RESEARCH ARTICLE

6-Dehydrogingerdione, an active constituent of dietary ginger, induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human breast cancer cells

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This study is the first to investigate the anticancer effect of 6-dehydrogingerdione (DGE), an active constituent of dietary ginger, in human breast cancer MDA-MB-231 and MCF-7 cells. DGE exhibited effective cell growth inhibition by inducing cancer cells to undergo G2/M phase arrest and apoptosis. Blockade of cell cycle was associated with increased levels of p21, and reduced amounts of cyclin B1, cyclin A, Cdc2 and Cdc25C. DGE also enhanced the levels of inactivated phosphorylated Cdc2 and Cdc25C. DGE triggered the mitochondrial apoptotic pathway indicated by a change in Bax/Bcl-2 ratios, resulting in caspase-9 activation. We also found the generation of reactive oxygen species is a critical mediator in DGE-induced cell growth inhibition. DGE clearly increased the activation of apoptosis signal-regulating kinase 1 and c-Jun N-terminal kinase (JNK), but not extracellular signal-regulated kinase 1/2 (ERK1/2) and p38. In addition, antioxidants vitamin C and catalase significantly decreased DGE-mediated JNK activation and apoptosis. Moreover, blocking JNK by specific inhibitors suppressed DGE-triggered mitochondrial apoptotic pathway. Taken together, these findings suggest that a critical role for reactive oxygen species and JNK in DGE-mediated apoptosis of human breast cancer.

Received: March 20, 2009
Revised: August 17, 2009
Accepted: September 1, 2009

Keywords:
6-Dehydrogingerdione / Apoptosis / Cell cycle / c-Jun N-terminal kinase / Reactive oxygen species

1 Introduction

Breast cancer is the most common form of cancer in women, comprising 23% of all cancers, with more than one million new cases *per* year [1]. This pathology is currently controlled by surgery and radiotherapy, and is frequently supported by adjuvant chemo- or hormonotherapies [2]. However, breast cancer is highly resistant to radiation and conventional chemotherapeutic agents, and this resistance is associated with a poor prognosis for this metastasis disease, especially in cases of hormone-independent cancer [2, 3]. About 30–40% of women with this form of cancer will develop metastases and

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Abbreviations: CMFDA, 5-chloromethylfluorescein diacetate; **DGE,** 6-dehydrogingerdione; **FBS,** fetal bovine serum; **GSH,** glutathione; **H2DCFDA,** 2′7′-dichlorodihydrofluorescein diacetate; **JNK,** c-Jun N-terminal kinase; **MAPK,** mitogen-activated protein kinase; **ROS,** reactive oxygen species; **TUNEL,** terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling; **XTT,** sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro)benzene-sulfonic acid hydrate

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eventually die from their disease [1]. Effective chemopreventive treatments for breast cancer are still needed to increase available regimens and advance the efficacy of chemotherapy.

All aerobic organisms are subjected to physiological oxidant stress as a consequence of aerobic metabolism. Superoxide anion (● O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (● OH) and hypochlorous acid are included among the reactive oxygen species (ROS) that are natural by-products of normal cell metabolism [4, 5]. The redox status of all aerobic cells is balanced by enzyme and nonenzyme systems [4–7]. Oxidative stress occurs when the imbalance between cellular oxidant species production and antioxidant capability [6, 7]. Evidence is accumulating which indicates that many chemotherapeutic agents may be selectively toxic to tumor cells because they increase oxidant stress and enhance these already stressed cells beyond their limit [8, 9]. Cytotoxic ROS signaling appears to be triggered by the activation of the mitochondrial-dependent cell death pathway through activation of the mitogen-activated protein kinase (MAPK) pathways and the proapoptotic Bcl-2 proteins Bax or Bak, with subsequent mitochondrial membrane permeabilization and cell death [4, 10, 11].

Ginger (*Zingiber officinale*) is a food plant known worldwide and is equally reputed for its medicinal properties [12]. It is an herbaceous, rhizomatous perennial plant widely distributed throughout the tropica land and the subtropical regions [13]. It is cultivated on a large scale in India, Bangladesh, Taiwan, Nigeria, Sri Lanka and East Asiatic countries. Rhizomes are aromatic, thick lobed and pale yellowish [12, 14]. There are several reports of the insecticidal activity of *Z. officinale* extracts [12, 15]. The pungent vanilloids of ginger, 6-gingerol and 6-paradol, exhibit antiproliferation activity in liver, pancreatic, prostate, gastric and leukemia cancer cells [16–18]. 6-Dehydrogingerdione (DGE; Fig. 1A), one of the major components of dietary ginger, is a potent antioxidant with 5-hydroxytryptamine receptor type 3 inhibitor [19]. This study is the first to determine the cell growth inhibition activity of DGE and examine its effect on cell cycle distribution and apoptosis in human breast cancer. In this study, we employed the human breast cancer MDA-MB-231 (ER-negative, p53 mutation, model of more advanced stage breast cancer) and MCF-7 (ER-positive, p53 wild type, model of early-stage breast cancer) cell lines to assess the molecular mechanisms responsible for the antiproliferative effect of DGE. We found that DGE caused cell cycle arrest at G2/M phase and induced an apoptotic response.

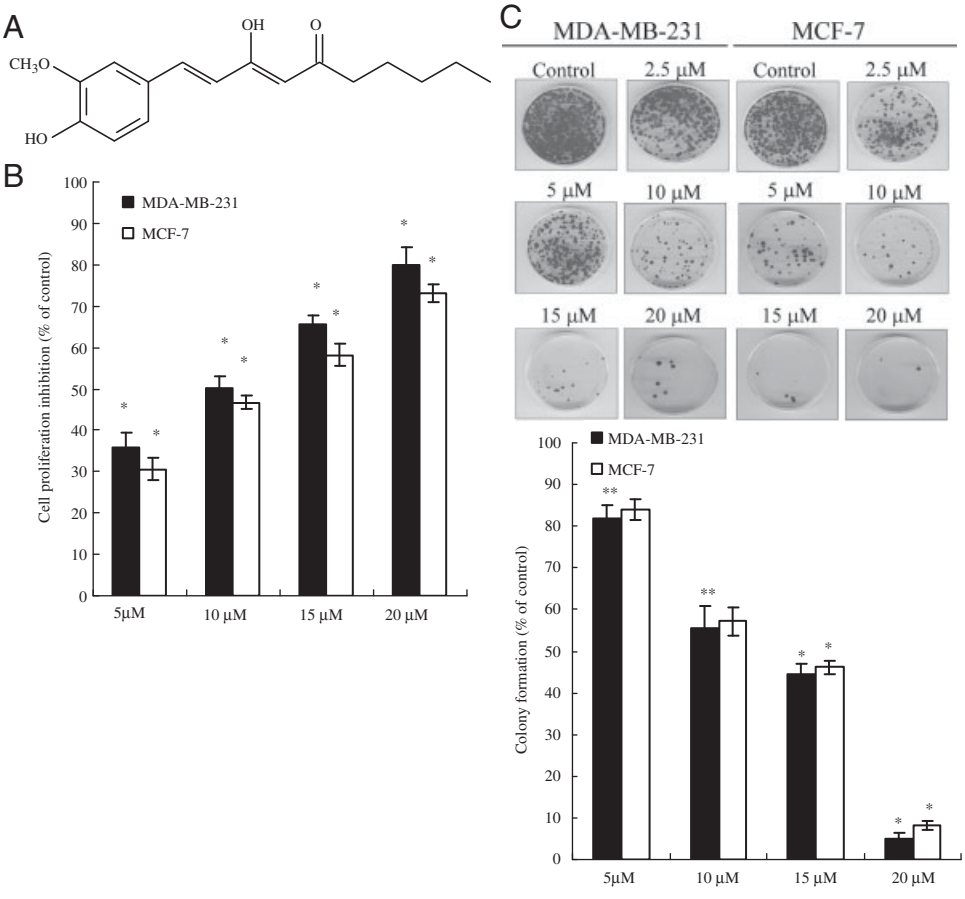

Figure 1. The effects of DGE on cell proliferative inhibition and colony formation in human breast adenocarcinoma MDA-MB-231 and MCF-7 cells. (A) Chemical structure of DGE isolated from the rhizomes of *Z. officinale.* (B) Cell proliferative inhibition effect of DGE in MDA-MB-231 and MCF-7 cells. (C) Influence of MDA-MB-231 and MCF-7 on the number of colony-forming cells, as evaluated by clonogenic assay. Cell growth inhibition activity of DGE was assessed by XTT. Cells were treated with various concentrations of DGE for 1 h; the clonogenic assay was performed as described in Section 2. Results are expressed as the percentage of cell proliferation relative to the proliferation of control. The data shown are the mean from three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and DGE-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

2 Materials and methods

2.1 Chemicals and reagents

Fetal bovine serum (FBS), penicillin G, streptomycin, amphotericin B, and DMEM were obtained from GIBCO BRL (Gaithersburg, MD, USA). DMSO, ribonuclease, and propidium iodide were purchased from Sigma Chemical (St. Louis, MO, USA). Sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). The antibodies to β-actin, cyclin B1, cyclin A, Cdc2, Cdc25C, phospho-Cdc2, phospho-Cdc25C, p21, Bax, Bak, Bcl-2, and Bcl-XL, ASK1, phospho-ASK1, c-Jun N-terminal kinase (JNK), phospho-JNK, p38, phospho-p38, ERK1/2, phospho-ERK1/2 were obtained from Cell Signaling Technology (Beverly, MA, USA).

2.2 Test compound

The rhizomes (25.6 kg) of *Z. officinale* were chipped and air-dried and extracted repeatedly with $CHCl_3$ (50 L × 4) at room temperature. The combined $CHCl_3$ extracts (896.5 g) were then evaporated and further separated into 20 fractions by column chromatography on silica gel (3.8 kg, 70−230 mesh) with gradients of *n*-hexane/$CHCl_3$. Fr. 8 (81.2 g), eluted with $CHCl_3$–MeOH (60:1). The fraction was next subjected to silica gel CC ($CHCl_3$–MeOH mixtures) and yielded DGE (163 mg). The purity of DGE was > 95% as determined by HPLC. The chemical structure of DGE was confirmed by NMR [20, 21].

2.3 Cell culture

MCF-7 cells and normal mammary epithelial cell H184B5F5/M10 cells were cultured in DMEM with non-essential amino acids, 0.1 mM sodium pyruvate, 10 μg/mL insulin and 10% FBS. MDA-MB-231 cells were maintained in DMEM supplemented with 10% FBS, 10 U/mL of penicillin, 10 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin, and was cultured in monolayer culture at 37°C and 5% CO_2.

2.4 Cell proliferation and clonogenic assay

Inhibition of cell proliferation by DGE was measured by XTT assay. Briefly, cells were plated in 96-well culture plates (1×10^4 cells/well). After 24 h incubation, the cells were treated with vehicle alone (0.1% DMSO) and DGE (5, 10, 15 and 20 μM) for 48 h. An amount of 150 μL XTT test solution, which was prepared by mixing 5 mL of XTT-labeling reagent with 100 μL of electron coupling reagent, was then added to each well. After 4 h of incubation, the absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

To determine the long-term effects, cells were treated with vehicle alone (0.1% DMSO) and DGE (2.5, 5, 10, 15 and 20 μM) for 1 h. After being rinsed with fresh medium, cells were allowed to grow for 14 days to form colonies that were then stained with crystal violet (0.4 g/L; Sigma).

2.5 Cell cycle analysis

To determine cell cycle distribution analysis, 5×10^5 cells were plated in 60 mm dishes and treated with vehicle alone (0.1% DMSO) and DGE (10 and 20 μM) for 12 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in PBS, resuspended in 1 mL of PBS containing 1 mg/mL ribonuclease and 50 μg/mL propidium iodide, incubated in the dark for 30 min at room temperature and analyzed by EPICS flow cytometer. The data were analyzed using Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

2.6 Apoptosis assay

Quantitative assessment of apoptotic cells was assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling (TUNEL) method, which examines DNA-strand breaks during apoptosis by using BD ApoAlertTM DNA Fragmentation Assay Kit. Briefly, cells were incubated with vehicle alone (0.1% DMSO) and DGE (10 and 20 μM) for the indicated times. The cells were trypsinized, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed, the cells were incubated with the reaction mixture for 60 min at 37°C. The stained cells were then analyzed with an EPICS flow cytometer and a fluorescence microscope at 20 × magnification.

2.7 Assay for caspase activity

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate LEHD-pNA (for caspase-9) and Ac-DEVD-pNA (for caspase-3 and caspase-7). The cell lysates was prepared and incubated with specific anti-caspase-7 and caspase-3 antibodies. Immunocomplexes were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, pH 7.4) for 2 h at 37°C. The release of *p*-nitroaniline was monitored at 405 nm. Results are represented as the percent change of the activity compared with the untreated control.

2.8 Measurements of ROS and glutathione

Levels of intracellular O_2^- and H_2O_2 were assessed spectrofluorimetrically by oxidation of specific probes: dihydroethidium (DHE, Molecular Probes, Leiden, The Netherlands) and 2′7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes). The amount of glutathione (GSH) was determined by 5-chloromethylfluorescein diacetate (CMFDA). Cells were plated at a density of 5×10^5 or 1×10^6, respectively, in 60-mm dishes, allowed to attach overnight, and exposed to catalase (1000 unit/mL) alone, vitamin C (100 μM) alone, DGE (20 μM) alone, catalase plus DGE or vitamin C plus DGE for specified time intervals. The cells were stained with 10 μM H2DCFDA, 20 μM DHE and 25 μM CMFDA for 10 min at 37°C and the fluorescence intensity in cells was determined using the flow cytometer (Becton Dickinson, Franklin Lakes, NY, USA).

2.9 Immunoblot assay and JNK kinase activity assay

Cells (8×10^6/dish) were seeded in a 10-cm dish. After 24 h of incubation, the cells were treated with 20 μM DGE for the indicated times. Total cell extracts were prepared in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 5 μg/mL Aprotinin and 5 μg/mL Leupetin). Equivalent amounts of protein (50 μg total protein) were resolved by SDS-PAGE (6–12%) and transferred to PVDF membranes. After the membrane was blocked in Tris-buffer saline containing 0.05% Tween-20 and 5% nonfat powdered milk, the membranes were incubated with primary antibodies specific to cyclin A, cyclin B1, Cdc2, Cdc25C, phospho-Cdc2, phospho-Cdc25C, p21, Bax, Bak, Bcl-2, Bcl-XL, JNK, ERK1/2, p38, ASK1, phospho-JNK, phospho-ERK1/2, phospho-p38 and phospho-ASK1 at 4°C for 1–6 h. After washing three times with Tris-buffer saline containing 0.05% Tween-20 for 10 min each, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection was performed using the enhanced chemiluminescence blotting detection system (Amersham, USA). The JNK activity was determined using kits from Cell Signaling Technology according to the manufacturer's instructions.

2.10 Statistical analysis

Data were expressed as means ± SD. Statistical comparisons of the results were made using analysis of variance. Significant differences ($p < 0.05$) between the means of control and DGE-treated cells were analyzed by Dunnett's test or Student's *t*-test.

3 Results

3.1 DGE inhibits cell proliferation and clonogenic survival in human breast adenocarcinoma MDA-MB-231 and MCF-7 cells

To investigate the potential cell proliferative inhibition activity of DGE in breast adenocarcinoma cells, we first examined the effect of DGE on cell proliferation and clonogenic survival in MDA-MB-231 and MCF-7 cells. As shown in Fig. 1B, exposure of MDA-MB-231 and MCF-7 to DGE for 48 h inhibited the growth of MDA-MB-231 and MCF-7 in a dose-dependent manner. The IC_{50} values of DGE were 9.8 μM for MDA-MB-231 and 11.7 μM for MCF-7. In contrast, the proliferation inhibitory effect of DGE on H184B5F5/M10 normal mammary epithelial cells was not significant at the same concentrations as on tumor cells (data not shown).

The anticancer activities of DGE inhibition were assessed by clonogenic assays, which correlate very well with *in vivo* assays of tumorigenicity in nude mice [22]. MDA-MB-231 and MCF-7 cells showed the ability to form clones in the untreated control wells. However, with the addition of DGE, a dose-dependent inhibition in clonogenicity was observed (Fig. 1C).

3.2 DGE inhibits the progression of cell cycle at G2/M phase by regulating cell cycle-related molecules

To investigate the mechanisms leading to loss of cell proliferation by DGE, we tested whether the observed inhibition effects of DGE on cell proliferation are due to induction of cell cycle arrest. As shown in Fig. 2A, treatment of MDA-MB-231 and MCF-7 cells with 10 and 20 μM. DGE increased the percentage of cells in G2/M phase after 12 h exposure (Fig. 2A).

We next examined the effect of DGE on cell cycle-regulatory molecules, including p21/WAF1, cyclin A, cyclin B1, Cdc2 and Cdc25C (Fig. 2B). The treatment of MDA-MB-231 and MCF-7 cells with DGE resulted in downregulation in the levels of cyclin A protein as well as the levels of cyclin B1. Downregulation of Cdc2 and Cdc25C was also observed in MDA-MB-231 and MCF-7 cells upon treatment with DGE. The level of phosphorylated cdc2 (Tyr15) and cdc25C (Ser216) increased markedly on treatment of MDA-MB-231 and MCF-7 cells with DGE. In addition, the expression of p21/WAF1 also increased in DGE-treated cells in comparison with the controls.

3.3 DGE induces apoptotic cell death by triggering mitochondrial apoptosis pathway in MDA-MB-231 and MCF-7 cells

A quantitative evaluation was also made using TUNEL to detect DNA-strand breaks. Compared with vehicle-treated

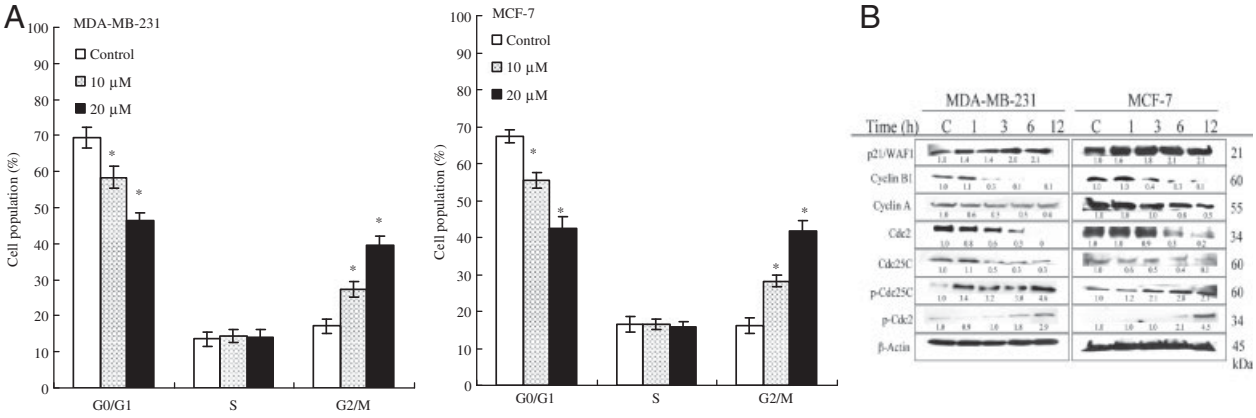

Figure 2. The effect of DGE on the regulation of cell cycle arrest. (A) The distribution of cell cycles in DGE-treated cells. (B) The expressions of cell cycle-related factors in DGE-treated MDA-MB-231 and MCF-7 cells. Cells were treated with vehicle and 20 μM DGE for 12 h, and cell cycle distribution was assessed by flow cytometry. For (B), cell cycle-related protein levels of 20 μM DGE-treated MDA-MB-231 and MCF-7 cells were determined by immunoblot. The data shown are the mean from three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and DGE-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

cells, DGE induced 52.28 and 48.36% of apoptotic cells at concentration 20 μM after 48 h treatment in MDA-MB-231 and MCF-7 cells, respectively (Fig. 3A).

To determine whether DGE induces apoptosis by triggering the mitochondrial apoptotic pathway, we measured the change of the expression of the Bcl-2 family protein and the activation of the caspase. Immunoblot analysis showed that treatment of MDA-MB-231 and MCF-7 cells with DGE increased Bax and Bak protein levels after 3 h exposure (Fig. 3B). In contrast, DGE decreased Bcl-2 and Bcl-XL levels, which led to an increase in the proapoptotic/anti-apoptotic Bcl-2 ratio (Fig. 3B). Hallmarks of the apoptotic process include the activation of cysteine proteases, which represent both initiators and executors of cell death. Upstream caspase-9 activities increased significantly, as shown by the observation that treatment with DGE increased caspase-9 activity in MDA-MB-231 and MCF-7 cells at 6 h treatment at 20-μM concentration (Fig. 3C). In addition, DGE also increased the activation of caspase-7 and caspase-3 in MDA-MB-231 cells, and increased activation of caspase-7 in MCF-7 cells after 12 h treatment (Figs. 3D and E).

3.4 DGE increases the generation of ROS, and decreases the level of GSH in MDA-MB-231 and MCF-7 cells

As ROS generation plays an important role in the pro-apoptotic activities of various anticancer agents [8–11], we tested the possibility that DGE induce apoptosis allowing for ROS accumulation. DHE-based FACS detection revealed that intracellular O_2^- level was increased in MDA-MB-231 and MCF-7 cells following treatment with 20 μM DGE. ROS was detected as early as 1 h after treatment with 20 μM DGE, peaked at 2 h (Fig. 4A). Similar results were obtained from H2DCFDA-based FACS detection data revealed that intracellular H_2O_2 levels increased in examined cells after 20 μM DGE treatment (Fig. 4B).

Because GSH scavenges ROS in cells by interacting with $OH\cdot$ and H_2O_2 [4–7], thus affecting ROS-mediated signaling pathways, we next examined the levels of GSH in DGE-treated cells. The result showed that intracellular GSH levels decreased in MDA-MB-231 and MCF-7 cells following treatment with 20 μM DGE. The depletion of GSH was detected as early as 1 h after treatment with 20 μM DGE (Fig. 4C).

3.5 DGE increases the activation of ASK1 and JNK, but not p38 and ERK1/2 in MDA-MB-231 and MCF-7 cells

Because it has been shown that ROS-mediated DNA damage triggers activation of MAPK and subsequent cell death [7, 9], we assessed the status of MAPK after DGE treatment. First, ASK1 activation (phosphorylation at the activation loop Thr845) was assessed by immunoblot. The activity of ASK1 is regulated by phosphorylation at different sites. Phosphorylation at Ser967 is essential for ASK1 association with 14-3-3 protein, which attenuates ASK1 activity. Oxidative stress induces dephosphorylation of Ser967 and phosphorylation of Thr845 in the activation loop of ASK1, and both are correlated with ASK1 activity and ASK1-dependent apoptosis [23]. Treatment of MDA-MB-231 and MCF-7 cells with DGE increased ASK1 activation (Thr845 phosphorylation). The change of active ASK1 was evident after 0.5–6 h of treatment (Fig. 5A). In addition, the result also showed that exposure of MDA-MB-231 and MCF-7 cells to 20 μM DGE resulted in activation of JNK. Activation (phosphorylation) of JNK and was determined after 0.5–6 h of treatment and persisted for

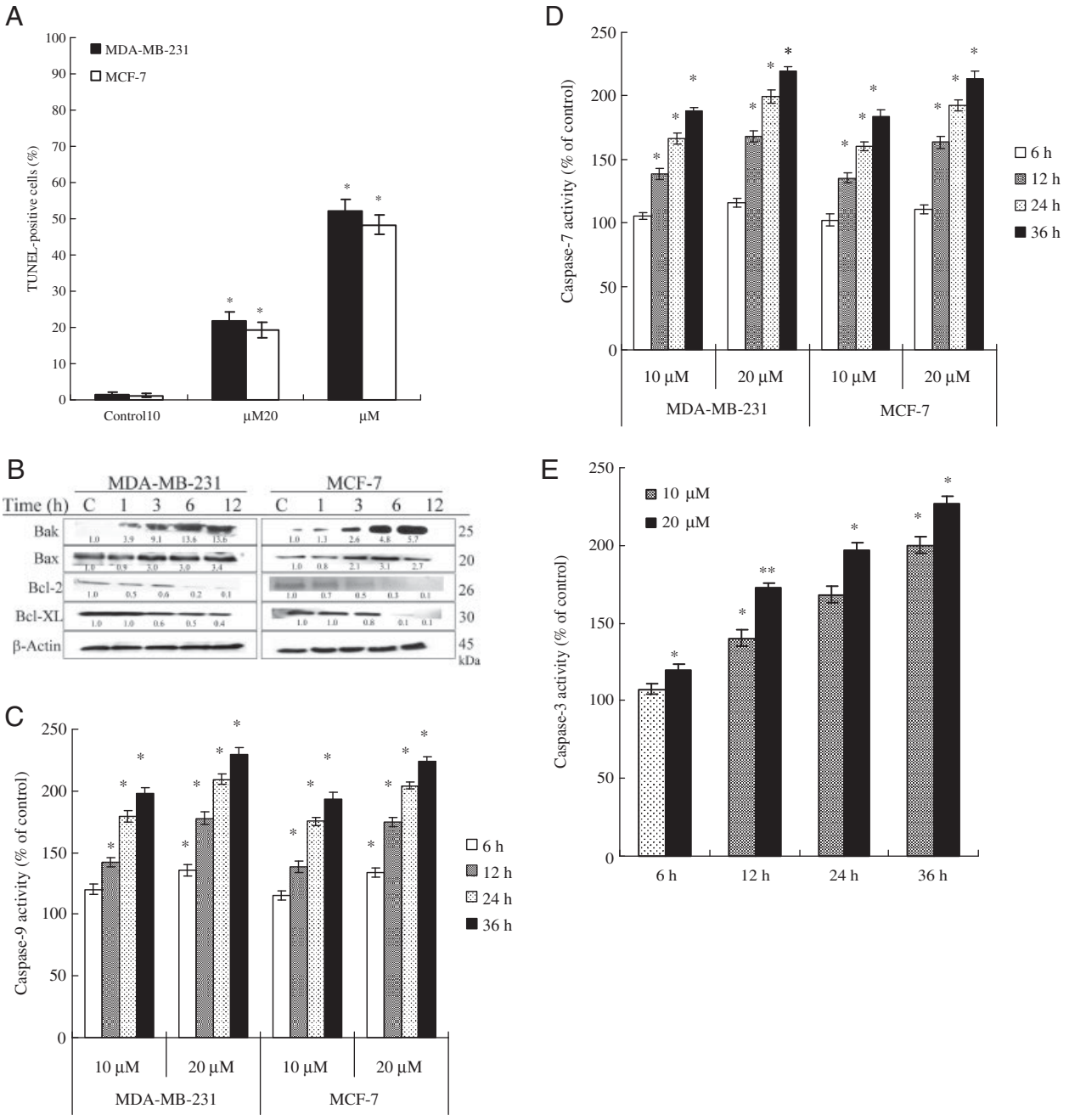

Figure 3. DGE induced apoptosis through the initiation of the mitochondrial pathway. Quantitative evaluations of TUNEL assay by flow cytometry (A). (B) The expression level of Bcl-2 family proteins. The activation of caspase-9 (C) and caspase-7 (D) in MDA-MB-231 and MCF-7 cells. The activation of caspase-3 (E) in MDA-MB-231 cells. For (A), cells were treated with various concentrations of DGE for 48 h. TUNEL-positive cells were examined by flow cytometry and were visible through fluorescence microscope. For (B), the Bcl-2 expression levels of 20 μM DGE-treated MDA-MB-231 and MCF-7 cells were determined by immunoblot. Caspase-9 was assessed by Caspase-9 Activity Assay Kit. Caspase-7 and caspase-3 was assessed by CaspSELECTTM Caspase-7 and -3 Immunoassay Kits. The data shown are the mean from three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and DGE-treated cells, as analyzed by Student's *t*-test ($p < 0.05$).

the duration of the experiment. On the other hand, the expression of JNK (unphosphorylated form) was not altered by DGE treatment. However, DGE failed to clearly affect the p38 and ERK1/2 activation in MDA-MB-231 and MCF-7 cells at any of the examined points in time (Fig. 5A). DGE-mediated activation of JNK was additionally confirmed by determining phosphorylation of one of its substrates (c-Jun). As shown in Fig. 5B, in comparison with the control, the Ser63

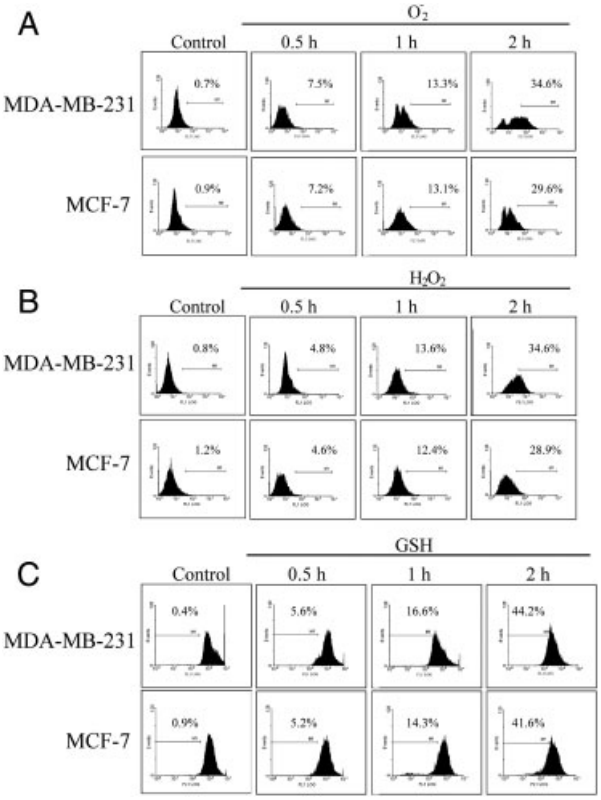

Figure 4. The effect of DGE on the production of ROS and the levels of GSH in MDA-MB-231 and MCF-7 cells. The effect of DGE on the production of O_2^- (A), the production of H_2O_2 (B) and the levels of cellular GSH (C). For (A–C), cells were treated with 20 μM DGE for indicated times. The amount of H_2O_2, O_2^- and GSH was assayed by H2DCFDA (for H_2O_2), DHE (for O_2^-) and CMFDA (for GSH) staining. For (C), data show the percentage of cells displaying intracellular GSH-negative cells. Results shown are representative of three independent experiments.

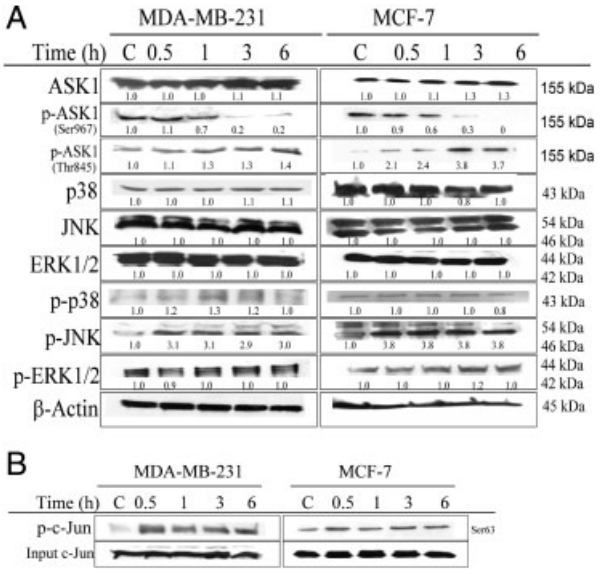

Figure 5. DGE increased the activation of ASK1 and JNK. (A) The effect of DGE on the activation of ASK1 and MAPK. (B) DGE increased the activity of JNK. Cells were treated with vehicle and 20 μM DGE for indicated times. Phosphorylated and unphosphorylated protein levels were assessed by immunoblot assay. The JNK activity was determined using JNK Activity Kit from Cell Signaling Technology according to the manufacturer's instructions. Results shown are representative of three independent experiments.

phosphorylation of c-Jun increased after a 0.5 h exposure of MDA-MB-231 and MCF-7 cells to 20 μM DGE (Fig. 5B).

3.6 The role of ROS on DGE-induced JNK activation and apoptosis

To investigate whether ROS generation is directly associated with DGE-induced JNK phosphorylation, caspase-9 activation and apoptosis, we assessed these events in MDA-MB-231 and MCF-7 cells pretreated with catalase (10 000 unit/mL) and vitamin C (100 μM) for 1 h followed by treatment with 20 μM DGE. As shown in Fig. 6A, pretreatment of MDA-MB-231 and MCF-7 cells with catalase and vitamin C could prevent the phosphorylation of JNK caused by DGE (Fig. 6A). We further assessed the effect of antioxidant agents in DGE-mediated caspase-9 activity and apoptosis. The results showed that the DGE-mediated caspase-9 activity and apoptosis in MDA-MB-231 and MCF-7 cells were significantly attenuated in catalase- or vitamin C-pretreated cells, compared with DGE-treated MDA-MB-231 and MCF-7 cells (Figs. 6B and C). These data confirm that DGE treatment of MDA-MB-231 and MCF-7 cells activates the mitochondrial apoptotic pathway *via* release of ROS, leading to the phosphorylation of JNK and activation of caspase-9.

3.7 The role of JNK on DGE-mediated apoptotic cell death

Next, we assessed the role of JNK on DGE-induced cell cycle arrest and apoptosis. As shown in Fig. 7A, the DGE-mediated JNK activation was effectively inhibited by 20 μM JNK inhibitor SP600125. Flow cytometric analysis showed that SP600125 had not any significant effect on the G2/M population, which was accumulated by the DGE treatment (Fig. 7B).

Figure 7A shows the effect of JNK inhibitor on Bax expression in MDA-MB-231 and MCF-7 cells. SP600125 decreased DGE-mediated Bax upregulation (Fig. 7A). Furthermore, the activation of caspase-9 induced by DGE was also attenuated by pretreatment with SP600125 (Fig. 7C). In addition, DGE-induced apoptosis were significantly prevented by SP600125 in MDA-MB-231 and MCF-7 cells. These results suggest that activation of JNK play an upstream role in DGE-mediated mitochondrial apoptotic pathways (Fig. 7D).

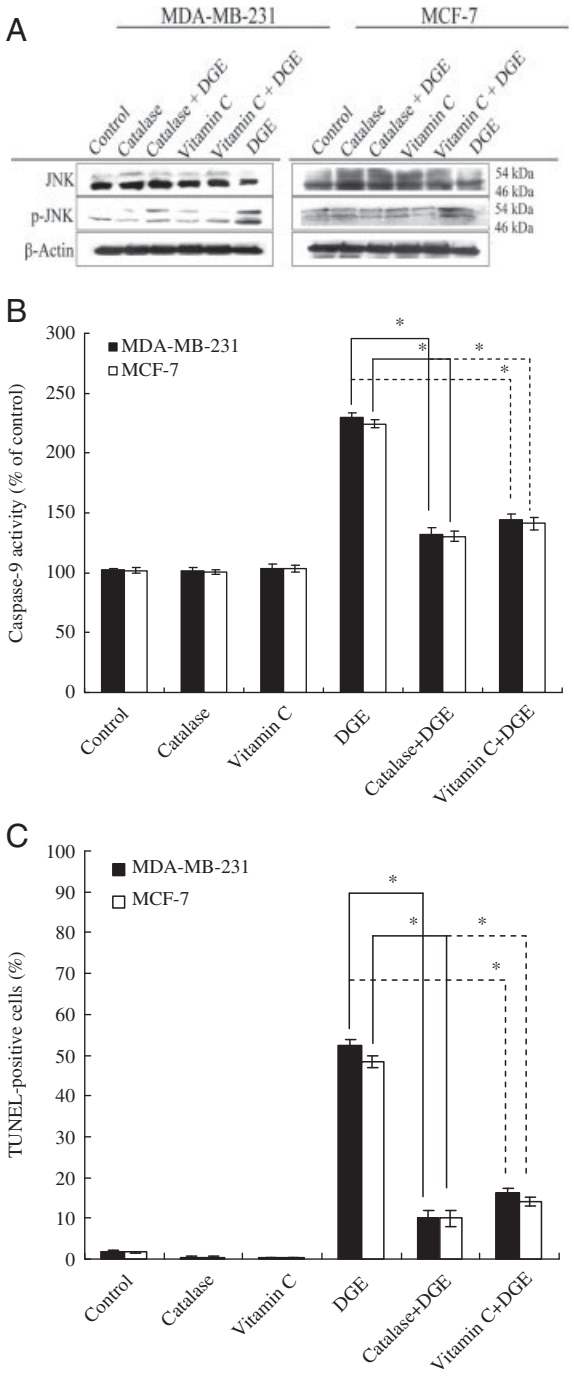

Figure 6. The role of ROS on DGE-mediated JNK activation and apoptosis. Antioxidant agents inhibited DGE-mediated JNK activation (A) and caspase-9 activity (B) and apoptosis (C). Cells were pre-treated with catalase (1000 units/mL) or vitamin C (100 μM) for 1 h; then 20 μM DGE was added and incubated for specific times (3 h for JNK activity, 36 h for caspase-9 and 48 h for apoptosis assay). The activation of JNK was measured by immunoblot assay. The induction of apoptosis was determined by TUNEL analysis. The data shown are the mean from three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between two test groups, as analyzed by Student's *t*-test ($p<0.05$).

4 Discussion

Breast cancer is the most common malignancy and the second leading cause of cancer mortality in women in both developed and developing countries [1]. This study is the first to show that DGE inhibits the growth of human breast adenocarcinoma MDA-MB-231 and MCF-7 cells, and did not exhibit any significant toxicity on normal breast cells. Both breast cancer cell lines treated with DGE accumulated in the G2/M phase of the cell cycle, and underwent apoptosis in a dose-and time-dependent manner.

Our results suggest that DGE blocked proliferation of tumor cells by arresting the cells in the G2/M phase of the cell cycle. It arrested the cell cycle progression by means of the downregulation of cyclin B1, cyclin A, and Cdc2, which are required for G2/M transition [24, 25]. In addition, DGE also increased the phosphorylation of Cdc25C, leading to increased accumulation of inactivated phospho-cdc2 (Tyr15). Moreover, the inhibition of cyclin-cdk complex might be amplified by simultaneous upregulation of the p21/WAF1 protein. Thus, we suggest that DGE arrests MDA-MB-231 and MCF-7 cells in the G2/M phase through CDK inhibitor upregulation and CDK-inhibition pathway.

Mitochondria are thought to be another pathway for apoptosis, and mitochondrial function is regulated through Bcl-2 family proteins comprising both anti-apoptotic (Bcl-2, Bcl-XL) and proapoptotic members (Bax, Bak) [26, 27]. DGE treatment results in a significant increase of Bax and Bak expression, and a decrease of Bcl-2 and Bcl-XL, suggesting that changes in the ratio of proapoptotic and antiapoptotic Bcl-2 family proteins might contribute to the apoptosis-promotion activity of DGE. In addition, our findings also showed the activation of caspase-9, caspase-7 and caspase-3 after MDA-MB-231 and MCF-7 cells were treated with DGE. Because the ratio of pro/anti-apoptotic Bcl-2 family protein was found to be significantly changed by treating breast cancer cell lines with DGE, and the change of Bax and Bak (at 3 h) occurred earlier than the activation of caspases (6 h for caspase-9 and 12 h for caspase-3 and caspase-7), this suggests that DEG increases caspase activities through the regulation of Bcl-2 family protein. These occurrences of mitochondrial apoptotic events play an important role in DGE-mediated apoptosis.

Cancer is usually survival in a hypoxic environment and it requires a coordinated adaptive response. The activation of HIF1-α was a critical regulator of hypoxic response and mechanism of tumor to support tumor cell survival under hypoxic conditions. Various oxygen species generated at complex III of the electron transport chain and xanthine/xanthine oxidase system can promote HIF1-α stabilization [28, 29]. Decreased ROS accumulation leading to tumor growth inhibition is one of the therapeutic strategies [28]. In contrast, enhancement of ROS production has long been associated with the apoptotic response induced by several anticancer agents [30, 31]. The status of intracellular redox is

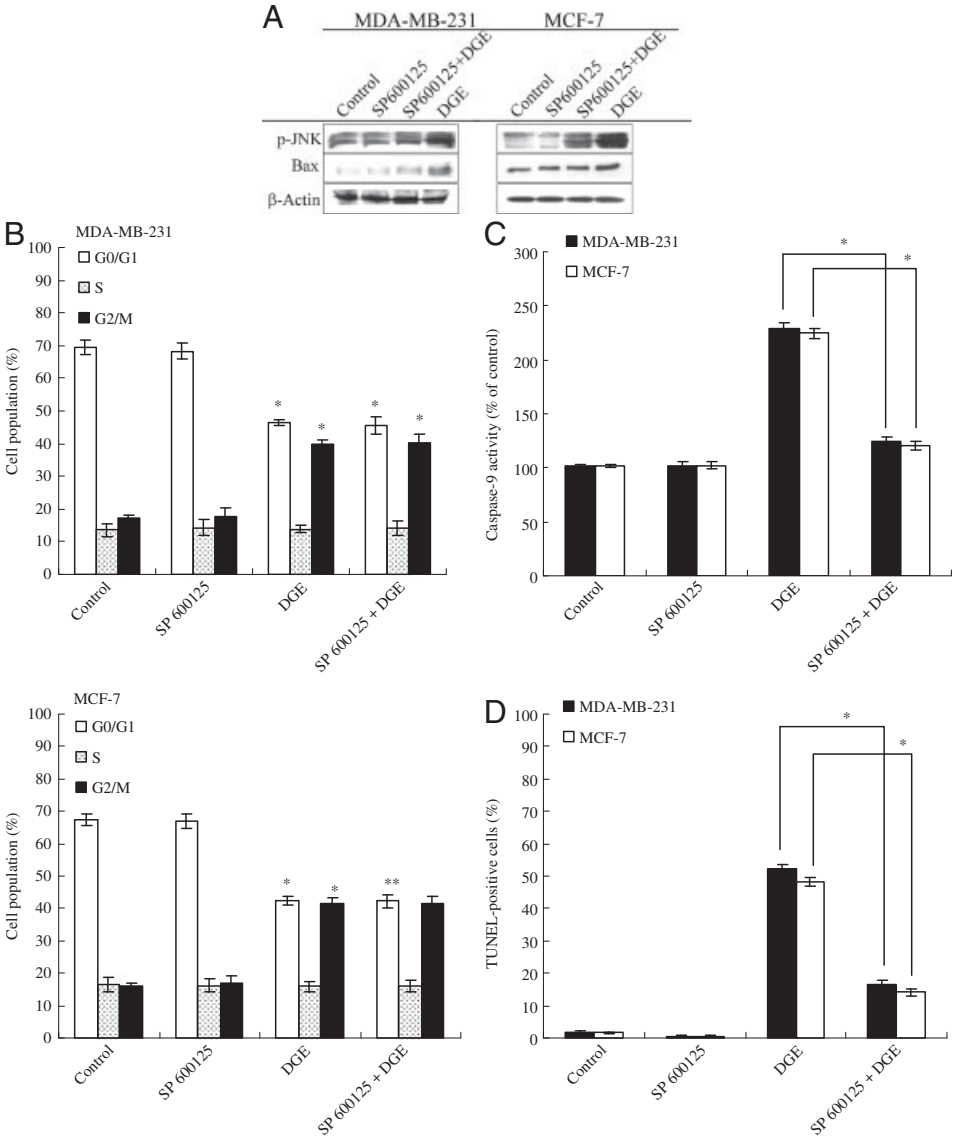

Figure 7. The role of JNK on DGE-triggered mitochondrial apoptotic pathway. (A) The effects of SP600125 on DGE-mediated JNK activation and Bax upregulation. (B) The effects of SP600125 on DGE-mediated cell cycle arrest. SP600125 inhibits DGE-mediated caspase-9 activity (C) and apoptosis (D). Cells were pre-treated with SP600125 (20 μM) for 1 h; then 20 μM DGE was added and incubated for specific times (12 h for Bax, 36 h for caspase-9 and 48 h for apoptosis assay). The data shown are the mean from three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between two test groups, as analyzed by Dunnett's test ($p < 0.05$).

regulated by antioxidant enzymes (SOD, catalase, GSH peroxidase) and nonenzymatic antioxidants (GSH, vitamin C) [6]. GSH is a major thiol-disulphide redox buffer that participates in redox reactions by maintaining a reducing environment in the cell. Upregulation of GSH levels is an important factor in protection against apoptosis and is associated with cancers resistant to therapy [32]. Consequently, low GSH levels are sometimes associated with mitochondrial dysfunction and induction of apoptosis, thus decreasing cancer's chemoresistance [32, 33]. ROS can cause apoptotic cell death *via* a variety of mechanisms, among which the activation of stress kinases JNK plays an important role. High levels of ROS also can induce apoptosis by triggering mitochondrial permeability transition pore opening, release of proapoptotic factors and activation of caspase-9 [4, 11, 34, 35]. O_2^- has also been indicated to regulate Bcl-XL expression, and inhibition of O_2^- by O_2^- scavenger *p*-benzoquinone prevents camptothecin-induced apoptosis [36]. Our study showed that DGE-mediated oxidative stress by increasing the production of O_2^- and H_2O_2 mainly resulted from GSH depletion. We further observed that DGE generates ROS and activates ASK1 as well as JNK, resulting in caspase-9 activation. Furthermore, these agents can regulate ROS detoxification, such as the scavengers of oxygen-free radicals vitamin C, and the

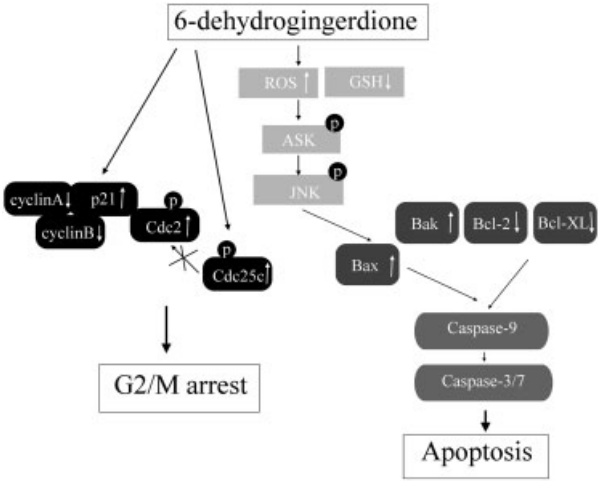

Figure 8. Models of action of DGE. Proposed models for how DGE affects various biochemical processes and events in MDA-MB-231 and MCF-7, resulting in G2/M arrest and apoptotic cell death, are shown in this schematic diagram.

H_2O_2-scavenging enzyme catalase, which inhibits JNK activation, blocks caspase-9 activation and decreases apoptosis induced by DGE. These data suggest that ROS accumulation contributes to DGE-induced cell death in human breast adenocarcinoma cells.

JNKs have been implicated in apoptotic response of cells exposed to chemotherapy and proinflammatory cytokines [30, 34, 35]. In malignant tissue samples from patients with primary breast cancer, JNK activity is less than in compared to normal breast tissue. The downregulation of JNK activation in breast cancer is due to the overexpression of MAPK phosphatases 1 and 2 [37]. A number of anticancer drugs have been reported to kill breast cancer cells *via* the JNK apoptotic pathway, and hence it is to be expected that blockade of JNK activation could inhibit anticancer activity [38]. The proapoptotic targets of the activated JNK are not clearly defined, but the phosphorylation of transcription factors such as c-Jun and p53, as well as pro- and antiapoptotic Bcl-2 family members, such as Bim, Bax and Bcl-2, have been suggested to be of importance [30, 34, 35]. In this report, we have shown that treatment of MDA-MB-231 and MCF-7 cells with DGE resulted in the accumulation of phospho-JNK. This JNK activation correlated well with the DGE-induced increase of JNK activity, as measured by the JNK substrate phospho-c-Jun. Furthermore, we observed that blocking the DGE-induced activation of JNK by chemical inhibitor could prevent Bax upregulation and caspase-9 activity, suggesting that DGE-induced JNK activation contributes to DGE-induced mitochondrial apoptotic pathways.

5 Concluding remarks

In conclusion, our data indicate that human breast adenocarcinoma cells are highly sensitive to growth inhibition and apoptosis induction by DGE. DGE-induced apoptosis is associated with mitochondrial cell death pathways, which is mediated by ROS generation and subsequently JNK activation (Fig. 8). The proposed working models for the molecular basis, if confirmed, would provide invaluable insights for approaches to the development of effective chemotherapy by targeting appropriate signal transducers.

This study was supported by grants from the Kaohsiung Medical University Research Foundation (KMU-Q098009) and Center of Excellence for Environmental Medicine, Kaohsiung Medical University (KMU-EM-98-3).

The authors have declared no conflict of interest.

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