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Involvement of Caspases and Bcl-2 Family in Nitric Oxide-Induced Apoptosis of Rat PC12 Cells

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This study was aimed to investigate the nitric oxide (NO)-induced cytotoxic mechanism in PC12 cells. Sodium nitroprusside (SNP), an NO donor, decreased the viability of PC12 cells in dose- and time-dependent manners. SNP enhanced the production of reactive oxygen species (ROS), and gave rise to apoptotic morphological changes including cell shrinkage, chromatin condensation, and DNA fragmentation. Expression of Bax was not affected, whereas Bcl-2 was downregulated in SNP-treated PC12 cells. SNP augmented the release of cytochrome *c* from mitochondria into cytosol and enhanced caspase −8, −9, and −3 activities. SNP upregulated both Fas and Fas-L, which are known to be components of death receptor assembly. These results suggest that NO induces apoptosis of PC12 cells through both mitochondria- and death receptor-mediated pathways mediated by ROS and Bcl-2 family.

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

INTRODUCTION

Nitric oxide (NO) is a highly diffusible, short-lived free radical gas that is generated in many mammalian tissues and has a role in both physiological and pathological functions. NO is a second messenger endogenously produced by the family of NO syntheses and has been shown to be involved in a variety of biological signaling processes, including apoptosis. In the central and peripheral nervous systems, NO acts as a neurotransmitter or neuromodulator, whereas it can be neurotoxic at high concentration (Gross & Wolin, 1995; Dawson & Dawson, 1996). NO is implicated in many neuronal pathological processes such as brain ischemia, neurodegeneration and inflammation (Jenner & Olanow, 1996; Bolanos et al, 1997; Peuchen et al, 1997), and 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. Apoptosis is characterized by early chromatin condensation, DNA fragmentation, and cell shrinkage.

In general, apoptosis is driven by the activation of a family of cysteine protease called caspases, which then cleave a critical set of cellular proteins to initiate apoptotic cell death (Crompton, 2000; Roth et al, 2000). These caspases are expressed as proenzyme and are activated by upstream stimuli. Among mammalian caspases of at least 14 known members, those involved in apoptosis can further be subdivided into the initiator caspases (−2, −8, −9, and −10) and the effector caspases (−3, −6, and −7) (Adams & Cory, 1998; Tsujimoto & Shimizu, 2000).

Two main pathways of activating caspases are death receptor-mediated mechanism and mitochondria-mediated mechanism. Both pathways share the activation of caspase-3, which activates DNase, causing apoptotic DNA fragmentation. Death receptor-mediated 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 activation with subsequent activation of caspase-3. The mitochondria-mediated pathway is initiated by the release of cytochrome *c* from the mitochondria into cytosol, subsequently resulting in the activation of caspase-9, which causes the activation of caspase-3.

Besides the caspases, members of the Bcl-2 family are also critical for the regulation of apoptosis. Bcl-2 family controls the release of mitochondrial cytochrome *c* by regulating the mitochondrial permeability transition (PT) pore, which is composed of the voltage-dependent anion channel (VDAC) in the outermembrane, the adenosine nucleotide translocated (ANT) in the innermembrane, and cyclophilin-D (Cyp-D) in the matrix assembles (Ankarcrona et al, 1995; Krajewski et al, 1999; Crompton et al, 2000). Bcl-2 family members are functionally divided into anti-apoptotic molecules (Bcl-2, Bcl-X_L, Bcl-W, Mcl-1 and A1) and pro-apoptotic molecules (Bax, Bcl-1s, Bid, Bad, Bim and Bik) (Adams & Cory, 1998; Tsujimoto & Shimizu, 2000). Among the Bcl-2 family, Bcl-2 and Bcl-X_L are prominent anti-apoptotic family whereas Bax and Bid are prominent pro-apoptotic family (Cheng et al, 1997). Even if many different mechanisms of NO-induced cell death have been proposed (Lee et al, 1997; Murphy, 1999), the detailed mechanisms of NO-induced cell death still remain unsettled.

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ABBREVIATIONS: NO, nitric oxide; ROS, reactive oxide species; VDAC, voltage-dependent anion channel; NAC, N-acetyl-L-cysteine; RT-PCR, reverse transcription polymerase chain reaction: Fas-L, Fas-ligand.

The present study is aimed to investigate the roles of signal molecules associated with mitochondria- and death receptor-mediated apoptotic pathways in NO-induced apoptosis of PC12 cells.

METHODS

Cell culture and SNP treatment

PC12 rat pheochromocytoma cells were maintained in RPMI 1640 media containing 10% heat inactivated horse serum, 5% heat inactivated fetal bovine serum (Gibco-BRL, USA) and gentamicin (50 μg/ml, Gibco-BRL, USA) under 5% CO_2 at 37°C. Sodium nitroprusside (SNP, Sigma ST. Louis, USA) was dissolved in distilled H_2O and sterilized through a 0.2 μm filter before use. Cells were treated with various concentrations of SNP for times specified.

Cell viability assay with MTT

The MTT assay depends on the fact 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, providing an indirect measurement of cell viability. PC12 cells were plated onto 96 well plates and exposed to SNP. After treatment, MTT was added to the culture medium at various time points at a final concentration of 0.1 mg/ml and incubated at 37°C for 4 h. The reaction product of MTT was dissolved in dimethylsulfoxide (DMSO), and optical density (O.D) was measured at 570 nm with DMSO as a blank using a ELISA reader (ELx800uv, Bio Tek Instruments, Inc).

Nuclear staining with Diff-Quick

Morphological changes of apoptotic cells were investigated by Diff-Quick (Kuk Jae, Japan) stain. Cells plated in 8-well chamber slide at a density of 1×10^5 were incubated for 18 h, subsequently followed by treatment with 500 μM SNP for 12 h. The cells were then washed with phosphate buffered saline (PBS) and fixed with acetone and methanol (1 : 1). After incubating for 20 min at −20°C, cells were stained with 10 μg/ml Diff-Quick in PBS and observed under a fluorescence microscope (Olympus, Japan).

Agarose gel electrophoresis for DNA fragmentation

Oligonucleosomal fragmentation of genomic DNA was assessed using the Apopladder kit (TaKaRa, Shuzo, Japan) according to the manufacture instruction. Briefly, cells were lysed in 200 μl of lysis buffer and centrifuged at 1,100 g for 10 min. After adding 20 μl of 10% SDS and 20 μl of proteinase K (20 mg/ml), the supernatant was incubated at 56°C for 1 h 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 with 12,000 g at 4°C. DNA pellets were resuspended in 20 μl of TE buffer (pH 8.0), and aliquots from each sample were electrophoresed at 80 V for 2 h on 1.8% agarose gels. DNA bands were visualized under UV light after staining with ethidium bromide.

Flow cytometric analysis

After incubation with 500 μM SNP, cells were pelleted by centrifugation at 300 g for 10 min and washed with PBS. Cell pellets were resuspended in 0.2 ml of PBS containing 1 mg/ml propidium iodide (PI) and 0.1% Triton X-100. Samples were kept in the dark for 1 h at 4°C and fluorescence emitted from the PI-DNA complex was quantitated by flow cytometry (Becton Dickinson. San, CA).

Detection of ROS production

ROS production was monitered by a fluoresence spectrometer (Hitachi F-4500, Japan) using 2', 7'- dichlorofluorescin diacetate (DCF-DA). Cells were plated on 96-well plate and treated with SNP. DCF-DA (25 μM) was added into the media for 10 min at 37°C. The fluorescent emission was measured at 530 nm.

Measurement of caspase assay

Caspase activity was assayed using the caspase-3, -9 activity assay kits (Calbiochem, CA) and caspase-8 activity kits (Santa Cruz, USA) according to the manufacturer's instructions. Briefly, PC12 cells were grown on 100 mm dishes and treated with SNP for the indicated times. The media were removed from the culture dishes, and the cells were collected, washed with PBS, and 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 (BCA assay, Pierce). Fifty microliters of cell lysates were 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 at 405 nm was measured by an ELISA reader.

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 spectrophotometrically quantified at 260 nm. For synthesis of cDNA, 1 μg of total RNA and 1 μl of oligodT (10 pmol) were mixed with 50 μl of 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 pmol each of primer using PCR-premix kits (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 extention 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'-CAGCCCCTGAATTACCCATG-3' (sense primer), 5'-CACTCCAGAGATCAAAGCAG-3' (antisense primer); for VDAC1, 5'-TGATACCACGTTAGACCTCC-3' (sense primer), 5'-ACAACCTGGAAGCTATTTCA-3' (antisense primer); for VDAC2, 5'-TGCAGTGGTGTGGAATTTT-3' (sense primer), 5'-ACGAGTGCAGTTGGTACCTGA-3' (antisense primer). The amplified products were analyzed on

1.5% agarose gels containing ethidium bromide and visualized by UVP Transilluminator/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'-TGCATCCTGCACCACCAACT-3' (sense primer) and 5'-CGCCTGCTTCACCACCTTC-3' (antisense primer). The intensities of the bands obtained were determined using the NIH Scion Image Software.

Western blot analysis

Cells were washed twice with PBS, and proteins were solubilized in a 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, USA). Protein extracts (100~500 μg) were boiled for 5 min in SDS-sample buffer and then subjected to electrophoresis on 12% polyacrylamide gel. Proteins were electroblotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, UK) overnight at 20 mA at 4°C, blocked with 5% skim milk (Becton Dickinson, USA) in Tris-buffered saline-0.1% Tween 20 (TBS-T) for 1 h and incubated with the respective primary antibody. Rat monocloned antibodies against cytochrome *c* (Pharmingen, CA), Bax (Santa Cruz, USA) , Bcl-2 (Santa Cruz, USA) and β-actin (Santa Cruz, USA) were applied. Blots were subsequently washed three times with TBS-T for 5 min 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).

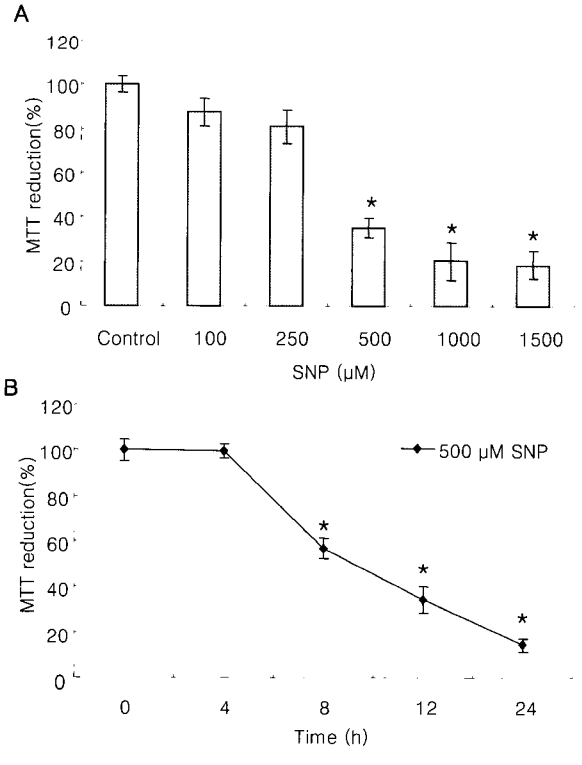

Fig. 1. The viability of SNP-treated PC12 cells. PC12 cells were incubated in media containing different concentrations of SNP for 12 h (A), and treated with 500 μM SNP for the time indicated (B). Cell viability was estimated by MTT assay. Each value represents mean±SD from 5 independent experiments. *$p<0.05$, compared with control.

RESULTS

NO induced ROS production and cell death in PC12 cells

As shown in Fig. 1, the cell viability was gradually reduced in dose- and time-dependent manners, when PC12 cells were exposed to SNP, a NO donor. The survival rate of PC12 cells was about 50% when the cells were treated with 500 μM SNP for 12 h. To determine the involvement of ROS in NO-induced cell death of PC12 cells, ROS production was measured using DCF-DA and, as seen in Fig. 2, SNP enhanced the ROS production in PC12 cells in a dose-dependent manner.

NO induced apoptosis in PC12 cells

Treatment of PC12 cells with 500 μM SNP for 12 h resulted in apoptotic morphologic changes, including chromatin condensation and cell shrinkage (Fig. 3A). DNA was isolated from SNP-treated PC12 cells, and the degradation of nuclear DNA into oligonucleosome fragments was assessed. The DNA ladder pattern was detectable in 500 μM SNP-treated PC12 cells (Fig. 3B). Furthermore, flow cytometric analysis indicated a specific DNA peak called apoptotic peak (or sub G1 peak) appeared when apoptotic cells were stained with PI (Fig. 3C). Since this peak represents one of the characteristics of apoptosis, these results demonstrate that the cell death of PC12 cells by SNP occurred via apoptosis.

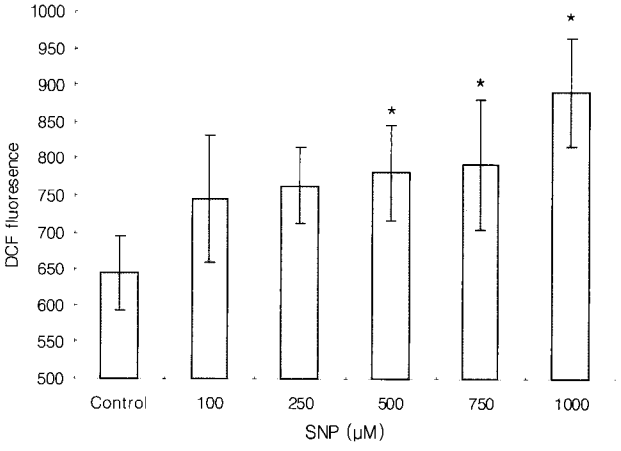

Fig. 2. ROS production was enhanced in SNP-treated PC12 cells. PC12 cells were treated with indicated concentrations of SNP at for 30 min. The intracellular levels of ROS were detected by measuring the DCF fluorescence. Each value represents mean±SD from 5 independent experiments. *$p<0.05$, compared with control.

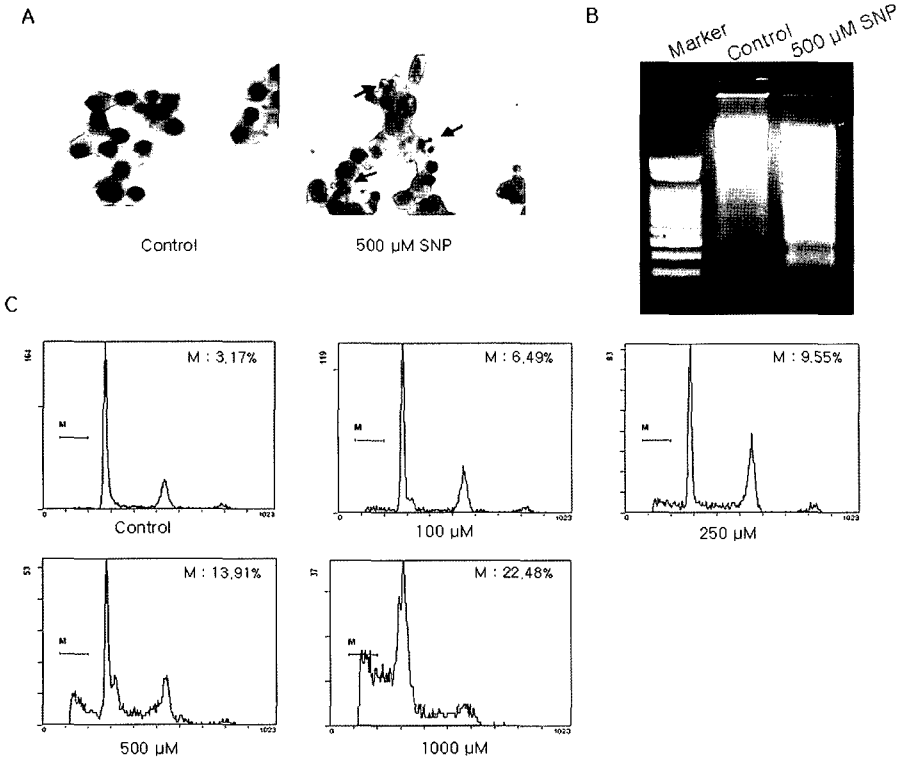

Fig. 3. NO-induced apoptosis in PC12 cells. PC12 cells were incubated in the absence or presence of 500 μM SNP for 12 h, and then were stained with Diff-Quick. Arrows indicate apoptotic bodies (A). DNA fragmentation assay was performed. M: 1 Kb ladder (B). PC12 cells were treated with indicated concentrations of SNP for 12 h. DNA contents in cells were measured by flow cytometry analysis (C).

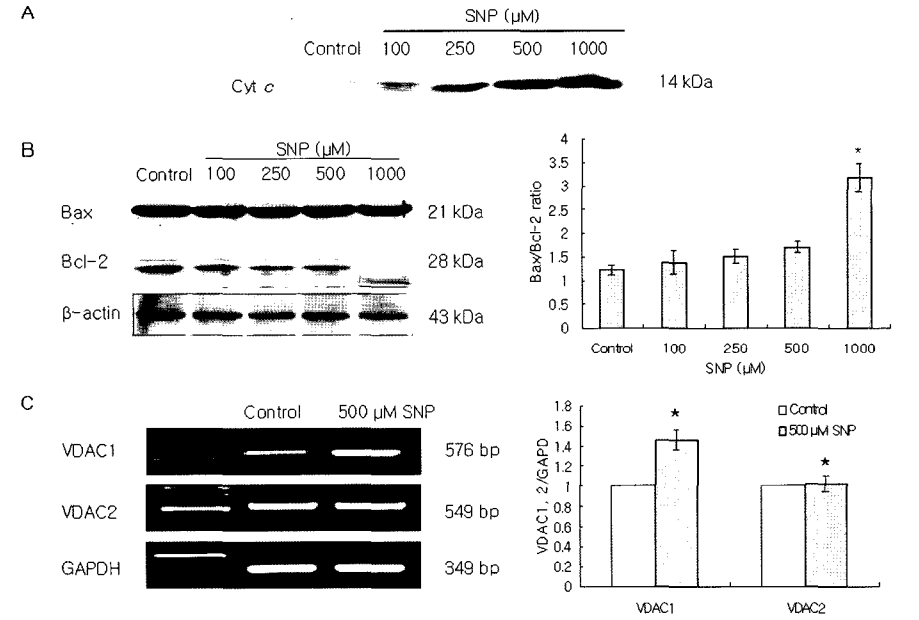

Fig. 4. NO-induced apoptosis of PC12 cells was mediated with mitochondria. PC12 cells were treated with indicated concentrations of SNP for 12 h. Protein levels of cytosolic cytochrome *c* (A), Bax and Bcl-2 (B) were analyzed by Western blot analysis. Expression of VDACs was detected by RT-PCR analysis. M: 100 bp ladder (C). Each value represents mean±SD from 5 independent experiments. *$p<0.05$, compared with control.

NO-induced apoptosis of PC12 cells was mediated with mitochondria

To evaluate whether mitochondria are involved in NO-induced apoptosis of PC12 cells, the amount of cytochrome *c* released from mitochondria into cytosol was determined by the method described previously. Thus, PC12 cells were incubated with different concentrations of SNP for different periods, and cytosolic fractions were subjected to Western-blot. As shown in Fig. 4A, cytosolic cytochrome *c* was increased in a dose-dependent manner in response to exposure of SNP. Expression ratio of Bax to Bcl-2 has proven to be significant for apoptosis determination, since a high ratio denotes a low apoptotic threshold for the cytochrome *c* release from mitochondria into cytosol, while a low ratio indicates a high apoptotic. Expression of Bcl-2, an antiapoptotic molecule, was downregulated in SNP- treated cells, while Bax, a proapoptotic molecule, was not affected in 500 μM SNP-treated cells for 12 h, achieving the low apoptotic threshold (Fig. 4B). VDAC1, a cytochrome *c* releasing channel in mitochondria, was upregulated in SNP-treated PC12 cells, whereas VDAC2 was not affected (Fig. 4C). The results demonstrate that cytochrome *c* is released from mitochondria into cytoplasm during NO-induced apoptosis of PC12 cells.

Caspases was involved in NO-induced apoptosis of PC12 cells

To determine whether caspases are involved in NO-induced apoptosis, executing caspase (caspase-3) activity and initiating caspases (caspase-8 and -9) activities were measured, because activated caspases subsequently cleave their substrate at a specific site. SNP (500 μM, 12 h) induced dramatic increment in all of caspase-3, -8 and -9 activities (Fig. 5). The level of caspase-8 activity was increased 5 fold, caspase-9 about 4.5 fold and caspase-3 about 4 fold after exposure to SNP, compared with control values.

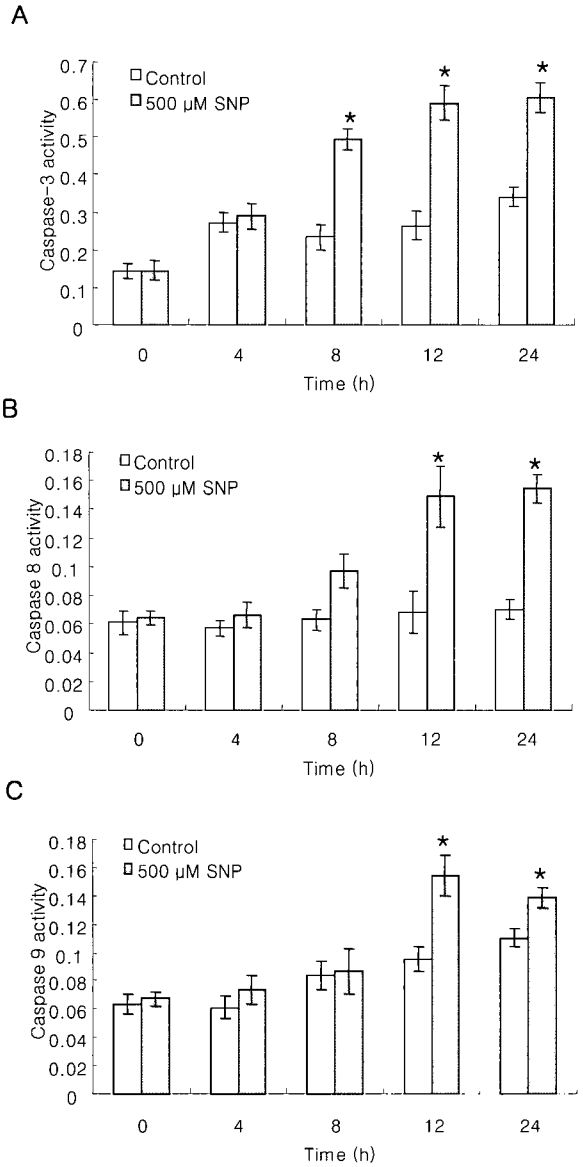

Fig. 5. NO-induced apoptosis occurred via caspases activation in PC12 cells. PC12 cells were treated with 500 μM SNP for indicated time periods. Extracts from NO-treated or untreated cells were assayed for caspase-3 (A), caspase-8 (B), caspase-9 (C) activity using colorimetric peptide substrate. Each value represents mean±SD from 5 independent experiments. *$p<0.05$, compared with control.

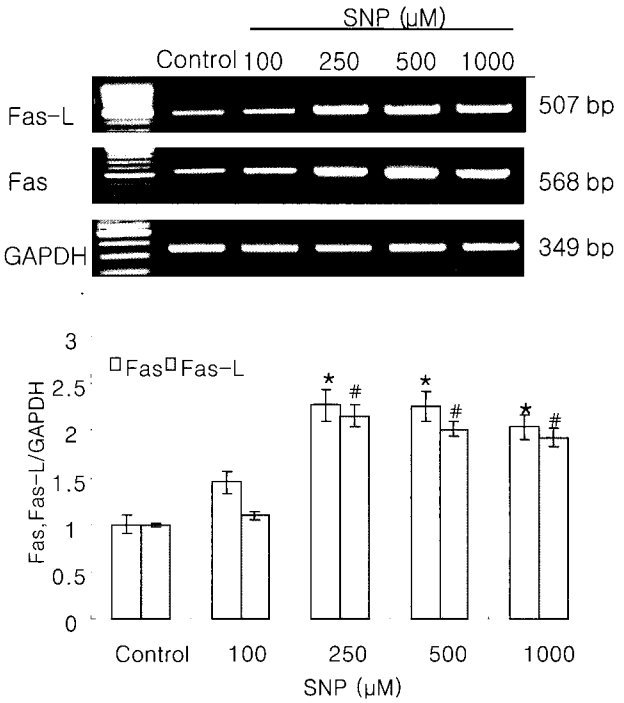

Fig. 6. Upregulated Fas and Fas-L in SNP-treated PC12 Cells. PC12 cells were treated with indicated concentrations of SNP for 12 h. The mRNA levels of Fas and Fas-L were determined by RT-PCR analysis. Each value represents mean±SD from 5 independent experiments. *$p<0.05$, compared with control (Fas), #$p<0.05$, compared with control (Fas-L).

Fas and Fas-L expressions were upregulated in SNP-treated PC12 cells

To know whether death receptor-mediated apoptosis pathways are activated in PC12 cells, the mRNA levels of Fas and Fas-L, the death receptor assembly, were determined using RT-PCR. Both Fas and Fas-L expressions were shown to increase about 3 fold and 2.8 fold, respectively, in SNP-treated cells (500 μM, 12 h) compared with controls (Fig. 6). These results suggest that death receptor mediated pathway may play a role in SNP-induced apoptosis of PC12 cells.

DISCUSSION

NO system has been implicated in a wide range of physiological functions in the nervous system. 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). In the present study, SNP were shown to result in a decrease of PC12 cell viability in dose- and time-dependent manners. Furthermore, SNP-treated cells showed not only DNA fragmentation, revealed by ladder pattern, but also apoptotic morphologic changes such as cell shrinkage, chromatin condensation and fragmented DNA. Taken together, the present results suggest that NO induces apoptosis 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).

It has been well documented that NO-induced apoptosis is driven through the production of ROS and the reaction with superoxide, resulting in the formation of peroxynitrite (Brown, 1999; Yuyama et al, 2003). In the present study, SNP enhanced the production of ROS in a dose-dependent manner in PC12 cells. These results suggest that NO-induced apoptosis of PC12 cells is likely to be driven through the production of ROS, in consistent with previous reports.

A variety of free radicals such as ROS and peroxynitrite are known to impair mitochondrial function, subsequently resulting in loss of mitochondrial transmembrane potential and release of mitochondrial pro-apoptotic molecules including cytochrome *c*, Smac and apoptosis-inducing factor (AIF) (Herrera et al, 2001; Fleury et al, 2002). The present study showed that SNP resulted in an increment of cytochrome *c* release from mitochondria into cytoplasm. In addition, SNP enhanced the activities of caspase-9. Taken together, mitochondria-dependent apoptotic pathway is clearly 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.

In general, caspase-3 is a key and common protease in both mitochondria- and death receptor-dependent pathways, and particularly is important in free radical-induced apoptosis (Earnshaw et al, 1999; Bal-Price & Brown, 2000). Previous studies have shown that caspase-3 is activated in response to various ROS (Leist et al, 1999; Bal-Price & Brown, 2000). The present study showed that caspase-3 activity was enhanced in SNP-treated PC12 cells, which is consistent with previous reports using other tissues and cells. The present study together with previous reports indicate that caspase-3 plays a pivotal role in NO-induced apoptosis of PC12 cells, even if caspase-independent cell death has been proposed to be involved in low concentration of NO-induced cell death of PC12 cells (Yuyama et al, 2003).

Another possible mechanism for activating caspase-3 involves caspase-8 mediated process activated by Fas and TNF receptor-1. Recent studies show that ROS such as H_2O_2 directly upregulates death receptor assembles such as Fas and Fas-L, subsequently activating caspase-8 (Facchinetti et al, 2002; Fleury et al, 2002). These previous reports suggest 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, a death receptor assembles, were shown to be upregulated and caspase-8 activity was enhanced in SNP-treated PC12 cells. Therefore, NO-induced apoptosis of PC12 cells is likely mediated by both mitochondria and death receptor-mediated pathways.

The Bcl-2 family is known to be well-characterized regulators of cytochrome *c* release from mitochondria into cytosol by regulating the mitochondrial PT pore which is composed of VDAC, ANT and Cyp-D. The Bcl-2 subfamily contains anti-apoptotic proteins such as Bcl-2 and Bcl-X_L, which reduce cytochrome *c* release and a loss of mitochodnrial transmembrane potential ($\Delta\psi_m$) (Gottlieb et al, 2000; Howard et al, 2002). On the other hand, 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). Bcl-2 proteins such as Bid, Bik and Bim are another subfamily of pro-apoptotic proteins, which are activated by caspase-8. Thus, the ratio of pro-apoptotic and anti-apoptotic Bcl-2 family may be pivotal cue for the release of cytochrome *c* from mitochondria. In the present study, therefore, the expressions of Bcl-2 and Bax were examined to elucidate the involvement of Bcl-2 family in NO-induced apoptosis: Bcl-2 mRNA was downregulated, whereas Bax mRNA was upregulated in SNP-treated PC12 cells. NO has earlier been reported in other tissues and cells to directly or indirectly regulate Bcl-2 family expression (Hemish et al, 2003; Huerta-Yepez et al, 2004). The present and previous results suggest that Bcl-2 family is involved in NO-induced apoptosis of PC12 cells. It is of interest to note in the present study that the expression of VDAC1, a major component of mitochondrial PT pore, was upregulated, subsequently resulting in enhanced release of cytochrome *c* release from mitochondria, but VDAC2 expression was not affected in SNP-treated PC12 cells. Some previous reports document that mitochondrial PT pore is opened in brain ischemia and related conditions such as hypoxia and hypoglycemia (Ankarcrona et al, 1995; Krajewski et al, 1999), although the expression of PT pore assembles was not studied in free radical-induced apoptotic condition. The present study demonstrated for the first time that the expression of VDAC, particularly VDAC1, was altered in NO-induced apoptosis of PC12 cells. The present and previous results indicate that altered Bcl-2 family and VDAC expression are involved in the increments of cytochrome *c* in NO-induced apoptosis. Further studies are needed to elucidate the underlying mechanisms of VDAC1 expression in NO-induced apoptosis. Another interesting result in the present study was that Bid was activated by SNP, since, unlike other Bcl-2 family, Bid is known to be activated by caspase-8. Based on the present data, it is speculated that there is a functional lineage between the death receptor-mediated apoptotic signals and the mitochondria-mediated apoptotic signals. Since Bcl-2 family regulates the produ-

ction of ROS, the cytochrome *c* release from mitochondria to cytosol and ROS could conversely regulate the expression of Bcl-X_L mRNA (Gottlieb et al, 2000; Herrera et al, 2001; Starkov et al, 2002). Further studies are required to determine the roles of the Bcl-2 family in NO-induced apoptosis of PC12 cells.

In summary, the present results suggest that NO-induced apoptosis of PC12 cells is mediated by both mitochondria- and death receptor-mediated pathways, involving ROS and Bcl-2 family.

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