

A simultaneous release of SOD1 with cytochrome c regulates mitochondria-dependent apoptosis

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Abstract To elucidate the significance of mitochondrial localization of Cu/Zn-SOD (SOD1), we studied the relationship between the release of mitochondrial SOD1 and apoptosis. Kinetic analysis using HL-60 cells showed that both mitochondria-dependent and mitochondria-independent pro-apoptotic drugs, such as staurosporine and actinomycin D, increased the generation of reactive oxygen species (ROS) and decreased mitochondrial membrane potential ($\Delta\psi$). ROS generation by these drugs was inhibited by Mn (III) tetrakis (5,10,15,20-benzoic acid) porphyrin (MnTBAP), a cell membrane-permeable SOD mimetic. However, MnTBAP inhibited the apoptosis induced by staurosporine but not by actinomycin D. MnTBAP failed to inhibit $\Delta\psi$ decrease and release of SOD1 and cytochrome c induced by actinomycin D. Moreover, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), an inhibitor of voltage-dependent anion channel (VDAC), inhibited the release of the two proteins and apoptosis induced by staurosporine but not actinomycin D. These results suggest that ROS plays an important role in mitochondria-dependent but not mitochondria-independent apoptosis and that the release of SOD1 increases the susceptibility of mitochondria to oxidative stress, thereby enhancing a vicious cycle leading to apoptosis.

Keywords Reactive oxygen species · Mitochondria · SOD1 · Cytochrome c · Apoptosis

Introduction

Apoptosis is characterized by cell shrinkage, cell surface blebbing, DNA fragmentation, and nuclear breakdown [1, 2]. Apoptotic stimuli generally induce the opening of membrane permeability transition (MPT) pores and swelling followed by release of pro-apoptotic proteins from the intermembranous space of mitochondria [3–5]. One such mitochondrial protein is cytochrome c, which is released into the cytosol in an early stage of apoptosis, forming an Apaf complex leading to activation of the caspase cascade [6, 7]. We previously reported that SOD1 localizes as a bound form in mitochondria of the brain and liver, and in various types of cells [8]. Neither cytochrome c nor SOD1 contains typical N-terminal presequences for mitochondrial uptake, yet both of them reside in the intermembranous space. Okado-Matsumoto et al. [9] also showed that SOD1 is localized in intermembranous space of rat liver mitochondria. Therefore, we investigated cellular distribution of SOD1 and cytochrome C during the process of apoptosis of human promyelocytic leukemia cells (HL-60).

The overproduction of superoxide and ROS induces oxidative stress, which is known to cause various forms of apoptosis [10–12]. In addition, the release of cytochrome c and consequent disruption of the electron transport chain also enhance generation of ROS [13]. Since superoxide is released into both sides of mitochondrial inner membranes [14, 15], removing superoxide in and around mitochondria would be very important in preventing apoptosis and cell death. The mitochondrial localization of the SOD1 seems to be required in protecting cells against mitochondria-derived oxidative damage and apoptosis. To elucidate the importance of subcellular localization of SOD1 in apoptosis, we investigated by using HL-60 cells the relationship between oxidative stress and release of the enzyme during

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apoptosis, and demonstrated that oxidative stress-triggered SOD1 release plays a critical role in mitochondria-dependent rather than mitochondria-independent apoptosis.

Materials and methods

Cell culture

HL-60 cells were grown in RPMI-1640 medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% heat inactivated fetal bovine serum (FBS). Cell culture was performed at 37°C and 5% CO₂ atmosphere.

Chemicals

RPMI-1640, N-ethylmaleimide (NEM), and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO). Staurosporine, actinomycin D, and DIDS were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MnTBAP was obtained from Calbiochem (San Diego, CA). Mouse anti-cytochrome c and sheep anti-SOD1 antibodies were purchased from PharMingen and Calbiochem (San Diego, CA), respectively. Rabbit anti-DNP/HRP antibody, HRP-conjugated anti-goat IgG, anti-mouse IgG, and anti-rabbit IgG were purchased from DAKO Glostrup (Denmark). Immunoprecipitation Starter Pack and an ECL kit, and 2',7'-dichlorofluorescein diacetate (DCFH-DA) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1) were obtained from Amersham Biosciences (UK) and Molecular Probes (Eugene, OR), respectively. Annexin V-APC was obtained from BD Pharmingen (San Diego, CA).

Assay for ROS generation using DCFH-DA

The intracellular generation of ROS was measured by using oxidation-sensitive fluorescent dye DCFH-DA [16]. HL-60 cells (1×10^6) were washed with ice-cold PBS and centrifuged at 1000 rpm for 5 min at 4°C. The cells were resuspended in 1 ml PBS and then 1 µl of DCFH-DA (5 mM in DMSO) was added to the cell suspension and incubated in a water-bath (37°C) for 15 min. Subsequently, the cells were kept on ice and analyzed with flow cytometry using FACS Calibur (Becton Dickinson Laboratory). Data analysis was performed using BD CELL Quest software.

Assay for mitochondrial membrane potential ($\Delta\psi$) using JC-1

Mitochondrial membrane potential ($\Delta\psi$) was monitored by using a lipophilic cationic probe JC-1 as described

previously in detail [17]. HL-60 cells (1×10^6) were suspended in 1 ml of RPMI-1640 medium, and loaded with JC-1 (10 µM) for 10 min at 37°C. Subsequently, the cells were detected with flow cytometry using FACS Calibur (Becton Dickinson Laboratory). Both green (FL-1) and red (FL-2) fluorescence were recorded, and $\Delta\psi$ was determined from the ratio of red/green (FL-1/FL-2).

Cytosolic and mitochondrial protein extraction

Both cytosolic and mitochondrial fractions were isolated from HL-60 cells (1×10^6) after washing in ice-cold PBS for 5 min at 1700 rpm. The washed cells were lysed using 100 µl of CLAMI (cell lysis and mitochondria intact buffer; 250 mM Sucrose and 70 mM KCl in PBS) containing 20 µg/ml digitonin at 4°C for 5 min. The cell suspension was centrifuged at 1100 rpm and 4°C for 5 min. The supernatant was removed and stored as the cytosolic fraction at -80°C . The pellet was re-suspended in 100 µl of a "universal immunoprecipitation buffer" (50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, 0.3% NP-40 and protease inhibitors) and placed at 4°C for 10 min. The suspension was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was stored as the mitochondrial fraction at -80°C , prior to analysis by Western blotting.

Detection of protein oxidation

Mitochondrial proteins were treated with 2,4-dinitrophenylhydrazine (DNPH) to produce DNP-derivatives as described earlier [18]. The treated proteins were used for the analysis by SDS polyacrylamide gel electrophoresis (PAGE) analysis. After electrophoresis, proteins were transferred onto immobilon-P membranes at 15 V (constant voltage, 1 h) using trans-blot transfer cell (BIO-RAD). The blots were incubated with rabbit anti-DNP/HRP antibody (diluted 1:500 in TBST containing 2% BSA) at room temperature for 1 h. After washing three times with TBST, the oxidized proteins were visualized using an ECL system (Amersham Biosciences, UK).

Analysis of SOD1 and cytochrome c release

The release of SOD1 and cytochrome c from mitochondria was detected using anti-SOD1 and anti-cytochrome c antibodies, respectively. Mitochondrial or cytosolic fractions were added with a 0.2 volume of 125 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 5% β -mercaptoethanol, 25% glycerol, and 0.002% bromophenol blue. After incubation at 95°C for 5 min, these samples were subjected to SDS-PAGE followed by Western blot analysis using specific antibodies.

Western blotting

After SDS-PAGE, proteins were transferred electrophoretically from the gel onto an Immobilon-P transfer membrane (Millipore, Bedford, MA). The membrane was blocked in TBST (0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Tween 20) containing 5% skimmed milk powder for 30 min, and then incubated with primary antibodies diluted in TBST containing 5% skimmed milk powder at room temperature for 1 h. After washing three times with TBST, the membrane was incubated at room temperature for 1 h with the HRP-conjugated secondary antibody diluted in TBST containing 5% skimmed milk powder. After washing with TBST, immunoreactive bands were visualized using an ECL system.

Measurement of cell viability and apoptosis

Apoptotic and necrotic cell deaths were distinguished by using Annexin V and PI as described previously [19]. HL-60 cells (1×10^6) were washed with ice-cold PBS and harvested by low-speed centrifugation, and resuspended in a binding buffer (10 mM HEPES buffer pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) at 1×10^6 cells/ml. To 0.1 ml aliquots containing 10^5 cells, Annexin V-APC and PI were added at the manufacturer's recommended concentrations. Cells were incubated for 15 min at room temperature in the dark and subsequently diluted with 0.4 ml binding buffer and analyzed by flow cytometry within 1 h. Flow cytometric analysis was performed on a Becton Dickinson FACSArray, and the data were collected and analyzed using the BD FACSArray software.

DNA fragmentation

HL-60 cells (1×10^6) were washed with ice-cold PBS and incubated in a lysis buffer (10 mM Tris-HCl buffer pH 7.4, 10 mM EDTA, 0.5% Triton X-100) at 4°C for 10 min. After centrifugation (at 15,000 rpm and 4°C for 20 min), the supernatant was collected. RNase A was added to the final concentration of 0.1 mg/ml, and the mixture was incubated at 37°C for 1 h. The cell lysate was then incubated at 37°C for 1 h with 0.1 mg/ml of proteinase K (Sigma-aldrich). DNA was extracted using 5 M NaCl and 2-propanol, followed by centrifugation at 15,000 rpm for 20 min. The DNA-extract was then dissolved in a TE buffer (10 mM Tris-HCl (pH 7.4), 10 mM EDTA) and separated on a 1.5% agarose gel. DNA fragmentation was visualized after ethidium bromide staining of the gel.

Statistical analysis

Values are expressed as mean \pm S.D. The differences between control and treated groups (staurosporin or

actinomycin D) were analyzed with Student's *t*-test. A *P* value $P < 0.05$ was considered significant.

Results

ROS generation in HL-60 cells with staurosporine or actinomycin D

Several studies documented that both staurosporine and actinomycin D induced oxidative stress-dependent apoptosis in various types of cells [20, 21]. In our experiment, HL-60 cells were treated with either staurosporine or actinomycin D for ≈ 3 h, and the time-course of intracellular production of ROS was monitored by DCFH-DA using flow cytometry. Shortly after the treatment, ROS level increases transiently followed by a decrease (Fig. 1). The increase of ROS was inhibited by treating cells with MnTBAP, a membrane-permeable SOD mimic.

Effects of staurosporine and actinomycin D on the induction of MPT

An increase of ROS level with either staurosporine or actinomycin D suggested that mitochondria would be under oxidative stress. Thus, we analyzed the effects of these drugs on the oxidation of mitochondrial proteins and induction of MPT. We detected protein oxidation using an anti-DNP antibody, which specifically binds to the carbonyl moieties of DNPH-treated mitochondrial proteins. When HL-60 cells were treated with either staurosporine or actinomycin D, a substantial increase in the carbonyl content of mitochondrial proteins was observed (Fig. 2). Protein carbonylation induced by either staurosporine or actinomycin D was inhibited by the treatment with MnTBAP. Under the same condition, we monitored the changes in $\Delta\psi$ using a fluorescent probe JC-1. The ratio of red/green (FL-1/FL-2) fluorescence reflecting changes in $\Delta\psi$ decreased in HL-60 cells treated with staurosporine or actinomycin D (Fig. 3). MnTBAP inhibited the decrease of $\Delta\psi$ induced by staurosporine, but not by actinomycin D.

Mitochondrial release of SOD1 and cytochrome c during staurosporine- and actinomycin D-induced apoptosis

We previously showed that oxidative stress induced MPT and released both SOD1 and cytochrome c from mitochondria [22]. To clarify the relationships among the time courses of MPT, SOD1, and cytochrome c releases during apoptosis, we analyzed the mitochondrial and cytosolic fractions of HL-60 cells treated with either staurosporine or actinomycin D. After the treatment, a time-dependent

Fig. 1 ROS generation assessed by flow cytometry after DCFH staining. HL-60 cells were cultured in the presence of either **a** 0.5 μ M staurosporine or **b** 0.5 μ g/ml actinomycin D for \approx 3 h and then stained with DCFH-DA. **c** HL-60 cells were also cultured in the presence or absence of MnTBAP (100 μ M) for 1 h, and then incubated with either staurosporine or actinomycin D for 3 h. Cellular generation of hydrogen peroxide was determined using DCFH-DA and flow cytometry. Values represent the mean \pm SE for three independent experiments and the significance was set at $P < 0.05$

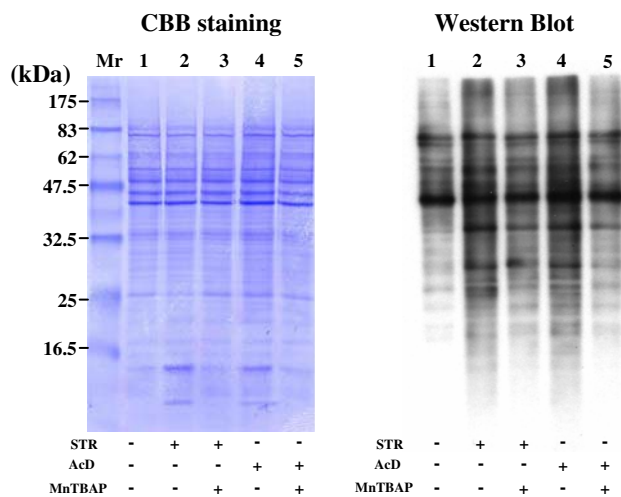
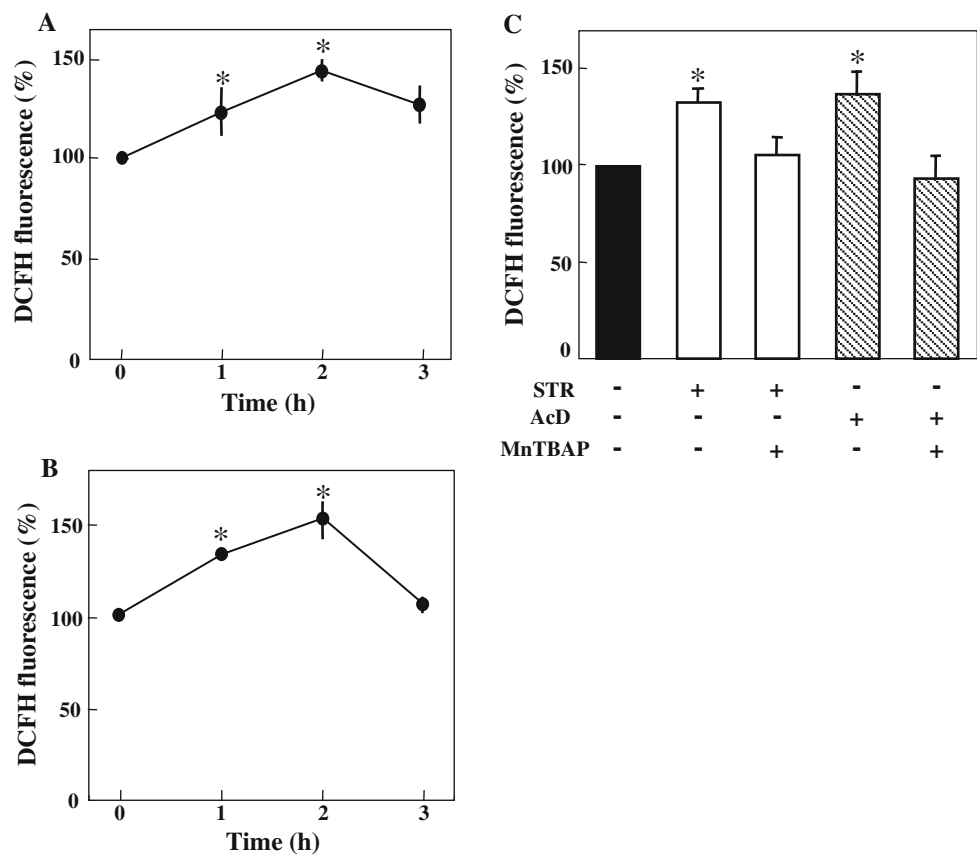


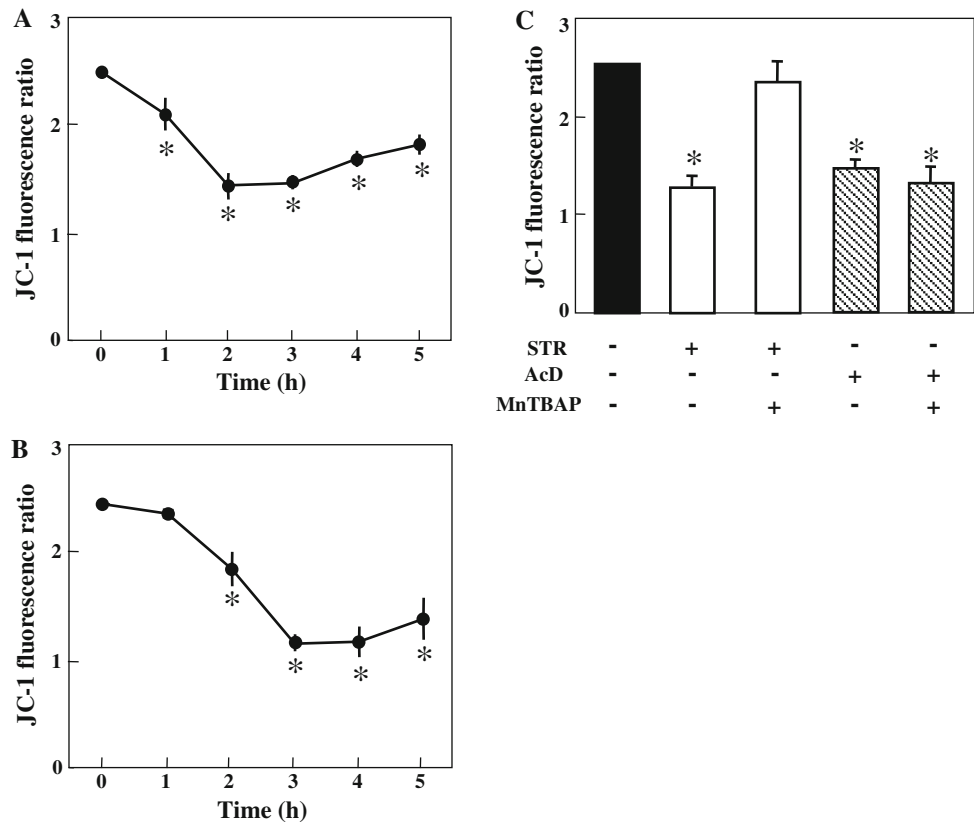
Fig. 2 The protein oxidation in mitochondria. HL-60 cells were cultured in the presence or absence of 100 μ M MnTBAP for 1 h and then incubated with either 0.5 μ M staurosporine or 0.5 μ g/ml actinomycin D for 2 h. Mitochondria was isolated from HL-60 cells and treated with 2,4-dinitrophenylhydrazine (DNPH). The DNPH-modified proteins in mitochondria were detected immunochemically using HRP-conjugated rabbit anti-DNP antibody as described in the text. Mr, molecular marker; 1, untreated control; 2, +0.5 μ M staurosporine, 3, +100 μ M MnTBAP, +0.5 μ M staurosporine; 4, +0.5 μ g/ml actinomycin D; 5, +100 μ M MnTBAP +0.5 μ g/ml actinomycin D

increase of both SOD1 and cytochrome c in the cytosolic fractions was observed (Fig. 4). At the 2 h timepoint, about 50% of SOD1 and 10% of cytochrome c were released to the cytosol. Both SOD1 and cytochrome c were completely released to cytosol after 5 h of treatment with staurosporine and actinomycin D. The release of the two proteins induced by staurosporine was inhibited by either MnTBAP or DIDS. In contrast, the release of the two proteins induced by actinomycin D was not affected by the two compounds. These results suggested that oxidative stress-induced MPT pore is required for staurosporine-stimulated but not actinomycin D-stimulated SOD1 and cytochrome c releases.

Phosphatidylserine externalization and DNA fragmentation in HL-60 treated with staurosporine and actinomycin D

During the early stage of apoptosis, phosphatidylserine (PS) was exposed to cell surface; this exposure could be detected by annexin V that binds to PS with high affinity [23, 24]. To clarify the relationship between the mitochondrial release of intermembranous proteins and apoptosis-related events, we analyzed the exposure of annexin V and DNA fragmentation in staurosporine- or actinomycin D-treated HL-60 cells. Figure 5 shows that the

Fig. 3 Effect on mitochondrial membrane potential. HL-60 cells were treated with **a** 0.5 μ M staurosporine or **b** 0.5 μ g/ml actinomycin D for \approx 5 h and then stained with JC-1. **c** HL-60 cells were pre-incubated in the presence or absence of 100 μ M MnTBAP for 1 h followed by treatment with staurosporine or actinomycin D for 3 h. The green and red fluorescence correspond to JC-1 monomers and its aggregates, respectively. The values of red and green fluorescence ratio (FI-1/FI-2) reflect the changes in and represented by mean \pm SE for three independent experiments and the significance was set at $P < 0.05$



proportion of annexin V-binding cells increased in a time-dependent manner. After externalization of PS, DNA fragmentation was induced by treating cells with either staurosporine or actinomycin D (Fig. 6). MnTBAP inhibited both PS externalization and DNA fragmentation induced by staurosporine but not by actinomycin D. DIDS also inhibited DNA fragmentation induced by staurosporine but not by actinomycin D.

Discussion

Recently, we reported that SOD1 is localized predominantly in mitochondria as a bound form in various types of cells [8]. We found that both SOD1 and cytochrome c were released simultaneously from mitochondria under the conditions when ROS generation and mitochondrial swelling took place [22]. Thus, we hypothesized that the release of SOD1 from mitochondria might participate in apoptosis in a similar manner to the release of cytochrome c. To validate this hypothesis, we investigated the subcellular localization and the role of SOD1 in ROS generation during mitochondria-dependent apoptosis. We demonstrated that ROS generation plays a key role in mitochondria-dependent apoptosis through increasing MPT

and triggering the releases of SOD1 and cytochrome c. In addition, kinetics analysis revealed that SOD1 released from mitochondria prior to cytochrome c. Thus, the release of SOD1 might contribute to mitochondria-dependent apoptosis by promoting ROS-triggered cytochrome c release through accelerating the accumulation of intracellular ROS.

Apoptosis or programmed cell death is characterized as type 1 (extrinsic, receptor-dependent, caspase-dependent) or type 2 (intrinsic, autophagic, mitochondria-dependent) [25, 26]. In this experiment, actinomycin D, an inhibitor of DNA-dependent RNA synthesis, was used to induce the type 1 form of apoptosis [25], whereas staurosporine, a potent protein kinase c inhibitor, was used to trigger type 2 apoptosis [21]. Several lines of evidence indicate that both staurosporine and actinomycin D increase the generation of ROS, thereby inducing oxidative stress-dependent apoptosis in various types of cell lines [20, 27–30]. In fact, the observation of our time course experiments in HL-60 cells suggested that both staurosporine and actinomycin D increased ROS generation. Although the enhanced generation of ROS by both staurosporine and actinomycin D was inhibited by the treatment of MnTBAP, only staurosporine-induced cell death was blocked by MnTBAP, suggesting that ROS is indispensable for mitochondria-dependent but not independent apoptosis.

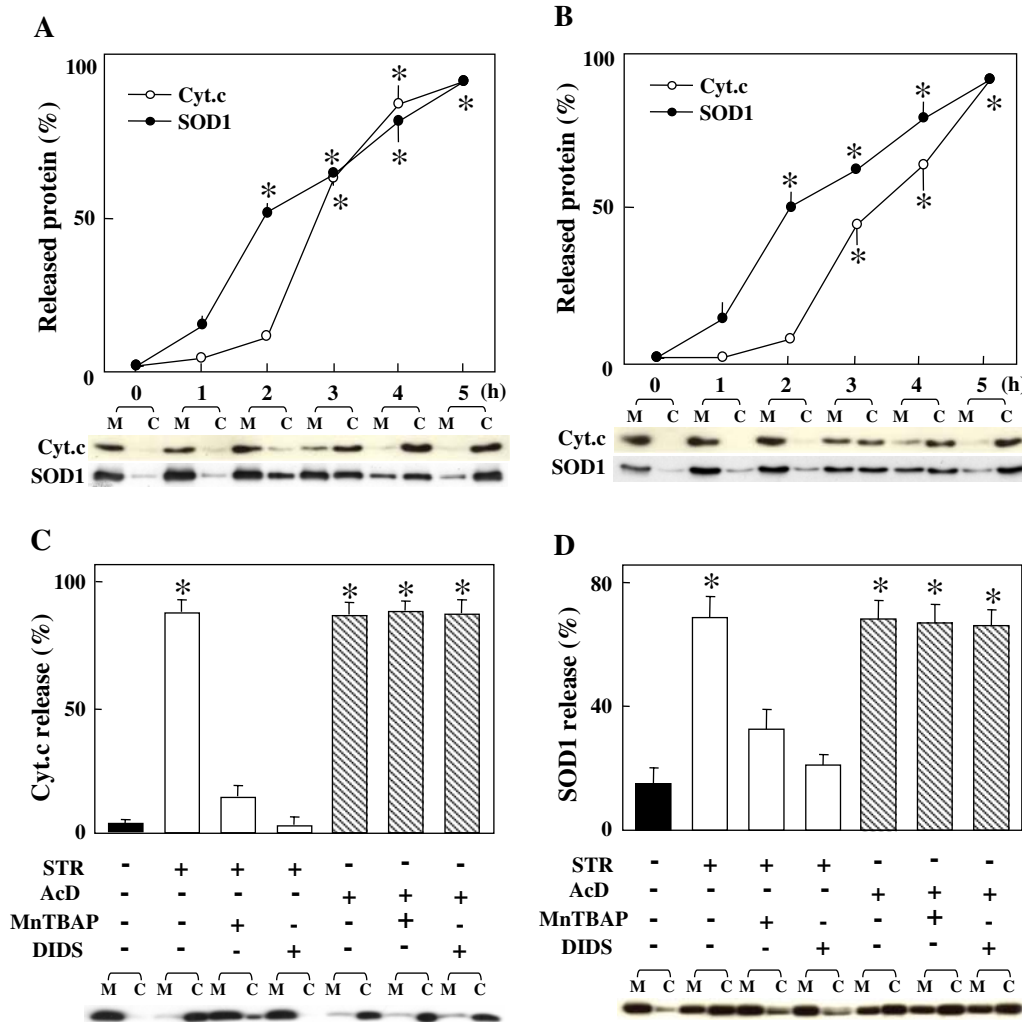


Fig. 4 Release of SOD1 and cytochrome c from mitochondria. HL-60 cells were cultured in the presence of **a** 0.5 μ M staurosporine or **b** 0.5 μ g/ml actinomycin D for \approx 5 h. Then, mitochondrial and cytosolic fractions were isolated as described in the text. Subcellular localizations of cytochrome c and SOD1 were determined by Western blot analysis using specific antibodies. Total proteins in mitochondrial and cytosolic fractions were calculated to 100%; proteins in cytosolic

fractions were calculated and blotted in figures. **c, d** HL-60 cells were pre-incubated with either 100 μ M MnTBAP or 500 μ M DIDS for 1 h and then incubated with staurosporine or actinomycin D for 5 h. Subcellular localizations of cytochrome c (**c**) and SOD1 (**d**) were determined by Western blot analysis using specific antibodies. Values represent the mean \pm SE ($n = 3$) and the significance was set at $P < 0.05$

Since endogenous oxidative stress is a central inducer in staurosporine-triggered apoptosis [21, 31, 32], MnTBAP significantly inhibited MPT and release of SOD1 and cytochrome c. In contrast, Actinomycin D not only induces oxidative damage but also activates caspase 8 to cleave and translocate Bid into mitochondria [25, 33, 34]. Thus, both the loss of $\Delta\psi$ and the release of the two proteins induced by actinomycin D were not inhibited by MnTBAP. In actinomycin D-triggered apoptosis, tBid induced the release of cytochrome c by a mechanism that is independent of MPT pore opening. In this paper, we analyzed the effects of the VDAC blocker (DIDS) on staurosporine- and actinomycin D-induced apoptosis. It was found that DIDS inhibited the release of those two proteins and DNA

fragmentation induced by staurosporine but did not inhibit the reactions induced by actinomycin D. Therefore, SOD1 and cytochrome c are released from different pathways in response to apoptosis signaling.

Oxidative stress-dependent mitochondrial degeneration and protein aggregation play principal roles in amyotrophic lateral sclerosis (ALS), a fatal, adult-onset neurodegenerative disease caused by mutations in SOD1 [35–39]. Mutations of SOD1 genes are found in 20% of patients with ALS [40]. It was also reported that a mutant SOD1 readily aggregates predominantly in the cytosol of motor neurons [41]. Preliminary experiments in our laboratory showed that the mutation of SOD1 decreased the affinity of the enzyme to mitochondria (unpublished

Fig. 5 Phosphatidylserine externalization induced by staurosporine and actinomycin D. At the indicated times, HL-60 cells were incubated with either **a** 0.5 μ M staurosporine or **b** 0.5 μ g/ml actinomycin D. **c** HL-60 cells were cultured in the presence or absence of 100 μ M MnTBAP for 1 h, and then incubated with staurosporine or actinomycin D for 5 h. Cell surface expression of PS was determined by the annexin V binding assay using flow cytometry. Values represent the mean \pm SE ($n = 3$) and the significance was set at $P < 0.05$

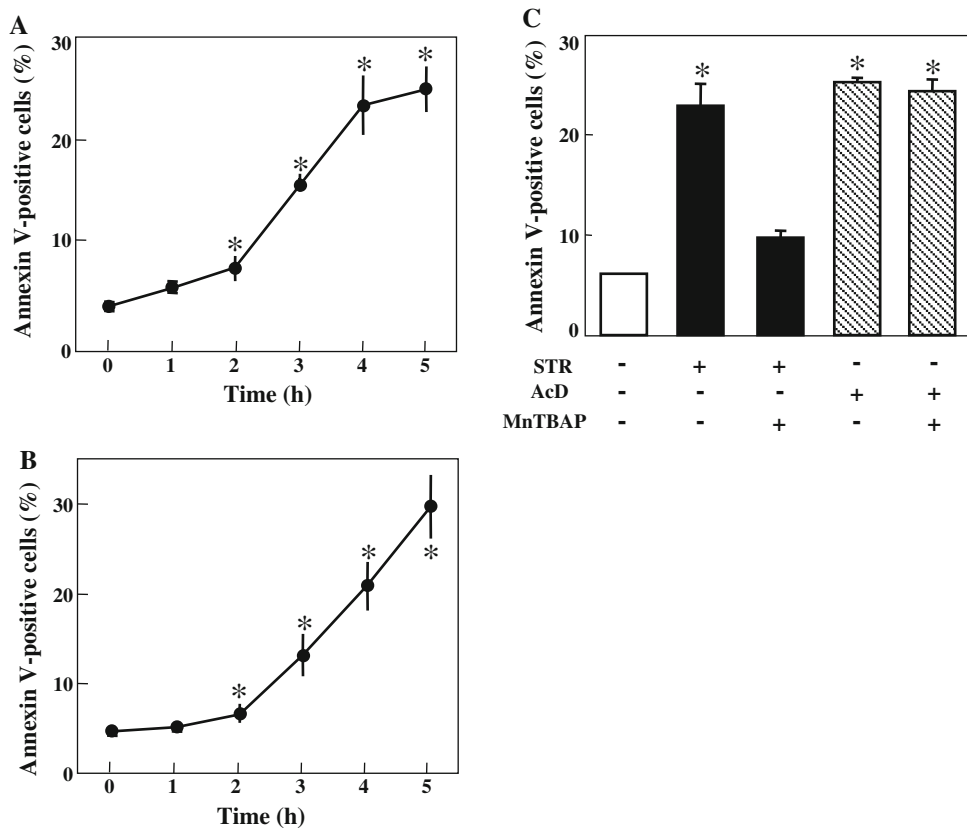
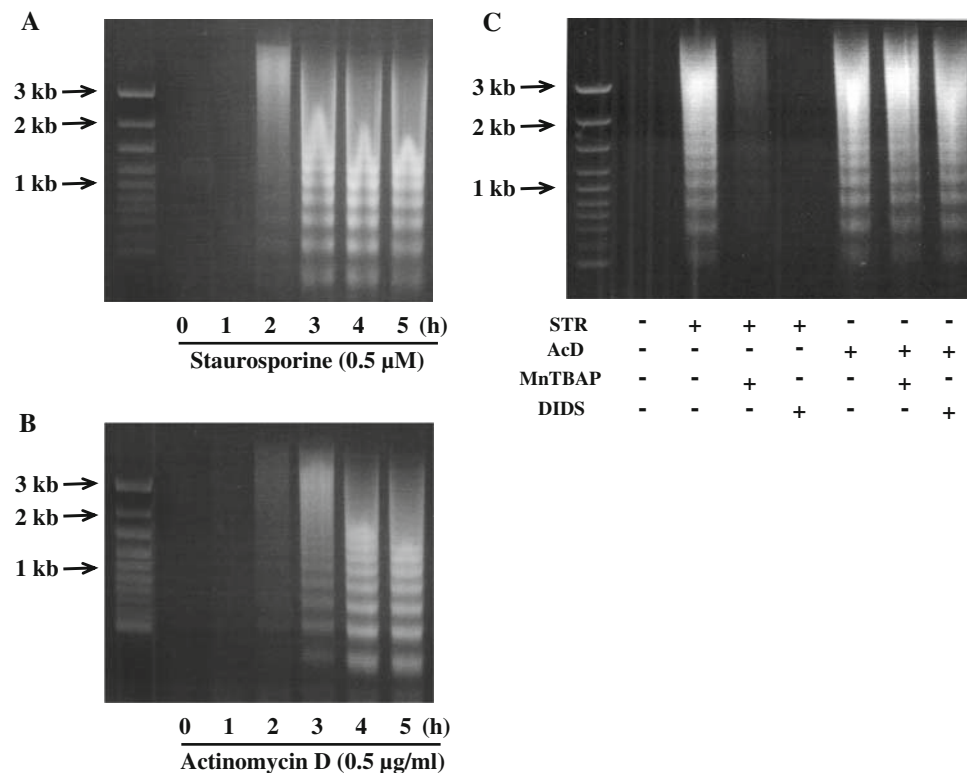


Fig. 6 DNA fragmentation induced by staurosporine and actinomycin D. HL-60 cells were treated with **a** 0.5 μ M staurosporine or **b** 0.5 μ g/ml actinomycin D. **c** HL-60 cells were pre-incubated with or without 100 μ M MnTBAP or 500 μ M DIDS for 1 h and then with staurosporine or actinomycin D for 5 h. Then, DNA fragmentation of cells was analyzed by agarose gel electrophoresis. Similar results were obtained in triplicate experiments



experiment). Since oxidative stress is enhanced in and around mitochondria lacking SOD1, this would lead to oxidation of critical thiol groups in ANT, thereby

triggering cytochrome c release. Guegan et al. [42] also reported that the release of cytochrome c from mitochondria has been observed in the spinal cord of

transgenic mice. This may be linked with a mutSOD1-mediated ROS generation.

In conclusion, the results of our time course studies demonstrate that both SOD1 and cytochrome c are released from mitochondria concurrently with the generation of ROS and the reduction of $\Delta\psi$. All these reactions are involved in oxidative stress-induced apoptosis. Since the delocalization of SOD1 from mitochondria would enhance mitochondrial oxidative injury, it would enhance apoptosis. Based upon these results, we propose that the release of SOD1 might be a critical initiator of mitochondria-dependent apoptosis. Further studies are required to fully elucidate the physiological implications of subcellular localization of SOD1 in mitochondria-dependent apoptosis.

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