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# Hydrogen Peroxide-induced Apoptosis in HL-60 Cells Requires Caspase-3 Activation

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Apoptosis has been associated with oxidative stress in biological systems. Caspases have been considered to play a pivotal role in the execution phase of apoptosis. However, which caspases function as executioners in reactive oxygen species (ROS)-induced apoptosis is not known. The present study was performed to identify the major caspases acting in ROS-induced apoptosis. Treatment of HL-60 cells with 50 μM hydrogen peroxide ($H_2O_2$) for 4 h induced the morphological changes such as condensed and/or fragmented nuclei, increase in caspase-3 subfamily protease activities, reduction of the procaspase-3 and a DNA fragmentation. To determine the role of caspases in $H_2O_2$-induced apoptosis, caspase inhibitors, acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone (Ac-YVAD-cmk), acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) and acetyl-Val-Glu-Ile-Asp-aldehyde (Ac-VEID-CHO), selective for caspase-1 subfamily, caspase-3 subfamily and caspase-6, respectively, were loaded into the cells using an osmotic lysis of pinosomes method. Of these caspase inhibitors, only Ac-DEVD-CHO completely blocked morphological changes, caspase-3 subfamily protease activation and DNA ladder formation in $H_2O_2$-treated HL-60 cells. This inhibitory effect was dose-dependent. These results suggest that caspase-3, but not caspase-1 is required for commitment to ROS-triggered apoptosis.

*Keywords:* Hydrogen peroxide, caspases, apoptosis, reactive oxygen species (ROS)

## INTRODUCTION

Many of the chemical and physical treatments capable of inducing apoptosis also evoke oxidative stress.[1] For example, both ionizing and ultraviolet (UV) irradiation are capable of inducing apoptosis and both generate reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) and hydroxyl radical.[1] ROS are also generated under physiological conditions:[2] oxidative phosphorylation in mitochondria; cytochrome P450 oxidation in microsomes; respiratory burst in phagocytes such as neutrophils, macrophages and monocytes; and, arachidonic acid cascade. These ROS, generated extracellularly and intracellularly, are considered to induce apoptosis in various mammalian cells, especially when their

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antioxidant capacities are surpassed.[3] This theory is primarily based on the following observations:[4] (i) the addition of ROS themselves or ROS generators, or the depletion of endogenous antioxidants can induce apoptosis; (ii) apoptosis can sometimes be inhibited by endogenous or exogenous antioxidants; (iii) apoptosis is sometimes associated with increases in the intracellular ROS levels. However, it has recently been reported that ROS are not necessary for the induction of apoptosis in some cases, and that Bcl-2, an anti-apoptotic protein, can protect cells from apoptosis even under conditions where ROS are unlikely to be produced.[5–7] Furthermore the observation that superoxide anion is a natural inhibitor of Fas-mediated cell death[8] is surprising. Probably, two pathways of apoptosis, ROS-dependent and ROS-independent pathways, exist.

Recently cysteine proteases, termed caspases,[9] which appear to play important roles in cellular apoptotic signalling have been identified.[10,11] Of these caspases, caspase-3 subfamily proteases have been well documented to be activated in various cells undergoing apoptosis and play critical roles in inducing their apoptosis.[12–15] However, recent report has provided evidence for caspase-independent apoptosis.[16] Therefore, it is conceivable that contribution of caspase to apoptosis depends on cell type used and/or apoptosis-inducing stimuli. For ROS-induced apoptosis, it is ill-defined how ROS induces apoptosis, and which caspase(s) play a critical role in ROS-dependent apoptosis.

In this context, we have initially confirmed whether treatment with $H_2O_2$ at a low concentration can induce a series of apoptotic events including morphological changes in nuclei, caspase activation and DNA fragmentation in an acute promyelocytic leukemia cell line, HL-60. We then used tetrapeptide caspase inhibitors, acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone (Ac-YVAD-cmk),[17] acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO)[18] and acetyl-Val-Glu-Ile-Asp-aldehyde (Ac-VEID-CHO),[19] selective for caspase-1 (ICE) subfamily proteases, caspase-3 (CPP32) subfamily proteases and caspase-6 (Mch2), respectively, to distinguish the potential roles of different caspases in ROS-induced apoptosis in HL-60 cells.

## MATERIALS AND METHODS

### Reagents

RPMI 1640 medium and a 123 bp DNA ladder were purchased from Gibco BRL (Grand Island, NY). Fetal calf serum (FCS) was purchased from Biological Industries (Haemek, Israel). $H_2O_2$ was purchased from Mitsubishi Gas Chemical Company Inc. (Tokyo, Japan). 7-Amino-4-methylcoumarin (AMC), Ac-YVAD-MCA, Ac-DEVD-MCA, Ac-YVAD-cmk, Ac-DEVD-CHO, and Ac-VEID-CHO were purchased from Peptide Institute, Inc. (Osaka, Japan). Hoechst 33342 was purchased from Molecular Probes, Inc. (Eugene, OR). Diphenylamine (DPA) was purchased from SIGMA Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade.

### Cells and Culture Conditions

Human promyelocytic leukemia HL-60 cells, kindly supplied by Dr. Kenzo Sato (Tottori University, Yonago, Japan), were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) (complete RPMI) at 37°C in a humidified incubator with 5% $CO_2$. The present experiments were performed using complete RPMI unless specified otherwise. Cells were seeded at a concentration of $2 \times 10^5$ cells/ml and logarithmic growth was maintained by passaging every 2 or 3 days.

### ROS-induced Apoptosis

Cells ($1 \times 10^6$ cells/ml, 5 ml/dish) were seeded into 60-mm culture dishes and then exposed to

$H_2O_2$ at a concentration of 1–100 μM, for periods of 0.5, 1, 2, 4 or 8 h at 37°C under 5% $CO_2$, 95% air. In some experiments using caspase inhibitors, those inhibitors were directly added to the medium 2 h prior to the addition of $H_2O_2$.

### Analysis of Nuclear Morphology

HL-60 cells were incubated with 50 μM $H_2O_2$ for 4 h at 37°C, washed with phosphate-buffered saline (PBS), fixed with 2% glutaraldehyde for 2 h. Samples were centrifuged, resuspended with PBS, stained with 1 mM Hoechst 33342, mounted on a glass slide, and observed under fluorescence microscopy.

### Detection of DNA Ladder

After $H_2O_2$ treatment, HL-60 cells were centrifuged at 300× *g* for 5 min, washed in ice-cold PBS twice, pelleted, and incubated in lysis buffer (10 mM EDTA, 50 mM Tris/HCl, pH 8.0, 0.5 mg/ml proteinase K, 0.5% sodium lauryl sulfate) for 1 h at 50°C. Then the lysates were extracted with phenol, followed by chloroform, and precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol. The pellets were rinsed with 70% ethanol, air-dried, dissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), and treated with DNase-free RNase for 2 h at 37°C. After re-extraction and reprecipitation, samples were dissolved in TE buffer. Approximately 10 μg of DNA in each well was electrophoresed on a 2% agarose gel, visualized under UV light after staining with ethidium bromide and photographed.

### Quantitation of DNA Fragmentation

DNA fragmentation was measured with the DPA assay. After incubation, HL-60 cells were centrifuged at 300× *g* for 5 min, washed in ice-cold PBS twice, pelleted, and incubated in 200 μl of lysis buffer (10 mM Tris/HCl, 10 mM EDTA, pH 8.0, 0.5% Triton X-100) for 10 min at 4°C. The lysates were centrifuged at 16,000× *g* for 20 min to separate the intact chromatin (pellet) from DNA fragments (supernatant). The supernatant was placed in a separate microfuge tube, and pellets were resuspended in 200 μl of lysis buffer. Both DNA fragments solution and the intact chromatin solution were precipitated for 30 min at 4°C in 200 μl of 1 N perchloric acid (PCA), pelleted at 16,000× *g* for 20 min and the supernatant was removed. Pellets were resuspended in 50 μl of 1 N PCA and incubated for 20 min at 70°C. DNA contents were quantitated using the DPA reagent.[20] The percentage of DNA fragmentation was calculated as the ratio of DNA fragments to the total DNA, the sum of DNA fragments and the intact chromatin.

### Measurement of Caspase Activity

Activities of caspase-1 subfamily and caspase-3 subfamily proteases, which we refer to below as YVADase and DEVDase, respectively, were measured as described previously.[21] In brief, cells were collected, washed in PBS and lysed for 20 min on ice in lysis buffer containing 10 mM HEPES-KOH (pH 7.4), 2 mM EDTA, 0.1% 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 1 mM PMSF and 5 mM dithiothreitol (DTT). After centrifugation at 2,000× *g* for 10 min, the supernatants were collected as lysates. For measurement of protease activity, 20 μg of lysate diluted to 20 μl with lysis buffer was mixed with 20 μl of 2 × ICE buffer (40 mM HEPES-KOH [pH 7.4], 20% [v/v] glycerol, 1 mM PMSF and 4 mM DTT) containing 40 μM Ac-DEVD-MCA or Ac-YVAD-MCA and incubated for 1 h at 37°C. After the addition of 1 ml of double distilled water (DDW), the fluorescence of the reaction mixture was determined with a spectrofluorometer. The excitation and emission wavelengths were 380 and 460 nm, respectively. One unit of protease activity was defined as the amount of enzyme required to release 1 pmol of AMC per minute.

### Western Blot Analysis

After treatment, cells were washed twice in PBS, lysed for 20 min on ice in lysis buffer containing 10 mM HEPES-KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 1 mM PMSF and 5 mM DTT. After centrifugation, the supernatants were transferred and protein content was analyzed. The supernatants were mixed with the same volume of 2 × SDS sample buffer (125 mM Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 12% $\beta$-mercaptoethanol and 0.04% bromophenol blue) and 40 μg protein from the supernatants was separated by SDS-PAGE using 12% polyacrylamide gel and electroblotted to polyvinylidine difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking nonspecific binding sites in wash buffer (10 mM Tris/HCl, pH 7.5, 100 mM NaCl and 0.1% Tween 20) containing 5% skim milk, the PVDF membrane was incubated for 1 h at room temperature with anti-human procaspase-3 antibody (Transduction Laboratories, Lexington, KT). It was then washed six times with wash buffer, incubated further with horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham, Buckinghamshire, England) for 1 h at room temperature, and then washed six times with wash buffer. The immunoblot was revealed using an enhanced chemiluminescence detection kit (Amersham).

### Loading of Cells with Caspase Inhibitors

A method utilizing osmotic lysis of pinosomes[22–24] was used to introduce the caspase inhibitors into cells efficiently. Various concentrations of caspase inhibitor peptides, Ac-YVAD-cmk, Ac-DEVD-CHO and Ac-VEID-CHO, and 1% dimethyl sulfoxide (DMSO, vehicle control), were dissolved in hypertonic medium: 0.5 M sucrose, 10% (w/v) polyethyleneglycol 1000, 10 mM HEPES in RPMI 1640 medium (pH 7.2). HL-60 cells ($5 \times 10^6$ cells) were pelleted in a conical tube and resuspended in 0.5 ml of prewarmed inhibitor-containing hypertonic medium for 10 min at 37°C. The suspension was diluted to 15 ml with prewarmed hypotonic medium (60% RPMI 1640 in DDW) and centrifuged at 300× $g$ for 3 min at room temperature. The pellets were resuspended in RPMI 1640 supplemented with 10% FCS at a concentration of $1 \times 10^6$ cells/ml and used for studies of $H_2O_2$-induced apoptosis. In some experiments, two other kinds of hypotonic medium (70% RPMI in DDW and 80% RPMI in DDW) were used.

## RESULTS

### Induction of DNA Fragmentation in HL-60 Cells by $H_2O_2$ Treatment

To determine the lowest concentration of $H_2O_2$ that induced apoptosis in HL-60 cells, we treated the cells with $H_2O_2$ at concentrations of 1–100 μM for 4 h. Electrophoresis showed that $H_2O_2$ induced typical ladder-like internucleosomal DNA cleavage at concentrations of 50 μM and above (Figure 1A). Percentages of DNA fragmentation in the cells treated with 50 and 100 μM $H_2O_2$ for 4 h were increased to 5 and 16 times that in control cells, respectively (Figure 1B). We then examined the period required to induce DNA ladder formation using 50 μM $H_2O_2$ in HL-60 cells. As shown in Figure 2A, cells showing typical DNA ladder formation were evident at 2 h of $H_2O_2$ treatment. Percentage of DNA fragmentation in the cells was maximal 4 h after 50 μM $H_2O_2$ exposure (Figure 2B).

### Activation of Caspase-3 in HL-60 Cells by $H_2O_2$ Treatment

DEVDase activities in the cells treated with 50 and 100 μM $H_2O_2$ for 4 h were increased to 2.7 and 8.2 times that in control cells, respectively, while YVADase activity remained unchanged at the concentrations of $H_2O_2$ used (Figure 3). A small increase in DEVDase activity was detected within 2 h after treatment of HL-60 cells with 50 μM

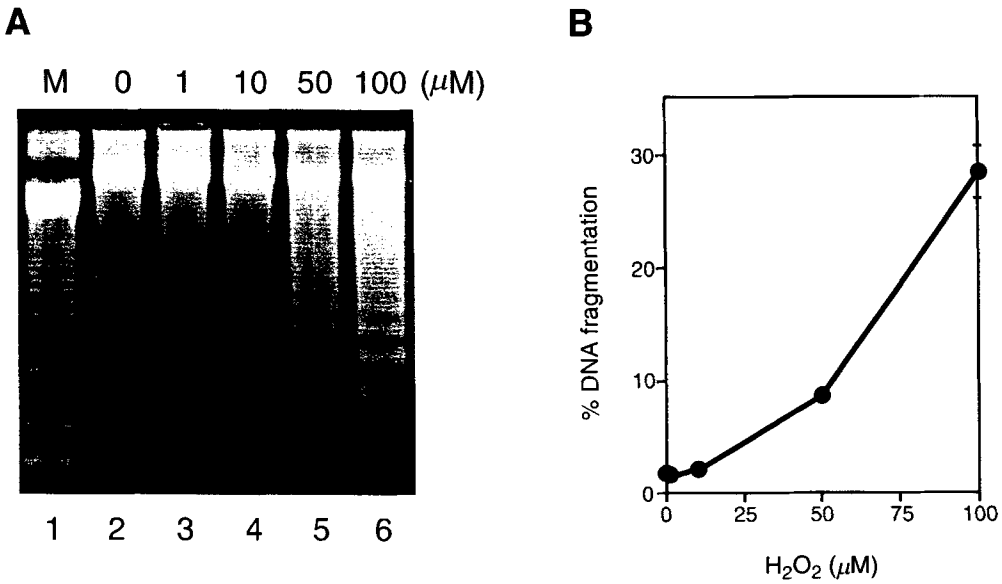


FIGURE 1 Internucleosomal DNA fragmentation in HL-60 cells exposed to various concentrations of $H_2O_2$. (A) Cells ($5 \times 10^6$) were seeded into dishes and incubated with 0, 1, 10, 50 or 100 μM $H_2O_2$ (lanes 2–6, respectively) for 4 h at 37°C under 5% $CO_2$, 95% air. Cellular DNA was extracted, and 10-μg aliquots were electrophoresed on a 2% agarose gel. Lane 1 is the 123-bp DNA ladder. Representative data from three separate experiments are shown. (B) After $H_2O_2$ treatment, DNA fragmentation in the cells was quantitated using the DPA assay. Data represent the mean ± standard error (SE) of three separate experiments. Some error bars are too small to visualize.

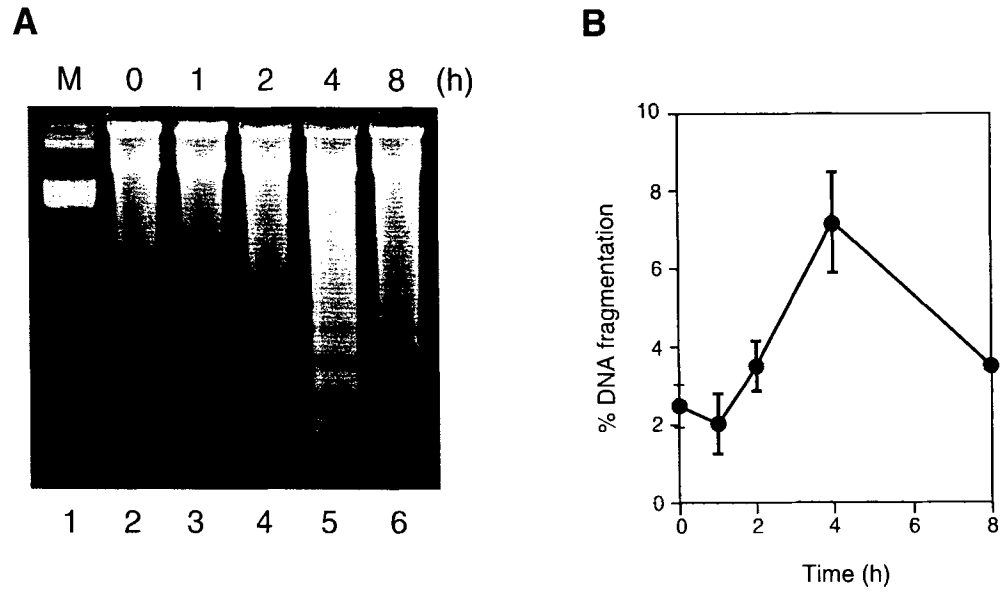


FIGURE 2 Internucleosomal DNA fragmentation in HL-60 cells exposed to 50 μM $H_2O_2$ for various periods. (A) Cells ($5 \times 10^6$) were incubated with 50 μM $H_2O_2$ for 0, 1, 2, 4 or 8 h (lanes 2–6, respectively) at 37°C under 5% $CO_2$, 95% air. DNA was electrophoresed as described in the legend to Figure 1. Lane 1 is the 123-bp DNA ladder. Representative data from three separate experiments are shown. (B) After treatment of cells with 50 μM $H_2O_2$, DNA fragmentation was quantitated using the DPA assay at the indicated times. Data represent the mean ± SE of at least three separate experiments. Some error bars are too small to visualize.

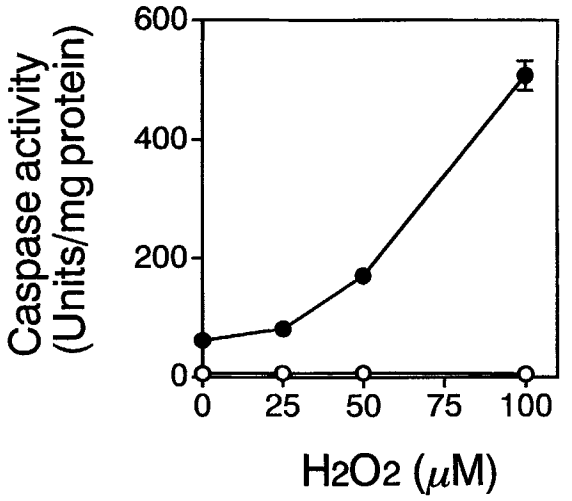


FIGURE 3 DEVDase and YVADase activities in HL-60 cells exposed to various concentrations of $H_2O_2$. Cells ($5 \times 10^6$) were seeded into dishes and incubated with 0, 25, 50 or 100 μM $H_2O_2$ for 4 h at 37°C under 5% $CO_2$, 95% air and then lysates were prepared. DEVDase (closed circles) and YVADase (open circles) activities in these lysates were measured using Ac-DEVD-MCA and Ac-YVAD-MCA as substrates, respectively. Data represent the mean ± SE of at least five separate experiments. Some error bars are too small to visualize.

$H_2O_2$, and the maximum increase in proteolytic activity occurred at 4 h (Figure 4A). YVADase activity remained unchanged throughout the incubation period (Figure 4A). The Western blot in Figure 4B revealed reduction of the procaspase-3 upon incubation with 50 μM $H_2O_2$ which paralleled the activation of a DEVDase (Figure 4A).

### $H_2O_2$-induced Morphological Changes in Nuclei of HL-60 Cells

Treatment with 50 μM $H_2O_2$ for 4 h, which apparently induced caspase-3 subfamily protease activation and DNA fragmentation, also caused chromatin condensation and/or nuclear fragmentation in HL-60 cells (Figure 5).

### Effect of Caspase Inhibitors on $H_2O_2$-induced Apoptosis in HL-60 Cells

To determine whether caspase inhibitors inhibit DNA ladder formation in HL-60 cells, three

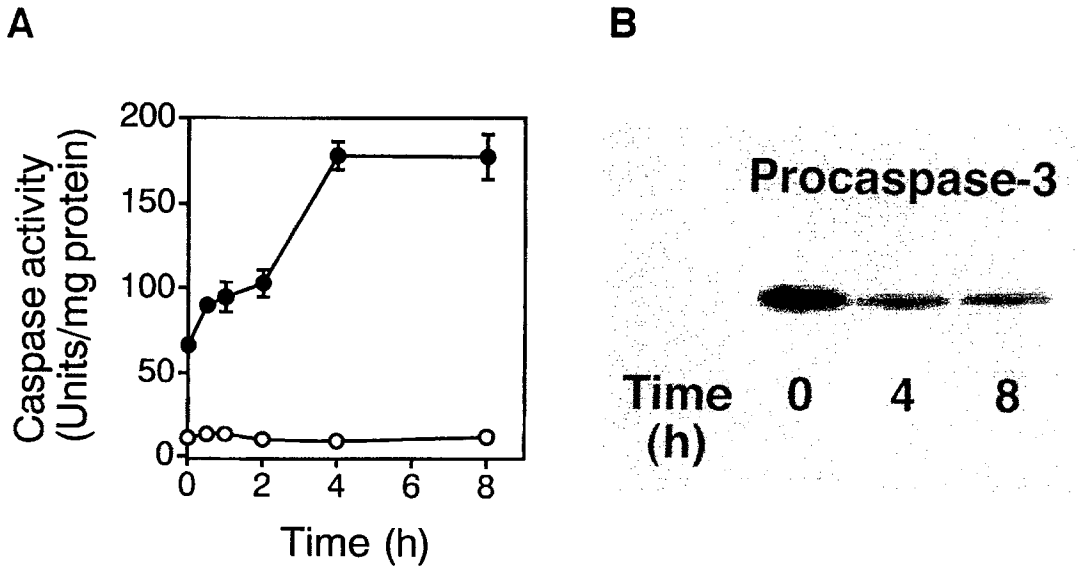


FIGURE 4 Time course of caspase-3 activation. Cells ($5 \times 10^6$) were incubated with 50 μM $H_2O_2$ for 0, 0.5, 1, 2, 4 or 8 h at 37°C under 5% $CO_2$, 95% air, and then lysates were prepared. (A) DEVDase (closed circles) and YVADase (open circles) activities in these lysates were measured as described in the legend to Figure 3. Data represent the mean ± SE of at least five separate experiments. Some error bars are too small to visualize. (B) The amounts of procaspase-3 were analyzed by Western blot analysis as described in Materials and Methods.

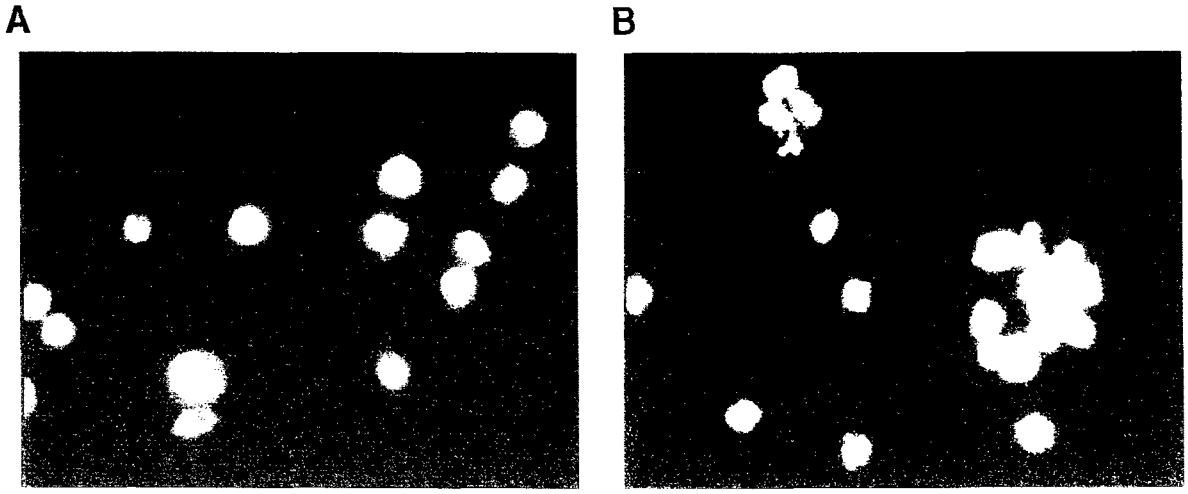


FIGURE 5 Nuclear morphology of HL-60 cells treated with $H_2O_2$. Cells were incubated in the absence (A) or presence (B) of 50 μM $H_2O_2$ for 4 h and then visualized with Hoechst 33342 dye for apoptotic nuclei.

tetrapeptide derivatives, Ac-YVAD-cmk, Ac-DEVD-CHO and Ac-VEID-CHO, which are selective inhibitors of caspase-1 subfamily proteases, caspase-3 subfamily proteases and caspase-6, respectively, were added directly to the culture medium 2 h before $H_2O_2$ treatment. However, these caspase inhibitors did not inhibit DNA ladder formation (data not shown). Since it is thought that penetration of Ac-YVAD-cmk, Ac-DEVD-CHO and Ac-VEID-CHO into HL-60 cells is very poor when they are added directly to the medium,[18, 24] we loaded the tetrapeptides into the cells using osmotic lysis of pinosomes[23, 24] as described in Materials and Methods. Cell viability just after this procedure was more than 98% when assessed by the trypan blue exclusion assay (data not shown). Then, apoptosis was induced by 4 h of treatment with 50 μM $H_2O_2$. Osmotic lysis of pinosomes alone, and vehicle (1% DMSO) alone, did not induce DNA fragmentation in HL-60 cells (Figure 6, lanes 2 and 3). The caspase inhibitors themselves also did not induce DNA ladder formation (Figure 6, lanes 4–6), whereas 50 μM $H_2O_2$ caused DNA fragmentation in HL-60 cells (Figure 6, lanes 7 and 8). Treatment with 500 μM Ac-DEVD-CHO, but not with 500 μM Ac-YVAD-cmk or 500 μM Ac-VEID-CHO, completely suppressed DNA ladder formation induced by 50 μM

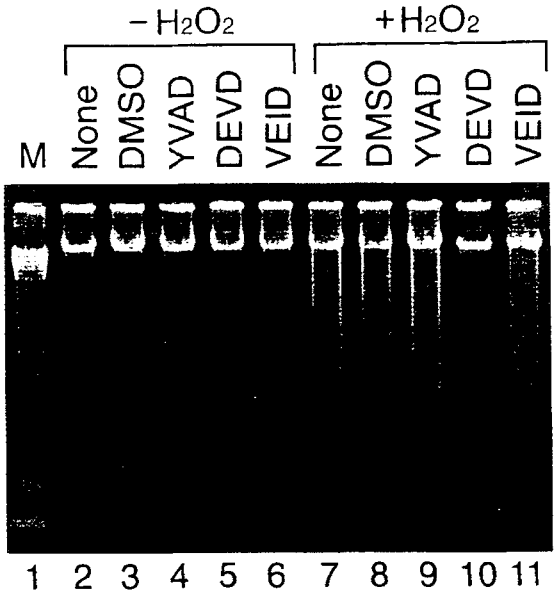


FIGURE 6 Effect of caspase inhibitors on internucleosomal DNA fragmentation in $H_2O_2$-treated HL-60 cells. Caspase inhibitors, 500 μM Ac-YVAD-cmk (lanes 4 and 9), 500 μM Ac-DEVD-CHO (lanes 5 and 10), and 500 μM Ac-VEID-CHO (lanes 6 and 11), and 1% DMSO (vehicle) (lanes 3 and 8) were loaded into cells ($5 \times 10^6$) using osmotic lysis of pinosomes, as described in Materials and Methods. Control cells were exposed to osmotic lysis of pinosomes without addition of inhibitors (None). Cells were then incubated in the absence (lanes 2–6) or presence (lanes 7–11) of 50 μM $H_2O_2$ for 4 h. DNA was electrophoresed as described in the legend to Figure 1. Lane 1 is the 123-bp DNA ladder. Representative data from three separate experiments are shown.

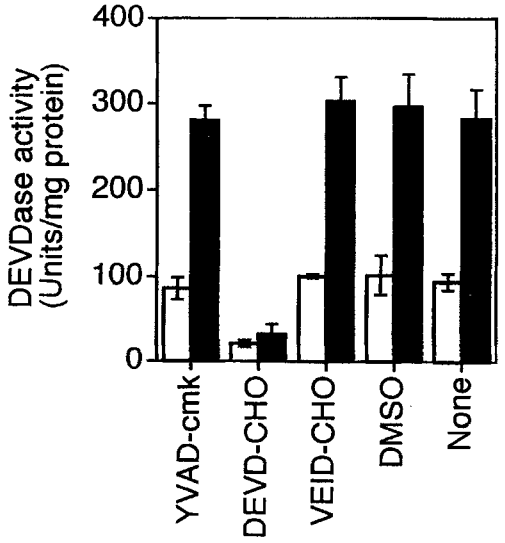


FIGURE 7 Effect of caspase inhibitors on DEVDase activity in $H_2O_2$-treated HL-60 cells. Caspase inhibitors, 500 μM Ac-YVAD-cmk, 500 μM Ac-DEVD-CHO, and 500 μM Ac-VEID-CHO, and 1% DMSO (vehicle) were loaded into cells ($5 \times 10^6$) using osmotic lysis of pinosomes, as described in Materials and Methods. Control cells were exposed to osmotic lysis of pinosomes without the addition of inhibitors (None). Cells were then incubated in the absence (open columns) or presence (closed columns) of 50 μM $H_2O_2$ for 4 h. DEVDase activity was measured as described in the legend to Figure 3. Data represent the mean ± SE of three separate experiments.

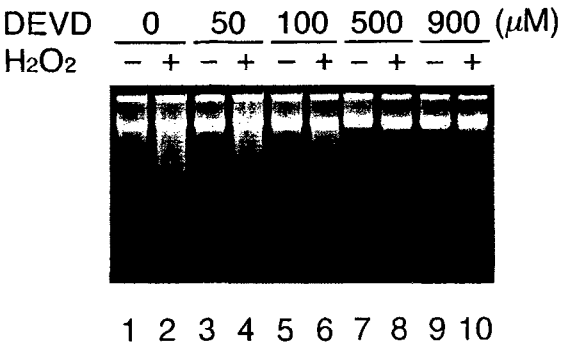


FIGURE 8 Effect of Ac-DEVD-CHO at various concentrations on $H_2O_2$-induced apoptosis. Ac-DEVD-CHO at concentrations of 0 (lanes 1 and 2), 50 (lanes 3 and 4), 100 (lanes 5 and 6), 500 (lanes 7 and 8) and 900 μM (lanes 9 and 10) was loaded into cells ($3 \times 10^6$) using osmotic lysis of pinosomes, as described in Materials and Methods. Cells were then incubated in the absence (odd numbered lanes) or presence (even numbered lanes) of 50 μM $H_2O_2$ for 4 h. DNA was electrophoresed as described in the legend to Figure 1. Representative data from three separate experiments are shown.

$H_2O_2$ in HL-60 cells (Figure 6, lanes 9–11). In the samples prepared using hypotonic solutions containing 70% or 80% RPMI, but not 60% RPMI, 500 μM Ac-DEVD-CHO did not inhibit DNA ladder formation (data not shown).

Treatment with 500 μM Ac-DEVD-CHO also completely suppressed DEVDase activation (Figure 7) and formation of fragmented nuclei (data not shown) in 50 μM $H_2O_2$-treated HL-60 cells. Furthermore, Ac-DEVD-CHO blocked DNA ladder formation induced by $H_2O_2$ in HL-60 cells in a dose-dependent manner (Figure 8).

## DISCUSSION

It has been reported that ROS such as $H_2O_2$,[25–29] nitric oxide (NO)[30–32] or peroxynitrite[33] can mediate apoptosis. However, NO[34] as well as the superoxide anion[8] described above can also function as cell death inhibitors. NO seems to inhibit diverse apoptogenic stimulus-induced caspase-3 activation via S-nitrosation and oxidation of critical thiol groups *in vitro*[34–36] and *in vivo*.[34] However, NO donor concentrations required for caspase inhibition are too high to allow assessment of the importance of endogenous NO formation to caspase inhibition.[35] $H_2O_2$ is a membrane-permeable and comparatively stable ROS, and therefore has been used in experiments examining redox regulation[37] and apoptosis.[25] Hampton and Orrenius[26] have reported that $H_2O_2$ only induces apoptosis in Jurkat T-lymphocytes by caspase activation at a low concentration (100 μM), while at higher concentrations it inactivates caspases by oxidizing their active site cysteines.

We initially showed here that treatment of HL-60 cells with $H_2O_2$ at a low concentration (50 μM) induced apoptosis, as confirmed by nuclear morphological changes, increase in caspase-3 subfamily protease activities, reduction of procaspase-3 and a typical DNA ladder pattern on electrophoresis. Although it may be argued

that the concentration of $H_2O_2$ (50 μM) used for the induction of apoptosis was unphysiologically high, concentrations of about 100 μM have been measured in abscess fluid,[38] and it has been reported that plasma $H_2O_2$ levels in patients with diabetes are 60–80 μM.[39] It has been reported previously that exposure of HL-60 cells to 15 μM $H_2O_2$ for 12 h leads to apoptosis,[25] although 10 μM $H_2O_2$ did not induce apoptosis in our experiment. This difference may be attributable to the shorter incubation period we employed.

The ICE family of cysteine proteases, recently renamed the caspases,[9] play a key role in apoptosis.[40] The mammalian caspase family has at least 10 members, which are divided into three subfamilies (the caspase-1, caspase-2 and caspase-3 subfamilies), based on their sequence homology.[9, 11] However, it is not known which caspases are the key components of the execution phase of ROS-induced apoptosis.

In the present study, treatment with 50 μM $H_2O_2$ caused a considerable increase in DEVDase activity which was accompanied by DNA laddering, while YVADase activity was not increased despite the existence of ICE in HL-60 cells.[24] These results indicated that caspase-3 subfamily proteases play a pivotal role in commitment to apoptotic execution induced by $H_2O_2$ in HL-60 cells. Together with the observation that peroxynitrite induces apoptosis via caspase-3 subfamily protease activation,[41] caspase-3 is likely to be one of the major executioners in ROS-induced apoptosis. To further confirm this possibility, we tried to inhibit $H_2O_2$-induced apoptosis using various selective caspase inhibitors. Inhibition of apoptosis in whole cells by peptide caspase inhibitors has been demonstrated in receptor-mediated apoptosis such as Fas-induced apoptosis.[12, 42] However, it has rarely been shown that in apoptosis caused by stress such as ROS, heat and UV irradiation, caspase inhibitors block apoptosis, especially DNA ladder formation.

Upon *in vivo*, but not *in vitro*, treatment with tetrapeptide inhibitors, the most plausible explanation is that the inhibitors are not efficiently introduced into the cells. The observation that direct addition of 100 μM Ac-YVAD-CHO and 100 μM Ac-DEVD-CHO did not prevent $H_2O_2$-induced DNA laddering in Ms-1 cells[29] appears to support the above possibility. Furthermore, we were unable to protect HL-60 cells against apoptosis by direct addition of Ac-DEVD-CHO. Therefore we used osmotic lysis of pinosomes[22–24] to introduce three caspase inhibitors into HL-60 cells, and clearly demonstrated that Ac-DEVD-CHO, a selective inhibitor of the caspase-3 subfamily proteases, completely inhibited DEVDase activation and nuclear and DNA fragmentation in HL-60 cells in the presence of 50 μM $H_2O_2$. Caspase-1 subfamily proteases seem to be located upstream from caspase-3.[43] Studies *in vitro*[43] and *in vivo*[44, 45] have shown that caspase-1 subfamily proteases activate downstream caspase-3 subfamily proteases, resulting in apoptosis. Moreover, caspase-1 subfamily proteases seem to be crucial for room temperature-induced apoptosis in HL-60 cells.[46] However, it has not been clarified whether a caspase-1-dependent step exists in $H_2O_2$-induced apoptosis. In a recent study,[43] YVADase activity was transiently stimulated within 30 min after treatment of W4 cells with anti-Fas antibody. Therefore, we examined YVADase and DEVDase activities over a period of 60 min after addition of 50 μM $H_2O_2$ to clarify the sequential activation of caspases. YVADase activity was not increased throughout this period (Figure 4A). Even 10 μM Ac-YVAD-cmk, a specific inhibitor of caspase-1 subfamily proteases, was unable to reduce any basal activity measured in these assays (data not shown), and therefore this was considered to be nonspecific activity. Furthermore, Ac-YVAD-cmk did not inhibit $H_2O_2$-induced *in vivo* apoptosis (Figure 6), suggesting that the apoptotic signal mediated by $H_2O_2$ might be transduced to caspase-3 subfamily proteases independently of caspase-1 subfamily proteases. In other words, it appeared that sequential activation such as YVADase-dependent DEVDase activation was not involved in $H_2O_2$-mediated apoptosis of HL-60 cells. Furthermore,

Ac-VEID-CHO, which preferentially inhibits caspase-6, a member of the caspase-3 subfamily, did not protect the cells from apoptosis (Figure 6), indicating that caspase-6 does not participate in $H_2O_2$-induced apoptosis, particularly DNA ladder formation, although caspase-3 and caspase-6 are considered to be the major active caspases present in apoptotic cells.[47] This discrepancy may be due to the use of different stimuli and cells. Further studies are required to determine the contribution of caspase-6 to ROS-induced apoptosis.

The present results suggest that ROS, such as $H_2O_2$, function as apoptosis inducers upstream of caspase-3, and that caspase-3 plays a leading role in commitment to ROS-mediated apoptosis. However, it remains to be shown whether ROS act directly on the upstream caspase(s) or via a second messenger, and further studies using specific inhibitors of individual caspases will be necessary to elucidate the molecular mechanism underlying ROS-induced apoptosis.



## *Acknowledgement*

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