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Cyclosporine enhances α_1-adrenoceptor-mediated nitric oxide production in C6 glioma cells

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Abstract

The present study was aimed at elucidating the effect of cyclosporine on phenylephrine-evoked nitric oxide (NO) production in C6 glioma cells using direct electrochemical NO monitoring. Phenylephrine (0.1–10 μM) dose-dependently stimulated NO production (0.8–12.9 μM) and this was blocked by NO synthase inhibitor, prazosin, Ca^{2+}-depletion and Xestospongin C (a blocker of the inositol 1,4,5-trisphosphate (IP_3) receptor), suggesting that the α_1-adrenoceptor signaling pathway mediates NO production in C6 cells. Cyclosporine (~10 μM) failed to evoke NO production but increased phenylephrine-evoked NO production by 20–120% of phenylephrine alone in a dose-dependent manner (1–5 μM). Xestospongin C, at a concentration which showed no effect on phenylephrine-induced NO production, significantly inhibited the cyclosporine-enhanced phenylephrine response. This finding suggests that cyclosporine may increase phenylephrine-induced NO production by accelerating IP_3 receptor function in the α_1-adrenoceptor signaling pathway in C6 cells. This enhanced NO production in glial cells may be operative for the occurrence of cyclosporine neurotoxicity including convulsions and encephalopathy.

Keywords: Immunosuppressant; Cyclosporine; Nitric oxide (NO); α_1-Adrenoceptor; C6 glioma cell

1. Introduction

Cyclosporine, a cyclic peptide of 11 amino acids, is a potent immunosuppressant that blocks calcineurin-mediated T cell activation. This compound is used to prevent allograft rejection after organ transplantation and to treat various autoimmune diseases (Kahan, 1989). Cyclosporine induces nephrotoxicity, hepatotoxicity and neurotoxicity. Among these adverse reactions, neurotoxicity including tremor, seizure, cortical blindness and encephalopathy are frequent in patients with high drug levels in serum, although within the therapeutic range (Gijtenbeek et al., 1999). We recently reported that cyclosporine produced convulsions by inhibiting γ-aminobutyric acid (GABA)-ergic neural activity and binding properties of $GABA_A$ 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). Cyclosporine injured brain capillary endothelial cells and inhibited the function and expression of P-glycoprotein, a multi-drug efflux pump (Kochi et al., 1999). A direct exposure to relatively high concentrations of cyclosporine killed cultured mouse oligodendrocytes and neurons, sparing astrocytes (McDonald et al., 1996). These findings may be interpreted as generating cyclosporine neurotoxicity, especially encephalopathy. Glial cells produce nitric oxide (NO) as an intracellular messenger, a free-radical gas synthesized by endothelial and neuronal constitutive NO synthase (cNOS) and inducible NOS (Huang and Fishman, 1996; Vincent et al., 1998). In addition to the physiological role of NO in the glial signaling pathway (Murphy et al., 1993), glia-derived NO has been strongly implicated in the pathogenesis of neurodegenerative disorders (Iadecola, 1997; Olanow, 1993). There is evidence suggesting that not only inducible

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NOS but also cNOS mediates brain injury (Bolaños and Almeida, 1999; Caggiano and Kraig, 1998; Hewett et al., 1994). Various biological mediators are involved in the NO production pathway in glial cells (Agulló and García, 1992a; Murphy et al., 1993). Noradrenaline is the most effective agonist activating cNOS, mainly through α_1-adrenoceptors (Agulló and García, 1992b).

In the present study, we present evidence that cyclosporine facilitates α_1-adrenoceptor-mediated NO production in C6 glioma cells, using direct electrochemical NO monitoring. A facilitatory action of cyclosporine on the stimulation-evoked NO production in glial cells may be related to the occurrence of cyclosporine neurotoxicity including convulsions and encephalopathy.

2. Materials and methods

2.1. Materials

C6 glioma cells (JCRB9096, Health Science Research Resources Bank, Osaka, Japan) were plated on organ culture dishes (2.5 cm^2) or 12-well culture plates (2.6 cm^2/well) (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal calf serum and 80 μg/ml of gentamycin under a humidified atmosphere of 95% air/5% CO_2 at 37°C for 2–4 days prior to the experiment. Stock cultures were maintained in flasks and passaged. Cells dissociated from stock cultures (less than the 12th subculture) with 0.25% trypsin were seeded on dishes (2.5×10^5 cells/dish). Culture medium and subculture reagents were obtained from GIBCO BRL (Life Technologies, Grand Island, NY, USA). Phenylephrine hydrochloride, prazosine hydrochloride and N^G-monomethyl-L-arginine acetate salt (L-NMMA) were obtained from Sigma (St. Louis, MO, USA). Xestospongin C and cyclosporine were from CALBIOCHEM (La Jolla, CA, USA) and Novartis Pharma (Tokyo, Japan), respectively. All remaining reagents, of analytical grade, were purchased from Wako (Osaka, Japan). NO standards were prepared by making serial dilutions of saturated NO solutions. To produce a saturated NO solution (typically containing approximately 1.4 mM NO), deionized water solution (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 until use.

2.2. Electrochemical monitoring of NO

Direct and continuous electrochemical measurement of NO was performed with a three-electrode potentiostatic EMS-100 system made by BIO-LOGIC (Grenoble, France) as previously described (Trevin et al., 1998). In brief, the working electrode was a carbon microfiber (7 μm diameter and approximately 0.5 mm in length), coated with tetrakis(3-methoxy-4-hydroxyphenyl)nickel(II) porphyrin (NiTMPP) (Midcentury, IL, USA) and Nafion (Aldrich, WI, USA) (Malinski and Taha, 1992). The reference and counter electrode were an aqueous Ag/AgCl electrode and a platinum wire, respectively. All electrodes were supplied from ASTEC (Fukuoka, Japan). Before the experiments, each NO biosensor was checked by differential normal pulse voltametry to locate the NO oxidation peak at the same potential as authentic NO. These NO oxidation potential values were contained between 630 and 750 mV versus the Ag/AgCl reference electrode and such variations were the result of different catalytic actions of NO oxidation by the NiTMHPP layer. Direct measurements of NO produced by cultured cells were realized by differential normal pulse amperometry at a NO oxidation peak potential value determined beforehand. At the start and/or the end of the experiment, each NO-biosensor calibration was performed by successive additions of NO standard solutions (0.35–4.20 μM). The specificity against NO_2^-, the main metabolite of NO in the aqueous solution (Butler et al., 1995), was checked and the NO biosensor showed no response for NO_2^- concentrations below 50 μM (Lantione et al., 1995). The current response of the NO biosensor was linear for NO concentrations ranging from 20 to 1500 nM, with a sensitivity between 0.3 and 1.3 nA/μM of NO and a detection limit of 20–50 nM in Krebs–Ringer solution.

2.3. Measurement of $NO2^-$ levels

The amounts of NO_2^-, a stable breakdown product of NO, were measured using an automated spectrophotometer-high performance liquid chromatography (HPLC) system (NO_x analyzer ENO-200, EICOM, Kyoto, Japan) according to Yamada and Nabeshima (1997). In brief, NO_2^- and NO_3^- in Krebs–Ringer solution were separated by a reverse-phase separation column (NO-PAK, EICOM, Kyoto, Japan) and NO_3^- was reduced to NO_2^- in a reduction column (NO-RED, EICOM, Kyoto, Japan). NO_2^- was mixed with a Griess reagent to form a purple azo dye in a reaction coil, the resulting dye measured using a flow-through spectrophotometer. The mobile phase (at a flow rate of 0.33 ml/min) was 10% methanol containing 0.15 M $NaCl/NH_4Cl$ and 1.3 mM 4Na–EDTA. The Griess reagent (at a flow rate of 0.1 ml/min) was 0.0125 N HCl containing 29 mM sulfanilamide with 1.3 mM *N*-naphthylethylenediamine. The contamination of NO_2^- and NO_3^- in Krebs–Ringer solution was examined in each experiment. In the present experiment, NO_2^- production was expressed as the amount of NO_2^- over the basal level (NO_2^- concentrations in cells exposed to the stimulation

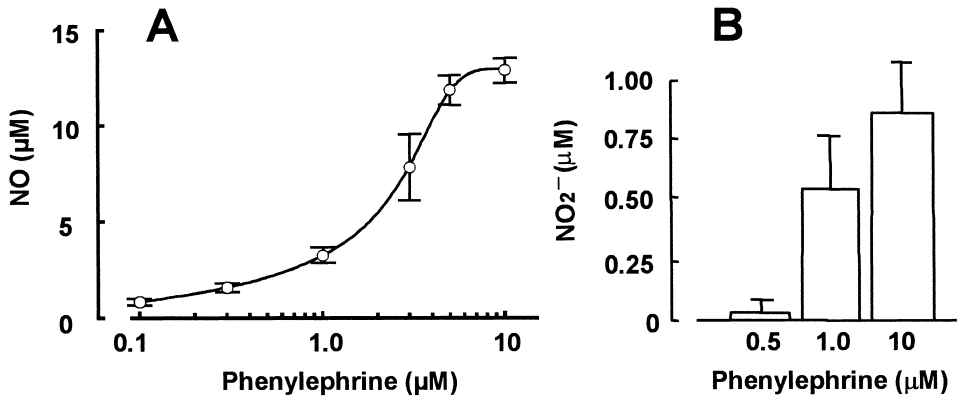

Fig. 1. (A) Concentration–response relationship of phenylephrine-induced NO production obtained using a direct electrochemical NO monitoring in C6 glioma cells. Values are means ± S.E.M. for three to five dishes. (B) Phenylephrine-induced NO_2^- formation by determining NO_2^- using the HPLC/Griess method in C6 cells. Bars represent means ± S.E.M. for four wells.

minus those in cells treated with vehicle). Measurements of NO_3^- production are not shown, since the variation of the basal NO_3^- level, including the contamination, was relatively large in cells exposed to vehicle in our laboratory.

2.4. Characterization of phenylephrine-induced NO production and effect of cyclosporine

Confluent C6 glioma cells in a 2.5-cm^2 dish were washed three times with Krebs–Ringer solution, pH 7.4 with the following composition (mM): NaCl 143.0, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1.2, NaH_2PO_4 1.0, and D-glucose 11.0. A dish was placed on the stage of an inverted microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) mounted with NO monitoring system. The NO biosensor was positioned about 10–20 μm above the cell surface using micromanipulator. After 10 min of treatment with L-arginine (1 mM), phenylephrine at a volume of 10 μl was added to cells in 1 ml of Krebs–Ringer solution with a transient mixing step to give the final concentration indicated. The evoked NO production was monitored for a 15-min period after the addition of phenylephrine. When the effects of various drugs or Ca^{2+}-free Krebs–Ringer solution were examined on phenylephrine-induced NO production, cells were pretreated with each drug or Ca^{2+}-depletion 30 min before the exposure to phenylephrine. To measure the accumulation of NO_2^-, cells in 12-well plates (2.6 cm^2/well) were washed three times with Krebs–Ringer solution and then incubated at 37°C for 10 min in 1 ml of Krebs–Ringer solution containing phenylephrine and/or cyclosporine.

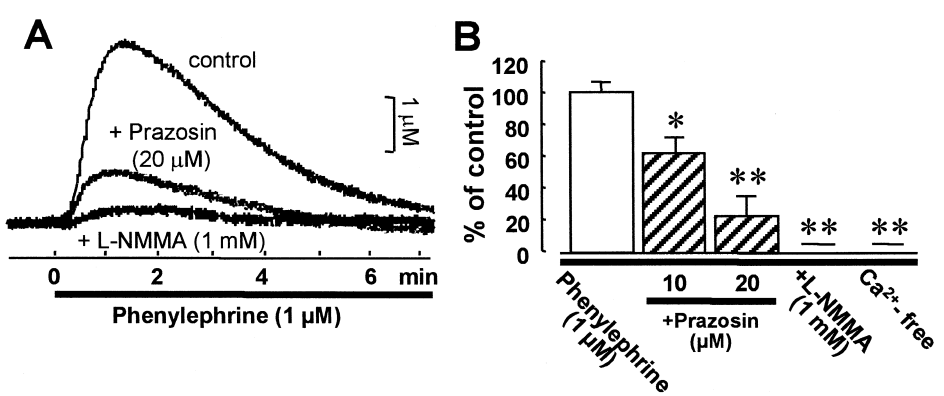

Fig. 2. (A) Representative differential pulse amperogram obtained using the NO biosensor, showing phenylephrine (1 μM)-evoked NO production (control, top trace) in C6 cells. L-arginine was added to cells 10 min before the injection of phenylephrine. Effects of prazosin (20 μM) and L-NMMA (1 mM) were examined in cells pretreated with each drug 30 min before the injection of phenylephrine (+prazosin (middle trace) and +L-NMMA (bottom trace), respectively). (B) Effects of prazosin, L-NMMA and Ca^{2+}-free on phenylephrine-evoked NO production in C6 cells. Values are expressed as % of phenylephrine (1 μM)-evoked NO production (control). A control was obtained in each experiment using the corresponding batch of C6 cells (four dishes in each batch) (2.8–3.7 μM). A representative control is shown. Bars represent means ± S.E.M. for three to five dishes. $^*P < 0.05$ and $^{**}P < 0.01$; significant difference from phenylephrine 1 μM.

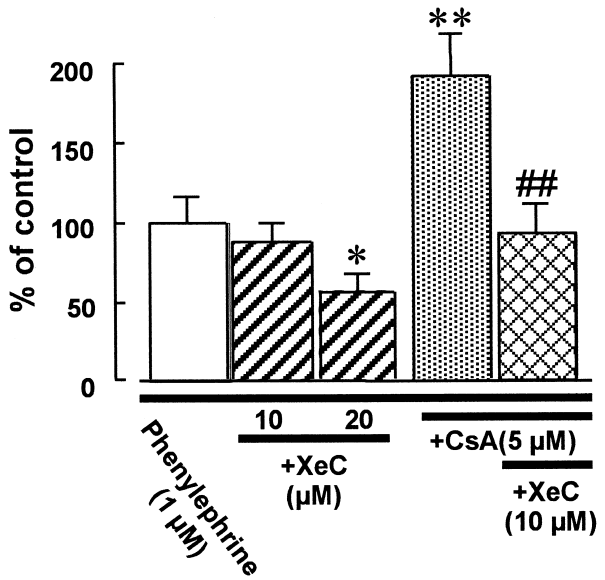

Fig. 3. Effects of Xestospongin C (XeC) on phenylephrine-evoked NO production and cyclosporine (CsA)-enhanced phenylephrine response in C6 cells. A control was obtained in each experiment using the corresponding batch of C6 cells (four dishes in each batch) (2.5–3.3 μM). A representative control is shown. Bars represent means ± S.E.M. for three to five dishes. $^{*}P < 0.05$ and $^{**}P < 0.01$; significant difference from phenylephrine (1 μM) alone. $^{\#\#}P < 0.01$; significant difference between phenylephrine (1 μM) + CsA (5 μM) in the absence of XeC (10 μM) and those in the presence of XeC.

2.5. Statistical analysis

Values are expressed as means ± S.E.M. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Bonferroni/Dunn's test for multiple comparisons (StatView, Abacus Concepts, CA, USA). Differences were regarded as statistically significant at $P < 0.05$.

3. Results

3.1. Characterization of phenylephrine-induced NO production in C6 glioma cells

Phenylephrine at concentrations ranging from 0.1 to 10 μM dose-dependently induced NO production in C6 glioma cells (0.8 ± 0.1–12.9 ± 0.5 μM) (Fig. 1A). The representative current–time curves obtained with the NO biosensor in C6 cells are shown in Fig. 2A. Traces represent the variation in current due to NO oxidation. When phenylephrine (1 μM) was added to cells first exposed to L-arginine (1 mM) 10 min before the stimulation, the NO biosensor signal immediately increased to reach a peak within 1–2 min and decreased slowly to a baseline 7–8 min after phenylephrine injection (top trace (control) in Fig. 2A). The difference in electrical current between the peak value and the baseline value (before phenylephrine addition) was directly proportional to the free NO concentration in the solution. The addition of phenylephrine in Krebs–Ringer solution without cells in the dish gave no significant signal except for a brief amperometric signal for a maximum of 30 s due to the mixing procedure. The basal accumulation of NO_2^- in Krebs–Ringer solution containing vehicle during a 10-min period was 2.1 ± 0.3 μM ($n = 4$). Phenylephrine 1 and 10 μM dose-dependently increased NO_2^- formation over the basal levels (0.6 ± 0.2 and 0.9 ± 0.2 μM, respectively) (Fig. 1B). Phenylephrine-induced NO production was significantly inhibited by prazosin (10 and 20 μM) in a dose-dependent manner and almost disappeared in cells exposed to L-NMMA 1 mM or Ca^{2+}-depletion (Fig. 2A and B). Xestospongin C (20 μM), a potent membrane permeable blocker of the inositol 1,4,5-trisphosphate (IP3) receptor (Gafni et al., 1997), significantly inhibited phenylephrine-induced NO production by $56.5 \pm 16.5\%$ (Fig. 3).

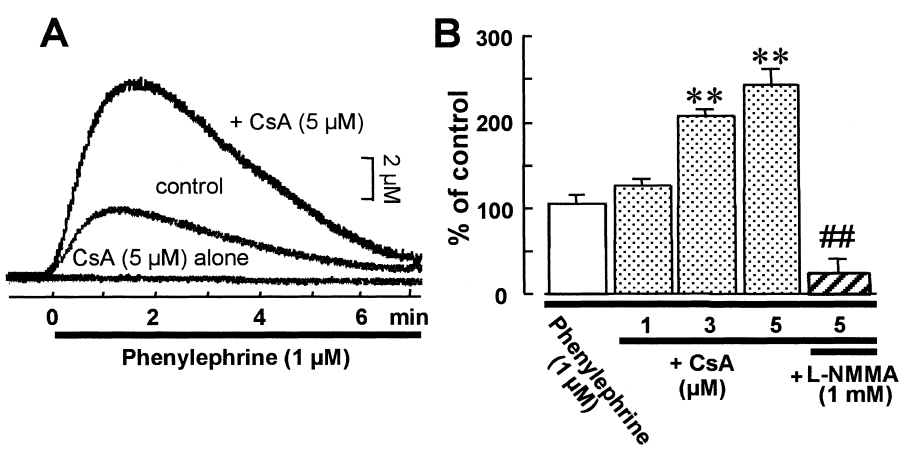

Fig. 4. (A) Traces showing the typical current–time curves of NO production evoked by phenylephrine (1 μM) in the absence (control, middle trace) and presence of cyclosporine (5 μM) (+cyclosporine, top trace) in C6 cells. The effect of cyclosporine (5 μM) alone on NO production were indicated in the bottom trace. (B) Concentration-dependent facilitatory action of cyclosporine on phenylephrine-evoked NO production in C6 cells. The effect of L-NMMA on cyclosporine-enhanced phenylephrine response is also shown. Values are expressed as % of phenylephrine (1 μM)-evoked NO production (control). A control was obtained in each experiment with the corresponding batch of C6 cells (three dishes in each batch) (2.6–4.2 μM). A representative control is shown. Bars represent means ± S.E.M. for four to five dishes. $^{*}P < 0.05$ and $^{**}P < 0.01$; significant difference from phenylephrine 1 μM. $^{\#\#}P < 0.01$; significant difference between cyclosporine (5 μM)-enhanced phenylephrine response in the absence of L-NMMA and that in the presence of L-NMMA.

3.2. Effect of cyclosporine on phenylephrine-induced NO production in C6 glioma cells

Cyclosporine alone, at concentrations up to 10 μM, failed to stimulate NO production over the detection limit (20–50 nM) (Fig. 4A). Cyclosporine (1, 3 and 5 μM) dose-dependently increased phenylephrine (1 μM)-evoked NO production by 19.3 ± 14.6%, 69.8 ± 11.9% and 119.5 ± 11.7% of the control, respectively (Fig. 4B). As shown in the representative traces (Fig. 4A), cyclosporine markedly increased the peak-high rather than the duration of the current. Phenylephrine (1 μM)-evoked NO_2^- formation (0.6 ± 0.2 μM, $n = 4$ wells) was also significantly enhanced by 5 μM cyclosporine (104.5 ± 14.0% of increase, $n = 4$ wells, $P < 0.01$). L-NMMA (1 mM) completely inhibited the cyclosporine-enhanced response (Fig. 4B). Xestospongin C, at a low concentration of 10 μM, which showed no influence on phenylephrine-evoked NO production, significantly inhibited the cyclosporine-increased phenylephrine response (Fig. 3).

4. Discussion

Phenylephrine acted on C6 glioma cells to stimulate NO production in a dose-dependent manner, a response blocked by the NOS inhibitor. This finding was further supported by measurement of NO_2^- formation using the HPLC/Griess method. Highly sensitive and specific microamperometric NO biosensors have been developed to measure small amounts of in situ NO production and this technique allows kinetic data acquisition for NO production (Lantione et al., 1995; Malinski and Taha, 1992). The concentration of phenylephrine required for the detection of the evoked NO production with the NO-biosensor was relatively low, compared with that using the HPLC/Griess method, because the NO biosensor is capable of detecting NO released much closer to C6 cells. The phenylephrine-evoked response was inhibited by prazosin and Ca^{2+}-depletion, supporting the findings obtained from measurement of cyclic GMP accumulation in cultured rat astrocytes (Agulló and García, 1991; Agulló et al., 1995). cNOS synthesizes NO in response to increased Ca^{2+} and functions in signal transduction cascades by linking temporal changes in the Ca^{2+} level to NO production, which serves as an activator of soluble guanylate cyclase (Knowles et al., 1989). These findings suggest that the stimulation of α_1-adrenoceptor may activate cNOS to produce NO in C6 glioma cells by increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_{IN}$). This notion was supported by our present observation that Xestospongin C, a potent membrane permeable blocker of the IP_3 receptor, significantly inhibited phenylephrine-induced NO production in C6 cells.

Cyclosporine alone failed to stimulate NO production. However, cyclosporine enhanced phenylephrine-evoked NO production and NO_2^- formation, this action blocked by NOS inhibitor in C6 cells. Cyclosporine exerts pharmacological properties 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 IP_3 receptor via FKBP12, 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). Tacrolimus (FK506) displaces FKBP12 and calcineurin from the IP_3 receptor and maintains the phosphorylation status of the receptor, leading to leaky Ca^{2+} channels where IP_3 causes increased Ca^{2+} flux at lower concentrations (Cameron et al., 1997, 1995; Snyder and Sabatini, 1995; Snyder et al., 1998). In contrast with FKBP12, cyclophilin does not bind to 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 the IP_3-stimulated Ca^{2+} flux was markedly increased (Cameron et al., 1995). This suggests that cyclosporine inhibits dephosphorylation of IP_3 receptor to maintain a leaky state of the IP_3 receptor channel that was phosphorylated by serine–threonine protein kinase such as protein kinase C. Although IP_3 receptor becomes leaky, cyclosporine alone appears incapable of elevating $[Ca^{2+}]_{IN}$ over the threshold for activation of cNOS. This may explain the present findings that cyclosporine alone failed to produce NO over the detection limit level of the NO biosensor, while the phenylephrine-evoked NO production in C6 cells was markedly facilitated by cyclosporine in the present study. Phenylephrine activates phospholipase C through the α_1-adrenoceptor to generate IP_3 and it stimulates the leaky Ca^{2+} channel (phosphorylation status of IP_3 receptor) maintained by cyclosporine. These events probably lead to the markedly higher level of $[Ca^{2+}]_{IN}$ than that induced by phenylephrine. This notion was supported by the present finding that Xestospongin C, an IP_3 receptor blocker, at a concentration which showed no influence on phenylephrine-evoked NO production, significantly inhibited an enhancement of the phenylephrine response induced by cyclosporine. 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 the astrocytes (Verkhratsky and Kettenmann, 1996). These stimulations-evoked NO productions are highly likely to be augmented by cyclosporine via a mechanism similar to that proposed here. This glia-derived NO is one of the neurotoxic factors in the brain (Iadecola, 1997; Olanow, 1993). In addition, cyclosporine produces dysfunction of the blood–brain barrier (Kochi et al., 1999).

We recently reported that cyclosporine-increased permeability of the brain endothelial cells was aggravated by the presence of C6 cells (Dohgu et al., 2000). These findings suggest the possibility that a facilitatory action of cyclosporine on the stimulation-evoked NO production in astrocytes causes dysfunction of the blood–brain barrier and neurotoxicity.

In conclusion, cyclosporine accelerated α_1-adrenoceptor-mediated NO production in C6 glioma cells, probably by inhibiting dephosphorylation of IP_3 receptor. This enhanced glial NO production that interacts with neurons and endothelial cells in the brain may be operative for the occurrence of cyclosporine neurotoxicity including convulsions and encephalopathy (Vincent et al., 1998).

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