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Caspase Activation and Cytochrome *c* Release During HL-60 Cell Apoptosis Induced by a Nitric Oxide Donor

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Nitric oxide (NO) from (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (NOC-18) induces apoptosis in human leukemia HL-60 cells. This effect was prevented by the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), thereby implicating caspase activity in the process. NOC-18 treatment resulted in the activation of several caspases including caspase-3, -6, -8, and -9(-like) activities and the degradation of several caspase substrates such as nuclear lamins and SP120 (hnRNP-U/SAF-A). Moreover, release of cytochrome *c* from mitochondria was also observed during NOC-18-induced apoptosis. This change was substantially prevented by Z-VAD-FMK, thereby suggesting that the released cytochrome *c* might function not only as an initiator but also as an amplifier of the caspase cascade. Bid, a death agonist member of the Bcl-2 family, was processed by caspases following exposure of cells to NOC-18, supporting the above notion. Thus, NO-mediated apoptosis in HL-60 cells involves a caspase/cytochrome *c*-dependent mechanism.

Keywords: Nitric oxide, apoptosis, caspase, cytochrome *c*, Bid

Abbreviations: NO, nitric oxide; NOC-18, (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; DEVD, Asp-Glu-Val-Asp; VEID, Val-Glu-Ile-Asp; IETD, Ile-Glu-Thr-Asp; LEHD, Leu-Glu-His-Asp; MCA, 4-methyl-coumaryl-7-amide; AMC, 7-amino-4-methyl-coumarin; PMSF, phenylmethylsulfonyl fluoride

INTRODUCTION

Apoptosis is a controlled form of cell death characterized by cell shrinkage, membrane blebbing, nuclear breakdown and DNA fragmentation.[1,2] Caspases, a family of cysteine proteases, play a central role in the apoptotic program.[3] A number of cellular substrates for caspases have been identified such as DFF45/ICAD and PAK2.[4–6] The cleavage of such proteins by caspases is responsible for several important biochemical and morphological changes of apoptosis.

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Caspases exist as dormant zymogens in healthy cells and become activated through proteolytic processing.[3] Various apoptotic stimuli cause the activation of the apical caspase-8 or -9; the former by recruitment to the death-inducing signaling complex (DISC) which consists of a ligated death receptor, like Fas, and its associated proteins, like FADD/MORT1 and FLASH,[7,8] and the latter by recruitment to the Apaf-1/cytochrome *c* complex in the presence of dATP.[9] The initiator caspases then trigger a caspase cascade by processing the effector caspases such as caspase-3.

Cytochrome *c* normally functions as a part of the respiratory chain, but once released into the cytosol from the mitochondria, this protein acts as an apoptogenic cofactor.[10] Two models for cytochrome *c* release have been proposed. One route is by rupture of the outer mitochondrial membrane, as a consequence of the opening of the permeability transition pore (PTP),[11] and a second route is via opening of the voltage-dependent anion channel (VDAC) stimulated by proapoptotic Bcl-2 family members such as Bax.[12]

Nitric oxide (NO) is a reactive free radical generated from L-arginine by constitutive or inducible NO synthase.[13] This radical has been implicated in some forms of apoptosis.[14] Previously, we reported that the initiation stage of apoptosis in human leukemia HL-60 cells induced by NOC-18, a chemical NO donor, is mediated by reactive oxygen species and derived molecules like peroxynitrite.[15] In this study, we investigated the mechanism of the execution stage of apoptosis, focusing on caspases and cytochrome *c* release. This paper contributes further to our knowledge of the molecular basis of NO-mediated apoptosis.

MATERIALS AND METHODS

Materials

(*Z*)-1-[*N*-(2-Aminoethyl)-*N*-(2-ammonioethyl)-amino]diazen-1-ium-1,2-diolate (NOC-18 or DETA/NO) was purchased from Dojindo Laboratories, Inc. (Kumamoto, Japan), and was freshly dissolved before use. Acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide (Ac-DEVD-MCA), Ac-Val-Glu-Ile-Asp-MCA (Ac-VEID-MCA), Ac-Ile-Glu-Thr-Asp-MCA (Ac-IETD-MCA), Ac-Leu-Glu-His-Asp-MCA (Ac-LEHD-MCA), and benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) were obtained from the Peptide Institute, Inc. (Osaka, Japan). Anti-cytochrome *c* antibody was purchased from Phar Mingen (San Diego, CA). Anti-SP120 antibody was kindly provided by Dr. K. Tsutsui (Okayama University, Japan). Other antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other materials used were of the highest grade commercially available.

Cell Culture

Human leukemia HL-60 cells were kindly donated by Dr. M. Saito (Hokkaido University, Japan). The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C under a 5% CO_2/95% air atmosphere.

DNA Fragmentation Assay

The extent of DNA fragmentation was determined by a spectrophotometric assay using diphenylamine,[16] as described in detail previously.[17] The laddering pattern of DNA was detected by agarose gel electrophoresis as described previously.[18]

Caspase Assay

HL-60 cells ($\sim 10^5$) were lysed in 50 μl of lysis buffer A (50 mM Tris pH 7.5, 0.5% Nonidet P-40, 0.5 mM EDTA, and 150 mM NaCl) on ice for 30 min and centrifuged at 15,000*g* for 10 min. The supernatant was incubated with 10 μM fluorogenic peptide substrate, Ac-DEVD-MCA,

Ac-VEID-MCA Ac-IETD-MCA, or Ac-LEHD-MCA in 200 µl of reaction buffer (20 mM HEPES pH 7.5 and 0.1 M NaCl) at 37°C for 1 h. The reaction was halted by the addition of stopper (0.2 M Gly-HCl, pH 2.8). The fluorescence of released 7-amino-4-methyl-coumarin (AMC) was measured by a fluorospectrophotometer (650-10 LC; Hitachi, Inc., Tokyo, Japan). The wavelengths for excitation and emission were 355 and 460 nm, respectively.

Subcellular Fractionation

After harvesting, HL-60 cells ($\sim 10^7$) were resuspended in 50 µl of ice-cold buffer A (250 mM sucrose, 20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF) and homogenized with Teflon homogenizer. The homogenate was centrifuged at 750*g* for 10 min at 4°C. The supernatant was then centrifuged at 10,000*g* for 15 min at 4°C. The resulting pellet was resuspended in buffer A and represented the mitochondrial (M) fraction. The supernatant was further centrifuged at 100,000*g* for 60 min at 4°C. The final supernatant (S-100) represented the cytosolic (S) fraction. Aliquots (50 µg) were used for Western blot analysis of cytochrome *c*.

Western Blot Analysis

HL-60 cells ($\sim 10^7$) were solubilized in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM PMSF), clarified by centrifugation, and the supernatant assayed for protein concentration by the method of Lowry *et al.*[19] The sample diluted in SDS-sample buffer (125 mM Tris–HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue) was subjected to SDS-polyacrylamide gel electrophoresis. After transfer to Immobilon filter (Millipore Co., Bedford, MA), the filter was incubated with a 1:1000 dilution of primary antibody followed by incubation with a 1:50,000 dilution of horseradish peroxidase-linked secondary antibody. Bound antibodies were developed using the ECLplus kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

RESULTS

NOC-18 Induces Apoptosis in HL-60 Cells

HL-60 cells undergo apoptotic cell death following exposure to NO donors, such as NOC-18, as described previously.[15,18] NOC-18 treatment of cells resulted in a dose- and time-dependent fragmentation of cellular DNA as determined by the diphenylamine assay (Figure 1A and B). Decomposed NOC-18 had no effect on the DNA status, thereby confirming the apoptosis-inducing action of NO itself. Agarose-gel electrophoresis showed a typical DNA ladder (Figure 1C). Characteristic morphological changes of apoptosis such as plasma membrane blebbing and cell shrinkage were also recognized in the NOC-18-treated HL-60 cells with a comparable time course to that of DNA fragmentation (not shown).

Caspase Activation in Response to NOC-18 Exposure

Since the caspase proteases are important for the execution of apoptosis,[3] we tested the effect of a cell-permeable inhibitor of caspases, Z-VAD-FMK, on the NOC-18-induced DNA fragmentation in HL-60 cells. Pretreatment of the cells with Z-VAD-FMK effectively suppressed the DNA fragmentation induced by NOC-18 in a dose-dependent manner (Figure 2). This result indicated that caspase activity was crucial for NOC-18 induction of apoptosis in HL-60 cells.

We next measured caspase-3, -6, -8, and -9(-like) activities in HL-60 cell extracts after different periods of NOC-18 treatment using fluorogenic peptide substrates (Figure 3A and B). Caspase-3-(-like) activity showed a large increase following

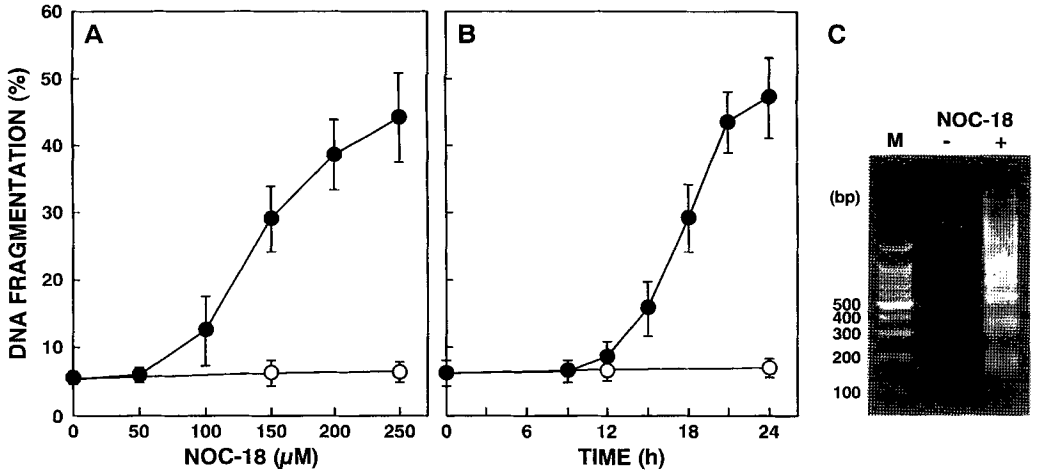

FIGURE 1 NOC-18-induced DNA fragmentation in HL-60 cells. The cells were treated with various concentrations of NOC-18 for 18 h (A) or with 150 μM NOC-18 for various times (B). Open circles indicate controls of decomposed NOC-18. Data are the mean ± SD of 3–5 independent experiments. (C) Electrophoretic patterns of DNA from the cells (5×10^5) treated for 21 h with 150 μM NOC-18 (+) or untreated (−). A representative photo is shown from two experiments with similar results.

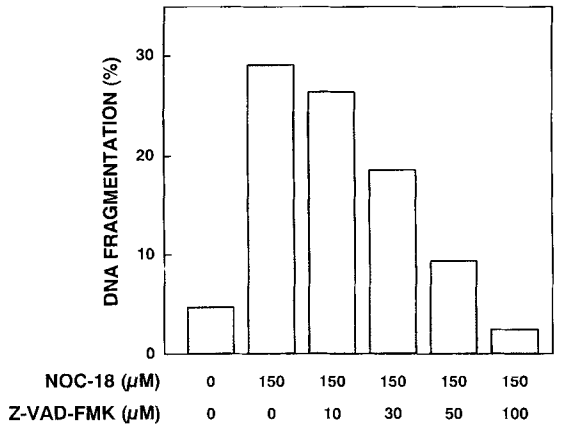

FIGURE 2 Effect of the pan-caspase inhibitor Z-VAD-FMK on NOC-18-induced DNA fragmentation in HL-60 cells. The cells were pretreated with the indicated concentrations of Z-VAD-FMK for 1 h, followed by treatment with 150 μM NOC-18 for 18 h. The extent of DNA fragmentation was determined by the diphenylamine method.

the addition of 150 μM NOC-18. Caspases-6, -8, and -9(-like) activities also increased but to a lesser extent than the caspase-3(-like) activity. Caspase-1(-like) activity was hardly detectable (not shown). Thus, NOC-18 treatment resulted in the activation of several caspases in the apoptotic program in HL-60 cells.

Degradation of Caspase Substrates in NOC-18-Treated HL-60 Cells

To demonstrate caspase activation *in vivo*, we assessed the proteolysis of endogenous caspase substrates, such as nuclear lamins and SP120 (hnRNP-U/SAF-A), by immunoblotting after SDS-polyacrylamide gel electrophoresis of the cell lysates from NOC-18-treated HL-60 cells. These proteins have previously been shown to be cleaved during apoptosis.[20–22] As shown in Figure 4, NOC-18 treatment resulted in a decrease in these intact proteins. In the case of lamin B, its cleaved product was detected with similar kinetics to the caspase activities. Therefore, the activation of caspases, such as caspases-3 and -6, was involved in the process of apoptosis induced by NOC-18 in HL-60 cells.

Cytochrome *c* Release from Mitochondria during NOC-18-Induced Apoptosis

The release of cytochrome *c* from the mitochondrial intermembrane space appears to play an important role in the induction of apoptosis.[11] The subcellular localization of cytochrome *c*

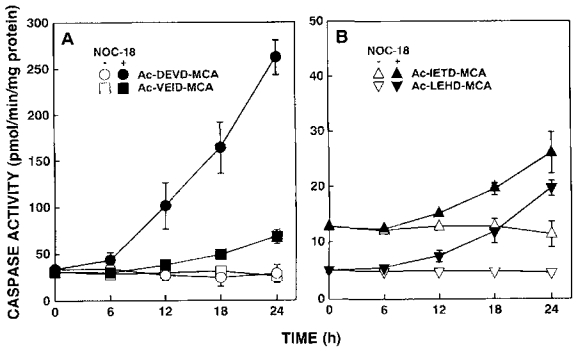

FIGURE 3 Caspase activities during NOC-18-induced apoptosis in HL-60 cells. The cells were either treated with 150 μM NOC-18 (dark symbols) or left untreated (light symbols) for the indicated periods. Total cell extracts were incubated with 10 μM fluorogenic peptide substrates, Ac-DEVD-MCA, Ac-VEID-MCA, Ac-IETD-MCA, or Ac-LEHD-MCA for caspase-3, -6, -8, or -9(-like) activities, respectively, at 37°C for 1 h The fluorescence of released AMC was measured.

during NOC-18-induced apoptosis in HL-60 cells was also assessed by immunoblotting (Figure 5A). In untreated cells, cytochrome *c* resided in the mitochondrial (M) fraction. However, at 18 h after the addition of NOC-18, half of the cytochrome *c* partitioned with the cytosolic (S) fraction, and most of the protein accumulated in the cytosol at 24 h. Thus, the redistribution of cytochrome *c* occurred in parallel with DNA fragmentation in response to NOC-18 treatment.

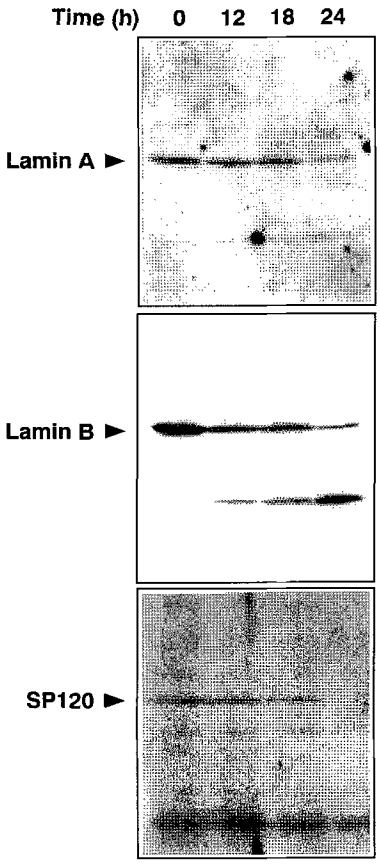

FIGURE 4 Western blot analyses of lamins and SP120 in NOC-18-treated HL-60 cells. The cells were treated with 150 μM NOC-18 for the indicated periods. Total cell lysates (50 μg) were analyzed by immunoblotting. Blots are representative of two experiments with similar results.

Caspases Mediate Cytochrome *c* Release Initiated by NOC-18

To elucidate whether cytochrome *c* release occurs prior to the activation of caspases or as a result of caspase activity, we tested the effect of Z-VAD-FMK on cytochrome *c* release in HL-60 cells after NOC-18 treatment. As shown in Figure 5B, pretreatment of the cells with 50 μM Z-VAD-FMK substantially decreased this release. This observation indicated that activated caspases also contributed to the release of cytochrome *c* during NOC-18-induced apoptosis in HL-60 cells.

Recent studies have shown that Bid, a death agonist member of the Bcl-2 family, contributes to cytochrome *c* release when cleaved by caspase-8 directly.[23,24] We therefore investigated whether or not this mechanism was implicated in cytochrome *c* release after NOC-18 treatment. Immunoblot analysis revealed that Bid was present as a ~22 kDa proform in untreated HL-60 cells and was time-dependently cleaved to a ~15 kDa fragment in response to NOC-18 exposure (Figure 6A). This cleavage was also prevented by Z-VAD-FMK (Figure 6B). Thus, the cleavage of Bid by caspases might be responsible for the release of mitochondrial cytochrome *c* during NOC-18-induced apoptosis in HL-60 cells.

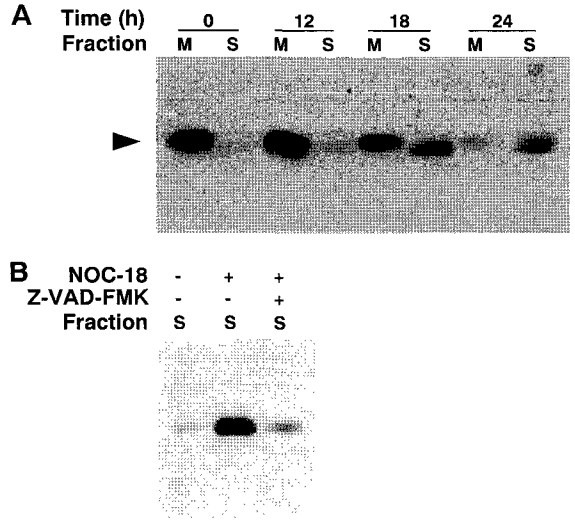

FIGURE 5 Western blot analysis of cytochrome *c* during NOC-18-induced apoptosis in HL-60 cells. (A) The cells treated with 150 μM NOC-18 for the indicated periods were fractionated into mitochondrial (M) and cytosolic (S) fractions. The cytochrome *c* in each fraction was determined by Western blotting. (B) The effect of the pan-caspase inhibitor Z-VAD-FMK (50 μM) on the NOC-18-induced cytochrome *c* release in HL-60 cells. Blots are representative of two experiments with similar results.

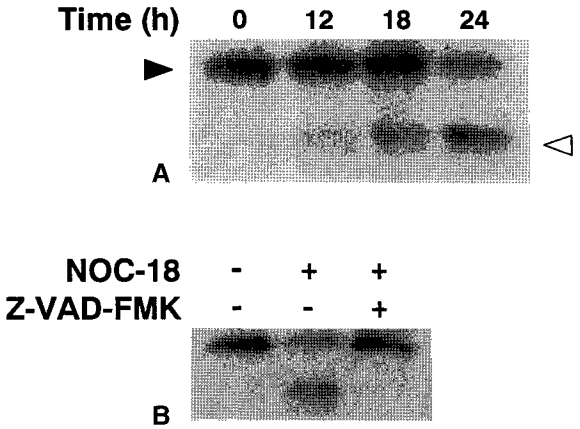

FIGURE 6 Western blot analysis of Bid during NOC-18-induced apoptosis in HL-60 cells. (A) The cells were incubated with 150 μM NOC-18 for the indicated periods. Total cell lysates (50 μg) were analyzed by immunoblotting. (B) The effect of the pan-caspase inhibitor Z-VAD-FMK (50 μM) on NOC-18-induced Bid cleavage in HL-60 cells. Blots are representative of two experiments with similar results. A light arrow indicate cleaved products of Bid.

DISCUSSION

NOC-18 induces apoptosis in human leukemia HL-60 cells. In this study, we showed that NOC-18 treatment of cells results in the activation of several caspases and the degradation of substrate proteins including lamins and SP120. From these observations, we concluded that the typical death protease cascade mediates in the apoptotic process of HL-60 cells induced by NOC-18.

On the basis of recent studies, it has been proposed that NO reversibly inhibits caspase activity by *S*-nitrosylation.[25] On account of this proposition, caspase activities in cell extracts were assayed in the absence of the reducing agent, dithiothreitol. However, an increase in activity of caspases was evident (Figure 3), thereby suggesting that another site(s) at which NO modulates the apoptotic process might exist upstream of the death proteases and differ according to the type of cell or apoptosis-inducing stimulus.

A number of reports support a role for factors derived from mitochondria, such as cytochrome *c*, in caspase activation.[11] Cytochrome *c* when released from mitochondria forms a complex with Apaf-1 in the presence of dATP to activate caspase-9.[9] Very recently, NO has also been reported to induce cytochrome *c* release during apoptosis of neuronal and leukemic cells.[26,27] Data presented in this paper show that cytochromc *c* release during NOC-18-induced apoptosis in HL-60 cells was also mediated by caspase activity. Therefore, we concluded that the released cytochrome *c* during NOC-18-induced apoptosis also contributes to the amplification of the activation of downstream caspases. Bid is one factor that can trigger cytochrome *c* release upon cleavage by caspases such as caspase-8.

We found that caspase activity contributed to cytochrome *c* release from mitochondria in NOC-18-treated HL-60 cells, but the mechanism by which NOC-18 triggers the first step in the caspase cascade is still obscure. Since some investigators have observed, in certain systems, caspase-3 activation without any accompanying

cytochrome *c* accumulation in the cytosol,[28,29] a pathway of cytochrome *c*-independent activation of caspases might exist and remains to be elucidated.

In conclusion, we showed that NOC-18-induced apoptosis in HL-60 cells involves caspase activity, as confirmed by: (1) the activation of several caspases; (2) the degradation of several caspase substrates; (3) the inhibition of NOC-18-induced DNA fragmentation by the pan-caspase inhibitor Z-VAD-FMK. In addition, we suggested that the NOC-18-induced release of mitochondrial cytochrome *c* also functions as an apoptotic amplifier, as supported by: (1) the prevention of cytochrome *c* release by Z-VAD-FMK; (2) the cleavage of Bid during the process. To understand the mechanism by which NO initiates the first step of a caspase cascade, further work will be required.

Acknowledgments

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