



Stimulation of tetrahydrobiopterin synthesis by cyclosporin A in mouse brain microvascular endothelial cells

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Abstract

We examined the effect of the immunosuppressant, cyclosporin A (CsA) on the synthesis of tetrahydrobiopterin (BH₄), a cofactor for nitric oxide (NO) synthase and a scavenger of reactive oxygen species (ROS), in mouse brain microvascular endothelial cells. Treatment with CsA increased the BH₄ content and the expression of mRNA level of GTP cyclohydrolase I, the rate-limiting enzyme of BH₄ synthesis. 2,4-Diamino-6-hydroxypyrimidine, an inhibitor of GTP cyclohydrolase I, strongly reduced the CsA-induced increase in BH₄ content. Cycloheximide (CHX), a protein synthesis inhibitor, also reduced CsA-induced BH₄ synthesis. These findings suggest that CsA stimulates BH₄ synthesis via a de novo pathway with the induction of GTP cyclohydrolase I. Moreover, CsA-induced the mRNA level of the inducible type of NO synthase, and stimulated the L-citrulline formation from L-arginine, which is a marker for NO synthesis. The CsA-stimulated L-citrulline formation was attenuated by the co-treatment with GTP cyclohydrolase I inhibitor. The expression of the endothelial type of NO synthase was low under basal condition, and was not affected by the treatment with CsA. These findings suggest that increase in BH₄ content induced by CsA is coupled with NO production by inducible type of NO synthase. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Cyclosporin A; Tetrahydrobiopterin; GTP cyclohydrolase I; Endothelial cells; Nitric oxide synthase

1. Introduction

Immunosuppressors including cyclosporin A (CsA) are widely used in organ transplantation to prevent graft rejection. However, it is known that CsA induces serious side effects such as encephalopathy, nephro-

toxicity and hypertension [1–4]. Several investigators reported that increased CsA levels in blood caused vascular endothelial cell injury through the production of reactive oxygen species (ROS) [5–7]. Brain microvascular endothelial cells may be a primary target of CsA toxicity in encephalopathy.

CsA has been shown to reduce the nitric oxide (NO) production in blood vessel [3,4]. On the other hand, some recent studies showed that CsA induces the expression of the endothelial isoform of NO synthase

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(eNOS) in bovine aortic endothelial cells (AECs) [8,9]. It has been reported that eNOS produces ROS, when tetrahydrobiopterin (BH4), one of the cofactors of NOS, is decreased [10,11]. Moreover, BH4 has a protective effect against vascular endothelial cell injury induced by ROS [11–13], which may be implicated in CsA-induced cytotoxicity. If CsA decreases the BH4 level in endothelial cells, it may be one of the mechanisms for endothelial cell injury and hypertension induced by CsA. Inversely, if CsA increases the BH4 level, the increased BH4 may have a protective role against CsA toxicity. However, the effect of CsA on BH4 synthesis has not been elucidated.

In the present study, we examined the effect of CsA on BH4 synthesis in mouse brain microvascular endothelial cells.

2. Materials and methods

2.1. Cell culture

Mouse brain microvascular endothelial cells (second passage) were purchased from Toyobo Co. (Tokyo, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were finally grown in 100-mm culture dishes to extract total RNA or in 6-well plates to measure biopterin content and L-arginine metabolism, and used throughout the experiments at 5–12 passages after they were purchased.

2.2. Measurement of biopterin level

Biopterin (BH4 and more oxidized species) was measured by reversed-phase high performance liquid chromatography (HPLC) with fluorometric detection as biopterin [11]. Confluent endothelial cells in 6-well plates were washed three times with physiological saline solution (PSS, pH 7.4) containing 118.5 mM NaCl, 4.74 mM KCl, 2.5 mM CaCl₂, 1.18 mM MgSO₄, 1.18 mM KH₂PO₄, 2.5 mM NaHCO₃, 11 mM glucose and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and treated with CsA at 37 °C in 2 ml of PSS. The cells were then treated with 10% trichloroacetic acid on ice for 30 min. The obtained

supernatant was oxidized with MnO₂, and injected into HPLC.

2.3. Measurement of mRNA levels of eNOS by reverse transcriptase RT-PCR

Confluent endothelial cells in 100-mm culture dishes were treated with CsA in 10 ml of PSS, and then total RNA was extracted by a modified guanidinium isothiocyanate method with ISOGENTM Reagent (Nippon Gene Co. Ltd., Tokyo, Japan). Reverse transcription and PCR amplification from 0.5 µg of total RNA were performed using *rTth* DNA polymerase (RT-PCR high Plus, Toyobo Co., Tokyo, Japan). Pair of primers used for amplification of eNOS was 5'-CTGGACACCAGGACAACC-3' and 5'-GCTGCTGTGCGTAGCTCT-3'. The thermocycler was programmed to give an initial cycle consisting of 60 °C reverse transcription for 30 min and 94 °C denaturation for 2 min, followed by 30 cycles of 94 °C denaturation for 1 min and annealing per extension at 54 °C for 1.5 min. To control for the amounts of total RNA, parallel RT-PCR of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was performed as a reference, using pair of primers of 5'-TCCACCACCCTGTTGCTGTA-3' and 5'-ACCA-CAGTCCATGCCATCAC-3'. PCR products were electrophoresed on a 3% NuSieve 3:1 agarose (FMC Co., ME, USA) gel containing ethidium bromide and visualized by UV-induced fluorescence [14].

2.4. Measurement of mRNA levels of GTPCH and iNOS by Northern blot hybridization

Confluent endothelial cells in 100-mm dishes were washed twice with PSS. The washed cells were then treated with CsA at 37 °C in 10 ml of PSS. Total RNA was extracted from the cells by a modified guanidinium isothiocyanate method with ISOGENTM Reagent. An amount of 20 µg of total RNA from each sample were treated with the solution containing 2.2 M formaldehyde, 50% formamide, 5 mM sodium acetate, 1 mM EDTA and 20 mM 3-morpholinopropanesulfonic acid (MOPS, pH 7.0) for 15 min at 65 °C, and the RNA was separated by electrophoresis through a 1% agarose gel containing 2.2 M formaldehyde in MOPS buffer containing 5 mM sodium acetate, 1 mM EDTA and 20 mM MOPS (pH 7.0). The RNA samples were

transferred to Hybond-*N* nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), and hybridized to the indicated random prime-labeled cDNA probe (Takara, Otsu, Japan). The cDNA probes of GTP cyclohydrolase I (GTPCH) and inducible NOS (iNOS) were prepared by RT-PCR using pairs of primers of 5'-GGATACCAGGAGACCATCTCA-3' and 5'-TAGCATGGTGCTAGTGACAGT-3', and 5'-CGCTACACTTCCAACGCAAC-3' and 5'-AGG-AAGTAGGTGAGGGCTTG-3', respectively, as previously described [15]. Hybridization reactions were carried out for 1 h at 68 °C in ExpressHyb™ hybridization solution (CLONTECH Lab. Inc., CA, USA). The membrane was washed in 20 mM Na₂HPO₄ (pH 7.2), 1 mM EDTA (pH 8) and 0.1% SDS. The washed membrane was exposed to Kodak Biomax™ film at -80 °C for 24–48 h. The membrane was re-hybridized by *GAPDH* cDNA, which is a constitutive gene. The cDNA probe of *GAPDH* was prepared by RT-PCR as previously described [15].

2.5. L-Arginine metabolism

L-Arginine metabolism was measured by using an adaptation of the protocol described previously by McDuffie et al. [16]. Confluent endothelial cells on 6-well plates were washed twice with PSS and subsequently treated with various concentrations of CsA in 2 ml of PSS for 24 h at 37 °C. The cells were then incubated with L-[³H]-arginine (74 kBq) for 30 min at 37 °C. The reaction buffer was rapidly aspirated and 1 ml of stop buffer containing 50 mM HEPES (pH 5.5), 5 mM EDTA and Dowex 50WX-8 resin (Na⁺ form) was added to the cells. The cells were scrapped with a cell scraper and then pipetted into 1.5 ml of centrifuge tube. The samples were transferred to centrifuge filter unit and centrifuged at 10,000 g for 10 min. Elutes were transferred to scintillation vials and added scintillation cocktail (Wako Pure Chemicals Industries Ltd., Osaka, Japan). The radioactivity was measured in a liquid scintillation counter.

2.6. Chemicals

CsA was obtained from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). L-N⁶-(1-iminoethyl)-lysine dihydrochloride (L-NIL) was obtained from Funakoshi Co. (Tokyo, Japan). Lipopolysaccharide

(LPS, *E. coli* type, Serotype 055: B5) was obtained from Sigma. DAHP was obtained from Aldrich. L-Arginine [2,3,4-³H] monohydrochloride (1.5 TBq/mmol) was purchased from NEN Life Science Products Inc. (Boston, MA, USA). All other reagents were of the highest grade commercially available.

2.7. Statistical analysis

Values are presented as mean ± S.E.M. of *n* observations. The statistical significance of observed differences was determined by analysis of variance followed by Bonferroni's method. Differences between means were considered significant when *P* < 0.05.

3. Results

We examined whether CsA affected the intracellular biopterin level in endothelial cells. As shown in Fig. 1A, the intracellular biopterin level was increased from 4 h after the addition of 10 μM CsA (Fig. 1A). Moreover, the increase in the biopterin level by the treatment with CsA (0.1–10 μM) was observed in a concentration-dependent manner (Fig. 1B). Thus, CsA increases the BH₄ level in brain microvascular endothelial cells. GTPCH is a key enzyme in the regulation of BH₄ synthesis. Changes in GTPCH mRNA levels by the treatment with CsA were measured by Northern blot analysis. The GTPCH mRNA level was increased from 3 h after the addition of CsA (10 μM) in an incubation period-dependent manner (Fig. 2A). The induction of GTPCH mRNA expression at 9 h after the treatment with CsA was observed at 10 and 30 μM, but not below 1 μM (Fig. 2B). Moreover, the CsA-induced increase in biopterin content was strongly reduced by the treatment with DAHP, a selective inhibitor of GTPCH activity (Fig. 3). CHX, a protein synthesis inhibitor, also suppressed the increase in the BH₄ content induced by CsA in endothelial cells (Fig. 4).

To determine whether CsA-induced BH₄ synthesis was accompanied by the induction of eNOS and/or iNOS expression, the effect of CsA on the expression of eNOS and iNOS mRNAs was examined. In mouse brain microvascular endothelial cells, the expression of eNOS mRNA was not detected by

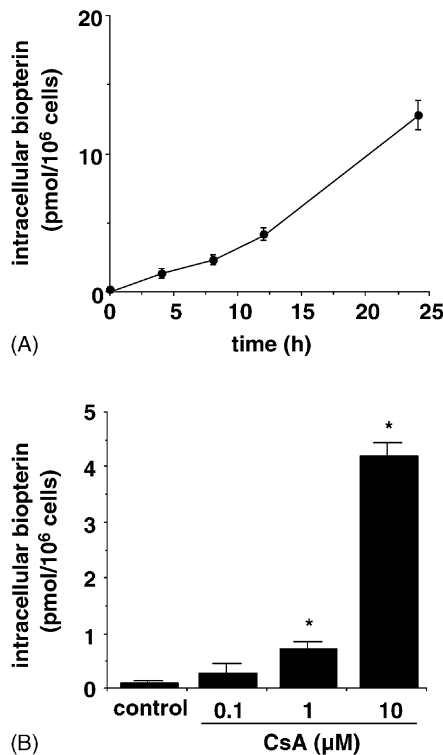


Fig. 1. The increase in biopterin content by CsA treatment. (A) Cells were incubated with CsA (10 μM) for various periods. At the indicated times after addition of the CsA, biopterin contents were determined. (B) Cells were incubated with various concentrations of CsA (0.1–10 μM) for 12 h. Values are the mean ± S.E.M. of four experiments. ‘*’ Significantly different from control group ($P < 0.05$).

Northern blot analysis, since its expression was very low. Therefore, PT-PCR method was employed for the measurement of eNOS mRNA level. The expression of eNOS was very low compared with mouse aortic endothelial cells (AECs), and was not significantly induced by the treatment with 10 μM CsA (Fig. 5A). However, CsA stimulated the expression of iNOS mRNA (Fig. 5B). The induction of iNOS mRNA by CsA (10 μM) was lower than that by 10 μg/ml LPS (Fig. 5B). Interestingly, in contrast to the expression of iNOS mRNA, CsA strongly stimulated BH4 synthesis compared with LPS-induction (Fig. 6).

We next examined the effect of CsA on L-citrulline formation from L-arginine, which is a marker for NOS activity. CsA increased L-citrulline formation

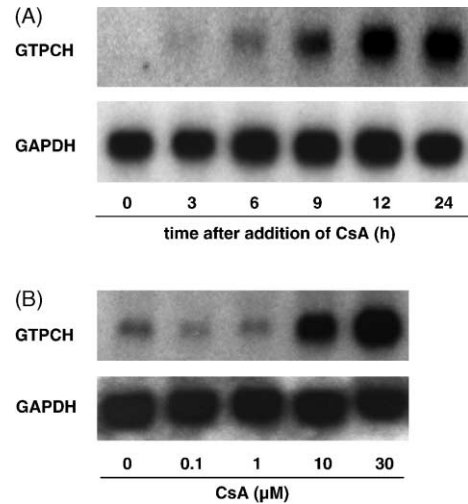


Fig. 2. The induction of the expression of GTPCH mRNA by CsA treatment. (A) Cells were incubated with CsA (10 μM) for various periods, and then total RNA was extracted from the cells. (B) Cells were incubated with CsA (0.1–30 μM) for 9 h, and then total RNA was extracted from the cells. After electrophoresis of 20 μg of total RNA per sample and transfer to a nylon membrane, the blot was sequentially hybridized with ³²P-labelled GTPCH cDNA and GAPDH cDNA.

in a concentration-dependent manner (Fig. 7A). The CsA-induced increase in L-citrulline formation was blocked by the treatment with NOS inhibitor L-NIL (Fig. 7B) or DAHP (Fig. 7C).

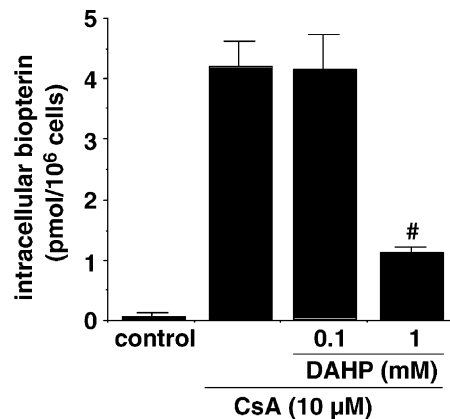


Fig. 3. Effect of GTPCH inhibitor on the CsA-induced increase in biopterin content. Cells were incubated with CsA (10 μM) in the presence or absence of DAHP (0.1 and 1 mM) for 12 h. Values are the mean ± S.E.M. of four experiments. ‘#’ Significantly different from the CsA alone group ($P < 0.05$).

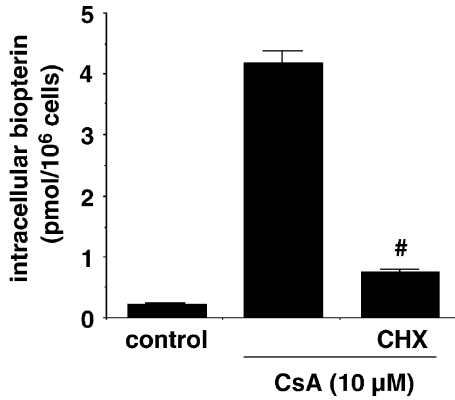


Fig. 4. Effect of CHX on the CsA-induced increase in biopterin content. Cells were incubated with CsA (10 μM) in the presence or absence of CHX (10 μM) for 12 h. Values are the mean ± S.E.M. of four experiments. '#' Significantly different from the CsA alone group ($P < 0.05$).

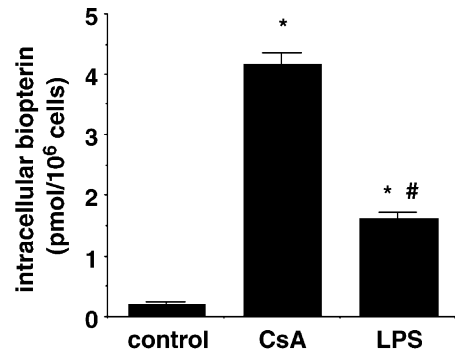


Fig. 6. The increase in biopterin content by CsA and LPS treatments. Cells were incubated with CsA (10 μM) or LPS (10 μg/ml) for 12 h. Values are the mean ± S.E.M. of four experiments. '*' Significantly different from control group ($P < 0.05$). '#' Significantly different from the CsA alone group ($P < 0.05$).

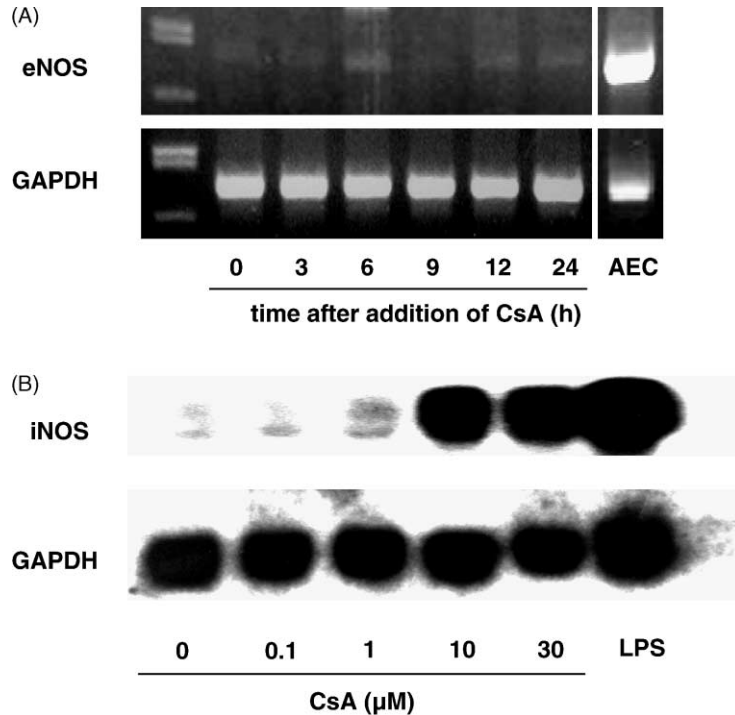


Fig. 5. Effects of CsA on the induction of eNOS and iNOS mRNA levels. (A) Mouse brain microvascular endothelial cells were treated with CsA (10 μM) for various periods, and then total RNA was extracted from the cells. Total RNA was also extracted from mouse AECs as a standard. Total RNA (0.5 μg) was amplified by RT-PCR using specific primers for eNOS or GAPDH. DNA size markers in the left-most lane correspond to 525, 500 and 400 bp. (B) Mouse brain microvascular endothelial cells were treated with various concentrations of CsA (0–30 μM) for 9 h or LPS (10 μg/ml) for 9 h, and then total RNA was extracted from the cells. After electrophoresis of 20 μg of total RNA per sample and transfer to a nylon membrane, the blot was sequentially hybridized with ³²P-labelled iNOS cDNA and GAPDH cDNA.

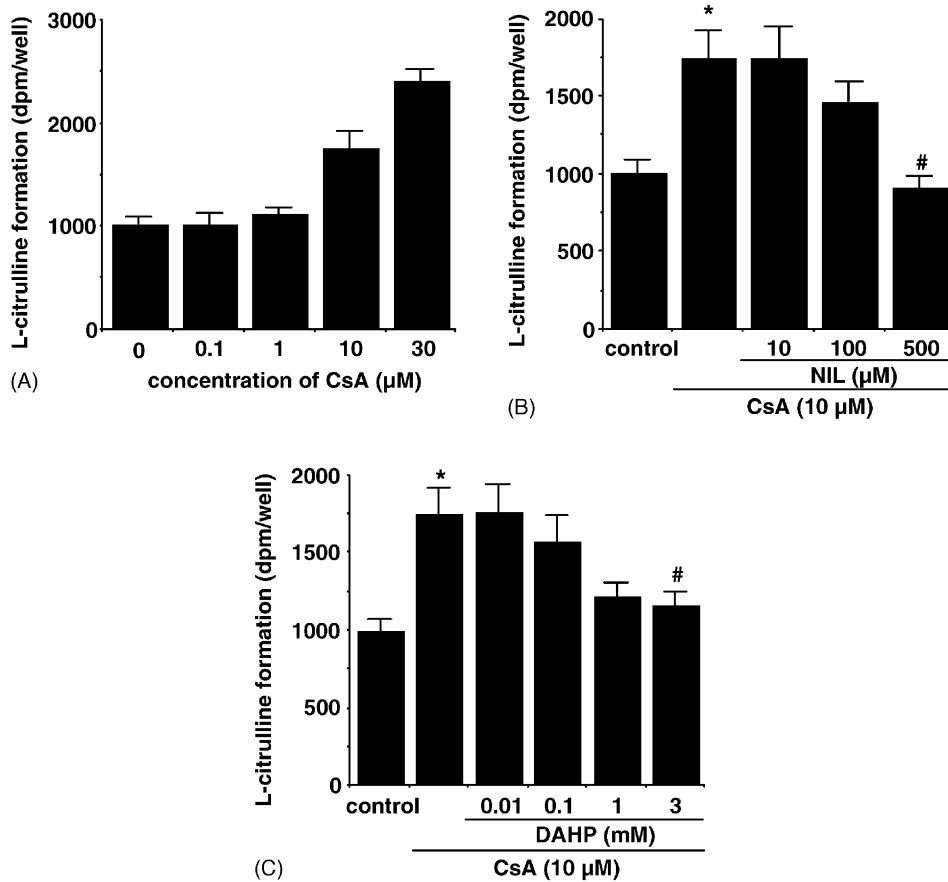


Fig. 7. Stimulation of L-citrulline formation by CsA. (A) Cells were incubated with various concentrations of CsA (0–30 μ M) for 12 h, and then incubated with L-[3 H]-arginine (74 kBq) for 30 min at 37 $^{\circ}$ C. (B) Cells were incubated with 10 μ M CsA for 12 h, and then incubated with L-[3 H]-arginine (74 kBq) for 30 min at 37 $^{\circ}$ C. L-NIL (10–500 μ M) were added to the cells 5 min before measurement of L-arginine metabolism. (C) Cells were incubated with 10 μ M CsA and DAHP (0.01–3 mM) for 12 h, and then incubated with L-[3 H]-arginine (74 kBq) for 30 min at 37 $^{\circ}$ C. Values are the mean \pm S.E.M. of four experiments. ‘*’ Significantly different from control group ($P < 0.05$). ‘#’ Significantly different from the CsA alone group ($P < 0.05$).

4. Discussion

In the present study, we showed that the addition of CsA to cultured mouse brain microvascular endothelial cells increased the intracellular BH₄ content. CsA-induced the expression of GTPCH, which is the rate-limiting enzyme for BH₄ synthesis, and DAHP, a specific inhibitor of GTPCH activity, inhibited the CsA-induced increase in the BH₄ level. Moreover, CHX, a protein synthesis inhibitor, also suppressed the increase in BH₄ content induced by CsA. These observations suggest that CsA induces BH₄ synthesis

in brain microvascular endothelial cells via a de novo pathway with induction of GTPCH.

CsA has been reported to induce the expression of eNOS in bovine AECs [8,9]. However, in the present study, CsA did not induce the expression of eNOS mRNA in mouse brain microvascular endothelial cells. Moreover, brain microvascular endothelial cells expressed much lower levels of eNOS mRNA than mouse AECs. Kimura et al. [17] suggested that the elevation of the intracellular calcium concentration with calcium ionophore did not induce NO synthesis in brain microvascular endothelial cells, using

DAF-2, an NO-sensitive fluorescence dye. NO production from eNOS in brain microvascular endothelial cells appeared to be very low or absent. Therefore, CsA-induced BH4 synthesis is likely to be uncoupled from NO production by eNOS in brain microvascular endothelial cells.

CsA-induced the expression of iNOS mRNA and stimulated the L-citrulline formation from L-arginine, which is a marker for NO production. The CsA-induced increase in L-citrulline formation was reduced by the co-treatment with GTPCH inhibitor. The increase in BH4 level has been shown to be required to produce NO by iNOS [18,19]. The CsA-induced increase in the BH4 level seems to be coupled with NO production from iNOS. It is known that CsA in its therapeutic concentration range (0.1–10 μ M) induces serious side effect such as encephalopathy, nephrotoxicity and hypertension [1–4]. The pathophysiological role of the stimulated NO production by CsA will be need to examine in the future studies.

LPS has been well-known to induce NO production through the induction of iNOS and BH4 synthesis in various types of cells [19–22]. The increase in the BH4 level induced by CsA was much higher than that by LPS, despite the induction of iNOS mRNA by CsA was lower than that by LPS. Therefore, it is possible that the CsA-induced increase in BH4 content has an another role other than as cofactor of NOS. We previously reported that the intracellular increased BH4 has a protective role against ROS- and NO-induced vascular endothelial cell injury [11–13,23–25]. Kojima et al. [26] reported that BH4 acts directly as a scavenger of superoxide anion generated by xanthine/xanthine oxidase or by the rat macrophage/phorbol myristate acetate radical-generating system. We also observed that the increase in the intracellular BH4 level reduces H₂O₂-induced intracellular oxidative stress using 2',7'-dichlorofluorescein, a hydroperoxide-sensitive dye, and strongly reduced H₂O₂-induced bovine AEC death [13]. Thus, BH4 functions not only as a cofactor of NOS, but also scavenger of ROS. CsA has been shown to cause cell injury through the production of ROS during its metabolism [27,28]. Therefore, although endothelial cell injury by CsA was not observed in the present study (data not shown), it is possible that the CsA-induced increase in the BH4 level may have a protective

role against CsA-induced cytotoxicity in vivo. Recently, Schmid et al. [29] reported that BH4 reduces allograft ischemia-reperfusion injury after lung transplantation. The increase in the BH4 level during transplantation with immunosuppressants may preserve ischemia-reperfusion injury and immunosuppressant-induced cytotoxicity.

In conclusion, the present findings suggested that CsA-induced the increase in BH4 content via a de novo pathway in mouse brain microvascular endothelial cells. The CsA-induced increase in BH4 content seems to be coupled with NO production by co-induced iNOS. The physiological role of the increase in the BH4 content and the NO production induced by CsA will be needed to examine in detail in future studies.

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