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ORIGINAL RESEARCH

Pitavastatin Strengthens the Barrier Integrity in Primary Cultures of Rat Brain Endothelial Cells

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Abstract Statins have a neuroprotective effect in neurological diseases, a pleiotropic effect possibly related to blood–brain barrier (BBB) function. We investigated the effect of pitavastatin on barrier functions of an in vitro BBB model with primary cultures of rat brain capillary endothelial cells (RBEC). Pitavastatin increased the transendothelial electrical resistance (TEER), an index of barrier tightness of interendothelial tight junctions (TJs), at a concentration of 10^{-8} M, and decreased the endothelial permeability for sodium fluorescein through the RBEC monolayer. The increase in TEER was significantly reduced in the presence of isoprenoid geranylgeranyl pyrophosphate, whereas farnesyl pyrophosphate had no effect on TEER. Our immunocytochemical and Western blot analyses revealed that treatment with pitavastatin enhanced the expression of claudin-5, a main functional protein of TJs. Our data indicate that pitavastatin strengthens the barrier integrity in primary cultures of RBEC. The BBB-stabilizing effect of pitavastatin may be mediated partly through inhibition of the mevalonate pathway and subsequent up-regulation of claudin-5 expression.

Keywords Pitavastatin · Statins · Blood–brain barrier · Tight junctions · Claudin-5 · Transendothelial electrical resistance · Geranyl geranylation · Brain endothelial cells (rat)

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Introduction

Statins reduce the serum level of low-density lipoprotein (LDL) cholesterol by inhibiting 3-hydroxyl-3-methyl coenzyme A (HMG-CoA) reductase. Crouse et al. (1998) previously reported that statins significantly decreased the incidence of cerebral, ischemic stroke, and cardiovascular events in patients without hypercholesterolemia. Based on a considerable number of clinical studies, Nassief and Marsh (2008) recently proposed that statins are a significant therapy for preventing stroke insults in coronary and cerebrovascular diseases. This clinical efficacy seems to depend on a vascular endothelial-cells-stabilizing effect, a pleiotropic effect beyond the reduction of serum LDL cholesterol (Sandoval and Witt 2008). In endothelial cells, statins have been found to deactivate platelet aggregation and procoagulation cascades by inducing thrombomodulin expression (Masamura et al. 2003). Statins also exert anti-inflammatory effects by decreasing the surface expression of vascular cell adhesion molecule-1 and E-selectin (Schonbeck and Libby 2004; Prasad et al. 2005), and by inhibiting the secretion of interleukin 6/8 and monocyte chemoattractant protein-1 (Rezaie-Majd et al. 2002). Of particular interest was the in vitro observation of

Kuhlmann et al. (2006) that fluvastatin has a vascular endothelial-cell-stabilizing effect in the blood–brain barrier (BBB), a functional phenotype of brain capillary endothelial cells.

The BBB provides ionic homeostasis and nutrients necessary for the proper functioning of the CNS and protects neurons from xenobiotics, blood-borne toxic substances, and stroke insults, as well as regulation of neuroactive mediator levels (Abbott et al. 2006; Zlokovic 2008). Therefore, disruption of the BBB is closely related to the onset and progress of cerebrovascular stroke (Cecchelli et al. 2007; Sandoval and Witt 2008). In the BBB, the tight junctions (TJs) consist of a huge architecture of cell–cell adhesion molecules composed of membrane proteins such as claudins, occludins, and ZO-1. TJs are located between the brain capillary endothelial cells and restrict the paracellular diffusion of water-soluble substances from the blood to the brain to protect neurons, which is an essential function of the BBB (Abbott 2005; Deli et al. 2005; Nakagawa et al. 2009). BBB properties are attributed mainly to the presence of the complex TJ network between endothelial cells. Thus, the BBB phenotype of brain endothelial cells is mainly due to the TJs, among which claudins are the key molecules in TJ assembly (Miyoshi and Takai 2005; Furuse and Tsukita 2006; Van Itallie and Anderson 2006).

Although beneficial effects of statins in neurological diseases have been demonstrated, few data are available on whether statins work on the TJs of the BBB. Hence, we studied the effect of pitavastatin, a strong statin with a longer action and less potential for drug interactions (Saito et al. 2002; Iglesias and Diez 2003), on claudin-5, a key protein of TJs, using an in vitro BBB model with primary cultures of rat brain capillary endothelial cells (RBEC).

Materials and Methods

All reagents were purchased from Sigma, USA, unless otherwise indicated. Wistar rats were obtained from Japan SLC, Inc., Japan. All animals were treated in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the Nagasaki University Animal Care Committee. Pitavastatin (LIVALO®) was kindly provided by Kowa Co., Ltd., Japan.

Rat Brain Capillary Endothelial Cells (RBEC)

Primary cultures of RBEC were prepared from 3-week-old rats, as previously described (Deli et al. 1997; Nakagawa et al. 2007, 2009). Meninges were carefully removed from forebrains and gray matter was minced into small pieces of approximately 1 mm^3 in ice-cold Dulbecco's modified Eagle's medium (DMEM), then dissociated by 25 up-and-down strokes with a 5-ml pipette in DMEM containing collagenase type 2 (1 mg/ml, Worthington Biochemical Corp., USA), 300-µl DNase (15 µg/ml), gentamycin (50 µg/ml), and 2-mM glutamine, and then digested in a shaker for 1.5 h at 37°C. The cell pellet was separated by centrifugation in 20% bovine serum albumin (BSA)-DMEM (1,000×*g*, 20 min). The microvessels obtained in the pellet were further digested with collagenase-dispase (1 mg/ml, Roche Applied Sciences, Switzerland) and DNase (6.7 µg/ml in DMEM for 1 h at 37°C).

Microvessel endothelial cell clusters were separated on a 33% continuous Percoll (Pharmacia, Sweden) gradient, then collected and washed twice in DMEM before plating on 35-mm plastic dishes coated with collagen type IV and fibronectin (both 0.1 mg/ml) (Day 0, Fig. 1).

RBEC cultures were maintained in DMEM/F12 supplemented with 10% plasma derived serum (PDS, Animal Technologies, Inc., USA), basic fibroblast growth factor (bFGF, Roche Applied Sciences, 1.5 ng/ml), heparin (100 µg/ml), insulin (5 µg/ml), transferrin (5 µg/ml), sodium selenite (5 ng/ml) (insulin–transferrin–sodium selenite media supplement), gentamycin (50 µg/ml), and puromycin (4 µg/ml) (RBEC medium I) at 37°C with a humidified atmosphere of 5% CO_2/95% air, for 2 days. Thus, according to the finding of Perriere et al. (2005), in the first 2 days we incubated cells in medium containing puromycin (4 µg/ml) to avoid the contamination of pericytes. After 2 days, the cells received new medium which contained all the components of RBEC medium except puromycin (RBEC medium II) (Day 2, Fig. 1).

When the cultures reached 80% confluency (Day 4), the purified endothelial cells were passaged by a brief

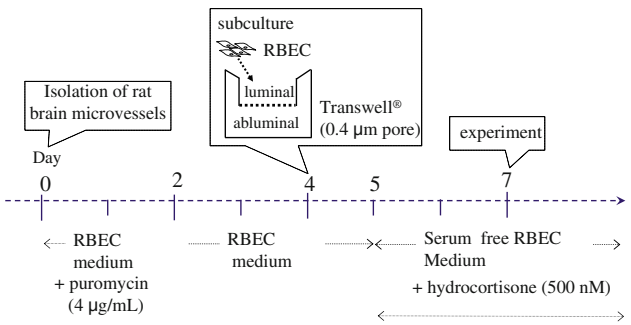

Fig. 1 Schematic drawing of the experiment with primary cultures of rat brain capillary endothelial cells (RBEC). RBEC were isolated 4 days before the establishment of the in vitro BBB model. To purify cultures, cells were kept in the presence of puromycin for 2 days. On Day 4, RBEC were added to the luminal compartment of the inserts and positioned in the 12-well plates. From Day 5, RBEC were grown in culture medium containing 500-nM hydrocortisone. Experiments were performed on Day 7

treatment with trypsin (0.05% w/v)-EDTA (0.02% w/v) solution, and an in vitro BBB model was constructed with Transwell® (Corning Incorporated Life Sciences, USA) inserts in 12-well culture plates. Endothelial cells (1.5×10^5 cells/cm^2) were seeded on the upper side of the collagen- and fibronectin-coated polyester membranes (0.4-μm pore size) of Transwell® inserts. This in vitro BBB model was maintained in RBEC medium II for 1 day, and on the next day the medium was changed to serum-free RBEC medium II containing 500-nM hydrocortisone (Hoheisel et al. 1998) (Day 5, Fig. 1). Under these conditions, the in vitro BBB model was established within 3 days after setting the cells. Experiments with pitavastatin were carried out on Day 7 (Fig. 1).

Evaluation of the Barrier Integrity

Transendothelial Electrical Resistance (TEER)

The TEER, reflecting mainly the flux of sodium ions through the TJs of endothelial cell layers in culture, was measured using an EVOM resistance meter (World Precision Instruments, USA). The extracellular matrix-treated Transwell® inserts were placed in a 12-well plate containing culture medium and then were used to measure the background resistance. The resistance measurements of these blank filters were then subtracted from those of filters with cells. The values are shown as $\Omega \times$ cm^2 based on culture inserts.

Transendothelial Permeability

The flux of sodium fluorescein (Na–F) and Evans' blue-albumin across the endothelial monolayer was determined as previously described (Honda et al. 2006; Nakagawa et al. 2007; Hiu et al. 2008). Cell culture inserts were transferred to 24-well plates containing 0.7-ml assay buffer (136-mM NaCl, 0.9-mM $CaCl_2$, 0.5-mM $MgCl_2$, 2.7-mM KCl, 1.5-mM KH_2PO_4, 10-mM NaH_2PO_4, 25-mM glucose, and 10-mM Hepes, pH 7.4) in the basolateral or lower compartments. In the inserts, the culture medium was replaced by 0.2-ml buffer containing 10-μg/ml Na–F (MW 376 Da) and 165-μg/mL Evans' blue-albumin (MW 67 kDa). The inserts were transferred at 20, 40, and 60 min to a new well containing assay buffer. The emission of Na–F was measured at 535 nm (Wallac 1420 ARVO Multilabel Counter, Perkin Elmer, USA; excitation: 485 nm), while the absorbance of Evans' blue was at 595 nm. The permeability of Na–F and Evans' blue-albumin was used as an index of paracellular and transcellular transport, respectively (Deli et al. 2005; Nakagawa et al. 2007). The apparent permeability coefficient, namely Papp (cm/s), derives from Fick's Law (Youdim et al. 2003).

Pitavastatin

Pitavastatin was added into the luminal (upper compartment) of the BBB model constructed with Transwell® at concentrations ranging from 10^{-11} to 10^{-6} M. At 48 or 24 h after pitavastatin treatment, barrier function was evaluated by TEER and Na–F/EBA permeability. As inhibition of isoprenoid intermediates of the mevalonate pathway are thought to be closely related to pleiotropic effects of statins (Park et al. 2002; Katsumoto et al. 2005; Kuhlmann et al. 2006), we used farnesyl pyrophosphate (FPP), a precursor of cholesterol, and geranylgeranyl pyrophosphate (GGPP), a small GTP-binding protein. At a concentration of 10^{-8} M, pitavastatin was also added in the presence or absence of 10^{-5} M GGPP or FPP. All experiments were repeated at least three times, and the number of parallel inserts was 4–8.

Claudin-5 Immunocytochemistry

To observe changes in brain endothelial TJs, BBB cells treated with pitavastatin were stained with primary antibody for claudin-5, a key protein responsible for barrier functions through the BBB (Honda et al. 2006).

At 48 h after pitavastatin administration, cells were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. Nonspecific reactions were blocked by 3% BSA in PBS for 30 min and then the cells were incubated with primary antibody (claudin-5, Zymed Laboratories, Inc., USA) overnight at 4°C. The cells were rinsed with PBS and incubated for 1 h at room temperature with secondary antibodies of Alexa Fluor 488 conjugated donkey anti-rabbit and anti-mouse immunoglobulins (Invitrogen Corp., USA). Between incubations cells were washed three times with PBS. Preparations were mounted in Gel Mount (Biomeda, USA), and staining was examined using a laser-scanning confocal microscope (LSM 5 Pa, Carl Zeiss, Germany) with excitation at 488 nm and a detection range from 500 to 535 nm. The primary antibody of claudin-5 was used in a dilution of 1:100 and the secondary antibodies with Alexa Fluor 488 in a dilution of 1:1000.

Western Blot Analysis of Claudin-5

RBEC were cultured in 3.5-mm plastic dishes coated with collagen type IV and fibronectin and then treated with 10^{-8} M pitavastatin for 48 h. Cells were harvested by scraping in CelLytic™ M cell lysis reagent supplemented with proteinase inhibitors (1-μg/ml aprotinin, 50-μg/ml phenylmethylsulfonyl fluoride, and 1-μg/ml leupeptin). Lysates were centrifuged at 12,000×*g* for 5 min at 4°C, supernatants were collected, and protein concentrations were determined with BCA protein assay reagent (Pierce, USA).

The samples were mixed with ×4 Laemmli sample buffer and heated at 95°C for 5 min. An equal amount of protein for each sample was separated by 12% SDS–PAGE and then transferred onto Hybond^{TM}-P (Amersham, UK). Nonspecific binding sites were blocked by Perfect-Block (MoBiTec GmbH, Germany) (1% w/v) in Tris-buffered saline (25-mM Tris, 150-mM NaCl, 2-mM KCl, pH 7.4) (TBS) containing 0.1% Tween-20. Anti-claudin-5 mouse monoclonal antibody (Zymed Laboratories, Inc., USA) and anti-β-actin rabbit polyclonal antibody (additional loading control) were used in dilutions of 1:5,000 and 1:10,000, respectively, in blocking solution for 1 h at room temperature. Peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulins (GE Healthcare, UK) were applied as secondary antibodies. Between incubations blots were washed three times with TBS. To reveal immunoreactive bands, the blots were incubated in SuperSignal West Femto Maximum Sensitivity Substrate SECL following the manufacturer's instructions (Pierce Biotechnology, USA) and detected by a FluorChem SP Imaging System (Alpha Innotech Corp., USA).

Quantitative RT–PCR mRNA Analysis

For total RNA extraction, RBEC were cultured in 3.5-mm plastic dishes coated with collagen type IV and fibronectin and then treated with pitavastatin (10^{-8} M) for 48 h. Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1-μg total RNA with the Reverse Transcription System (Promega, USA). Polymerase chain reaction (PCR) fragments for claudin-5 and GAPDH were amplified using primer pairs. Primer sequences are as follows: sense 5′-ACG GGA GGA GCG CTT TAT GC-3′, antisense 5′-TTG ACT GGA AAA CTG AAC AC-3′ for claudin-5; sense 5′-ACA TCA AGA AGG TGG TGA AG-3′, antisense 5′-TTG GAG GCC ATG TAG GCC ATG-3′ for GAPDH. PCR was performed in a final volume of 20 μl containing 0.5-μl template cDNA, 1.6-μl dNTP mixture, 2-μl of ×10 PCR buffer, 0.5-U Takara Taq^{TM} Hot Start Version polymerase (Takara Bio, Inc., Japan), and 1 μM each of primer using a PCR Express II thermal cycler (Thermo Electron Corp., USA). PCR was performed with 27 cycles of denaturation at 98°C for 10 s, annealing at 51°C for 30 s, and extension at 72°C for 15 s. PCR products were analyzed by electrophoresis on 1.5% agarose gels, stained by ethidium bromide and then visualized using FluorChem^{TM} SP (Alpha Innotech, USA).

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). We used the *t* test and ANOVA (analysis of variance) of the SAS system for calculations. A P value of less than 0.05 was considered to be statistically significant.

Results

The TEER of the RBEC monolayer model displayed more than 100 Ω × cm^2 (the control value; 114 ± 7.0 Ω × cm^2, $n = 4$). In order to analyze the potential beneficial effects of pitavastatin on BBB integrity at concentrations ranging from 10^{-11} to 10^{-6} M, the TEER of the in vitro monolayer BBB model was measured (Fig. 2). At a concentration of 10^{-8} M, the 48-h treatment of pitavastatin significantly elevated the TEER to 130 ± 1.0% of the control level (Fig. 2a). Interestingly, compared to at 10^{-8} M, the statin exerted a significant but lesser effect of 115 ± 4.2% at a higher concentration of 10^{-7} M, and no effect at 10^{-6} M. The 24-h treatment of pitavastatin for RBEC showed a smaller elevation of the TEER level when compared to the 48-h treatment (Fig. 2b). Since the strongest effect of pitavastatin was observed at 10^{-8} M, this concentration was used in subsequent experiments.

Pitavastatin significantly decreased the permeability of Na–F (3.82 ± 0.86 vs. 2.82 ± 0.77 × 10^{-6} cm/s at 48 h; $P < 0.01$) (Fig. 3a), supporting the idea that pitavastatin works on TJs in RBEC to reduce paracellular transport and strengthen BBB integrity. The permeability of Evans' blue-albumin, an index of transcellular transport, was not affected by pitavastatin treatment (Fig. 3b).

Our immunohistochemical study revealed that pitavastatin increased the expression of claudin-5, a key protein composing TJs (Fig. 4). There was a discontinuous and linear staining of claudin-5 marginally localized in the cell border between RBEC (Fig. 4a). Immunostaining became clearer and sharper in RBEC treated with pitavastatin at 10^{-8} M for 48 h (Fig. 4b) when compared to that in the control. These linear appearances of caludin-5 observed in pitavastatin-treated cells seemed to be in a good agreement with the strong barrier properties measured by TEER (Fig. 3).

Changes in caludin-5 levels in RBEC treated with pitavastatin were investigated by Western blot analyses. As shown in Fig. 5a, pitavastatin apparently increased the expression of claudin-5. However, the administration of pitavastatin had no apparent effect on the total mRNA level of claudin-5 (Fig. 5b).

Since GGPP and FPP are important downstream effectors of the HMG-CoA reductase pathway, we examined whether the addition of these isoprenoids antagonizes the protective effect of pitavastatin. The pitavastatin-induced increase in TEER was abolished in the presence of GGPP (10 μM), whereas the addition of FPP (10 μM) did not block the effect of pitavastatin (Fig. 6).

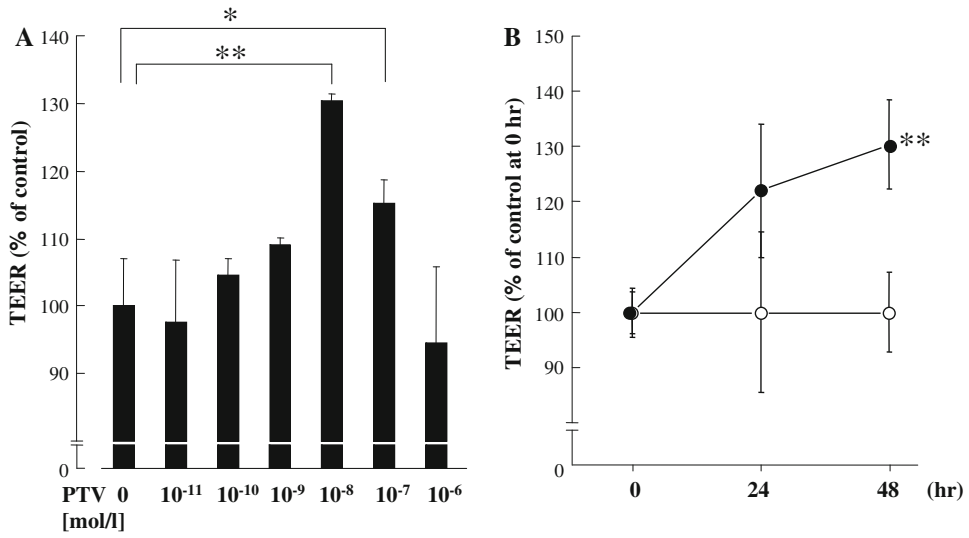

Fig. 2 Effects of pitavastatin (PTV) on the transendothelial electrical resistance (TEER) in rat brain capillary endothelial cell (RBEC) monolayers of the in vitro BBB model. **a** Confluent RBEC cultures were exposed to PTV at concentrations of 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M for up to 48 h. The TEER values are expressed as a percent of the control value (100% = 115 ± 7.0 Ω × cm^2, $n = 4$). Significantly higher levels of TEER were observed at 48 h after addition of 10^{-8} M (** $P < 0.01$ vs. control) and 10^{-7} M (* $P < 0.05$ vs. control) pitavastatin (PTV), compared with the control. No differences were observed in TEER of cells treated with 10^{-6} M PTV. **b** Confluent RBEC cultures were treated with 10^{-8} M PTV for up to 24 or 48 h. At 48 h, the TEER was 130 ± 1.0% (** $P < 0.01$ vs. control). All data are presented as mean ± SD ($n = 4$)

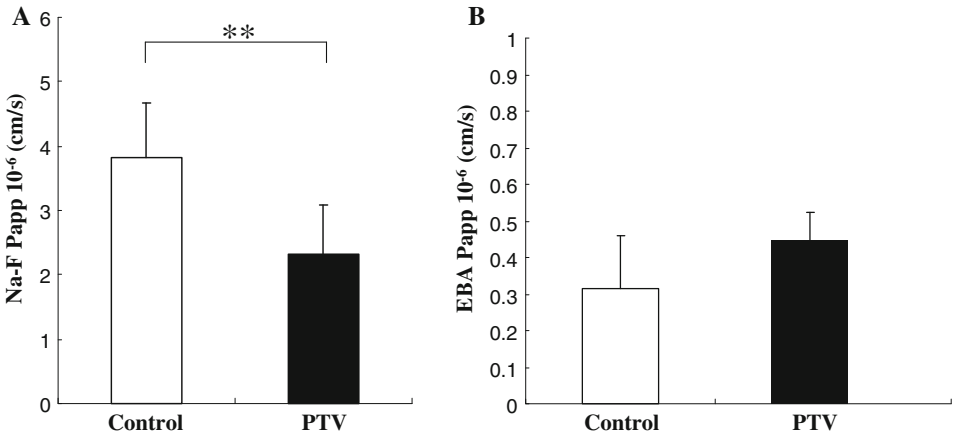

Fig. 3 Transendothelial permeability (Papp) changes for paracellular marker sodium fluorescein (Na–F) (**a**) and transcellular marker Evans' blue-albumin (EBA) (**b**) in rat brain capillary endothelial cell monolayers of the in vitro BBB model at 48 h after treatment of 10^{-8} M pitavastatin (PTV). A significantly higher level of Papp for Na–F was observed in cells treated with 10^{-8} M PTV (** $P < 0.01$ vs. control). All data (10^{-6} cm/s) are presented as mean ± SD ($n = 8$, Na–F; $n = 4$, EBA)

Discussion

As the BBB protects neurons from xenobiotics and regulates the level of neuroactive mediators (Abbott 2005; Zlokovic 2008), the maintenance of a functional BBB is essential for the treatment of neurological disorders such as cerebrovascular stroke and neurodegenerative disease. There is clinical evidence that statins have a beneficial, pleiotropic effect of stroke prevention in cerebrovascular diseases (Amarenco et al. 2006; Nassief and Marsh 2008). Kuhlmann et al. (2006) noted for the first time in an in vitro study that fluvastatin has a BBB stabilizing effect. In this study, we found that pitavastatin, a "new generation" statin with a stronger cholesterol-lowering effect (Kajinami et al. 2000; Bolego et al. 2002; Katsumoto et al. 2005), augmented the barrier integrity of an in vitro BBB model of RBEC by increasing the expression of claudin-5, a key protein organizing the functional barrier of TJs in the BBB. This finding supports the hypothesis that the therapeutic efficacy of statins is due to the maintenance of

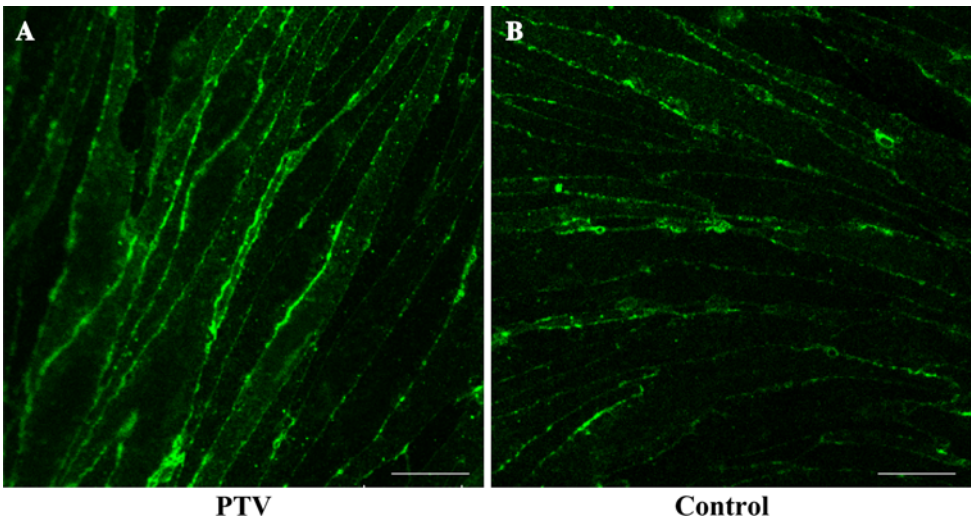

Fig. 4 Immunofluorescent staining for the tight junction protein claudin-5 in rat brain capillary endothelial cells (RBEC) of the in vitro BBB model. A stronger staining of continuous, smooth, pericellular, and belt-like patterns is noted in cells treated with 10^{-8} M pitavastatin (PTV) for 48 h (**a**), compared with the control cells (**b**), *bar* = 20 μm

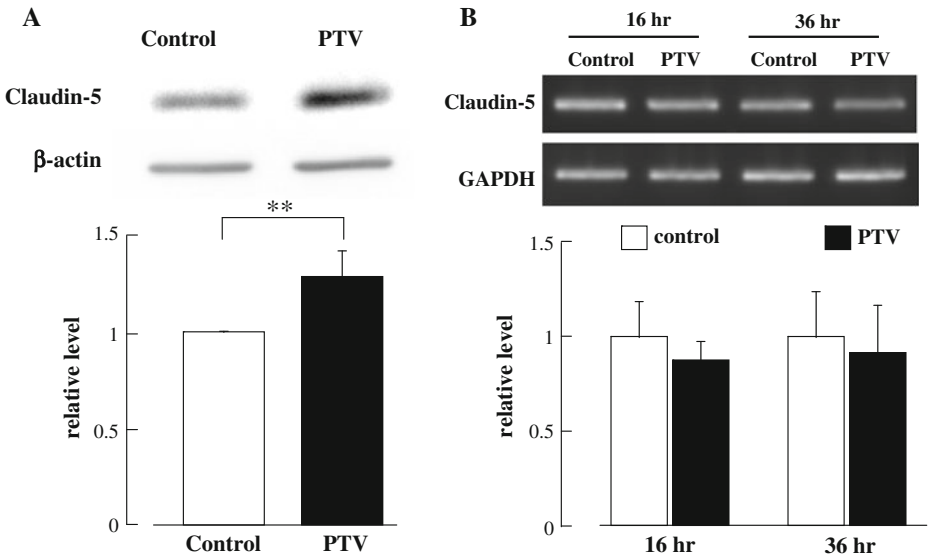

Fig. 5 Expression of the tight junction protein caludin-5 (**a**) and claudin-5 mRNA (**b**) in rat brain capillary endothelial cells (RBEC) of the in vitro BBB model treated with pitavastatin (PTV). **a** Western blot analysis of claudin-5 detected a stronger density in cells treated with 10^{-8} M PTV for 48 h that in control cells (** $P < 0.01$). The level of caludin-5 expression was relative to the internal control (β-actin). **b** Quantitative RT–PCR analysis detected no differences in expression of claudin-5 mRNA between cells treated with 10^{-8} M PTV for 16 and 36 h and control cells. GAPDH was used as an internal reference. Data obtained by densitometric analyses of Western blots and RT–PCR are presented as mean ± SD ($n = 3$)

BBB functions to defend neurons from neurological disorders.

In this study, pitavastatin increased TEER at concentrations of 10^{-7} and 10^{-8} M and decreased the permeability of Na–F at 10^{-8} M. These concentrations are equivalent to the clinical serum level of pitavastatin in humans (Katsumoto et al. 2005). Simultaneously, we also detected a pitavastatin-induced increase in claudin-5 expression. The BBB function mainly depends on the presence of a complex TJ network between endothelial cells. The TJs consist of a complex of transmembrane and cytoplasmic proteins. Accumulative evidence has revealed that claudins, a multigene family of more than 20 members, are key molecules in TJ assembly (Miyoshi and Takai 2005; Furuse and Tsukita 2006; Van Itallie and Anderson 2006). The pattern of expression of the different claudin family members varies among tissue types, which confers tissue-specific properties to TJs (Gonzalez-Mariscal et al. 2003). The claudins expressed in endothelial cells within the brain are claudin-1, -3, -5, and -12, and they are suggested to be the candidate molecules responsible for endothelial barrier function (Morita et al. 1999; Wolburg et al. 2003). In fact, a previous study with claudin-5-deficient mice found that claudin-5 was indispensable for the

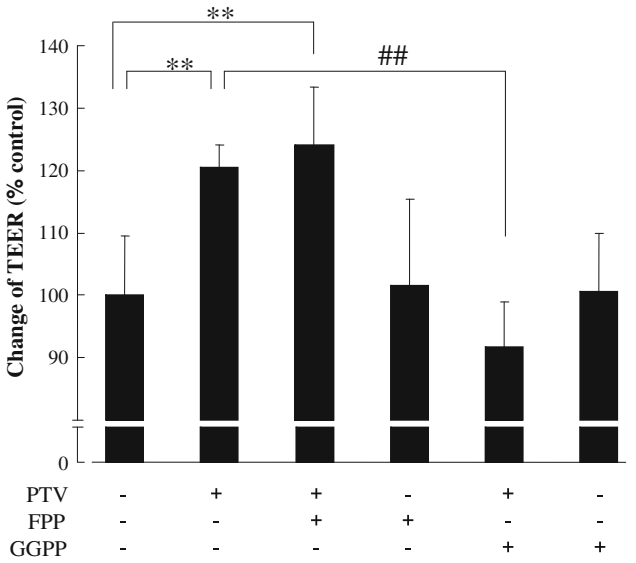

Fig. 6 Effects of geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) on the pitavastatin (PTV)-induced increase in the transendothelial electrical resistance (TEER) in rat brain capillary endothelial cell (RBEC) monolayers of the in vitro BBB model. GGPP or FPP was added to the luminal compartment of the inserts of the BBB model at a concentration of 10^{-5} M [GGPP (+)], and then RBEC were treated with the drug for 48 h in the presence [column PTV (+)] or absence [column PTV (−)] of 10^{-8} M PTV. The PTV-induced increase in TEER was abolished in the presence of GGPP, as indicated by column PTV (+), FPP (−), and GGPP (+), whereas 10^{-5} M FPP did not block the effect of pitavastatin [columns PTV (+), FPP (+), and GGPP (−)]. All data are presented as mean ± SD ($n = 4$). ** $P < 0.01$, ## $P < 0.01$

barrier function of the BBB (Nitta et al. 2003). Thus, the TEER, an index of barrier tightness of interendothelial TJs, is closely related to the amount of claudin-5 expressed as a TJ protein (Koto et al. 2007).

A pitavastatin-induced increase in the protein level of claudin-5 was also detected in this study, while the level of claudin-5 mRNA was unchanged. Therefore, the pitavastatin-induced change in the expression of claudin-5 is likely due to posttranscriptional regulation such as enhanced translation or reduced degradation of claudin-5 molecules. Furthermore, pitavastatin may translocate claudin-5 from the cellular cytoplasm to the plasma membrane, resulting in increasing functional TJs in the BBB.

As the increase in barrier tightness induced by pitavastatin was reversed by GGPP but not by FPP, GGPP-targeting molecules, or a GGPP-dependent signaling mechanism seems to be responsible for the pharmacological potency of pitavastatin in the BBB. In fact, among GGPP-dependent noncholesterol signals in the mevalonate pathway, endothelial nitric oxide (NO) synthase (eNOS) is stimulated via RhoA activation by statins (Laufs et al. 1998), and simvastatin and rosuvastatin protected against ischemia-induced endothelial permeability of the heart in a NO-dependent manner (Di Napoli et al. 2000). Our present data on isoprenoids is in accord with the experimental data of Kuhlmann et al. (2006) obtained from fluvastatin and an in vitro BBB model composed of cell lines ECV304 and C6. Hence the mechanism of NO-dependent dephosphorylation of the myosin light chain (Kuhlmann et al. 2006) may also be operative in our in vitro BBB model with primarily cultured RBEC.

Katsumoto et al. (2005) found that in a case of human epidermal microvessel endothelial cells (HUVEC), peripheral endothelial cell line-activating effects such as migration, proliferation, and viability induced by a low concentration (3×10^{-7} M) of pitavastatin were blocked by both FPP and GGPP, a finding which does not concur with our present data showing that FPP failed to reverse the barrier-strengthening effect of pitavastatin (10^{-8} M). As FPP targets Ras for cell proliferation and viability, and GGPP is pertinent to RhoA activation, pitavastatin-induced barrier tightness seems to be related to phosphorylated claudin-5, an inactive type of claudin-5 linked to barrier function at TJs. In addition to previous findings on cAMP/protein kinase A-dependent phosphorylation of claudin-5 (Ishizaki et al. 2003; Soma et al. 2004), claudin-5 was also recently found to be phosphorylated at position T207 by Rho kinase, a downstream effector of Rho A (Yamamoto et al. 2008). Thus, our findings along with findings that phosphorylated claudin-5 are inactive in maintaining barrier tightness in TJs (Yamamoto et al. 2008), and that RhoA/Rho kinase activation results in functional impairment of the BBB (Persidsky et al. 2006), indicates that the beneficial effect of pitavastatin on the BBB is related to the proportion of functional claudin-5 and phosphorylated claudin-5.

We observed here that pitavastatin worked on our in vitro BBB model RBEC to elevate the TEER at a concentration of 10^{-8} M, a level equivalent to serum levels observed clinically in patients (Kajinami et al. 2000); at a higher concentration of 10^{-6} M, there was no obvious effect. This biphasic phenomenon between high and low concentrations has been noted in vitro for pitavastatin-induced cell-activating effects such as migration, proliferation, and viability on HMVEC (Katsumoto et al. 2005). Mechanisms related to the discrepancy between low and high concentrations on barrier tightness remain to be elucidated. Cells surrounding brain capillary endothelial cells such as astrocytes and pericytes contribute to the formation and maintenance of a functional BBB (Abbott 2005). Astrocytes induce the formation of interendothelial TJs (Abbott et al. 2006), a fundamental characteristic of the BBB. Brain pericytes, the nearest neighbors of brain capillary endothelial cells sharing a common basal membrane in capillaries, also have a fundamental role in the development, maintenance, and regulation of the BBB (Hayashi

et al. 2004: Nakagawa et al. 2007). Therefore, the pharmacological significance of statins in barrier functions can be better elucidated when more is known of the molecular mechanisms underlying our BBB model consisting of a triple co-culture of primary REBC, astrocytes, and pericytes (Nakagawa et al. 2007, 2009).

To conclude, we obtained what seems to be the first evidence that pitavastatin elevates claudin-5 expression with an increase of barrier tightness in primary cultures of RBEC. Clinically, therapeutic approaches using statins with novel strategies need to be designed. Our present finding sheds light on the pharmacological significance of statins in various kinds of CNS diseases.

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