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ORIGINAL PAPER

The Role of Ca^{2+} on the DADS-induced Apoptosis in Mouse–Rat Hybrid Retina Ganglion Cells (N18)

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Abstract Diallyl disulfide (DADS), a component of garlic, has been shown to induce growth inhibition and apoptosis in human cancer cell types. The present studies were designed to investigate the effects of DADS on mouse–rat hybrid retina ganglion cells (N18) to better understand its effect on apoptosis and apoptosis-related genes in vitro. Cell viability, cell cycle analysis, reactive oxygen species (ROS), Ca^{2+} production, mitochondria membrane potential, apoptosis induction, associated gene expression and caspases-3 activity were examined by flow cytometric assay and/or Western blot. After 24-h treatment with DADS, a dose- and time-dependent decrease in the viability of N18 cells was observed and the approximate IC_{50} was 27.6 μM. The decreased percentage of viable cells are associated with the production of ROS then followed by the production of Ca^{2+} which is induced by DADS. DADS induced apoptosis in N18 cells via the activation of caspase-3. DADS increased the protein levels of p53, cytochrome *c* and phosphated JNK within 24 h of treatment and it decreased the levels of Bcl-2 and those factors may have led to the mitochondria depolarization of N18 cells. DADS induced apoptosis were accompanied by increased levels of Ca^{2+} and decreased mitochondrial membrane potential which then led to release the cytochrome *c*, cleavage of pro-caspase-3. Deleted levels of Ca^{2+} by BAPTA-AM 10 μM (intracellular calcium chelator) then led to decrease DADS-induced apoptosis. Inhibition of caspase-3 activation by inhibitor (*z*-VAD-fmk) completely blocked DADS-induced apoptosis on N18 cells. The results indicated that oxidative stress modulates cell proliferation and Ca^{2+} modulates the cell death induced by DADS.

Keywords Diallyl disulfide · Apoptosis · Caspase-3 · Mitochondria · Human retina ganglion cells

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Introduction

Cell growth including tumor cells, is now recognized to represent a balance between actively regulated processes (cell division and cell death) [1]. Apoptosis is a kind of cell death, which causes specific morphological changes

and DNA fragmentation that are considered as the major cytopathologic hallmarks of the apoptotic process [2, 3]. Many studies have demonstrated that the inappropriate regulation of apoptosis is associated with a variety of diseases [4–6]. Therefore, how to induce apoptosis of cancer cells is a major strategy for cancer chemotherapy.

Garlic is a plant commonly used as food and has been reported to have medicinal properties. Garlic and its products have received increased focus because of their possible beneficial effects in reducing the risk of heart disease such as decreasing plasma cholesterol and triglyceride levels in humans [7, 8]. Enhanced garlic consumption is closely related with reduced cancer incidence [9, 10]. Diallyl disulfide (DADS), one of a component of garlic (*Allium sativum*), contains growth inhibition of human cancer cells (colon, lung, skin, and breast) [11–14]. It was reported that DADS could significantly inhibit the proliferation of HepG2 cells [15]. DADS significantly increased the cell cycle arrest at G2/M phase of human colon tumor cells (HCT-15) via the inhibition of Cdc2 (cyclin-dependent kinase 1, Cdk1) activity [16].

DADS induced apoptosis of H460 cells by the generation of H_2O_2 and induction of p53 and activation of caspase-3 [11, 12]. Recently, it also was reported that DADS induced cell death through reactive oxygen species-dependent c-jun NH2-terminal kinase/c-jun signaling cascade mediates neuroblastoma [17]. It has also been reported that the elevation of cytosolic Ca^{2+} by exposure to Ca^{2+} ionophores or thapsigargin is sufficient to induce apoptosis in many different cell types [18, 19]. But if agents suppress Ca^{2+} influx or if buffer intracellular Ca^{2+} can prevent apoptosis have been shown in differential systems [20–22]. Sustained increases in intracellular Ca^{2+} trigger apoptosis in in vivo and in vitro systems have been reported [23, 24]. However, elevated rod photoreceptor Ca^{2+} plays a key role in the process of apoptotic cell death in humans and animal models during inherited retinal degeneration, retinal diseases and injuries, and even in chemical exposure.

So far DADS as an antitumor agent has been established, however the role of Ca^{2+} on the apoptosis caused by DADS within the N18 cells remains to be evaluated. Therefore, the aim of the present study was to dissect the mechanisms underlying DADS induced cytotoxicity and apoptosis and Ca^{2+} production in human retina ganglion cells (N18) cancer cells.

Materials and methods

Chemicals and reagents

Diallyl disulfide (DADS) was purchased from Fluka Chemika Co. (Bucha, Switzerland). Tris–HCl, triton X-100, propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BAPTA, Potassium phosphates and dimethyl sulfoxide (DMSO) were purchased from Merck Co. (Darmstadt, Germany). RPMI-1640, penicillin–streptomycin, trypsin-EDTA, fetal bovine serum (FBS), and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3 activity assay kit was bought from Boehringer Mannheim (Mannhein, Germany).

Mouse–rat hybrid retina ganglion cell line (N18)

N18 cell line was obtained from a Japanese Collection of Research Bioresources Bank. N18 cell line is a hybrid of retina ganglion cells with lymphoma cells. The cells are cultured for several generations, and have been checked for viability. The cells were placed into 75 cm^3 tissue culture flasks and grown at 37°C under a humidified 5% CO_2 and 95% air at one atmosphere in DMEM medium supplemented with 10% FBS, 1% penicillin–streptomycin (10 ng/ml penicillin and 10 ng/ml streptomycin) and 1% glutamine.

Cell viability of N18 cells treated with or without DADS was determined by using flow cytometry

The N18 cells were plated in 12 well plates at a density of 5×10^5 cells/well and grown for 24 h. They were then added to different concentrations of DADS for final concentration 0, 0.5, 5, 10, 25 and 50 μM, while only adding DMSO (solvent) for the control regimen and grown at 37°C, 5% CO_2 and 95% air for a different period of time. For determining cell viability, the flow cytometry assay was used as described previously [25–27].

Flow cytometry analysis of DNA content for apoptosis from N18 cells treated with different concentrations of DADS

About 5×10^5 cells/well of N18 cells in 12-well plate with concentrations (0, 0.5, 5, 10, 25, and 50 μM) of DADS were incubated in an incubator for different time periods, and then the cells were harvested by centrifugation. The cells were fixed gently (drop by drop) by putting 70% ethanol (in PBS) in ice overnight and were then resuspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase (Sigma, St. Louis, MO, USA) and 0.1% triton X-100 in dark room. After 30 min at 37°C, the cells were analyzed with a flow cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argonion laser at 488 nm wave-length. Then the cell cycle was determined and analyzed [25–27].

Caspase-3 activity determination of N18 cells treated with or without DADS

The N18 cells were plated in 6 well plates at a density of 5×10^5 cells/well and grown for 24 h. Then various concentrations of DADS with solely added DMSO (solvent) for the control regimen were grown at 37°C in a humidified 5% CO_2 for 12 h. About 5×10^6 cells were lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris–HCl, pH 8.0, 2 mM dithiothreitol, 1 mM PMSF, 1 μg/ml aprotinin, 1 mg/ml leupeptin) for 30 min at 4°C followed by centrifugation at $10{,}000 \times g$ for 30 min. For caspase-3 activity determination, 50 μl of reaction mixtures with fluorogenic report substrate peptides was specifically used for caspase-3. The substrate peptide (200 μM) was incubated at 37°C with cytosolic extracts (15 μg of total protein) in reaction buffer (100 mM HEPES, 10% sucrose, 10 mM DTT, 0.1% 3-[3-chloamidopropyl] dimethylammonio) 1-propanesulfonate. Fluorescence was determined after 2 h (excitation wavelength, 400 nm; emission wavelength, 505 nm) with a fluorescence plate reader (Fluoroskan Ascent; Labsystems) [27].

Inhibition of DADS-induced apoptosis by the caspase-3 inhibitor (Ac-DEVD-CHO) on N18 cells

In order to examine whether or not caspase-3 activation is involved in the apoptosis triggered by DADS, the N18 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor (*z*-VAD-fmk) 3 h prior to treatment with DADS. Then, we determined the apoptosis and caspase-3 activity as described above.

Detection of reactive oxygen species (ROS) in N18 cells by flow cytometry

The level of ROS of the N18 cells was examined by flow cytometry (Becton Dickinson FACS Calibur), using the 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). The N18 cells were treated with or without various concentrations (0.5, 5, 10, 25 and 50 μM) of DADS for 24 h to detect the changes of ROS. The cells were harvested and washed twice, re-suspended in 500 μl of 2,7-Dichlorodihydrofluorescein diacetate (10 μM) and incubated at 37°C for 30 min and analyzed by flow cytometry [27].

Detection of Ca^{2+} concentrations in N18 cells after cotreated with DADS by flow cytometry

The level of Ca^{2+} of the N18 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using the Indo 1/AM (Calbiochem; La Jolla, CA). Cells were pre-treated with or without 10 μM BAPTA-AM (intracellular calcium chelator) for 3 h then with various concentrations (0.5, 5, 10, 25 and 50 μM) of DADS for 24 h to detect the changes of Ca^{+2} concentration. The cells were harvested and washed twice, and for apoptosis analysis and the others were re-suspended in Indo 1/AM (3 μg/ml) and incubated at 37°C for 30 min and analyzed by flow cytometry [28].

Detection of mitochondrial membrane potential in N18 cells by flow cytometry

The level of mitochondrial membrane potential of the N18 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using the $DiOC_6$ (4 mol/l). Cells were treated with or without various concentrations (0.5, 5, 10, 25 and 50 μM) of DADS for 24 h to detect the changes of mitochondrial membrane potential. The cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 mol/l) and incubated at 37°C for 30 min and analyzed by flow cytometry [27].

Western blotting for examing the effect of DADS on p53, JNK, Bcl-2, and cytochrome *c* of N18 cells

The total proteins were collected from N18 cells treated with or without various concentrations of DADS for 48 h before the p53, JNK, Bcl-2, and cytochrome *c* were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described previously [27].

Statistical analysis

Statistical calculations of the data were performed using an unpaired Student's *t*-test and ANOVA analysis. Statistical significance was at $P < 0.05$.

Results

Effects of DADS on the percentage of viable cells from N18 cells

To investigate the effects on the percentage of viable cells caused by DADS, the N18 cells after treated with or without various concentrations of DADS were measured by flow cytometry. About 10–50 μM DADS reduced the percentage of N18 cells in dose-dependent effects and 25 μM DADS induced time-dependent effects as shown in Fig. 1a, b. In these experiments, we verified that DADS induced cytotoxicity to N18 cells in vitro.

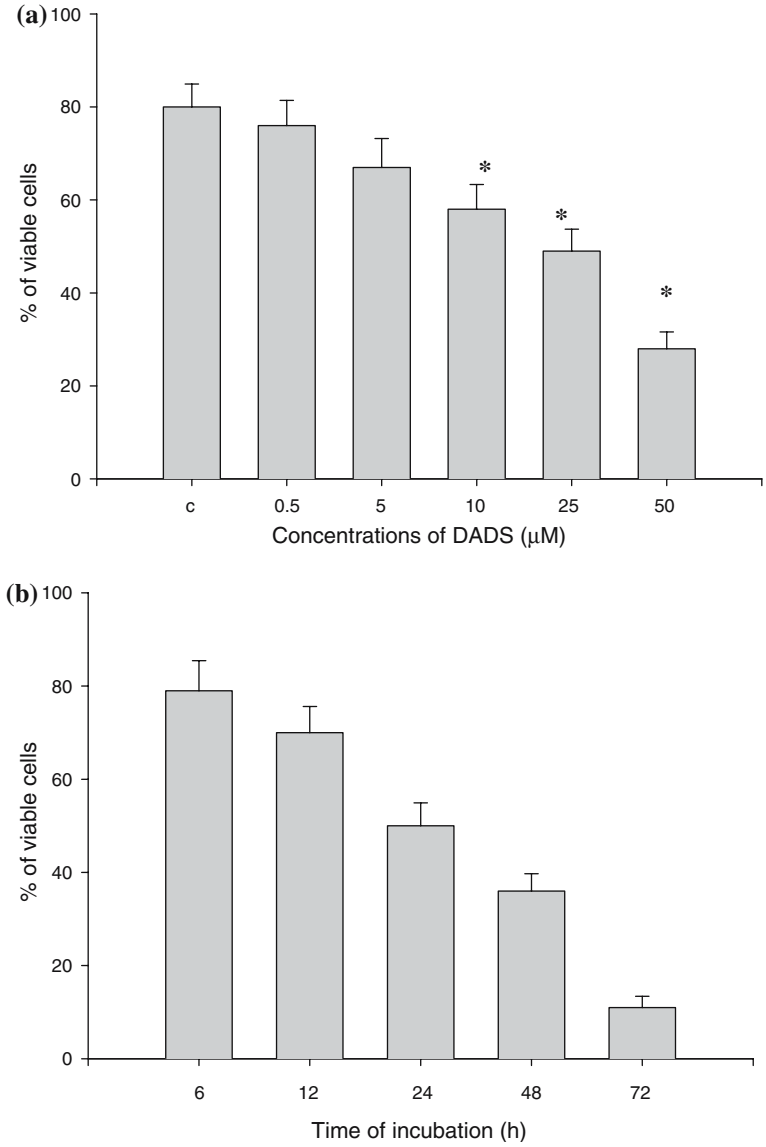

Fig. 1 The percentage of the viable N18 cells after DADS treatment with 24 h incubation. The N18 cells (2×10^5 cells/well; 12 well plates) were plated in RPMI 1640 medium + 10% FBS with different concentrations of DADS for 24 h (panel **a**) or 25 μM DADS for 6, 12, 24, 48 and 72 h (panel **b**). Then cells were collected by centrifugation and the viable cells were determined by flow cytometry as described in Materials and Methods. Each point is mean ± SD of three experiments. $*P < 0.05$

Effects of DADS on the production of reactive oxygen species (ROS) from N18 cells

As shown in Table 1, the treatment of N18 cells with 25 μM DADS induced ROS production when using DCFH-DA staining follows were analyzed by flow cytometry. The ROS production increased dramatically in less than 0.5 h and reached its maximum level at about 2 h. After DADS treated up to 120 min, the ROS production decreased in N18 cells. Those results indicated that ROS may be an early event in DADS-modulated cell death.

Effects of DADS on the production of Ca^{2+} on N18 cells

As shown in Table 2, the data revealed that DADS induced significant Ca^{2+} production compared to the control in examined N18 cells. The effects of DADS on the Ca^{2+} production are dose-dependent manner when using Indo 1/AM staining follows that were analyzed by flow cytometry.

Effects of DADS on the mitochondria membrane potential (MMP) from N18 cells

As shown in Table 3, the data revealed that DADS decreased the levels of MMP compared to the control in examined N18 cells. The effects of DADS on the levels of MMP are dose-dependent manner when using $DiOC_6$ staining follows that were analyzed by flow cytometry.

Induction of apoptosis by DADS on N18 cells

Apoptosis was detected by PI stained and annexin V method after 48 h of continuous exposure to various con-

Table 1 Flow cytometric analysis of reactive oxygen species in mouse–rat hybrid retina ganglion cells (N18) with or without 25 μM DADS treatment

Time of incubation (min)	Percentage of cells stained by DCFH-DA (% control)
0	0.3 ± 0.1
15	2.2 ± 0.3
30	8.3 ± 1.0[a,b]
45	12.5 ± 1.4[a–c]
60	16.4 ± 1.8[a–d]
90	21.1 ± 2.4[a–e]
120	14.1 ± 2.0[a–f]
180	9.5 ± 1.2[a–e,g]

The retina ganglion cells (N18) cells (5×10^5 cells/ml) were treated with 25 μM DADS. The zero concentration was defined as control. The percentage of cells that were stained by DCFH-DA dye, and the stained cells were determined by flow cytometry as described in the Materials and Methods section. The results are expressed as mean ± SD of 9 determinations. F=128.226, $P < 0.001$

[a]Significantly different, $P < 0.05$, vs. 0 min

[b]Significantly different, $P < 0.001$, vs.15 min

[c]Significantly different, $P < 0.05$, vs. 30 min

[d]Significantly different, $P < 0.05$, vs. 45 min

[e]Significantly different, $P < 0.05$, vs. 60 min

[f]Significantly different, $P < 0.001$, vs. 90 min

[g]Significantly different, $P < 0.001$, vs. 120 min

centrations of DADS then analyzed by flow cytometry (Fig. 2a, b). As shown in Fig. 2, DADS induced significant apoptosis compared to the control and those effects are concentration-dependent manner. The percentage of

Table 2 Flow cytometric analysis of Ca^{2+} concentration in mouse–rat hybrid retina ganglion cells (N18) with or without DADS treatment

DADS (μM)	Percentage of cells stained by indo-1/AM (% control)
0	1.0 ± 0.2
0.5	3.1 ± 0.6
5	12.4 ± 1.8[a,b]
10	18.9 ± 2.1[a–c]
25	26.4 ± 2.6[a–d]
50	41.2 ± 3.2[a–e]

The N18 cells (5×10^5 cells/ml) were treated with various concentrations of DADS. The zero concentration was defined as control. The percentage of cells that were stained by DCFH-DA dye, and the stained cells were determined by flow cytometry as described in the Materials and Methods section. The results are expressed as mean ± SD of 7 determinations. F =73.84, $P < 0.001$

[a]Significantly different, $P < 0.05$, vs. 0 μM

[b]Significantly different, $P < 0.001$, vs. 5 μM

[c]Significantly different, $P < 0.05$, vs. 10 μM

[d]Significantly different, $P < 0.05$, vs. 25 μM

Table 3 Flow cytometric analysis of mitochondrial membrane potential in mouse–rat hybrid retina ganglion cells (N18) with or without various concentrations of DADS treatment for 24 h

DADS (μM)	Percentage of cells stained by DiOC6
0 (control)	91.2 ± 8.9
0.5	84.8 ± 8.4
5	71.2 ± 7.1[a,b]
10	46.6 ± 4.8[a–c]
25	31.1 ± 2.4[a–d]
50	16.3 ± 1.2[a–e]

The N18 cells (5×10^5 cells/ml) were treated with various concentrations of DADS. The zero concentration was defined as control. The percentage of cells that were stained by DiOC6 dye, and the stained cells were determined by flow cytometry as described in the Materials and Methods section. The results are expressed as mean ± SD of 7 determinations. F=71.06, $P < 0.001$

[a]Significantly different, $P < 0.05$, vs. 0 μM

[b]Significantly different, $P < 0.001$, vs. 5 μM

[c]Significantly different, $P < 0.05$, vs. 10 μM

[d]Significantly different, $P < 0.05$, vs. 25 μM

[e]Significantly different, $P < 0.05$, vs. 50 μM

apoptosis increased significantly in N18 cells by the co-treatment of DADS.

Inhibition of DADS-induced caspase-3 activity and apoptosis by the caspase inhibitor *z*-VAD-fmk from N18 cells

The purpose of these experiments was for examining whether or not caspase-3 activation is involved in the apoptosis of N18 cells triggered by DADS. The results indicate that DADS increased caspase-3 activity (Fig. 3a). The N18 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor (*z*-VAD-fmk), 3 h prior to the treatment with DADS before assayed for caspase-3 activity. The results demonstrated that *z*-VAD-fmk decreased caspase-3 activity (Fig. 3b) which was accompanied by the marked attenuation of DADS-induced apoptotic cell death (Fig. 3a, b). The result indicated that the activation of caspase-3 contributes to DADS-induced apoptosis in N18 cells.

Inhibition of DADS-induced Ca^{2+} concentrations and apoptosis by the Ca^{2+} chelator (BAPTA-AM) from N18 cells

The above results already demonstrated that DADS induced significant apoptosis compared to the control. The aim of these experiments was for examining whether or not Ca^{2+} production is involved in the apoptosis of N18 cells triggered by DADS. However, pretreatment of

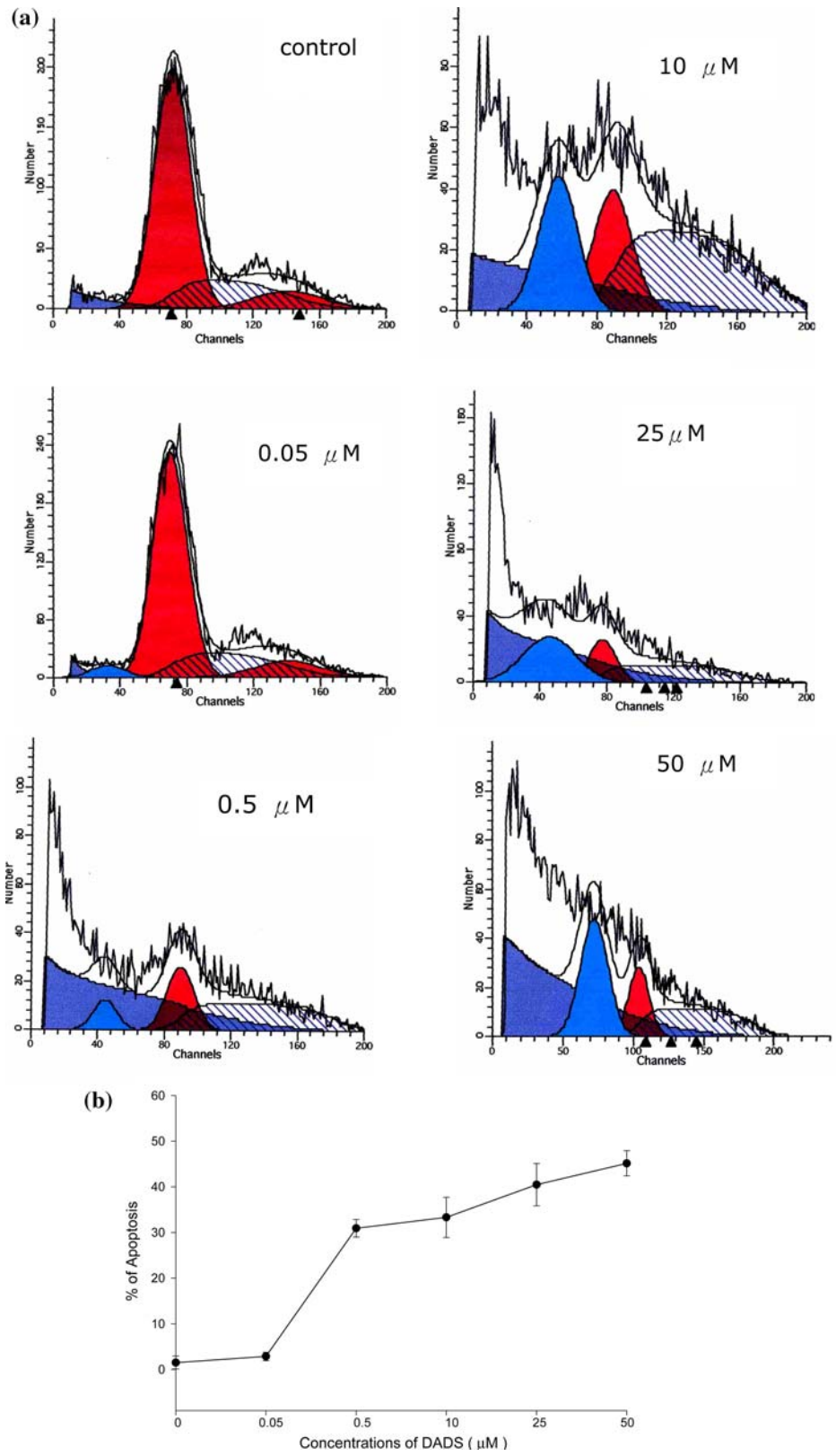

Fig. 2 Flow cytometric analysis of the effects of DADS on the N18 sub-G1 group. The N18 cells were exposed to various concentrations of DADS for 48 h, and the cells were harvested and analyzed for sub-G1 group (panel **a**: cell cycle analysis panel **b**: the percent of cells in apoptosis) and were analyzed by flow cytometry as described in Materials and Methods. Data represents mean ± SD of three experiments. $^{*}P < 0.05$

the N18 cells cultures with chelator BAPTA-AM was done to capture Ca^{2+} then to examine the apoptosis occurrences. The data indicates that BAPTA-AM abolished Ca^{2+} production and blocked the percentage of apoptosis in examined N18 cells significantly (Fig. 4a, b).

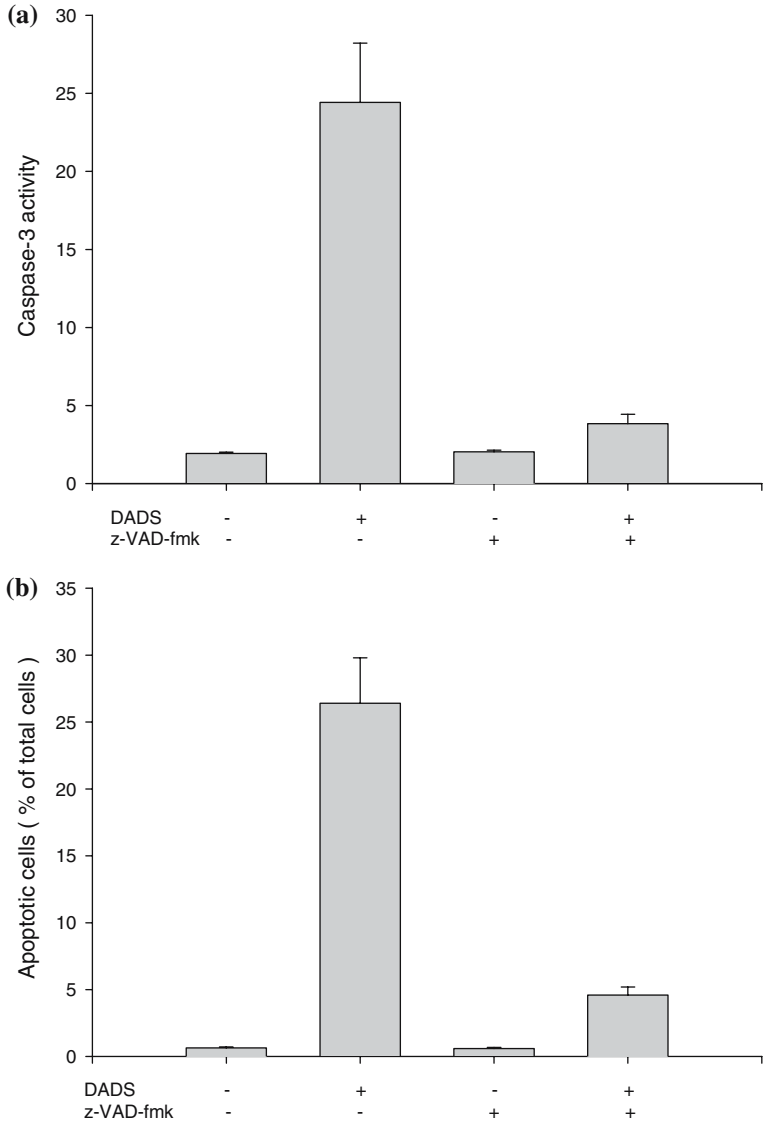

Fig. 3 Flow cytometric analysis of the effects of DADS on N18's caspase-3 activity and apoptosis. The N18 cells were incubated with 25 μM DADS and/or with or without *z*-VAD-fmk treatment for caspase-3 activity (panel **a**) and apoptosis determination (panel **b**) as described in Materials and methods. Data represents mean ± SD of three experiments. $^{*}P < 0.05$

Western blotting for examing the effect of DADS on p53, JNK, Bcl-2, and cytochrome *c* of N18 cells

The N18 cells were treated with or without various concentrations of DADS for 24 h then cells were harvested for examining the amounts of p53, JNK, Bcl-2 and cytochrome *c* by using Western blotting. The results indicated that DADS increased the expression of p53 (Fig. 5a), JNK phosphorylation (Fig. 5b) and cytochrome *c* (Fig. 5d) release but DADS decreased the expression of Bcl-2 in examined N18 cells (Fig. 5c). Those observations are associated with apoptosis.

Discussion

Much evidence has demonstrated that garlic has been used as a therapeutic agent for a long period and high intake of garlic is associated with a protective effect against cancers such as stomach and colorectal polyps in human [29, 30]. Among garlic derivatives, DADS represents the majority (60%) of oil-soluble compounds, and it has been demonstrated to induce cell cycle arrest and apoptosis in human leukemia HL-60 [14] and colon carcinoma HT-29 [31]. In the present results, it was demonstrated that DADS induces growth arrest and apoptosis in mouse–rat hybrid retina ganglion cells (N18) through the mitochondria-dependent pathway (JNK up regulation, p53 expression, Bcl-2 down regulation, decreased mitochondria membrane potential, cytochrome *c* release and activation of caspase-3). We also found out that DADS induced ROS production in earlier treated time and increased the levels of Ca^{2+} in examined N18 cells. It has been reported that DADS induces apoptosis as well as the tumor suppressor gene p53 in non small cell lung cancer cell lines [12] and in human colorectal HCT 116 cells [32].

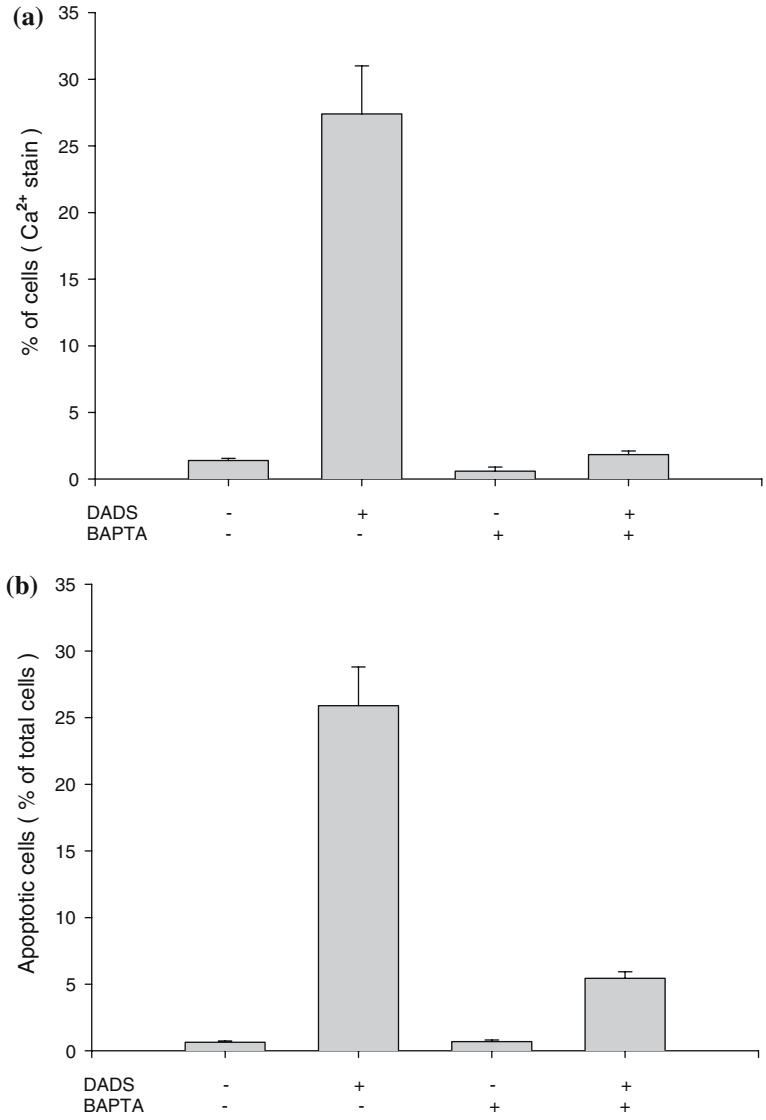

Fig. 4 Flow cytometric analysis of the effects of DADS on N18's Ca^{2+} concentration and apoptosis. The N18 cells were incubated with various concentrations of 25 μM DADS and/or with or without BAPTA treatment for Ca^{2+} concentration (panel **a**) and apoptosis determination (panel **b**) as described in Materials and methods. Data represents mean ± SD of three experiments. $^{*}P < 0.05$

The ROS production in N18 cells represents the earlier step of events occurring on DADS treatment during the first 15 min (Table 1), then up to 90 min before slightly decreasing (180 min). This is in agreement with the report from DADS treated neuroblastoma cell line SH-SY5Y by Filomeni et al. [17]. Both results suggested that DADS mediated oxidative stress in both examined cell lines (N18 and SH-SY5Y). Although we added antioxidant (human superoxide dismutase: hSOD) that led to decrease the levels of DADS cytotoxicity (increased percentage of viable cells), the amounts of ROS is still slightly higher than control (data not shown). That mean that hSOD did not completely abolish the levels of ROS. Therefore, other different pathways or modes of action by DADS inductions should be taken into account in the cascade of events leading to cell death. For example histones acetylation, Ca^{2+} production and a plausible widespread thiol oxidizing action.

It was reported that neural cell death is often mediated by the activation of JNK, the c-Jun upstream MAP kinase [33]. Our results from Western blot demonstrated that DADS increased the levels of JNK phosphorylation (Fig. 5) and that these effects are also dose-dependent. Normally, the JNK levels are maintained very low by the formation of a heterocomplex with GST [33]. JNK had been suggested to might be able to enable apoptosis by interfering directly with mitochondria, resulting in release of cytochrome *c* [34] through destabilization of members of the Bcl-2 family [34, 35]. The present results demonstrated that DADS induced ROS production, and then led to increase the levels of JNK phosphorylation. In other words, ROS caused GST and JNK dissociate leading to JNK activation then formed JNK phosphorylation and led to Bcl-2 destabilization before finally leading to the apoptosis. Our data demonstrated that DADS induced down-regulation of Bcl-2 also may be due to the result of

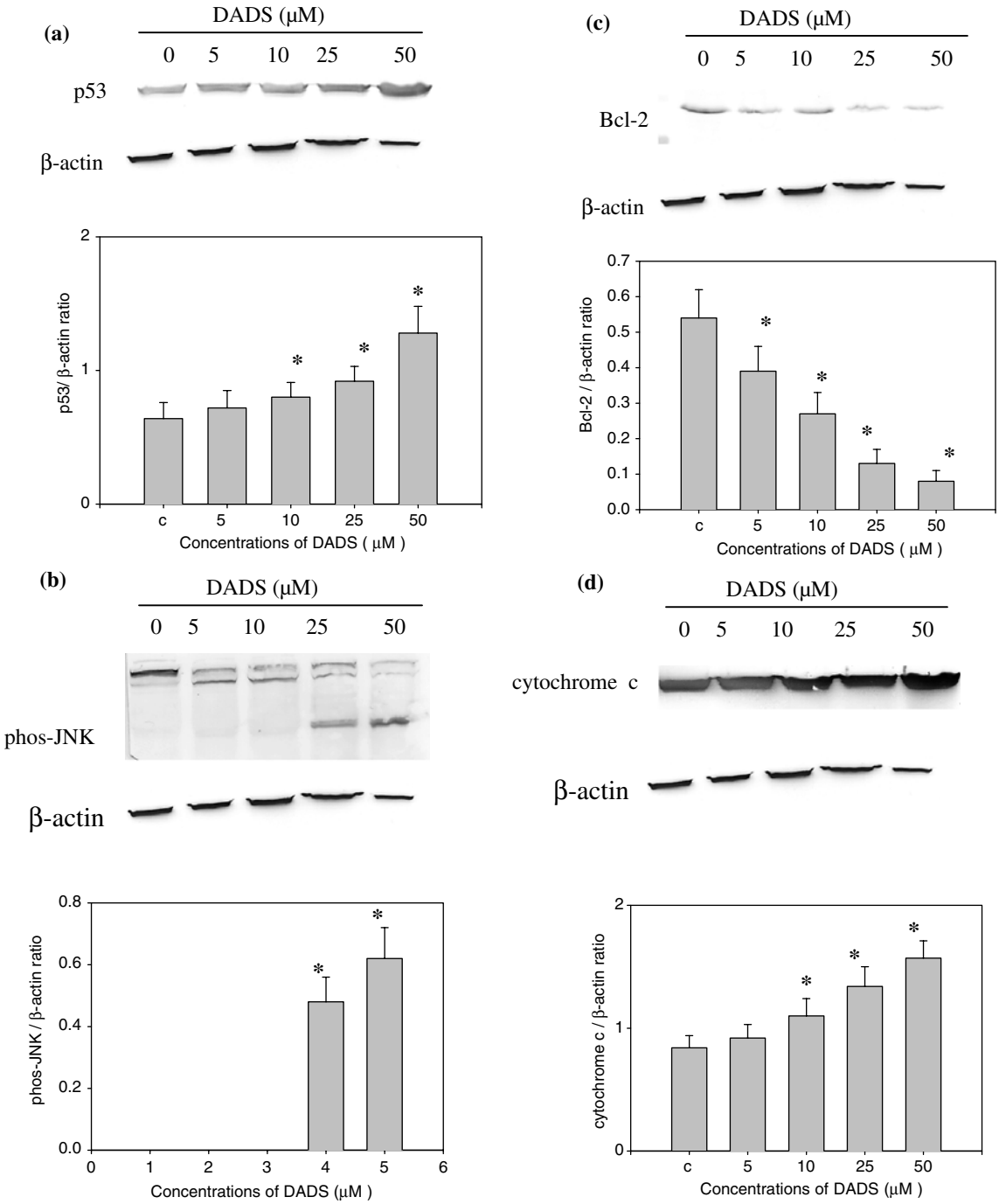

Fig. 5 Representative Western blot showing changes on the levels of p53, JNK, Bcl-2 and cytochrome *c* in N18 cells after exposed to DADS. The N18 cells (5×10^6/ml) were treated with 0, 0.5, 5, 10, 25 and 50 μM DADS for 24 h then cytosolic fraction and total protein were prepared and determined as described in Materials and methods. Then followed by evaluation of the levels of p53 (panel **a**), JNK (panel **b**), Bcl-2 (panel **c**) and cytochrome *c* (panel **d**) expressions were estimated by Western blotting as described in Materials and methods

JNK activation. Moreover, our results also show that DADS-induced the Ca^{2+} production and capture of Ca^{2+} by chelator (BAPTA) before leading to decrease the apoptosis in N18 cells (Fig. 4a, b). Ca^{2+} plays an important role of DADS induced apoptosis. Disruption of cellular Ca^{2+} homeostasis is a critical event in apoptosis. It had been suggested that Ca^{2+} overload my play a fundamental role in the process of photoreceptor apoptosis in chemical-induced and inherited retinal degenerations. The involvement of increases in [Ca^{2+}] was also reported in cells undergoing apoptosis in many different settings [36, 37].

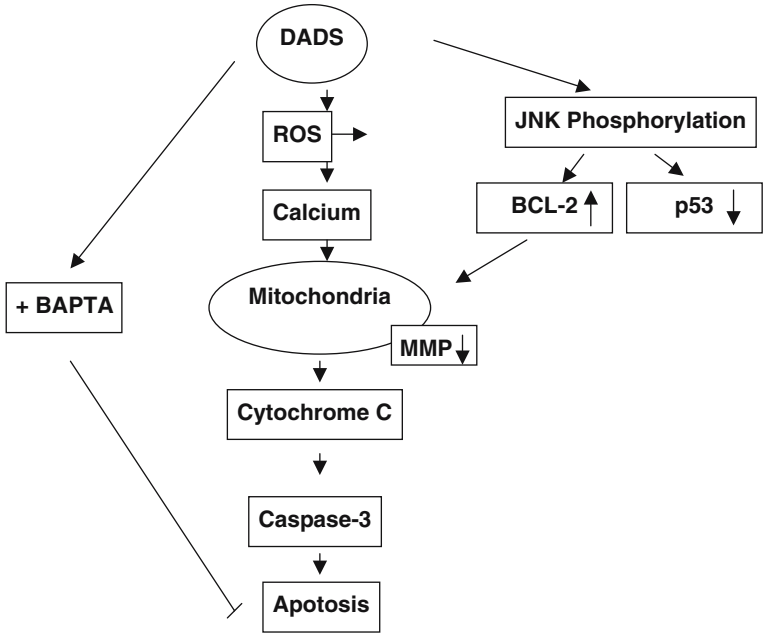

Fig. 6 Proposed model of DADS mechanism of action for apoptosis in N18 cells. DADS induced ROS, Ca^{+2} production and decreased MMP levels which led to caspase-3 activity then caused apoptosis in N18 cells

Much evidence has demonstrated that the overexpression of Bcl-2 results in reduced levels of Ca^{2+} production on endoplasmic reticulum (ER) and $[Ca^{2+}]_{ER}$, whereas the down-regulation of Bcl-2 led to the increasing of $[Ca^{2+}]_{ER}$ [21, 38–40]. Our results showed that DADS decreased the levels of Bcl-2 in N18 cells (Fig. 5) and that may be the reason why in DADS treated N18 cells, there was on increase of the expression of Ca^{2+} production. However, it is not known whether Bcl-2 conducts Ca^{2+} production per se or activates an endogenous leak. It is well documented that massive depletion of the ER Ca^{2+} store is an ER stress condition that initiates execution of apoptosis [37]. Our data is in agreement with this concept because we use BAPTA (Ca^{2+} chelator) to deplete the concentration of Ca^{2+} that led to a blockage of the caspase-3 dependent apoptosis pathway in DADS treated N18 cells. It had been suggest that mitochondria and caspases play a fundamental role in the execution of Ca^{2+}-induced rod apoptosis. Caspase-3 plays an important role in retinal apoptosis of N18 cells. Ac-DEVD-CHO can effectively ameliorate retinal apoptosis by blocking the effect of caspase-3. This is in agreement with other reports which show that 14 caspases have been identified, but it seems that caspase-3 is the main caspase involved in the process of neuronal cell death [41].

It has been reported that physiological stimuli trigger transient and partial discharge of the ER Ca^{2+} store and Bcl-2 seems to utilize a partial decrease in ER Ca^{2+} to promote cell survival [37]. Thus, depletion of ER calcium per se promotes activation of an apoptotic cascade. We also use catalase and *N*-acetylcystein treatment to prevent the accumulation of ROS and these radical scavengers did block the elevation of intracellular Ca^{2+} (data not shown). In conclusion (Fig. 6), the increase of $[Ca^{2+}]$ is a key mediator in DADS-induced apoptosis in N18 cells because (i) after DADS exposure to N18 cells, there were rapid and sustained increases in $[Ca^{2+}]$, (ii) the increase of $[Ca^{2+}]$ preced ROS production and caspase-3 activation, (iii) an intracellular Ca^{2+} chelator (BAPTA), abolished DADS-induced $[Ca^{2+}]$ elevation and ROS production, which further prevented caspases-3 activation and apoptosis.

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