<table>
<thead>
<tr>
<th>Title</th>
<th>Pitavastatin Strengthens the Barrier Integrity in Primary Cultures of Rat Brain Endothelial Cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Morofuji, Yoichi; Nakagawa, Shinsuke; So, Gohei; Hiu, Takeshi; Horai, Shoji; Hayashi, Kentaro; Tanaka, Kunihiko; Suyama, Kazuhiko; Deli, Maria A; Nagata, Izumi; Niwa, Masami</td>
</tr>
<tr>
<td>Citation</td>
<td>Cellular and molecular neurobiology, 30(5), pp.727-735; 2010</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2010-07</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10069/23013">http://hdl.handle.net/10069/23013</a></td>
</tr>
<tr>
<td>Rights</td>
<td>© Springer Science+Business Media, LLC 2010; The original publication is available at <a href="http://www.springerlink.com">www.springerlink.com</a></td>
</tr>
</tbody>
</table>
Pitavastatin Strengthens the Barrier Integrity in Primary Cultures of Rat Brain Endothelial cells

Yoichi Morofuji · Shinsuke Nakagawa · Gohei So · Takeshi Hiu · Shoji Horai · Kentaro Hayashi · Kunihiko Tanaka · Kazuhiko Suyama · Maria A. Deli · Izumi Nagata · Masami Niwa

Y. Morofuji ( ) · G. So · K. Hayashi · N. Kitagawa · K. Suyama · I. Nagata
Departments of Neurosurgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

S. Nakagawa · S. Horai · K. Tanaka · M. Niwa
Departments of Pharmacology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

S. Nakagawa · M. A. Deli · M. Niwa
BBB Laboratory, PharmaCo-Cell Co. Ltd., 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

M. A. Deli
Laboratory of Molecular Neurobiology, Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, Temesvári körút 62, H-6726 Szeged, Hungary
Abstract Statins have a neuroprotective effect in neurological diseases, a pleiotropic effect possibly related to the 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 RBEC monolayer. The increase in the TEER was significantly reduced in the presence of isoprenoid geranylgeranyl pyrophostate, whereas farnesyl pyrophostate had no effects for the TEER. Our immunocytochemical and Western blot analyses revealed that the treatment of 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 possibility that the BBB stabilizing-effect of pitavastatin is mediated partly through inhibition of mevalonate pathway, subsequently up-regulating the expression of claudin-5, a key protein in TJs would have to be considered.

Keywords Pitavastatin · Statins · Blood–brain barrier · Tight junctions · Claudin-5 · Transendothelial electrical resistance · Geranyl geranylation · Brain endothelial cells (rat)
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 amount of clinical studies, Nassief and Marsh (2008) recently proposed that statins are a significant therapy for preventing stroke insults with coronary and cerebrovascular diseases. This clinical efficacy of statins 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 were found to deactivate platelet aggregation and procoagulation cascades by inducing a 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 have an vascular endothelial cells-stabilizing effect in the blood-brain barrier (BBB), a functional phenotype of endothelial cells of cerebral capillary working in the brain.
The BBB provides ionic homeostasis and nutrients necessary for the proper functioning of the CNS and it protects neurons from xenobiotics, blood-borne, toxic substances and stroke insult and also regulates the level of neuroactive mediators (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 et al. 2008). In the BBB, the tight junctions (TJs), a huge architecture of cell-cell adhesion molecule composed of membrane proteins such as claudins, occludins and ZO-1 and located between the brain capillary endothelial cells restrict the paracellular diffusion of water-soluble substances from blood to brain to protect neurons, which is essential for a functional BBB (Abbott 2005; Deli et al. 2005, Nakagawa et al. 2009). The BBB properties are attributed mainly to the presence of complex TJs network between endothelial cells. Thus, the BBB phenotype of brain endothelial cells mainly includes TJs and among TJs proteins claudins are key molecules in the TJs 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 TJs of the BBB. Hence, we studied here the effect of pitavastatin, a strong statin with a longer action and a less potential of drug interactions (Saito et al. 2002; Iglesias and Diez 2003) on caludin-5, a main and key protein of TJs, by 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 as 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 rat brain capillary endothelial cells (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-times of 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, 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-transferin-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% CO₂/95% air, for 2 days. Thus, according to the finding of Perrière et al. (2005), in the first two days we incubated cells in the medium containing puromycin (4 μg/ml) to avoid the contamination of pericytes. After 2 days, the cells received a 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 treatment with trypsin (0.05% wt/vol)-EDTA (0.02% wt/vol) solution, and an in vitro BBB model was constructed with Transwell® (Corning Incorporated Life Sciences, USA) in the well of the 12-well culture plates. The endothelial cells (1.5 × 10⁵ cells/cm²) were seeded on the upper side of the collagen- and fibronectin-coated polyester membrane (0.4 μm pore size) of Transwell® inserts. This in vitro model of the BBB 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 were established within 3 days after setting of the cells. Experiments of 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 TJs of endothelial cell layers in culture conditions 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 $\Omega \times \text{cm}^2$ 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$_2$PO$_4$, 10 mM NaH$_2$PO$_4$, 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 376Da) and 165 μg/mL Evans’ blue-albumin (MW 67kDa). 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 absorbency of Evans’ blue is 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 the concentrations ranging from 10^{-11} to 10^{-6} M. Forty-eight or 24 h after pitavastatin treatment, the 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; Kulmann 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, pitvastatin was also added in the presence or absence of 10^{-5} M
GGPP or FPP. All experiments were repeated at least 3 times, and the number of parallel inserts was 4-8.

Claudin-5 Immunocytochemistry

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

Forty-eight 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) lasted for 1 h. Between incubations cells were washed three times with PBS. Preparations were mounted in Gel Mount (Biomed, USA) and staining was examined using a laser-scanning confocal microscope (LSM 5 PASCAL, 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 1:100 and secondary antibodies with Alexa Fluor 488 were in a dilution 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 CellLytic\textsuperscript{TM} M cell lysis reagent supplemented with proteinase inhibitors (1μg/mL aprotinin, 50 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin). Lysates were centrifuged at 12,000×g for 5 min at 4°C and 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 was transferred onto Hybond\textsuperscript{TM}-P (Amersham, UK). Non-specific 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 a dilution of 1:5,000 and 1:10,000 in blocking solution to incubate blots for 1 h at room temperature, respectively. 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 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 were treated with pitavastatin (10^{-8} M) for 48 h. The total RNA was isolated with the RNeasy Mini Kit (Qiagen KK, Japan) according to the manufacturer’s instructions. First strand cDNA was synthesized from 1 μg of total RNA with the Reverse Transcription System (Promega, USA). Polymerase chain reaction (PCR) fragments for claudin-5 and GAPDH were amplified using the primer pairs. The sequences of primers as follows: sense primer 5’- ACG GGA GGA GCG CTT TAT GC -3’ and antisense primer 5’- TTG ACT GGA AAA CTG AAC AC -3’ for claudin-5; sense primer 5’- ACA TCA AGA AGG TGG TGA AG -3’ antisense primer 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 of template cDNA, 1.6 μl of dNTP mixture, 2 μl of 10xPCR buffer, 0.5 unit of TaKaRa Taq™ Hot Start Version polymerase (TAKARA BIO INC, Japan) and 1 μM of each primer using PCR Express II thermal cycler (Thermo Electron Corp, USA). PCR was performed with 27 cycles of denaturation at 98°C for 10 sec, annealing at 51°C for 30 sec and extension at 72°C for 15 sec. The PCR products were analyzed by electrophoresis on 1.5% agarose gels and stained by ethidium bromide and
were then visualized using FluorChem™ SP (Alpha Innotech, USA).

Statistical Analysis

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

Results

The TEER of this RBEC monolayer model displayed more than 100 $\Omega \times \text{cm}^2$ (the control value; 114 ± 7.0 $\Omega \times \text{cm}^2$, mean ± SD, n = 4). In order to analyze potential beneficial effects of pitavastatin at concentrations ranging from $10^{-11}$ to $10^{-6}$ M on the BBB integrity, the TEER of in vitro monolayer BBB model was measured (Fig. 2). At a concentration of $10^{-8}$ M, 48-h treatment of pitavastatin significantly elevated the TEER to 130 ± 1.0 % of the control level (Fig. 2A). Interestingly, at a higher concentration of $10^{-7}$ M the statin exerted a significant but a lesser effect of 115 ± 4.2 %, as compared to the experiment done at $10^{-8}$ M, and no effect was observed at $10^{-6}$ M. Twenty-four h treatment of pitavastatin for RBEC had a smaller elevation of the TEER level, as compared to 48-h treatment (Fig. 2B). Since the strongest effect of pitavastatin was observed at $10^{-8}$ M, this concentration was used in the following 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), a finding supporting the idea that pitavastin worked on TJs in RBEC to reduce a paracellular transport and then strengthen the 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). The immunostaining became more clear and sharp in RBEC treated with pitavastatin at 10⁻⁸ M for 48 h (Fig. 4B), as compared to the control RBEC. These linear appearances of caludin-5, a key protein of TJs observed in pitavastatin-treated cells seemed to be in a good agreement with the strong barrier properties measured by the TEER (Fig. 3).

The change of caludin-5 level in RBEC treated with pitavastatin was investigated by Western blotting analyses. As shown in Fig. 5A, pitavastatin apparently increased the expression of claudin-5. However, the administration of pitavastatin had no apparent effects on total mRNA level of claudin-5 (Fig. 5B).

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

**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 an essential idea for the treatment of neurological disorders such as cerebrovascular stroke and neurodegenerative disease. There is clinical evidence revealed that statins have a beneficial, pleiotropic effect of stroke prevention on cerebrovascular diseases (Amarenco et al. 2006; Nassief and Marsh 2008). Kuhlmann et al. (2006) noted in an in vitro study for the first time that fluvastatin have 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 an expression of claudin-5, a key protein organizing functional barrier of TJs in the BBB, a finding which supports the idea on therapeutic potency of statins in maintaining BBB functions to defend neurons from neurological disorders.

In the present study, pitavastatin increased the 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 pitavastatin-induced increase of claudin-5 expression. The BBB
function mainly depends on the presence of complex TJs network between the 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 twenty members, are a key molecule in the TJs 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 the tissue types, which confers the tissue-specific properties to TJs (Gonzalez-Mariscal et al. 2003). The claudins expressed in endothelial cells within the brain are claudin-1, claudin-3, claudin-5 and claudin-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, the previous study with claudin-5-deficient mice disclosed that claudin-5 was indispensable for the 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 TJs protein (Koto et al. 2007).

Pitavastatin-induced increase in the protein level of claudin-5 was detected in this study, while the level of claudin-5 mRNA was unchanged. Therefore, pitavastatin-induced change in the expression of claudin-5 is due to the posttranscriptional regulation such as enhanced translation and 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 and GGCP-dependent signaling mechanism seems to be responsible for the pharmacological potency of pitavastatin in the BBB. In fact, among GGCP-dependent non-cholesterol signaling in the mevalonate pathway, endothelial nitric oxide (NO) synthase (eNOS) is stimulated via RhoA activation by statins (Laufs et al. 1998), and Di Napoli et al. (2000) reported that in their studies on endothelial permeability of the heart, simvastatin and rosuvastatin protected against ischemia-induced endothelial permeability in a NO-dependent way. Our present data on isoprenoids is in accord with the experimental data of Kulmann et al. (2006) obtained from fluvastatin and an in vitro BBB model composed of the cell lines ECV304 and C6, hence the mechanism of NO-dependent dephosphorylation of myosin light chain (Kulmann 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 microvascular endothelial cells (HUVEC), a peripheral endothelial cells line the cell activating effect such as migration, proliferation and viability induced by a low concentration (3 x 10^{-7}M) of pitavastatin was blocked by both FPP and GGPP, a finding which does not concur with our present data 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, a pleiotropic effect of statins seems to be related to phosphorylated claudin-5, an inactive type of claudin-5 functionally linked to the barrier function at
the Tjs. In addition to the previous finding on cAMP/protein kinase A-dependent phosphorylation of claudin-5 (Ishizaki et al. 2003; Soma et al. 2004), claudin-5 was recently found to be also phosphorylated at a position of T207 by Rho kinase, a downstream effector of Rho A (Yamamoto et al. 2008). Thus, when the present data are taken together in conjunction with the findings that phosphorylated claudin-5 is inactive in maintaining barrier tightness of Tjs (Yamamoto et al. 2008) and RhoA/Rho kinase activation resulted in functional impairments of the BBB (Persidsky et al. 2006), it may be that the beneficial effect of pitavastatin on the BBB is related to the proportion of functional claudin-5 and phosphorylated claudin-5, an inactive form at interendothelial Tjs.

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 being equivalent to the serum level observed clinically in patients (Kajinami et al. 2000), whereas at a higher concentration of $10^{-6}$M there was no obvious effect. The biphasic phenomenon between high and low concentrations was noted in vitro in pitavastin-induced cell-activating effect such as migration, proliferation and viability on HMVEC (Katsumoto et al. 2005). Mechanisms related to participation to the discrepancy between the effect of low and high concentrations of pitavastatin on barrier tightness of Tjs remains to be elucidated. Furthermore, cells surrounding brain capillary endothelial cells such as astrocytes and pericytes contribute to the formation and maintenance of a functional BBB (Abbott 2005). Astrocytes induces 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 capillary 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 of the BBB will be precisely elucidated when more is known of molecular mechanisms on our BBB model consisting of the triple co-culture of primary rat brain capillary endothelial cells, astrocytes and pericytes (Nakagawa et al. 2007: Nakagawa et al. 2009).

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

**Acknowledgments** Pitavastatin was obtained as a generous gift from Kowa Co., Ltd., Japan. We wish to thank Yasuko Yamashita, Takanori Shimono, and Tsuyoshi Izumo for their critical reviews of the manuscript and outstanding professional guidance. This work was supported by Grant-in-Aid for Scientific Research (C).


Honda M, Nakagawa S, Hayashi K, Kitagawa N, Tsutsumi K, Nagata I,


dephosphorylation of myosin light chains. Neuropharmacology 51:907-913


monocyte chemoattractant protein-1 in circulating monocytes from hypercholesterolemic patients. Arterioscler Thromb Vasc Biol 22:1194-1199


Figure legends

**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 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-wl plates. From Day 5, RBEC were grown in culture medium containing 500 nM hydrocortisone. Experiments were performed on Day 7.

**Fig. 2** Effects of pitavastatin (PTV) on the transendothelial electrical resistance (TEER) in rat brain capillary endothelial cells (RBEC) monolayers of 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 hr. The TEER value are expressed in the percent of the control value ($100\% = 115 \pm 7.0 \ \Omega \times cm^2$, means ± SD, n = 4). Significant higher levels of the TEER were observed in rat brain capillary endothelial cells monolayer of in vitro BBB model 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), as compared with the control. We noted no differences in the TEER of cells treated with $10^{-6}$ M PTV. (B) Confluent RBEC cultures were treated with $10^{-8}$ M PTV for up to 24 h or 48 h. At 48 h, the TEER were $130 \pm 1.0 \%$ ($**P < 0.01$ vs. control). All data are presented as means ± 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 cells monolayer of in vitro BBB model at 48 h after addition of $10^{-8}$ M pitavastatin (PTV). A significant higher level of the Papp for Na-F was observed in the cells treated with $10^{-8}$ M PTV (**$P < 0.01$ vs. control). All data ($10^{-6}$ cm/s) are presented as means ± SD ($n = 8$, Na-F; $n = 4$, EBA).

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

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

**Fig. 6** Effects of geranylglycerol pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) on pitavastatin (PTV)-induced increase in the transendothelial electrical resistance (TEER) in rat brain capillary endothelial cells (RBEC) monolayers of in vitro BBB model. GGPP or FPP were added to the luminal compartment of the inserts of BBB model at a concentration of 10⁻⁵ M (GGCP (+)), 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. PTV-induced increase in the TEER was abolished in the presence of GGPP, as indicated by column PTV (+), FPP (-), GGPP (+), whereas 10⁻⁵ M FPP did not block the effect of pitavastatin (columns of PTV (+), FPP (+), GGPP (-)). All data are presented as means ± SD (n = 4).

*P < 0.05, ##P < 0.01
subculture

Isolation of rat brain microvessels

Day
0 2 4 5 7

Transwell® (0.4 μm pore)

Fig. 1

Serum free RBEC II
Medium
+ hydrocortisone (500 nM)

RBEC I
+ medium
(4 μg/mL)

RBEC II
medium
Fig. 2
Fig. 3
Fig. 4
Fig. 5

A

Control  PTV

Claudin-5

β-actin

**

B

16 hr  36 hr

Control  PTV  Control  PTV

Claudin-5

GAPDH

control  PTV

0.5  1.5

relative level

Control  PTV

16 hr  36 hr

control  PTV
Fig. 6