Mechanism of nitric oxide-induced apoptosis in human neuroblastoma SH-SY5Y cells

Ryuichi Moriya, Takashi Uehara, Yasuyuki Nomura*

Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

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Abstract We have attempted to elucidate the precise mechanism of nitric oxide (NO)-induced apoptotic neuronal cell death. Enzymatic cleavages of DEVD-AFC, VDVAD-AFC, and LEHD-AFC (specific substrates for caspase-3-like protease (caspase-3 and -7), caspase-2, and caspase-9, respectively) were observed by treatment with NO. Western blot analysis showed that pro-forms of caspase-2, -3, -6, and -7 are decreased during apoptosis. Interestingly, Ac-DEVD-CHO, a caspase-3-like protease inhibitor, blocked not only the decreases in caspase-2 and -7, but also the formation of p17 from p20 in caspase-3 induced by NO, suggesting that caspase-3 exists upstream of caspase-2 and -7. Bongkrekic acid, a potent inhibitor of mitochondrial permeability transition, specifically blocked both the loss of mitochondrial membrane potential and subsequent DNA fragmentation in response to NO. Thus, NO results in neuronal apoptosis through the sequential loss of mitochondrial membrane potential, caspase activation, and degradation of inhibitor of caspase-activated DNase (CAD) (CAD activation).

Key words: Nitric oxide; Apoptosis; Mitochondrion; Caspase

1. Introduction

Caspases comprise a structurally related group of cysteine proteases that share a dominant primary specificity for cleaving peptide bonds following Asp residues. To date, 14 mammalian homologues of caspases have been identified and classified into three groups based on their homology, structure, and substrate specificity [1,2]. Caspases are typically constitutively present within cells as inactive zymogens that require proteolytic processing to achieve their active, two-chain configurations [3–6]. Caspases are known to cleave a number of proteins, including other caspases. In consequence, it is believed that apoptosis occurs in a caspase-sensitive manner.

A direct activation of caspase-mediated apoptosis has been found to involve cytochrome *c*, an essential component of the mitochondrial respiratory chain [7]. In many systems, the release of cytochrome *c* from mitochondria to the cytosol has been demonstrated to be a crucial step in the activation of apoptosis [8–10]. Once released from mitochondria, cytochrome *c*, in interaction with apoptotic protease activity factor-1 (Apaf-1) and caspase-9, initiates the cleavage and activation of caspase-3 [11]. Active caspase-3 in turn activates other executive caspases and leads to the subsequent characteristic features of apoptosis, including the cleavage of fodrin and lamin, chromatin condensation, and nuclear fragmentation [12]. In particular, it has recently been reported that inhibitor of caspase-activated DNase (ICAD) and acinus, important factors in DNA fragmentation and condensation, have been isolated and identified to be substrates for caspase-3 [13–15]. On the other hand, Bcl-2 and other related anti-apoptotic proteins can prevent cytochrome *c* release and inhibit caspase activation and apoptosis [8,9,16]. Although the mechanisms by which cytochrome *c* is released from the mitochondria remain unknown, some observations suggest that cytochrome *c*-mediated apoptosis is probably distinct from that mediated by AIF [17].

It is widely accepted that inducible nitric oxide (NO) synthase, which produces a large amount of NO, is expressed in response to several stresses, including brain ischemia and treatment with bacterial endotoxin and inflammatory cytokines [18–20]. Although NO is involved in neurotoxicity after several stresses such as ischemia, the crucial mechanism of NO-induced cytotoxicity is still unclear. Some mechanisms regarding NO-induced cytotoxicity in neurons have been proposed. It has been reported that NO can enhance ADP-ribosylation or S-nitrosylation of an increasing number of proteins, and two of these proteins have been identified as NO-target proteins [21,22]. One is glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is S-nitrosylated by NO, thereby inhibiting enzyme activity. GAPDH is known to be a key enzyme in the glycolytic conversion of glucose to pyruvic acid and represents an important pathway for carbohydrate metabolism in most organisms [23]. Hence, the inhibition of GAPDH activity by NO would diminish the amount of ATP formed from glycolysis and decrease the flow of substrates to the electron transport chain. NO also activates poly ADP-ribosyl synthetase (PARS) in association with damage to DNA [22]. The damage may be a critical event in NO neurotoxicity through the *N*-methyl-D-aspartate receptor. DNA damage stimulates PARS activity, resulting in the addition of a large number of ADP-ribose groups to substrates such as histones and PARS itself. Once massive DNA damage and

*Corresponding author. Fax: (81)-11-706 4987.
E-mail: nomura@pharm.hokudai.ac.jp

Abbreviations: DEVD-AFC, Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICAD, inhibitor of caspase-activated DNase; LEHD, Leu-Glu-His-Asp; NO, nitric oxide; SDS, sodium dodecyl sulfate; VDVAD, Val-Asp-Val-Ala-Asp

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the associated extensive activation of PARS occur, β-nicotinamide adenine dinucleotide, which is the donor of the ADP-ribose groups, is depleted. Consequently, it is believed that ATP is also depleted, with cell death occurring due to energy depletion. In addition to the indicated above actions of NO, a small GTP-binding protein Ras, mitogen-activated protein kinase (MAPK), stress-activated protein kinase, and p38 MAPK have been found to be activated by NO in several cells [24–28]. These findings indicate that NO can modulate the intracellular signal transduction activated by growth factors, cytokines, or stresses.

We have recently demonstrated that NO triggers apoptotic neuronal death via caspase activation following mitochondrial damage [29]. Moreover, NO elicits the release of cytochrome *c* into the cytosol during apoptosis. These results suggest that both the stimulation of caspase activity and cytochrome *c* release are heavily involved in NO-induced neuronal apoptosis. However, a detailed mechanism for the NO activation of the death signal has not yet been determined. In the current study, we attempted to elucidate the mechanism of NO-induced neuronal apoptosis.

2. Materials and methods

2.1. Materials

Synthetic peptide-based substrates for caspase-3-like protease (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC)), caspase-2 (*N*-acetyl-Val-Asp–Val-Ala-Asp-7-amino-4-trifluoromethylcoumarin (Ac-VDVAD-AFC)), and caspase-9 (*N*-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (Ac-LEHD-AFC)) were purchased from MBL (Nagoya, Japan). NOC18 was from Dojindo Laboratory (Kumamoto, Japan). Anti-caspase-2, -3, -6, -7 antibodies were purchased from Pharmingen (USA). Cyclosporin A (CsA) and bongkrekic acid (BA) were from Sigma (USA) and BIOMOL (USA), respectively.

2.2. Cell culture

Human SH-SY5Y cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C in humidified 5% CO_2/95% air.

2.3. Assay for caspase activity

Caspase-2, -3, -7, and -9 activities were measured using caspase-2, caspase-3, and caspase-9 fluorometric protease assay kits. This assay is based on detecting the cleavage of substrate VDVAD-AFC (caspase-2), DEVD-AFC (caspase-3 and -7), or LEHD-AFC (caspase-9), as described previously [30,31]. Briefly, cells were treated with 250 μM NOC18 for the indicated periods. At the appropriate time, the medium was aspirated, and the cells were washed with phosphate-buffered saline and added to 50 μl of ice-cold cell lysis buffer. The cells were incubated for 10 min on ice and then the cell lysates were centrifuged for 30 min at 15 000 rpm at 4°C. Next, cleared lysate (100 μg of protein) was incubated at 37°C for 1 h with adding to 50 μl of reaction buffer containing 50 μM substrate. The amounts of released AFC were measured with a fluorescent spectrophotometer with excitation at 400 nm and emission at 505 nm.

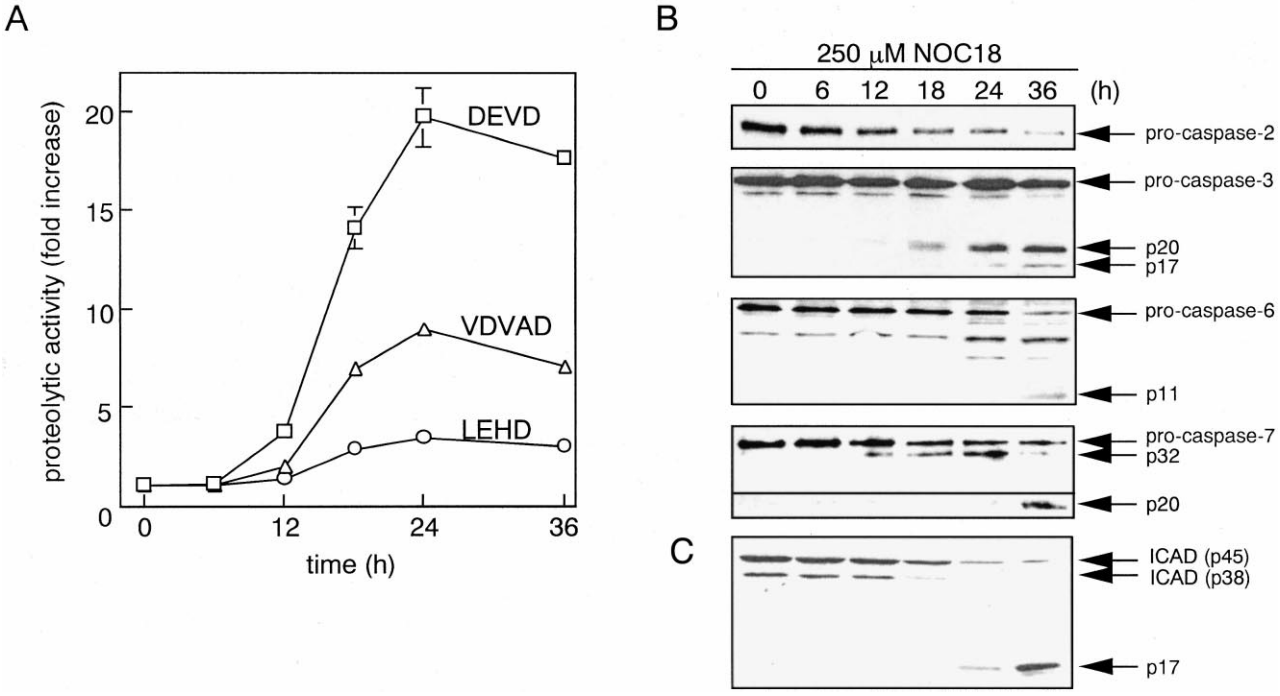

Fig. 1. NO activates caspases. A: Time-course of the activation of substrate cleaving activity in cell extracts after NOC18 challenge. The cells were stimulated with 250 μM NOC18 for the indicated periods (0–48 h). Cell lysates were then prepared, and an aliquot (100 μg) was used for caspase-2, -3-like proteases, and caspase-9 assay. A comparison of the fluorescence of AFC with that of an unstimulated sample allows for the determination of the fold increase in caspase activity. Data are the means of triplicate runs in parallel. B: Decrease in pro-forms of caspase-2, -3, -6, and -7 in response to NOC18 in SH-SY5Y cells. The cells were incubated with 250 μM NOC18 for various periods. Total cell lysates were prepared and then subjected to Western blot analysis using specific antibodies for caspase-2 (C-20, Santa Cruz), caspase-3, -6, and -7 (Pharmingen). Kinetic analyses of the levels of caspases are shown. Each cleaved fragment during apoptosis is indicated by an arrow. C: Western blot analysis of the cleavage of ICAD amino-terminal region by caspase(s) during NOC18-mediated apoptosis in SH-SY5Y cells. The same cell lysates shown in B were examined by Western blotting with an ICAD amino-terminal-specific. The p17 fragment, DFF-45 (NT), generated is indicated, as well as the DFF-45 and DFF-45-partial (in this case referring to the alternatively spliced, partial length DFF-45 protein) products. Only the DFF-45 amino-terminal-specific antiserum detects the second, smaller DFF-45 alternatively spliced product.

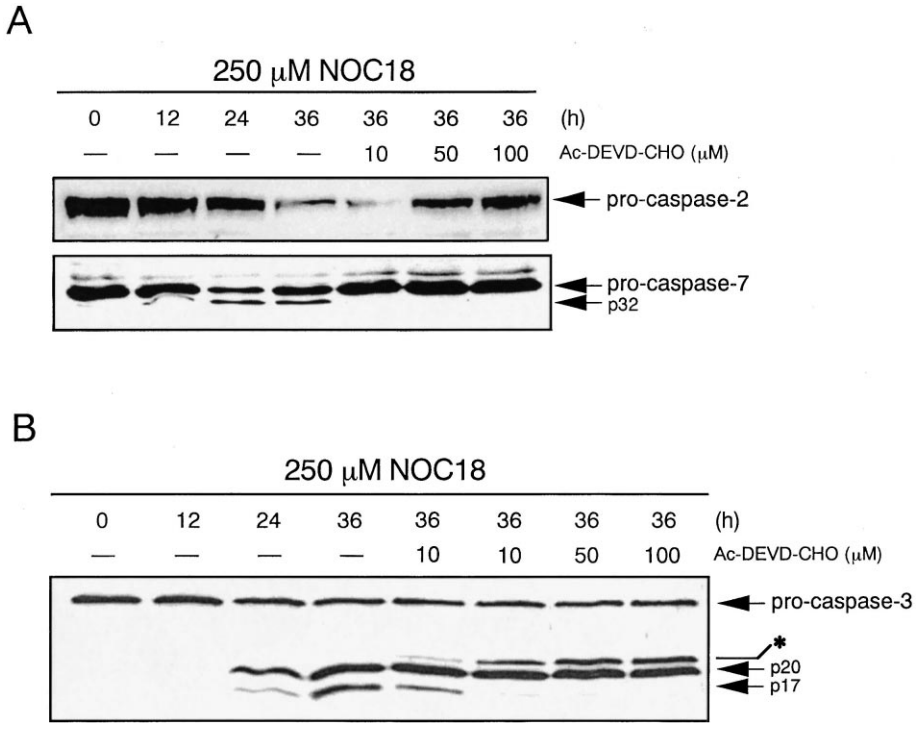

Fig. 2. Sensitivity of caspase-2, -3, and -7 activation to an irreversible inhibitor of caspase-3-like protease (Ac-DEVD-CHO) in NOC18-treated SH-SY5Y cells. SH-SY5Y cells were treated or not treated with several concentrations of Ac-DEVD-CHO, a cell-permeable caspase-3-like protease inhibitor, for 30 min before NOC18 challenge. The cells were then incubated with 250 μM NOC18 for various periods (0–36 h) and subjected to Western blot analysis. Effects of Ac-DEVD-CHO on the processing of caspase-2 and -7 (A), and caspase-3 (B) are shown. In B, asterisk shows the cleavage product 22 kDa in size, which reacts with anti-caspase-3 antibody.

2.4. Western blot analysis

The cells were collected and suspended in 50 mM Tris–HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The cells were then disrupted by a Dounce homogenizer with 20 strokes and centrifuged at 15 000 rpm for 20 min at 4°C, as described [32,33]. The supernatants were used for the immunoblotting of caspase-2, -3, -6, and -7, cytochrome *c*, and ICAD, as described previously [30,34]. 20 μg of protein was separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred onto a nitrocellulose membrane. The following were used as primary antibodies: anti-human caspase-2, -3, -6, and -7, cytochrome *c* (Pharmingen), and anti-human DFF-40 (ICAD) (MBL, Japan). Horseradish peroxidase-conjugated anti-mouse or rabbit IgG (Amersham) was used as the secondary antibody. Detection of the bands was performed using the ECL system (Amersham).

2.5. Detection of mitochondrial membrane potential

Mitochondrial membrane potential was measured by the incorporation of the cationic fluorescent dye rhodamine 123. Briefly, cells were incubated for 15 min at 37°C in the presence of 10 μM rhodamine 123. Fluorescent intensity was analyzed with a FACScan Flow Cytometer (Becton Dickinson). As a control experiment, cells were incubated with 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (mCICCP), an uncoupler.

2.6. Detection of reactive oxygen species (ROS) generation

To measure the ROS generation in response to NOC, cells were exposed to 5 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 10 min at 37°C. The fluorescent intensity was analyzed with a FACScan Flow Cytometer (Becton Dickinson).

2.7. Assessment of apoptosis by deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL)

The apoptotic cells were detected by TUNEL of nucleosomal fragments generated by endonuclease cleavage using a MEBSTAIN Apoptosis kit II (MBL), as reported previously [35]. All images were taken on a laser-scanning confocal microscope (LSM510; Carl Zeiss).

3. Results

3.1. NO-induced caspase activation in SH-SY5Y cells

We initially investigated the effect of NOC18, an NO donor, on caspase activation. Detergent extracts prepared from the cells at various periods after a 250 μM NOC18 treatment were tested for the cleaving activity of three fluorogenic peptide substrates (DEVD-AFC, VDVAD-AFC, and LEHD-AFC). DEVD-AFC is a fluorogenic, tetrapeptide substrate that is cleaved by caspase-3-like protease (mainly caspase-3 and -7). VDVAD-AFC and LEHD-AFC are specific substrates for caspase-2 and caspase-9, respectively. All enzymatic cleavages of DEVD-AFC, VDVAD-AFC, and LEHD-AFC were detected 12 h after the NOC18 challenge. Their cleaving activities were clearly elevated and sustained with NOC18 treatment. Maximal DEVD-AFC, VDVAD-AFC, and LEHD-AFC cleaving activities were detected at 24 h following NOC18 treatment (Fig. 1A). We next evaluated the cleavage of each caspase in response to NOC18 by Western blot analysis. Caspase-2, -3, -6, and -7 are synthesized as precursor molecules, and are approximately 48, 32, 34, and 34 kDa in size, respectively. Immunoblotting analysis revealed that proforms of each caspase decrease in NOC18-treated cells in a time-dependent manner (Fig. 1B). In particular, new bands corresponding to p20 and p17 of caspase-3 were detected at 18 h after NOC18 challenge in samples in which the processing of pro-caspase-3 was evident. The p11 active subunit of caspase-6 was detected at 36 h after treatment with NOC18. Moreover, the intermediate band p32 of caspase-7 (lack of prodomain) was visible after a 12 h incubation period with 250 μM NOC18 with the use of anti-caspase-7 antibody, a

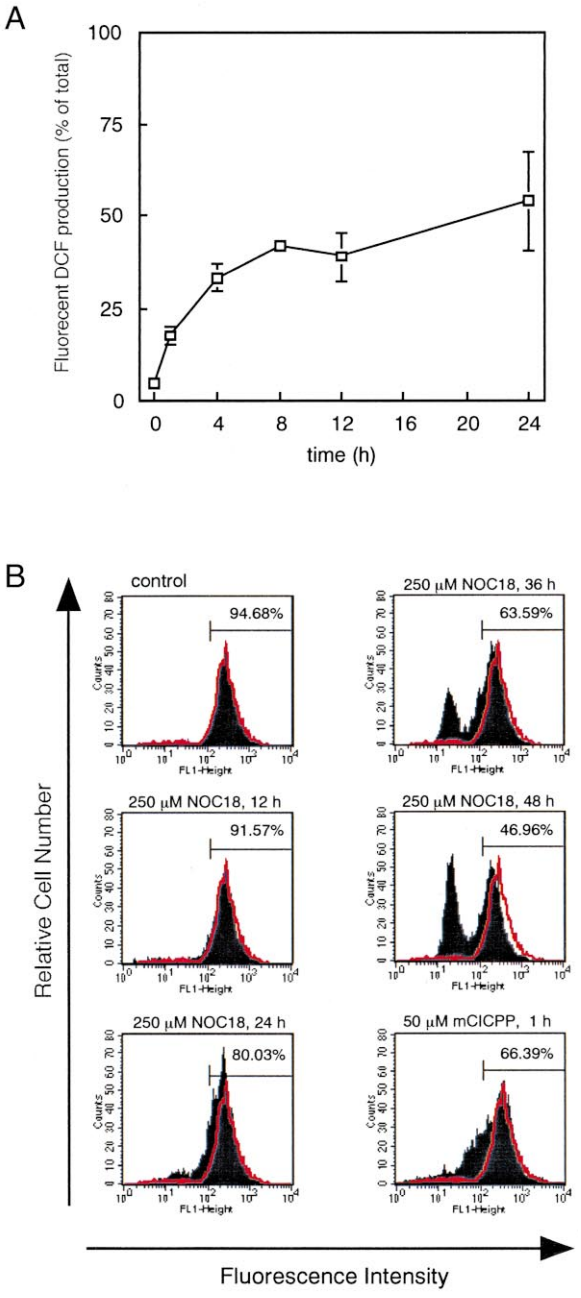

Fig. 3. ROS production and the reduction of mitochondrial membrane potential after NOC18 treatment in neuronal cells. A: Kinetic analysis of ROS production by NOC18 treatment. Cells were incubated with 250 μM NOC18 for various periods (0–24 h) and then stained for 10 min with 5 μM DCFH-DA before assay. Thereafter, the cells were washed twice, placed into suspension, and evaluated on the flow cytometer (fluorescence-activated cell sorting). The percentages reflect the production of peroxides (DCF fluorescence). Data are typical results from three independent experiments. B: Changes in the mitochondrial membrane potential during NOC18-induced apoptosis. Cells were incubated for 15 min at 37°C with the potential-sensitive dye rhodamine 123 (10 μM). After incubation, cells were immediately analyzed by a flow cytometer. The results are representative of one of four separate experiments. The percentage was quantitated using a FACScan Flow Cytometer. As a control, cells were incubated before analysis with 50 μM mCICCP for 1 h.

peptide corresponding to amino acids 25–42 of human caspase-7, as an immunogen (Fig. 1B). Further, immunoblotting analysis using anti-cleaved caspase-7 (20 kDa) antibody detected the presence of p20 in caspase-7 36 h after that treatment. We next examined whether or not NOC18 cleaves the ICAD, a substrate for caspase-3 and -7, using these same cell lysates by Western blotting with an ICAD amino-terminal-specific antibody. In the quiescent state, ICAD 45 and 38 kDa in size were found to exist in SH-SY5Y cells (Fig. 1C). Each full-length ICAD (45 and 38 kDa) was decreased in response to NOC18 in a time-dependent manner. A new band corresponding to a breakdown product of ICAD 17 kDa in size was detected 24 h after NOC18 challenge in samples in which the cleaving of ICAD was evident.

3.2. *Effects of Ac-DEVD-CHO on caspase processing by NOC18*

We investigated the effects of Ac-DEVD-CHO, a caspase-3-like protease inhibitor, on caspases processing in response to NOC18. Treatment with Ac-DEVD-CHO was found to suppress the processing of pro-forms of caspase-2 and -7 in a concentration-dependent manner (Fig. 2A). Interestingly, the cleavage of pro-caspase-3 to a p20 fragment was insensitive to Ac-DEVD-CHO; the processing of p20 to p17, however, was inhibited by the treatment with Ac-DEVD-CHO (Fig. 2B).

3.3. *ROS generation and the mitochondrial permeability transition by NOC18*

The generation of ROS was detected by FACScan analysis using DCFH-DA. NOC18 treatment for 1 h caused a progressive increase in the production of ROS in SH-SY5Y cells (Fig. 3A). Kinetic analysis showed that ROS generation in response to NOC18 is significantly elevated 8 h after treatment, with these levels continuing for up to 24 h. Variations in the cellular content of the cationic lipophilic fluorescent dye rhodamine 123 were determined by FACScan analysis to evaluate the changes in mitochondrial membrane potential. Twelve hours after NOC18 challenge, the cells in which the fluorescent intensity of rhodamine 123 had decreased were detected (Fig. 3B). Exposure of SH-SY5Y cells to NOC18 produced decreases in the percentage of cells with high rhodamine 123 staining, representing a fall in the mitochondrial membrane potential in a time-dependent manner (Fig. 3B).

3.4. *Effects of permeability transition inhibitors on NOC18-induced loss of mitochondrial function*

CsA and BA are known to inhibit cyclophilin D and adenine nucleotides translocator in the mitochondrial permeability transition pore complex (PTPC), respectively. Both BA and CsA, but not FK506 (data not shown), significantly recovered from the loss of mitochondrial membrane potential in response to NOC18 (Fig. 4A). In this case, treatment with CsA was capable of attenuating the release of cytochrome *c* into the cytosol fraction in response to NOC18 (Fig. 4B). We evaluated the effects of BA on NOC18-induced neuronal apoptosis. Exposure of SH-SY5Y cells to NOC18 for 24 h resulted in apoptotic cell death, as estimated by TUNEL staining. In contrast, the numbers of DNA-fragmented (TUNEL-positive) cells were remarkably reduced in the BA-pretreated cells (Fig. 4C).

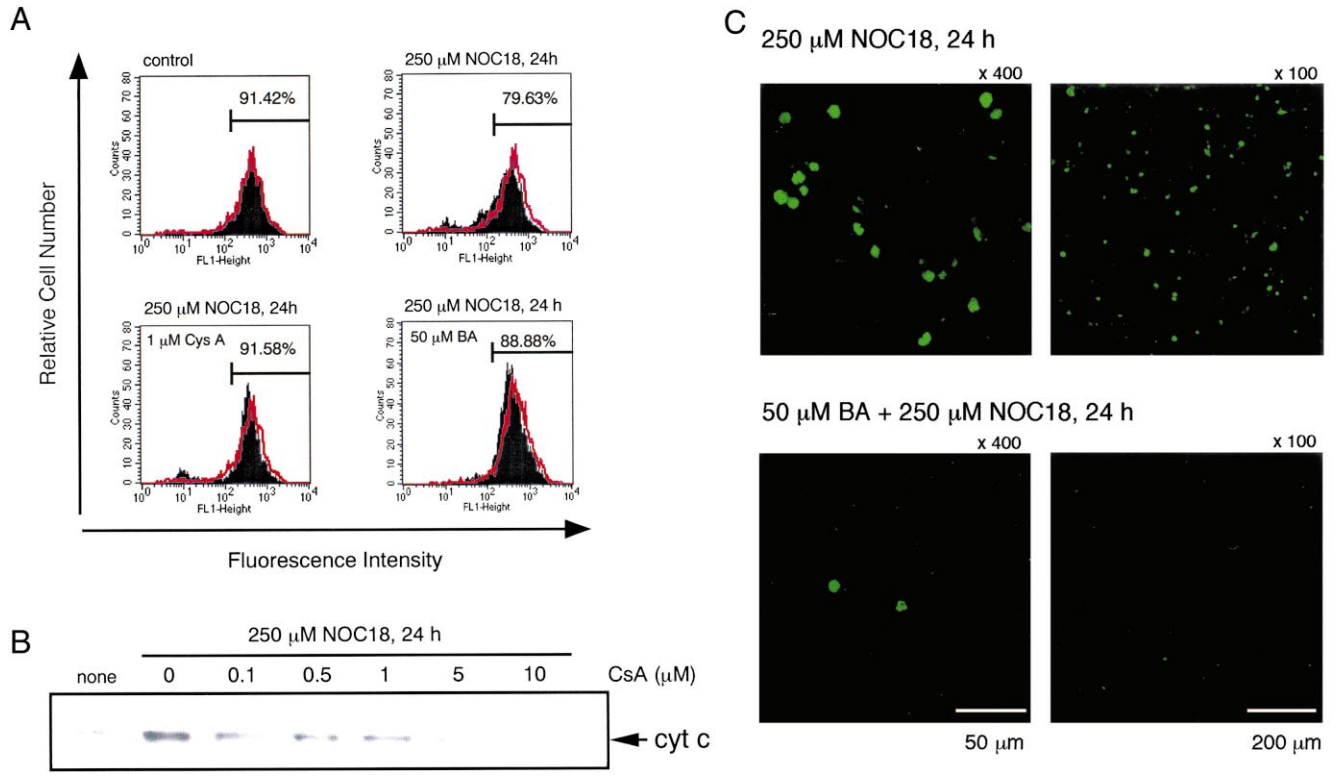

Fig. 4. Effects of permeability transition inhibitors on NOC18-induced changes in mitochondrial function. A: Effects of CsA and BA on the loss of mitochondrial membrane potential by NOC18. Cells were pretreated with 1 μM CsA or 50 μM BA for 30 min prior to NOC18 challenge. The cells were then stimulated with 250 μM NOC18 for 24 h when exposed to similar concentrations of CsA or BA. The mitochondrial membrane potential was analyzed as described in the legend for Fig. 3. B: Inhibitory effects of CsA on NOC18-induced cytochrome *c* release into the cytosol fraction. The cytosol fractions were separated by SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Cytochrome *c* was detected by Western blot analysis using a monoclonal antibody (7H8.2C12). C: Protective effects of BA on NOC18-induced DNA fragmentation. The apoptotic cells were detected by TUNEL of nucleosomal fragments generated by endonuclease cleavage. Scale bars: 50 μm in left panels; 200 μm in right panels.

4. Discussion

4.1. NO activates caspases

The purpose of the present study was to elucidate a detailed mechanism for NO-induced apoptotic cell death in SH-SY5Y neuroblastoma cells. We have reported previously that NO induces apoptosis via cytochrome *c* release into the cytosol and caspase activation in SH-SY5Y cells [29]. In the current study, we assessed the mitochondrial function and characterized the caspase isoforms activated by NOC18 treatment. Initially, we investigated the cleavage activity of the peptide substrate for each caspase. As shown in Fig. 1A, significant caspase-2 (VDVAD-AFC cleaving activity), caspase-3 and -7 (DEVD-AFC cleaving activity), and caspase-9 (LEHD-AFC cleaving activity) activities were detected in the extracted cytosol fractions with the same time-courses. Enzymatic cleavages of each fluorescent peptide substrate increased slightly 12 h after the addition of NOC18 and thereafter became elevated for up to 36 h. These results suggest that caspases, including at least caspase-2, -3, -7, and -9, are involved in NOC18-induced apoptosis.

Western blot analysis revealed that the level of pro-caspase-2 is decreased by NOC18 challenge in a time-dependent manner, as reported previously. In addition, the active forms of caspase-3 (p17 and p20), caspase-6 (p11), and caspase-7 (p20) were also detected during apoptosis (Fig. 1B). These findings suggest that NO activates several isoforms of caspases (at least caspase-2, -3, -6, -7, and -9) in SH-SY5Y cells. In this same study, we attempted the detection of caspase-9 by using anti-caspase-9 antibody. Unfortunately, we could not detect pro-caspase-9 even in the quiescent state, probably due to the lower concentrations of this molecule (data not shown). In agreement with this result, the cleaving activity of LEHD-AFC was lower than those of DEVD-AFC and VDVAD-AFC (Fig. 1A). Once apoptosis initiating factor (for example, cytochrome *c* release into the cytosol) activates, even the lower levels of caspase-9 could be sufficient to activate the sequential caspases and following apoptosis. We have found that NO cleaves several intracellular proteins such as PARP, lamin, D4-GDI in a caspase-sensitive manner ([29], data not shown). Under these conditions, NO results in apoptotic cell death accompanying DNA fragmentation. We therefore attempted to assess whether the activation of caspases in response to NOC18 leads to the cleavage of ICAD by Western blot analysis with ICAD antibody. ICAD serves as both a specific inhibitor of CAD and as a molecular chaperone to ensure proper folding of the endonuclease [13,14,36], and is a substrate for caspase-3 and -7 that must be cleaved before apoptotic internucleosomal DNA fragmentation can proceed [7,13]. As shown in Fig. 1C, NOC18 promoted ICAD cleavage, as demonstrated by Western blot analysis, suggesting that NO-induced apoptosis in SH-SY5Y cells occurs through the cleavage and inactivation of ICAD by caspases.

To elucidate the cascade of caspase activation induced by

NOC18, we next examined the effects of Ac-DEVD-CHO, a caspase-3-like protease inhibitor, on the cleavages of caspase-2, -3, and -7. Both the decrease in pro-caspase-2 and pro-caspase-7 levels and the appearance of the cleaved product of caspase-7 by challenge with NOC18 were recovered from in response to the treatment with Ac-DEVD-CHO in a concentration-dependent manner. These results strongly indicated that a DEVD-sensitive protease, possibly caspase-3, acts upstream of caspase-2 and -7. Interestingly, this inhibitor attenuated only the NO-induced formation of the p17 product from p20, but not p20 from pro-caspase-3 (p32). Thus, pro-caspase-3 may be cleaved to a p20 form by other caspases (possibly caspase-9) relatively insensitive to Ac-DEVD-CHO. Subsequent cleavage from p20 to p17 is presumably autocatalytic, as it is completely inhibited by Ac-DEVD-CHO in a concentration-dependent manner. Moreover, under these conditions, a 22 kDa cleavage product was detected during NOC18 challenge in the presence of Ac-DEVD-CHO (denoted by an asterisk in Fig. 2B). Similar results have been reported from studies in which the 22–23 kDa fragment has been observed after B cell antigen receptor ligation in B104 cells in the presence of low concentrations of Z-VDVAD-fmk [37]. Although the characteristics of the cleavage product have been investigated, it has been found to lack caspase-3 activity. Taken together, these results indicate that the 22 kDa fragment is generated via an Ac-DEVD-CHO-insensitive caspase.

Studies with the cleavage of fluorogenic peptide substrates, Western blot analysis, and caspase inhibitors strongly suggest that caspase-3 activation occurs upstream of caspase-2 and -7. In particular, as it is well known that caspase-3 is activated by caspase-9, our experiments suggest that (1) a DEVD-insensitive caspase (possibly caspase-9) initially generates p20 and p12, and then (2) DEVD-sensitive caspases (caspase-3 itself and other caspases) generate p17 from p20 in the next step.

4.2. *Effects of NO on mitochondrial function*

In this report, we assessed the mitochondrial function and role of NO-induced apoptosis. NO results in mitochondrial damage and mitochondrial respiratory chain inhibition [38,39]. In particular, NO binds reversibly to cytochrome *c* oxidase (complex IV) in mitochondrial respiration [40]. Thereafter, superoxide, which is also generated under these conditions, can interact with NO to form peroxynitrite ($ONOO^-$) [41]. $ONOO^-$ inhibits mitochondrial respiratory enzymes in an irreversible manner, suggesting that $ONOO^-$ may play a major role in the inhibition of respiration occurring under long-term exposure to NO. In our study, we demonstrated that significant ROS generation is detected 1 h after NOC18 treatment, peaks at 6 h, and continues until 24 h (Fig. 3A). This event was observed earlier than caspase activation and the following apoptosis. Thus, it was suggested that NO from NOC18 directly influences the mitochondrial respiratory chain in its early stages. However, it is still unknown whether ROS production leads to a death signal for apoptosis.

As another key molecule, cytochrome *c* released from the mitochondria has been identified as an important factor that can activate caspase-9. In fact, we have observed that the levels of cytosolic cytochrome *c* are increased in response to NOC18, and that the amounts of this protein in the membrane fraction containing mitochondria show a corresponding decrease in a time-dependent manner [29]. Unfortunately, the precise mechanism for cytochrome *c* translocation by an apoptosis inducer is still unclear. Kim et al. [42] have reported that cytochrome *c* release to the cytosol in response to treatment with cytosine β-D-arabinofuranoside occurs with an accompanying decline in the mitochondrial transmembrane potential. In contrast, the cytochrome *c* release induced by staurosporine precedes the loss of mitochondrial membrane potential [8]. In view of these results and other findings [43], the relationship between mitochondrial membrane potential and cytochrome *c* release remains controversial. On the other hand, Bcl-2 and other related anti-apoptotic proteins have been found to prevent cytochrome *c* release and to inhibit caspase activation and apoptosis [44]. In contrast, pro-apoptotic Bax and Bak stimulate cytochrome *c* release and induce the loss of mitochondrial membrane potential [45–50]. It has recently been reported that Bax and Bak, but not Bid and Bik, elicit cytochrome *c* release accompanying the loss of mitochondrial membrane potential in the presence of Ca^{2+} in isolated mitochondria [51]. However, it is still unknown how native Bcl-2 family proteins regulate the mitochondrial function. Hortelano et al. [52] have reported that NO triggers the permeability transition in isolated mitochondria and that this event can be detected 4 h after GSNO (an NO donor) challenge. The relationship between the mitochondrial permeability transition and the following cytochrome *c* release, however, was not described. In the present study, we attempted to determine whether or not NOC18 induces mitochondrial membrane potential loss and cytochrome *c* release. As shown in Fig. 3B, NOC18 evokes the loss of mitochondrial membrane potential in a time-dependent manner with this loss of mitochondrial membrane potential being detected slightly 12 h after NOC18 treatment (Fig. 3B). Under these conditions, a significant release of cytochrome *c* has been observed 12 h after NOC18 challenge [29]. In addition, in the present study, there were no changes in the levels of Bcl-2 family proteins such as Bcl-2, Bcl-XL, Bax, Bak, and Bid (data not shown). These results showed that NOC18 elicits both cytochrome *c* release to the cytosol from mitochondria and a loss of mitochondrial membrane potential with a similar time-course, ROS production, however, precedes these events.

4.3. *Relationship between mitochondrial membrane potential loss and apoptosis via caspases*

We next attempted to elucidate the relationship between the loss of mitochondrial membrane potential and apoptosis through cytochrome *c* release and caspase activation using two compounds: CsA, a cyclophilin D inhibitor, and BA, an adenine nucleotide translocator (ANT) inhibitor [53,54]. Both inhibitors reversed the loss of mitochondrial membrane potential triggered by NOC18 (Fig. 4A,B). In that case, treatment with CsA attenuated the release of cytochrome *c* to the cytosol from mitochondria in response to NOC18 in a concentration-dependent manner (Fig. 4C). In addition, BA suppressed NOC18-induced DNA fragmentation in SH-SY5Y cells (Fig. 4D). These findings suggest that the inhibition of mitochondrial membrane potential loss induced by prolonged exposure to NO could attenuate the activation of a death signal such as cytochrome *c* release and DNA fragmentation. It is obvious that the mitochondrial PTPC consists of cyclophilin D, ANT, voltage-dependent anion channel (VDAC), and several possible proteins. It is considered that the mitochondrial membrane potential is primarily regulated by PTPC [55,56]. In view of our results, the compound that protects

against mitochondrial membrane potential loss may be capable of attenuating neuronal apoptosis. Shimizu et al. [57] have reported that the Bcl-2 family of proteins affects VDAC and regulates the mitochondrial membrane potential and release of cytochrome *c* during apoptosis. However, we have no idea how Bcl-2 family proteins are involved in the release of cytochrome *c* in response to NO. Inhibitors of PTPC, however, seem to be effective against NO-induced neuronal apoptosis.

In summary, we here show that NO sequentially induces (1) ROS production, (2) the loss of mitochondrial membrane potential and cytochrome *c* release into the cytosol, (3) caspase activation, and (4) DNA fragmentation. Treatment with non-selective caspase inhibitor such as Z-Asp-CH_2-DCB can block NO-induced apoptosis [29]. Although the selective peptide inhibitors for caspases can attenuate the processing of each proform of caspase, they cannot completely prevent the apoptosis in response to NO (data not shown). In addition, our investigation showed that the overexpressed caspase-7 (C186G) mutant does not function as a dominant-negative, possibly due to the existence of intrinsic caspase, unlike the signal transduction triggered by cytokine or growth factor (T. Uehara, R. Moriya, and Y. Nomura, unpublished data). Since the death signal is amplified as it moves downstream as activated caspase cleaves and activates its own and many other caspases, the overexpression of mutant caspase seems to be ineffective. Therefore, as reported here, the inhibition of the death signal at the initiation stage or upstream is sufficient to block the apoptosis induced by NO. In particular, PTPC selectively attenuates NO-induced apoptosis (Fig. 4). Thus, the inhibition of PTPC, which leads to the recovery of mitochondrial membrane potential loss, is likely to render neuronal cells more resistant to the damaging effects of NO and brain ischemia.

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