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Induction of apoptosis by diallyl disulfide through activation of caspase-3 in human leukemia HL-60 cells

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Abstract

Diallyl disulfide (DADS), a component of garlic (*Allium sativum*), has been known to exert potent chemopreventative activity against colon, lung, and skin cancers. However, its molecular mechanism of action is still obscure. The present study demonstrated that DADS induces apoptosis of human leukemia HL-60 cells in a concentration- and time-dependent manner with an IC_{50} for cell viability of less than 25 μM. DADS activated caspase-3 as evidenced by both the proteolytic cleavage of the proenzyme and increased protease activity. Activation of caspase-3 was maximal at 3 hr and led to the cleavage of 116 kDa poly(ADP-ribose) polymerase (PARP), resulting in the accumulation of an 85 kDa cleavage product. Both activation of caspase-3 and cleavage of PARP were blocked by pretreatment with either antioxidants or a caspase-3 inhibitor, but not a caspase-1 inhibitor. DADS increased the production of intracellular hydrogen peroxide, which was blocked by preincubation with catalase. These results indicate that DADS-induced apoptosis is triggered by the generation of hydrogen peroxide, activation of caspase-3, degradation of PARP, and fragmentation of DNA. The induction of apoptosis by DADS may be the pivotal mechanism by which its chemopreventative action against cancer is based.

Keywords: Diallyl disulfide; Garlic; Caspase-3; HL-60 cell; Apoptosis; Reactive oxygen intermediates

1. Introduction

Garlic is a plant commonly used for seasoning food in many different cultures of the world, and its medicinal properties have been touted since ancient times. Epidemiological studies have shown that enhanced garlic consumption is closely related with reduced cancer incidence [1,2]. DADS, an oil-soluble organosulfur compound in processed garlic, inhibits the proliferation of human colon, lung, and skin cancer cells [3]. Sundaram *et al.* [4] suggested that DADS induces apoptosis of human colon tumor cells by increasing intracellular calcium concentration.

Apoptosis is a physiologically programmed mechanism by which cells die. It is characterized by chromatin condensation, membrane blebbing, cell shrinkage, and DNA fragmentation [5]. It can be initiated by oxidative stress, which in turn is mediated by the generation of ROIs [6]. Oxidative stress activates caspases, a family of cysteine proteases that are involved in the induction of cell death by apoptosis [7,8]. Generally, caspases exist in cells as inactive precursor forms [9,10]. Caspase activation is tightly regulated by an apoptosis activating complex, requiring proteolytic removal of an amino-terminal domain of procaspase to produce an active caspase [11–14]. Once activated, caspase-3 cleaves many substrate proteins including PARP, ICAD, and structural proteins such as actin, fodrin, or lamin to generate the characteristic apoptotic morphology [15–18]. Cleavage of

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Abbreviations: Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-CHO (aldehyde); Ac-DEVD-AFC, *N*-acetyl-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethyl-coumaine); Ac-YVAD-CHO, *N*-acetyl-Tyr-Val-Ala-Asp-CHO (aldehyde); CAD, caspase-activated deoxyribonuclease; CM-H_2DCFDA, 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DADS, diallyl disulfide; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; ICAD, inhibitor of caspase-activated deoxyribonuclease; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; and ROI, reactive oxygen intermediate.

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ICAD leads to the activation of CAD, which in turn cleaves genomic DNA within internucleosomal regions and generates multimers of nucleosomal domain-sized fragments [19].

In this study, we demonstrated that DADS induces apoptosis in human leukemia HL-60 cells through the generation of ROIs and subsequent activation of the caspase-3 pathway.

2. Materials and methods

2.1. Culture conditions

The human leukemia cell line HL-60 was purchased from the American Type Culture Collection. Cells were placed into 75 cm^2 tissue culture flasks and grown at 37° under a humidified, 5% CO_2 atmosphere in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum and 2 mM glutamine, 10,000 units/mL of penicillin, 10 mg/mL of streptomycin, and 2.5 μg/mL of amphotericin B.

2.2. MTT assay for cell viability

The viability of cultured cells was determined by assaying for the reduction of MTT to formazan [20]. In brief, after 24 hr of incubation with DADS, cells (10^4/well) in 96-well plates were washed twice with PBS. MTT (100 μg/0.1 mL of PBS) was added to each well. Cells were incubated at 37° for 1 hr, and DMSO (100 μL) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a model Spectra MAX PLUS (Molecular Devices).

2.3. Caspase activity assay

After treatment with DADS (Fluka), cells were washed with ice-cold PBS and lysed in Triton X-100 buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris–HCl, pH 7.5) for 30 min on ice. Cell lysates were mixed with caspase assay buffer (10% glycerol, 2 mM dithiothreitol, and 20 mM HEPES, pH 7.5) containing 20 μM Ac-DEVD-AFC (Pharmingen Inc.), a caspase substrate, and incubated for 1 hr at 37°. Enzyme catalyzed release of AFC was monitored using a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

2.4. Western blot analysis of procaspase (CPP32) and PARP cleavage

Cell extract proteins were separated by SDS–PAGE. Subsequently the proteins were transferred onto a nitrocellulose membrane (Millipore) using a semi-dry blotting apparatus (Bio-Rad). Prior to incubation with antibodies against caspase-3 and PARP (Transduction Lab.), membranes were blocked with 2% BSA for 30 min. After washing the membranes, an alkaline-phosphatase-coupled secondary antibody was added. The target proteins became visible following the addition of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), a substrate of alkaline phosphatase.

2.5. Flow-cytometric analysis of annexin V-FITC binding

Cells (1×10^6) were incubated with DADS for 12 hr and then harvested. Specific binding of annexin V-FITC was performed by incubating the cells for 15 min at room temperature in a binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) containing a saturating concentration of annexin V-FITC and PI. After incubation, the cells were pelleted and analyzed in a FACScan analyzer (Becton Dickinson). Annexin$^+$/PI$^+$ cells were defined as necrotic (or late apoptotic), while annexin$^+$/PI$^-$ cells were defined as apoptotic.

2.6. Detection of DNA fragmentation by gel electrophoresis

Cell pellets (3×10^6 cells) were resuspended in 500 μL of lysis buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris–HCl, pH 8.0) at room temperature for 15 min and centrifuged at 16,000 *g* for 10 min. DNA was then extracted twice with phenol:chloroform (1:1), precipitated with ethanol, and resuspended in Tris/EDTA buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA). DNA was analyzed after separation by gel electrophoresis (2% agarose).

2.7. Measurement of intracellular ROIs generation

The intracellular formation of hydrogen peroxide was detected using the fluorescent probe CM-H_2DCFDA (Molecular Probes). Control cells and cells treated with 25 μM DADS were analyzed for changes in fluorescence. Cells (1×10^5/mL) were washed with PBS, and loaded with 10 μM CM-H_2DCFDA at 37° for 30 min in the dark. During the loading, the acetate groups on CM-H_2DCFDA were removed by intracellular esterases, trapping the probe inside the HL-60 cells. Production of ROIs was measured by changes in fluorescence at an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

2.8. Protein determination

The cytosolic protein concentration in HL-60 cells was determined by the method of Bradford [21] with bovine serum albumin as the standard. All of the samples were assayed in triplicate.

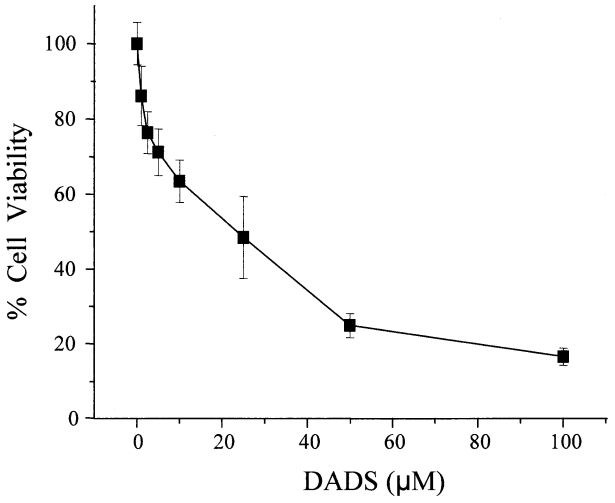

Fig. 1. Effects of DADS on cell viability. HL-60 cells (1×10^5) were treated with DADS for 24 hr, and their viability was determined by MTT assay. The percentage of viable cells was calculated as a ratio of A_{570} of treated to control cells (treated with 0.05% DMSO vehicle). Each value is the mean ± SEM of four independent experiments.

2.9. Statistical analysis

Statistical analysis of the data was performed with Student's *t*-test and ANOVA. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Concentration-dependent reduction of cell survival

We used the MTT conversion assay to determine the total number of living HL-60 cells exposed to DADS. As shown in Fig. 1, the number of live HL-60 cells was less than 20% after exposure to 100 μM DADS for 24 hr. The IC_{50} of DADS for cell viability was less than 25 μM.

3.2. Induction of apoptosis

Redistribution of membrane phosphatidylserine from the inner leaflet of the plasma membrane to the outer surface is common in many apoptotic cells [22]. HL-60 cells were treated with 25 μM DADS for 12 hr and costained with annexin V-FITC and PI. As shown in Fig. 2B, treatment with DADS resulted in an increase of the cell population that was positive for annexin V staining. A substantial number of the annexin V-positive cells were not stained by PI, indicating that they were viable under this specific experimental condition. DNA extracts from HL-60 cells treated with DADS for 24 hr displayed a characteristic ladder pattern of discontinuous DNA fragments on agarose gel electrophoresis (Fig. 2G).

3.3. Activation of caspase-3 during DADS-induced apoptosis

To directly address the involvement of caspase-3 in DADS-induced apoptosis, caspase activity was determined in DADS-treated HL-60 cells using a fluorogenic caspase-3 substrate, Ac-DEVD-AFC. As shown in Fig. 3A, DADS caused a concentration-dependent activation of caspase-3. This protease activity was maximal at 3 hr (Fig. 3B), and it preceded apoptosis, which is consistent with other reports that caspase activation is required for DNA fragmentation [23]. This result was confirmed by western blot analysis of caspase-3 protein (Fig. 4), which demonstrates that the DADS-induced proteolytic cleavage of procaspase-3 occurs in a time-dependent manner.

3.4. Degradation of PARP, an endogenous substrate of caspase-3

Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. This cleavage leads to its inactivation, thus preventing futile DNA repair cycles [15]. Although PARP is not essential for cell viability, the cleavage of PARP is another hallmark of apoptosis [24]. Treatment of HL-60 cells with 25 μM DADS induced the proteolytic cleavage of PARP (116 kDa), resulting in the accumulation of the 85 kDa cleavage product (Fig. 5). PARP cleavage was apparent 4 hr after DADS treatment, immediately following the appearance of caspase activity and preceding DNA fragmentation.

3.5. Prevention of apoptosis by a caspase-3 inhibitor

Pretreatment of HL-60 cells for 1 hr with a caspase-3 inhibitor, Ac-DEVD-CHO, inhibited both the appearance of apoptosis (Fig. 2, C and G) and the activation of caspase-3 (Fig. 6A) by DADS. In contrast, Ac-YVAD-CHO, a caspase-1 inhibitor, had little effect at a similar concentration (19 ± 2.8% for apoptosis and 16.5 ± 3.1 pmol AFC/min/mg protein for caspase activity), consistent with the characteristically high substrate specificity of different caspases. Western blot analysis revealed that Ac-DEVD-CHO blocks PARP cleavage (Fig. 6B).

3.6. Triggering of apoptosis by oxidative stress

Several reports suggest an involvement of ROIs, upstream of caspase-3 activation, in the signal transduction pathways leading to apoptosis. [25]. To determine the involvement of ROIs in DADS-induced apoptosis, hydrogen peroxide levels were determined in DADS-treated HL-60 cells. As shown in Fig. 7, DADS caused the cells to generate hydrogen peroxide. Production was linear, i.e. time-dependent, up to 30 min and persisted for 2 hr. Furthermore, when HL-60 cells were pretreated with either 10 mM NAC (an antioxidant) or 400 units of catalase (a scavenger for hy-

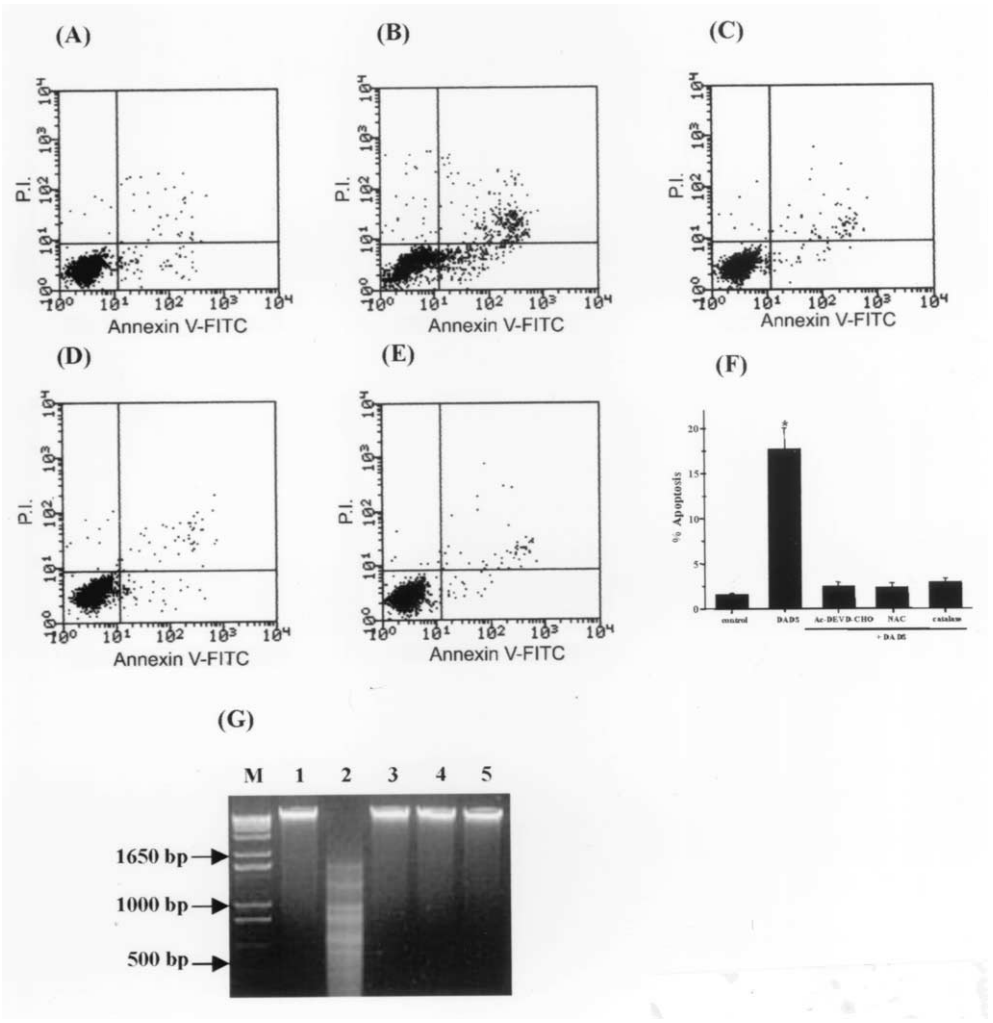

Fig. 2. Flow cytometric analysis of HL-60 cells. HL-60 cells (4×10^6) incubated for 12 hr in the absence (A) or presence (B) of 25 μM DADS were stained with FITC-conjugated annexin V and PI for flow cytometry. To determine the role of caspase-3 and ROIs in DADS-induced phosphatidylserine externalization and cell death, HL-60 cells were pretreated with 5 μM Ac-DEVD-CHO (C), 10 mM NAC (D), or 400 units catalase (E) before treatment with DADS. (F) Representation of results shown in A–E (means $\pm$ SEM from three independent experiments). Key: (*) significantly different from control, $P < 0.05$ by Student's *t*-test. HL-60 cells (5×10^6) were incubated with DADS with or without 5 μM Ac-DEVD-CHO, 10 mM NAC, or 400 units catalase for 24 hr, and DNA fragmentation was analyzed by 2% agarose gel electrophoresis (G); Lane 1, control; Lane 2, 25 μM DADS; Lanes 3–5, 25 μM DADS following 5 μM Ac-DEVD-CHO (lane 3), 10 mM NAC (lane 4), or 400 units catalase (lane 5).

drogen peroxide) for 1 hr before incubation with DADS, they were protected from undergoing apoptosis (Fig. 2, D and E). In addition, both catalase and NAC prevented caspase-3 activation and PARP cleavage (Fig. 6, A and B).

4. Discussion

DADS, an oil-soluble sulfur compound, and allyl mercaptan are formed in individuals eating raw garlic. The major components of cooked garlic are DADS and diallyl trisulfide, which is reduced to allyl mercaptan in blood [26]. Allyl mercaptan at a relatively low concentration (5 μM) increased caspase-3 activity to a level comparable to that of 25 μM DADS. DADS is reported to comprise about 60% of garlic oil [27], indicating that it is the most appropriate compound to use in the study of the possible effects of raw and cooked garlic.

The present study indicates that DADS is an effective inducer of apoptosis in HL-60 cells. Apoptosis is an evolutionarily conserved suicide program residing in cells. It leads to cell death through a tightly regulated process resulting in the removal of damaged or unwanted tissue. It also plays an important role in the development of various diseases including cancer [28,29]. Recently, interest has focused on the manipulation of the apoptotic process for the treatment and prevention of cancer. Thus, much effort has been directed toward the search for compounds that influence apoptosis and understanding their mechanism of action. The processes that elicit activation of the apoptotic program are diverse. A family of caspases plays a pivotal role in the execution of apoptosis. Caspase-3 has been

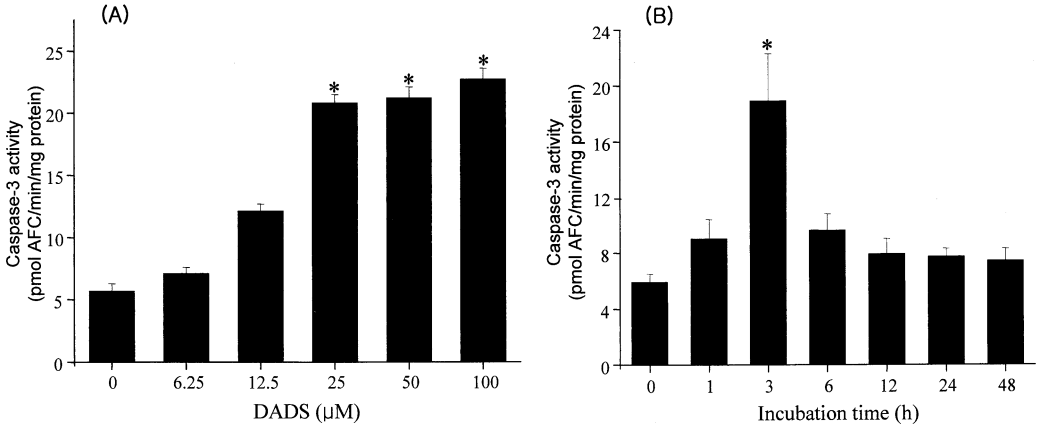

Fig. 3. DADS-induced apoptosis through activation of caspase-3. HL-60 cells (5×10^6) were treated with either (A) a range of concentrations (0–100 μM) of DADS for 3 hr, or (B) 25 μM DADS for a period of 1, 3, 6, 12, 24, and 48 hr. Cytosolic extracts were prepared and assayed for caspase-3 activity as described in "Materials and methods." Values represent means ± SEM of six separate experiments. Key: (*) $P < 0.05$ when compared with control by one-way ANOVA using Turkey's multi-comparison procedures.

shown to play an important role in chemotherapy- [30], growth factor withdrawal- [31], Fas- [32], and retinoic acid-induced apoptosis [33].

Caspase-3 is a most likely candidate to mediate DADS-induced apoptosis, as evidenced by both the increased protease activity of caspase-3 and the proteolytic cleavage of the proenzyme in DADS-treated HL-60 cells (Figs. 3 and 4). Using both flow cytometry analysis and the DNA fragmentation assay, we observed that HL-60 cell apoptosis can be prevented by inhibiting caspase activity with a caspase-3 inhibitor, Ac-DEVD-CHO, but not with a caspase-1 inhibitor, Ac-YVAD-CHO. Using a fluorogenic substrate, we showed that DADS increases caspase-3 activity in a time- and concentration-dependent manner, without affecting the activity of caspase-1. Although our findings point to a critical role for caspase-3 in DADS-induced HL-60 cell apoptosis, we do not rule out the possibility that other caspase family proteases may also play a crucial role in HL-60 cell apoptosis. In fact, caspase-6, caspase-7, and caspase-8 also may be inhibited by Ac-DEVD-CHO.

The execution phase of programmed cell death involves the activation of caspases and the subsequent cleavage of several cellular substrates such as PARP, gelsolin, actin, lamins, and fodrin. The increased caspase-3 activity in DADS-treated cells was accompanied by cleavage of PARP. PARP is a 116-kDa protein, which converts NAD to nicotinamide and protein-linked ADP-ribose polymers. In response to growth factor withdrawal or upon exposure to a variety of chemotherapeutic compounds, PARP is cleaved to generate an 85-kDa fragment. The caspase-3 inhibitor, Ac-DEVD-CHO, prevented both the DADS-induced cleavage of PARP and apoptosis of HL-60 cells, establishing an essential role for caspase-3 activation in DADS-induced apoptosis.

Apoptosis is sometimes associated with increased levels of intracellular ROIs. Addition of exogenous antioxidants such as NAC can inhibit apoptosis [6]. In this study, we showed that DADS-induced apoptosis in HL-60 cells was prevented by the presence of either NAC or catalase. Both NAC and catalase also prevented caspase-3 protease activity, proteolytic activation of procaspase-3, and cleavage of PARP in DADS-treated HL-60 cells. This would suggest that DADS-induced apoptosis is mediated by ROIs. The specific molecular mechanisms involved, however, remain to be elucidated.

In conclusion, we present evidence that incubation of HL-60 cells with DADS stimulates the generation of ROIs, activation of caspase-3, and induction of apoptosis. Hence,

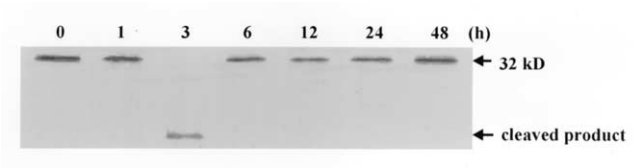

Fig. 4. Time course of procaspase-3 cleavage in HL-60 cells in response to DADS treatment. Cytosolic extracts (20 μg of protein) from HL-60 cells treated with 25 μM DADS for the indicated times were resolved by SDS–PAGE and probed for caspase-3. This experiment was repeated three times with similar results, and typical data are presented.

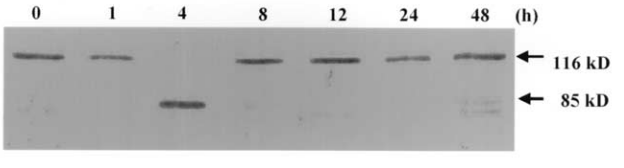

Fig. 5. Time course of PARP cleavage by DADS. HL-60 cells were treated for the indicated times with 25 μM DADS, and the cleavage of PARP was analyzed by western blotting as described in "Materials and methods." This experiment was repeated three times with similar results, and typical data are presented.

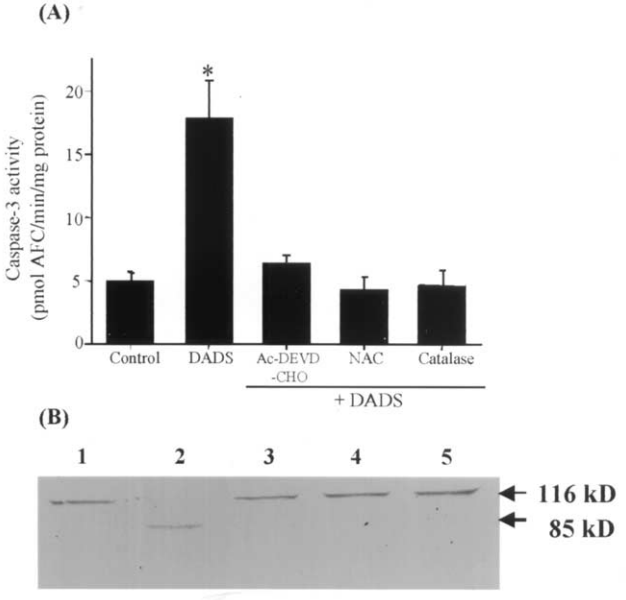

Fig. 6. Inhibition of DADS-induced caspase-3 activation and PARP cleavage by a caspase-3 inhibitor or scavengers of ROIs. HL-60 cells (5×10^6) were pretreated with Ac-DEVD-CHO (5 μM), NAC (10 mM), or catalase (400 units) for 1 hr followed by treatment with 25 μM DADS for a further 3 hr. Cytosolic extracts were prepared and assayed for caspase-3 activity (A) and PARP cleavage (B) as described in "Materials and methods." Values represent means ± SEM of six separate experiments. Key: (*) $P <$ 0.05 when compared with control by one-way ANOVA using Turkey's multi-comparison procedures. Lane 1, control; Lane 2, 25 μM DADS; Lanes 3–5, 25 μM DADS following 5 μM Ac-DEVD-CHO (lane 3), 10 mM NAC (lane 4), or 400 units catalase (lane 5).

our data raise the possibility that DADS may have some chemopreventative value for human myeloid leukemia.

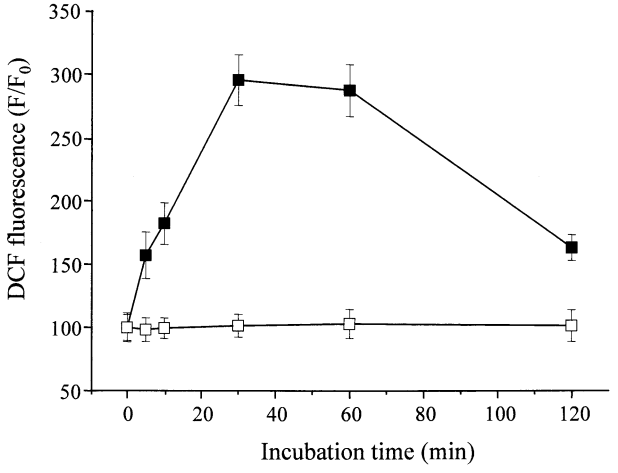

Fig. 7. Time course of the effect of DADS on intracellular hydrogen peroxide production. HL-60 cells (5×10^6) preloaded with the fluorogenic probe (CM-H_2DCFDA) were exposed to either 25 μM DADS alone (■) or in the presence of 400 units catalase (□), and the change in fluorescence of the oxidized probe was determined as indicated under "Materials and methods." Each value is the mean ± SEM of four independent experiments.

Acknowledgments

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