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Diallyl disulfide induces apoptosis in human leukemia HL-60 cells through activation of JNK mediated by reactive oxygen

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Diallyl disulfide (DADS) is a chemopreventive agent that can induce apoptosis in many tumor cells. Reactive oxygen species (ROS) are important mediators in apoptosis induced by various stimuli, including chemopreventive agents. The phosphotransferase c-JUN N-terminal kinase (JNK) has been shown to regulate apoptosis. In this study, we found that DADS-induced apoptosis in human leukemia HL-60 cells is mediated by ROS-activated JNK. The DADS-treated HL-60 cells showed a dose- and time-dependent decrease in cell viability and proliferation. Agarose gel electrophoresis of cells treated with 10.0 or 20.0 mg/L DADS for 24 h showed a characteristic ladder pattern in their DNA. Levels of DADS-induced ROS, as measured by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) fluorescence, also showed dose- and time-dependent increases in HL-60 cells. Activity of JNK was induced by DADS in a dose-dependent manner; HL-60 cells exposed to10.0 mg/L DADS for 8 h showed maximum levels of phosphorylated JNK, which decreased when exposed for additional 4 h. In contrast, Sp600125, a specific inhibitor of JNK, blocked apoptosis of HL-60 cells exposed to DADS. *N*-Acetylcysteine (NAC), a known antioxidant, also decreased ROS generation, effectively blocked apoptosis, and decreased DADS-induced phosphorylated JNK levels. These results suggest that JNK is involved in DADS-induced ROS-mediated apoptosis in HL-60 cells.

1. Introduction

Apoptosis depends on the sequential activation of different processes involving cellular boundary alteration, protein phosphorylation, and gene transcription (Carson and Ribeiro 1993). It is a critical protective mechanism against damaged, improperly produced or tumorigenic cells (Yu et al. 2002); many studies have focused on selectively killing tumor cells through its induction (Ferreira et al. 2002).

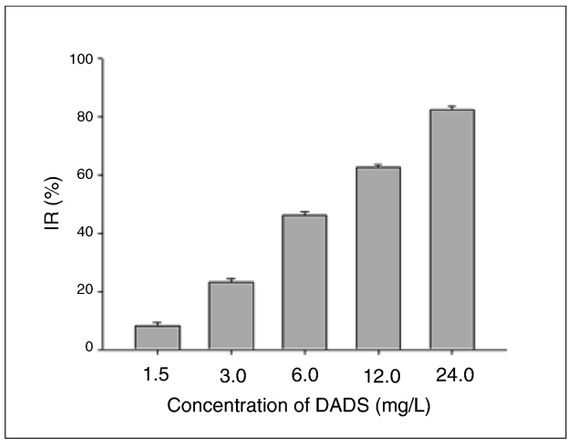
Studies have suggested the main active component of the cancer-fighting allyl sulfides in garlic is diallyl disulfide (DADS), which can induce apoptosis in many tumor cells, including gastric cancer, lung cancer, colon cancer and others (Park et al. 2002; Filomeni et al. 2003; Lu et al. 2004; Wen et al. 2004). Previous studies in our laboratory confirmed that DADS was able to induce differentiation and apoptosis in human gastric cancer cells and human leukemia cells. The mechanism of inducing apoptosis involves caspase activation, G_2/M phase cell cycle arrest, histone acetylation, inhibition of extracellular signal-regulated kinases (ERKs), activation of p38 signaling pathway, up-regulation of Ras-related C3 botulinum toxin substrate 2 (i.e., Rac2, a GTPase) etc. (Yuan et al. 2004; Ling et al. 2006; Zhao et al. 2006; Tan et al. 2008; Yi et al. 2009). However, the exact mechanism by which DADS induces tumor cell apoptosis needs further study.

Reactive oxygen species (ROS) are important second messengers in many cellular processes including apoptosis (Gabbita et al. 2009). Accumulating evidence suggests that ROS may act as signaling molecules for the initiation and execution of the apoptotic processes (Carmody and Cotter 2001; Wu et al. 2005). While ROS levels have been shown to increase when some tumor cells are exposed to DADS (Yang et al. 2009), the exact role of ROS and its interaction with other signaling molecules in apoptosis are not completely clear. Recent reports suggest that ROS closely affect regulation of mitogen-activated protein kinases (MAPKs) signal-transduction pathways (Hancock 2001). Tsai et al. (2007) confirmed that diallyl disulfide up-regulates expression of glutathione S-transferase-P via an AP-1-dependent pathway when apoptosis is induced in human liver adenocarcinoma Clone 9 cells. Lei et al. (2008) considered mechanisms to include ERK inhibition of ERK and activation of the SAPK/JNK and p38 pathways in human breast cancer cell line MCF-7. Wu et al. (2009) showed mechanisms of apoptosis induction by diallyl disulfide in human lung adenocarcinoma cells to include activation of C-Jun N-terminal kinase (JNK), up-regulation of p53, and down-regulation of bcl-2 expression; JNK also affects diverse cellular functions such as cell proliferation, differentiation, and apoptosis (Kyriakis and Avruch 1996). While the mechanism of JNK activation is not fully elucidated, different stimuli may initiate different mediators to activate the JNK signaling pathway (Yujiri et al. 1998; Xia et al. 2000). Studies have shown ROS to be effective apoptosis mediators of JNK in many tumor cells (Shen and Liu 2006; Zou 2008). This study aims to show that ROS are principal regulators of the JNK signal pathway during DADS-induced apoptosis in human leukemia HL-60 cells.

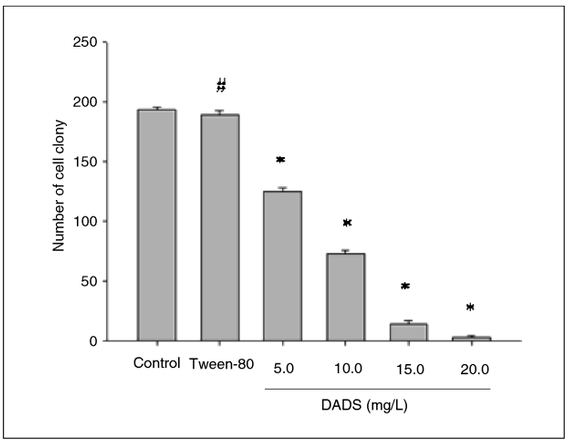
2. Investigations and results

2.1. Effects of DADS on cell viability, proliferation and apoptosis

The HL-60 cells were treated with various concentrations of DADS for 48 h for cell viability test. The results of an



(A)



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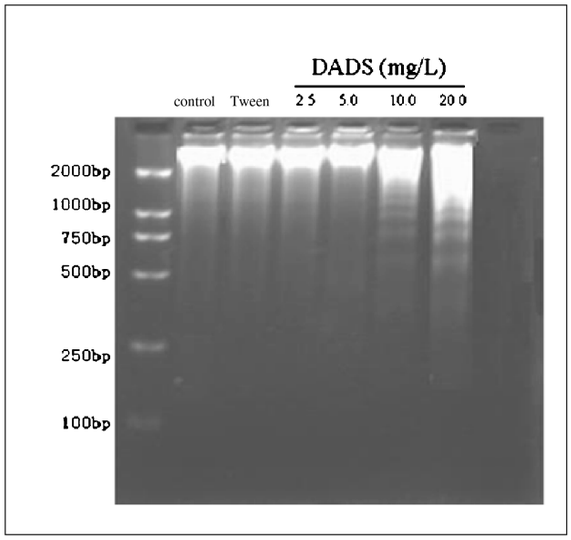

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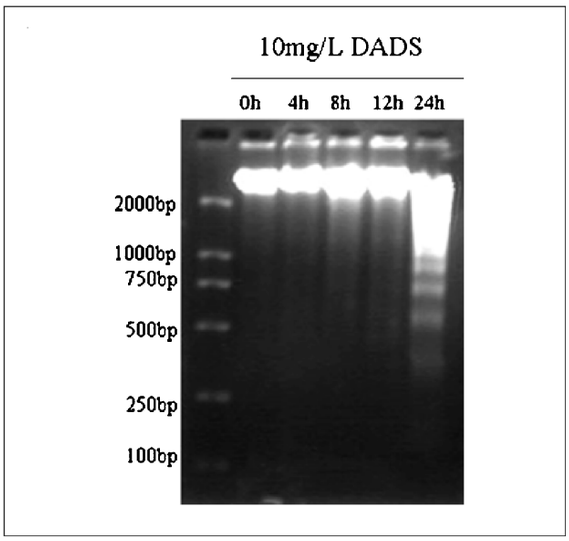


(D)

Fig. 1: Effects of DADS on cell viability, proliferation and apoptosis. (A) Effects of different concentrations DADS on HL-60 cells, as shown by cell inhibition rates (%). Values are mean ± SD from three independent experiments. (B) Soft-agar cell colony numbers of HL-60 cells treated with different concentrations of DADS after 2–3 weeks. Values are mean ± SD from three independent experiments. $^{\#}P > 0.05$ vs control; $^{*}P < 0.05$ vs control. (C) DNA agarose electrophoresis of HL-60 cells treated with DADS at different concentrations for 24 h. (D) DNA agarose electrophoresis of HL-60 cells treated with 10.0 mg/L DADS for different lengths of time

MTT assay showed that, in the presence of DADS, cell inhibition rates ranged from 23.06% to 82.31% after treatment with 3.0–24.0 mg/L DADS for 48 h. This finding suggests that DADS exerts a dose-dependent cytotoxic effect on HL-60 cells (Fig. 1A). The DADS-treated cells showed decreased colony proliferation in soft agar, as shown in Fig. 1B, indicating that DADS inhibits HL-60 cell proliferation in a dose-dependent manner.

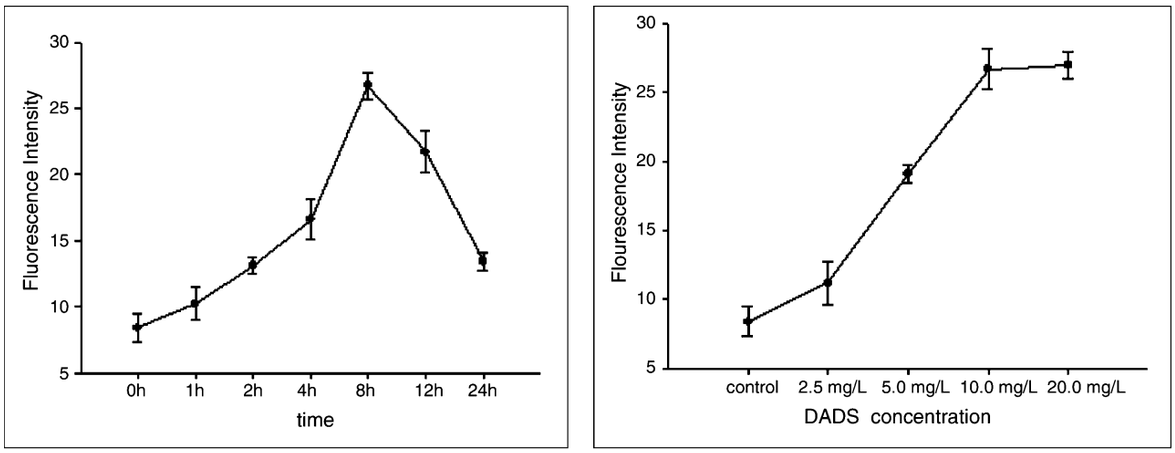
The nuclear DNA of late-stage apoptotic cells degraded to regular nucleosome-sized fragments in multiples of 180 base pairs, as shown in Fig. 1C and D; after treatment with 10.0 and 20.0 mg/L DADS for 24 h, DNA extracted from cells displayed a characteristic ladder pattern in agarose gel electrophoresis, while other groups showed normal bands.

2.2. Effects on levels of intracellular ROS induced by DADS

Because 2′,7′-dichlorofluorescein-H (DCFH) is rapidly oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS, intensity of DCF fluorescence is proportional to the amount of intracellular ROS generated. HL-60 cells treated with DADS showed increased intensity of DCF fluorescence, suggesting that intracellular ROS was increased. As shown in Fig. 2A, when HL-60 cells were exposed to 10.0 mg/L of DADS, ROS production levels increased dramatically in 1 h, peaked at about 8 h, and began to decrease over the next 4 h. The increase in ROS was dose-dependent, and ROS increased after cells were exposed to 2.5–20.0 mg/L DADS for 8 h (Fig. 2B).

2.3. Effect of NAC and sp600125 on HL-60 cells apoptosis induced by DADS

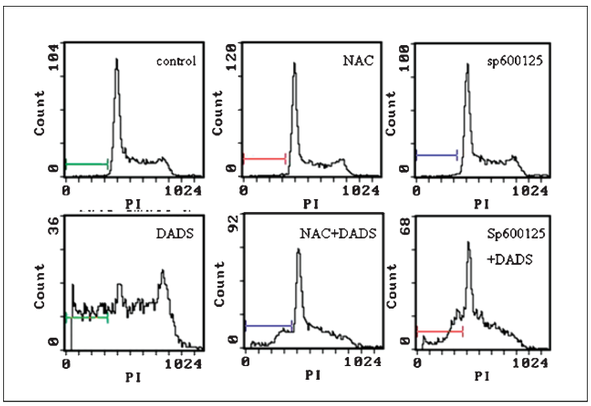
In the DNA histogram, the amplitude of sub-G_1 DNA peak, which is lower than G_1 DNA peak, represents the number of apoptotic cells. As shown in Fig. 3, after HL-60 cells were treated with 10 mg/L DADS for 24 h, the percentage of apoptotic cells were 36.8%. Compared with controls, 10 mg/L DADS induced apoptosis in HL-60 cells, while 10 mM NAC and 10 μM sp600125 did not. When HL-60 cells were exposed to DADS in the presence of the antioxidants NAC or the kinase inhibitor



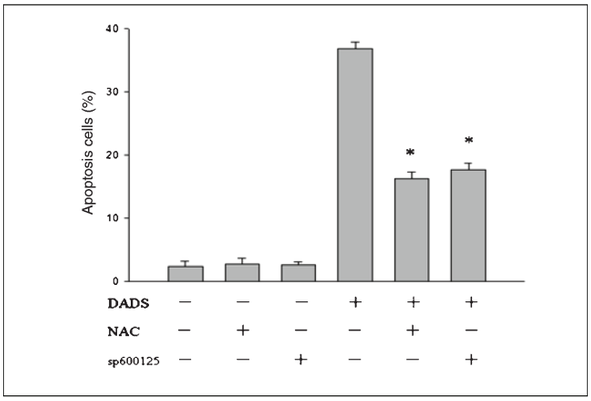
(A) (B)

Fig. 2: Flow cytometry (FCM) analysis of fluorescence intensity of DCFH-DA-stained HL-60 cells. (A) FCM analysis of HL-60 cells treated with 10.0 mg/L DADS for different lengths of time. (B) FCM analysis of HL-60 cells treated with different concentrations of DADS after 8 h

sp600125, the percentages of apoptotic cells were 16.2% and 17.8%, respectively, showing that pretreatment of HL-60 cells with NAC and sp600125 blocked apoptosis induced by DADS ($P < 0.05$).



(A)



(B)

Fig. 3: Flow cytometry (FCM) analysis of apoptosis in HL-60 cells induced by 10 mg/L DADS, with and without pretreatment with 10 mM NAC or 10 μM sp600125. (A) Representative experiment of sub-diploid DNA content was measured by FCM to estimate apoptosis. (B) Data are expressed as mean values ± standard deviations and represent three determinations. *$P < 0.05$ vs 10 mg/L DADS treatment

2.4. JNK1/2 activation in DADS-induced, ROS-mediated apoptosis in HL-60 cells

The kinases JNK1/2 were activated during DADS-induced apoptosis. In Western blot analyses (Fig. 4A), after HL-60 cells were exposed to DADS for 8 h, the amounts of JNK1/2 increased as doses of DADS increased; phospho-JNK1/2 levels also increased in a dose-dependent manner. Fig. 4B shows time-dependent activation of JNK by DADS in HL-60 cells. Cells exposed to 10 mg/L DADS for 4 h had increased phospho-JNK1/2 levels compared with unexposed control cells. The activity of JNK peaked after an 8-h treatment but decreased after cells were treated with DADS for an additional 4 h.

To evaluate the effect of sp600125 and NAC on JNK activation in DADS-induced apoptosis, HL-60 cells were treated with NAC or sp600125 for 30 min prior to exposure to 10 mg/L DADS. Western blot results also showed sp600125 and NAC to significantly diminish expression of phosphor-JNK (especially phosphor-JNK1) when HL-60 cells were exposed to 10 mg/L DADS for 8 hours ($P < 0.05$) (Fig. 4C).

3. Discussion

Stimulating or inducing tumor cell apoptosis has become a new possibility for tumor treatment (Schulze-Bergkamen and Krammer 2004). Large numbers of cytokines and drugs have been shown to induce tumor cell apoptosis; among these, the role of allyl sulfides (found in garlic) in the prevention and treatment of tumors has attracted some attention (Kwon et al. 2002). The most active anti-cancer ingredient in garlic, DADS, induces apoptosis in several tumor types, including HL-60 cells, but the exact mechanism has been unclear. We investigated this mechanism in the present study.

We previously reported that low doses (1.25 mg/L) of DADS have significant anti-proliferative effects, and can induce differentiation in leukemia HL-60 cells (Wu et al. 2004). The present study used moderate doses (2.5–20 mg/L) of DADS to induce apoptosis in HL-60 cells. Based on the preliminary work, this study further shows that the growth of HL-60 cells is inhibited when they are exposed to DADS. The results of MTT assays and soft agar colony-forming experiments showed that DADS inhibits the viability and proliferation of the HL-60 cells in a dose-dependent manner. When HL-60 cells were treated with 10.0 and 20.0 mg/L DADS for 24 h, their DNA fragments displayed a characteristic ladder pattern on agarose gel

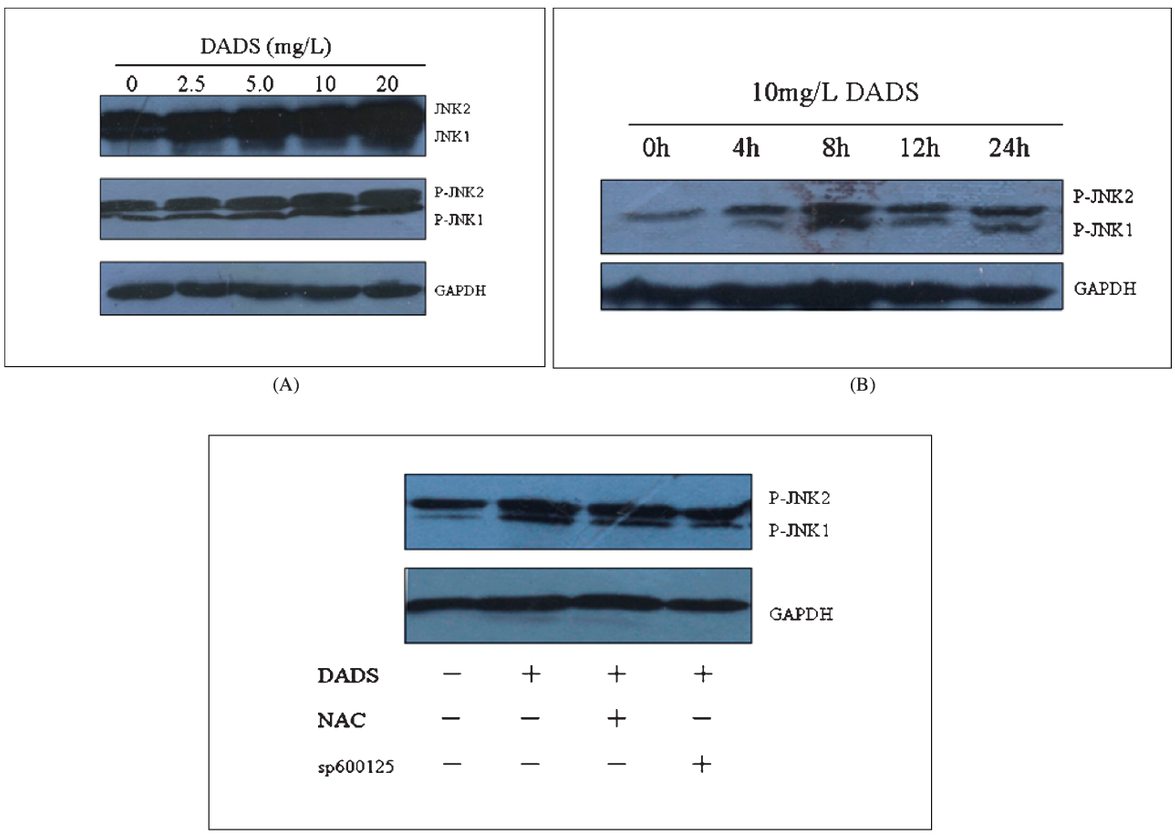


Fig. 4: Western blot analysis of JNK activation in HL-60 cells induced by DADS. (A) HL-60 cells were treated with different concentrations of DADS for 8 h. Levels of amounts of JNK1/2 and phospho-JNK1/2 were determined by western blot analysis, and GAPDH was the corresponding lanes. (B) JNK activation in HL-60 cells after treatment for different lengths of time with 10.0 mg/L DADS. (C) Effects of pretreatment with NAC and sp600125 on JNK activation in DADS-treated HL-60 cells

electrophoresis. This result indicates that DADS is an effective inducer of apoptosis in HL-60 cells.

A number of studies have shown that ROS are closely linked to apoptosis of tumor cells. In mammalian cells, mitochondria leak of ROS as a consequence of damage incurred during the process of apoptosis is a primary trigger of further apoptosis; exogenous ROS, such as provided by moderate levels of H_2O_2, can induce apoptosis in many types of cells (Ueda et al. 2002). Some studies have suggested that endogenously produced ROS—triggered by many stimuli, such as cancer drugs, UV light, ionizing radiation etc.—play a key role in the apoptotic processes (Ding et al. 2002; Chan et al. 2003). In this case, we studied the role of intracellular ROS formation in DADS-induced apoptosis of HL-60 cells. When HL-60 cells were exposed to DADS, ROS levels increased significantly in dose- and time-dependent modes. When HL-60 cells were exposed to 10.0 mg/L of DADS, ROS production increased dramatically in 1 h, reached its maximum level at about 8 h, and began to decrease in the next 4 h. This result indicates that oxidative burst is an early event in HL-60 cells induced by DADS. Moreover, our results show that DADS induces apoptosis in HL-60 cells, while pretreating cells with NAC decreases the percentage of apoptotic cells, which suggests (a) DADS increases ROS levels to initiate apoptosis; and (b) DADS-induced apoptosis is ROS-dependent in HL-60 cells.

Many lines of evidence have suggested that members of MAPK pathway, including ERKs, JNKs, and p38 MAPK, play important roles in cell survival and death (Shen et al. 2006). Several studies have shown that phosphorylation of Bax mediated by p38 MAPK leads to initiation of mitochondria-dependent apoptosis of cells treated with a variety of cell-death stimulants (Bong-Jo et al. 2002). Treatment of HL-60 cells with DADS led to profound changes in the levels of activated ERKs and p38 MAPK by phosphorylation. DADS decreased the levels of activated pERK and increased the levels of the activated p38 MAPK, supporting reports of cell apoptosis in the presence of their respective inhibitors (Tan et al. 2008). At present, there are also many studies supporting JNK's place as an important proapoptotic factor in many tumor cells, the suppression of which, by either genetic or pharmacological approaches, discourages apoptosis (Thannickal and Fanburg 2000) In this study, our results have shown that the kinase inhibitor specific for JNK, sp600125, can significantly decrease apoptosis rates in cells induced by DADS. They also show that the JNK signaling pathway is involved in DADS-induced apoptosis in HL-60 cells. Reactive oxygen species are known to be apoptosis triggers and modulators but cannot directly activate caspases themselves; ROS-induced apoptosis therefore requires other participants, including JNK (Chandra et al. 2000). In Western blot analyses, after HL-60 cells were treated with DADS for 8 h, expression of JNK1/2 and phospho-JNK1/2 increased as doses of DADS increased. Western blot analyses also showed increased phospho-JNK1/2 activity over time during DADS treatment. Cells exposed to DADS for 4 h had increased phospho-JNK1 and phospho-JNK2 levels compared with unexposed control cells. Activity of JNK induced by DADS in HL-60 cells peaked after an 8-h treatment but decreased after cells were treated with DADS for additional 4 h, suggesting that increased ROS proceeds JNK1/2 activation. Our results also show that NAC can inhibit JNK1 phosphorylation as well as decrease rates of apoptosis in cells induced by

DADS. Therefore, our results indicate that DADS may modulate apoptosis by altering ROS, which in turn activates JNK1/2.
In conclusion, our study clearly shows, first, that DADS not only restrains HL-60 cells' viability and proliferation, but also can induce HL-60 cells apoptosis; second, increased intracellular ROS and activation of JNK are involved in apoptosis of HL-60 cells induced by DADS; and third, ROS is an effective moderator of JNK during DADS induction of HL-60 cells apoptosis. It also appears that increased ROS may precede the activation of JNK1/2. However, the exact mechanisms of DADS induction of tumor cells apoptosis needs further investigation.

4. Experimental

4.1. Materials and reagents

The DADS reagent came from Fluka Chemika Company (Buchs, Switzerland). JNK antibodies, phospho-JNK antibodies and relative secondary antibodies were purchased from Abcam Biotechnology; 2′,7′-dichlorofluorescin diacetate (DCFH-DA), *N*-acetyl-L-cystein (NAC), sp600125 and Western Blot Detection Kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.2. Cell culture

Human acute myeloid leukemia HL-60 cells were purchased from Central South University, routinely cultured in RPMI-1640 culture medium with 10% calf serum, in a 37 °C, saturated humidity, 5% CO_2 incubator. Cells in logarithmic growing phase were used for experiments.

4.3. Evaluation of cell viability and cell proliferation

Cell viability was determined by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay. MTT was performed as previously described (Qin et al. 2008). Results were expressed as percentage of MTT reduction: Cell Inhibition rate (IR%) = [A570 (control) − A570 (treatment)]/(A570 (control) × 100%. A570 (control) is the absorbance of cancer cells without drug treatment; A570 (treatment) is the absorbance of cancer cells exposed to the drug.
Cell proliferation inhibition was evaluated by soft agar colony-forming experiments. Logarithmic growing-phase cells were collected, centrifuged and resuspended in culture medium; cell density was adjusted to 1.0×10^3/mL. Different cell suspensions were rapidly mixed with agar, and the mixture immediately poured into 24-well plates at 1 mL each. The agar was allowed to solidify at room temperature. Then 24-well plates were then transferred into a CO_2 incubator, at 37 °C, 5% CO_2 and saturated humidity, and cultured 2–3 weeks. Colonies with diameters > 75 μm were counted with the inverted microscope.

4.4. DNA fragmentation

The HL-60 cells that were exposed to various concentrations of DADS, or to 10.0 mg/L DADS for different times, were collected. Cellular genomic DNA was isolated according to the manufacturer's instructions of the Apoptosis Cells DNA Ladder Assay Kit (Abcam, USA). Isolated DNA fragments were analyzed by electrophoresis in 2% agarose gel containing ethidium bromide. Results were analyzed by a gel imaging system.

4.5. Measurement of ROS levels

To evaluate ROS production levels, a control group and HL-60 cells treated with DADS were stained with DCFH-DA which is normally a non-fluorescent compound but transforms into fluorescent DCF when oxidized by ROS. Cells were collected and resuspended in a serum-free culture medium; 10 mM DCFH-DA was added, and the mixture incubated at 37 °C for 30 min. to assure probes and cells contacted sufficiently. Cells were then washed with serum-free culture medium and analyzed with a flow cytometer (Beckman-Coulter Co, USA).

4.6. Antioxidant and kinase inhibitors treatment and FCM analysis

To explore whether generation of ROS and JNK signaling pathways were the underlying mechanisms in DADS-induced apoptosis, the antioxidant N-acetylcysteine (NAC) and the kinase inhibitor specific for JNK, sp600125, were added to cells 30 min before DADS treatment. After 24 h DADS treatment, cells were collected and fixed with 70% ethanol. Before analysis with a flow cytometer, cells were washed with PBS; 15 μl 10 g/L RNase A was added to them. They were then gently mixed with 400 μl 50 mg/L propidium iodide and incubated in the dark for 30 min.

4.7. Western blot analysis

After cells were lysed in lysis buffer (10 mM Tris-HCl pH 7.6, 100 mM NaCl, 1.0 mM EDTA and 100 mg/L PMSF), cell debris was removed using centrifugation at 12,000 rpm for 30 min at 4 °C. Protein concentration was determined by bicinchoninic acid (BCA) protein quantification assay (Pierce, Rockford, IL, USA). Cellular proteins were loaded, at 30 μg in each well, in 10% sodium dodecylsulfate (SDS)-polyacrylamide gels. After running the gels, protein bands were transferred electrophoretically to polyvinylidene fluoride (PVDF) membrane. The membranes were saturated and blocked with 5% fat-free milk for 3 h at room temperature, then incubated with primary antibody for 2 h, with continuous rocking. After washing with PBS, the membranes were soaked in secondary antibody solution for an additional hour. The membranes were then treated with an enhanced chemiluminescence reagent. Signals were detected by exposing membranes to X-ray films; relative signal intensity was quantified with a Bio Image Intelligent Quantifier.

4.8. Statistics

SPSS10.0 statistical software was used to process results. Experimental data were presented as ± SD; differences between experimental and control groups were detected by *t* test; $P < 0.05$ was set for the criteria of statistical significance.

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