Involvement of Antiapoptotic Signals in Rat PC12 Cells Proliferation by Cyclosporin A Treatment

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Cyclosporin A (CsA) plays an important role in clinical medicine and basic biology as an immunosuppressant and a mitochondrial permeability blocker, respectively. It was reported that CsA has a protective role by preventing apoptosis and promoting the proliferation in severed neurons. However, the molecular mechanisms for CsA-induced neuronal cell proliferation are unclear. In this study, we examined the mechanisms underlying the CsA-induced proliferation of PC12 cells. CsA increased the viability of PC12 cells in a dose (over 0.1~10 µM) -and time-dependent manner. The level of ROS generation was decreased in the CsA-treated PC12 cells. Expression of Bcl-2, an antiapoptotic molecule that inhibits the release of cytochrome c from the mitochondria into the cytosol, was upregulated, whereas Bax, a proapototic molecule, was not changed in the CsA-treated PC12 cells. CsA downregulated the mRNA expression of VDAC 1 and VDAC 3, but VDAC 2 was not changed in the CsA-treated PC12 cells. The level of cytosolic cytochrome c released from the mitochondria and the caspase-3 activity were attenuated in the CsA-treated PC12 cells. These results suggest that the mitochondria-mediated apoptotic signal and Bcl-2 family may play an important role in CsA-induced proliferation in PC12 cells.

Keywords: Cyclosporin A, PC12 cells, Cytochrome c, Caspase, Bcl-2 family

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Introduction

Cyclosporin A (CsA) is a lipophilic cyclic peptide derived from the fungus *Tolypocladium inflatum*, and has been used as an immunosuppressive drug to prevent organ transplant rejection (Faulds *et al.*, 1993). In addition to its immunosuppressive properties, CsA may have potent neuroprotective and neurotrophic activity. Previous studies have reported that CsA decreases the infarct and edema size when administrated orally before or during transient ischemia. In addition, CsA dramatically ameliorates the CA1 hippocampal damage after transient forebrain ischemia in rats (Ucjino *et al.*, 1995).

The mechanisms underlying the neuroprotective effect of CsA have not been established and many hypotheses have been put forward to explain these effects. It was demonstrated that CsA has neuroprotective effect through an anti-inflammatory response by downregulating cytokine expression or suppressing calcineurin, a mediator of the death-signaling pathway (Folbergrova *et al.*, 1997). A recent report using a model of global ischemia suggested that the pro-survival pathway activated by neurotrophic factor (NTF) might participate in the neuroprotective effect of CsA (Miyata *et al.*, 2001). Furthermore, one hypothesis underlying the neuroprotective effect of CsA suggested that CsA acts as specific inhibitor of the mitochondrial permeability transition pore (mPTP). CsA directly inhibits the formation of mPTP and prevents the changes in the permeability of the mitochondrial membranes, and the loss of the transmembrane potential and subsequently results in the prevention of various apoptotic signals (Bernardi *et al.*, 1994). Other reports on the neuropro- tective effect of CsA suggest the possibility that CsA stimulates neuronal cell survival by mediating the antiapoptotic signals because the cell survival rate depends on

the balance between cell proliferation and cell death.

Surprisingly, one of the significant clinical side effects in CsA therapy is neurotoxicity, which occurs in up to 60% of transplant patients (Paul, 2001). Despite the extensive studies over the decades, there is some controversy between CsA-induced neurotoxicity and neuroprotection during the long-term treatment within the therapeutic range. Serkova et al.(2004) reported that the effect of CsA on the mitochondria is essential for determining the level of neurotoxicity and neuroprotection and the net effect depends on the concentrations of CsA and oxygen. However, the roles of the apoptotic signals involved with the effect of CsA on the mitochondria and the growth regulation in neuronal cells in vitro was not completely understood.

Apoptosis is a gene-regulated cell death that is involved in the control of the cell number and removal of inappropriate or damaged cells in physiological and pathological processes (Arends et al., 1990). Apoptosis is an important process in a variety of biological systems including normal cell turnover, the immune system, embryonic development and metamorphosis, as well as in chemical-induced cell death (Ellis, 1991). The main players in the proteolytic cascade activated during apoptosis are caspases (Earnshaw et al., 1999). The caspases are a family of highly conserved aspartate-specific cysteine proteases related to the mammalian interleukin-1β-converting enzyme, which is involved in the final execution phase of apoptosis (Nicholson and Thronberry, 1997). Among the mammalian caspases of at least 14 known members, those involved with apoptosis can be further subdivided into the initiator caspases (−2, −8, −9 and −10) and the effector caspases (−3, −6 and −7). In addition to the caspases, the members of the Bcl-2 protein family are also essential for the regulation of apoptosis. The Bcl-2 family controls the release of mitochondrial cytochrome c by regulating the permeability of the outer mitochondrial membrane. The Bcl-2 family is functionally divided into antiapoptotic molecules (Bcl-2, Bcl-X$_L$, Bcl-W, Mcl-1, and A1) and proapoptotic molecules (Bax, Bcl-1s, Bid, Bad, Bim, and Bik) (Adams and Cory, 1998; Tsujimoto and Shimizu, 2000a).

Recent reports have shown that the mitochondria play an important role in apoptosis by releasing the apoptogenic cytochrome c from the inter-membrane space into the cytosol from the mitochondria (Soeda et al., 2001). Once in the cytoplasm, cytochrome c activates caspase-9, resulting in the activation of caspase-3, a marker of the apoptotic executing stage (Li et al., 1997; Thronbery et al., 1997). PC12 is a cell line derived from rat adrenal pheochromocytoma and widely used as an in vitro model for investigation neuronal apoptosis, oxygen sensor mechanism, and neuronal differentiation (Kroll and Czyzyk-Krzeska, 1998). This study examined the roles of the mitochondria-mediated apoptotic signals and the Bcl-2 family in CsA-induced proliferation of PC12 cells through investigating the caspase-3 activity and expression of VDAC, cytosolic cytochrome c, Bcl-2 and Bax.

Materials and Methods

Cell culture and cell viability assay

PC12 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA) under 5% CO_2 at 37°C. CsA (Sigma, St. Louis, MO, USA) was dissolved in RPMI 1640 and sterilized through the 0.2 μmfilter. The cell viability was determined using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetra zolium bromide) assay (Sigma).

Detection of ROS production and caspase activity

The production of reactive oxygen species (ROS) was monitored by a fluorescence spectrometer (Hitachi F-4500, Tokyo, Japan) using 2', 7'-dichlorofluor -bescin diacetate (DCF-DA). The cells were plated on a 96-well plate and treated with N-acetyl-cysteine (NAC; Sigma), a ROS scavenger and CsA. DCF-DA (25 μM) was added to the media for a further 10 min at 37°C. The level of excitation was measured at a wavelength of 480 nm and the level of emission was measured at 530 nm. An ELISA reader was used to assess the caspase activities using the caspase-3 activity assay kit containing the DEVD-pNA substrate (Calbiochem, La Jolla, CA, USA) according to the manufacturer's instructions.

Isolation of total RNA and reverse transcription-polymerase chain reaction (RT-PCR)

For the extraction of the total RNA, the cells were homogenized with a polytron homogenizer in a Trizol reagent (Gibco-BRL). The RNA samples were quantified by spectrophotometry at 260 nm. For the synthesis of cDNA, 2 μg of the total RNA and 2 μl of Oligo-dT (10 pmoles) were mixed with 50μl RNase-free water, and incubated at 42°C for 1 h and 94°C for 5 min. The PCR products were generated in PCR buffer containing 10 pmoles of each primer using a PCR-premix kit (Bioneer, Deajoen, Korea). After the first denaturation step (5 min at 95°C), the samples were subjected to 30 cycles consisting of 40 sec at 95°C, 40 sec at 55°C, and 1 min 30 sec at 72°C, with a final extension step of 10 min, on a GeneAmp PCR system (Perkin-Elmer 2400, Rodgau-Jugeshein, Germany). The following primer pairs were used: for VDAC 1, 5'-TGATACCACGTTAGACCTCC-3' (sense), 5'-ACAACCTGGAAGCTATTTCA-3' (anti sense), for VDAC 2, 5'-TGCAGTGGTGTGGAATTTT-3' (sense), 5'-ACGAGTGCA GTTGGTACCTGA-3' (antisense), for VDAC 3, 5'-GCTG CTAAGTATAGGCTGGA-3' (sense), 5'-CCACTGGATGGAT CTGTAAT-3' (antisense). The amplified products were analyzed on 1.5% agarose gels containing ethidium bromide and visualized using a UVP Transilluminator/Polaroid camera System (UVP Laboratories, CA, USA). The following primer pair for GAPDH (control) was used: 5'-TGCATCCTGCA CCACCAACT-3' (sense) and 5'-CGCCTGCTTCACCACT-TC-3' (antisense). The intensities of the bands were determined using NIH Scion Image Software.

Western blot analysis

The cells were washed twice with PBS and the proteins were dissolved a lysis buffer (1% NP-40, 500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Benzamid, 1 µg/ml Trypsin inhibitor) containing a cocktail of Complete protease inhibitors (Boehringer Mannheim, Indianapolis, IN, USA). The level of cytosolic cytochrome c was determined by resuspending the pellet in an extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-NaOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, and 1 mM DTT. The lysates were incubated for 30 min at 4°C, centrifuged at 11,000 × g for 20 min and the protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL, USA). The protein extracts (100 – 300 µg) were boiled with the SDS-sample buffer for 5 min and subjected to electrophoresis on 12% polyacrylamide gel. The proteins were electroblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) and blocked with 5% skim milk (Becton Dickinson, USA) in Tris-buffered saline-0.1% Tween-20 (TBS-T). The primary antibodies used were rat monoclonal anti-cytochrome c (Pharmingen, San Diego, CA, USA), Bcl-2, Bax and anti-ß-Actin (Santa Cruz, CA, USA). The blots were washed three times in TBS-T for 5 min and incubated with the specific peroxidase-coupled secondary antibodies (Sigma). The bound antibodies were visualized using an enhanced chemiluminescent detection system (Amersham Pharmacia Biotech).

Statistical analysis

Results are expressed as mean±standard deviations (SD) and analyzed by Student's t-test. Mean values were considered significantly different at $p < 0.05$.

Results

Effects of CsA on proliferation and ROS generation in PC12 cells

The effect of CsA on the proliferation of PC12 cells was assessed by the MTT assay. As shown in Fig. 1A, the cell viability was gradually increased in a dose-dependent manner when the PC12 cells were exposed to 0.5~50 µM CsA for 24 h. CsA increased the number of PC12 cells by approximately 120~200% relative to the control at concentrations 0.5~50 µM. The number of cells doubled at 5 µM CsA compared with the control. Survival rate by CsA treatment showed the maximum at 5 µM, whereas decreased slightly at 50 µM CsA. As shown in Fig. 1B, 5 µM CsA increased proliferation of PC12 cells in a time-dependent manner. In order to determine whether the CsA reduces the cytosolic ROS level in PC12 cells, the level of cytosolic ROS was measured in the CsA-treated PC12 cells using the DCF-DA. Fig. 2 showed that the generation of cytosolic ROS was reduced by the CsA treatment in a dose-dependent manner.
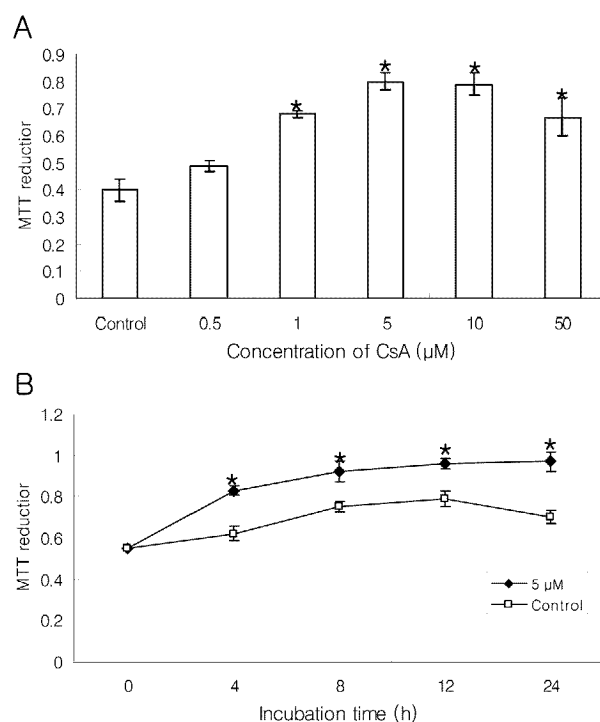

Fig. 1. Effect of CsA on proliferation of PC12 cells. The level cell proliferation was determined by the MTT assay, as described in materials and methods. The PC12 cells were incubated with different concentration of CsA for 24 h (A) and 5 µM CsA for the indicated times (B). Data are expressed as a mean ± SD from 3 independent experiments. *$p < 0.05$, compare to CsA-untreated cells.
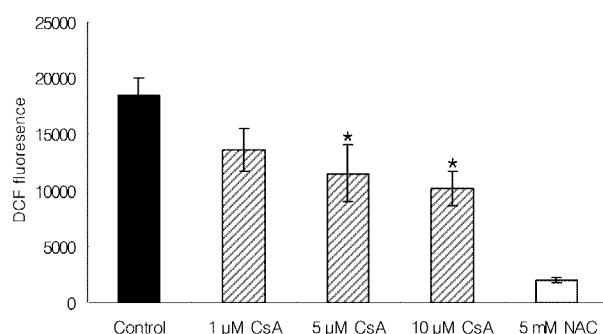

Fig. 2. Cytosolic ROS level was reduced in the CsA-treated PC12 cells. The PC12 cells loaded with DCF were incubated for 12 h at different concentrations (1, 5, and 10 µM) of CsA or for 1 h with 5 mM NAC, a free radical scavenger. The intracellular levels of ROS were detected by measuring the DCF-DA fluorescence. Data are expressed as the mean ± SD from 3 independent experiments. *$p < 0.05$, compare to CsA-untreated cells.

Effects of CsA on cytosolic levels of cytochrome c and VDAC expression

In order to determine wether the mitochondria are involved in the CsA-induced proliferation of PC12 cells, the amount of cytochrome c released from the mitochondria into the cytosol was examined using the Western blot

analysis in the cytosolic fractions according to a previous method (Boulares *et al.*, 2002). The cytosolic levels of cytochrome *c* was assumed to be a consequence of the cytochrome *c* released from the mitochondria into the cytosol. The level of cytosolic cytochrome *c* was reduced in response to the exposure to CsA for 12 h, whereas the level of mitochondrial cytochrome *c* was increased (Fig. 3). To determine the mechanism by which CsA inhibits the release of cytochrome *c* from the mitochondria into the cytosol, the effects of CsA on expression of the voltage-dependent anion channels (VDAC) were examined using the RT-PCR. The level of VDAC 1 and VDAC 3 was downregulated but VDAC 2 expression was slightly increased in the CsA-treated PC12 cells (Fig. 4).

Effects of CsA on the ratio of Bcl-2 to Bax expression

Bax and Bcl-2 are important regulators of cell survival
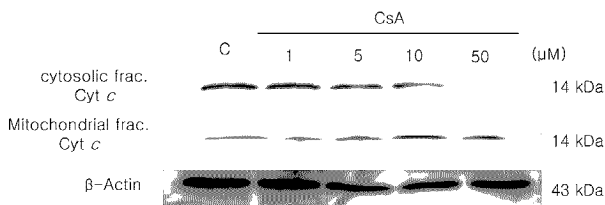

Fig. 3. Decrease in the level of cytosolic cytochrome *c* in the CsA-treated PC12 cells. After exposing the cells to different concentrations (1, 5, 10 and 50 μM) of CsA for 12 h, the levels of cytosolic and mitochondrial cytochrome *c* were determined using the Western blot analysis.

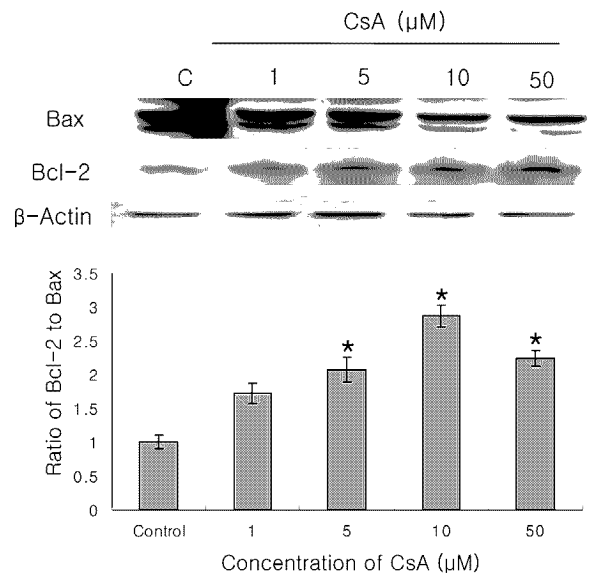(Tsujimoto and Shimizu. 2000a). Bax induces the release of cytochrome *c* from the mitochondria, leading to apoptotic

Fig. 5. CsA decreased the ratio of Bcl-2 to Bax expression. After incubating the PC12 cells with various concentrations of CsA for 12 h, the protein levels of Bcl-2 and Bax were determined using the Western blot analysis. The histograms showed the density of the Bcl-2 to Bax expression ratio, relative to the control group. Data are expressed as the mean ± SD from 3 independent experiments. $*p < 0.05$, compare to CsA-untreated cells.
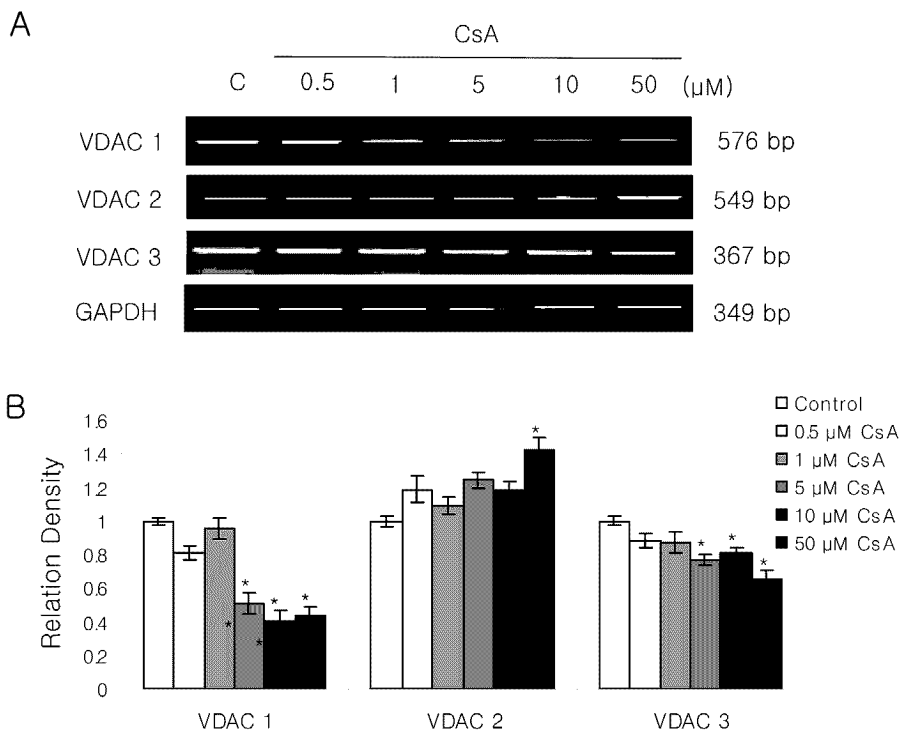

Fig. 4. Downregulation of VDAC in the CsA-treated PC12 cells. (A) After incubating the PC12 cells with CsA (0.5, 1, 5, 10 and 50 μM) for 12 h, the mRNA levels of VDAC 1, VDAC 2, and VDAC 3 were determined using the RT-PCR analysis. (B) Densitometric analysis of the RT-PCR products. Data are expressed as the mean ± SD from 3 independent experiments. $*p < 0.05$, compare to CsA-untreated cells.
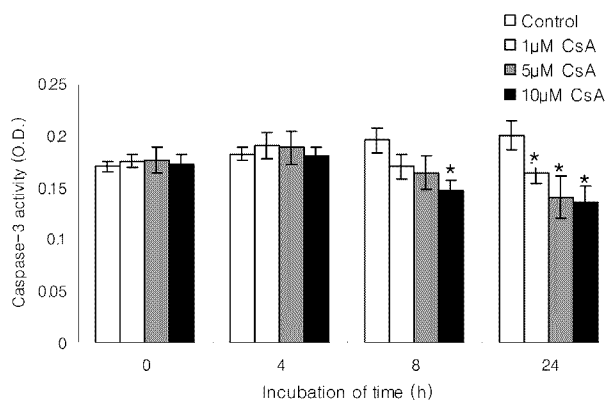
Fig. 6. Effect of CsA on the caspase-3 activity in the CsA-treated PC12 cells. The cells were incubated in the absence and the presence with different concentrations of CsA (1, 5 and 10 μM) for different time. The absorbance for caspase-3 activity was measured at 405 nm using an ELISA reader, as described in materials and methods. The results are expressed as the mean ± SD from 5 independent experiments. *$p < 0.05$, compare to CsA-untreated cells.

cell death, whereas Bcl-2 inhibits the release of cytochrome c from the mitochondria, leading to an antiapoptotic effect. After treating the PC12 cells with CsA (1~50 μM) for 12 h, the protein expression of Bax and Bcl-2 in the PC12 cells was determined using the Western blot analysis. Fig. 5 showed that the expression of Bax was decreased in a dose-dependant manner, whereas CsA upregulated Bcl-2. Therefore, CsA increased the Bcl-2/Bax ratio, which means to favor survival.

Effect of CsA on caspase-3 activities in PC12 cells

PC12 cells were incubated in the absence and presence of different concentrations of CsA (1, 5, and 10 μM) with different time (4, 8, and 24 h). The caspase-3 activity, a marker of apoptosis, was determined on the basis that active caspases consequently cleave their substrate at a specific site. As shown in Fig. 6, the 4 h treatment of CsA did not significantly activate caspase-3, whereas the 8 h and 24 h treatment induced the decrease of activity relative to control in a dose-dependant manner.

Discussion

Up to date, there is some controversy regarding the effect of CsA as to whether it acts in neuroprotection or neurotoxicity on neuronal cells. One of the prominent side effects in CsA therapy is the neurotoxicity associated with all types of organ transplantation, as well as with autoimmune disorders (Paul et al., 2001). In contrast, many reports have shown that CsA ameliorates the brain injury induced by cerebral ischemic-reperfusion (Shiga et al., 1992; Folbergrova et al., 1997). The adverse effects of CsA may be due to the subpopulation of neuronal cells that have different characteristics in response to CsA. Previous reports have shown that CsA has different effects on cell proliferation according to the cell

types. CsA interferes with T cell proliferation whereas it promotes hair epithelial cell proliferation (Mascarell et al., 2004; Takahashi and Kaminura, 2001). In this study, CsA induced the PC12 cell proliferation in a dose- and time-dependent manner, suggesting that CsA has a stimulating effect on the PC12 cell proliferation. The concentrations (0.5~10 μM) of CsA used in this study are similar to the plasma concentrations (100~200 ng/ml) of patients undergoing CsA treatment (Hassell and Hefti, 1991).

It has been well known that the mitochondria play a key role in neuronal cell survival or cell death because they are regulators ofthe both energy metabolisms and the apoptotic pathways (Fiskum, 2000; Wieloch, 2001; Friberg and Wieloch, 2002). Some reports have shown that the mechanism of neurotoxicity by CsA might be mediated by a mitochondrial dysfunction, resulting in an increase in ROS production, the inhibition of ATP synthesis, the release of cytochrome c, the loss of the membrane potential and the induction of various mitochondrial permeability transitions (Bernardi et al., 1994; Sullivan et al., 2005). In contrast, another study reported that CsA has a potent neuroprotective role by blocking the mitochondrial dysfunction, leading to the enhancement of survival in CNS trauma (Sullivan et al., 2005). This previous reports suggested that the mechanism underlying the neuroprotectionby CsA may be involved with the functional mitochondria including the inhibition of a mitochondrial dysfunction as well as the apoptotic pathway in damaged neurons.

The mitochondria-mediated apoptotic pathway is initiated when cytochrome c is released through VDAC from the space between the inner mitochondria membrane (IMM) and the outer mitochondria membrane (OMM) (Crompton, 2000). In the cytosol, the cytochrome c forms apoptosome complexes with Apaf-1 and procaspase-9 in the presence of dATP, and the auto-activation of caspase-9 activates procaspase -3 into caspase-3 (Li et al., 1997; Thronberry et al., 1997). CsA is a specific VDAC-permeability transition pore (PTP) blocker, which inhibits the release of cytochrome c from the mitochondria into the cytosol (Cesura et al., 2003). In the present study, the release of cytosolic cytochrome c was moderately diminished in the CsA-treated PC12 cells. This result suggests the possibility that the inhibition of mitochondria -mediated apoptotic signals might be involved in CsA-induced PC12 cell proliferation, even though it need further study on the relationship to other regulators of mitochondria -mediated apoptotic signal. In addition, the mRNA expression of VDAC 1 and VDAC 3 among the VDAC family is down-regulated in the CsA-treated PC12 cells, suggesting that CsA inhibit the release of cytochrome c from the mitochondria into the cytosol through regulating VDAC expression.

Moreover, the opening and closing ofVDAC-PTP is modulated by the Bcl-2 family proteins (Tsujimoto and shimizu, 2000b; ZamZami and Kroemer, 2001). Bax directly interacts with VDAC and increases the opening the VDAC-PTP, whereas Bcl-2 blocks the opening of VDAC-PTP by heterodimerizing

with Bax. Therefore, a high Bcl-2/Bax ratio reduces the release of cytochrome c resulting in the stimulation of cell survival, not the apoptotic cell death. Interestingly, the present results showed that Bcl-2 expression was moderately increased, whereas Bax, a proapoptotic molecule, remained unchanged in the CsA-treated PC12 cells. From the previous reports and the present results, it is assumed that CsA reduces the level of cytosolic cytochrome c in PC12 cells by directly blocking VDAC-PTP opening and/or regulating the expression of the Bcl-2 family in PC12 cells. Indeed, the overexpression of Bcl-2 that inhibits the release of cytochrome c from the mitochondria into the cytosol, results in promoting the proliferation of myocyte or vascular endothelial cells (Nor et al., 2001; Limana et al., 2002). Besides, Bcl-2 promotes the regeneration of severed axons in mammalian CNS (Chen et al., 1997). In practice, the expression of Bcl-2 in the lumbar ganglion cells of rats increased significantly after the CsA treatment, showing the neuroprotective role of CsA (Rezzani et al., 2004). A recent report showed that Bcl-2 reduces the level of cytosolic ROS, a potent apoptotic stimulator, inducing cell proliferation (Deng et al., 2003). In this study, the cytosolic ROS levels were significantly decreased in the CsA-treated PC12 cells. From the previous reports and these results, it is suggested that reducing the cytosolic ROS level under controlling by Bcl-2 family may be mediated with the proliferation by CsA in PC12 cells.

To confirm the antiapoptotic effect of CsA, activity of caspase-3, a key emzyme of the apoptotic process, was determined after CsA treatment in PC 12 cells. In accordance with the release of cytosolic cytochrome c, the caspase-3 was significantly activated in a dose-dependant manner compared with control. These findings are consistent with the recent studies showing the neuroprotection of CsA through the inhibition of caspase-3 activity in the ischemia-reperfusion injury (Duan et al., 2004).

In conclusion, these results suggest that the inhibition of mitochondria -mediated apoptotic signal by regulating Bcl-2 family, downregulating VDAC, and lower releasing the cytosolic cytochrome c play an important role in CsA-induced proliferation in PC12 cells.

Acknowledgement

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