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Inhibition of Nitric Oxide-induced Neuronal Apoptosis in PC12 Cells by Epigallocatechin Gallate

Ji Yeon Jung, Yeon Jin Jeong, Chang Ryoung Han, Sun Hun Kim, Hyun Jin Kim, Ki Heon Lee, Ha Ok Park, and Won Jae Kim

Dental Science Research Institute, School of Dentistry, Chonnam National University, Gwangju 500-757, Korea

In the central nervous system, nitric oxide (NO) is associated with many pathological diseases such as brain ischemia, neurodegeneration and inflammation. The epigallocatechin gallate (EGCG), a major compound of green tea, is recognized as protective substance against neuronal diseases. This study is aimed to investigate the effect of EGCG on NO-induced cell death in PC12 cells. Administration of sodium nitroprusside (SNP), a NO donor, decreased cell viability in a dose- and time-dependent manner and induced genomic DNA fragmentation with cell shrinkage and chromatin condensation. EGCG diminished the decrement of cell viability and the formation of apoptotic morphologenic changes as well as DNA fragmentation by SNP. EGCG played as an antioxidant that attenuated the production of reactive oxygen species (ROS) by SNP. The cells treated with SNP showed downregulation of Bcl-2, but upregulation of Bax. EGCG ameliorated the altered expression of Bcl-2 and Bax by SNP. The release of cytochrome c from mitochondria into cytosol and expression of voltage -dependent anion channel (VDAC)1, a cytochrome c releasing channel in mitochondria, were increased in SNP-treated cells, whereas were attenuated by EGCG. The enhancement of caspase-9, preceding mitochondria-dependent pathway, caspase-8 and death receptor-dependent pathway, as well as caspase-3 activities were suppressed by EGCG. SNP upragulated Fas and Fas-L, which are death receptor assembly, whereas EGCG ameliorated the expression of Fas enhanced by SNP. These results demonstrated that EGCG has a protective effect against SNP-induced apoptosis in PC12 cells, through scavenging ROS and regulating the mitocondria- and death receptor-mediated signal pathway. The present study suggest that EGCG might be a natural neuroprotective substance.

Key Words: Nitric oxide, PC12 cell, Apoptosis, Caspase, Bcl-2 family, EGCG

INTRODUCTION

Nitric oxide (NO), a highly diffusible and short-lived free radical gas, has both physiological and pathological function in many mammalian tissues. In the central and peripheral nervous systems, NO acts as a neurotransmitter or neuromodulator, whereas it can be neurotoxic at high concentration level (Gross & Wolin, 1995; Dawson & Dawson, 1996). NO is implicated in a variety of neuronal pathological process such as brain ischemia, neurodegeneration and inflammation (Jenner & Olanow, 1996; Bolanos et al, 1997; Peuchen et al, 1997). NO can cause cell death by either necrosis or apoptosis. Necrosis is often characterized by swelling of the cell and cytoplasmic organelles, followed by rupture of the plasma membrane. However, apoptosis is characterized by chromatin condensation, DNA fragmentation, and cell shrinkage.

In general, apoptosis is triggered by a variety of stimuli. These include the mitochondrial response, ER stresses, and cell surface receptors such as Fas. The cysteine protease called caspases, which cleave a critical set of cellular proteins to initiate apoptotic signal, includes several representatives invovled in apoptosis (Crompton, 2000; Roth et al, 2000). Among mammalian caspases of at least 14 known members, those involved in apoptosis can be further subdivided into the initiator caspases (−2, −8, −9, and −10) and the effector caspases (−3, −6, and −7) (Adams & Cory, 1998; Tsujimoto & Shimizu, 2000). According the pathways of activating caspases, main cell death mechanism are divided into death receptor-mediated mechanism and mitochondria-mediated mechanism. Both pathways share the activation of caspase-3 as an executor caspase, which is a critical determinant of whether or not a cell commits to death. The mitochondrial pathway is initiated from release of cytochrome *c* from mitochondria into cytosol, subsequently resulting in caspase-9 activation which causes the activation of caspase-3. Death receptor pathway is stimulated by cell surface death receptors such as tumor necrosis factor (TNF) receptor and Fas (Beer et al, 2000). The receptors activated by ligands lead to caspase-8 ac-

Corresponding to: Won Jae Kim, Department of Oral Physiology, Chonnam National University School of Dentistry, 300 Yongbong-dong, Buk-gu, Gwangju 500-757, Korea. (Tel) 82-62-530-4881, (Fax) 82-62-530-4885, (E-mail) wjkim@ chonnam.ac.kr

ABBREVIATIONS: NO, nitric oxide; EGCG, epigallocatechin gallate; ROS, reactive oxide species; VDAC, voltage-dependent anion channel; NAC, N-acetyl-L-cystein; sodium nitroprusside, SNP; Fas-ligand, Fas-L; pheochromocytoma cells, PC12 cells.

tivation, with subsequent activation of caspase-3.

Besides the caspases, the Bcl-2 family are also critical for the regulation of apoptosis. Bcl-2 family control the release of mitochondrial cytochrome *c* by regulating the mitochondrial permeability transition (PT) pore composed of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenosine nucleotide translocated (ANT) in the inner membrane, and cyclophilin-D (Cyp-D) in the matrix assembles (Ankarcrona et al, 1995; Krajewski et al, 1999; Crompton et al, 2000). In the Bcl-2 family, pro-apoptotic molecules such as Bax promote the formation of membrane pore and the release of cytochrome *c* and other pro-apoptotic factors from mitochondria, while the anti-apoptotic molecules Bcl-2 and Bcl-X_L prevent these effect (Adams & Cory, 1998; Tsujimoto & Shimizu, 2000). Among the Bcl-2 protein family, Bcl-2 and Bcl-X_L are prominent anti-apoptotic molecules, whereas Bax and Bid are prominent pro-apoptotic molecules (Cheng et al, 1997). Even if many different mechanisms of NO-induced cell death have been proposed (Murphy, 1999), the detail molecular mechanisms of NO-induced cell death have been unsettled.

On the other hand, tea polyphenols are natural plant flavonoids found in the leaves and stems of tea plant. The green tea polyphenols have showed a variety of pharmacological properties such as anti-inflammatory, anticarcinogenic, and antioxidant effects (Stoner et al, 1995; Gensler et al, 1996; Shi et al, 2000). The green tea polyphenols comprise (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin, (−)-epicatechin, (+)-gallocatechin, and catechin. Among these polyphenols, EGCG is a major strong component responsible for biological effects. EGCG has been reported to display a potent antioxidant property because it possesses two triphenolic groups in its structure (Jin et al, 2000). Thus, EGCG is expected to have beneficial effects on free radical-induced cell death including the production of reactive oxygen species (ROS) in nervous tissues and cells. This study was designed to investigate the effect and mechanism of EGCG against NO-induced cell death in PC12 cells.

METHODS

Cell culture with SNP and EGCG treatment

Rat pheochromocytoma PC12 cells were cultured in RPMI 1640 medium containing 10% horse serum, 5% fetal bovine serum (Gibco-BRL, USA) inactivated by heat and gentamicin (50 μg/ml, Gibco-BRL, USA) under 5% CO_2 at 37°C. Sodium nitroprusside (SNP, Sigma, USA) and EGCG (Sigma, USA) was dissolved in distilled H_2O and sterilized through a 0.2 μm filter before use. EGCG was pretreated for 30 min before SNP treatment.

Cell viability assay

The MTT assay relies on the observation that viable cells with active mitochondria reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT) into a visible dark-blue formazan reaction product and providing an indirect measurement of cell viability. PC12 cells were plated onto 96 well plates and exposed to SNP alone or pretreated with different concentration of EGCG for 30 min. After treatments, MTT was added to the culture medium at a final concentration of 0.1 mg/ml and incubated at 37°C for 4 h. The reaction product was dissolved in dimethylsulfoxide (DMSO) and optical density (OD) was spectrophotometrically measured at 570 nm with DMSO as a blank using a ELISA reader (ELx800uv, BIO Tek Instruments, USA).

Nuclear staining with Diff-Quick

Morphological changes of apoptotic cells were investigated by Diff-Quick® stain. Cells were plated in 8-well chamber slide at a density of 1×10^5 was incubated for 18 h, subsequently followed by treatment of 5 mM SNP for 12 h. The cells were then washed with PBS and fixed with acetone and methanol (1 : 1). After incubating for 20 min at −20°C, cells were stained with solution containing 10 μg/ml Diff-Quick® (Kuk Jae, Japan) in PBS and observed under fluorescence microscope (Olympus, Japan).

DNA fragmentation assay

Oligonucleosomal fragmentation of genomic DNA was assessed using the Apopladder® kit (TaKaRa Shuzo, Japan) according to the manufacturer's instruction. Briefly, cells were lysed in 200 μl of lysis buffer and centrifuged at 1,100 g for 10 min. The supernatant was incubated at 56°C for 1 h after adding 20 μl of 10% SDS and 20 μl of proteinase K (20 mg/ml), and then treated with 1 μg of RNase at 37°C for 1 h. DNA was extracted and precipitated overnight at −20°C in a precipitant mixture containing 0.95 ml of ethanol and pelleted by centrifugation for 15 min at 12,000 g at 4°C. DNA pellets were resuspended in 20 μl of Tris-EDTA buffer (pH 8.0) and aliquots from each sample were electrophoresed at 80 V for 2 h on 2% agarose gels. DNA bands were visualized under UV light after staining with ethidium bromide.

Determination of ROS production

ROS production was monitered by fluorescence spectrometer (Hitachi F-4500, Japan) using 2', 7'- dichlorofluorescin diacetate (DCF-DA). Cells were plated on 96-well plate and treated with SNP alone, or N-acetyl-L-cystein (NAC) or EGCG with SNP. DCF-DA (25 μM) was added into the media for 10 min at 37°C. Excitation at 485 and emission at 530 nm was measured by fluorometer (Hitachi, Japan).

Caspase substrate cleavage assay

Caspase activity was assayed using the caspase-3, -9, and -8 activity assay kit (Calbiochem, CA) according to the manufacturer's instructions. Briefly, PC12 cells were grown on 100 mm dishes and treated with SNP alone or pretreated with EGCG for the indicated time. The media were removed from the culture dishes and the cells were collected and washed with PBS, then resuspended in cell lysis buffer. After incubation on ice for 10 min, the lysates were centrifuged for 20 min at 12,000 g, and the supernatants were collected and protein concentrations were determined by BCA assay. Fifty microliters of cell lysates was mixed with reaction buffer containing the DEVD-*p*NA substrate (200 μM) for caspase-3 activity, LEHD-*p*NA substrate (200 μM) for caspase-9 activity and IETD-*p*NA substrate for caspase-8 activity. After incubation for 24 h at 37°C, absorbance was measured in the wells at 405 nm by

ELISA reader. The enzyme activity is expressed as arbitrary units of a relative value.

Reverse transcription-polymerase chain reaction (RT-PCR)

For extraction of total RNA, cells were homogenized with a polytron homogenizer in Trizol reagent (Gibco-BRL, USA). RNA samples were quantified by spectrophotometry at 260 nm wavelength. For synthesis of cDNA, 1 μg of total RNA and 1 μl of Oligo-dT (10 pmole) were mixed with 50 μl RNase-free water, and then incubated at 42°C for 1 h and 94°C for 5 min. PCR products were generated in PCR buffer containing 10 pmoles of each primer using PCR-premix kit (Bioneer, Korea). After the first denaturation step (5 min at 95°C), 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, USA). The following primer pairs were used: for Fas, 5'-CAAGGGACTGATAGCATCTTTGAGG-3' (sense primer), 5'-TCCAGATTCAGGGTCACAGGTTG-3' (antisense primer); for Fas-L, 5'-CA GCCCCTGAATTACCCATG-3' (sense primer) 5'-CACTCCAGAGATCAAAGC AG-3' (antisense primer); for VDAC1, 5'-TGATACCACGTTAGACCTCC -3' (sense primer) 5'-ACAACCTGGAAGCTATTTCA-3' (antisense primer); for VDAC2, 5'-TGCAGTGGTGTGGAATTTT-3' (sense primer) 5'-ACGAGTGCA GTTGGTACCTGA-3' (antisense primer). The amplified products were analyzed on 1.5% agarose gels containing ethidium bromide and visualized by UVP Trans-illuminator/Polaroid camera system (UVP Laboratories, CA). RT-PCR was performed with primers for the housekeeping gene, GAPDH, as a control. The following primer pairs for GAPDH were used: 5'-TGCATCCTGCACCACC AACT-3' (sense primer) and 5'-CGCCTGCTTCACCACCTTC-3' (antisense primer). The intensities of the obtained bands were determined using the NIH Scion Image Software.

Western blotting

Cells were washed twice with PBS and proteins solubilized in the lysis buffer (500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM benzamiden, 1 μg/ml trypsin inhibitor) containing a cocktail of protease inhibitor (Roch, Germany). Lysates were incubated for 30 min at 4°C, centrifuged at 12,000 g for 20 min and protein concentrations were determined by BCA protein assay (Pierce, IL, USA). Protein extracts (100~500 μg) were subjected to electrophoresis on 12% polyacrylamide gel and electroblotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, UK) at 20 mA at 4°C overnight. The membrane was blocked with 5% skim milk (Becton Dickinson, USA) in Tris-buffered saline-0.1% Tween 20 (TBS-T) and incubated with the primary antibody. Rat monocloned antibodies against cytochrome *c* (Pharmingen, CA), Bax (Santa Cruz, USA) and Bcl-2 (Santa Cruz, USA) were applied. Blots were subsequently washed in TBS-T and incubated with specific peroxidase-coupled secondary antibodies (anti-mouse IgG horseradish peroxidase [HRP], anti-Rabbit IgG-HRP, Sigma Aldrich, USA). Bound antibodies were visualized using an enhanced chemiluminescent detection system (Amersham Pharmacia Biotech, UK).

Statistical analysis

Values were expressed as means±SD. The data were analyzed by student's *t*-test. Mean values were considered significantly different when $p<0.05$.

RESULTS

Effect of EGCG on cell viability and ROS production in SNP-treated cells

The cell viability was greatly reduced in a dose- and time-dependent manner when PC12 cells were exposed to SNP, a NO donor (Fig. 1A, 1C). The survival rate of PC12 cells was about 40% in the cells treated with 500 μM SNP

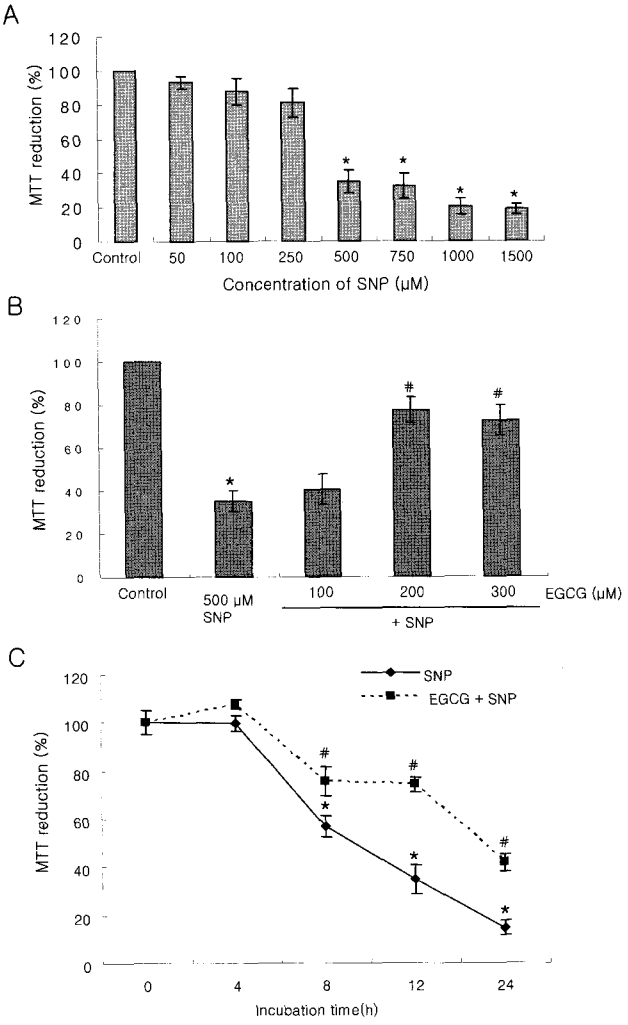

Fig. 1. Effects of SNP and EGCG on the viability of PC12 cells. After PC12 cells were treated with different concentrations of SNP for 12 h (A), or treated with different concentrations of EGCG for 30 min followed by 500 μM SNP (B), or 200 μM EGCG for 30 min before 500 μM SNP treatment (C), cell viability was determined by MTT assay. Each values are mean ±SD from 5 independent experiments. $^{*}p<0.05$, compared with control, $^{\#}p<0.05$, compared with 500 μM SNP-treated group.

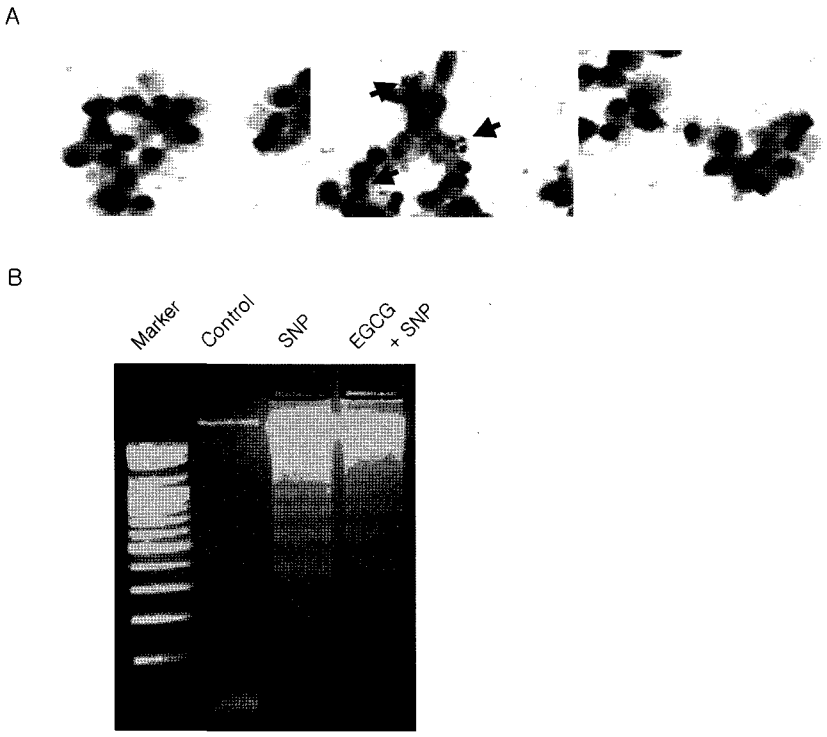

Fig. 2. Effect of EGCG on SNP-induced morphological changes of PC12 cells. PC12 cells were incubated in the absence (left) or presence of 500 μM SNP for 12 h with (right) or without pretreatment of EGCG for 30 min (middle). The cells were stained with Diff-Quick (1 μg/ml) and nuclear morphology was observed by fluorescence microscopy ($\times$100) (A). DNA was prepared from the cells treated with 500 μM SNP alone for 12 h or pretreated with 200 μM EGCG for 30 min before SNP treatment and electrophoresed and visualized with ethidium bromide (B).

for 12 h. The percentage of cell viability was near zero at 24 h after exposing to 500 μM SNP. The decreased cell viability was rescued in a dose-dependent manner by pretreatment of EGCG for 30 min (Fig. 1B).

EGCG inhibited NO-induced apoptosis in PC12 cells

The SNP induced the characteristics of apoptotic cell death over the staining with Diff-Quick and DNA fragmentation assay. Exposing the PC12 cells to 500 μM SNP for 12 h gave rise to the chromatin condensation, cell shrinkage and apoptotic body (Fig. 2A). DNA fragmentation, the biochemical hallmark of apoptosis, was shown by electrophoresis of genomic DNA in SNP-treated PC12 cells (Fig. 2B). The pretreatment of 200 μM EGCG for 30 min before SNP exposure attenuated the apoptotic morphologic changes in SNP-treated PC12 cells (Fig. 2). Fig. 3 showed that SNP induced the production of ROS in a dose-dependent manner, while pretreatment with 200 μM EGCG for 30 min ameliorated the SNP-induced ROS production in PC12 cells.

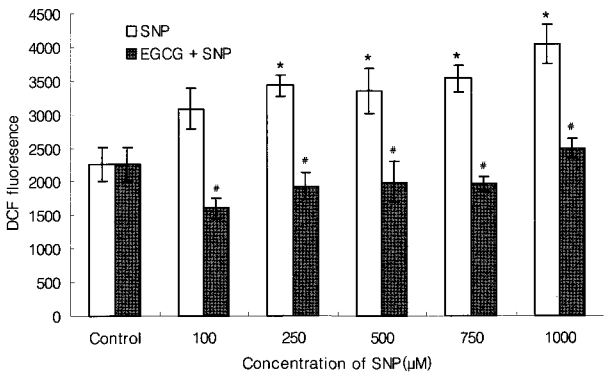

Fig. 3. Effect of EGCG on the enhanced ROS production in SNP-treated PC12 cells. PC12 cells loading DCF-DA were incubated with different concentration of SNP in the presence or absence of 200 μM EGCG for 30 min. The intracellular levels of ROS were detected by measuring the DCF fluorescence. Values are mean$\pm$SD from 5 independent experiments. $^{*}p<0.05$, compared with control. $^{\#}p<0.05$, compared with 500 μM SNP-treated group.

Effect of EGCG on the release of cytochrome c from mitochondria into cytosol and the expression of VDAC in SNP-treated PC12 cells

To evaluate whether mitochondrial dysfunction is involved in NO-induced apoptosis or not, the release of cytochrome *c* from mitochondria into cytosol was detected by western blotting in SNP-treated cells. As shown as Fig. 4A, treatment with 500 μM SNP for 12 h increased the release of cytochrome *c* from mitochondria into cytosol in PC12 cells. Besides, the expression of VDAC 1, a cytochrome *c* releasing channel in mitochondria, was upregulated after

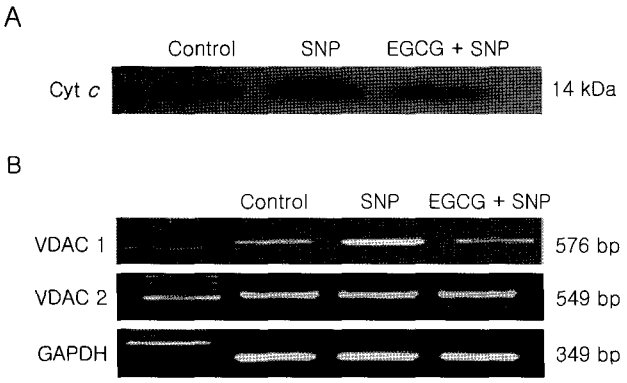

Fig. 4. Effect of EGCG on altered expression of cytochrome c and VDACs by SNP in PC12 cells. Levels of cytosolic cytochrome *c* (Cyt *c*) were determined using immunoblot after PC12 cells exposed to 500 μM SNP for 12 h with or without pretreatment of 200 μM EGCG for 30 min (A). Expression of VDACs were detected by RT-PCR (B).

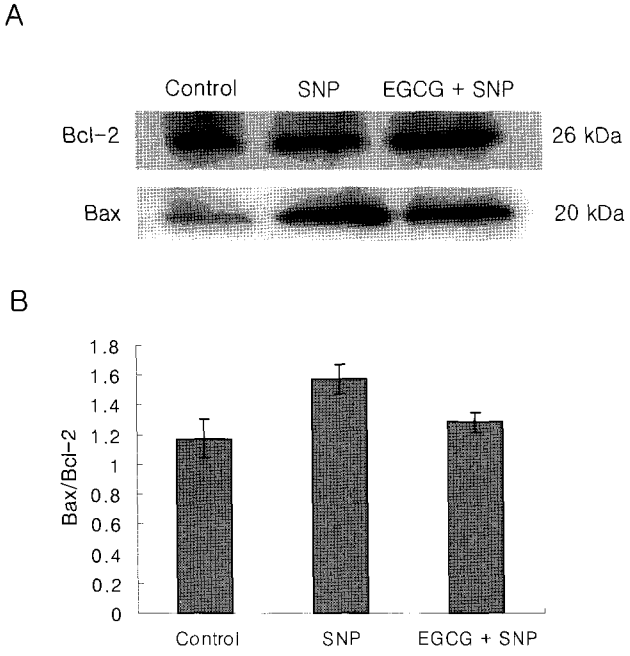

Fig. 5. Effect of EGCG on Bcl-2 and Bax expression in SNP-treated PC12 cells. The mRNA levels of Bax and Bcl-2 were determined by RT-PCR in 500 μM SNP-treated PC12 cells for 12 h with or without pretreatment of 200 μM EGCG for 30 min (A). The ratio of BAX and Bcl-2 determined by densitometer was calculated and graphed (B).

500 μM SNP treatment for 12 h, whereas VDAC 2 was not affected in SNP-treated PC12 cells (Fig. 4B). Pretreatment with 200 μM EGCG for 30 min suppressed the release of cytochrome *c* from mitochondria into cytosol as well as the upregulation of VDAC 1 by SNP.

Effects of EGCG on the expression of Bax and Bcl-2 in SNP-treated cells

Expression ratio of Bax to Bcl-2 has proven to be significant for apoptosis determination, meaning a high ratio denotes a low apoptotic threshold, or while a low ratio indicates a higher apoptotic threshold (Fennel and Cotter, 2000). In SNP-treated cells, expression of Bcl-2, an antiapoptotic molecule, was downregulated, whereas, Bax, a proapoptotic molecule, was upregulated indicating the low apoptotic threshold. However, pretreatment with 200 μM EGCG for 30 min ameliorated the altered expression of Bcl-2 and Bax by SNP, resulting in reduction of the ratio of Bax to Bcl-2 (Fig. 5).

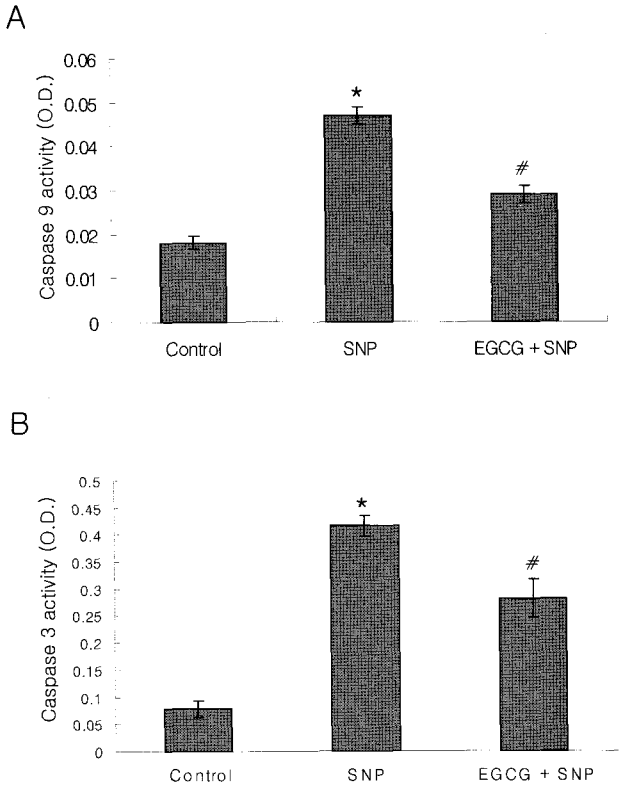

Fig. 6. Effect of EGCG on SNP-induced caspase-9 and caspase-3 activity in PC12 cells. Caspases activities were determined in 500 μM SNP treated PC12 cells for 12 h with or without pretreatment of 200 μM EGCG for 30 min. Enzymatic activity of caspase-9 protease was determined by incubation of 50 μg of total protein with LEHD-*p*NA substrate (200 μM) (A). Enzymatic activity of caspase-3 protease was determined by incubation of 50 μg of total protein with DEVD-*p*NA substrate (200 μM) (B).Values are mean$\pm$ SD from independent 5 experiments. $^{*}p<0.05$, compared with control. $^{\#}p<0.05$, compared with 500 μM SNP-treated group.

EGCG reduces the increased activity of caspases (-9 and -3) in SNP-treated cells

To determine whether mitocondrial dependent apoptotic signaling are involved in NO-induced apoptosis, the activities of initiating caspase (caspase-9) and executing caspase (caspase-3) were measured on the basis that activated caspases subsequently cleave their substrate at a specific site. SNP (500 μM, 12 h) increased the caspase-9 and -3 activities about 2.7 and 4.3 folds related to control, respectively. Pretreatment with 200 μM EGCG for 30 min significantly attenuated the SNP-induced caspase-9 and -3

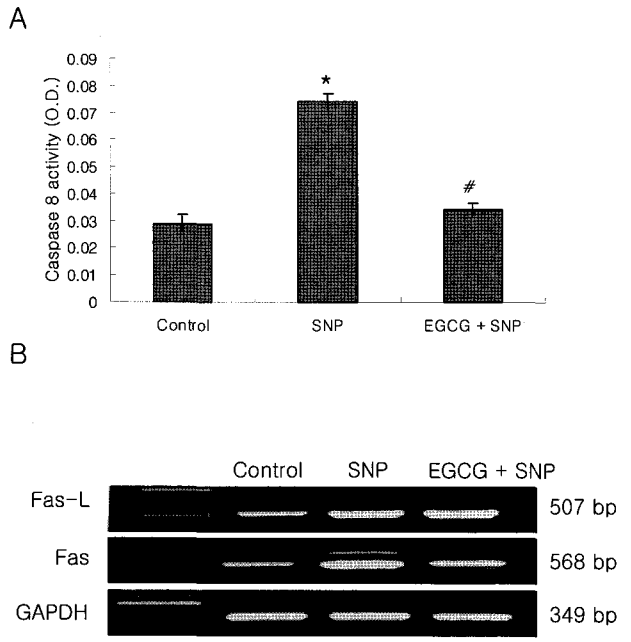

Fig. 7. Effect of EGCG on SNP-induced caspase-8 activity and Fas and Fas-L expression in SNP-treated PC12 cells. Cells were incubated in media containing 500 μM SNP for 12 h with or without pretreatment of 200 μM EGCG for 30 min. Enzymatic activity of caspase-8 protease was determined by incubation of 50 μg of total protein with IETD-pNA substrate (200 μM) (A). The mRNA levels of Fas and Fas-L, components of death receptor assemblies, were determined by RT-PCR (B). Values are mean±SD from independent 5 experiments. $^{*}p<0.05$, compared with control. $^{\#}p<0.05$, compared with 500 μM SNP- treated group.

activities in PC12 cells (Fig. 6).

Effect of EGCG on the expression of Fas/Fas-L and the activity of caspase-8 in SNP-treated PC12 cells

To know whether death receptor-mediated apoptosis pathway are activated in NO-induced apoptosis, expression of Fas and Fas-L, death receptor assemblies, and activity of caspase-8, death receptor mediated-initiating caspase, were observed. As shown in Fig. 7, treatment with 500 μM SNP for 12 h in the PC12 cells resulted in the upregulated mRNA expression in Fas and Fas-L, and the enhanced caspase-8 activity. While pretreament with 200 μM EGCG for 30 min ameliorated the upregulation of Fas and the activation of caspase-8 induced by SNP, but not Fas-L (Fig. 7).

DISCUSSION

NO-induced cell death in nervous systems is a major concern in various clinical entities such as brain ischemia, neurodegeneration and inflammation (Bolanos et al, 1997; Peuchen et al, 1997). PC12 is a cell line derived from rat pheochromocytoma and widely used as an *in vitro* model for investigating neuronal apoptosis, oxygen sensor mechanism, and neuronal differentiation (Kroll & Czyzyk-Krzeska, 1998). In the present experiment, SNP, a NO donor, decreased PC12 cell viability in a dose- and time-dependent manner. Besides, SNP-treated cells demonstrated DNA fragmentation showing ladder pattern, and apoptotic morphologic changes such as cell shrinkage and chromatin condensation. These results suggest that NO induces apoptotic cell death in PC12 cells because gross nuclear changes and DNA fragmentation patterns are critical events to differentiate between typical apoptosis and necrosis (Estman, 1995; Fraker et al, 1995).

NO-induced apoptosis is well documented to be driven through the production of ROS, resulting in formation of peroxynitrite (Brown, 1999; Yuyama et al, 2003). In the present study, SNP enhanced the production of ROS in PC12 cells in a dose-dependent manner. From these results, it is speculated that NO-induced apoptosis is likely to be driven from the production of ROS in PC12 cells, in consistent with those of previous reports.

In general, caspase-3 is a key and common protease in both mitochondria- and death receptor-dependent pathways and particularly an important in free radical-induced apoptosis (Earnshaw et al, 1999; Bal-Price et al, 2000). Previous studies have shown that caspase-3 is activated in response to various ROS (Leist et al, 1999; Bal-Price et al, 2000). The present study showed that caspase-3 activity was enhanced in SNP-treated PC12 cells, assuming that caspase-3 plays a pivotal role in NO-induced apoptosis in PC12 cells, even if caspase-independent cell death is proposed to involve in low concentration of NO-induced cell death in PC12 cells (Yuyama et al, 2003). The caspase-3 is activated via caspase-9 activation in response to the release of cytochrome *c* from the mitochondria, in concert Apaf-1 and dATP (Du et al, 2000). The present study showed that SNP increased cytochrome *c* release from mitochondria into cytoplasm. Besides, SNP enhanced the activity of caspase-9. Taken together, mitochondria-dependent apoptotic pathway is definitely proven to be involved in NO-induced apoptosis of the PC12 cells since cytochrome *c* and caspase-9 are major molecules associated with mitochondria-dependent pathway. Another possible mechanism for activating caspase-3 is caspase-8 mediated process activated by Fas and TNF receptor-1. Recent studies have reported that ROS such as H_2O_2 directly induces upregulation of death receptor assembles such as Fas and Fas-L, subsequently activating caspase-8 (Facchinetti et al, 2002; Fleury et al, 2002). From these previous reports, there is a possibility that death receptor-dependent apoptosis pathway may be involved in caspase-3 activation in NO-induced apoptosis of PC12 cells. In the present study, Fas and Fas-L was upregulated and caspase-8 activity was enhanced in SNP-treated PC12 cells, suggesting that death receptor-dependent pathway may be involved in NO-induced apoptosis of PC12 cells. From the present results, NO-induced apoptosis is likely to be mediated by both mitochondria and death receptor-mediated pathways in PC12 cells.

The Bcl-2 family are known to be well-characterized regulators of cytochrome *c* release from mitochondria into cytosol by regulating the mitochondrial PT pore composed of VDAC, ANT and Cyp-D. The Bcl-2 subfamily contains antiapoptotic proteins such as Bcl-2 and Bcl-X_L, which reduce cytochrome *c* release and a loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) (Gottlieb et al, 2000; Howard et al, 2002). The Bax subfamily contains pro-apoptotic proteins such as Bax and Bak, which induce cytochrome *c* release and a loss of $\Delta\Psi_m$ (Starkov et al, 2002). Thus, ratio of pro-apoptotic to anti-apoptotic Bcl-2 may be a pivotal cue to release of cytochrome *c* from mitochondria.

In NO-treated PC12 cells, these ratio of Bcl-2 family was increased, showing upregulated Bax and downregulated Bcl-2. These results elucidate the involvement of Bcl-2 family in NO-induced apoptosis of PC12 cells, which might be an important role in consistent with previous reports (Hemish et al, 2003; Huerta-Yepez et al, 2004). In addition, the expression of VDAC 1, a major component of mitochondrial PT pore, was upregulated, but VDAC 2 expression was not affected in SNP-treated PC12 cells. Some previous reports have documented that mitochondrial PT pore is opened in brain ischemia and related conditions such as hypoxia and hypoglycemia, subsequently resulting in the enhancement of cytochrome *c* release from mitochondria (Ankarcrona et al, 1995; Krajewski et al, 1999), whereas the expression of PT pore assembles was not studied in free radical-induced apoptotic condition. This present study demonstrates the first evidence that the expression of VDAC, particularly VDAC 1, was altered in NO-induced apoptosis of PC12 cells. From these present and previous results, it is assumed that the altered Bcl-2 family and VDAC expression are involved in the increased release of cytochrome *c* in NO-induced apoptosis. Although Bcl-2 family regulates the production of ROS and cytochrome *c* release from mitochondria (Gottlieb et al, 2000; Starkov et al, 2002), ROS conversely regulates the expression of Bcl-X_L mRNA (Herrera et al, 2001). Therefore, further researches will be required to determine the roles of the Bcl-2 family in NO-induced apoptosis of PC12 cells.

On the other hands, EGCG has been known to display a potent antioxidant property and its effects have been extensively studied. Previous several *in vitro* and *in vivo* reports have showed that EGCG has a neuroprotective effect against neuronal injury and hippocampal neuronal damage after transient global ischemia (Guo et al, 1996; Kondo et al, 1999; Lee et al, 2000). Besides, green tea extract has been demonstrated to protect brain, liver, and kidney from lipid peroxidation injury. More recently, several reports have documented that EGCG attenuates β-amyloid-induced neurotoxicity in cultured hippocampal neurons and reduces 6-hydroxydopamine-induced apoptosis in PC12 cells (Jin et al, 2000; Choi et al, 2001; Nie et al, 2002; Peng et al, 2002). Nagai *et al* (2002) reported the neuroprotective effects of EGCG against NO-induced neuronal damage after ischemia by acting as an anti-oxidant. However, its exact molecular mechanism is little known. EGCG remarkably reduced the cell death by SNP in PC12 cells. Moreover, EGCG inhibited apoptotic morphologic changes such as cell shrinkage, DNA fragmentation, and chromatin condensation in SNP-treated cells. The present study demonstrated that EGCG has a neuroprotective effect against SNP-induced neuronal apoptosis.

One of possible antiapoptotic mechanisms of EGCG may be driven from its direct scavenging of ROS produced by NO, since polyphenols such as EGCG can scavenge different kinds of ROS and organic free radicals, for example, superoxide anion, hydroxyl radicals, and lipid free radicals (Nie *et al*, 2002). In the present study, EGCG significantly ameliorated the increase of ROS production by SNP in PC12 cells. This result demonstrates that EGCG has antiapoptotic effect against NO-induced apoptosis through scavenging the ROS produced by NO in PC12 cells.

Another antiapoptotic mechanism of EGCG may be driven by regulating the proteins participated in NO-induced apoptosis. In SNP-treated PC12 cells, EGCG ameliorated the altered expression of Bcl-2 family and VDCA1, which was associated with cytochrome *c* release from mitochondria into cytosol. The antiapoptotic action of EGCG may be driven by regulating the expression of Bcl-2 family and VDAC. The recent some reports have documented that EGCG directly regulates the expression of Bcl-2 family such as Bax, Bad, Mdm2, Bcl-2, Bcl-W and Bcl-X_L (Levites et al, 2002; Mandel et al, 2004). Since it is unknown that EGCG directly or indirectly regulates the expression of Bcl-2 family and VDAC1 through scavenging ROS, further researches will be required to elucidate the regulating mechanisms of Bcl-2 family and VDAC by EGCG. In addition, the present study showed that EGCG ameliorated the increased release of cytochrome *c* from mitochondria, and also the enhanced caspase-9 and then caspase-3 activity by SNP. These results suggest that EGCG suppressed the release of cytochrome *c*, which activate caspase-9 and caspase-3 as downstream in mitochondria-dependent pathway, subsequently result in inhibition of NO-induced apoptosis. In addition, EGCG attenuated the enhanced expression of Fas, a death receptor assemble, but did not affect the enhanced expression of Fas-L in SNP-treated cells. Furthermore, caspase-8 activity, preceding death receptor-dependent apoptotic pathway, was suppressed by EGCG. Further research will be required to determine the regulation mechanism of Fas by EGCG.

Taken together, the present results suggest that NO-induced apoptosis is mediated by both mitochondria- and death receptor-dependent pathways and EGCG prevents NO-induced neuronal death through the scavenging of ROS and the regulation of Bcl-2 family, VDAC, caspases and Fas, which play an essential role in NO-induced apoptosis. Recently, it was reported that green tea polyphenols can pass through the brain-blood barrier to exert neuroprotective effects, since EGCG was present in brain, liver and plasma 60 min after the administration of green tea polyphenols (Levites et al, 2002; Mandel et al, 2003). Therefore, it is speculated that administration of green tea may prevent NO-induced neurotoxicity.

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