Apolipoprotein E (apoE) is a major apolipoprotein in the brain. The e4 allele of apoE is a major risk factor for Alzheimer disease, and apoE deficiency in mice leads to blood-brain barrier (BBB) leakage. However, the effect of apoE isoforms on BBB properties is as yet unknown. Here, using an in vitro BBB model consisting of brain endothelial cells and pericytes prepared from wild-type (WT) mice, and primary astrocytes prepared from human apoE3- and apoE4-knock-in mice, we show that the barrier function of tight junctions (TJs) was impaired when the BBB was reconstituted with primary astrocytes from apoE4-knock-in mice (apoE4-BBB model). The phosphorylation of occludin at Thr residues and the activation of protein kinase C (PKC) at TJs or interact directly with TJ proteins (16–18). Among these, PKC is a major risk factor for Alzheimer disease (AD) (2, 3). ApoE is expressed in several organs, with the liver showing the highest expression level, followed by the brain. In the brain, apoE is a major apolipoprotein and plays a major role in the transportation of lipids as a lipid acceptor (1). ApoE-containing lipoprotein particles are mainly produced by astrocytes and deliver cholesterol and other essential lipids to neurons through low density lipoprotein (LDL) receptor family members (4–6). A number of studies revealed that astrocytes are involved in the control of endothelium blood-brain barrier (BBB) properties (7, 8) and that apoE deficiency leads to BBB leakage (9–11).

BBB is formed by brain endothelial cells and is essential for the protection of the central nervous system from harmful blood molecules and cells (12). Brain endothelial cells form tight junctions (TJs), which are the fundamental characteristics of BBB (13, 14). The assembly of TJs requires at least three types of transmembrane protein, namely, occludin, claudin, and junctional adhesion molecule (15). Protein kinases are localized at TJs or interact directly with TJs. Among protein kinases, PKC is a major role in the transportation of lipids as a lipid acceptor (1). ApoE-containing lipoprotein particles are mainly produced by astrocytes and deliver cholesterol and other essential lipids to neurons through low density lipoprotein (LDL) receptor family members (4–6). A number of studies revealed that astrocytes are involved in the control of endothelium blood-brain barrier (BBB) properties (7, 8) and that apoE deficiency leads to BBB leakage (9–11).
RESULTS

The results of in vitro studies showed that BBB permeability was higher in apoE4-knock-in mice than in apoE3-knock-in mice.

EXPERIMENTAL PROCEDURES

Materials—A mouse monoclonal anti-LDL receptor-related protein 1 (LRP1) antibody, a rabbit polyclonal anti-LDL receptor antibody, a rabbit monoclonal anti-low-density lipoprotein (VLDL) receptor antibody, and an anti-phosphorylated PKC\(\eta\) (pPKC\(\eta\)) antibody were purchased from Abcam Inc. (Cambridge, MA). A mouse monoclonal anti-occludin antibody was purchased from Invitrogen, and a rabbit polyclonal anti-actin antibody was from Sigma. A goat polyclonal anti-apoE antibody was purchased from Millipore (Billerica, MA). Anti-phosphorylated Tyr (Tyr(\(P\))) antibody, anti-phosphorylated Thr (Thr(\(P\))), and rabbit polyclonal anti-pPKC\(\eta\) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse control IgG was from Millipore Corp. (Bedford, MA), and rabbit control IgG was from Southern Biotech (Birmingham, AL). Recombinant receptor-associated protein (RAP) was produced and purified as described previously (22, 23).

Animals—Mice expressing human apoE were generated by the gene-targeting technique taking advantage of homologous recombination in embryonic stem cells (knock-in) (24). Three-week-old C57BL/6 mice were purchased from SLC Inc. (Hamamatsu, Japan). For astrocyte culture, pregnant C57BL/6 mice were purchased from SLC Inc., and newborn mice at postnatal day 2 were used for the experiment. ApoE-KO mice were obtained from the Jackson Laboratories (Bar Harbor, ME). The National Center for Geriatrics and Gerontology Institutional Animal Care and Use Committee approved the animal studies.

Evans Blue Assay—BBB permeability was quantified using the established Evans blue dye technique. Two hundred microliters of 20% mannitol (Sigma) was injected into 6-month-old apoE knock-in mice through the tail vein. After 30 min, 200 \(\mu\)l of 2% Evans blue (Sigma) was injected intraperitoneally. Mice were killed at 3 h after injection. The cerebellum and cerebral cortex were collected and then incubated in 500 \(\mu\)l of formamide for 72 h in the dark. Subsequently, the absorption (A) of the extracted dye was measured at 630 nm by spectrophotometry.

Cell Cultures—Primary cultures of mouse brain capillary endothelial cells (mBECs) were prepared from 3-week-old mice in accordance with the method described previously (21). The mice were killed, and the gray matter was dissected out. The gray matter was minced in ice-cold Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) and then dissociated into single cells by 25 times of up- and down-strokes with a 5-ml pipette in 10 ml of DMEM containing 100 \(\mu\)l of collagenase type 2 (100 mg/ml; Sigma), 150 \(\mu\)l of DNase I (1 mg/ml; Roche Applied Science) followed by digestion for 1.5 h at 37 °C. The digest in 20% bovine serum albumin (BSA) (Sigma) in DMEM was centrifuged at 1,000 \(\times\) g for 20 min to obtain cell pellets. The microvessels obtained from the pellets were further digested with collagenase and dispase (1 mg/ml; Roche Applied Science) for 1 h at 37 °C. Microvessel endothelial cell clusters were separated on a 33% continuous Percoll (Pharmacia) gradient, collected, and washed twice in DMEM before plating on 60-mm plastic dishes coated with collagen type IV (Nitta Gelatin) and fibronectin (Calbiochem) (both 0.1 mg/ml). mBEC cultures were maintained at 37 °C for 2 days in DMEM/F12 (Invitrogen) supplemented with mBEC medium I containing 10% FBS, basic fibroblast growth factor (1.5 ng/ml; Roche Applied Science), heparin (100 \(\mu\)g/ml; Sigma), insulin (5 \(\mu\)g/ml; Sigma), transferrin (5 \(\mu\)g/ml; Sigma), sodium selenite (5 ng/ml; Sigma) (insulin-transferrin-sodium selenite media supplement), penicillin, streptomycin (Invitrogen), and puromycin (4 \(\mu\)g/ml; Sigma). On the 3rd day, the medium was replaced with a new medium that contained all of the components of mBEC medium I except puromycin (mBEC medium II). When the cultures reached 80% confluence (4th day in vitro), the purified endothelial cells were passaged and used. Pure cultures of mouse cerebral pericytes were obtained by a 2-week culture of isolated brain microvessel fragments, which contain pericytes beside endothelial cells. When the cultures reached confluence, cells were treated with trypsin (Invitrogen), replated onto uncoated dishes, and cultured in DMEM supplemented with 10% FBS. Culture medium was changed every 3 days. Highly astrocyte-rich cultures were prepared in accordance with a method described previously (25). In brief, brains of day 2 postnatal human apoE-knock-in mice, WT mice, or apoE-KO mice were removed under anesthesia. The cerebral cortices from the mice were dissected, freed from meninges, and diced into small pieces; the cortical fragments were incubated in 0.25% trypsin and 20 mg/ml DNase I in PBS at 37 °C for 20 min. The fragments were then dissociated into single cells by pipetting. The cells were seeded in 75-cm\(^2\) dishes with DMEM-containing 10% FBS at a density of 5 \(\times\) 10\(^7\) cells/dish. After 10 days of incubation in vitro, flasks were shaken at 37 °C overnight, and the remaining astrocytes in the monolayer were trypsinized (0.1%) and reseeded. The astrocyte-rich cultures were maintained in DMEM-containing 10% FBS until use.

Construction of in Vitro BBB Models—To construct in vitro models of BBB, pericytes (1.5 \(\times\) 10\(^7\) cells/cm\(^2\)) were seeded on the bottom side of the polyester membrane of the Transwell inserts (Corning Inc., Corning, NY) coated with collagen type IV and fibronectin. The cells were allowed to adhere firmly overnight, then endothelial cells (1.5 \(\times\) 10\(^7\) cells/cm\(^2\)) were seeded on the upper side of the inserts placed in the wells of 24-well culture plates (for measurement of TEER) or 6-well plates (for Western blotting). Astrocytes (1 \(\times\) 10\(^7\) cells/cm\(^2\)) on the 6-well plates or 24-well plates were maintained in mBEC medium II. Finally, the Transwell inserts with mBECs and pericytes were placed into the 6-well or 24-well plates with astrocytes and maintained for 7 days. For the experiment to examine the effect of apoE-containing medium on BBB integrity, the double co-cultured model using pericytes and mBECs in the absence of astrocytes was used. For the preparation of conditioned media, primary astrocytes prepared from apoE3- or apoE4-knock-in mice were cultured in mBEC medium II for 48 h, and the conditioned media of apoE3-expressing astrocytes (apoE3-CM) or apoE4-expressig astrocytes (apoE4-CM) were collected. To determine the effect of apoE3-CM or apoE4-CM on BBB integrity, each CM was added only to the abluminal side of the double co-cultured model, and the abluminal side was filled with mBEC medium II. These culture media were replaced with newly prepared CM or fresh mBEC medium II on the 3rd and 5th days, and TEER was determined on the 7th day.
ApoE Regulates Tight Junctions

Measurement of TEER—Barrier integrity in in vitro BBB models was analyzed by measurement of TEER. TEER was measured using an epithelial-volt-ohm meter and Endohm-24 chamber electrodes (World Precision Instruments). TEER of coated but cell-free filters was subtracted from the measured TEER of models shown as $\Omega \times \text{cm}^2$.

Real-time PCR Analysis—The mRNA levels of TJ proteins were evaluated by real-time PCR analysis. Total RNA was extracted using a CellAmp™ Direct RNA Prep kit (Takara Bio Inc., Shiga, Japan) and reverse-transcribed with oligo(dT) and random primers using a PrimeScript RT reagent kit (Takara Bio Inc.). Relative real-time PCR was carried out using SYBR Premix Ex Taq™ II (Takara Bio Inc.) and Thermal Cycler Dice Real-time system TP-800 (Takara Bio Inc.) in accordance with the manufacturer’s protocols. The oligonucleotide sequences used for the primer sets were 5'-CGCTTATCTTGAGCTGGAC-3' and 5'-GTCATTGCTGGTTGCAATAGATTG-3' for occludin, 5'-CACCACTCCACGGAGTCACTCAC-3' and 5'-GTGCTGTCGTCACCATCTGGAA-3' for claudin 3, 5'-AGTTAAAGGCAGGACTAGCAC-3' and 5'-CAACGATGTTGGCAGAACAGCAGAT-3' for claudin 5, and 5'-GCCAATCACAATTCGAGGCTGATC-3' and 5'-GCCAATCAGGCTGATCTG-3' for apoE.

Western Blotting—The protein expression levels of occludin, PKCζ, and phosphorylated PKCζ in in vitro BBB models were determined by Western blotting. Cells were washed with PBS three times, harvested using a cell scraper, and lysed by sonication in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA). Cell lysates were subjected to SDS-PAGE with 7.5% gels (Wako Pure Chemicals, Osaka, Japan) and transferred to polyvinylidene difluoride membranes (Millipore). The blot was blocked with a primary antibody followed by a horseradish peroxidase-labeled secondary antibody (Cell Signaling Technology, Inc., Beverly, MA). The blot was developed using chemiluminescent substrate ECL Plus (GE Healthcare). Signals were visualized and quantified using a LAS-3000 miniuluminescent image analyzer (Fujiﬁlm, Tokyo, Japan). The protein level in cell lysates was normalized to the expression level of the actin protein. The phosphorylation state of occludin was analyzed by immunoprecipitation. For immunoprecipitation, cell lysates were incubated with magnetic protein G beads (Dynal, Hamburg, Germany) linked with an anti-Thr(P) or anti-Tyr(P) antibody. The obtained precipitates were washed three times with PBS and incubated at 70°C for 10 min in SDS sample buffer. Dissociated occludin in the supernatant was analyzed by Western blotting as described above.

Statistical Analyses—The collected data were analyzed by one-way analysis of variance (ANOVA) including appropriate variables followed by Dunnett’s test or unpaired Student’s t test (comparison between two groups). Results were considered significant when $p < 0.05$.

RESULTS

BBB Integrity Was Impaired in ApoE4-BBB Model—First, we examined whether mBECs and primary pericytes express apoE. We confirmed that the mRNA level of apoE in mBECs was about 1,000 times lower than that in primary astrocytes (data not shown), and we did not detect apoE in the culture media of mBECs and primary pericytes by Western blotting (data not shown). Thus, we prepared mBECs and primary pericytes from wild-type mice and astrocytes from apoE3- or apoE4-knock-in mice, and we cultured for 7 days. TEER was measured on the indicated culture days and presented as $\Omega \times \text{cm}^2$. The data presented are means ± S.D. (error bars) (n = 3), *, p < 0.001 compared with the values of apoE3-BBB models on day 7 or day 5 (unpaired Student’s t test).

ApoE Regulates Tight Junctions

Measurement of TEER—Barrier integrity in in vitro BBB models was analyzed by measurement of TEER. TEER was measured using an epithelial-volt-ohm meter and Endohm-24 chamber electrodes (World Precision Instruments). TEER of coated but cell-free filters was subtracted from the measured TEER of models shown as $\Omega \times \text{cm}^2$.

Real-time PCR Analysis—The mRNA levels of TJ proteins were evaluated by real-time PCR analysis. Total RNA was extracted using a CellAmp™ Direct RNA Prep kit (Takara Bio Inc., Shiga, Japan) and reverse-transcribed with oligo(dT) and random primers using a PrimeScript RT reagent kit (Takara Bio Inc.). Relative real-time PCR was carried out using SYBR Premix Ex Taq™ II (Takara Bio Inc.) and Thermal Cycler Dice Real-time system TP-800 (Takara Bio Inc.) in accordance with the manufacturer’s protocols. The oligonucleotide sequences used for the primer sets were 5’-CGCTTATCTTGAGCTGGAC-3’ and 5’-GTCATTGCTGGTTGCAATAGATTG-3’ for occludin, 5’-CACCACTCCACGGAGTCACTCAC-3’ and 5’-GTGCTGTCGTCACCATCTGGAA-3’ for claudin 3, 5’-AGTTAAAGGCAGGACTAGCAC-3’ and 5’-CAACGATGTTGGCAGAACAGCAGAT-3’ for claudin 5, and 5’-GCCAATCACAATTCGAGGCTGATC-3’ and 5’-GCCAATCAGGCTGATCTG-3’ for apoE.

Western Blotting—The protein expression levels of occludin, PKCζ, and phosphorylated PKCζ in in vitro BBB models were determined by Western blotting. Cells were washed with PBS three times, harvested using a cell scraper, and lysed by sonication in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA). Cell lysates were subjected to SDS-PAGE with 7.5% gels (Wako Pure Chemicals, Osaka, Japan) and transferred to polyvinylidene difluoride membranes (Millipore). The blot was blocked with a primary antibody followed by a horseradish peroxidase-labeled secondary antibody (Cell Signaling Technology, Inc., Beverly, MA). The blot was developed using chemiluminescent substrate ECL Plus (GE Healthcare). Signals were visualized and quantified using a LAS-3000 miniuluminescent image analyzer (Fujiﬁlm, Tokyo, Japan). The protein level in cell lysates was normalized to the expression level of the actin protein. The phosphorylation state of occludin was analyzed by immunoprecipitation. For immunoprecipitation, cell lysates were incubated with magnetic protein G beads (Dynal, Hamburg, Germany) linked with an anti-Thr(P) or anti-Tyr(P) antibody. The obtained precipitates were washed three times with PBS and incubated at 70°C for 10 min in SDS sample buffer. Dissociated occludin in the supernatant was analyzed by Western blotting as described above.

Statistical Analyses—The collected data were analyzed by one-way analysis of variance (ANOVA) including appropriate variables followed by Dunnett’s test or unpaired Student’s t test (comparison between two groups). Results were considered significant when $p < 0.05$.

RESULTS

BBB Integrity Was Impaired in ApoE4-BBB Model—First, we examined whether mBECs and primary pericytes express apoE. We confirmed that the mRNA level of apoE in mBECs was
in the WT-BBB model was comparable with that in the apoE3-BBB model, whereas TEER in the apoE-KO-BBB model was significantly lower than that in the apoE3-BBB model (Fig. 1B). Furthermore, we performed experiments to determine the effect of apoE-containing media on BBB integrity. The conditioned media of primary astrocytes expressing apoE3 (apoE3-CM) or apoE4 (apoE4-CM) were added to the luminal side of the co-culture model with pericytes and mBECs in the absence of astrocytes. TEER was significantly lower in these models than in the apoE3-BBB model (Fig. 1B).

ApoE Isoforms Do Not Affect Expression Levels of TJ Proteins—Next, we analyzed the expression levels of occludin, claudin 3, and claudin 5, all of which were reported to be major constituents of TJ strands (15). Each BBB model was cultured for 7 days, and the mRNA levels of these proteins in mBECs were analyzed by real-time PCR. Results are shown as relative ratios to actin. The expression levels of occludin (Fig. 2A), claudin 3, and claudin 5 (data not shown) in the apoE3-BBB model were comparable with those in the apoE4-BBB, WT-BBB, and apoE-KO-BBB models.

Thr Phosphorylation of Occludin Was Regulated in an ApoE Isoform-dependent Manner—The phosphorylation of occludin at Tyr residues is reported to negatively regulate TJ integrity (26) whereas phosphorylation at Thr residues is required for the assembly of occludin into TJs (19). We then determined whether the phosphorylation of occludin is regulated in an ApoE isoform-dependent manner. On day 7, mBECs were harvested using a cell scraper, and the level of phosphorylated occludin was determined by immunoprecipitation followed by Western blotting. We did not detect the phosphorylation of occludin at Tyr residues (data not shown). The levels of phosphorylated occludin at Thr residues were significantly lower in the apoE-KO-BBB model compared with those in the apoE4-BBB, WT-BBB, and apoE-KO-BBB models.

LRP1 Is Involved in the Regulation of the TJ Integrity in the ApoE3-BBB Model—ApoE is a ligand for receptors of the LDL receptor (LDLR) superfamily, several of which act both as endocytic receptors (28) and signaling receptors (5). Thus, we examined the effect of the RAP on the phosphorylation of PKC. RAP, a 39-kDa protein, is a specialized chaperone of members of the LDLR family, including LRP1 (29, 30). RAP binds to LRP1 at multiple sites (29, 30) with high affinity ($K_d = 1–10$ nM) (31). RAP has also been used extensively as an antagonist of LRP1 (32). On day 7, RAP (1 µM) was added to the media of astrocytes in an in vitro BBB model and cultured for 4 h at 37 °C. After
incubation, the phosphorylation of PKC\(_\eta\) in mBECs was analyzed by Western blotting. The treatment of the apoE3-BBB model with RAP attenuated the phosphorylation of PKC\(_\eta\) to a level similar to that of the apoE4-BBB model (Fig. 3A). Because RAP inhibits LRP1 as well as the LDLR (29) and because apoE-containing particles can bind to the very low-density lipoprotein (VLDL) receptor (33), we next treated the apoE3-BBB model with the anti-LRP1 antibody (25 \(\mu\)g/ml), which recognizes the extracellular domain of LRP1, LDLR, or VLDLR, respectively, on day 7 for 4 h at 37 °C, to determine whether specific apoE receptors are involved in PKC\(_\eta\) phosphorylation. Among these antibodies, only the anti-LRP1 receptor antibody suppressed the phosphorylation of PKC\(_\eta\) (Fig. 3, B–D). Consistent with this suppression of PKC\(_\eta\) phosphorylation, the treatment of the apoE3-BBB model with the anti-LRP1 antibody also suppressed the phosphorylation of occludin at Thr residues (Fig. 3E). Next, we examined whether treatment of the apoE3-BBB model with the anti-LRP1 antibody impairs TJ integrity. The prepared apoE3-BBB model was cultured for 7 days, and the anti-LRP1 antibody (25 \(\mu\)g/ml) was added to the astrocyte culture medium. After further incubation of the cultures for 4 h at 37 °C, TJ integrity was evaluated by measuring TEER. Treatment of the apoE3-BBB model with the anti-LRP1 antibody significantly decreased TEER (Fig. 3F), indicating that TJ integrity requires the function of apoE receptor LRP1.

**BBB Integrity Is Impaired in ApoE4-knock-in Mice**—Finally, we evaluated the BBB integrity in human apoE3-, apoE4-knock-in mice, and apoE-KO mice. BBB integrity was evaluated using an Evans blue dye technique. Consistent with the results shown in Fig. 1, BBB integrity was impaired in the apoE4-knock-in mice compared with the apoE3-knock-in mice (Fig. 4). Consistent with previous studies (9, 11), BBB integrity was impaired in the apoE-KO mice (Fig. 4). We found a higher amount of leakage in the cerebellum of human apoE-knock-in mice than in the cerebrum, which is consistent with the findings of previous studies using apoE-KO mice (9, 11).

**DISCUSSION**

In this report, we provided evidence that TEER is lower in the apoE4-BBB and apoE-KO models than in the apoE3-BBB and WT-BBB models. The activation of PKC\(_\eta\) and the phosphory-
lation of occludin at its Thr residues, which regulates TJ integrity, were dependent on the apoE isoform. Furthermore, the treatment of the apoE3-BBB model with the anti-LRP1 antibody diminished the activation of PKCζ and phosphorylation of occludin, suggesting that LRP1 may be involved in the regulation of TJ integrity through the activation of PKCζ and phosphorylation of occludin at its Thr residues in the apoE3-BBB model. Our results also indicate that this pathway is impaired in the apoE4-BBB model. Consistent with the results of in vitro studies, BBB permeability was increased in the apoE4-knock-in mice and apoE-KO mice compared with the apoE3-knock-in mice. As revealed in previous studies (7, 8), astrocytes are involved in the control of BBB integrity. Astrocytes are considered to be the major source of apoE in the CNS (4 – 6), and apoE deficiency leads to BBB leakage (9 – 11). These lines of evidence suggest that astrocytes may regulate BBB integrity through apoE. In the present study, our findings indicate that apoE secreted from astrocytes is involved in the regulation of TJ integrity through the activation of PKCζ and phosphorylation of occludin at its Thr residues in an apoE3-BBB model and that this pathway is impaired in the apoE4-BBB model. We provide the first evidence that TJ integrity in BBB is regulated in an apoE isoform-dependent manner.

ApoE-containing particles act as ligands of LDLR family members such as LRP1 and play critical roles in maintaining brain lipid homeostasis and associated synaptic and neuronal integrity (4, 34). Previous studies have shown that apoE induces lipid release to generate HDL-like particles from macrophages and astrocytes in an isoform-dependent manner; apoE3 induces a greater lipid release than apoE4 (35 – 39). In addition to functioning as lipid carriers, a recent study showed that apoE-containing particles act as signaling molecules through LRP1 to activate PKCδ, a novel PKC isoform (40), and protect neurons from apoptosis in an apoE isoform-dependent manner (41). Here, we showed that apoE regulates the activation of PKCζ, which is also a novel PKC isoform (40), through LRP1 in mBECs in the in vitro BBB model. Thus, our results suggest that apoE-containing particles also act as signaling molecules to regulate the activation of PKCζ, the subsequent phosphorylation of occludin at its Thr residues, and TJ integrity. Our findings also indicate that apoE4-containing particles might be less efficient as signaling molecules than apoE3-containing particles.

ApoE4 is a major risk factor for AD. Although how apoE4 influences AD onset and progression has not been fully understood yet, recent studies suggest that the differential effects of apoE isoforms on amyloid-β (Aβ) aggregation and clearance could play important roles in AD pathogenesis. Human apoE-transgenic mice display an isoform-specific pattern of Aβ deposition (E3 > E4) (42 – 46). Previous studies have shown that lipid-poor apoE4 and lipid-free apoE4 enhance Aβ production by increasing LRP1- and apoER2-mediated endocytosis of an amyloid precursor protein (47, 48). ApoE also facilitates the proteolytic clearance of Aβ from the brain in an apoE isoform-dependent manner and requires its lipidation (49). Considering BBB as a pathway of Aβ clearance in the brain (50 – 52), a recent study has shown that Aβ complexed to apoE2 and apoE3 is cleared out of the brain at a significantly higher rate than Aβ complexed to apoE4 (53). Interestingly, several studies have demonstrated that there is an increased permeability in the BBB of the AD model mice compared with age-matched control mice (54, 55) and that Aβ fibrils could increase the permeability of bovine pulmonary arterial endothelial cells, as detected by TEER measurement (56). Furthermore, a recent study using pericyte-deficient mice has shown that BBB breakdown is associated with the accumulation of neurotoxic and/or vasoconstrictor serum proteins in the brain, which leads to secondary neurodegenerative changes (57). In this study, we provide evidence that TJ integrity in BBB is impaired in the apoE4-BBB model and apoE4-knock-in mice. Thus, these lines of evidence suggest that apoE4 may affect AD pathogenesis and/or neurodegeneration through its effects on BBB. We have shown using in vitro and in vivo models that apoE regulates the activation of PKCζ, the phosphorylation of occludin at Thr residues, and TJ integrity in an apoE isoform-dependent manner. Further study will be needed to elucidate the contribution of BBB impairment to AD pathogenesis.

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