

Garlic Compounds Generate Reactive Oxygen Species Leading to Activation of Stress Kinases and Cysteine Proteases for Apoptosis in Human Glioblastoma T98G and U87MG Cells

Arabinda Das, PhD
Naren L. Banik, PhD
Swapan K. Ray, PhD

Department of Neurosciences, Medical University of South Carolina, Charleston, South Carolina.

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Address for reprints: Swapan K. Ray, PhD, Department of Neurosciences, Medical University of South Carolina, 96 Jonathan Lucas Street, Suite 325E, Charleston, SC 29425; Fax: (843) 792-8626; E-mail: raysk@musc.edu

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BACKGROUND. Garlic-derived organosulfur compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) provide significant protection against carcinogenesis.

METHODS. Dose-dependent cytotoxic effects of the garlic compounds (DAS, DADS, and DATS) were tested in human glioblastoma T98G and U87MG cells. Wright staining and ApopTag assay confirmed induction of apoptosis. Measurements showed that production of reactive oxygen species (ROS) and an increase in intracellular free $[Ca^{2+}]$ promoted apoptosis. Western blot analysis indicated that increased expression and activities of the stress kinases and cysteine proteases caused apoptosis. Use of JC-1 showed changes in mitochondrial membrane potential ($\Delta\psi_m$) for mediation of apoptosis. Use of the specific inhibitors monitored the activation of different kinases and proteases in apoptosis.

RESULTS. Treatment of glioblastoma cells with garlic compounds triggered production of ROS that induced apoptosis with the phosphorylation of p38 MAPK and activation of the redox-sensitive JNK1 pathway. Pretreatment of cells with ascorbic acid attenuated ROS production, p38 MAPK phosphorylation, and JNK1 activation. Pretreatment with JNK1 inhibitor I also significantly reduced cell death. Increases in intracellular free $[Ca^{2+}]$, expression of calreticulin, and activation of caspase-4 indicated involvement of endoplasmic reticulum (ER) stress in apoptosis. Other events in apoptosis included overexpression of Bax, down-regulation of Bcl-2 and some BIRC proteins, mitochondrial release of cytochrome c and Smac into the cytosol, and activation of calpain, caspase-9, and caspase-3.

CONCLUSIONS. Garlic compounds induced apoptosis in glioblastoma cells due to production of ROS, increase in ER stress, decrease in $\Delta\psi_m$, and activation of stress kinases and cysteine proteases. *Cancer* 2007;110:1083–94. © 2007 American Cancer Society.

KEYWORDS: apoptosis, diallyl sulfide, diallyl disulfide, diallyl trisulfide, glioblastoma, oxidative stress.

Glioblastoma is the most malignant type among all primary brain tumors.¹ The median survival, even with aggressive multimodal-ity treatments, does not exceed 1 year. Thus, new therapeutic strategies need to be explored. Garlic (*Allium sativum* L.) is a widely consumed herb in foodstuffs and medicines.² Epidemiologic, clinical, and laboratory studies have shown that crushed or processed garlic and its active principles such as diallyl sulfide (DAS; ie, $CH_2=CH-CH_2-S-CH_2-CH=CH_2$), diallyl disulfide (DADS; ie, $CH_2=CH-CH_2-S-S-CH_2-CH=CH_2$), and diallyl trisulfide (DATS;

ie, $\text{CH}_2=\text{CH}-\text{CH}_2-\text{S}-\text{S}-\text{S}-\text{CH}_2-\text{CH}=\text{CH}_2$) possess diverse biologic activities including antitumorigenesis effects.³ Increasing evidence suggests that the mechanism of anticancer action of garlic compounds may involve modulation of signal transduction pathways.⁴

Induction of apoptosis by garlic products can be dependent on production of reactive oxygen species (ROS).⁵ Some inducers of apoptosis work via production of ROS that can activate the mitogen-activated protein kinase (MAPK) pathway.⁶ The MAPK kinases (MAPKKs) phosphorylate and activate downstream MAPK kinases (MAPKKs) that ultimately activate the MAPK pathway. Many extracellular stimuli are transduced to the cells through these intracellular signaling cascades. Activation of p38 MAPK is generally associated with induction of apoptosis, whereas activation of p42/44 MAPK exerts cytoprotective effects.^{7,8} DADS induces apoptosis in leukemia HL-60 cells and breast cancer MDA-MB-231 cells with activation of caspase-3,^{6,9} and suppresses colon tumor cell proliferation and cell cycle arrest.^{10,11} The antitumor effects of DADS may be related to its ability to inhibit the proliferation of tumor cells in vitro and in vivo.¹² However, the role of the p38 MAPK pathway in inducing apoptosis in glioblastoma cells after exposure to garlic compounds is unclear.

The current study was conducted to understand the mechanism of induction of apoptosis by garlic compounds in human glioblastoma T98G and U87MG cells. We revealed activation of stress kinases, calpain, and caspases for apoptosis in human glioblastoma cells treated with DAS, DADS, and DATS.

MATERIALS AND METHODS

Cell Culture

Cells were grown in RPMI-1640 medium with 10% fetal bovine serum (FBS) at 37°C in a fully humidified incubator containing 5% carbon dioxide. Before treatments, cells were starved in RPMI-1640 medium with 0.5% FBS for 24 hours. Stock solutions of DAS and DADS (Sigma Chemical Company, St. Louis, Mo) and DATS (LKT Laboratories, St. Paul, Minn) were prepared in dimethyl sulfoxide (DMSO) just before the experiments. An equal amount of DMSO (0.01%) was added to untreated control cells. Dose-response studies were conducted to determine that treatment of cells with 100 μM DAS, 100 μM DADS, and 25 μM DATS for 24 hours could induce apoptosis in glioblastoma cells. After treatments, cells were used for the determination of amounts of apoptosis and specific proteins involved in this process.

Trypan Blue Dye Exclusion Test

Residual cell viability in the attached and detached cell populations was estimated by Trypan blue dye exclusion test.¹² At least 600 cells were counted in 4 different fields for determination of residual cell viability.

Wright Staining and ApopTag Assay

The cells from each treatment were sedimented onto the microscopic slide and fixed in methanol before examination of apoptosis by Wright staining¹² and ApopTag assay.¹² Wright staining detected characteristic apoptotic features such as chromatin condensation, cell-volume shrinkage, and membrane-bound apoptotic bodies. ApopTag assay kit (Intergen, Purchase, NY) was used for biochemical detection of DNA fragmentation in apoptotic cells. The nuclei containing DNA fragments were stained dark brown with ApopTag assay and were not counterstained with methyl green that, however, stained normal nuclei pale to medium green. After ApopTag assay, cells were counted to determine percentage of apoptosis.

Determination of ROS Production

The fluorescence probe 2',7'-dichlorofluorescein diacetate (DCF-DA) was used for assessment of ROS production¹³ in T98G and U87MG cells. Briefly, cells were seeded (1×10^5 cells/well) in 6-well culture plates and the next day cells were washed twice with Hank balanced salt solution (GIBCO-BRL, Grand Island, NY) and loaded with 500 μl Hank balanced salt solution containing 5 μM DCF-DA and different concentrations of DAS, DADS, and DATS. Cells were then incubated at 37°C for different timepoints (0–1440 minutes) and the intracellular fluorescence intensity was measured at 530 nanometers (nm) after excitation at 480 nm in Spectramax Gemini XPS (Molecular Devices, Sunnyvale, Calif). The increase in fluorescence intensity was used to assess the net production of intracellular ROS.

Fura-2 Assay

The fluorescence Ca^{2+} indicator fura-2/AM was used, as we described previously,¹² for determination of intracellular free $[\text{Ca}^{2+}]$ in T98G and U87MG cells. The value of K_d , a cell-specific constant, was determined experimentally to be 0.387 μM for the T98G cells and 0.476 μM for the U87MG cells, using standards of the Calcium Calibration Buffer Kit with Magnessium (Molecular Probes, Eugene, Ore).

TABLE 1
Primers Used for Examining Levels of mRNA Expression of Specific Genes

Gene	Primer sequence	Product size (base pairs)
β -actin	Sense: 5'-GTG GGG CGC CCC AGG CAC CA-3' Antisense: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'	436
bax	Sense: 5'-GCA GGG AGG ATG GCT GGG GAG A-3' Antisense: 5'-TCC AGA CAA GCA GCC GCT CAC G-3'	352
bcl-2	Sense: 5'-CCG GGA GAT CGT GAT GAA GTA-3' Antisense: 5'-CAT ATT TGT TTG GGG CAT GTC T-3'	708
calpastatin	Sense: 5'-AAT GCT GCT TTG GAT GAC CTG-3' Antisense: 5'-ACC TGT ACT CAG CAG GTA CTG-3'	230

Analysis of mRNA Expression

Extraction of total RNA, reverse transcription-polymerase chain reaction (RT-PCR), and agarose gel electrophoresis were performed as we described previously.¹⁴ All primers (Table 1) for the RT-PCR experiments were designed using Oligo software (National Biosciences, Plymouth, Minn). The level of β -actin gene expression served as an internal control.

Antibodies

Monoclonal antibody against β -actin (Sigma Chemical Company) was used to standardize cytosolic protein loading on the SDS-PAGE. Anti-cytochrome *c* oxidase subunit IV (COX4) antibody (Molecular Probes) was used to standardize the mitochondrial protein loading. The inner mitochondrial membrane protein COX4 remains in the mitochondria regardless of activation of apoptosis.¹⁵ Antibodies against cytochrome *c* (BD Biosciences, San Jose, Calif), α -spectrin (Affiniti, Exeter, UK), phospho-p38 MAPK (Promega, Madison, Wis), and phospho-p44/42 MAPK (New England Biolabs, Beverly, Mass) were also used. All other primary immunoglobulin (Ig)G antibodies were purchased from Santa Cruz Biotech (Santa Cruz, Calif). We used horseradish peroxidase-conjugated goat antimouse IgG secondary antibody (ICN Biomedicals, Aurora, Ohio) for detecting all primary IgG antibodies and horseradish peroxidase-conjugated goat antirabbit IgG secondary antibody (ICN Biomedicals) for detecting calpain and α -spectrin.

Western Blot Analysis

Western blot analysis was performed as we described previously.¹² The autoradiograms were scanned using Photoshop software (Adobe Systems, Seattle, Wash) and the optical density (OD) of each band was determined using Quantity One software (BioRad, Hercules, Calif).

Measurement of $\Delta\psi_m$

For measurement of $\Delta\psi_m$, cells were treated with DAS, DADS, or DATS in medium containing 5 μ g/mL JC-1 from 0 to 24 hours.¹³ After JC-1 staining, the cells were washed twice with phosphate-buffered saline (PBS) and resuspended. After excitation at 488 nm, the fluorescence emission of JC-1 was measured in a fluorescent plate reader (Molecular Devices, Sunnyvale, Calif) at wavelengths corresponding to its monomer (530 nm) and *J* aggregate (590 nm) forms. Any decrease in the JC-1 monomer (530 nm): *J* aggregate (590 nm) ratio indicates a drop in $\Delta\psi_m$ in the cells.

Analyses of Cytosolic, Mitochondrial, and Nuclear Proteins

Preparations of cytosolic, mitochondrial, and nuclear fractions were performed by standard procedures.¹² Cytochrome *c* in the supernatant fluids and pellets and also caspase-3 activated DNase (CAD) in nuclear fractions were analyzed by Western blotting.

Colorimetric Assays for Measuring Caspase-9 and Caspase-3 Activities

Caspase-9 and caspase-3 activities in the cells were measured based on the release of *p*-nitroanilide (*p*-NA) from LEHD-*p*-NA and DEVD-*p*-NA, respectively, using the commercially available colorimetric assay kits (Sigma Chemical Company). Concentration of the *p*-NA released from the substrate was calculated from the absorbance at 405 nm.

Treatment With Stress Kinase Inhibitors

The cell-permeable JNK inhibitor I and SB203580 (Calbiochem, La Jolla, Calif) were used at 10 μ M for specific inhibition of phosphorylation of JNK1 and p38 MAPK, respectively. Lower concentrations did not show significant inhibition of phosphorylation and higher concentrations were toxic. Inhibitor was added 1 hour before the addition of a garlic compound and maintained throughout the experiment.

Treatment With Protease Inhibitors

Cells were pretreated (1 hour) with either 10 μ M calpeptin (Calbiochem), or 10 μ M caspase-9 inhibitor I (Calbiochem), or 10 μ M caspase-3 inhibitor IV (Calbiochem) at 37°C for prevention of cell death due to subsequent treatment with a garlic compound. Control cultures were pretreated (1 hour) with an equivalent amount of DMSO ($\leq 0.01\%$) or left untreated. The residual viable cells did not uptake Trypan blue dye and the percentage of viability was calculated.

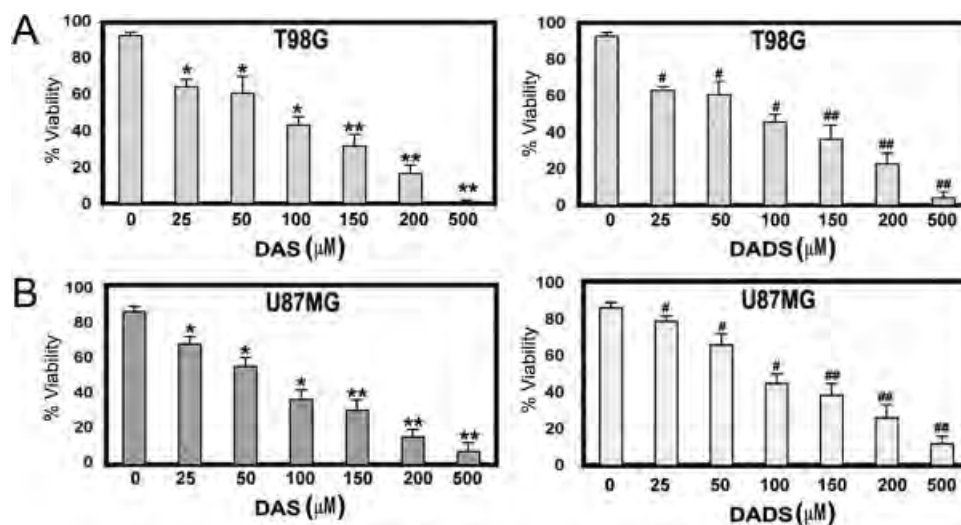


FIGURE 1. Trypan blue dye exclusion test to assess residual cell viability in (A) T98G cells and (B) U87MG cells after exposure to different doses of diallyl sulfide (DAS) and diallyl disulfide (DADS).

Measurement of Glutathione S-Transferase Activity

We measured total glutathione S-transferase (GST) activity in cell homogenates by using the standard GST assay kit (Sigma Chemical) that contained 1-chloro-2,4-dinitrobenzene (CDNB) for covering the total activity of the broadest range of GST isozymes. On conjugation of the thiol group of glutathione to the substrate CDNB, there is an increase in the absorbance at 340 nm.

Statistical Analysis

Results obtained from different treatments were analyzed using StatView software (Abacus Concepts, Berkeley, Calif). Data were expressed as mean \pm standard deviation (SD) of separate experiments ($n \geq 3$) and compared by 1-way analysis of variance (ANOVA) followed by the Fisher post hoc test. Significant differences from control values are indicated by $P < .05$ (*, #, or †) and $P < .001$ (**, ##, or ††).

RESULTS

Garlic Compounds Decreased Cell Viability and Increased Apoptotic Death

Garlic compounds (DAS and DADS) dose-dependently decreased residual cell viability in both T98G and U87MG cells (Fig. 1). Apoptotic death (Fig. 2) was determined using Wright staining (Fig. 2A) and ApopTag assay (Fig. 2B). We found that treatment of the cells with 100 μM DAS or 100 μM DADS was optimum to induce apoptosis. Cells from the ApopTag assay were counted for determination of amounts of apoptosis. Compared with control (CTL) cells, treat-

ment with DAS and DADS increased the percentage of apoptosis (Fig. 2C). Notably, DADS was more effective than DAS for induction of apoptosis.

DAS and DADS Triggered ROS Production

We measured ROS production in T98G and U87MG cells after exposure to DAS and DADS for different times (Fig. 3). Time-dependently, DAS and DADS increased the ROS production in T98G cells (Fig. 3A) as well as in U87MG cells (Fig. 3B) and ROS production could be completely blocked by pretreatment of cells with ascorbic acid (Asc). These results indicated that DAS and DADS induced apoptosis by a mechanism that required increase in intracellular ROS levels in T98G and U87MG cells.

ROS Induced p38 MAPK Phosphorylation Leading to Apoptosis

Because ROS production could activate the stress kinase pathway, we examined phosphorylation of stress kinases (Fig. 4). Treatment of glioblastoma cells with DAS and DADS induced an increase in phosphorylation of p38 MAPK (p-p38 MAPK) selectively because no increase in phosphorylation of p42 MAPK or p44 MAPK occurred (Fig. 4A). Compared with CTL cells, DAS and DADS caused a significant increase in p-p38 MAPK (Fig. 4B). Asc was used as an antioxidant to examine its effect on the level of expression of p-p38 MAPK (Fig. 4C). Asc completely blocked an increase in expression of p-p38 MAPK (Fig. 4D), indicating the involvement of ROS in inducing p38 MAPK phosphorylation. The levels of expression of p38 MAPK remained almost the same

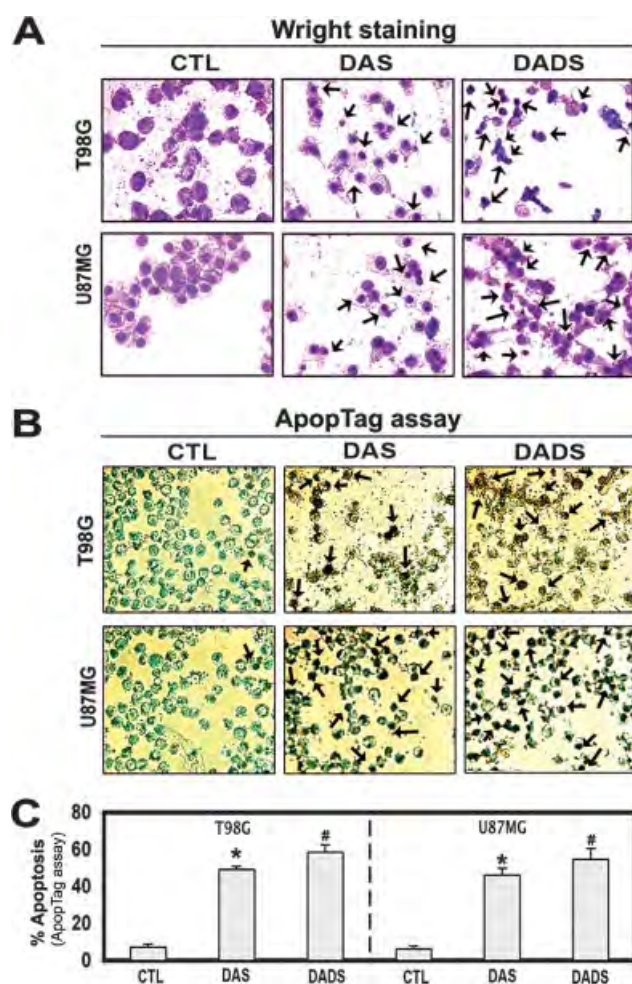


FIGURE 2. Morphologic and biochemical staining to examine induction of apoptosis in T98G and U87MG cells. Treatments (24 hours): control (CTL), 100 μ M diallyl sulfide (DAS), and 100 μ M diallyl disulfide (DADS). (A) Wright staining. (B) ApopTag assay. (C) Bar diagram to show percent apoptosis based on ApopTag assay.

in all treatments. To determine whether p38 MAPK phosphorylation was required for induction of apoptosis, we tested the effect of SB203580 that could specifically inhibit p38 MAPK phosphorylation.¹⁶ Pretreatment of the cells with 5 μ M SB203580 almost completely blocked apoptosis in glioblastoma cells (data not shown). Together, these results suggested that ROS production induced selective phosphorylation of p38 MAPK for apoptosis in glioblastoma cells after exposure to DAS and DADS.

DAS and DADS Activated JNK1 Pathway for Cell Death

The active form of JNK1 is phosphorylated JNK1 (p-JNK1) that regulates the transcription of several genes for induction of cell death. We performed Western blot analysis to examine levels of expression of

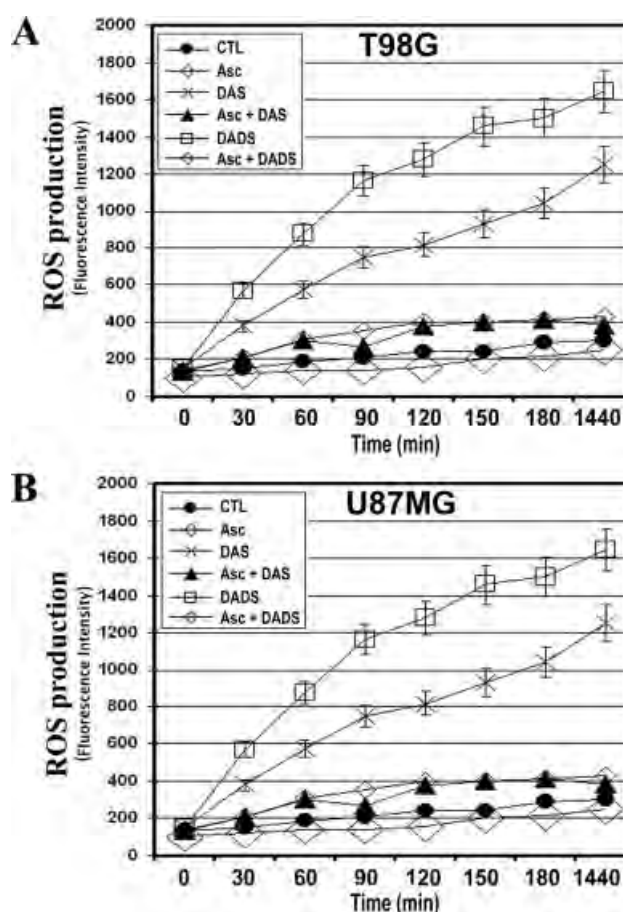


FIGURE 3. Determination of reactive oxygen species (ROS) production in (A) T98G cells and (B) U87MG cells. Treatments (0, 30, 60, 90, 120, 150, 180, and 1440 minutes) in the presence of 5 μ M 2',7'-dichlorofluorescein diacetate (DCF-DA): control (CTL), 10 μ M ascorbic acid (Asc), 100 μ M diallyl sulfide (DAS), 10 μ M Asc (1-hour pretreatment) + 100 μ M DAS, 100 μ M diallyl disulfide (DADS), and 10 μ M Asc (1-hour pretreatment) + 100 μ M DADS.

p-JNK1 (Fig. 5). Increased expression of p-JNK1 occurred in glioblastoma cells after treatments with DAS and DADS (Fig. 5A). The involvement of activated JNK1 pathway in cell death was confirmed using the cell-permeable JNK inhibitor I (Fig. 5B). Pretreatment of the cells with 10 μ M JNK inhibitor I provided protection from cell death (Fig. 5B), indicating activation of the JNK1 pathway in cell death.

DAS and DADS Increased Intracellular Free $[Ca^{2+}]$

An abrupt increase in intracellular free $[Ca^{2+}]$ after treatment with anticancer agents could activate Ca^{2+} -dependent proteases for induction of apoptosis. We used the fura-2 assay to determine the intracellular free $[Ca^{2+}]$ in T98G and U87MG cells (Fig. 6). Compared with CTL cells, treatment with DAS or

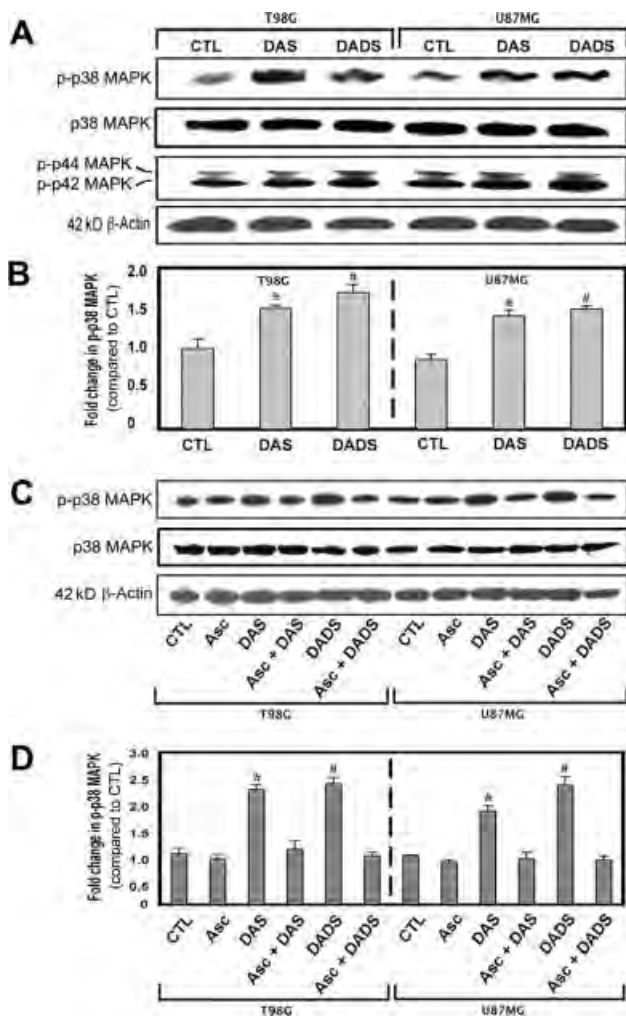


FIGURE 4. Phosphorylation of p38 MAPK during apoptosis in T98G and U87MG cells. (A) Western blot analysis showing levels of p-p38 MAPK, p38 MAPK, p-p42/44 MAPK, and β -actin after the treatments (24 hours): control (CTL), 100 μ M diallyl sulfide (DAS), and 100 μ M diallyl disulfide (DADS). (B) Bar diagram to show fold change in p-p38 MAPK. (C) Western blot analysis showing levels of p-p38 MAPK, p38 MAPK, and β -actin after the treatments (24 hours): control (CTL), 10 μ M ascorbic acid (Asc), 100 μ M DAS, 10 μ M Asc (1-hour pretreatment) + 100 μ M DAS, 100 μ M DADS, and 10 μ M Asc (1-hour pretreatment) + 100 μ M DADS. (D) Pretreatment with Asc prevented phosphorylation of p38 MAPK.

DADS caused a significant increase in intracellular free $[Ca^{2+}]$ in T98G and U87MG cells (Fig. 6), suggesting that garlic compounds caused Ca^{2+} influx leading to apoptosis.

Apoptosis With an Increase in the Bax:Bcl-2 Ratio

A commitment of cells to apoptosis was measured by examining any increase in the Bax expression and/or decrease in Bcl-2 expression (Fig. 7). Our RT-

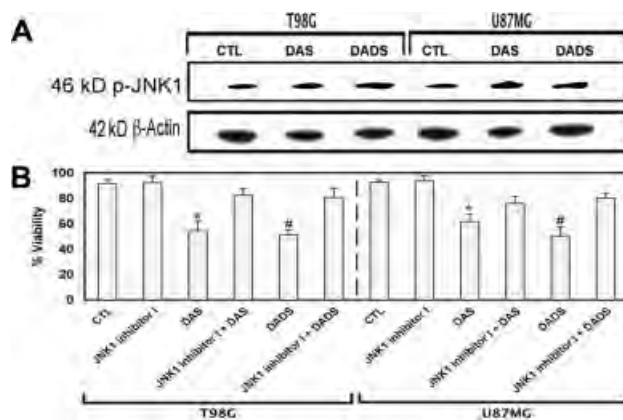


FIGURE 5. Activation of redox-sensitive JNK1 pathway in T98G and U87MG cells. (A) Western blot analysis showing levels of p-JNK1 and β -actin after the treatments (24 hours): control (CTL), 100 μ M diallyl sulfide (DAS), and 100 μ M diallyl disulfide (DADS). (B) JNK inhibitor I prevented cell death. Treatments (24 hours): control (CTL), 10 μ M JNK inhibitor I, 100 μ M DAS, 10 μ M JNK inhibitor I (1-hour pretreatment) + 100 μ M DAS, and 100 μ M DADS, and 10 μ M JNK inhibitor I (1-hour pretreatment) + 100 μ M DADS.

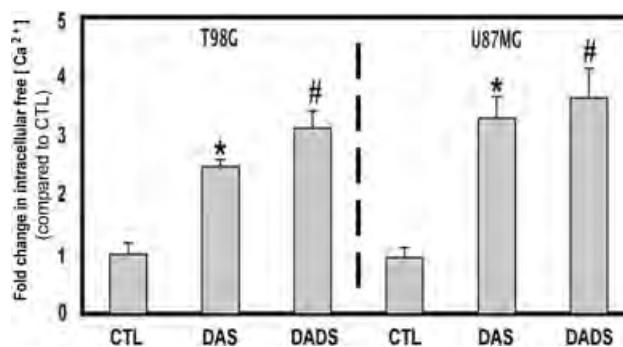


FIGURE 6. Fura-2 assay to determine percent increase in intracellular free $[Ca^{2+}]$ in T98G and U87MG cells. Treatments (24 hours): control (CTL), 100 μ M diallyl sulfide (DAS), and 100 μ M diallyl disulfide (DADS).

PCR experiments demonstrated that garlic compounds increased mRNA expression of the bax gene and slightly decreased mRNA expression of the bcl-2 gene (Fig. 7A). Also, Western blot analysis with a monoclonal antibody capable of recognizing both 21-kilodalton (kD) Bax α and 24-kD Bax β bands showed an increase in Bax levels, whereas another antibody detected almost no change in 26-kD Bcl-2 levels after exposure to garlic compounds (Fig. 7B). Densitometric analysis of the Western blots confirmed that, compared with CTL cells, garlic compounds significantly increased the Bax:Bcl-2 ratio (data not shown), making a commitment of the glioblastoma cells to apoptosis.

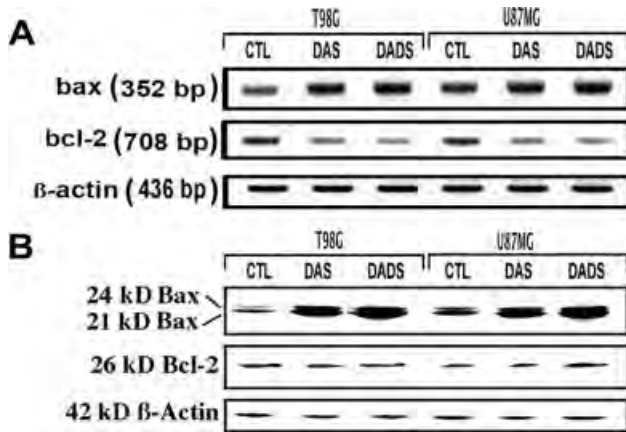


FIGURE 7. Expression of Bax and Bcl-2 at mRNA and protein levels in T98G and U87MG cells. Treatments (24 hours): control (CTL), 100 μ M diallyl sulfide (DAS), and 100 μ M diallyl disulfide (DADS). (A) Agarose gels showing mRNA levels of bax- α , bcl-2, and β -actin. bp indicates basepairs. (B) Western blot analysis showing protein levels of Bax, Bcl-2, and β -actin.

Loss of $\Delta\psi_m$ and Mitochondrial Release of Cytochrome c for Activation of Caspase-9

We examined mitochondrial events leading to activation of caspase-9 for cell death (Fig. 8). Loss of mitochondrial membrane potential ($\Delta\psi_m$) is readily measured using the JC-1 staining of mitochondria in the cells.¹³ As expected, a high JC-1 ratio (590 nm: 530 nm), indicating normal $\Delta\psi_m$ in control T98G (Fig. 8A) and U87MG cells (Fig. 8B). Treatments with DAS and DADS dropped slowly the mean red and green fluorescence ratio of the mitochondria in a biphasic way, indicating loss of the $\Delta\psi_m$ for cell death. The loss of $\Delta\psi_m$ was associated with the disappearance of 15-kD cytochrome c from the mitochondrial fraction and its subsequent appearance in the cytosolic fraction (Fig. 8C). We monitored expression of COX4 as an internal control in mitochondrial fraction. The mitochondrial release of cytochrome c into the cytosol aided formation of the active 37-kD

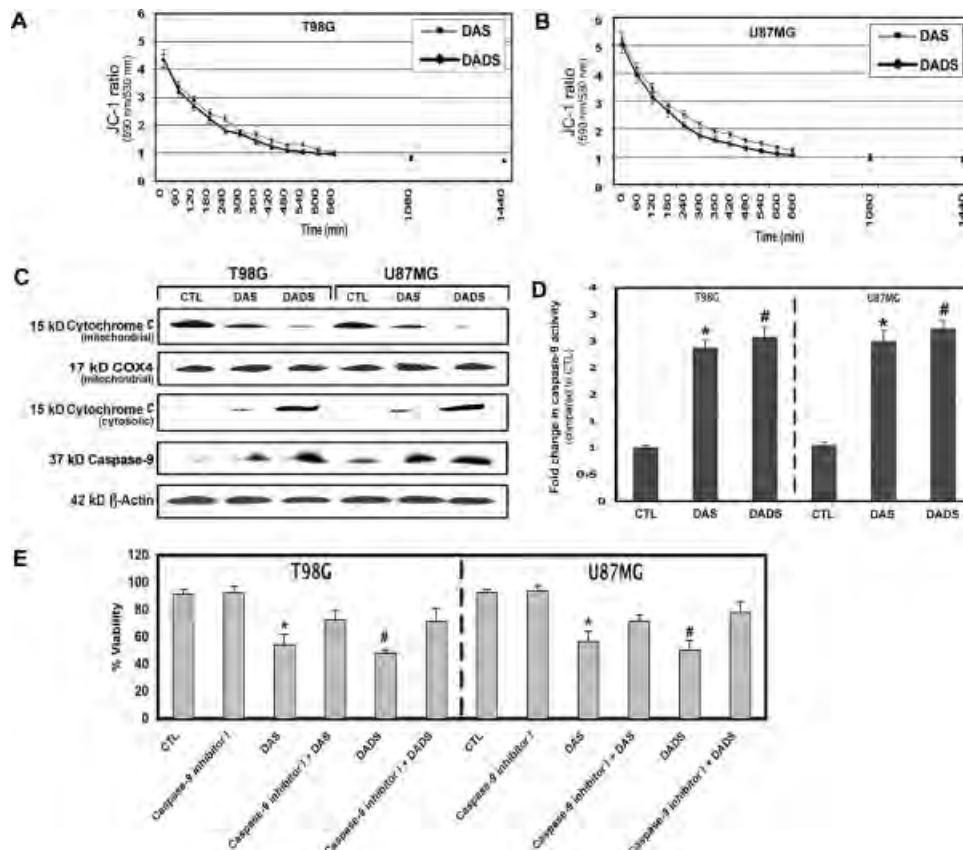


FIGURE 8. Changes in JC-1 ratio (590 nanometers [nm]/530 nm) in (A) T98G and (B) U87MG cells after the treatments (60, 120, 180, 240, 300, 360, 420, 480, 540, 600, 660, and 1440 minutes): 100 μ M diallyl sulfide (DAS) and 100 μ M diallyl disulfide (DADS). (C) Western blot analysis showing levels of cytochrome c, COX4, caspase-9, and β -actin. (D) Determination of caspase-9 activity using a colorimetric assay. (E) Determination of percent cell viability after the treatments (24 hours): control (CTL), 10 μ M caspase-9 inhibitor I, 100 μ M DAS, 10 μ M caspase-9 inhibitor I (1-hour pretreatment) +100 μ M DAS, 100 μ M DADS, and 10 μ M caspase-9 inhibitor I (1-hour pretreatment) +100 μ M DADS. *,# indicate a significant difference from the CTL value ($p < .05$).

caspase-9 fragment in T98G and U87MG cells after treatment with DAS and DADS (Fig. 8C). Further, colorimetric assay demonstrated a significant increase in caspase-9 activity in both glioblastoma cells after treatments with garlic compounds (Fig. 8D). Pretreatment of cells with caspase-9 inhibitor I prevented cell death (Fig. 8E), suggesting the involvement of caspase-9 in cell death. Together, these results suggested that loss of $\Delta\psi_m$, mitochondrial release of cytochrome *c*, and subsequent activation of caspase-9 played key roles in cell death.

Mitochondrial Release of Smac Into the Cytosol for Suppression of BIRC

In response to apoptotic stimuli, Smac is released from mitochondria into the cytosol to block the inhibitory function of inhibitor-of-apoptosis proteins (IAPs) and thereby promote caspase-9 activation.¹⁷ Because all IAPs bear 1 or more characteristic zinc-finger motif termed baculovirus IAP repeat (BIR), the whole family of these proteins is now more precisely known as BIR-containing (or BIRC) proteins rather than IAPs. We examined the levels of Smac and BIRC proteins in apoptosis of T98G and U87MG cells after treatments with DAS and DADS (Fig. 9). Both DAS and DADS induced mitochondrial release of Smac into the cytosol (Fig. 9A) and thereby significantly decreased the level of mitochondrial Smac (Fig. 9B). The protein levels of BIRC-2, -3, -4, and -5 were examined by Western blot analysis (Fig. 9C). The mitochondrial release of Smac into the cytosol correlated well with the decrease in BIRC-2, -3, -4, and -5 levels. These results indicated that DAS and DADS induced apoptosis in T98G and U87MG cells with mitochondrial release of Smac into the cytosol and suppression of BIRC proteins.

Association of Endoplasmic Reticulum Stress With Activation of Caspase-4, Calpain, and Caspase-3

We monitored endoplasmic reticulum (ER) stress in the overexpression of calreticulin (an ER luminal protein)¹⁸ and activation of caspase-4 (localized in ER and cleaved to the active form in response to ER stress)¹⁹ and calpain in glioblastoma cells after exposure to garlic compounds (Fig. 10). An increase in expression in calreticulin was associated with activation of caspase-4 in glioblastoma cells (Fig. 10A). Degradation of 270-kD α -spectrin to 145-kD spectrin breakdown product (SBDP) and 120-kD SBDP has been attributed to proteolytic activities of calpain and caspase-3, respectively.¹² Treatment of cells with DAS and DADS caused increases in 145-kD SBDP and 120-kD SBDP, indicating increases in activities of calpain and caspase-3, respectively (Fig. 10A). In-

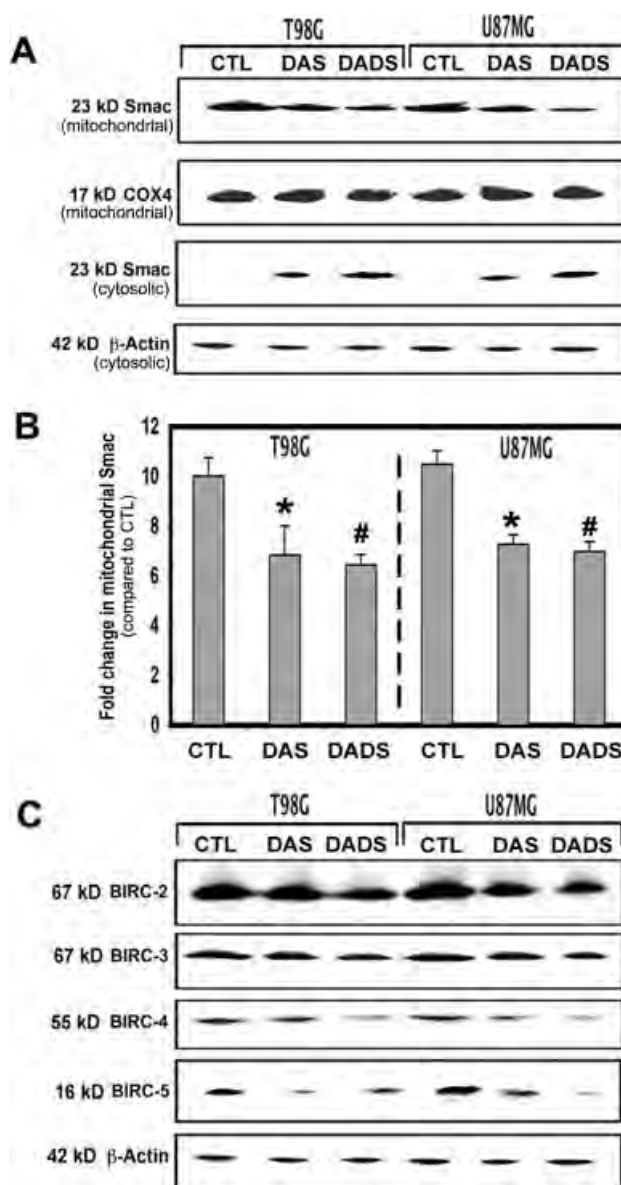


FIGURE 9. Mitochondrial release of Smac into the cytosol for suppression of BIRC proteins in T98G and U87MG cells. Treatments (24 hours): control (CTL), 100 μ M diallyl sulfide (DAS), and 100 μ M diallyl disulfide (DADS). (A) Western blot analysis showing protein levels of Smac, COX4, and β -actin. (B) Densitometric analysis showing fold change in Smac in mitochondrial fractions. (C) Western blot analysis showing protein levels of BIRC-2, -3, -4, and -5, and β -actin. *,# indicate a significant difference from the CTL value ($p < .05$).

creased calpain activity was also associated with Bid cleavage to tBid (Fig. 10A). Caspase-3 activation was further confirmed in the generation of active 20-kD caspase-3 fragment (Fig. 10A). Garlic compounds significantly increased caspase-3 activity, as determined by a colorimetric assay (Fig. 10B). Pretreatment of the cells with calpeptin (Fig. 10C) and caspase-3

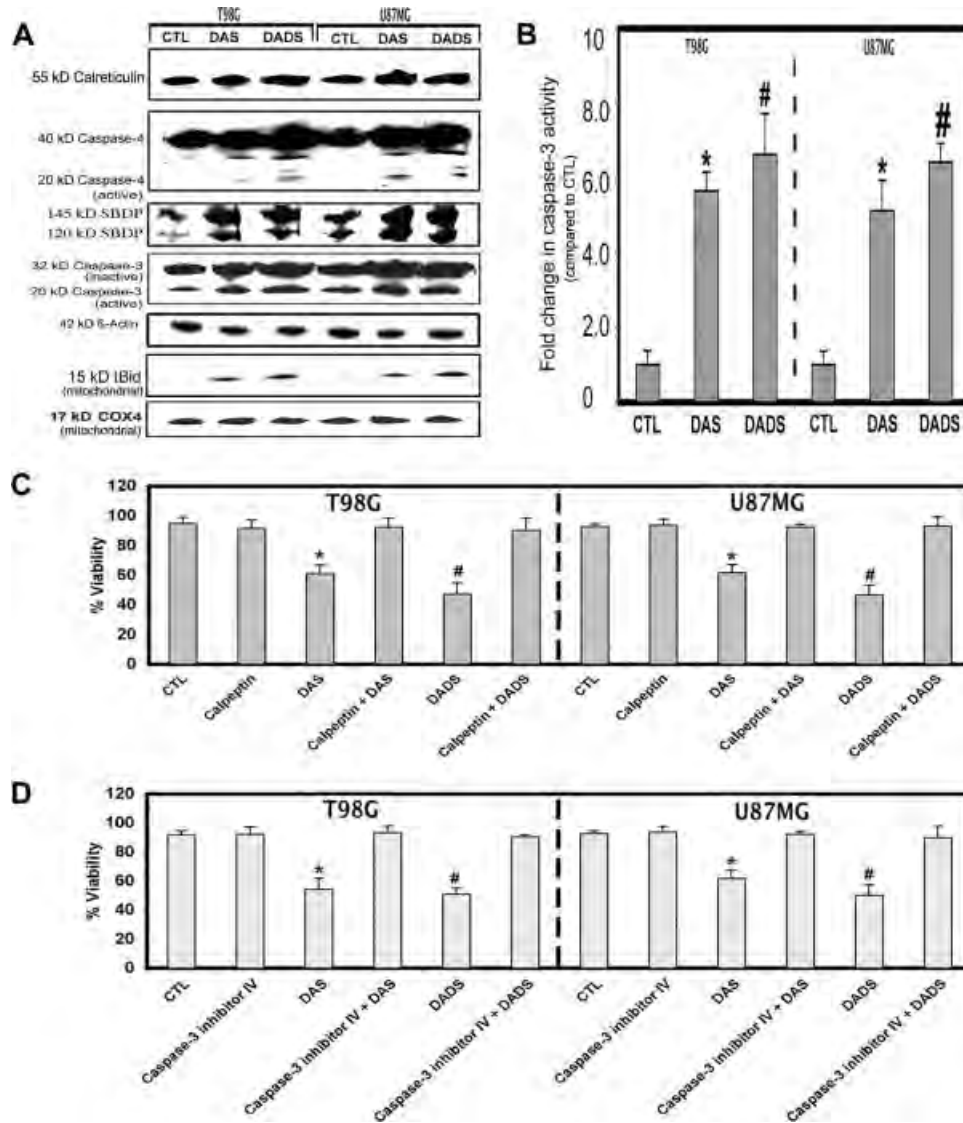


FIGURE 10. Roles of calreticulum, caspase-4, calpain, and caspase-3, and Bid cleavage in cell death in T98G and U87MG cells. Treatments (24 hours): control (CTL), 100 μ M diallyl sulfide (DAS), and 100 μ M diallyl disulfide (DADS). (A) Western blot analysis showing levels of calreticulum, caspase-4, spectrin breakdown product (SBDPs), active caspase-3, tBid, and β -actin. (B) Determination of caspase-3 activity by a colorimetric assay. (C) Determination of percent cell viability after the treatments (24 hours): control (CTL), 10 μ M calpeptin, 100 μ M DAS, 10 μ M calpeptin (1-hour pretreatment) + 100 μ M DAS, 100 μ M DADS, 10 μ M calpeptin (1-hour pretreatment) + 100 μ M DADS. (D) Determination of percent cell viability after the treatments (24 hours): control (CTL), 10 μ M caspase-3 inhibitor IV, 100 μ M DAS, 10 μ M caspase-3 inhibitor IV (1-hour pretreatment) + 100 μ M DAS, 100 μ M DADS, 10 μ M caspase-3 inhibitor IV (1-hour pretreatment) + 100 μ M DADS. *,# indicate a significant difference from the CTL value ($p < .05$).

inhibitor IV (Fig. 10D) inhibited cell death, indicating requirement of activities of calpain and caspase-3, respectively, for garlic compound-mediated cell death in T98G and U87MG cells.

DAS and DADS Decreased Calpastatin Expression at mRNA and Protein Levels

The proteolytic activity of calpain is controlled by calpastatin (the endogenous calpain inhibitor) that,

however, may be degraded by calpain and caspases during apoptosis.²⁰ We examined the levels of calpastatin in both T98G and U87MG cells after treatments with DAS and DADS (Fig. 11). Our RT-PCR results demonstrated a substantial reduction in calpastatin at the mRNA level (Fig. 11A). In addition, Western blot analysis demonstrated a similar decrease in calpastatin at the protein level (Fig. 11B). A decreased level of calpastatin might not be sufficient to effi-

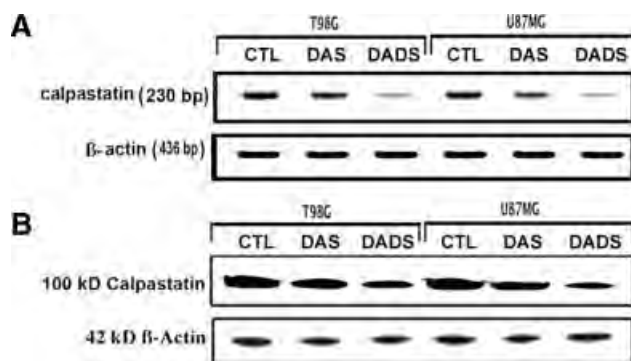


FIGURE 11. Determination of expression of calpastatin at mRNA and protein levels in T98G and U87MG cells. Treatments (24 hours): control (CTL), 100 μ M diallyl sulfide (DAS), and 100 μ M diallyl disulfide (DADS). (A) Agarose gels showing mRNA levels of calpastatin and β -actin. bp indicates basepairs. (B) Western blot analysis showing protein levels of calpastatin and β -actin.

ciently inhibit the increased calpain activity in T98G and U87MG cells after treatment with DAS and DADS.

DAS and DADS Induced CAD Activation

In apoptosis, activation of caspase-3 cleaves the inhibitor of caspase-3-activated DNase (ICAD) to release CAD.¹² Free CAD is active and can enter the nucleus to degrade chromosomal DNA. We performed Western blot analysis for estimation of CAD in nuclear fraction (Fig. 12). One set of gel was stained with Coomassie Blue to ensure equal amounts of nuclear protein loading in all lanes. The levels of nuclear CAD were increased in T98G and U87MG cells after treatment with DAS and DADS.

Low Dose of DATS Induced Cell Death in Glioblastoma Cells

In addition to DAS and DADS, we examined the efficacy of DATS (another garlic compound) for induction of apoptosis in glioblastoma cells (Fig. 13). We found that DATS induced cell death in a dose-dependent manner in T98G (Fig. 13A) and U87MG (Fig. 13B) cells. Notably, DATS was more potent than DAS and DADS for induction of cell death. The mechanism of DATS-mediated cell death in T98G (Fig. 13C) and U87MG (Fig. 13D) cells was associated with ROS production, which was inhibited by pretreatment of cells with Asc. We also observed that treatment of glioblastoma cells with a low dose of DATS caused loss of $\Delta\psi_m$ (Fig. 13E) and increased activities of caspase-9 (Fig. 13F) and caspase-3 (Fig. 13G), indicating involvement of mitochondria in mediation of cell death. Because up regulation of GST activity

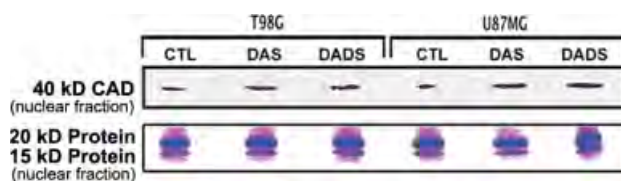


FIGURE 12. Levels of CAD in nuclear fractions of T98G and U87MG cells. Treatments (24 hours): control (CTL), 100 μ M diallyl sulfide (DAS) and 100 μ M diallyl disulfide (DADS). (A) Western blot analysis showing levels of CAD in nuclear fractions. (B) A representative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was stained with methylene blue to monitor equal amounts of loading of nuclear protein in each lane.

in the cells could be a potential mechanism of resistance to alkylating agents such as garlic compounds, we measured the levels of GST activity in both T98G and U87MG cells after exposure to DAS, DADS, and DATS (Fig. 13H). Our data showed nonsignificant increases in GST activity in glioblastoma cells exposed to all 3 garlic compounds (Fig. 13H), suggesting that any nonsignificant increase in GST activity would not be sufficient to protect the cells from significantly increased ROS-mediated cell death.

DISCUSSION

This study demonstrates that the garlic compounds (DAS, DADS, and DATS) are effective for the induction of apoptosis in human glioblastoma T98G and U87MG cells (Figs. 1–13). Based on the results, we propose a schematic diagram to show different components and pathways leading to apoptosis in glioblastoma cells after treatment with garlic compounds (Fig. 14).

Production of ROS may play a role in causing apoptosis via death receptor and mitochondria.^{5,21} Phosphorylation of p38 MAPK induces apoptosis, whereas phosphorylation of p42/44 MAPK exerts cytoprotective effects.^{22,23} We detected phosphorylation of p38 MAPK but not p42/44 MAPK in glioblastoma cells after exposure to DAS and DADS. A decrease in phosphorylation of p38 MAPK by a specific inhibitor (SB203580) markedly decreased induction of apoptosis (data not shown). The addition of Asc completely blocked the phosphorylation of p38 MAPK, suggesting the involvement of ROS in phosphorylation of p38 MAPK. Thus, production of ROS provided a signal for selective phosphorylation of p38 MAPK and induction of apoptosis in glioblastoma cells after treatment with garlic compounds. Pretreatment of cells with the JNK inhibitor I (a synthetic peptide) demonstrated a reduction in cell death, suggesting an essential role of JNK1 in induc-

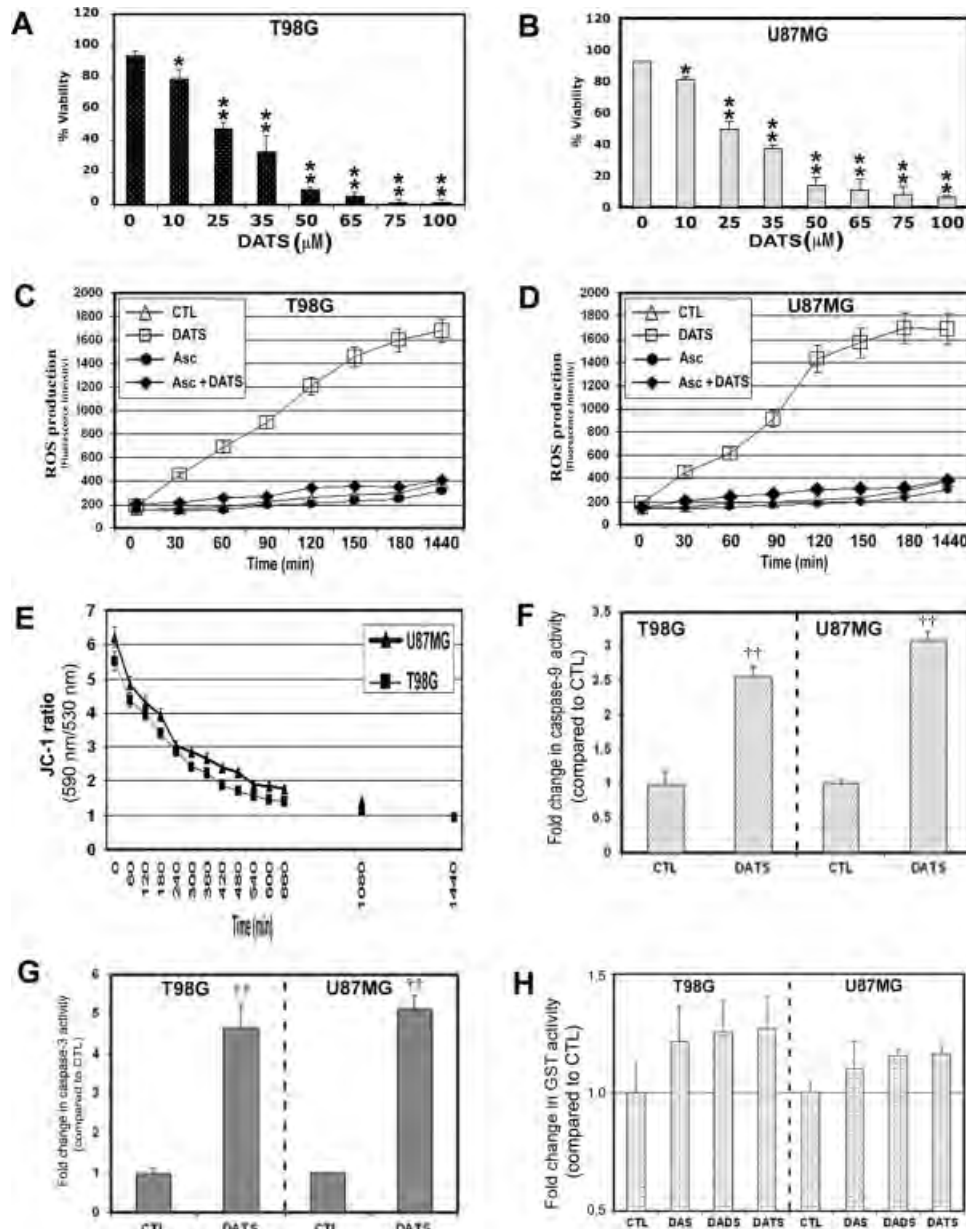


FIGURE 13. The garlic compound diallyl trisulfide (DATS) induced cell death via reactive oxygen species (ROS) production and a mitochondria-mediated pathway. Determination of residual cell viability in (A) T98G cells and (B) U87MG cells after treatment (24 hours) with different doses of DATS. Determination of ROS production in (C) T98G cells and (D) U87MG cells after the treatments (0, 30, 60, 90, 120, 150, 180, and 1440 minutes) in the presence of 5 μ M 7 2',7'-dichlorofluorescein diacetate (DCF-DA): control (CTL), 25 μ M DATS, 10 μ M ascorbic acid (Asc), and 10 μ M Asc + 100 μ M 7 DATS. (E) Changes in the JC-1 ratio (590 nanometers [nm]/530 nm) in T98G and U87MG cells after treatment with 25 μ M DATS for different times. Colorimetric assays for determination of fold change in (F) caspase-9 activity and (G) caspase-3 activity. (H) Determination of total glutathione S-transferase (GST) activity in T98G and U87MG cells after the treatments (24 hours): control (CTL), 100 μ M diallyl sulfide (DAS), 100 μ M diallyl disulfide (DADS), and 25 μ M DATS.

tion of apoptosis in glioblastoma cells treated with garlic compounds. The JNK inhibitor I inhibits apoptosis due to specific inhibition of phosphorylation of JNK1, indicating involvement of the JNK1 pathway in apoptosis.^{22,23} In addition, an increase in intracellular free $[Ca^{2+}]$ is known to cause apoptosis in

several cell culture models.¹² Garlic compounds increased intracellular free $[Ca^{2+}]$ in glioblastoma cells, suggesting that induction of apoptosis also required activation of Ca^{2+} -dependent pathways.

Proapoptotic and antiapoptotic members of the Bcl-2 family regulate the release of cytochrome *c*

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