of secreted apoE3 and apoE4 was enhanced by the overexpression of LR11/SorLA. In contrast, the cellular uptake of apoE2 was not affected by the expression of LR11/SorLA. Co-immunoprecipitation assay revealed that apoE-isoform-dependent differences were observed in the formation of an apoE-LR11 complex (apoE4 > apoE3 > apoE2). ApoE-isoform-dependent differences in cellular uptake of FAM-labeled Aβ were further investigated by coculture assay in which donor cells secrete one of the apoE isoforms and recipient cells express FL-LR11. The cellular uptake of extracellular Aβ into the recipient cells was most prominently accentuated when cocultured with the donor cells secreting apoE4 in the medium, followed by apoE3 and apoE2. Taken together, our results provide evidence for the mechanism whereby human-apoE-isoform-dependent differences modulate the cellular uptake of Aβ mediated by LR11/SorLA.

1. Introduction

Alzheimer's disease (AD) has emerged as the most prevalent form of dementia in adults. The cardinal pathological features of AD brain are the loss of synapses and neurons as well as abnormal accumulation of misfolded proteins such as β-amyloid (Aβ) and phosphorylated tau. The deposition of Aβ in the brain is considered to be the earliest event in AD [1] and is substantially affected by the *APOE* genotype, which has been shown to be a strong genetic risk factor for AD in various ethnic populations [2,3]. The presence of the *APOE* ε4 allele markedly increases the risk of developing AD and decreases the age at onset by 10 to 15 years; in contrast, the ε2 allele confers protection against AD development [4,5]. Although there have been numerous studies attempting to elucidate the mechanism underlying the increase or decrease in the risk of AD posed by different apoE isoforms [6], the exact mechanisms are still not completely understood. Thus, to address the differential effects of apoE isoforms on the amyloid pathology and Aβ metabolism is important in the elucidation of the AD pathogenic pathway.

LR11, also known as SorLA, is a type I membrane protein that has homology to members of the LDL receptor family and vacuolar

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; APP, amyloid precursor protein; VPS10, vacuolar protein sorting 10; TACE, tumor necrosis factor-α-converting enzyme; FL, full-length; CSF, cerebrospinal fluid; PCR, polymerase chain reaction; GFP, green fluorescent protein; HEK, human embryonic kidney; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; co-IP, coimmunoprecipitation.

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protein sorting 10 (VPS10) protein family. LR11/SorLA is predominantly expressed on neurons in the cerebral cortex and hippocampus [7]. Reduced expression of LR11/SorLA in the brains of AD patients and subjects with mild cognitive impairment has been reported [8]. Furthermore, Aβ deposition is more prominent in *LR*11 knockout mice crossed with a mouse AD model [9]. These findings suggest the protective role of LR11/SorLA against AD pathology. In addition, some of the single-nucleotide polymorphisms (SNPs) in *SORL*1 encoding LR11/SorLA were found to be associated with a risk of sporadic AD in various ethnic backgrounds [3]. Full-length (FL) LR11/SorLA is cleaved by tumor necrosis factor-α-converting enzyme (TACE) to generate soluble LR11 [10]. We previously reported that the level of soluble LR11/SorLA was significantly high in the cerebrospinal fluid (CSF) of patients with AD [11]. The *APOE* ε4 carriers among AD patients showed higher levels of soluble LR11/SorLA than the ε4 noncarriers [11]. This suggests that the level of soluble LR11/SorLA in CSF is associated with AD in an apoE-isoform-dependent manner.

LR11/SorLA functions as the neuronal lipoprotein endocytic receptor of ApoE [12] and the sorting receptor of the amyloid precursor protein (APP) to regulate amyloidogenesis in endosomal and Golgi compartments [13]. LR11/SorLA interacts with newly synthesized APP in the Golgi apparatus and prevents the trafficking of APP into the cellular compartment where secretases reside. Consequently, the overexpression of LR11/SorLA in cultured cells reduces amyloidogenic processing [14]. Very recently, a novel function of LR11/SorLA has been reported. Caglayan et al. reported that LR11/SorLA plays a role in the lysosomal targeting of newly generated Aβ through the binding of Aβ to the amino-terminal VPS10 protein domain of LR11 [15]. This binding enhances Aβ clearance in lysosomes by internalization of extracellular Aβ through the receptor LR11/SorLA. With this as a background, we here attempted to determine the apoE-isoform-dependent effects on the cellular uptake of Aβ through the formation of a complex between an spoE isoform and LR11/SorLA.

2. Materials and methods

2.1. cDNA cloning and construction of expression plasmids

Complementary DNA (cDNA) of human full-length LR11 was amplified by PCR using pCMV6 plasmid cDNA containing human LR11 (Origene, Rockville, USA) and cloned into the pcDNA3.1 vector (Life Technologies, Carlsbad, USA). Human *APOE* ε3 cDNA was cloned and inserted into the pcDNA3.1 vector. We generated two allelic variants of human *APOE*, namely, *APOE* ε2 and ε4, by mutagenesis. Each isoform of *APOE* was cloned into the pEGFP vector (Clontech, Mountain View, USA). All constructs generated from PCR products were verified by DNA sequencing.

2.2. Cell culture and transfection

Mouse neuroblastoma Neuro2a (N2a) and human embryonic kidney (HEK)293T cells were cultured as previously described [16]. Transient transfection of plasmid DNA into cells was carried out by using Lipofectamine 2000 (Life Technologies). To generate stable cell lines, N2a cells transfected with cDNA encoding human apoE2, apoE3, apoE4 or FL-LR11 were selected using G418. Peptides of Aβ40 were purchased from Wako (Tokyo, Japan) and FAM-labeled Aβ40 from AnaSpec (Fremont, USA). They were dissolved in 1% ammonium hydroxide.

2.3. Coculture system

Coculture experiments were performed essentially as previously described [16]. Donor HEK293T cells transiently transfected with cDNA encoding human apoE2, apoE3, apoE4, apoE2-GFP, apoE3-GFP, or apoE4-GFP were cultured on a dish with a 1.0 μm filter insert (BD Bioscience, San Diego, USA) for 24 h. The donor HEK293T cells on the dish were placed in a six-well culture dish containing the recipient N2a cells mock-transfected or transiently transfected with the FL-LR11 cDNA construct. After 24 h of coculture, the lysate of the recipient cells and the medium were subjected to Western blot analysis.

2.4. Western blot analysis

The cells were solubilized using a lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% NP-40, 0.5% sodium deoxycholate, and 5 mM EDTA). The protein concentration of the detergent-extracted lysates was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, USA). Equal amounts of protein were boiled in Laemmli sample buffer and then subjected to Tris–glycine or Tris-tricine SDS–PAGE system. The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, USA) and then incubated with appropriate primary antibodies. FL-LR11 and sLR11 were detected using the monoclonal antibody 48/LR11 (BD Biosciences), which recognizes the extracellular domain of LR11. ApoE was visualized by the monoclonal antibody E6D7 (Sigma, St. Louis, USA). Aβ polypeptides were detected by the monoclonal antibody 6E10 (Covance, Berkeley, USA). GFP-fusion proteins were detected by the monoclonal antibody1E4 (MBL, Japan). Actin was detected using the goat anti-actin antibody I-19 (Santa Cruz Biotechnology, Dallas, USA). Immunoreactive bands were detected using an Immobilon Western Chemiluminescent HRP substrate (Millipore). The band intensities were quantified using a LAS system (GE Healthcare, Pittsburgh, USA).

2.5. Co-immunoprecipitation assays

HEK293T cells were cotransfected with the cDNA encoding apoE2, apoE3, or apoE4 and with FL-LR11 cDNA. The cells were solubilized using co-IP buffer (1% CHAPS, 150 mM NaCl, 50 mM HEPES, pH 7.5). The supernatant was incubated with either an anti-LR11 antibody (48/LR11) or an anti-apoE antibody (AB947, Millipore) at 4 °C for 16 h. To immunoprecipitate the protein complex, 25 μL of protein G Mag Sepharose beads (GE Healthcare) was added to the mixture, and the mixture was incubated on a rotator at 4 °C. The beads were collected on a magnetic stand and washed three times with the solubilization buffer. The immunoprecipitated proteins were released from the beads by incubation in Laemmli sample buffer. The obtained samples were analyzed by SDS–PAGE followed by Western blot analysis.

2.6. Fluorescence confocal laser scanning microscopy and image analysis

N2a cells treated with GFP-labeled apoE3 or FAM-labeled Aβ40 were cultured. All images were obtained using an inverted microscope (TE-300NT, Nikon, Japan) and a confocal microscope (CSU-10, Yokogawa Electric Corp, Japan). The obtained images were further analyzed with a quantitative analysis system (AquaCosmos, Hamamatsu Photonics, Japan).

2.7. Statistical analyses

Data are shown as the mean ± standard error of the mean (SEM). Statistical comparison between two groups was carried out by the Student *t*-test. For statistical comparison among several groups, we used one-way analysis of variance (ANOVA) followed

by the Tukey post hoc test. The statistical analyses were performed using SPSS ver. 12.0 J (SPSS Japan).

3. Results

3.1. Isoform-dependent change in level of secreted apoE by expression of LR11/SorLA

We first determined whether the expression of FL-LR11/SorLA affects the levels of apoE in the lysate and medium. N2a cells stably expressing human apoE3 were mock-transfected or transiently transfected with human FL-LR11 cDNA. The levels of apoE3 in the medium and lysate were determined using the anti-apoE antibody at different time points from 2 to 24 h after the medium was replaced. The level of apoE3 migrating at ~34 kDa and secreted into the medium increased after the medium replacement in a time-dependent manner (Fig. 1A). The level of apoE3 in the medium was significantly lower in N2a cells transfected with FL-LR11 24 h after the medium change compared with mock-transfected cells, whereas the level of apoE in the lysate was comparable between the mock- and FL-LR11-transfected cells (Fig. 1B).

Next, we investigated whether apoE-isoform-dependent differences are observed in this study. N2a cells stably expressing human apoE2, apoE3, or apoE4, were mock-transfected or transiently transfected with FL-LR11, and the levels of apoE in the medium and lysate were determined by immunoblot analysis. The apoE ratio (medium/lysate) significantly decreased in apoE3- and E4-expressing cells that were transiently transfected with FL-LR11 (Fig. 1C and D), whereas the apoE ratio (medium/lysate) was unchanged in apoE2-expressing cells transfected with FL-LR11. These findings suggest that the effect of LR11/SorLA on apoE ratio (medium/lysate) is dependent on the apoE isoform.

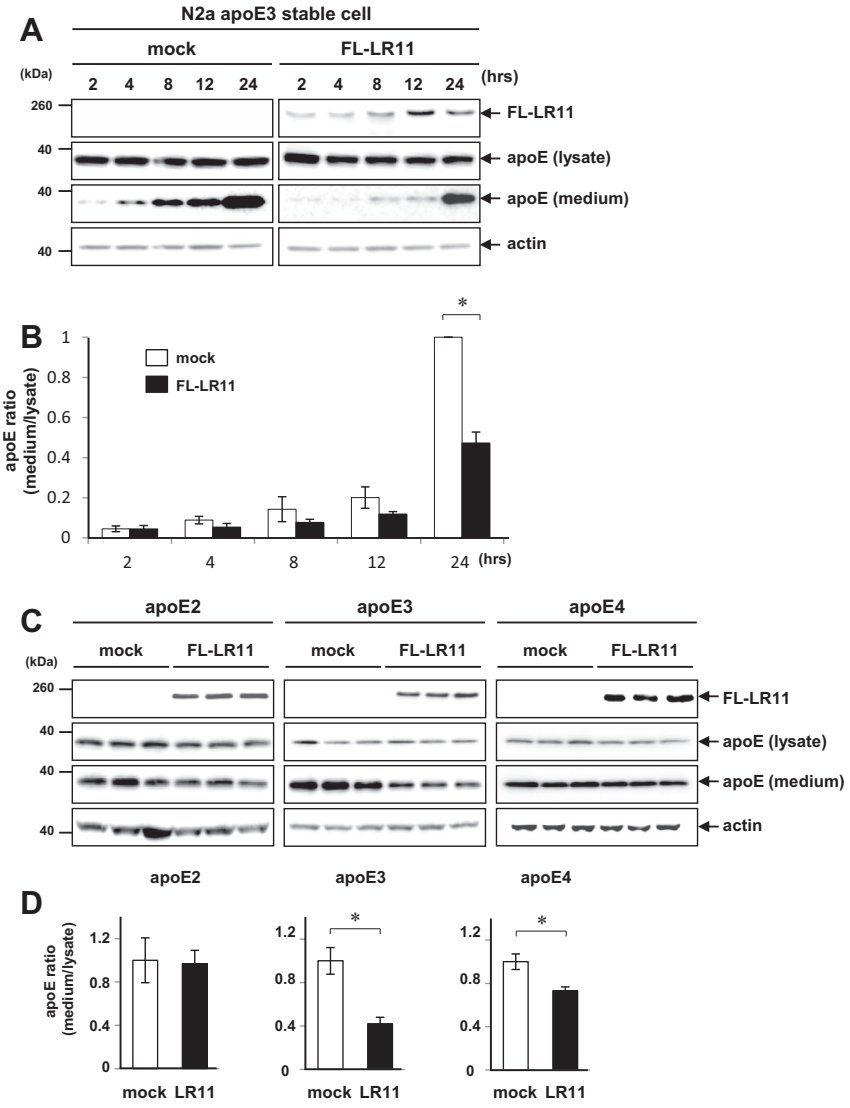

Fig. 1. Human-apoE-isoform-dependent differences in apoE ratio mediated by LR11/SorLA. (A) N2a cells stably expressing human apoE3 were mock-transfected or transiently transfected with human FL-LR11. The levels of apoE3 in the medium and cell lysate were determined by immunoblot analysis at different time points ranging from 2 to 24 h after the medium change. Note that the level of apoE in the medium of cells expressing FL-LR11 was decreased compared with mock-transfected cells. The equivalence of protein loading is shown in the β-actin blot. (B) Results of semiquantitative analysis by densitometry of apoE level. The apoE ratio (medium/lysate) was defined as the ratio of the level of apoE in the medium to that in the cell lysate. The apoE ratio (medium/lysate) significantly decreased 24 h after the medium change in cells transfected with FL-LR11 compared with mock-transfected cells. Results of three independent experiments are shown as mean ± SEM. $^{*}p < 0.05$ by Student *t*-test. (C) N2a cells stably expressing human apoE2, apoE3, or apoE4 were mock-transfected or transiently transfected with human FL-LR11. The levels of apoE in the medium and the lysate of cells expressing each of the apoE isoforms were determined 24 h after the medium change. (D) Results of semiquantitative analysis of the apoE ratio (medium/lysate). The apoE ratio (medium/lysate) was significantly decreased in apoE3- or apoE4-expressing cells transfected with FL-LR11, whereas the apoE ratio did not change in apoE2-expressing cells. $^{*}p < 0.05$.

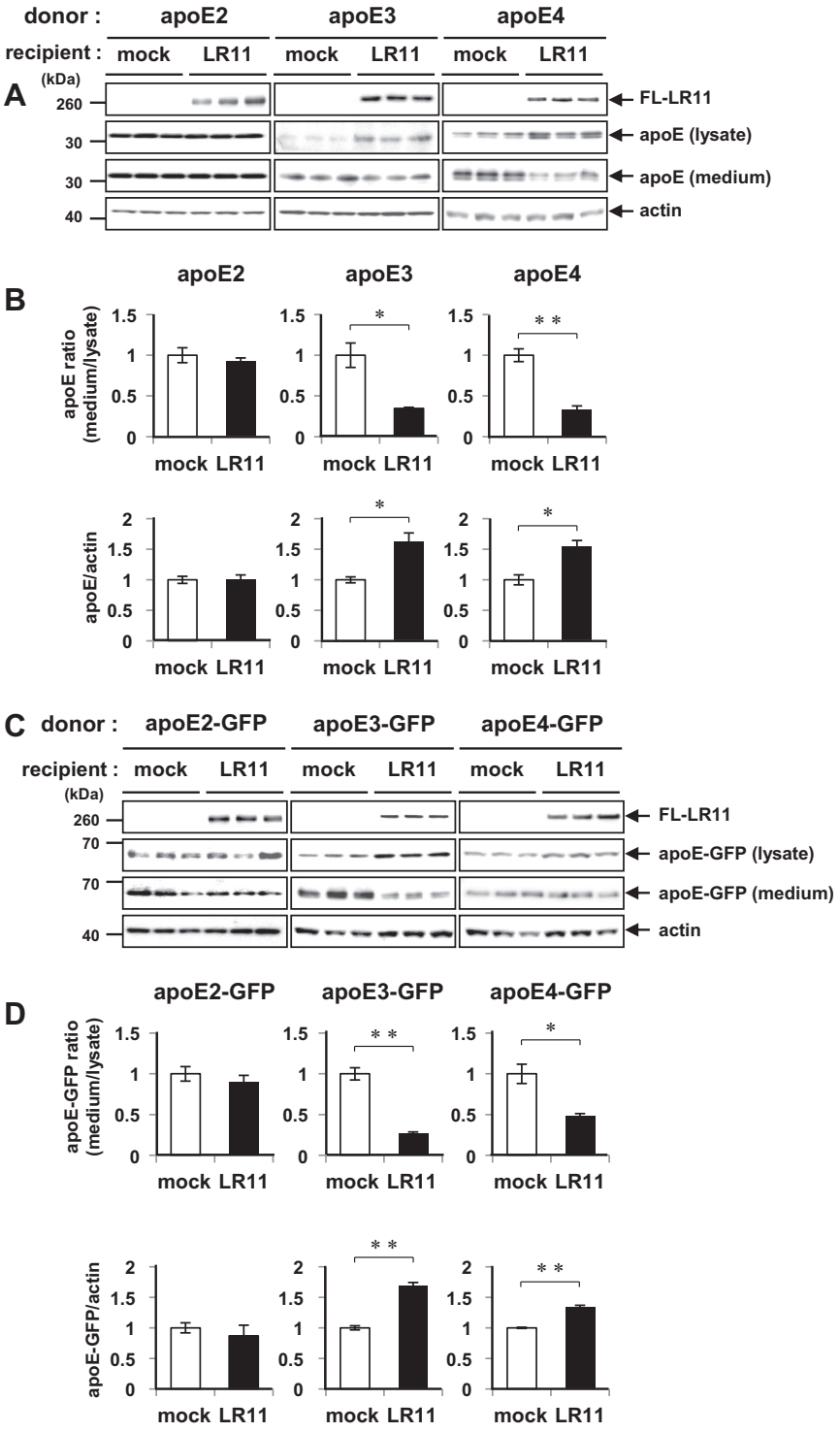

Fig. 2. ApoE isoform-dependent cellular uptake of apoE by LR11/SorLA in coculture system. (A) The recipient N2a cells mock-transfected or transiently transfected with FL-LR11 were cocultured with the donor HEK293T cells that were transiently transfected with apoE2, apoE3, or apoE4 for 24 h. Cellular uptake of secreted apoE from the medium into the recipient N2a cells was examined by Western blot analysis. (B) Results of semiquantitative analysis of cellular uptake of apoE are shown. The apoE ratio (medium/lysate) significantly decreased in the recipient cells transfected with FL-LR11 compared with the mock-transfected recipient cells when the recipient cells were cocultured with the donor HEK293T cells expressing apoE3 or apoE4 (*upper panels*). The apoE level normalized by β-actin level in the lysate of the recipient cells transfected with FL-LR11 showed a significant increase compared with that of mock-transfected recipient cells when the recipient cells were cocultured with the donor HEK293T cells expressing apoE3 or apoE4 (*lower panels*). $^{*}p < 0.05$; $^{**}p < 0.01$. (C) The recipient N2a cells mock-transfected or transiently transfected with FL-LR11 were cocultured with the donor HEK293T cells that secreted apoE2-GFP, apoE3-GFP, or apoE4-GFP in the medium for 24 h. Cellular uptake of apoE from the medium into the recipient N2a cells was examined. (D) Results of semiquantitative analysis of cellular uptake of apoE-GFP are shown. The apoE ratios (medium/lysate) for the apoE isoforms are shown (*upper panels*). The levels of apoE-GFP normalized by β-actin level for the apoE isoforms are shown (*lower panels*). $^{*}p < 0.05$; $^{**}p < 0.01$.

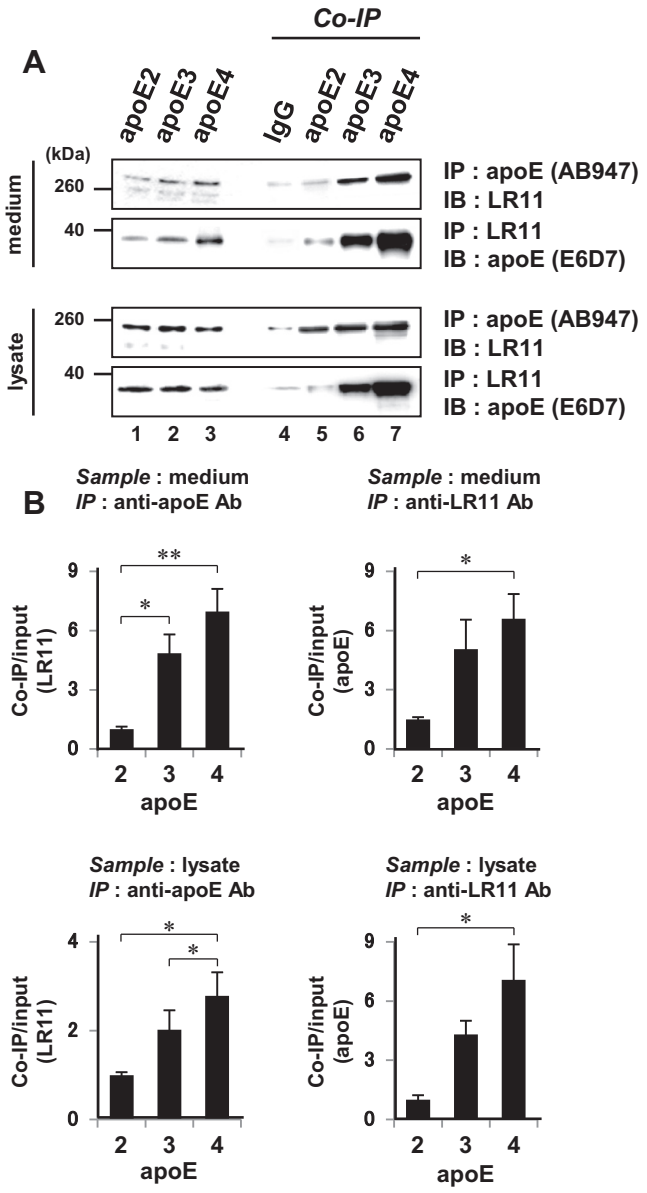

Fig. 3. Human-apoE-isoform-dependent differences in formation of complex between apoE and LR11/SorLA examined by co-IP assay. (A) HEK293T cells were cotransfected with each of the human apoE isoforms and human FL-LR11. The formed complex between apoE and LR11/SorLA was immunoprecipitated using the anti-apoE antibody (AB947) or anti-LR11 antibody (48/LR11), followed by immunoblot analysis. The lysates (*lanes* 1–3) and immunoprecipitated samples (*lanes* 4–7) were run on the same gel. (B) Results of semiquantitative analysis of immunoprecipitated apoE and LR11/SorLA. $^{*}p < 0.05$; $^{**}p < 0.01$ by Tukey test following ANOVA.

3.2. Cellular uptake of apoE examined by fluorescence confocal laser microscopy

The finding that the level of apoE in the medium of cells transfected with FL-LR11 decreased raises the possibility that apoE in the medium was taken up into cells by the lipoprotein receptor LR11/SorLA. To explore this possibility, N2a cells were incubated with the medium prepared from cells that transiently expressed the apoE3-GFP fusion protein. Fluorescence confocal microscopy revealed apoE-GFP signals inside the cells transfected with FL-LR11 and incubated with the medium of cells expressing apoE3-GFP (Supplementary Fig. 1B). This finding suggests that the cellular uptake of apoE from the medium was enhanced by the expression of LR11/SorLA.

3.3. ApoE-isoform-dependent cellular uptake of apoE in coculture system

We next examined whether the enhanced uptake of apoE by FL-LR11 is modified by apoE isoforms using the coculture system. First, we attempted to confirm whether apoE3 and GFP-labeled apoE3 secreted from the donor HEK293T cells are taken up into the recipient cells. This experiment revealed that apoE3 and GFP-labeled apoE3 were detected in the lysate of the recipient cells (Supplementary Fig. 2B), whereas GFP alone without apoE was not detected in the recipient cells.

Having established that apoE secreted from the donor cells is taken up into the recipient cells, we next asked if isoforms of apoE differently affect the cellular uptake of apoE in the recipient N2a cells mock-transfected or transiently transfected with FL-LR11. The level of apoE taken up into the recipient N2a cells was determined by immunoblot analysis using the anti-apoE antibody (Fig. 2A). The cellular uptake levels of apoE3 and apoE4 were significantly higher in the recipient cells that express FL-LR11 compared with the mock-transfected cells (Fig. 2B). In a similar experiment using donor cells that express apoE2-GFP, apoE3-GFP, or apoE4-GFP, the cellular uptake levels of apoE3-GFP and apoE4-GFP were increased by the expression of FL-LR11 (Fig. 2C and D). These results suggest that FL-LR11 enhances the cellular uptake of apoE3 and apoE4 but not that of apoE2.

3.4. ApoE-isoform-dependent binding affinity to LR11/SorLA

We speculated that the differential cellular apoE uptake in the recipient cells may be explained by a difference in binding affinity between LR11/SorLA and each of the apoE isoforms. To test this hypothesis, we examined the ability of LR11/SorLA to form a complex with each of the apoE isoforms by co-immunoprecipitation (co-IP) assay. We collected the detergent-extracted lysate of HEK293T cells that were cotransfected with FL-LR11 and each of the apoE isoforms. Co-IP assay using the anti-apoE or anti-LR11 antibody was performed using the medium and cell lysate. This assay revealed that LR11/SorLA bound to each isoform of apoE with different efficiencies (Fig. 3A). ApoE4-expressing cells showed the highest level of the LR11-apoE complex, followed by apoE3- and apoE2-expressing cells (Fig. 3B).

3.5. ApoE-isoform-dependent cellular uptake of Aβ

Given the physical interaction between LR11/SorLA and apoE, we next considered the possibility that FL-LR11 may be involved in the cellular uptake of the apoE-Aβ complex, because apoE is known to form a complex with Aβ. To address this issue, N2a cells were incubated with FAM-labeled Aβ40 at various concentrations of ranging from 0.5 to 3.0 μM. By fluorescence confocal laser microscopy, we detected the cellular uptake of FAM-labeled Aβ at concentrations above 1.0 μM (Supplementary Fig. 3A). We observed the cellular uptake of FAM-labeled Aβ at a low concentration of 0.25 μM when the recipient cells were transfected with FL-LR11 and cocultured with the donor cells expressing apoE4 (Supplementary Figs. 3B, 4A and 4B). The finding suggests that the cellular uptake of Aβ was enhanced in the presence of both FL-LR11 and apoE.

We finally performed coculture experiments in which the recipient N2a cells mock-transfected or transiently transfected with FL-LR11 were cocultured with the donor cells expressing apoE2, E3, or E4 in the presence of the Aβ peptides at 0.25 μM. The cellular uptake level of apoE and Aβ were highest when the recipient cells were cocultured with the donor cells expressing apoE4, followed by apoE3 and apoE2 (Fig. 4A and B).

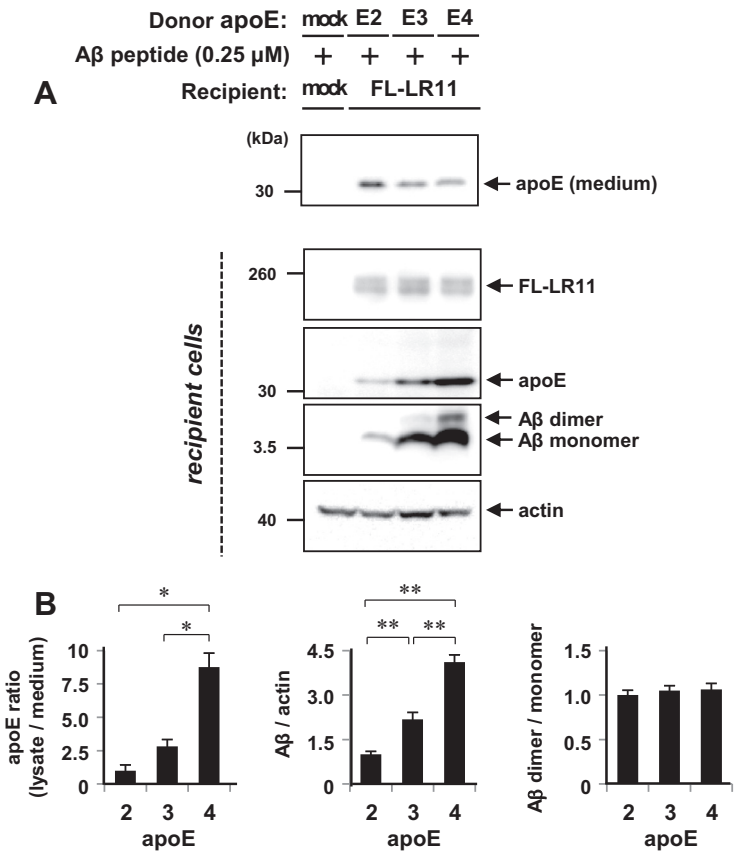

Fig. 4. ApoE-isoform-dependent differences in cellular uptake of Aβ in cells expressing LR11/SorLA. (A) Recipient N2a cells stably expressing human FL-LR11 were cocultured with donor N2a cells stably expressing human apoE2, apoE3, or apoE4 in the presence of Aβ40 peptides at 0.25 μM for 24 h. The cellular uptake of Aβ and apoE into the recipient cells was examined by Western blot analysis. (B) Results of semiquantitative analysis of cellular uptake of Aβ and apoE are shown as -fold increase compared with the apoE2 isoform. $^{*}p < 0.05$; $^{**}p < 0.01$ by Tukey test following ANOVA.

4. Discussion

Despite significant advances in our understanding of the pathological events occurring in the AD brain, the factors leading to Aβ accumulation in sporadic AD patients are not well understood. A wealth of evidence has confirmed that *APOE* ε4 is the strongest genetic risk factor for sporadic AD [2,3]. Although it is not fully understood how human apoE isoforms differentially affect the various pathogenic processes implicated in AD, several lines of evidence suggest that the effects of apoE on Aβ accumulation, aggregation, clearance, neurotoxicity, and neuroinflammation may play a role in AD pathogenesis [6]. Hence, understanding how each of the apoE isoforms differentially plays a pathophysiological role in AD is an important question to address. In this regard, this study provided new insights into how apoE isoforms differentially modify the AD pathogenic pathway with particular focus on the cellular uptake of Aβ via the lipoprotein receptor LR11/SorLA.

First, we demonstrated that the overexpression of LR11/SorLA enhanced the cellular uptake of apoE3 and apoE4. This finding suggests that LR11/SorLA functions as the apoE receptor. This notion was suggested by previous studies showing that FL-LR11 at the cell surface was capable of cellular uptake of apoE [12]. Importantly, we here showed that the cellular uptake of apoE is modified by different apoE isoforms. The cellular uptake of apoE4 and apoE3 was significantly enhanced by the expression of LR11/SorLA. In support of this notion, the co-IP assay revealed that the apoE-LR11/SorLA complex formation showed apoE-isoform-dependent differences in efficiencies, that is, apoE4 > apoE3 > apoE2. Major apoE receptors belong to the LDL receptor family including the LDL receptor and LDLR-related protein (LRP1). Different efficiencies in the formation of complexes between the LDL receptor and the apoE isoforms were observed. Similar to our finding, the cellular uptake levels of apoE3 and apoE4 were previously found to be higher than that of apoE2 in cells expressing the LDL receptor [17].

Secondly, our cell culture experiments revealed that extracellular Aβ was taken up into cells overexpressing LR11/SorLA in an apoE-isoform-dependent manner. A recent study has shown that LR11/SorLA binds to Aβ directly through the VPS10 protein domain of LR11/SorLA [15]. Importantly, we demonstrated that the cellular uptake of extracellular Aβ was apoE-isoform-dependent, that is, apoE4 > apoE3 > apoE2. Although this finding is potentially interesting, how this finding is relevant to AD pathogenesis is not presently understood. Enhancement of cellular uptake of Aβ may have two different consequences. A harmful consequence would be that an increased intracellular Aβ concentration through the uptake of extracellular Aβ may provide seeds of Aβ oligomers, a toxic forms of Aβ [18]. Some studies suggested that intracellular Aβ oligomers may be a major cause of synaptic dysfunction [19]. On the other hand, a beneficial consequence would be that the cellular uptake of extracellular Aβ may enhance the clearance of Aβ in lysosomes [20]. Our finding favors the former possibility, that is, cellular uptake of Aβ may exert a detrimental effect on neurons because this phenomenon is most closely associated with the presence of apoE4. Further studies delineating the precise consequences of increased intracellular Aβ level caused by enhanced cellular uptake of Aβ will be required.

ApoE is predominantly generated by astrocytes and microglia in the brain and is subsequently lipidated by ABCA1 to form lipoprotein particles [21]. It has been demonstrated that lipoprotein receptors including LDLR and LRP on the neuronal surface are able to take apoE-Aβ complexes into neurons after apoE is lipidated [22]. The lipidation of apoE has been shown to influence its isoform-specific affinity to Aβ. The efficiency of complex formation between lipidated apoE and Aβ is in the order of apoE2 > apoE3 > apoE4 [23]. In addition, the variable levels of apoE oxidation may affect the properties of apoE binding to Aβ. In this study, we did not fully address the lipidation or oxidation status of apoE secreted from HEK293T cells, which should be taken into account when interpreting our results. To minimize confounding factors, it is desirable to use apoE prepared under conditions that preserve apoE lipidation and oxidation status found in the brain. Collectively, our study indicated that LR11/SorLA is capable of cellular uptake of Aβ in an apoE-isoform-dependent manner; however, its pathophysiological role requires further investigation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.111.

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