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Aqueous *Ocimum gratissimum* Extract Induces Apoptotic Signalling in Lung Adenocarcinoma Cell A549

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ABSTRACT

Ocimum. gratissimum (OG) is widely used as a traditional herb for its antibacterial activity in Taiwan. Recently, antitumor effect of OG on breast cancer cell is also reported; however, the effects of OG on human pulmonary adenocarcinoma cell A549 remain unclear. Therefore, we aimed to investigate whether aqueous OG extract (OGE) affects viability of A549 cells and the signals induced by OGE in A549 cells. Cell viability assays revealed that OGE significantly and dose-dependently decreased the viability of A549 cell. Morphological examination and DAPI staining indicated that OGE induced cell shrinkage and DNA condensation for A549 cells. Further investigation showed that OGE enhanced activation of caspase-3, caspase-9 and caspase-8 and increased protein level of Apaf-1 and Bak, but diminished the level of Bcl-2. Additionally, OGE inhibited the phosphorylation of extracellular signal-regulated kinase (ERK) yet enhanced the phosphorylation of c-Jun N-terminal kinase (JNK) and p38 MAP kinase (p38). In conclusion, our findings indicate that OGE suppressed the cell viability of A549 cells, which may result from the activation of apoptotic signaling and the inhibition of anti-apoptotic signaling, suggesting that OGE might be beneficial to lung carcinoma treatment.

Key words: Ocimum gratissimum; Apoptosis; ERK; JNK; p38; Bcl-2; Bak

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Introduction

Lung adenocarcinoma is the major cause of cancer-related mortality worldwide (1, 2). Despite success of concurrent therapeutic approaches treating lung cancer including chemotherapy and radiotherapy, marked chances of undesirable and adverse side effects caused by existing therapies need to be managed. Use of medicinal plants in chemoprevention is considered as an ideal treatment with good efficacy and few side effects compared with allopathic medicine (3). Mounting evidences have shown that dietary intake of phytochemicals, an important group of chemopreventive agents, reduces the risk of cancer (4) and has antitumor potential against lung cancer (5).

Chemopreventive agents are compounds that prevent development of cancer. Their preventive effects are attributed to (1) intervening in interaction of the carcinogen with cellular DNA, (2) altering intracellular signaling pathways as results of stopping progression of an initiated cell through preneoplastic changes into a malignant cell, (3) inhibiting angiogenesis, (4) inducing cell cycle arrest and (5) triggering apoptosis. It is believed that the apoptosis induced by chemopreventive agents not only may inhibit the carcinogenesis induced by carcinogens, but also may suppress the growth of tumor and enhance the cytotoxic effects of antitumor drug on tumor, which plays a pivotal role in the antitumor therapies (6).

The genus *Ocimum*, belonging to the family *Labiatae*, is widely found in tropical

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and subtropical regions. The widespread plant is known for its chemopreventive, anticarcinogenic, free radical scavenging and others pharmacological properties and used as a traditional herb in European and Asian countries since ancient times (7). It has been prepared in a variety of forms for consumption. The aqueous leaf extract and seed oil are reported to show chemopreventive and anti-proliferative activity on Hela cells (8). Ethanolic extract of *Ocimum* leaf also has been shown to have significant modulatory influence on carcinogen metabolizing enzymes including cytochrome P450, cytochrome b5 and aryl hydrocarbon hydroxylase, glutathione-s-transferase (9, 10). Additionally, *Ocimum sanctum* prepared in the form of fresh leaf paste, aqueous and ethanolic extract has been reported to reduce the incidence of papillomas and squamous cell carcinoma in carcinogen-treated hamsters with a observation that the aqueous extract excerts more profound effect that the other two forms (11). Nevertheless, the mechanisms of aqueous extract of *Ocimum gratissimum* (OGE) underlying it anticancer property remain sketchy.

In the present study, the anticancer effects of OGE were investigated using human lung carcinoma A549 cells. The effects of OGE on cell viability and apoptosis of A549 cell were determined by measuring the activity of mitochondrial malate dehydrogenase and the DNA fragmentation, respectively. The OGE-induced caspase activation cascades and kinase signaling, including caspase-3, caspase-8, caspase-9,

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apaf-1, Bcl-2, Bak, extracellular signal-regulated kinase (ERK), Akt, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase (p38), were elucidated using immunological approaches.

Methods

Materials

[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 4'-6-Diamidino-2-phenylindole (DAPI), penicillin and streptomycin were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsin-EDTA were purchase from Gibco BRL (Gaithersburg, MD). Antibodies against caspase-3, caspase-8, caspase-9, apaf-1, Bak, Bcl-2, phosphorylated-ERK1/2, ERK1/2, phosphorylated-JNK, JNK, phosphorylated-p38 and p38 were purchased from Cell Signaling Technologies (Beverly, MA). Antibodies against β-actin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Sigma. HRP-conjugated secondary antibodies against mouse IgG and rabbit IgG were purchased from Abcam Inc. (Cambridge, UK). The lung adenocarcinoma cell A549 was obtained from American Type Culture Collection (ATCC; Rockville, MD).

Preparation of OGE

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Leaves of *Ocimum gratissimum* Linn were harvested and washed with distilled water followed by homogenization with distilled water using polytron. The homogenate was incubated at 95°C for 1 hour (h) and then filtered through two layers of gauze. The filtrate was centrifuged at 20,000 g, 4ºC for 15 min to remove insoluble pellets and the supernatant (OGE) was thereafter collected, lyophilized and stored at -70°C until use.

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Cell Culture and Experimental Treatments

Lung adenocarcinoma cell A549 were maintained in DMEM supplemented with 10% FBS and 100 µg/ml penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded in 6-well culture plates at an initial density of $1x10^5$ cells/ml and grown to approximately 80% confluence. The culture was treated with OGE at indicated concentrations for 48 hrs and subsequently washed with phosphate-buffered saline (PBS; 25 mM sodium phosphate, 150 mM NaCl, pH 7.2). the resulting cells were collected for following analyses.

DAPI Staining

DAPI staining was performed to assess morphological changes in the chromatin structure of A549 cells undergoing apoptosis as previously described (12). Briefly, cells were trypsinized, mounted on glass slides, and fixed in 4% paraformaldehyde

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for 30 min followed by staining with 1 μg/ml DAPI for 30 min. Apoptosis was characterized by chromatin condensation and fragmentation when examined by fluorescence microscopy. The incidence of apoptosis in each treatment was analyzed by counting 300 cells and presented in the percentage of apoptotic cells.

Cell Viability Assay

Cell viability was determined by MTT assay (13) in the absence or presence of 50 or 100 μg/ml OGE. After the 48 h treatments, medium was removed and the A549 cells were incubated with MTT (0.5 mg/ml) at 37°C for 4 h. The viable cell number was directly proportional to the production of formazan, which was dissolved in isopropanol and determined by measuring the absorbance at 570 nm using a microplate reader (SpectraMAX 360 pc, Molecular Devices, Sunnyvale, CA).

Immunoblotting

A549 cells were washed with PBS and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 10 μg/ml aprotinin and leupeptin). The lysates were incubated on ice for 30 min and centrifuged at 20,000g for 15 min. The supernatants were collected and measured for protein concentration using Bradford method. Crude proteins (30 μg per lane) were subjected to a 12.5% SDS-polyacrylamide gel, and

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then transferred onto a nitrocellulose membrane (Millipore, Bedford ,MA). The blotted membrane was blocked with 5% w/v skimmed milk in PBS, and then incubated for 2 h with 1/1000 dilution of antibodies against human caspase-3, caspase-8, caspase-9, apaf-1, Bak, Bcl-2, phosphorylated-ERK1/2, ERK1/2, phosphorylated-JNK, JNK, phosphorylated-p38, p38, β-actin and GAPDH, respectively. Antigen-antibody complex were detected using 1/2000 dilution of peroxidase-conjugated secondary antibodies and displayed using ECL chemiluminescence reagent (Millipore).

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Statistical Analysis

Statistical analysis was performed using the SigmaStat version 3.5 for Windows (Systat Software Inc. San Jose, CA). The results are presented as mean ± SD. The statistical significance between groups was determined using Student's t test. A *p* value less than 0.05 was considered statistically significant.

Results

OGE Suppressed the Cell Viability of A549 Cells

To examine the cytotoxic effects of OGE on A549 cells, the cells were treated with a serial concentration of OGE (100, 200, 300, 500 and 800 μg/ml) for 48 hrs, and then the cell viability was determined. As shown in Fig. 1, the cell viability in presence of

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OGE was found decreased in association with the concentration of OGE in a does-dependent fashion. The viability was significantly decreased to 87.2 ± 1.2, 70.6 ± 3.3, and 27.5 ± 1.4% of control with 300, 500 and 800 μg/ml OGE, respectively ($p < 0.05$ as compared to control). Accordingly, the findings showed that 500 and 800 μg/ml OGE treatment significantly suppressed the viability of A549 cells.

OGE Altered the Morphology of A549 cells and Induced the DNA Condensation

To investigate whether cell death in presence of OGE is attributed to apoptotic events, cell morphology and DNA fragmentation for OGE-treated A549 cells was examined. As shown in Fig. 2A, OGE treatment altered the morphology of A549 cells, which were shrunk into circle and lost the ability to attach (left panel). Additionally, OGE treatment also induced the DNA condensation in the A549 cells, which were dose-dependently increased with the concentration of OGE (right panel). The incidence of DNA condensation was quantitated as shown in Fig. 2B, which revealed that 500 and 800 μg/ml OGE treatment resulted in significant apoptosis (17.5 ± 1.7% and 56.8± 2.3%, $p < 0.01$ as compared to control) in A549 cell.

OGE Activated both Intrinsic and Extrinsic Apoptotic Pathway in A549 Cells

To investigate the apoptotic mechanisms induced by OGE treatment, the activation of intrinsic and extrinsic caspase cascades was investigated. As shown in Fig. 3, the level of procaspase-3, an important effecter caspase, was decreased by OGE treatment in a

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does-dependent manner. Concomitant with the decrease of procaspase-3, the level of cleaved caspase-3 was dose-dependently increased by OGE treatment. The upstream activators of procaspase-3 in intrinsic pathway, caspase-9 and Apaf-1, were further investigated. The levels of procaspase-9 and cleaved caspase-9 were dose-dependently decreased and increased respectively upon OGE treatment. The level of Apaf-1 was also dose-dependently increased by OGE treatment (Fig.3).

The effect of OGE treatment on caspase-8, an upstream activator of caspase-3 in extrinsic pathway, was also investigated. Although the level of procaspase-8 showed no significant change by OGE treatment, the levels of two cleaved caspase-8 (43 kDa and 12 kDa) were remarkably increased by OGE treatment (Fig. 4).

OGE Increased Level of Bak and Decreased Level of Bcl-2 in A549 Cells

To further elucidate the putative mechanism underlying the OGE-associated apoptotic signaling, the level of proapoptotic proteins Bak and anti-apoptotic proteins Bcl-2 at various concentrations of OGE was examined. After being normalized and verified with GAPDH, expression of Bax increased remarkably in a dose-dependent manner. Moreover, there was an obvious decrease in the Bcl-2 protein level in the OGE-treated A549 cells as compared to the control (Fig. 5A).

Quantitative Bak and Bcl-2 expression after being standardized to GAPDH (n=3) was shown in Fig. 5B. Expression of Bak was increased significantly upon OGE treatment,

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whereas the significant decrease in expression of Bcl-2 in A549 cells. With 800 μg/ml OGE treatment, the level of Bak and Bcl-2 was increased to 321.3 ± 11.4% and decreased to 50.2 ± 2.2%, respectively ($p < 0.01$ as compared to control).

OGE Induced the Phosphorylation of JNK and p38, but Diminished the Phosphorylation of ERK in A549 Cells

MAP kinases have been widely reported for their involvements in the survival, proliferation, differentiation and apoptosis in different cancer cells (14). Therefore, the influence of OGE treatment on activation of three important MAP kinases, ERK, JNK and p38, were further investigated. As shown in Fig. 6, remarkable phosphorylation of ERK, but not JNK and p38, was detected in the control A549 cells. OGE treatment significantly inhibited the phosphorylation of ERK and enhanced the phosphorylation of JNK and p38.

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Discussion

Aberrant cells such as mutated or proliferating neoplastic cells are removed by programmed cell death, namely apoptosis (15). Two well-known pathways, extrinsic and intrinsic pathways, are respobsible for triggering apoptosis (16). In the case of the intrinsic pathway, a release of cytochrome C from mitochondria results in binding to Apaf-1 and subsequently leads to activation of procaspase-9 and following caspase-3

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(17). Activated caspase-3 exerts as the key executioner of apoptosis to induce the cleavage and inactivation of key cellular protein (17, 18). In present study, it is demonstrated that OGE treatment increased the Apaf-1 expression level and activated the caspase 9 and 3 cascade. Additionally, it is known that caspase-3 can be activated by caspase-8 through the extrinsic pathway (19). Our results showed that OGE treatment simultaneously induced the activation of caspase-8. These findings indicate that both activations of the intrinsic and extrinsic pathway are of responses to exposure to presence of OGE in A549 as results of apoptosis.

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MAP kinase cascades consist of a core of three protein kinases such as ERK1/2, p38 and JNK pathways (20, 21). Thus, to understand the molecular mechanism of OGE, the potential involvement of MAP kinase pathway was investigated in OGE-induced apoptosis by immunoblotting. The dysregulation of Akt and ERK is known as a prominent feature of many human cancers including non-small cell lung cancer (22). Our findings are consistent with the aberration that a relative high level of ERK phosphorylation in the control A549 cells, which may contribute to the malignancy and high frequency of metastasis of lung cancer. Interestingly, our result that ERK phosphorylation in A549 cells was significantly inhibited in presence of OGE suggests that OGE treatment may have suppressive influence on the constitutive survival signaling for A549 cells. Moreover, the JNK and p38 phosphorylation in

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A549 cells was found enhanced in reponse to OGE treatment, which play an important role in apoptotic signaling through regulating the activities of pre-existing Bcl-2 family proteins and mediating caspase activation (23).

Recently, several lines of evidence indicate that extracts of *Ocimum* species possess antitumor effects. Ethanolic extract of *Ocimum sanctum* has been reported to induce apoptosis of A549 cells mainly via the intrinsic/mitochondria-dependent pathway and suppress the growth of lewis lung carcinoma in mice (24). However, our findings show that OGE activates both intrinsic and extrinsic pathway in A549 cell. The differences might be attributed to the species variation and/or the extraction approaches used in each study. Aqueous extract of OG leaf is also reported to inhibit the growth and the migration of breast cancer cell MDA-MB-231 (25). With the treatment of 0.5% (5 mg/ml) aqueous extract of OG leaf for 72 hrs, the cell viability of MDA-MB-231 was reduced to approximate 60% of the control. Our results reveal that the 500 and 800 μg/ml OGE treatment significantly diminishes the cell viability of A549 to 47% and 36% of the control. These findings suggest that lung adenocarcinoma is more susceptible to OGE treatment than breast cancer.

In conclusion, the present study provides evidences that OGE treatment significantly alter viability of lung adenocarcinoma A549 through a synergy of induction of apoptotic signaling and suppression of anti-apoptotic signaling.

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Moreover, OGE treatment simultaneously inhibits the activation of ERK and enhances the activation of JNK and p38, which is consistent with the enhanced apoptotic signaling and reduced anti-apoptotic signaling basing on their well-known effects on these signal cascades. By manipulating both arms of apoptotic and anti-apoptotic pathway, OGE represents a promisingly effective chemopreventive agent for lung adenocarcinoma.

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Figure legends

Figure 1. OGE diminished cell viability of A549 cell. The cell viability of A549 cells treated with a series concentration of OGE (10, 50, 100, 200 and 300 μg/ml) for 48 h was determined. Data were expressed as mean ± SEM for 3 independent experiments.*, $p < 0.05$ and **, $p < 0.01$ as comparing to control (C).

Figure 2. OGE altered cell morphology and induced DNA condensation of A549 cell. A549 cells were treated with 0, 500 and 800 μg/ml OGE for 48 h and then stained with DAPI. (A) The cell morphology and the DNA condensation was photographed by fluorescence microscopy (200X). The cells presented DNA condensation were indicated by arrow. (B) The incidence of DNA condensation was determined for the A549 cells with different treatments. Data were expressed as mean ± SEM for 3 independent experiments.

Figure 3. OGE induced activation of intrinsic/mitochondrial apoptotic pathway. A549 cells were treated with 0, 500 and 800 μg/ml for 48 hr, and then were lyzed for the determination of protein levels of caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9 and Apaf-1 by immunoblotting. β-actin was used as control. The apparent molecular weights for detected proteins were indicated.

Figure 4. OGE induced activation of caspase-8. A549 cells were treated with 0, 500 and 800 μg/ml for 48 hr, and then were lyzed for the determination of protein levels

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of caspase-8 and cleaved caspase-8 by immunoblotting. β-actin was used as control. The apparent molecular weights for detected proteins were indicated.

Figure 5. OGE enhanced protein level of Bak and diminished protein level of Bcl-2. A549 cells were treated with 0, 500 and 800 μg/ml for 48 hr, and then were lyzed for the determination of protein levels by immunoblotting. (A) The expression levels of Bak and Bcl-2 were determined. GAPDH was used as control. (B) The expression levels of Bak and Bcl-2 were quantitatively expressed after being standardized to GAPDH. Data are expressed as mean ± SEM for 3 independent experiments for each concentration point. **, $p < 0.01$ as compared with control.

Figure 6. OGE inhibited phosphorylation of Erk but enhanced phosphorylation of JNK and p38. A549 cells were treated with 0, 500 and 800 μg/ml for 48 hr, and then were lyzed for the determination of protein levels by immunoblotting. The levels of phosphorylated-ERK1/2 (p-ERK1/2), ERK1/2, p-JNK, JNK, p-p38 and p38 were presented.

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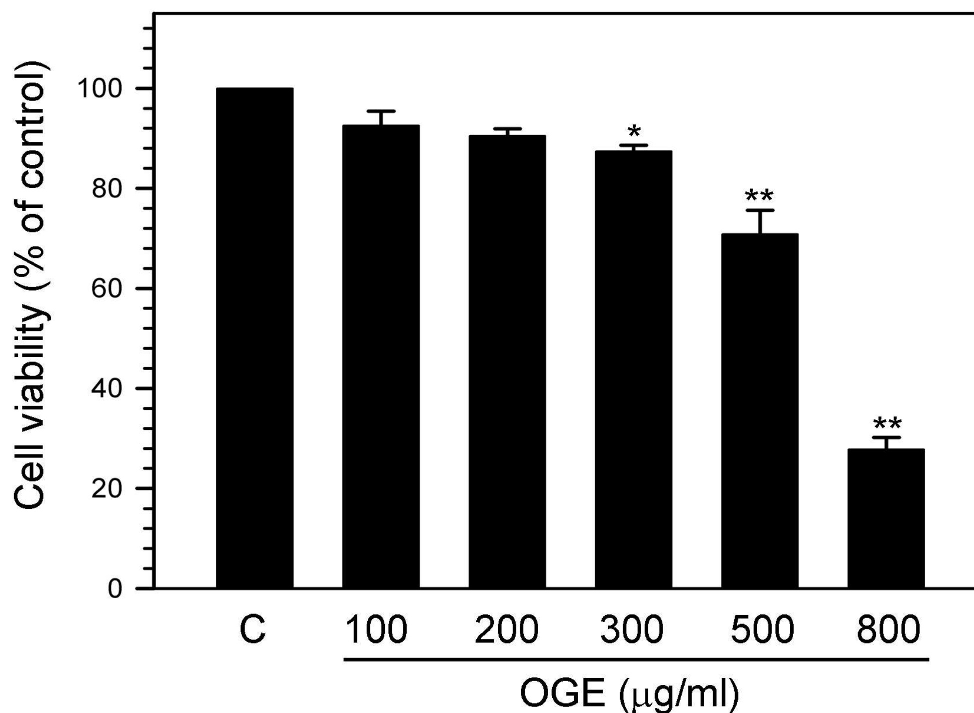

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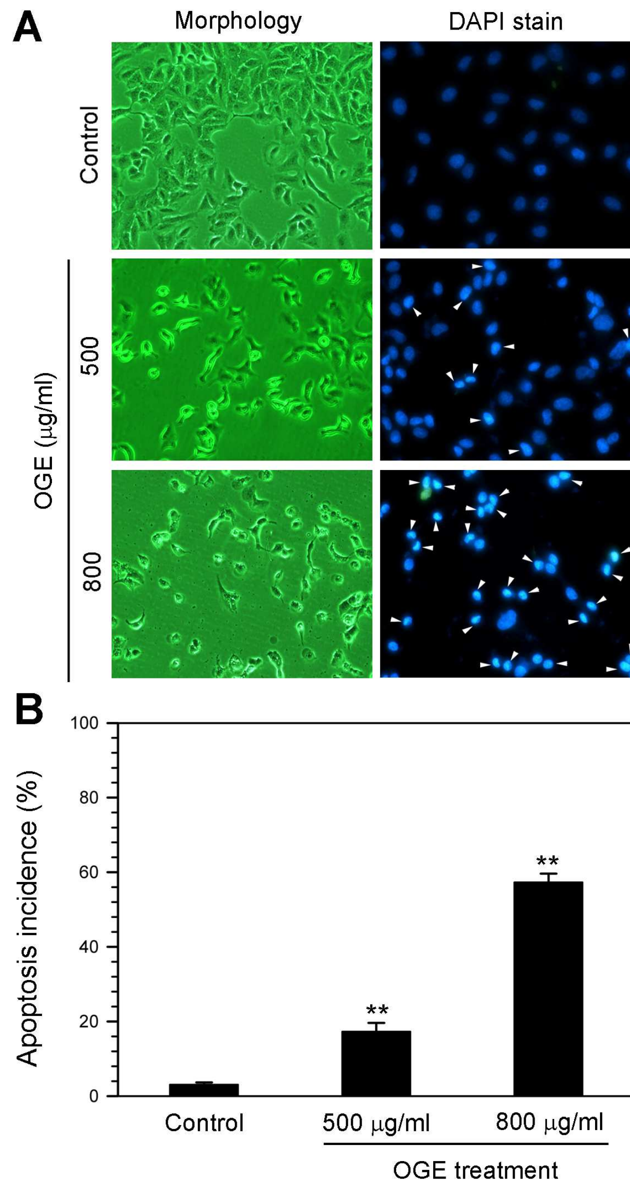

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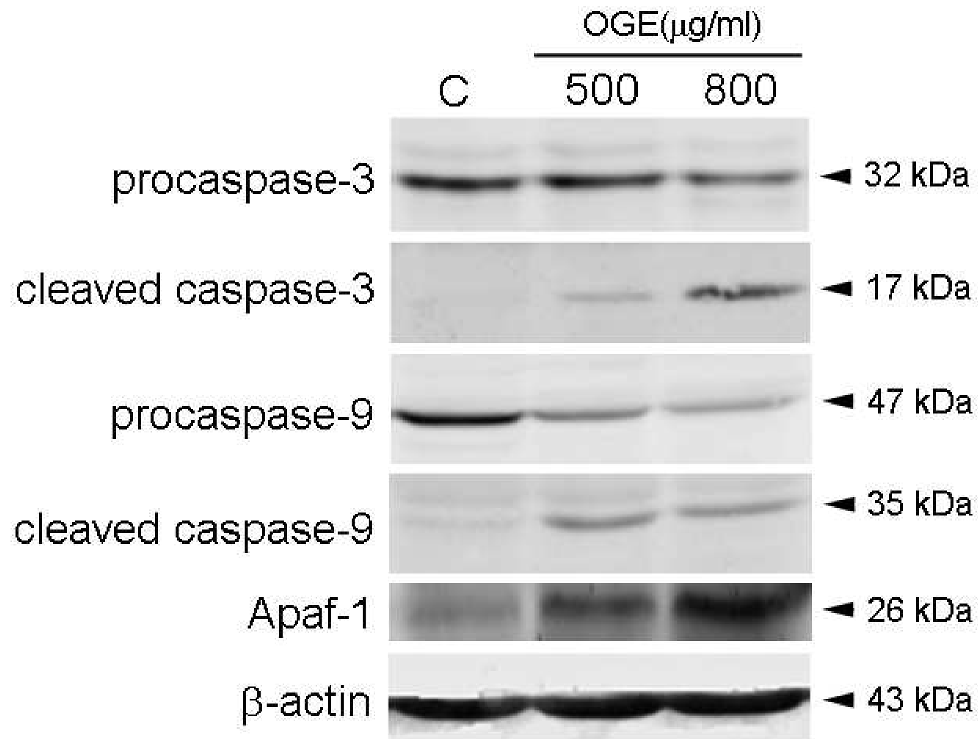

OGE induced activation of intrinsic/mitochondrial apoptotic pathway. A549 cells were treated with 0, 500 and 800 ug/ml for 48 hr, and then were lyzed for the determination of protein levels of caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9 and Apaf-1 by immunoblotting. β-actin was used as control. The apparent molecular weights for detected proteins were indicated.
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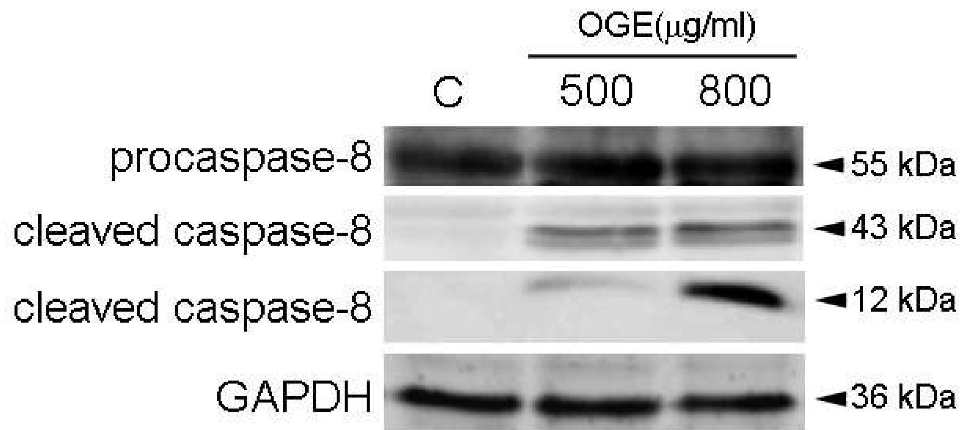

OGE induced activation of caspase-8. A549 cells were treated with 0, 500 and 800 ug/ml for 48 hr, and then were lyzed for the determination of protein levels of caspase-8 and cleaved caspase-8 by immunoblotting. β-actin was used as control. The apparent molecular weights for detected proteins were indicated.
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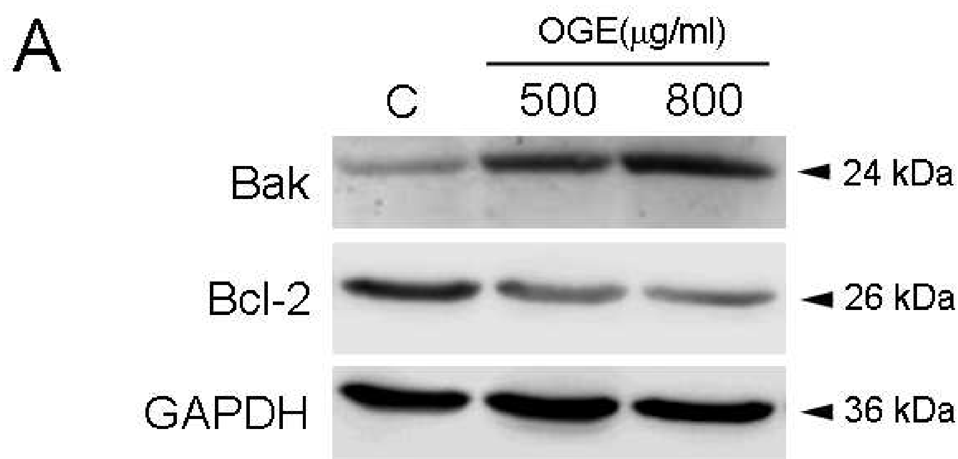

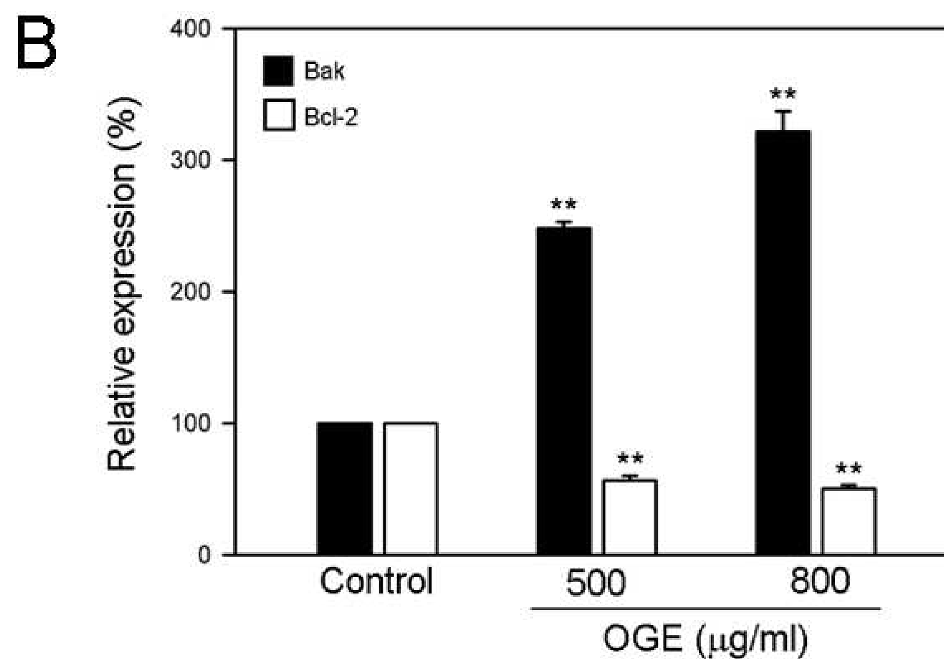

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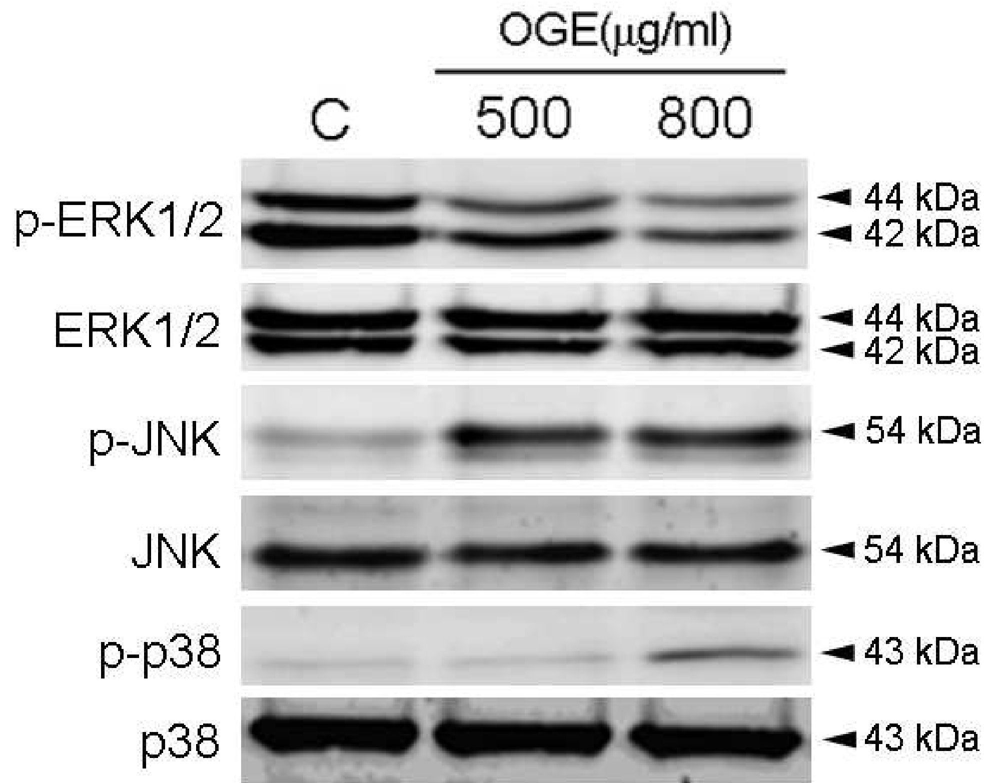

OGE inhibited phosphorylation of Erk but enhanced phosphorylation of JNK and p38. A549 cells were treated with 0, 500 and 800 ug/ml for 48 hr, and then were lyzed for the determination of protein levels by immunoblotting. The levels of phosphorylated-ERK1/2 (p-ERK1/2), ERK1/2, p-JNK, JNK, p-p38 and p38 were presented.
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