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Role of Ca^{2+} in diallyl disulfide-induced apoptotic cell death of HCT-15 cells

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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); BAPTA-AM, 1,2-bis (2-aminophenoxyethane)-N,N,N-tetraacetic acid tetrakis (acetoxymethyl ester); BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular Ca^{2+} level; DADS, diallyl disulfide; DCF, dichlorodihydrofluorescein; EDTA, ethylenediaminetetraacetic acid; GDI, GDP-dissociation inhibitor; HEPES, N-[2-hydroxyethyl]piperazine-N-[4-butanesulfonic acid]; ICAD, an inhibitory protein for the caspase-activated deoxyribonuclease (CAD); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine; PARP, poly (ADP-ribose) polymerase; ROIs, reactive oxygen intermediates

Abstract

Diallyl disulfide (DADS) induced apoptosis through the caspase-3 dependent pathway in leukemia cells was earlier reported from this laboratory. In this study, we investigated the involvement of Ca^{2+} in DADS-induced apoptotic cell death of HCT-15, human colon cancer cell line. DADS induced the elevation of cytosolic Ca^{2+} by biphasic pattern; rapid Ca^{2+} peak at 3 min and following slow and sustained elevation till 3 h after the addition of DADS. Production of H_2O_2 was also observed with its peak value at 4 h. Apoptotic pathways including the sequence of caspase-3 activation, poly(ADP-ribose) polymerase cleavage, and DNA fragmentation by DADS were completely blocked by various inhibitors such as specific caspase-3 inhibitor, free radical scavenger, and intracellular Ca^{2+} chelator. N-acetylcystein and catalase treatment prevented the accumulation of H_2O_2 and later caspase-3 dependent apoptotic pathway. However, these radical scavengers did not block the elevation of intracellular Ca^{2+}.

Treatment of cells with 1,2-bis(2-aminophenoxyethane)-N,N,N-tetraacetic acid tetrakis -acetoxymethyl ester (BAPTA-AM), cellular Ca^{2+} chelator, resulted in a complete blockage of the caspase-3 dependent apoptotic pathway of HCT-15 cells. It abolished the elevation of intracellular Ca^{2+}, and furthermore, completely inhibited the production of H_2O_2. These results indicate that cytosolic Ca^{2+} elevation is an earlier signaling event in apoptosis of HCT-15 cells. Collectively, our data demonstrate that DADS can induce apoptosis in HCT-15 cells through the sequential mechanism of Ca^{2+} homeostasis disruption, accumulation of H_2O_2, and resulting caspase-3 activation.

Keywords: diallyl disulfide, Ca^{2+}, H_2O_2, caspase-3, apoptosis, HCT-15 cells

Introduction

Garlic is a plant commonly used as a food item in many different cultures of the world, and its medicinal properties have been touted since ancient times. Epidemiological studies show that enhanced garlic consumption is closely related with reduced cancer incidence (Haenszel *et al*., 1972; Buiatti *et al*., 1989). Dially disulfide (DADS), an oil-soluble organosulfur compound in processed garlic, inhibited the proliferation of human colon, lung, and skin cancer cells (Sundaram and Milner, 1996a, Sundaram and Milner, 1996b). Recently, we reported that DADS induced apoptosis of human leukemia HL-60 cells through the generation of H_2O_2 and subsequent activation of caspase-3 (Kwon *et al*., 2002).

Apoptosis is a cell death program originally characterized by specific morphological and biochemical modifications in higher eukaryotic cells. These structural changes such as plasma and nuclear membrane blebbings, chromatin condensation, proteases activation, and DNA fragmentation are considered as landmarks of the apoptotic process (Jacobson *et al*., 1997; Fadeel *et al*., 1999). Specific proteases called caspases have been shown to be the key elements of the executive phase of apoptosis (Chinnaiyan *et al*., 1997; Cohen, 1997; Li *et al*., 1997; Thonberry and Lazebnik, 1998). Among these proteases, caspase-3 (CPP32) has been proposed as a

mediator of mammalian apoptosis. Inhibition of caspase-3 activity attenuates apoptosis in several models (Enari *et al*., 1996; Kirsch *et al*., 1999; Kwon *et al*., 2001). Activated caspase-3 proteolytically cleaves important nuclear and cytoskeletal proteins during apoptosis. Substrates include poly (ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair (Lazebnik *et al*., 1994), structural proteins such as actin, fodrin, or lamin to generate characteristic apoptotic morphology (Kothakota *et al*., 1997; Mashima *et al*., 1997), ICAD, an inhibitory protein for the caspase-activated deoxy-ribonuclease (CAD) (Sakahira *et al*., 1998), and GDP dissociation inhibitor for the ras-related Rho family GTPase (D4-GDI) (Kwon *et al*., 2002). The functional significance of these cleavages has not yet been determined.

Numerous data have shown the involvement of Ca^{2+} homeostasis in apoptosis and, in particular, the prelethal increase of its intracellular concentration (McConkey and Orrenius, 1996; McConkey and Orrenius, 1997). Indeed, the elevation of cytosolic Ca^{2+} by exposure to Ca^{2+} ionophores or thapsigargin is sufficient to induce apoptosis in many different cell types (Takadera and Ohyashiki, 1997; Nakamura *et al*., 2000), and agents that suppress Ca^{2+} influx or buffer intracellular Ca^{2+} can prevent apoptosis in several different systems (Scoltock *et al*., 2000; Shen *et al*., 2001; Zhang *et al*., 2001). However, despite the accumulation of data, the link between intracellular Ca^{2+} homeostasis and the activation of the apoptotic program remains unknown.

In this study, we demonstrated that DADS induced apoptosis in human colon HCT-15 cells, which was accelerated by increase of cytosolic Ca^{2+}.

Materials and Methods

Culture conditions

The human colon cancer cell line HCT-15 was purchased from the American Type Culture Collection. Cells were placed into 75 cm^2 tissue culture flasks and grown at 37°C 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 penicillin, 10 mg/ml streptomycin, and 2.5 μg/ml amphotericin B.

MTT assay for cell viability measurement

The viability of cultured cells was determined by assaying for the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Oez *et al*., 1990). In brief, after 48 h of incubation with DADS, cells (1×10^5/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°C for 1 h, 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).

Measurement of intracellular free calcium levels $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ in HCT-15 cells was quantified by fluorescence imaging of the Ca^{2+} indicator dye fura-2. All experiments were performed at 37°C. Cells (5×10^6) were incubated for 60 min in the presence of 5 μM fura-2 AM (Molecular Probes), followed by two washes with fresh medium. Immediately before imaging, the normal culture medium was replaced with Hanks balanced salt solution. Fluorescence was measured at 510 nm at two excitation wavelengths, 340 and 380 nm. The ratio of the fluorescence (*R*, 340:380) was calculated using the FeliX software in a RatioMaster fluorescence spectrophotometer (Photon Technology International). The maximum, R_{max}, and minimum, R_{min}, values of the fluorescence ratio were obtained by the addition of 10 μM ionomycin and 4 mM of Ca^{2+} or 4 mM of EGTA, respectively. Standard Ca^{2+}-EGTA buffers (Molecular Probes) were used for calibration (Grynkiewicz *et al*., 1985).

Measurement of intracellular H_2O_2 generation

The intracellular formation of H_2O_2 was detected using the fluorescent probe 5-(and -6)-chlroromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes). Control cells and cells treated with 100 μM DADS were analyzed for changes in fluorescence. Cells (5×10^6) were washed with PBS, and loaded with 10 μM CM-H_2DCFDA at 37°C 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 HCT-15 cells. Production of H_2O_2 was measured by changes in the fluorescence of dichlorofluorescein (DCF) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

Caspase activity assay

After treatment with DADS (Fluka), cells (5×10^6) were washed with ice-cold PBS and lysed in Triton X-100 buffer (10 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, and 10 mM EDTA) for 30 min on ice. Cell lysates were mixed with caspase assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, and 2 mM dithithretiol) containing 20 μM Ac-DEVD-AFC (Pharmingen Inc.), a caspase-3 substrate and incubated for 1 h at 37°C. Enzyme catalyzed release of AFC was monitored using a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Western blot analysis of 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

antibody against PARP antibody (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.

Detection of DNA fragmentation by gel electrophoresis

Cell pellets (1 x 10^7) 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 (1.8% agarose).

Protein determination

The cytosolic protein concentration in the HCT-15 cell was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard. All of the samples were assayed in triplicate.

Statistical analysis

Statistical analysis of the data was performed with Students *t*-test and ANOVA. Differences with $P < 0.05$ were considered statistically significant.

Results

Elevation of $[Ca^{2+}]_i$ in HCT-15 cells after treatment with DADS

Disruption of cellular Ca^{2+} homeostasis has been proposed to be a critical event in both apoptosis and necrosis (Nicotera and Orrenius, 1992; Trump and Berezesky, 1995). Therefore the changes of $[Ca^{2+}]_i$ was monitored after the addition of DADS by fluorescence ratio imaging of the Ca^{2+} indicator dye fura-2. When exposing cells onto 100 ìM DADS, the $[Ca^{2+}]_i$ was increased in a biphasic patterns. The rapid elevation of $[Ca^{2+}]_i$ was observed and reached its peak level within 3 min (Figure 1A, inset). Then, the second, slow and sustained elevation of $[Ca^{2+}]_i$ was occurred until 3 h with concentration dependency (Figure 1). The $[Ca^{2+}]_i$ rose from a basal level of approximately 50 nM to a level of nearly 100 nM at the 3 h time point. Our finding is consistent with previously published results (Sundaram and Milner, 1996). DATS (10 μM) caused a marked and progressive increase in intracellular Ca^{2+} in A549 cells during the first four hours after treatment and exposure to 1 μM DATS to A549 cells for 24 hours significantly induced apoptosis, as indicated by increased DNA fragmentation (Sakamoto *et al.*, 1997). Thus, sensitivity toward the DADS might be different among different cell types.

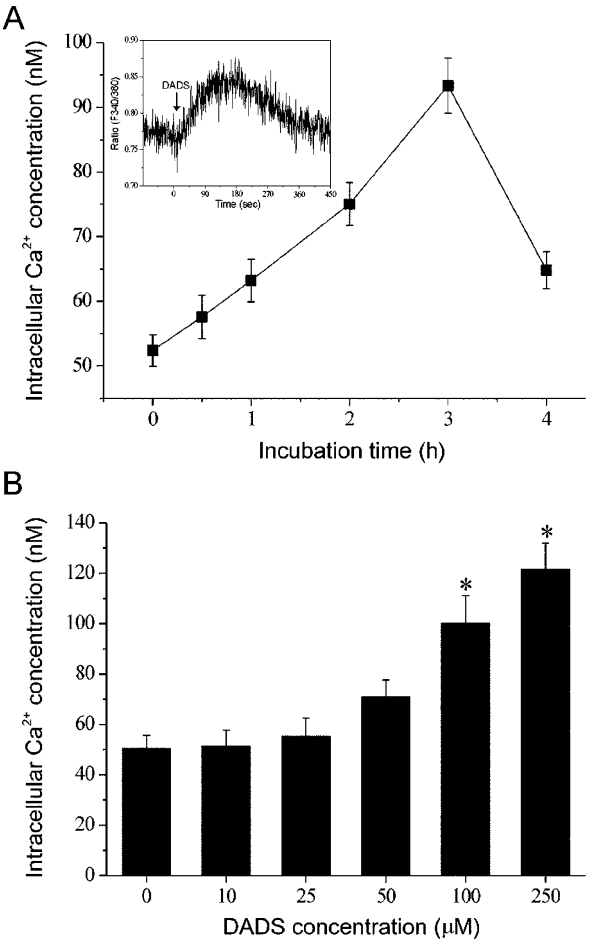

Figure 1. Effect of DADS on $[Ca^{2+}]_i$ in HCT-15 cells. (A) After treatment of HCT-15 cells (5 x 10^6) with either (A)100 mM DADS for a period up to 4 h (inset, the changes of $[Ca^{2+}]_i$ for initial 7 min), or (B) a range of concentrations (0 - 250 μM) of DADS were toeated for 3 h, cells were loaded with Fura-2 AM for 30 min, and the fluorescence ratio of 340 and 380 nm (F340/380) were analyzed. Intracellular Ca^{2+} concentrations were quantitated by formula described in Materials and Methods and values are mean ± SEM of determinations made in 3 separate experiments.

Generation of H_2O_2 and activation of caspase-3 by DADS

Our previous study suggests an involvement of reactive oxygen intermediates (ROIs) which lie upstream of caspase-3 activation in the signal transduction pathways leading to apoptosis in HL-60 cells (Kwon *et al.*, 2002). To determine the involvement of ROIs in DADS-induced apoptosis of HCT-15 cells, H_2O_2 generation was assayed. As shown in Figure 2, DADS caused H_2O_2 generation linearly up to 4 h in a time-dependent manner.

The kinetics of caspase-3 activation in HCT-15 cells treated with DADS is shown in Figure 3. Caspase

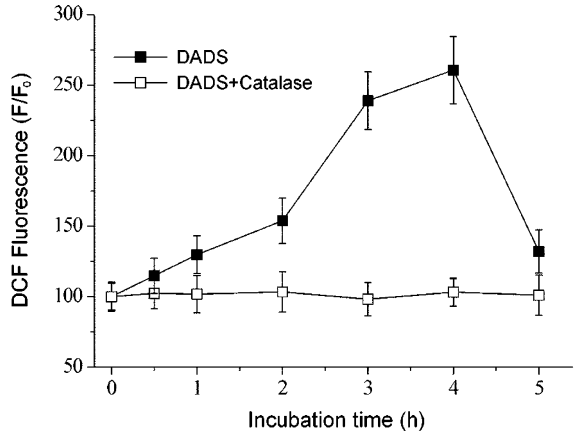

Figure 2. Effect of DADS on H_2O_2 production in HCT-15 cells. HCT-15 cells (5×10^6) preloaded with the fluorogenic probe (CM-H_2DCFDA) were exposed to either 100 mM DADS alone (■) or in the presence of 400 units catalase (□), and the fluorescence change of the oxidized probe was determined as indicated under Materials and Methods. Results are presented as the changes of DCF fluorescence and each value is the mean ± SEM of four independent experiments.

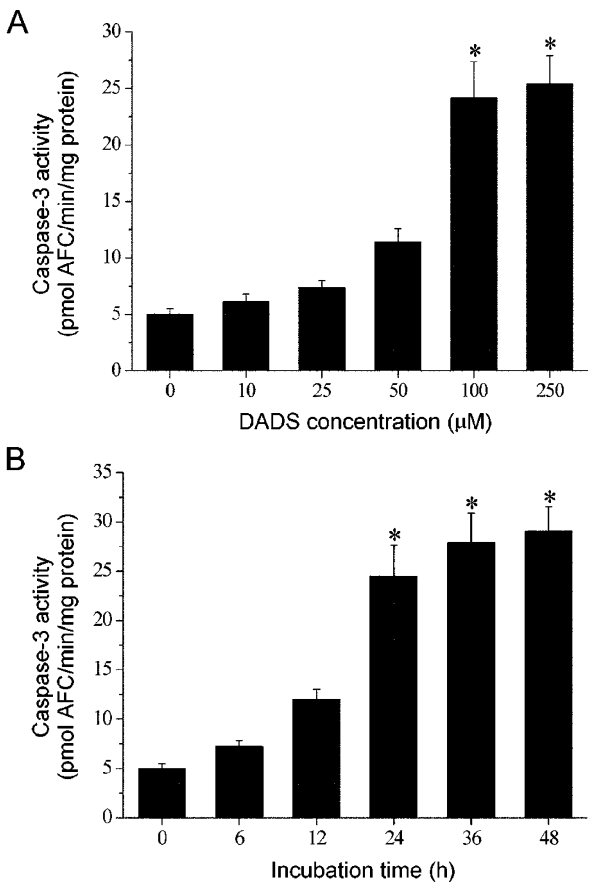

Figure 3. DADS-induced caspase-3 acitivation. HCT-15 cells (5×10^6) were treated with either (A) a range of concentrations (0-250 μM) of DADS for 24 h, or (B)100 mM DADS for a period up to 48 h. Cytosolic extracts were prepared and assayed for caspase-3 activity as described in Materials and Methods. Each value represents mean ± SEM of six separate experiments. * $P < 0.05$ compared with control.

activity was determined using fluorogenic caspase-3 substrate, Ac-DEVD-AFC in HCT-15 cell lysate following DADS treatment. As shown in Figure 3A, DADS caused a concentration-dependent activation of caspase-3. The significant increase of caspase-3 activity started at 12 h and reached its peak level at 24 h (Figure 3B). Compared to the time course of apoptosis determined by agarose gel electorphoresis, it is noted that the elevation of caspase-3 activity occurred more or less concurrently with DNA fragmentation (data not shown). The activation of caspase-3 was further confirmed by the cleavage of PARP, one of its well-known substrate proteins as determined by Western blot (Figure 4B, lane 2). DNA extract from HCT-15 cells treated with DADS for 48 h generated a characteristic ladder pattern of discontinuous DNA fragments on agarose gel electrophoresis (Figure 5A, lane 2).

Similar results were obtained for the Ca^{2+} ionophore-treated cells. When HCT-15 cells were treated with A23187 (2 μM) or ionomycin (250 nM), generation of H_2O_2 as well as caspase-3 activation were observed (Figure 6), suggesting the roles of Ca^{2+} in apoptosis of HCT-15 cell damage.

Effect of inhibitors on DADS-induced apoptosis

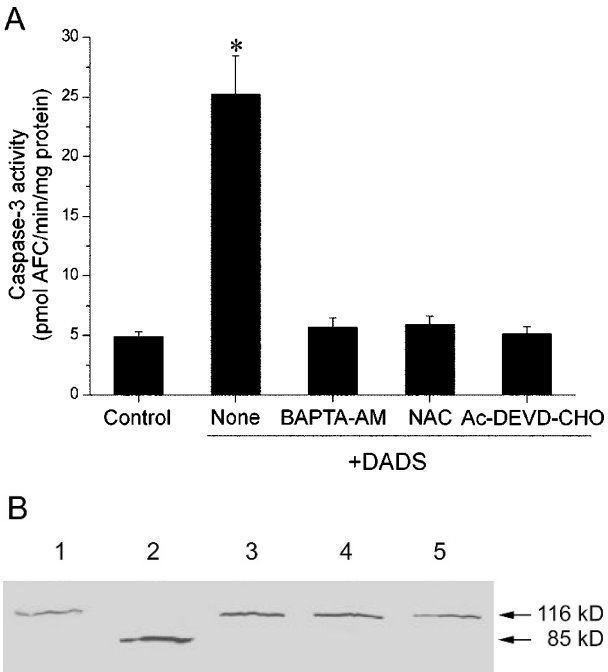

Figure 4. Inhibition of DADS-induced caspase-3 activation and PARP cleavage HCT-15 cells (5×10^6) were pretreated with BAPTA-AM (5 μM), NAC (10 mM), or Ac-DEVD-CHO (5 μM) for 1 h followed by treatment with 100 μM DADS for further 24 h. Cytosolic extracts were prepared and assayed for caspase-3 activity (A) and PARP cleavage (B) as described in Materials and Methods. Values represent means ± SEM of six separate experiments. * $P < 0.05$ compared with control; Lane 1, control; Lane 2, 100 μM DADS; Lane 3-5, 100 μM DADS following 5 μM BAPTA-AM (lane 3), 10 mM NAC (lane 4), or 5 μM Ac-DEVD-CHO (lane 5), respectively.

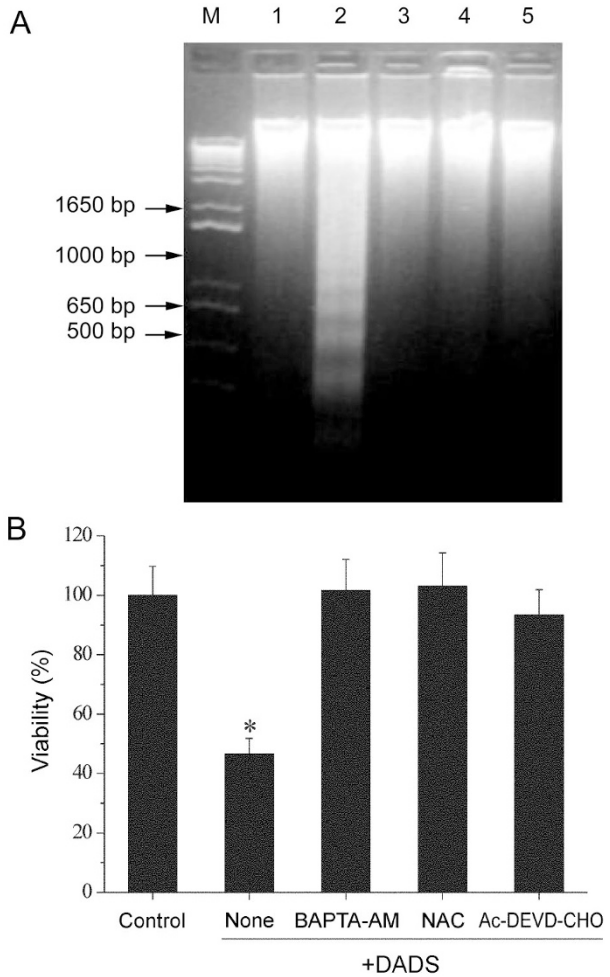

Figure 5. Prevention of DADS-induced apoptotic cell death. HCT-15 cells (1×10^7) were incubated with 100 μM DADS with or without various inhibitors for 48 h and DNA fragmentation was analyzed by 1.8% agarose gel electrophoresis; Lane 1, control; Lane 2, 100 μM DADS; Lane 3-5, 100 μM DADS following 5 μM BAPTA-AM (lane 3), 10 mM NAC (lane 4), or 5 μM Ac-DEVD-CHO (lane 5), respectively. (B) MTT assay for cell viability. HCT-15 cells (1×10^5) were treated with 100 μM DADS with or without 5 μM BAPTA-AM, 10 mM NAC, or 5 μM Ac-DEVD-CHO for 48 h, 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 separate experiments.

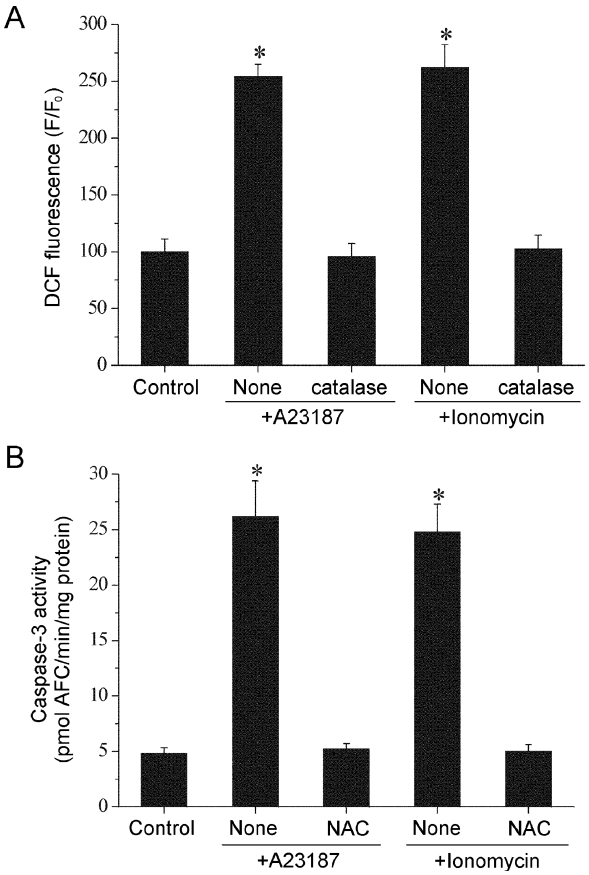

Figure 6. Calcium ionophore-induced generation of H_2O_2 and caspase-3 activation (A) HCT-15 cells (5×10^6) were treated with either 2 μM of A23187 or 250 nM ionomycin in the presence or absence of 400 units catalase. The changes of DCF fluorescence were measured as described in Materials and Methods. (B) HCT-15 cells (5×10^6) were treated with Ca^{2+} ionophore as described above in the presence or absence of 10 mM NAC. Cytosolic extracts were prepared and assayed for caspase-3 activity. Each value represents mean ± SEM of three separate experiments. $^*P < 0.05$ compared with control.

We examined the specific components of the apoptotic pathway in the presence of various inhibitors. Pretreatment of HCT-15 cells for 1 h with caspase-3 inhibitor Ac-DEVD-CHO or commonly used antioxidant N-acetylcysteine (NAC), completely blocked the DADS-induced apoptotic pathway including caspase-3 activation and PARP cleavage (Figures 4 and 5). BAPTA-AM, which penetrates into the cell and subsequent to hydrolysis to BAPTA, serves as an intracellular Ca^{2+}-chelator, also completely abolished the caspase-3 activation, PARP cleavage and DNA fragmentation by DADS (Figures 4 and 5). These results indicate that both $[Ca^{2+}]_i$ elevation and ROIs formation play the important roles in DADS-induced apoptosis of HCT-15 cells.

We next determined the sequential events of H_2O_2 generation and the elevation of $[Ca^{2+}]_i$. Treatment with BAPTA-AM which can buffer the increase of $[Ca^{2+}]_i$, suppressed the DCF fluorescence to DADS (Figure 7A). In contrast, the increase of $[Ca^{2+}]_i$ induced by DADS was unaffected by pretreatment of either NAC or catalase (Figure 7B). These findings suggest that DADS-induced H_2O_2 generation is Ca^{2+}-dependent and may act at a point subsequent to elevation of $[Ca^{2+}]_i$.

Discussion

Disruption of cellular Ca^{2+} homeostasis has been proposed to be a critical event in both apoptosis and necrosis. In the case of necrosis, increases of $[Ca^{2+}]_i$

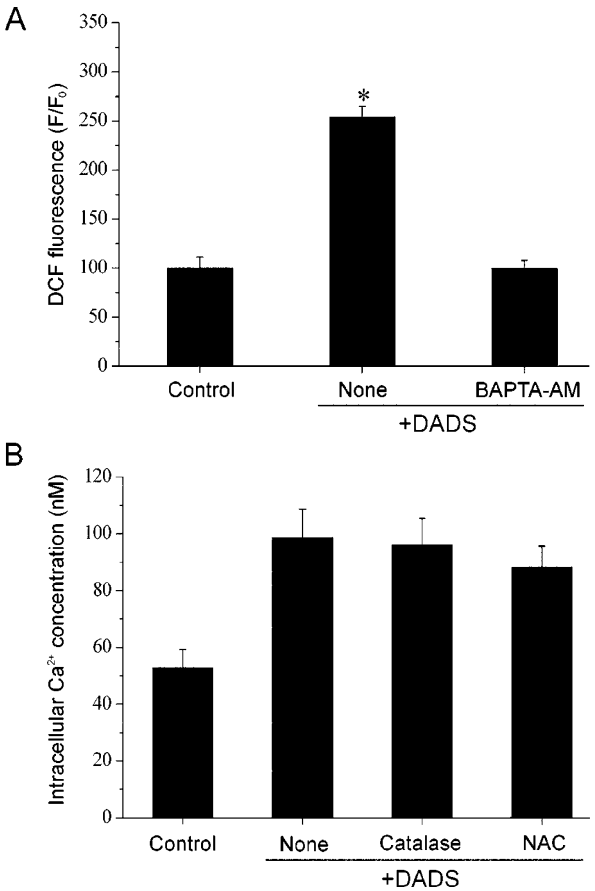

Figure 7. Relationship between increase of $[Ca^{2+}]_i$ and H_2O_2 production (A) HCT-15 cells were pretreated with 5 μM BAPTA-AM for 1 h, and then DADS-induced changes of DCF fluorescence were analyzed (A). (B) HCT-15 cells were pretreated with catalase (400 U) or NAC (10 mM), and then followed by DADS exposure for 3 h. The assay methods of $[Ca^{2+}]_i$ and H_2O_2 production were the same as described in Figure 1 and Figure 2, respectively. * $P < 0.05$ compared with control.

have been widely reported. Exposure of cultured pancreatic α-cells to alloxan results in rapid increase of $[Ca^{2+}]_i$, which triggers cytotoxic signaling events in pancreatic α-cells, and blockade of Ca^{2+} influx prevents such necrotic cell death (Kim *et al*., 1994; Park *et al*., 1996). The involvement of increases in $[Ca^{2+}]_i$ was also reported in cells undergoing apoptosis in many different settings (Orrenius, 1996; McConkey and Gwag *et al*., 1999; Shen *et al*., 2001).

We demonstrated that engagement of the DADS in HCT-15 cells results in rapid but small, and subsequent sustained increases in $[Ca^{2+}]_i$, suggesting that alterations in Ca^{2+} homeostasis may be involved apoptosis. In the case of HL-60 cells, no increase of $[Ca^{2+}]_i$ was observed after treatment with DADS (data not shown). This suggests that DADS-induced cell damage by disrupting Ca^{2+} homeostasis is not a universal phenomenon, but is rather dependent on cell types. In light of this finding, we inhibited the DADS-induced rise in $[Ca^{2+}]_i$ in HCT-15 cells by chelating intracellular Ca^{2+} with BAPTA-AM, which completely prevented the activation of caspase-3 and DNA fragmentation. So far, the exact signaling pathway from $[Ca^{2+}]_i$ elevation to caspase-3 activation is largely unknown. Theoretically, there are several mechanisms linking Ca^{2+} overloading with caspase-3 activation in the apoptotic process: involvement of oxidative stress, alterations of mitochondrial function, and the direct activation of protease (McConkey and Orrenius, 1997; Chakraborti *et al*., 1999).

In a wide variety of multicellular organisms, ROIs production, in conjunction with changes in Ca^{2+} homeostasis, is a common feature of apoptosis and necrosis (Nicotera and Orrenius, 1992; Richter *et al*., 1995; Lipton and Nicotera, 1998). We demonstrated the increase of DCF fluorescence correlating with the production of H_2O_2 in the cells treated with DADS. The ROIs, appear to play a central role in subsequent caspase-3 activation and nuclear apoptosis as evidenced by the complete blocks of these processes by NAC or catalase.

DADS-induced increase of H_2O_2 production was Ca^{2+} dependent because intracellular Ca^{2+} chelator abolished the DADS-induced H_2O_2 generation and subsequent apoptotic pathway. This suggests that the production of H_2O_2 is a late step in Ca^{2+}-mediated cell death. Indeed, NAC has been shown to inhibit Ca^{2+} ionophore-induced apoptosis in cultured cortical neurons (Hatanaka *et al*., 1996). It is interesting to investigate the relationship between intracellular Ca^{2+} elevation and H_2O_2 production. Actually, the increase of intracellular Ca^{2+} does not always precede the production of H_2O_2. The reverse pathway has been also reported. For example, gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol, increased both the elevation of intracellular Ca^{2+} and production of H_2O_2 in HL-60RG cells (Isuzugawa *et al*., 2001). However, the change of intracellular Ca^{2+} levels was secondary to H_2O_2 generation. In this study, we observed Ca^{2+} ionophore-induced generation of H_2O_2 and caspase-3 activation, which were abolished by pretreatment with antioxidant. Cytoplasmic Ca^{2+} overload can result in increased production of ROIs mediated by several mechanisms: activation of nitric oxide synthase (Yun *et al*., 1996), conversion of xanthine dehydrogenase to xanthine oxidase (Atlante *et al*., 1997), activation of phospholipase A_2 (Chan and Fishman, 1980), and increase of mitochondrial production of superoxide (Castilho *et al*., 1995). The ROIs-evoked membrane damage may induce further Ca^{2+} influx and resultant accentuated Ca^{2+} influx in turn will induce the generation of further ROIs. Thus, initial Ca^{2+} influx at 3 min after DADS treatment is likely to initiate a cascade of ROIs generation and cytosolic Ca^{2+} accumulation.

Using a fluorogenic substrate, we showed that DADS

increased caspase-3 activity in a time- and concentration-dependent manner. The increased caspase-3 activity in DADS-treated cells was accompanied by cleavage of PARP. The caspase-3 inhibitor, Ac-DEVD-CHO, prevented both DADS-induced cleavage of PARP and apoptosis of HCT-15 cells, establishing an essential role for caspase-3 activation in DADS-induced apoptosis. Once again, caspase-3 activation and PARP cleavage were de-pendent on the increase of $[Ca^{2+}]_i$ and Ca^{2+}-de-pendent H_2O_2 generation.

Recently, Bottone *et al*. (2002) reported that DADS induced apoptotic cell death of colorectal HCT116 cells by p53-dependent induction of antitumorigenic NSAID-activated gene (NAG-1). Although we used different cell line, we cant exclude the possibility that NAG-1 is activated in our system. In the present study, we have provided strong evidence to the conclusion that the increase of $[Ca^{2+}]_i$ is a key mediator in DADS-induced apoptosis in HCT-15 cells, based on the following observations: (i) Upon DADS exposure, there were rapid and sustained increases in $[Ca^{2+}]_i$, (ii) the increase of $[Ca^{2+}]_i$ preceded H_2O_2 production and caspase-3 activation, (iii) BAPTA, an intracellular Ca^{2+} chelator, abolished DADS-induced $[Ca^{2+}]_i$ elevation and H_2O_2 production, which further prevented casapase-3 activation and DNA fragmentation. Although no direct evidence is available, it is believed that $[Ca^{2+}]_i$ elevation may trigger caspase-3 activation through the generation of H_2O_2.

Acknowledgments

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