When MBEC4 cells were co-cultured with pericytes, the Na-F permeability of MBEC4 cells was increased from 3.26 ± 0.13 × 10^{-4} cm/min in monolayers to 3.86 ± 0.23 × 10^{-4} cm/min (Fig. 1C). The presence of pericytes decreased rhodamine 123 accumulation in MBEC4 cells from 0.51 ± 0.12 nmol/mg protein in monolayers to 0.43 ± 0.10 nmol/mg protein (Fig. 1D). A 12 h-exposure to CsA at 5 or 10 μM showed no effect on cell viability as determined by mitochondrial dehydrogenase activities (WST-8 assay) of MBEC4 cells in MBEC4 monolayers (100.0 ± 1.8 and 103.1 ± 7.8% of vehicle, respectively, $n = 4$ inserts) and pericyte co-cultures (99.2 ± 2.5 and 106.8 ± 2.3% of vehicle, respectively, $n = 4$ inserts). Figure 1C and D shows the effect of CsA (5 μM) on the permeability of MBEC4 cells to Na-F and the accumulation of rhodamine 123 in MBEC4 cells, respectively, in both MBEC4 monolayers and pericyte co-cultures. The Na-F permeability of MBEC4 cells was time-dependently increased during a period of 1–6 h after the addition of CsA (5 μM), reaching a peak at 6 h (Fig. 1B). A significant difference in CsA-induced hyperpermeability was observed between MBEC4 monolayers and pericyte co-cultures, with the effect being most apparent after a 6 h-exposure [$F(1, 24) = 10.51$, $P < 0.01$] (Fig. 1C). At the peak time, CsA increased the Na-F pearmeability of MBEC4 cells by 10.3 ± 3.4 and 31.9 ± 3.6% of each corresponding vehicle treatment in MBEC4 monolayers and pericyte co-cultures, respectively (Fig. 1C). Following exposure of cells to CsA for 9–12 h, permeability of the MBEC4 monolayers was gradually increased. However, the effect of CsA on pericyte co-cultures permeability became more moderate, reducing to the same level seen in MBEC4 monolayers (Fig. 1B). To clarify the role of brain pericytes in CsA-induced dysfunction of BBB, a 6 h-exposure of cells to CsA (5 μM) was employed in the following experiment. The accumulation of rhodamine 123 in MBEC4 cells was increased by 13.9 ± 5.9 and 43.4 ± 8.5% of each corresponding vehicle treatment in MBEC4 monolayers and pericyte co-cultures, respectively, after a 6 h-exposure to CsA (Fig. 1D). A significant difference in CsA-induced decrease in P-gp activity was observed between MBEC4 monolayers and pericyte co-cultures [$F(1, 28) = 4.65$, $P < 0.05$]. CsA-induced inhibition of P-gp function was more potent in pericyte co-cultures than in MBEC4 monolayers.

When pericyte co-cultures were treated with CsA (5 μM) for 6 h, the levels of TGF-β1 mRNA in brain pericytes were significantly decreased to 81.0 ± 7.3% of vehicle (Fig. 2A) [$F(2, 13) = 5.05$, $P < 0.05$]; however, a 12 h exposure to CsA failed to decrease levels of TGF-β1 mRNA in brain pericytes (103.8 ± 10.8% of vehicle). In pericyte co-cultures, TGF-β1 (0.01–1 ng/mL) dose-dependently inhibited the elevation of Na-F permeability (Fig. 2B), and rhodamine 123 accumulation (Fig. 2C), in MBEC4 cells, induced by CsA (132.9 ± 11.4 to 105.4 ± 2.5% and 140.3 ± 9.9 to 98.6 ± 6.4% of vehicle, respectively).

DISCUSSION

In the present study, CsA (5 μM) time-dependently increased the Na-F permeability of MBEC4 cells in pericyte co-cultures, this effect reaching a peak at 6 h

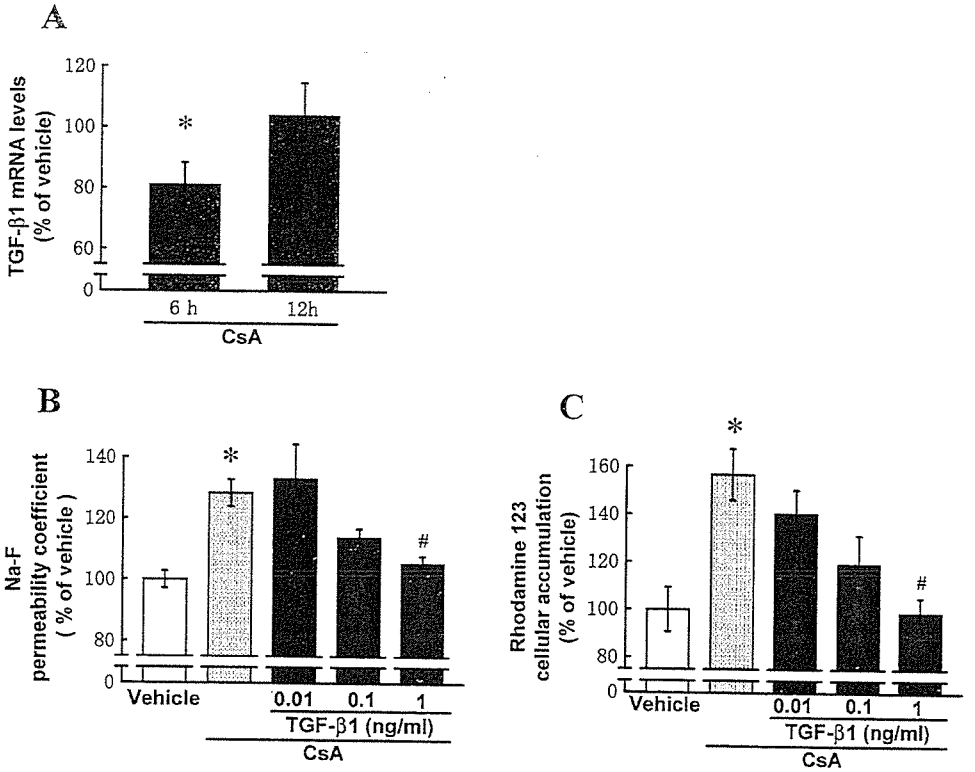

Fig. 2 (A) Effect of CsA (5 μM) on TGF-β1 mRNA expression in brain pericytes at 6 and 12 h after the addition of CsA in pericyte co-cultures. Total RNA of brain pericytes was extracted and subjected to real-time PCR analysis. Fold changes in TGF-β1 mRNA are normalized to GAPDH and compared with each corresponding vehicle treatment. Values are the means ± SEM (n = 3–5). $^{*}p < 0.05$, significant difference from vehicle. (B) Effect of TGF-β1 on CsA-increased Na-F permeability of MBEC4 cells in pericyte co-cultures. Results are expressed as % of vehicle (vehicle; 3.28 ± 0.22 × 10^{-4} cm/min). Values are the means ± SEM (n = 8–16). $^{*}p < 0.05$, significant differences from vehicle.$^{\#}p < 0.05$, significant difference from CsA treatment. (C) Effect of TGF-β1 on CsA-increased rhadamine 123 accumulation of MBEC4 cells in pericyte co-cultures. Results are expressed as % of each corresponding vehicle treatment (vehicle; 1.16 ± 0.24 nmol/mg protein). Values are the means ± SEM (n = 4–12). $^{*}p < 0.05$, significant differences from vehicle.$^{\#}p < 0.05$, significant difference from CsA treatment.

after the addition of CsA (Fig. 1B and C). A 6 h exposure to CsA also decreased P-gp function in MBEC4 cells in pericyte co-cultures, this effect being more apparent than that in MBEC4 monolayers (Fig. 1D). These findings suggest that CsA-induced hyperpermeability of, and P-gp dysfunction in, MBEC4 cells, was aggravated in pericyte co-cultures. We previously reported that treatment with CsA (0.5–10 μM) for 24 h dose-dependently decreased the viability of MBEC4 cells (Kochi *et al.*, 1999). The present study demonstrated that a 12 h-exposure to CsA at concentrations up to 10 μM had no effect on the viability of MBEC4 cells in MBEC4 monolayers and pericyte co-cultures. Thus, the exposure time (6 h) and the submaximum concentration (5 μM) of CsA without cytotoxicity were selected here.

The significance of brain pericytes in the regulation of the BBB was suggested by our previous study using a primary culture of rat brain pericytes. We reported

that brain pericyte-derived TGF-β1 contributed to the induction and up-regulation of BBB function (Dohgu *et al.*, 2004b, 2005). In the present study, the presence of human brain pericytes decreased the function of tight junctions and increased the function of P-gp, in MBEC4 cells, by about 20%. An interjunctional property of MBEC4 cells was lowered by co-culturing with human brain pericytes, this event being inconsistent with our previous report using a primary culture of rat brain pericytes. The different backgrounds of human brain pericytes commercially supplied and species-different cell type may be compounding factors in this discrepancy (Lai and Kuo, 2005); however, further studies are required to confirm this. Here, we employed a convenient *in vitro* model with MBEC4 cells and human brain pericytes.

To test whether TGF-β1 production of the brain pericytes participates in the mediation of CsA-induced dysfunction of the BBB, we examined the effect of CsA on the expression of TGF-β1 in brain pericytes. A 6 h exposure to CsA (5 μM) significantly decreased the levels of TGF-β1 mRNA in brain pericytes in pericyte co-cultures (Fig. 2A). Treatment with TGF-β1 dose-dependently inhibited CsA-induced hyperpermeability and P-gp dysfunction in MBEC4 cells in pericyte co-cultures (Fig. 2B and C). These findings suggest that CsA decreases BBB function by inhibiting TGF-β1 production in the brain pericytes. In pericyte co-cultures, an aggravation of CsA-induced hyperpermeability of MBEC4 cells occurred with a peak at 6 h after the addition of CsA, becoming more moderate at 9–12 h (Fig. 1B). In parallel with these events, TGF-β1 mRNA levels were significantly decreased by a 6 h exposure to CsA but not by a 12 h exposure (Fig. 2A), suggesting that CsA-induced hyperpermeability is ameliorated by the compensatory secretion of TGF-β1 from brain pericytes during the later period of CsA exposure. These data further support a critical role of pericyte-derived TGF-β1 in mediating CsA-induced BBB dysfunction.

RT-PCR analysis demonstrated the expression of TGF-β receptor I and II in MBEC4 cells and brain pericytes (Fig. 1A). TGF-β1 mRNA was also detected in brain pericytes (Fig. 2A) and MBEC4 cells (data not shown). Considering these observations, TGF-β is likely to participate in the up-regulation of BBB function through an autocrine and/or paracrine pathway in brain endothelial cells and pericytes. Autoinduction of TGF-β1 is mediated by binding of the transcription factor activator protein-1 (AP-1) complex to homologous elements in two regions of the TGF-β1 promoter (Kim *et al.*, 1990). In this positive autoregulation process, CsA is known to directly inhibit activation of the JunD isoforms in the AP-1 complex responsible for TGF-β signaling in lung fibroblasts (Eickelberg *et al.*, 2001). Calcineurin, a molecular target of CsA, has been shown to be involved in AP-1 activation in immune cells (Pfeuffer *et al.*, 1994; Tsuboi *et al.*, 1994). Based on this evidence, CsA may be interpreted as lowering TGF-β1 expression in brain pericytes due, at least in part, to an inhibition of the AP-1 activation step during TGF-β1 autoinduction. We previously reported that CsA increased NO production in MBEC4 cells (Dohgu *et al.*, 2004a) and elevated levels of NO have been known to reduce TGF-β1 production in the heart (Smith *et al.*, 2005). Therefore, CsA may also act on MBEC4 cells to lower TGF-β1 expression in brain pericytes.

In conclusion, CsA-induced hyperpermeability and P-gp dysfunction of MBEC4 cells were markedly aggravated in co-cultures with brain pericytes. This aggravation appears to occur due to CsA-induced inhibition of TGF-β1 expression in brain pericytes. These findings suggest that an inhibition of brain pericyte-derived TGF-β1 contributes to the occurrence of CsA-induced dysfunction of the BBB, thereby triggering neurotoxicity.

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Rapid Communication

An Inhibitory Role of Nitric Oxide in the Dynamic Regulation of the Blood-Brain Barrier Function

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SUMMARY

1. The present study aimed at elucidating the effects of nitric oxide (NO) on blood-brain barrier (BBB) function with mouse brain capillary endothelial (MBEC4) cells.

2. Histamine (20–100 μM) evoked NO production (1.6–7 μM) in MBEC4 cells in a dose-dependent manner.

3. The permeability coefficient of sodium fluorescein for MBEC4 cells and the cellular accumulation of rhodamine 123 in MBEC4 cells were increased dose-dependently by addition of NO solutions (14 and 28 μM) every 10 min during a 30-min period.

4. The present study demonstrated that NO increased the permeability and inhibited the P-glycoprotein efflux pump of brain capillary endothelial cells, suggesting that NO plays an inhibitory role in the dynamic regulation of the BBB function.

KEY WORDS: nitric oxide; blood-brain barrier (BBB); permeability; P-glycoprotein; mouse brain endothelial cells .

INTRODUCTION

The blood-brain barrier (BBB) contributes to brain homeostasis and fulfills a protective function by regulating the access of solutes and toxic substances to the central nervous system. The BBB is formed by brain capillary endothelial cells, which are closely sealed by tight junctions (Pardridge, 1999). The tight junctions in the

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BBB prevent significant passive movement of small hydrophilic molecules from the blood to the brain, but specialized transport systems mediate the entry of essential substances such as glucose, amino acids, choline, monocarboxylic acids, amines, thyroid hormones, purine bases, and nucleosides (Tsuji and Tamai, 1999; Kusuhara and Sugiyama, 2001). The efflux transporter P-glycoprotein (P-gp) is a key element of the molecular machinery that confers special permeability properties on the BBB. P-gp, which was initially recognized for its ability to excrete anticancer drugs from multidrug-resistant cancer cells, is strongly expressed in brain capillaries. Its expression in the BBB limits the accumulation of many hydrophobic molecules and potentially toxic substances in the brain.

Nitric oxide (NO) is a transient product of inflammatory processes, generated from L-arginine by the enzyme NO synthase (NOS). NO appears to be involved in numerous vital cellular functions including neurotransmission, blood-pressure control, and the regulation of vascular tone. The basal production of NO appears to be required for biological regulation, and yet an excess of this same molecule can be cytotoxic to organism. But the molecular mechanisms mediating NO-induced tissue injury and breakdown of the BBB are not completely understood.

In the present study, to clarify the role of NO in the dynamic regulation of the BBB, we examined effects of NO on the function of tight junctions and P-gp in mouse brain capillary endothelial (MBEC4) cells. MBEC4 cells show the highly specialized characteristics of brain microvascular endothelial cells including P-gp expression (Tatsuta *et al.*, 1992, 1994).

MATERIALS AND METHODS

A saturated NO solution (typically containing approximately 1.4 mM NO) was prepared according to Ikesue *et al.* (2000). Deionized water (2 mL) was bubbled with argon for 20 min to remove oxygen. Then, the solution was bubbled with pure NO gas for 20 min and kept in a glass flask with a rubber septum under a NO atmosphere prior to use.

MBEC4 cells, which were isolated from BALB/c mouse brain cortices and immortalized by SV40-transformation (Tatsuta *et al.*, 1992), were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. They were grown in 2.5-cm^2 dish, 12-well Transwells (Costar, MA) and 24-well plates in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Direct and continuous electrochemical measurement of NO was performed with a three-electrode potentiostatic EMS-100 system (BIO-LOGIC, Grenoble, France), as previously described (Ikesue *et al.*, 2000; Trevin *et al.*, 1998). In brief, confluent MBEC4 cells in a 2.5-cm^2 dish were washed three times with Krebs–Ringer solution (143.0 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, and 11.0 mM D-glucose, pH 7.4). The dish was placed on the stage of an inverted microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) mounted with an NO monitoring

system. The NO-biosensor (ASTEC, Fukuoka, Japan) was positioned about 10 μm above the cell surface. Ten minutes after treatment with 1-mM L-arginine (Sigma, St. Louis, MO), histamine (Wako, Osaka, Japan) in a volume of 10 μL was added to the cells in 1 mL of Krebs–Ringer solution with a transient mixing step to give the final concentration indicated. The level of production of NO in MBEC4 cells was monitored for a 15-min period after the addition of histamine.

MBEC4 cells (42,000 cells/cm^2) were cultured on the collagen-coated polycarbonate membrane of the Transwell insert (3.0-μm pore size, 12-well type). After 3 days, they were washed three times with serum-free medium. Cells were exposed to 5 or 10 μL of NO solution (final concentration, 14 and 28 μM, respectively) injected into the inside of the insert (luminal side) every 10 min during a 30-min period. To initiate the transport experiments, the medium was removed, and cells were washed three times with Krebs–Ringer buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose, pH 7.4). Krebs–Ringer buffer (1.5 mL) was added to the outside of the insert (abluminal side). Krebs–Ringer buffer (0.5 mL) containing 100 μg/mL of sodium fluorescein (Na-F; Sigma) was loaded on the luminal side of the insert. Samples (0.5 mL) were removed from the abluminal chamber at 10, 20, 30, and 60 min and were immediately replaced with fresh Krebs–Ringer buffer. Aliquots (5 μL) from the abluminal chamber samples were mixed with 200 μL of Krebs–Ringer buffer, and then the concentration of Na-F was determined using a multiwell fluorometer ($E_x(\lambda)$ 485 nm; $E_m(\lambda)$ 530 nm; CytoFluor Series 4000, PerSeptive Biosystems, Framingham, MA). The permeability coefficient and clearance were calculated according to the method described by Dehouck *et al.* (1992). Clearance was expressed as microliters of tracer diffusing from the luminal to abluminal chamber and was calculated from the initial concentration of tracer in the luminal chamber and final concentration in the abluminal chamber: Clearance (μL) = $[C]_A \times V_A/[C]_L$ where $[C]_L$ is the initial luminal tracer concentration, $[C]_A$ is the abluminal tracer concentration, and V_A is the volume of the abluminal chamber. During a 60-min period of the experiment, the clearance volume increased linearly with time. The average volume cleared was plotted versus time, and the slope was estimated by linear regression analysis. The slope of clearance curves for the MBEC4 monolayer was denoted by PS_{app}, where PS is the permeability-surface area product (in microliters per minute). The slope of the clearance curve with a control membrane was denoted by $PS_{membrane}$. The real PS value for the MBEC4 monolayer (PS_{trans}) was calculated from $1/PS_{app} = 1/PS_{membrane} + 1/PS_{trans}$. The PS_{trans} values were divided by the surface area of the Transwell inserts to generate the permeability coefficient (P_{trans}, in centimeters per minute).

The functional activity of P-gp was determined by measuring the cellular accumulation of rhodamine 123 (Sigma) according to the method of Fontaine *et al.* (1996). MBEC4 cells (21,000 cells/cm^2) were cultured on collagen-coated 24-well plates. Three days after seeding, they were washed three times with serum-free medium and then exposed to 14–28 μM of NO solution every 10 min during a 30-min period. The medium was removed, and the cells were washed three times with assay buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, and 11 mM D-glucose, pH 7.4). The cells were incubated

with 0.5 mL of assay buffer containing 5 μM of rhodamine 123 for 60 min. Then, the solution was removed, and the cells were washed three times with ice-cold phosphate-buffered saline and solubilized in 1 M NaOH (0.2 mL). The solution was neutralized with 1-M HCl (0.2 mL), and the rhodamine 123 content was determined using a multiwell fluorometer ($E_x(\lambda)$ 485 nm; $E_m(\lambda)$ 530 nm). The cellular protein was measured by the method of Bradford (Bradford, 1976).

The effect of the NO solution on cell viability was assessed using a WST-8 assay (Cell Counting Kit, DOJINDO, Kumamoto, Japan). A highly water-soluble formazan dye, reduced by mitochondrial dehydrogenase, was measured by determining the absorbance of each sample with a 450-nm test wavelength and a 700-nm reference wavelength.

The values are expressed as means ± SEM. Statistical analysis was performed using Student's *t*-test. One-way analysis of variance (ANOVA) followed by the Dunnett test was applied to multiple comparisons. The differences between means were considered to be significant when P values were less than 0.05.

RESULTS

Figure 1A shows a representative current-time curve obtained with the NO biosensor in MBEC4 cells. When L-arginine (1 mM) was added to cells 10 min before the addition of histamine, the signal became stable within 1–2 min. The NO-biosensor signal increased rapidly to reach a peak within 1 min after the injection of histamine. Following this period, the signal decreased slowly to the baseline about 5–7 min post-injection. Histamine at concentrations of 20–100 μM dose-dependently increased NO production (1.55 ± 0.65–6.94 ± 1.43 μM) in MBEC4 cells (Fig. 1B).

To evaluate effect of NO on the BBB function, the concentration and the exposure time of NO were determined on the basis of NO production evoked by histamine. When the NO solution at the final concentrations of 14 and 28 μM was added every 10 min during a 30-min period, the permeability coefficients of Na-F for MBEC4 cells increased dose-dependently to 113.6 ± 12.9 and 123.7 ± 2.8%, respectively (Fig. 2A). The exposure to NO solution had no effect on cell viability assessed with the WST-8 assay (14 μM: 93.7 ± 3.3%, 28 μM: 101.3 ± 3.2% of control).

As shown in Fig. 2B, the accumulation of rhodamine 123 in MBEC4 cells increased dose-dependently to 108.3 ± 4.8 and 164.5 ± 14.6% of the control value after a 30-min exposure to the NO solution at concentrations of 14 and 28 μM, respectively.

DISCUSSION

As shown in Fig. 1, direct and continuous electrochemical monitoring revealed that histamine stimulated MBEC4 cells to release NO over a short period (about 5 min). This phenomenon appears to be due to an activation of the constitutive endothelial form of NO synthase (eNOS) through a Ca^{2+}/calmodulin-dependent pathway in response to H_1 receptor-mediated increases in intracellular Ca^{2+} (Daum

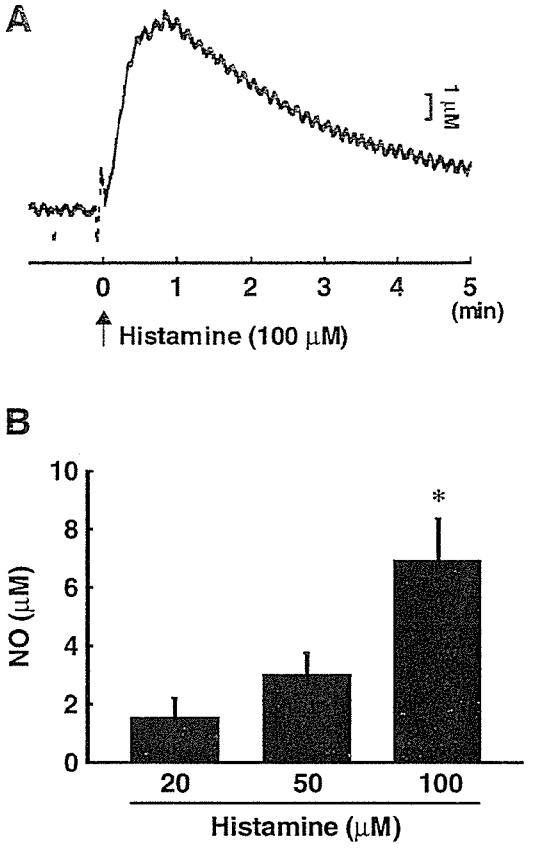

Fig. 1. Histamine-induced NO production in MBEC cells. (A) A representative differential pulse amperogram obtained using a NO biosensor shows the level of NO production evoked by histamine (100 μM). (B) Concentration-response relationship of histamine-induced NO production using direct electrochemical monitoring in MBEC cells. Values are means ± SEM ($n = 3$). $^{*}p < 0.05$, significant difference from 20 μM histamine.

et al., 1983; Kishi *et al.*, 1996). Various endogenous substances including histamine stimulate NO production in the microvascular endothelium and/or glial and neuronal cells in the brain (Mayhan, 1996), probably contributing to dynamic regulation of the BBB function.

The permeability of MBEC4 cells to Na-F was apparently increased by a brief exposure to NO solutions at 2- to 4-fold the concentrations induced by histamine (Fig. 2A). The NO solutions employed here are more prominent than convenient NO donors such as sodium nitroprusside with respect to biological properties including distribution and degradation. The cell viability was not influenced by NO at the concentrations employed here (14–28 μM), suggesting that the increased paracellular permeability of MBEC4 cells was not due to the cytotoxicity of NO. Therefore, NO is highly likely to lower the functions of the tight junctions at the

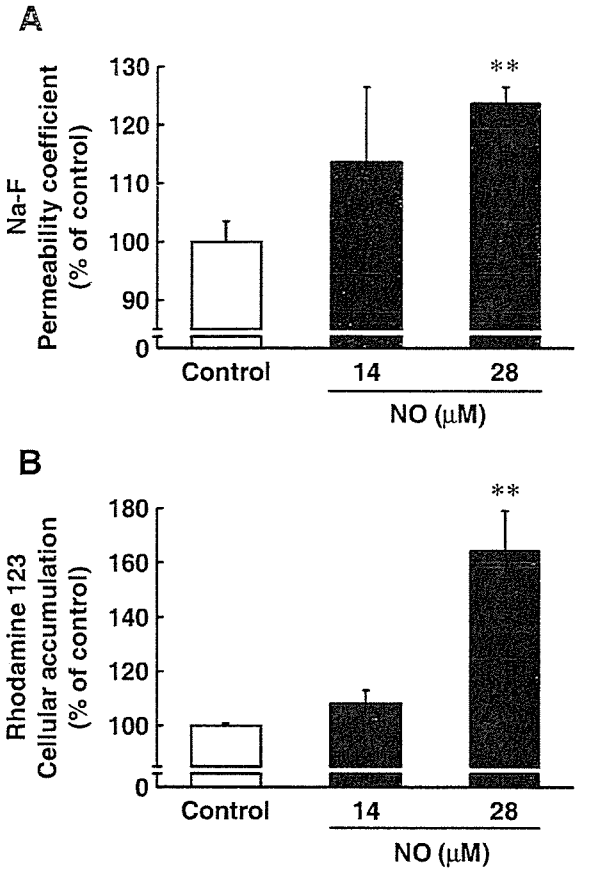

Fig. 2. Changes in the permeability coefficient of Na-F (A) and the cellular accmulation of rhodamine 123 (B) in MBEC4 cell monolayers after addition of NO solutions every 10 min during a 30-min period. Data are expressed as a percentage of the corresponding control value (A: 1.47 ± 0.21 × 10^{-4} cm/min, B: 0.95 ± 0.07 nmol/mg protein). Values are shown as means ± SEM (n = 3–18). $^{**}P < 0.01$, significant difference from control.

BBB, supporting early reports (Hurst and Fritz, 1996; Mayhan, 2000; Shukula *et al.*, 1996). The mechanisms by which NO donors increased vascular endothelial permeability involved an increase in the level of cyclic guanosine monophosphate (cGMP) A1 (Gimeno *et al.*, 1998) or the formation of peroxynitrite (Menconi *et al.*, 1998). These substances conceivably influence the intrinsic tight junction proteins and the associated actin cytoskeleton through a direct or second signaling pathway. Further studies are required to clarify this mechanism.

In the present study, the most important finding was that the accumulation of rhodamine 123, a substrate of P-gp (Fontaine *et al.*, 1996), in MBEC4 cells was increased by treatment with NO solutions (Fig. 2B). P-gp is an energy-dependent efflux pump mediating the transportation of substances from the basement membrane to the luminal surface. NO induced a depletion of endothelial ATP by

inhibiting glyceraldehyde-3-phosphate dehydrogenase (glycolytic enzyme) activity (Hurst *et al.*, 2001). This inhibition of energy metabolism is probably associated with the NO-triggered inhibition of P-gp function.

In the present study, NO induced hyperpermeability in MBEC4 cells and inhibition of the P-gp efflux pump. These findings suggest that NO in the brain plays an inhibitory role in the dynamic regulation of the BBB function.

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Short Communication

Protective Action of Indapamide, a Thiazide-Like Diuretic, on Ischemia-Induced Injury and Barrier Dysfunction in Mouse Brain Microvascular Endothelial Cells

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Abstract. The aim of the present study was to elucidate the effects of indapamide on ischemic damage to the blood-brain barrier (BBB) in vitro. The ischemia/reperfusion conditions employed here significantly decreased the viability of mouse brain capillary endothelial (MBEC4) cells, an effect ameliorated by indapamide. Ischemia increased the permeability of MBEC4 cells to two cellular transport markers, sodium fluorescein and Evan's blue-albumin. Indapamide reduced the ischemia-induced hyperpermeability of cells. These results suggest that indapamide may have a protective role against ischemia-induced injury and dysfunction of the BBB.

Keywords: indapamide, ischemia, blood-brain barrier

The blood-brain barrier (BBB) greatly restricts the transport of substances between the blood and the central nervous system. The BBB is primarily formed by brain capillary endothelial cells, which are sealed closely with tight junctions (1). Disruption of the BBB can lead to edema and central nervous system pathology in conditions such as stroke. In vitro, hypoxia has been shown to increase the permeability of brain microvascular endothelial cells (2, 3). Targeting the BBB may enhance our understanding of the mechanisms that mediate ischemic brain damage and might aid the development of future treatments for stroke (4, 5).

Indapamide is an indoline derivative of chlorosulfonamide that has both diuretic and antihypertensive properties. Indapamide protects against myocardial ischemia due to its antioxidant action (6). A randomized controlled clinical trial showed that in combination, therapy with perindopril, an angiotensin-converting-enzyme inhibitor, and indapamide reduced the risk of stroke in hypertensive and non-hypertensive patients to a greater degree, compared to single drug therapy with perindopril alone (7). This clinical evidence suggests a possible involvement of indapamide in protecting against recurrent cerebrovascular disease. The present study was, therefore, aimed at evaluating the effect of indapamide on ischemia/reperfusion-induced damage to cerebral microvascular endothelial cells in vitro.

The mouse brain capillary endothelial cells used in the current study (MBEC4) show highly specialized characteristics of brain microvascular endothelial cells (8). MBEC4 cells were isolated from BALB/c mouse brain cortices and immortalized by SV40-transformation (8). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 units /mL of penicillin, and 100 μg/mL of streptomycin. Cells were grown in collagen-coated 24-well plates (21,000 cells/cm^2, 1.8 cm^2/well; Corning, Acton, MA, USA) in a humidified atmosphere of 5% CO_2 / 95% air at 37°C. Ischemia was initiated in vitro by incubating cells with D-glucose-free Krebs-Ringer buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM

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NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM sucrose, pH 7.4) in an anoxic incubator replaced with 5% CO_2 / 95% N_2 for 10 h at 37°C (ischemia conditions). Subsequently, cells were incubated with serum-free DMEM in 5% CO_2 / 95% air at 37°C for 1 h (reperfusion conditions). As a control, cells were incubated with normal Krebs-Ringer buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose, pH 7.4) for 10 h and subsequently incubated with serum-free DMEM for 1 h in 5% CO_2 / 95% air at 37°C (normal conditions). To test whether indapamide protects against ischemia/reperfusion-induced cell death, cells were exposed to 10 – 50 μM of indapamide (Kyoto Pharmaceutical Industries, Kyoto) or vehicle (0.25% dimethyl Sulfoxide) during conditions of ischemia and reperfusion or normal conditions. Cell viability was measured by the WST-1 assay (Cell Counting Kit-8; Dojindo, Kumamoto).

Endothelial barrier function was evaluated by measuring permeability of cells to sodium fluorescein (Na-F) and Evan's blue-albumin (EBA) as previously described (9). MBEC4 cells were grown on the collagen-coated membrane (1.1 cm^2, 0.4-μm pore size) of a Transwells™ insert (42,000 cells/cm^2, Corning) and subsequently exposed to the above-mentioned ischemia conditions for 7 h. As a control, MBEC4 cells were incubated with normal Krebs-Ringer buffer for 7 h (normal conditions). Cells were exposed to 10 – 100 μM of indapamide or vehicle during ischemia conditions or normal conditions. To initiate transport experiments, the medium was removed and cells were washed with normal Krebs-Ringer buffer. Krebs-Ringer buffer containing 100 μg /mL of Na-F (MW 376; Sigma, St. Louis, MO, USA) and 670 μg/mL EBA bound to 0.1% BSA (MW = 67 kDa) were loaded on to the luminal side of the insert. Samples were removed from the abluminal chamber at 30, 60, 90, and 120 min and immediately replaced with Krebs-Ringer buffer. The concentrations of Na-F and EBA were determined with a CytoFluor Series 4000 fluorescence multiwell plate reader [Ex(λ) 485 ± 10 nm and Em(λ) 530 ± 12.5 nm; PerSeptive Biosystems, Framingham, MA, USA] and an Opsys MR microplate reader (630 nm; DYNEX Technologies, Chantilly, VA, USA), respectively. The permeability coefficient and clearance were calculated according to the method of Dehouck et al. (10), as previously described (9).

Data are expressed as the mean ± S.E.M. Statistical analysis was performed using one-way analyses of variance (ANOVA) followed by Tukey-Kramer's post-hoc test. The difference in means was considered to be significant when the P value was less than 0.05.

As shown in Fig. 1, the ischemia (10 h)/reperfusion

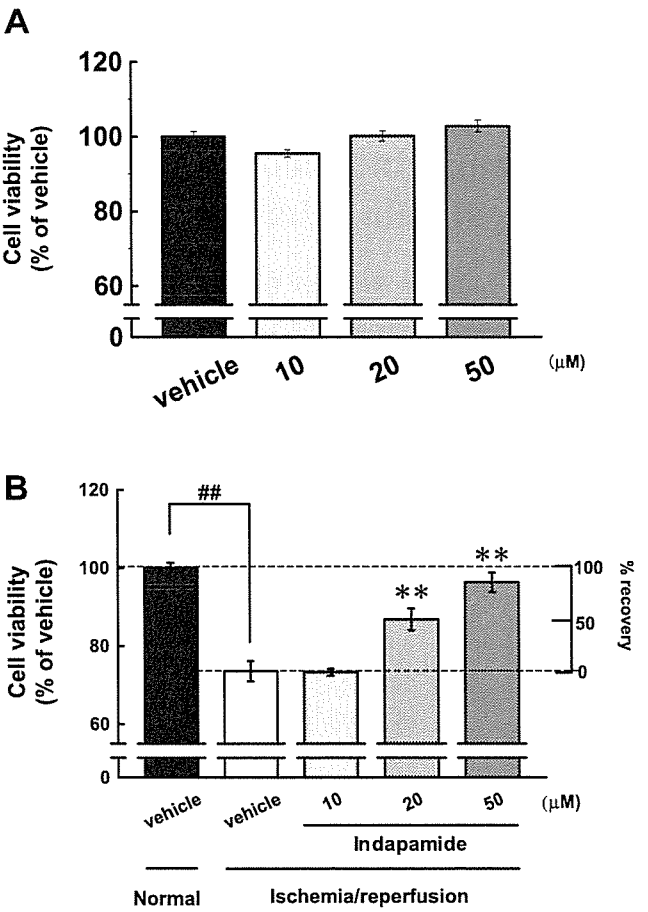

Fig. 1. Effects of indapamide on the viability of MBEC4 cells subjected to normal (A) or ischemia/reperfusion (B) conditions. Results are expressed as % of cell viability under normal conditions with vehicle treatment (% of vehicle). The % recovery is shown on the right-side of panel B and was calculated using the following equation: [% of vehicle under ischemia/reperfusion conditions with indapamide treatment – % of vehicle under ischemia/reperfusion conditions with vehicle treatment] / [% of vehicle under normal conditions with vehicle treatment – % of vehicle under ischemia /reperfusion conditions with vehicle treatment] × 100. Data are expressed as the mean ± S.E.M. (n = 8 – 20). $^{\#\#}P<0.01$, significant difference from vehicle under normal conditions. $^{**}P<0.01$, significant difference from the vehicle under ischemia/reperfusion conditions.

(1 h) conditions significantly decreased the viability of MBEC4 cells grown on collagen-coated wells, by 26.5% of cells subjected to normal conditions. The effect of indapamide on ischemia/reperfusion-induced damage in MBEC4 cells was concentration-dependent: 20 – 50 μM indapamide improved recovery by 50% – 86%. These concentrations have no effect on cell viability under normal conditions. As shown in Fig. 2, a 7-h period of ischemia resulted in increased permeability of MBEC4 cells grown on collagen-coated membranes to Na-F (paracellular transport marker) and EBA (transendothelial transport marker). Following ischemia, the perme-

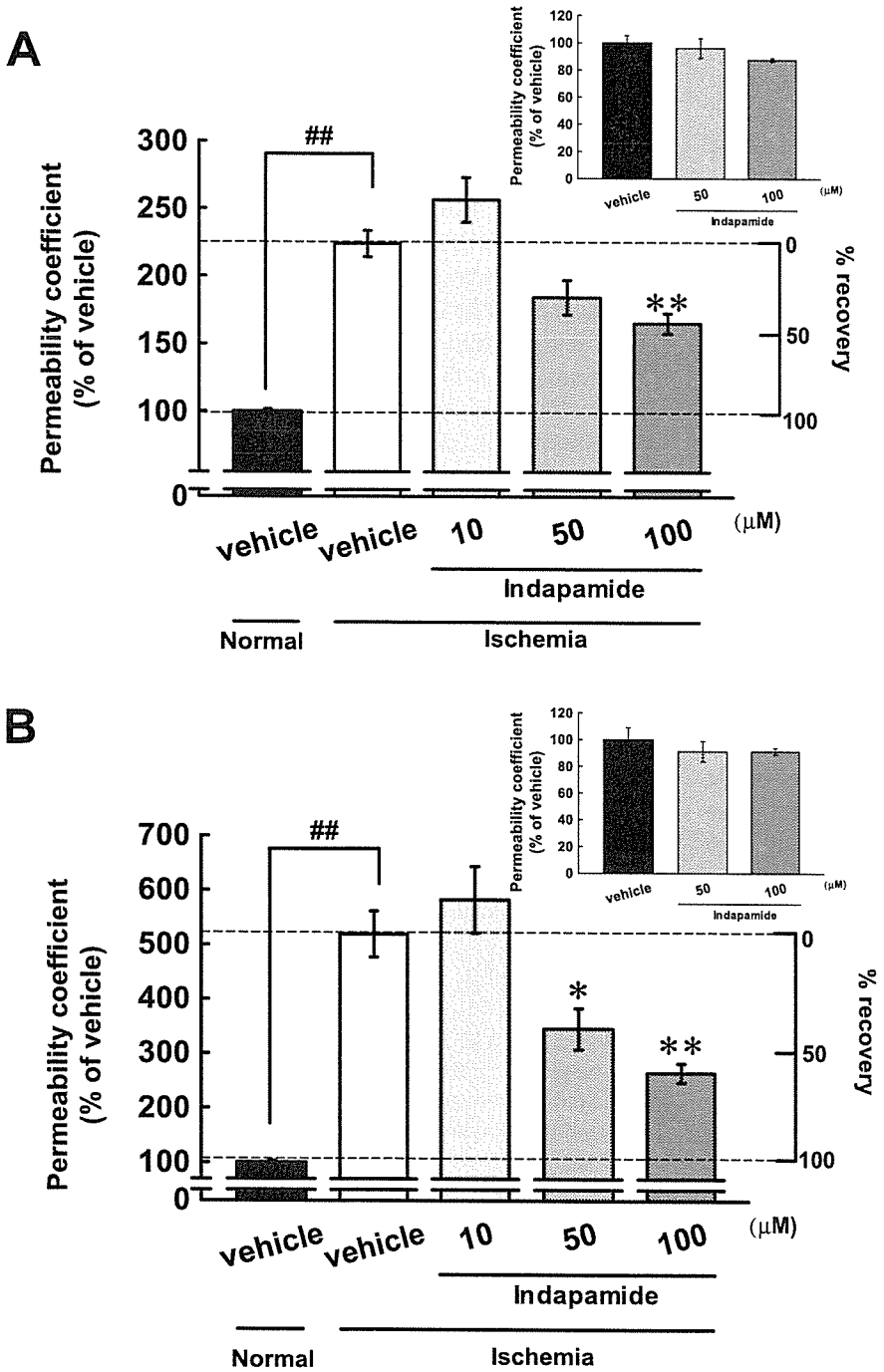

Fig. 2. Effects of indapamide on Na-F (A) and EBA (B) permeability in MBEC4 cell monolayer after a 7-h exposure to normal or ischemic conditions. The inset in each panel shows the effects of indapamide on permeability of the MBEC4 cell monolayer to Na-F and EBA after a 7-h exposure to normal conditions. The permeability coefficients of Na-F and EBA in the MBEC4 monolayer, after a 7-h exposure to normal conditions with vehicle treatment, were $3.109 \pm 0.012 \times 10^{-4}$ and $0.798 \pm 0.003 \times 10^{-4}$ cm/min, respectively. Results are expressed as % of vehicle under normal conditions with vehicle treatment. The % recovery is shown on the right-side of each panel and was calculated by the following equation: [% of vehicle under ischemia condition with indapamide treatment − % of vehicle under ischemia condition with vehicle treatment] / [% of vehicle under normal condition with vehicle treatment − % of vehicle under ischemic condition with vehicle treatment] × 100. Data are expressed as the mean ± S.E.M. (n = 8). $^{\#\#}P<0.01$, significant difference from vehicle under normal conditions. $^{*}P<0.05$, $^{**}P<0.01$, significant difference from vehicle under ischemic conditions.

ability coefficients of Na-F and EBA were significantly increased to 223.7 ± 9.8% and 518.9 ± 42.5% of the vehicle under normal conditions, respectively. The viability of MBEC4 cells grown on membranes after a 7-h exposure to ischemic conditions and following termination of the permeability test (2 h) were 72.8 ± 3.6% and 71.3 ± 1.0% of cells subjected to normal conditions, respectively; there was no difference in cell viability before and after the permeability test. Following a 7-h exposure to indapamide (50 − 100 μM) under ischemic conditions, hyperpermeability of MBEC4 cells to Na-F and EBA was concentration-dependently reduced by 31.9% − 47.4% and 41.4% − 60.6% of the vehicle, respectively. This effect was not accompanied by a change in cell viability (vehicle: 72.8 ± 3.6%, 100 mM: 75.9 ± 2.3%). Under normal conditions, these concentrations of indapamide had no effect on the permeability coefficients of Na-F and EBA in MBEC4 cells. These findings suggest that indapamide may efficiently inhibit ischemia-induced hyperpermeability rather than inhibit

ischemic cell death. MBEC4 cells that were grown on membranes exhibited a higher vulnerability to ischemic cytotoxicity and lower sensitivity to the cytoprotective action of indapamide than those grown on the smooth plastic surface of a well. Further experiments will be required to clarify these issues.

The in vitro ischemia/reperfusion conditions used in the present study significantly reduced the viability of MBEC4 cells, and this effect was ameliorated by indapamide. Ischemic conditions increased the permeability of MBEC4 cells to Na-F and EBA, but this hyperpermeability was reduced by indapamide.

Disruption of the BBB is a critical event during cerebral ischemia as it is followed by the passive diffusion of water, leading to vasogenic edema and secondary brain injury. Cerebral edema is a major and fatal complication of acute ischemic stroke. Free radical overproduction after brain ischemia and reperfusion contributes to brain edema and neuronal damage. Thus, an inhibition of free radical formation is likely to prevent the occurrence of brain edema and neuronal damage. Indapamide has an antioxidant effect and has the potential to scavenge oxygen free radicals and their derivatives. Boucher et al. reported that indapamide treatment has a protective effect on cardiac tissue during the early stages of postischemic reperfusion (6). The present findings suggest that indapamide may protect cerebral endothelial cells from ischemic damage due to antioxidation and/or free radical scavenging. This phenomenon may contribute, at least in part, to the mechanisms by which indapamide facilitated protection of perindopril against recurrent stroke in a recent clinical study (7).

Brain capillary endothelial cells form a metabolic and physical barrier that separates the periphery from the brain to maintain cerebral homeostasis. The lack of fenestrations and the presence of tight junctions differentiate brain microvessel endothelial cells from peripheral microvascular endothelium. While adherent junctions and other junctional proteins contribute to cell-to-cell contacts in the paracellular cleft, tight junctions are critical for restricting paracellular diffusion in the cerebral microvasculature. Increased cerebrovascular permeability is a principal factor in the development of cerebral edema after brain ischemia. Several studies have shown a relationship between cyclic AMP (cAMP) levels and permeability of endothelial cell monolayers. For example, elevation of intracellular cAMP concentrations induces high transendothelial electrical resistance and increases P-glycoprotein function in brain capillary endothelial cells (11, 12). In addition, when bovine aortic endothelial cells were cultured in hypoxic conditions, cellular cAMP levels decreased and this phenomenon was associated with an increase in cellular permeability (13). Furthermore, a decrease in cAMP levels was detectable after brain microvascular endothelial cells were exposed to hypoxic conditions for 3 h (14). Moreover, indapamide was shown to augment cAMP production induced by forskolin, an adenylyl cyclase activator, but did not alter basal cAMP levels in cardiomyocytes alone (15). Therefore, indapamide may protect cerebral microvascular endothelial cells from ischemic dysfunction by increasing intracellular cAMP levels. The effects of indapamide on the expression of tight junction-related proteins and on intracellular messengers are now being investigated in rat brain primary cultured endothelial cells.

We present here in vitro evidence to suggest a possible protective action of indapamide against ischemia/reperfusion-induced injury and dysfunction of the BBB.

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Two different clinical phenotypes of Creutzfeldt-Jakob disease with a M232R substitution

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Running title: Clinical features of CJD232

Key words: Creutzfeldt-Jakob disease, M232R, clinical phenotype, uncommon variant, diffusion-weighted MRI

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