

Nitric oxide mediates cyclosporine-induced impairment of the blood–brain barrier in cocultures of mouse brain endothelial cells and rat astrocytes

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Received 8 October 2004

Available online 30 October 2004

Abstract

The present study was designed to clarify the involvement of nitric oxide (NO) signaling in the adverse effect of cyclosporine on the blood–brain barrier. Cyclosporine increased the permeability of sodium-fluorescein and the cellular accumulation of rhodamine 123, a substrate of P-glycoprotein, in mouse brain endothelial (MBEC4) cells. This effect was markedly enhanced two- to threefold when MBEC4 cells were cocultured with rat astrocytes or C6 glioma cells. Direct and continuous electrochemical measurement of NO demonstrated that cyclosporine dose-dependently increased histamine- and phenylephrine-evoked NO production in MBEC4 cells and astrocytes, respectively. A NO synthase inhibitor (*N*^G-monomethyl-L-arginine) blocked slightly and markedly cyclosporine-induced impairment of the endothelial barrier in the monolayer and coculture system, respectively. These findings suggest that cyclosporine impairs the brain endothelial barrier function by accelerating NO production in the brain endothelial and astroglial cells. This event may be interpreted as triggering the occurrence of cyclosporine neurotoxicity.

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Keywords: Cyclosporine; Neurotoxicity; NO (nitric oxide); Blood–brain barrier; Permeability; P-glycoprotein

1. Introduction

Cyclosporine, a cyclic 11-amino acid peptide, is widely used as a potent immunosuppressant to prevent allograft rejection in solid organ transplantation and in fatal graft-vs.-host disease after bone marrow transplantation, and to treat various autoimmune diseases including rheumatoid arthritis (Kahan, 1989). Despite its high efficacy, cyclo-

sporine has adverse effects including renal dysfunction, cardiovascular disorders, gastrointestinal disorders and neurological complications. These events occur with a relatively high frequency (20–40%) in organ-transplanted patients (Gijtenbeek et al., 1999; Pirsch et al., 1997; U.S. Group, 1994).

The entry of cyclosporine into the brain is prevented by the tight junctions and P-glycoprotein, a multi-drug efflux pump, of the brain microvascular endothelial cells. But the adverse neurological effects of cyclosporine, including tremors, seizures and encephalopathy, strongly suggest the possibility of cyclosporine transport across the blood–brain

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barrier. We previously reported that cyclosporine produced convulsions by inhibiting γ -aminobutyric acid (GABA)-ergic neural activity and binding properties of the GABA_A receptor (Shuto et al., 1999). The inhibition of GABAergic neurotransmission by cyclosporine may lead to an activation of serotonergic neural activity and consequently produce tremors (Shuto et al., 1998). These findings in vivo are considered to be due to a direct action of cyclosporine transported across the blood–brain barrier rather than an indirect effect through the periphery. In fact, we demonstrated that cyclosporine at a high concentration decreased the function and expression of P-glycoprotein in brain capillary endothelial cells (Kochi et al., 1999; 2000). The blood–brain barrier is primarily formed by brain capillary endothelial cells, which are closely sealed by tight junctions (Partridge, 1999). P-glycoprotein is abundantly expressed in the brain endothelial cells and limits the accumulation of many hydrophobic molecules and toxic substances in the brain (Schinkel, 1999). Recently, we demonstrated that nitric oxide (NO) increased the permeability and inhibited the P-glycoprotein efflux pump of brain capillary endothelial cells, suggesting that NO impairs the dynamic regulation of the blood–brain barrier function (Yamauchi et al., in press). Astrocytes and pericytes are cellular components of the blood–brain barrier. Astrocytes surround the cerebral capillaries and regulate blood–brain barrier function through cell-to-cell contact and secretion of soluble factors (Terasaki et al., 2003).

The present study was designed to clarify the involvement of NO signaling in the adverse effect of cyclosporine on the blood–brain barrier. We first evaluated the effect of cyclosporine on the permeability and the P-glycoprotein function of mouse brain endothelial (MBEC4) cells alone and cocultured with rat astrocytes or C6 glioma cells. Second, the effect of cyclosporine on the stimulation-evoked NO production was examined in MBEC4 cells and rat astrocytes using direct electrochemical NO monitoring.

2. Materials and methods

2.1. Materials

Cyclosporine was kindly supplied by Novartis Pharma (Bazel, Switzerland). Sodium fluorescein (Na-F, MW 376), rhodamine 123, phenylephrine hydrochloride, histamine, L-arginine and *N*^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma (St. Louis, MO, USA). Culture medium and subculture reagents were obtained from Invitrogen (Carlsbad, CA, USA). All remaining reagents of analytical grade were purchased from Wako (Osaka, Japan).

2.2. Animals

Wistar rats aged 3 days old were used in this study. All the procedures involving experimental animals adhered to

the law (No. 105) and notification (No. 6) of the Japanese Government, and were approved by the Laboratory Animal Care and Use Committee of Fukuoka University.

2.3. Cell culture

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) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. C6 glioma cells (JCRB9096, Health Science Research Resources Bank, Osaka, Japan) were cultured in DMEM supplemented with 10% fetal calf serum, and 50 μ g/ml gentamicin. Newborn rat astrocytes were isolated according to the method of McCarthy and de Vellis (1980) and Sastradipura et al. (1998) with a slight modification. Briefly, the cerebral cortex from 3-day-old rats was minced and treated with papain (90 units/ml; Worthington, Lakewood, NJ) and DNase I (2000 units/ml; Sigma) at 37 °C for 15 min. The mechanically dissociated cells were seeded into plastic flasks in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. After 10–14 days in culture, floating cells and weakly attached cells on the mixed primary cultured cell layer were removed by vigorous shaking of the flask. Then, astrocytes on the bottom of the culture flask were trypsinized and seeded into new culture flasks. The primary cultured astrocytes were maintained in DMEM. They were grown in a humidified atmosphere of 5% CO₂/95% air at 37 °C.

The preparation of the in vitro blood–brain barrier models has been described previously (Dohgu et al., 2000). In brief, C6 cells or rat astrocytes (40,000 cells/cm²) were first cultured on the outside of the collagen-coated polycarbonate membrane (3.0 μ m pore size) of the Transwell™ insert (12-well type, Costar, MA, USA) directed upside down in the well. Two days later, MBEC4 cells (42,000 cells/cm²) were seeded on the inside of the insert placed in the well of the 12-well culture plate (Costar) (C6 coculture and rat astrocyte coculture). The monolayer system was also made with MBEC4 cells alone (MBEC4 monolayer).

2.4. Treatment with cyclosporine and nitric oxide (NO) synthase inhibitor

Cyclosporine was first dissolved in ethanol and diluted with serum-free culture medium (0.1% as the final ethanol concentration). MBEC4 cells were cultured for 3 days, and these inserts were washed three times with serum-free medium. Then cells were exposed to 1–5 μ M cyclosporine injected into the inside of the insert (luminal side) for 12 h. When the effect of NO synthase (NOS) inhibitor was examined, L-NMMA (1 mM) was loaded both inside and outside of the insert (luminal and abluminal side). In

parallel, cells were treated with serum-free medium containing the corresponding amount of ethanol as the vehicle.

2.5. Paracellular transport of Na-F

To initiate the transport experiments, the medium was removed and MBEC4 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 $\mu\text{g/ml}$ of Na-F 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 immediately replaced with fresh Krebs–Ringer buffer. Aliquots (5 μl) of the abluminal medium were mixed with 200 μl of Krebs–Ringer buffer and then the concentration of Na-F was determined using a fluorescence multiwell plate reader ($\text{Ex}(\lambda)$ 485 nm; $\text{Em}(\lambda)$ 530 nm) (CytoFluor Series 4000, PerSeptive Biosystems, Framingham, MA, USA). The permeability coefficient and clearance were calculated according to the method described by Dehouck et al. (1992). Clearance was expressed as microliters (μl) 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 vs. time, and the slope was estimated by linear regression analysis. The slope of clearance curves for the MBEC4 monolayer or coculture systems was denoted by PS_{app} , where PS is the permeability-surface area product (in $\mu\text{l/min}$). The slope of the clearance curve with a control membrane was denoted by $\text{PS}_{\text{membrane}}$. In the coculture system, the control membrane is the C6 cell- or rat astrocyte-layered membrane. The real PS value for the MBEC4 monolayer and the coculture system (PS_{trans}) was calculated from $1/\text{PS}_{\text{app}} = 1/\text{PS}_{\text{membrane}} + 1/\text{PS}_{\text{trans}}$. The PS_{trans} values were divided by the surface area of the Transwell inserts to generate the permeability coefficient (P_{trans} , in cm/min).

2.6. Functional activity of P-glycoprotein

The functional activity of P-glycoprotein was determined by measuring the cellular accumulation of rhodamine 123 (Sigma) according to the method of Fontaine et al. (1996). MBEC4 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). In both coculture systems, C6 cells and rat astrocytes on the outside of the membrane were removed

with a cell scraper. MBEC4 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 fluorescence multiwell plate reader ($\text{Ex}(\lambda)$ 485 nm; $\text{Em}(\lambda)$ 530 nm, CytoFluor Series 4000). The cellular protein was measured by the method of Bradford (1976).

2.7. Electrochemical monitoring of NO

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 or rat astrocytes in a 2.5 cm^2 dish (BD FALCON™, BD Biosciences, NJ, USA) were washed three times with Mg^{2+} -free 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 a NO monitoring system. The NO-biosensor (ASTEC, Fukuoka, Japan) was positioned about 10 μm above the cell surface. Ten minutes after treatment with L-arginine (1 mM), histamine or phenylephrine in a volume of 10 μl was added to the cells in 1 ml of Mg^{2+} -free Krebs–Ringer solution with a transient mixing step to give the final concentration indicated. The level of production of NO in MBEC4 cells or rat astrocytes was monitored for a 15-min period after the addition of histamine or phenylephrine. Cyclosporine was added 20 min before treatment with L-arginine.

2.8. Assessment of cell viability

The effect of cyclosporine on the viability of cells in the MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems was assessed using a WST-8 assay (Cell Counting Kit-8, DOJINDO, Kumamoto, Japan). A highly water-soluble formazan dye (WST-8), 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 using a microplate reader (Opsys MR, DYNEX technologies, Chantilly, VA, USA).

2.9. Measurement of NO Production using NO-specific dye

An accumulation of NO production during a 12 h period was assessed using a NO-specific fluorescent dye, 4,5-diaminofluorescein diacetate (DAF-2 DA, Sigma) (Nakatsubo et al., 1998). MBEC4 and C6 cells were seeded on wells of the 24-well culture plate. Cells were

incubated with DAF-2 DA at a final concentration of 10 μM for 1 h at 37 °C and then rinsed three times with serum-free medium. Cells containing DAF2 DA were exposed to cyclosporine (1–5 μM) for 12 h and they 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 fluorescence was measured using a fluorescence multiwell plate reader (Ex(λ) 485 nm; Em(λ) 530 nm, CytoFluor Series 4000). Then, cells were solubilized with 250 μl of 1 M NaOH. Aliquots of the cell solution were removed for protein assay according to the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) (Bradford, 1976). Data for each experiment were normalized to the cellular protein.

2.10. Statistical analysis

The values are expressed as the means \pm S.E.M. Statistical analysis was performed using Student's *t*-test. One way and two way analyses of variance (ANOVAs) followed by Tukey–Kramer's tests were applied to multiple comparisons. The differences between means were considered to be significant when *P* values were less than 0.05.

3. Results

3.1. Permeability and P-glycoprotein function of MBEC4 cells in MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems

After MBEC4 cells were cultured for 3 days, the basal permeability and P-glycoprotein efflux pump of MBEC4 cells were evaluated in three in vitro blood–brain barrier models (Fig. 1). The permeability coefficient of Na-F for MBEC4 cells was increased by 57.6% in the C6 coculture and reduced by 45.6% in the rat astrocyte coculture, when compared to that in the MBEC4 monolayer (Fig. 1A). The accumulation of rhodamine 123 in MBEC4 cells was significantly increased by 16.8% in the C6 coculture and significantly reduced by 17.9% in the rat astrocyte coculture relative to the MBEC4 monolayer (Fig. 1B).

3.2. Effect of cyclosporine on permeability and P-glycoprotein function of MBEC4 cells in MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems

As shown in Figs. 2 and 3, the exposure to cyclosporine (1–5 μM) for 12 h dose-dependently increased the permeability of Na-F and the cellular accumulation of rhodamine 123 in the MBEC4 monolayer and C6 coculture. The Na-F permeability and the rhodamine 123 accumulation of MBEC4 cells in the rat astrocyte coculture were increased to 127.7 \pm 7.2% and 126.7 \pm 3.0% of vehicle by 5 μM cyclosporine. These adverse effects of cyclosporine in the

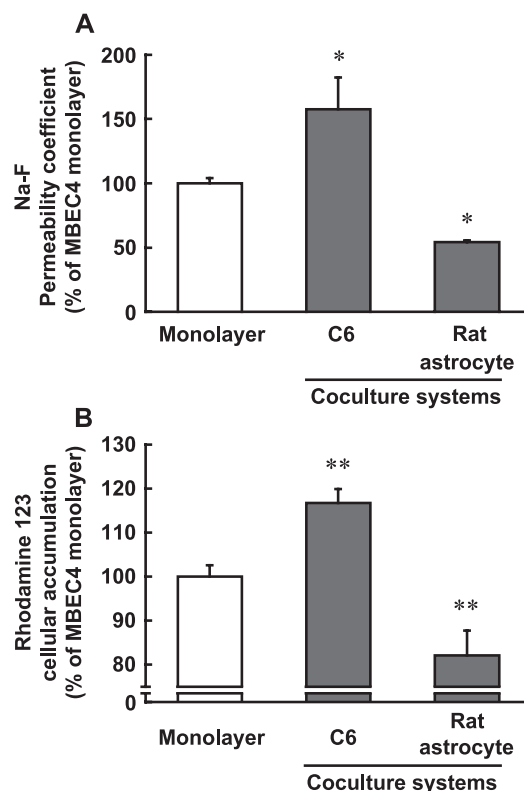


Fig. 1. Permeability and P-glycoprotein function of MBEC4 cells in MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems. (A) MBEC4 permeability coefficients of Na-F. Results are expressed as % of the MBEC4 monolayer ($1.88 \pm 0.10 \times 10^{-4}$ cm/min). Values are the means \pm S.E.M. ($n=3-7$). * $P<0.05$, significant difference from MBEC4 monolayer. (B) Rhodamine 123 accumulation in MBEC4 cells. Results are expressed as % of the MBEC4 monolayer (2.19 ± 0.16 nmol/mg protein). Values are the means \pm S.E.M. ($n=4-17$). ** $P<0.01$, significant difference from MBEC4 monolayer.

presence of C6 cells and astrocytes were two- to threefold more potent than the effects in the MBEC4 monolayer.

The WST-8 assay showed that cyclosporine at the highest concentration tested (5 μM) had no effect on cell viability in any of the three culture systems (MBEC4 monolayer: 98.2 ± 1.00 , C6 coculture: 101.2 ± 1.79 , rat astrocyte coculture: $103.9 \pm 7.5\%$ of the corresponding vehicle).

3.3. Effect of cyclosporine on stimulation-evoked NO production and spontaneous NO production in MBEC4 cells, rat astrocytes and C6 cells

Cyclosporine alone at concentrations less than 5 μM failed to stimulate NO production to a detectable level (Fig. 4). In the absence of cyclosporine, histamine (100 μM) and phenylephrine (1 μM) produced small amounts of NO in MBEC4 cells and rat astrocytes (9.27 ± 0.69 and 0.32 ± 0.036 μM), respectively (Fig. 4). Cyclosporine (1, 2 and 5 μM) dose-dependently increased histamine (100 μM)- and phenylephrine (1 μM)-evoked NO production in MBEC4 cells and astrocytes, respectively (Fig. 4). The increases induced by 5 μM cyclosporine reached

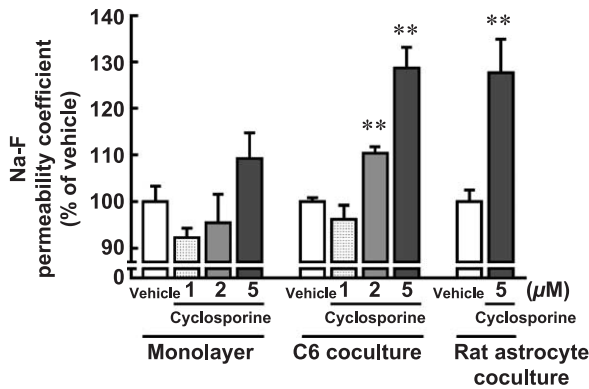


Fig. 2. Effect of cyclosporine on MBEC4 permeability of Na-F in MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems. Transport experiments were performed after 12 h of exposure to cyclosporine. Results are expressed as % of each corresponding vehicle (monolayer; $2.35 \pm 0.24 \times 10^{-4}$ cm/min, C6 coculture; $2.34 \pm 0.25 \times 10^{-4}$ cm/min, rat astrocyte coculture; $1.16 \pm 0.03 \times 10^{-4}$ cm/min). Values are the means \pm S.E.M. ($n=3-24$). ** $P<0.01$, significant difference from each corresponding vehicle.

174.1 \pm 7.7% and 198.2 \pm 15.0% of vehicle in MBEC4 cells and astrocytes, respectively.

When total amounts of NO production during a 12 h period was measured using a NO-specific fluorescent dye, an exposure of cyclosporine (5 μ M) for 12 h significantly increased spontaneous (basal) NO production by 70 and 190% in MBEC4 and C6 cells, respectively (Fig. 5).

3.4. Effect of NO synthase inhibitor on cyclosporine-increased permeability of Na-F and accumulation of rhodamine 123 in MBEC4 monolayer and rat astrocyte coculture systems

L-NMMA (1 mM) (a NOS inhibitor) alone had no effect on the permeability of Na-F and the accumulation of rhodamine 123 in the MBEC4 monolayer and C6 coculture.

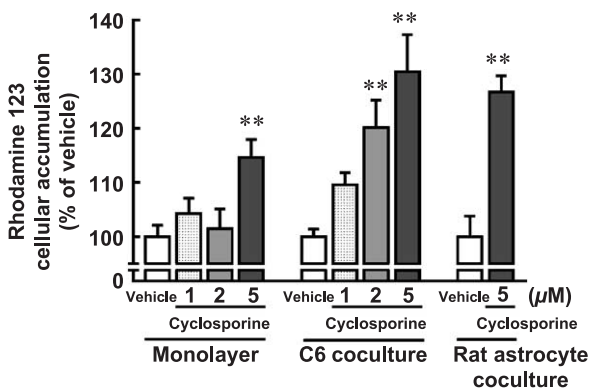


Fig. 3. Effect of cyclosporine on rhodamine 123 accumulation in MBEC4 cells of the MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems. P-glycoprotein function was evaluated after 12 h of exposure to cyclosporine. Results are expressed as % of each corresponding vehicle (monolayer; 3.50 ± 0.56 nmol/mg protein, C6 coculture; 3.79 ± 0.47 nmol/mg protein, rat astrocyte coculture; 0.85 ± 0.04 nmol/mg protein). Values are the means \pm S.E.M. ($n=4-20$). ** $P<0.01$, significant difference from each corresponding vehicle.

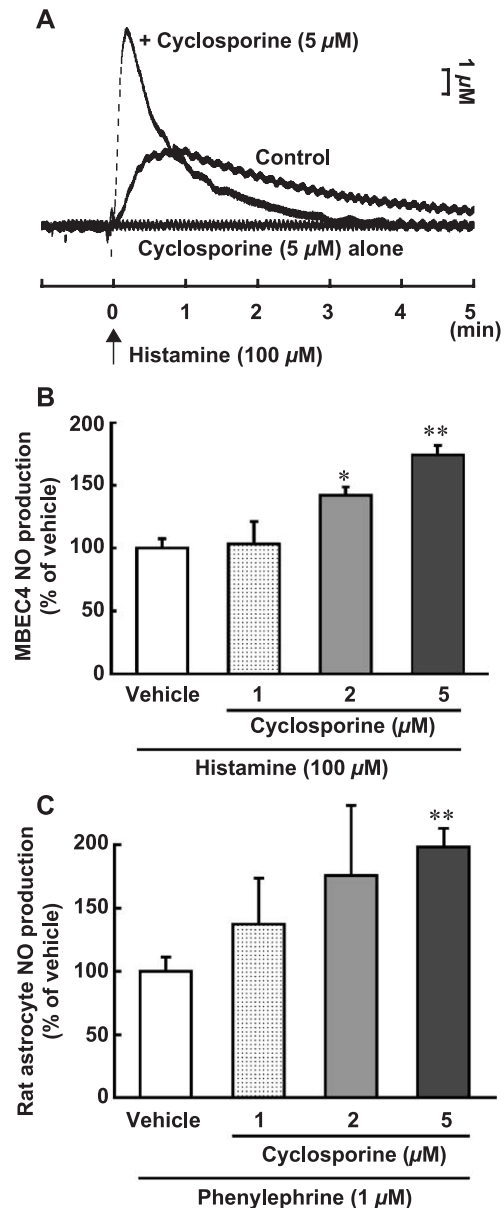


Fig. 4. Effect of cyclosporine on stimulations-evoked NO production. (A) Representative differential pulse amperogram obtained using NO biosensor shows NO production evoked by histamine (100 μ M) in the absence (Control, middle trace) and presence of cyclosporine (5 μ M) (+cyclosporine, top trace) in MBEC4 cells. The effect of cyclosporine (5 μ M) alone on NO production was indicated in the bottom trace. (B) Concentration-dependent facilitatory effect of cyclosporine on histamine-evoked NO production in MBEC4 monolayer. Results are expressed as % of histamine (100 μ M)-evoked NO production (vehicle; 9.27 ± 0.69 μ M). Values are the means \pm S.E.M. ($n=3$). * $P<0.05$ and ** $P<0.01$, significant differences from vehicle. (C) Concentration-dependent facilitatory effect of cyclosporine on phenylephrine-evoked NO production in rat astrocyte coculture system. Results are expressed as % of phenylephrine (1 μ M)-evoked NO production (vehicle; 0.32 ± 0.036 μ M). Values are the means \pm S.E.M. ($n=4-7$). * $P<0.05$ and ** $P<0.01$, significant differences from vehicle.

Cyclosporine (5 μ M)-increased permeability of Na-F and accumulation of rhodamine 123 in MBEC4 cells were significantly decreased to 114.3 \pm 5.9% and 112.6 \pm 3.7% of

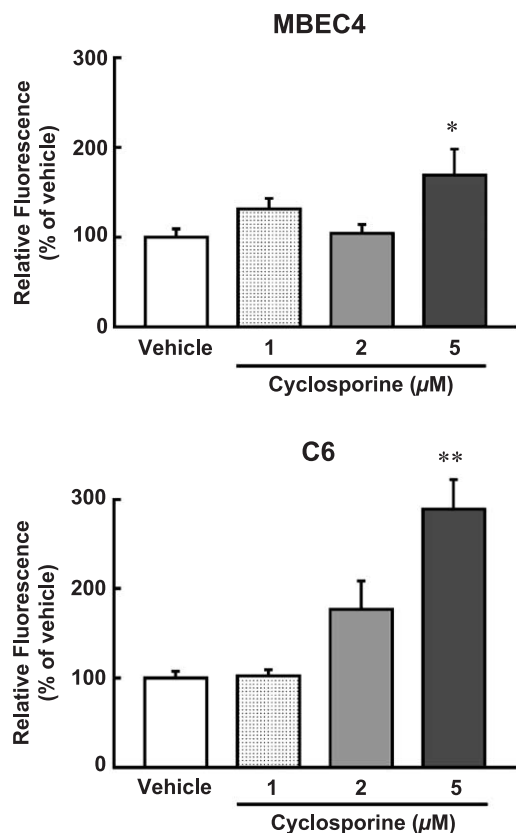


Fig. 5. Effect of cyclosporine on spontaneous NO production in MBEC4 and C6 cells. Cells were loaded with a NO-specific fluorescent dye (DAF-2 DA) and then treated with 1–5 μM cyclosporine for 12 h. The fluorescence intensity of DAF-2 DA was increased by NO produced by cells and this was normalized to the cellular protein. Relative fluorescence is expressed as the ratio (%) to the control value obtained in the absence of cyclosporine (vehicle). Values are the means±S.E.M. ($n=8$). * $P<0.05$, significant differences from vehicle.

vehicle, respectively, by 1 mM L-NMMA in the C6 coculture (Fig. 6). In contrast, L-NMMA (1 mM) produced a slight inhibition of the permeability of Na-F and the accumulation of rhodamine 123 induced by Cyclosporine (5 μM) in the MBEC4 monolayer (Fig. 6).

4. Discussion

We made three types of the in vitro blood–brain barrier models; MBEC4 monolayer, C6 coculture, and rat astrocyte coculture systems. MBEC4 cells alone show the highly specialized characteristics of brain microvascular endothelial cells including the integration of tight junctions and expression of P-glycoprotein (Tatsuta et al., 1992, 1994). Astrocytes, a cellular component of the blood–brain barrier, induce and maintain the functioning of the blood–brain barrier through cell-to-cell contact and the secretion of soluble factors (Rubin and Staddon, 1999). The barrier function in these models was evaluated based on the permeability of Na-F and the P-glycoprotein efflux pump

of MBEC4 cells. Na-F was used as a marker of permeability through the paracellular route. The permeability and the accumulation of rhodamine 123 in MBEC4 cells were markedly decreased in the presence of rat astrocytes (Fig. 1). These findings suggest that astrocytes participate in tightening the intercellular junctions and facilitating P-glycoprotein function of brain endothelial cells. A positive role for astrocytes in the expression and maintenance of endothelial tight junctions has been documented (Dehouck et al., 1992; Gaillard et al., 2001; Isobe et al., 1996; Rauh et al., 1992; Hayashi et al., 1997), although there are few reports concerning astrocyte-enhanced P-glycoprotein function. The C6 cell line, which originated from rat glioma cells, is commonly used as an experimental model of astrocytes due to convenient handling (Zhang et al., 2004). The barrier function of MBEC4 cells was speculated to be

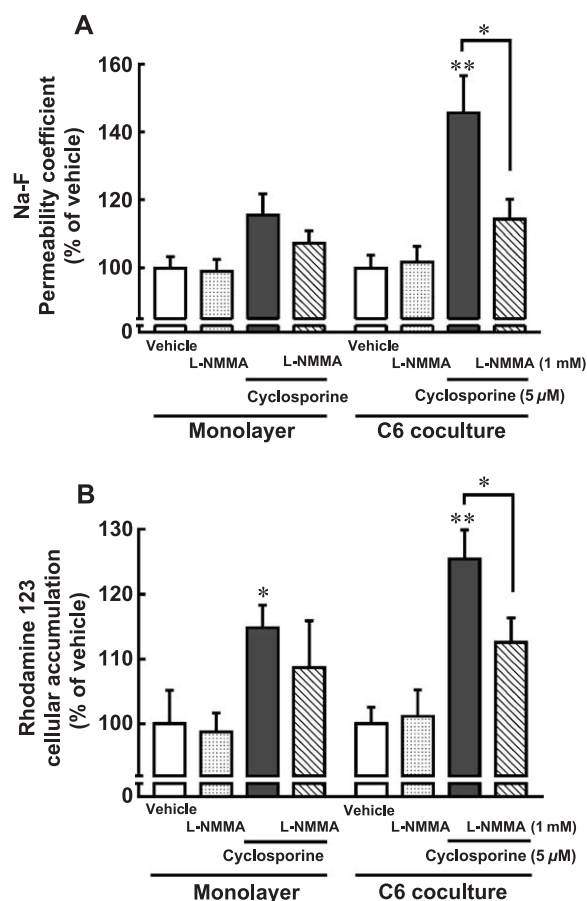


Fig. 6. Effect of NO synthase inhibitor (L-NMMA) on cyclosporine-increased MBEC4 permeability of Na-F (A), and rhodamine 123 accumulation in MBEC4 cells (B) in MBEC4 monolayer and C6 coculture system. (A) Results are expressed as % of each corresponding vehicle (monolayer; $1.73 \pm 0.10 \times 10^{-4}$ cm/min, C6 coculture; $2.95 \pm 0.55 \times 10^{-4}$ cm/min). Values are the means±S.E.M. ($n=7-11$). * $P<0.05$, ** $P<0.01$, significant difference from each corresponding vehicle. (B) Results are expressed as % of each corresponding vehicle (monolayer; 14.2 ± 3.94 nmol/mg protein, C6 coculture; 9.05 ± 2.00 nmol/mg protein). Values are the means±S.E.M. ($n=7-12$). * $P<0.05$, ** $P<0.01$, significant differences from each corresponding vehicle.

enhanced by the C6 cells similar the rat astrocyte coculture. However, the present findings indicated hyperpermeability of Na-F and reduced P-glycoprotein function in the C6 coculture. These results may be due to neoplastic changes in the glial characteristics. The precise mechanism by which C6 cells affect the barrier function of MBEC4 cells is now under investigation. There were no apparent differences in the endothelial cell response to cyclosporine between the C6 coculture and rat astrocyte coculture (Figs. 2 and 3). This confirms that the C6 coculture model is suitable for pharmacological research.

Cyclosporine-induced neurotoxicity including tremors, convulsions and encephalopathy occurred frequently in patients with high blood concentrations of cyclosporine, although these concentrations were within the therapeutic range (Gijtenbeek et al., 1999). The maximal blood concentrations of cyclosporine in patients with renal or liver transplantation are known to be in the range varying from 1 to 1.5 μM (Kahan et al., 1995; Keown and Niese, 1998; Grant et al., 1999). Therefore, the concentrations (1–5 μM) of cyclosporine used in the present study are less than three- to fivefold level of the maximal concentrations in patients. Cyclosporine (5 μM) significantly increased the permeability of Na-F and the accumulation of rhodamine 123 in the MBEC4 monolayer. These effects were potentiated markedly in the coculture with C6 cells or rat astrocytes (Figs. 2 and 3). Then, we substituted C6 cells for the primary culture of rat astrocytes to save the experimental animals, costs and labor in the subsequent pharmacological study. The presence of C6 cells increased by two- to threefold the facilitatory and inhibitory effects of cyclosporine (1–5 μM) on the paracellular permeability and P-glycoprotein activity of the MBEC4 monolayer, respectively. These effects were not due to the direct cytotoxicity of cyclosporine. These findings indicated that cyclosporine reduces the barrier function of brain endothelial cells to penetrate into the brain, leading to further aggravation of the blood–brain barrier function by interacting with astrocytes.

A 12-h exposure of cyclosporine (5 μM) in the same schedule as the blood–brain barrier function test significantly increased the accumulation of spontaneous NO production during a 12-h period, when measured using a NO-specific fluorescent dye (Fig. 5). A direct electrochemical NO monitoring failed to detect cyclosporine-increased basal NO production (Fig. 4). This monitoring method is capable of detecting a rapid NO production in response to the stimulation as a sharp current–time curve, even if amounts of NO production are small. However, NO biosensor is relatively difficult to detect a slow NO production with a low peak and a long-lasting plateau phase, even if total amounts during a long period is large. Cyclosporine dose-dependently enhanced histamine- and phenylephrine-evoked NO production in MBEC4 cells and rat astrocytes, respectively (Fig. 4). Cyclosporine exerts pharmacological effects by binding to cyclophilin (peptidyl-propyl isomerase), a highly basic and abundant cytosolic

protein (Marks, 1996). This cyclosporine/cyclophilin complex inhibits calcineurin, a serine–threonine phosphatase 2B, thereby blocking its phosphatase activity (Marks, 1996; Yakel, 1997). Calcineurin regulates the activity of ion channels and neurotransmitter release. Calcineurin anchored to the inositol 1,4,5-triphosphate (IP_3) receptor via FKBP12, a FK506-binding protein, regulates the phosphorylation status of the receptor, resulting in a dynamic Ca^{2+} -sensitive regulation of IP_3 -mediated Ca^{2+} flux (Cameron et al., 1997). In contrast with FKBP12, cyclophilin does not bind to the IP_3 receptor (Cameron et al., 1995). However, when rat cerebellar microsomes were treated with cyclosporine and cyclophilin, protein kinase C-induced IP_3 receptor phosphorylation and IP_3 -stimulated Ca^{2+} flux were markedly increased (Cameron et al., 1995). This suggests that cyclosporine inhibits the dephosphorylation of the IP_3 receptor to maintain a leaky IP_3 receptor channel that was phosphorylated by serine–threonine protein kinases such as protein kinase C. Although the IP_3 receptor becomes leaky, cyclosporine alone appears incapable of elevating $[\text{Ca}^{2+}]_{\text{IN}}$ over the threshold for activation of the constitutive NO synthase. This may explain the present findings that cyclosporine alone failed to raise NO production above the detection limit of the NO biosensor, while the histamine and phenylephrine-evoked NO production in MBEC4 and C6 cells, respectively, was markedly facilitated by cyclosporine. Histamine and phenylephrine activate phospholipase C through the H_1 receptor and α_1 -adrenoceptor, respectively, to generate IP_3 (Lum and Malik, 1994; Daum et al., 1983) and stimulate the leaky Ca^{2+} channel (phosphorylation status of IP_3 receptor) maintained by cyclosporine. These events probably lead to the markedly higher level of $[\text{Ca}^{2+}]_{\text{IN}}$ than that induced by histamine and phenylephrine in MBEC4 cells and rat astrocytes, respectively. We previously reported that cyclosporine also enhanced α_1 -adrenoceptor-mediated NO production in C6 cells (Ikese et al., 2000). In the brain, various biological substances including noradrenaline, glutamate, histamine and endothelin stimulate G protein-coupled receptors that have a common intracellular signaling pathway (IP_3 /diacylglycerol) in astrocytes (Verkhatsky and Kettenmann, 1996). This endogenous stimulator-evoked NO production is highly likely to be augmented by cyclosporine via a mechanism similar to that proposed here. In fact, our *in vivo* microdialysis experiment showed that an intraperitoneal injection of cyclosporine significantly increased NO production in the rat dorsal hippocampus (Fujisaki et al., 2002).

The present study demonstrated that cyclosporine impaired the barrier function of brain endothelial cells and this effect was remarkably potentiated by co-culturing MBEC4 cells with C6 cells or rat astrocytes. A NO synthase inhibitor, L-NMMA at a concentration of 1 mM showed no effect on the Na-F permeability and the rhodamine 123 accumulation of MBEC4 cells in both culture systems (Fig. 6), suggesting that L-NMMA (1 mM)

has not nonspecific effect on the basal blood–brain barrier functions of MBEC4 cells. L-NMMA (1 mM) significantly blocked the cyclosporine-induced increase in the permeability of Na-F and accumulation of rhodamine 123 in the C6 coculture. This protective effect was moderate in the MBEC4 monolayer (Fig. 6). These findings suggest that cyclosporine-enhanced NO production in astrocytes largely contributes to an impairment of the blood–brain barrier. This notion is supported by our previous findings that NO lowered the function of tight junctions and P-glycoprotein at the blood–brain barrier (Yamauchi et al., in press). The mechanisms by which NO donors increased vascular endothelial permeability involved an increase in the level of cGMP (Gimeno et al., 1998) or the formation of peroxynitrite (Menconi et al., 1998). These substances conceivably influence intrinsic tight junction proteins and the associated actin cytoskeleton through a direct or second signaling pathway (Burgstahler and Nathanson, 1995; Liu and Sundqvist, 1997). It is, therefore, likely that cyclosporine passes through the slightly impaired barrier of brain endothelial cells and then acts on astrocytes to enhance NO production, leading to further aggravation of the blood–brain barrier impairment.

In conclusion, cyclosporine accelerated stimulation-evoked NO production in brain endothelial and astroglial cells. This enhanced production of NO that interacts with each cellular component of the blood–brain barrier is involved in the sequential process of blood–brain barrier functional impairment induced by cyclosporine.

Acknowledgements

This work was supported, in part, by Grants-in-Aid for Scientific Research ((B)(2) 14370789) and ((C)(2) 15590475) from JSPS, Japan, by a Grant-in-Aid for Exploratory Research (16659138) from MEXT, Japan and by funds (No.: 031001) from the Central Research Institute of Fukuoka University.

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