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12-*o*-Tetradecanoylphorbol 13-acetate prevents baicalein-induced apoptosis via activation of protein kinase C and JNKs in human leukemia cells

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Abstract In the present study, we found that baicalein (BE), but not its glycoside baicalin (BI), induced apoptosis in human leukemia HL-60 and Jurkat cells, but not in primary murine peritoneal macrophages (PMs) or human polymorphonuclear (PMN) cells, by the MTT assay, LDH release assay, and flow cytometric analysis. Activation of the caspase 3, but not caspase 1, enzyme via inducing protein processing was detected in BE-induced apoptosis. The ROS-scavenging activity of BE was identified by the anti-DPPH radical, DCHF-DA, and in vitro plasmid digestion assay, and none of chemical antioxidants including allpurinol (ALL), *N*-acetyl-cystein (NAC), and diphenylene iodonium (DPI) affected BE-induced apoptosis in HL-60 cells. This suggests that apoptosis induced by BE is independent of the production of ROS in HL-60 cells. Interestingly, the apoptotic events such as DNA ladders formation and activation of the caspase 3 cascade were significantly blocked by TPA addition in the presence of membrane translocation of PKCα, and TPA-induced protection was reduced by adding the PKC inhibitors, GF-109203X and staurosporin. TPA addition induces the phosphorylation of JNKs and ERKs, but not p38, protein in HL-60 cells, and incubation of HL-60 cells with JNKs inhibitor SP600125, but not ERKs inhibitor, PD98059 or the p38 inhibitor SB203580, suppressed the protective effect of TPA against BE-induced apoptotic events including DNA ladders, apoptotic bodies, caspase 3 and D4-GDI protein cleavage in according with blocking JNKs protein phosphorylation. In addition, PKC inhibitor GF-109203X treatment blocks TPA-induced ERKs and JNKs protein phosphorylation, which indicates that activation of PKC locates at upstream of MAPKs activation in TPA-treated HL-60 cells. Additionally, a loss in mitochondrial membrane potential with a reduction in Bcl-2 protein expression, the induction of Bad protein phosphorylation, and translocation of cytochrome *c* from mitochondria to the cytosol were observed in BE-treated HL-60 cells, and these events were prevented by the addition of TPA. GF-109203X and SP600125 suppression of TPA against cytochrome *c* release induced by BE was identified. This suggests that activation of PKC and JNKs participate in TPA's prevention of BE-induced apoptosis via suppressing mitochondrial dysfunction in HL-60 cells.

Keywords Apoptosis · Baicalein · ROS · TPA · PKC · JNKs

Abbreviations

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NBT	nitroblue tetrazolium
BCIP	5-bromo-4-chloro-3-indolyl phosphate
ERKs	extracellular regulatory kinases
MAPKs	mitogen-activated protein kinases
JNKs	c-Jun N-terminal kinases
PKC	protein kinase *c*

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TPA	12-*o*-Tetradecanoylphorbol 13-acetate
ALL	Allopurinol
NAC	*N*-acetyl-cystein
DPI	diphenylene iodonium
PARP	poly (ADP-ribose) polymerase
PM	peritoneal macrophages
PMN	polymorphonuclear cells
DPPH	1,1-dephenyl-2-picrylhydrazyl
DCHF-DA	dichlorodihydrofluorescein diacetate

Introduction

Flavonoids are a group of low-molecular-weight polyphenolic compounds, and several biological effects of flavonoids have been identified including anti-inflammation, apoptosis induction, and antioxidation. Baicalein (5,6,7-trihydroxyflavone; BE) is one of the major components of *Scutellaria baicalensis*, and anti-tumor [1], antiapoptosis [2], and anti-inflammatory [3] activities induced by BE have been reported. BE has been shown to exert anti-inflammatory effects via blocking lipopolysaccharide (LPS)-induced nitric oxide (NO) production in macrophages [4], and to inhibit thrombin-induced adhesion molecule expression in cultured human umbilical vein endothelial cells (HUVECs) [5]. BE is also reported to have reactive oxygen species (ROS)-scavenging activity in different cells. BE protected against hydrogen peroxide (H_2O_2)-induced oxidative stress in human neuroblastoma HS-SY5Y cells [6], and inhibited β-amyloid-induced cell death in rat cortical neurons [7]. The protective effects of BE are associated with its ROS-scavenging activity. In contrast to its cytoprotective effects, BE has been reported to induce apoptosis and inhibit proliferation in several cell types. BE induced apoptosis in gastric, pancreatic, and prostate cancer cells via as a 12-lipoxygenase inhibitor [8–10]. In MCF-7 cells, BE suppressed 17β-estradiol-induced transactivation, and induced apoptosis [11]. In lung squamous carcinoma CH27 cells, BE induced cell cycle arrest at the S-phase, followed by the induction of apoptosis [12]. Although several biological activities of BE have been reported, intracellular molecules involved in modulation of apoptosis induced by BE are still undefined.

Several molecular events can enable cells to escape the apoptosis induced by proapoptotic stimuli, and investigating the antiapoptotic mechanism can provide evidence for the action of stimuli. 12-*o*-Tetradecanoylphorbol-13-acetate (TPA) is a well-known tumor promoter in skin carcinogenesis, and induces differentiation in human myeloid leukemia cells in vitro. Activation of protein kinase C (PKC) followed by activation of mitogen-activated protein kinases (MAPKs) participates in TPA-induced cellular responses including cell death, cell survival, cell-cycle progression, proliferation, and differentiation [13–15]. Several previous studies indicated that activation of PKC by TPA induced apoptosis in LNCaP prostate carcinoma cells [16, 17]. Gonzalez-Guerrico and Kazanetz reported that TPA induced apoptosis in prostate cancer cells via the release of the death receptor ligand and activation of the apoptotic cascade [18]. Schaar et al. concluded that TPA reduced cell viability by reducing the activity of extracellular-regulated protein kinases (ERKs) in leukemia cells [19]. In contrast to apoptosis induction, TPA has been shown to inhibit singlet oxygen-induced apoptosis via activation of PKCs and a decrease in caspase 8 activation [20]. In human leukemia HL-60 cells, TPA treatment suppressed DNA fragmentation induced by polychlorinated biphenyls [21]. Although TPA possesses proapoptotic and antiapoptotic effects in cells, the effect of TPA on flavonoid-induced apoptosis in human leukemia HL-60 cells is still unclear.

In the present study, BE but not its glycoside, baicalin (BI), induced apoptosis in human leukemia HL-60 and Jurkat cells, but not in primary mice peritoneal macrophages (PMs) or human polymorphonuclear (PMN) cells. The preventive mechanism of TPA against BE-induced apoptosis via modulating the intracellular kinase cascades is elucidated herein.

Materials and methods

Cell culture

Human promyeloleukemic HL-60 and Jurkat cells were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). Mouse primary cultured PMs were obtained as in a previous study [22]. Human PMN cells were obtained from healthy male donors after Ficoll-Hypaque density gradient separation. All cells were grown in RPMI1640 containing 10% heat-inactivated fetal bovine serum, and were maintained at 37°C in a humidified incubator containing 5% CO_2. Exponentially growing cells were exposed to drugs for the indicated time periods. All culture reagents were purchased from Gibco/BRL.

Chemicals

The colorigenic synthetic peptide substrates, Ac-DEVD-pNA and Ac-YVAD-pNA, the protease inhibitors for Ac-DEVD-FMK and Ac-YVAD-FMK, and the PKC inhibitor, GF-109203X, were purchased from Calbiochem (La Jolla, CA, USA). 12-*o*-Tetradecanoylphorbol 13-acetate (TPA), propidium iodide, stuarosporine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), baicalein, baicalin, and other chemicals were obtained from Sigma

Chemical (St. Louis, MO, USA). Antibodies for detecting PARP, caspase 3, and D4-GDI protein were obtained from IMGENEX. Antibodies for detecting Bcl-2 family proteins and α-tubulin were purchased from Santa Cruz (Santa Cruz, CA, USA). Dichlorodihydrofluorescein diacetate (DCHF-DA) and DiOC6(3) were obtained from Molecular Probe.

Cell viability

Cell viability was assessed by MTT staining as described previously [23]. Briefly, cells were plated at a density of 10^5 cells/well in 24-well plates. After overnight growth, cells were treated with different concentrations of BE or BI under different conditions for 12 h, with the addition of the tetrazolium compound, MTT, into the medium at the end of the experiments. After incubation for 4 h at 37°C, 200 μl of 0.1 N HCl in 2-propanol was placed in each well to dissolve the tetrazolium crystals, and the absorbance at a wavelength of 600 nm was detected.

Determination of ROS production

ROS production was measured by flow cytometric analysis via DCHF-DA staining. Cells were treated with the indicated compounds for 2 h followed by washing with cold PBS to remove the extracellular compounds, and after the addition of DCHF-DA (100 μM) with or without H_2O_2 (200 μM), the mixture was allowed to sit for 30 min. The intensity of green fluorescence in cells was detected by flow cytometric analysis.

Western blotting

Total cellular extracts (20 μg) were prepared and separated on 8% SDS-polyacrylamide mini gels for PARP protein detection, and 12% SDS-polyacrylamide gels for caspase 3, cleaved D4-GDI, Bcl-2 family protein, and α-tubulin detection. The proteins in the gels were transferred to immobilon polyvinylidenedifluoride membranes (Millipore), and incubated with 1% bovine serum albumin at room temperature for 1 h, followed by incubation with the indicated antibodies for a further 3 h at room temperature. Expression of the indicated proteins was detected by incubation with an alkaline phosphatase-conjugated anti-mouse IgG antibody, and visualized by staining with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as described in our previous papers [24–26].

Release of cytochrome *c* from mitochondria to the cytosol

Both mitochondrial and cytosolic fractions were prepared for detection of cytochrome *c* (Cyt c) protein expression by Western blotting. Cells under different treatments were washed once with ice-cold PBS, and resuspended in 5 volumes of hypotonic buffer (20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose). Cells were homogenized and centrifuged at $750 \times g$ for 10 min at 4°C. The supernatants were centrifuged at $10{,}000 \times g$ for 15 min at 4°C, and the supernatant (cytosolic fraction and the pellets (mitochondrial fraction) were collected. The pellet was dissolved in 0.1 ml of lysis buffer (10 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycoltetraacetic acid (EGTA), 0.15 M NaCl, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 mM PMSF, 2 mM sodium orthovanadate, and 1% SDS). The expression of Cyt *c* in both mitochondrial and cytosolic fractions was analyzed by Western blotting.

DNA gel electrophoresis

Cells (10^6/ml) under different treatments were collected and washed with PBS, and the cell lysate was dissolved in 100 μl of lysis buffer (50 mM Tris (pH 8.0); 10 mM EDTA; 0.5% sodium sarkosinate, and 1 mg/ml proteinase K) for 3 h at 56°C, followed by the addition of RNase A (0.5 mg/ml) and further incubation for another hour. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) before agarose electrophoresis, and DNA in the gel was detected by ethidium bromide staining under UV illumination.

Measurement of the mitochondrial membrane potential

Cells under different treatments were incubated with DiOC6(3) (40 nM) at 37°C for 30 min, and washed with cold PBS. The cell pellet was collected by centrifugation at $500 \times g$ for 10 min, and resuspended in 500 μl of PBS. Fluorescence intensities of DiOC6(3) in cells were analyzed by flow cytometric analysis (FACScan, Becton Dickinson, San Jose, CA, USA) with excitation and emission settings of 484 and 500 nm, respectively.

Analysis of respective caspase activities

Ac-DEVD-pNA and Ac-YVAD-pNA were used as colorimetric substrates for detecting the activities of caspases 3 and 1, respectively. Cells under different treatments were collected and resuspended in extraction buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA). The cell lysates containing 100 μg of protein were incubated with 100 μM of Ac-DEVD-pNA and Ac-YVAD-pNA at 37°C for

1 h, and the enzyme activity was measured by detecting the cleavage of the pNA colorimetric substrate at an absorbance of 405 nm.

DPPH radical-scavenging activity assay

The scavenging activities of BE and BI against DPPH radicals were measured. In brief, different concentrations of BE and BI were added to 0.1 ml of 1 M Tris-HCl buffer (pH 7.9), and then mixed with 1.2 ml of 500 mM DPPH in methanol for 20 min while the sample was protected from light. The absorbance at a wavelength of 517 nm was determined, and the decrease of absorbance at 517 nm was calculated to determine the scavenging activity. The addition of deionized water instead of BE or BI was used as a blank in the present study.

Analysis of hypodiploid cells

Trypsinized cells were washed with ice-cold PBS and fixed in 70% ethanol at –20°C for at least 1 h. After fixation, cells were washed twice, incubated in 0.5 ml of 0.5% Triton X-100/PBS at 37°C for 30 min with 1 mg/ml of RNase A, and stained with 0.5 ml of propidium iodide (50 mg/ml) for 10 min. Fluorescence emitted from the propidium-DNA complex was quantitated after excitation of the fluorescent dye by FACScan flow cytometry (Becton Dickenson, San Jose, CA, USA).

Plasmid digestion assay

Covalently closed circular plasmid pBR322 DNA (0.25 μg) in a final volume of 30 μl was treated with varying concentrations of BE or BI in the presence and absence of Cu(II) ions plus H_2O_2 (40 μM) for 1 h. To this, 6 μl of 5 × tracking dye (40 mM EDTA, 0.05% bromophenol blue, and 50 vol.% glycerol) was added, followed by agarose electrophoresis. DNA in the gel was observed by ethidium bromide staining under a UV-transilluminator.

Statistical analysis

All experiments were performed in triplicate, and values are expressed as the mean ± S.E. Student's *t*-test was used to compare the indicated two groups, and the significance of the difference was described. *p* values of <0.01 or <0.05 were regarded as indicating a significant difference.

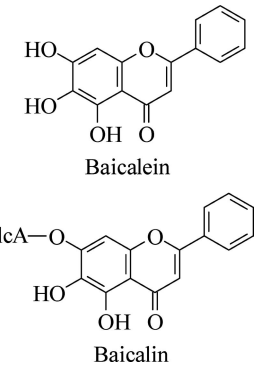

Fig. 1 Chemical structures of baicalein (BE) and baicalin (BI). BI is a structural analogue of BE, and contains a glycoside at C7 of BE

Results

Baicalein but not baicalin reduced the viability of human leukemia HL-60 and Jurkat cells, but not that of primary mice peritoneal macrophages (PMs) or human polymorphonuclear (PMN) cells

Baicalein (BE) and baicalin (BI) possess similar chemical structures, and BI contains a glycoside at C7 of BE (Fig. 1). Data of the DNA fragmentation assay showed that BE but not BI dose-dependently induced DNA ladder formation in HL-60 cells (Fig. 2(A;) left panel). In the same part of the experiment, BE, at a dose of 80 μM, time-dependently induced DNA ladders in HL-60 cells (Fig. 2(A) right panel). Additionally, BE but not BI dose-dependently induced DNA fragmentation in human leukemia Jurkat cells (Fig. 2(B)). In order to examine the differential cytotoxic effects of BE between normal and leukemia cells, mice PMs and human PMN cells were both used in the study. As illustrated in Fig. 2(C), slight but not significant DNA ladders was observed in BE-treated PM or PMN cells (Fig. 2(C)). The cytotoxicity elicited by BE was evaluated by the MTT assay and LDH release assay in the present study. Data in Fig. 2(D) show that BE (but not BI) dose-dependently reduced the viability of HL-60 cells according to the MTT assay. However, BE showed no effect on the viability of PM and PMN cells according to the MTT assay (Fig. 2(E)). An increase in the ratio of dead cells (dark ones) was detected in BE-treated HL-60 cells by trypan blue exclusion assay (Data not shown). Data of the LDH release assay indicated that BE but not BI elevated the release of LDH to the medium in HL-60 and Jurkat cells, but not in PM or PMN cells (Fig. 2(F)). In order to examine if BE reduces the viability via apoptosis induction, apoptotic bodies and hypodiploid cells in BE- and BI-treated HL-60 cells were detected via microscopic observation and flow cytometric analysis, respectively. Data in Fig. 2(G) show that the occurrence of apoptotic bodies was found in BE- but not BI-treated HL-60 cells under microscopic observation. Results of flow cytometric analysis

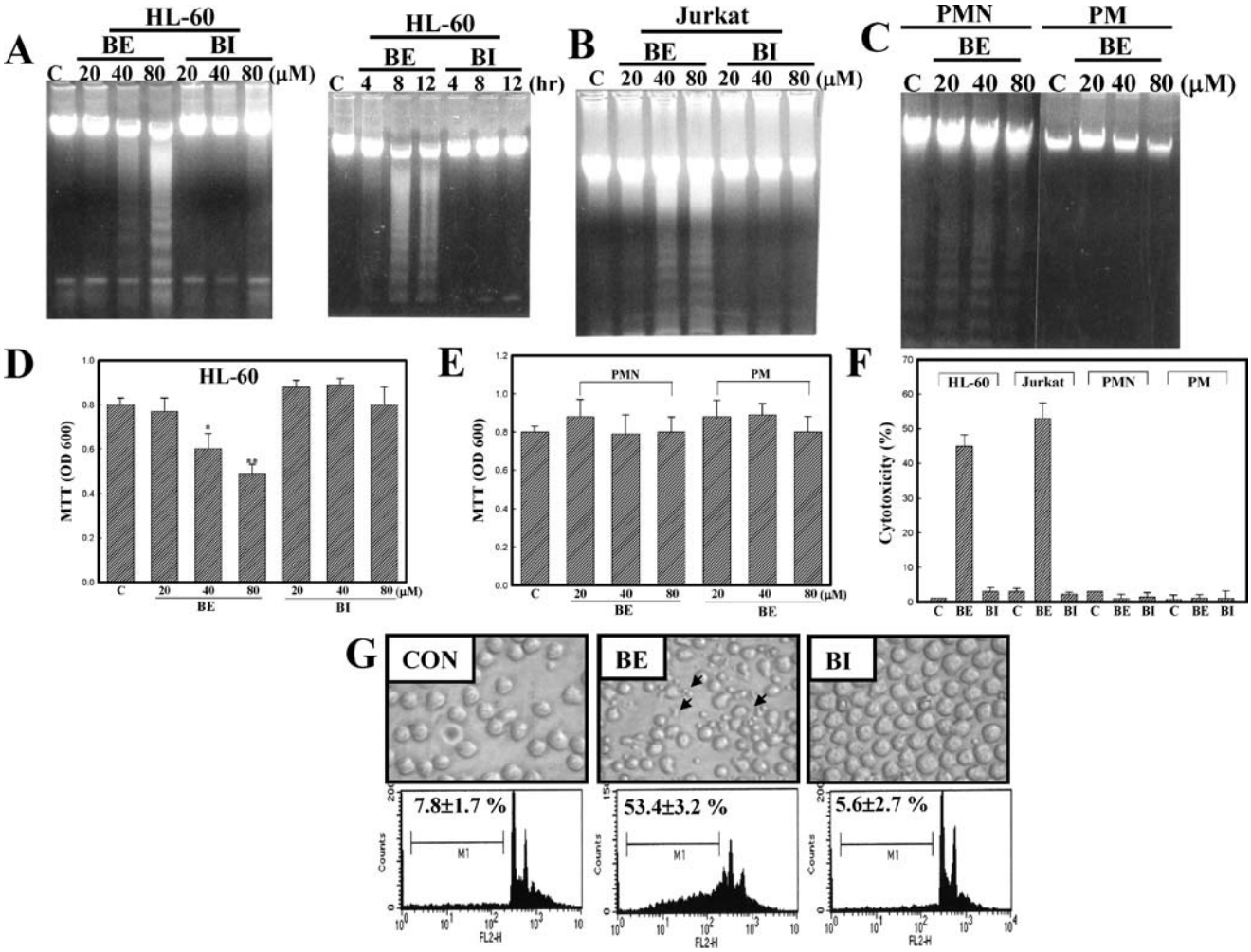

Fig. 2 Baicalein (BE) but not baicalin (BI) induced DNA ladder formation and reduced viability in human leukemia HL-60 and Jurkat cells, but not in primary mice peritoneal macrophages (PMs) or human polymorphonuclear (PMN) cells. (A) (*Right panel*) HL-60 cells were treated with different concentrations of BE and BI (20, 40, and 80 μM) for 12 h, and the integrity of DNA was analyzed by agarose electrophoresis. (*Left panel*) Cells were treated with BE or BI (80 μM) for different times (4, 8, and 12 h), and the integrity of DNA was analyzed. (B) As described in (A), human leukemia Jurkat cells were used in the present study. Jurkat cells were treated with different concentrations of BE and BI (20, 40, and 80 μM) for 12 h, and the integrity of DNA was analyzed by agarose electrophoresis. (C) As described in (A), two normal cell types, including PMN cells and PMs respectively derived from mice and humans, were used to examine the effect of BE. Both cell types were treated with different concentrations of BE (20, 40, and 80 μM) for 12 h, and the integrity of DNA was analyzed. (D) As described in (A), the viability of HL-60 cells under different treatments was evaluated by the MTT assay, as described in "Materials and Methods". (E) The cytotoxic effect of BE on both PMN cells and PMs was evaluated by the MTT assay. (F) Indicated cells were treated with BE (80 μM) or BI (80 μM) for 12 h, and the amount of LDH released into the medium was detected by an ELISA kit at OD595 nm. The percentage of cytotoxicity was examined using the equation of [(Treated group − Control group)/(Triton X-100-treated group − Control group)] × 100%. $^{*}p < 0.05$ and $^{**}p < 0.01$, significantly different from the control group as analyzed by Student's *t*-test. (G) Occurrence of apoptotic bodies and hypodiploid cells in baicalein (BE) (but not baicalin (BI))-treated HL-60 cells. HL-60 cells were treated with BE or BI (80 μM) for 12 h, and the morphological changes and apoptotic bodies were examined under a microscope (*Upper panel*). Arrows indicate the apoptotic bodies. the (*Lower panel*) Identical to the conditions in upper panel, hypodiploid cells (sub-G1) of HL-60 in the presence or absence of BE or BI treatment were examined by flow cytometric analysis via PI staining

indicated that an increase in the ratio of hypodiploid cells (at the sub-G1 peak) was observed in BE- but not BI-treated HL-60 cells. These data indicated that (a) BE exhibits cytotoxic effect in leukemia cells, but is less effective in normal cells; and (b) BE's reduction of the viability of leukemia cells occurs via apoptosis induction.

Activation of caspase 3 but not caspase 1 participates in BE-induced apoptosis in human leukemia cells

The roles of caspases 1 and 3 in BE-treated in human leukemia cells were investigated in the present study. As illustrated in Fig. 3(A), BE but not BI dose-dependently

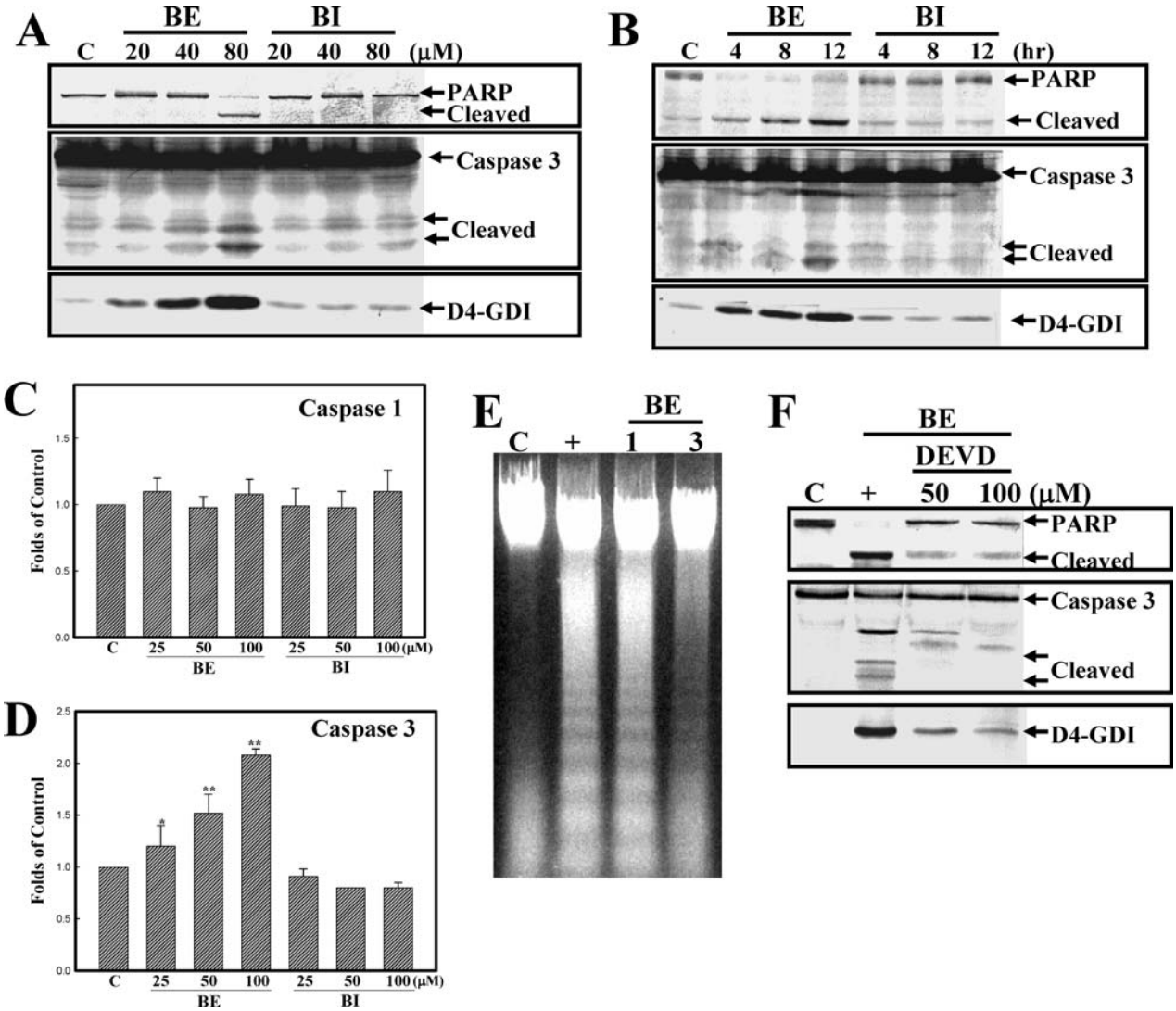

Fig. 3 Activation of caspase 3 is an essential event in baicalein (BE)-induced apoptosis. (A) BE but not baicalin (BI) induced caspase 3 activation via induction of cleavage. HL-60 cells were treated with different concentrations of BE or BI for 12 h, and the expressions of pro- and cleaved fragments of caspase 3 protein and its substrates, PARP and D4-GDI, were examined by Western blotting using specific antibodies. (B) As described in (A), cells were treated with BE and BI (80 μM) for different times, and the expressions of pro- and cleaved fragments of caspase 3 protein and its substrates, PARP and D4-GDI, were examined by Western blotting using specific antibodies. (C) The enzyme activity of caspase 1 in the lysates extracted from HL-60 under different treatments was examined using the specific peptidyl substrate, Ac-YVAD-pNA. (D) The enzyme activity of caspase 3 in lysates extracted from BE- and BI-treated HL-60 cells was examined using the specific peptidyl substrate, Ac-DEVD-pNA. (E) With the addition of caspase 3, but not caspase 1, the peptidyl inhibitor Ac-DEVD-FMK significantly attenuated BE-induced DNA ladder formation in HL-60 cells. Cells were treated with the caspase 1 inhibitor (1, Ac-YVAD-FMK; 100 μM) or the caspase 3 inhibitor (3, Ac-DEVD-FMK; 100 μM) for 30 min, followed by BE (80 μM) treatment for a further 12 h. The integrity of DNA in cells was analyzed by agarose electrophoresis. (F) The caspase 3 inhibitor, Ac-DEVD-FMK, significantly attenuated BE-induced protein processing of caspase 3, PARP, and D4-GDI. (F) As described in (E), the expression of indicated proteins was examined by Western blotting. $^{*}p < 0.05$ and $^{**}p < 0.01$, significantly different from the control as analyzed by Student's *t*-test

induced caspase 3 protein processing, characterized by a decrease in the caspase 3 protein (32 kDa) and an increase in the cleaved fragments (15 and 17 kDa). Inducing the cleavage of two caspase 3 downstream substrates, PARP and D4-GDI, through increasing the cleaved form of PARP (85 kDa) and D4-GDI (23 kDa) protein was observed in BE-treated HL-60 cells. In the same part of the experiment, BE (but not BI) (80 μM) time-dependently induced the cleavage of caspase 3, PARP, and D4-GDI protein in HL-60 cells (Fig. 3(B)). Furthermore, the activity of the caspases enzyme in BE- and BI-treated HL-60 cells was evaluated by an activity assay using specific substrates including Ac-DEVD-pNA for caspase 3 and Ac-YVAD-pNA for caspase 1. Data of Fig. 3(C) and (D) show that the addition of BE dose-dependently induced the enzyme activity of caspase 3, but not caspase 1, in HL-60 cells; however neither caspase 3 nor

caspase 1 enzyme activity was altered by BI treatment. In order to verify if the activation of caspase 3 is an essential event in BE-induced apoptosis, two specific peptidyl substrates including Ac-DEVD-FMK for caspase 3 and Ac-YVAD-FMK for caspase 1 were used in the present study. Results in Fig. 3(E) indicate that the addition of Ac-DEVD-FMK, but not Ac-YVAD-FMK, significantly prevented HL-60 cells from undergoing BE-induced DNA fragmentation. BE-induced protein procession in caspase 3, PARP, and D4-GDI was significantly blocked by Ac-DEVD-FMK (Fig. 3(F)).

BE-induced apoptosis is independent of the production of ROS in human leukemia HL-60 cells

In order to examine the role of free radicals in BE-induced apoptosis, three methods including anti-DPPH radical, hydroxyl radical-induced plasmid digestion, and DCHF-DA assays were applied in the study. Data of the anti-DPPH radical assay showed that both BE and BI exhibited scavenging activity on DPPH radicals in vitro, and BE seemed more effective than BI. The IC_{50} values of BE and BI were 17.8 ± 0.3 and 46.7 ± 1.2 μM, respectively (Fig. 4(A)). Additionally, the effects of BE and BI on hydroxyl radicals elicited by H_2O_2 plus Cu^{+2}-induced damage were examined by the plasmid digestion assay. As illustrated in Fig. 4B, hydroxyl radical-induced DNA damage was characterized by a decrease in the level of the supercoiled form and an increase in the level of the open-circle form of the plasmid, and BE but not BI dose-dependently inhibited the damage induced by hydroxyl radicals via the re-occurrence of the supercoiled plasmid in the present study. Because the above two methods are in vitro studies, we investigated the level of intracellular peroxide in HL-60 cells under different treatments via the DCHF-DA assay. In the absence of H_2O_2, BE treatment showed no effect on the level of intracellular peroxide; interestingly, BE reduction of H_2O_2-induced peroxide production in HL-60 cells was observed (Fig. 4C). Chemical antioxidants including allopurinol (ALL; a xanthine oxidase inhibitor), diphenylene iodonium (DPI; an NADPH oxidase inhibitor), and *N*-acetyl cysteine (NAC) addition exhibited no effect on BE-induced DNA ladder formation (Fig. 4D). Suppression of H_2O_2-induced DNA ladders by chemical antioxidants such as NAC was identified in our previous study as a positive control [27].

TPA suppression of BE-induced apoptosis occurs via PKC activation in HL-60 cells

Previous studies indicated that activation of PKC might reduce apoptosis in several types of cells, however the effect of PKC activation in BE-induced apoptosis is still unclear. TPA is a well-known tumor promoter via PKC activation, and our previous study [13] showed that TPA-induced transformation is mediated by activation of PKC and MAPKs. As described in Fig. 5(A), TPA, at the doses of 25, 50, and 100 ng/ml, significantly protected HL-60 cells from BE-induced DNA fragmentation. An induction of the protein processing of caspase 3, PARP, and D4-GDI in BE-treated HL-60 cells was attenuated by TPA incubation (Fig. 5(B)). A translocation of PKCα protein from cytosol to membrane was detected in TPA-treated HL-60 cells by Western blotting (Fig. 5(C)) GF-109203X (GF; a PKC inhibitor) and staurosporin (ST; a protein kinase inhibitor) were used in the present study to investigate the role of PKC activation in TPA's prevention of apoptosis induced by BE. Data in Fig. 5(D) show that GF and ST alone exhibited no effect on the integrity of DNA, and TPA prevention of DNA ladder formation induced by BE was blocked by the addition of GF and ST in HL-60 cells. Data of the MTT assay indicated that TPA treatment inhibited a reduction in the viability induced by BE, and that was reversed by adding GF and ST to HL-60 cells (Fig. 5(E)).

Activation of JNKs is involved in TPA prevention of apoptosis induced by BE

We further investigated the role of MAPKs including ERKs, p38, and JNKs in the protective effect of TPA against BE-induced apoptosis. Three specific chemical inhibitors including PD98059 (PD) for ERKs, SB203580 (SB) for p38, and SP600125 (SP) for JNKs were used in the present study. Data of morphological observation show that SP (5 μM) reverses the protective effect of TPA on BE-induced apoptotic bodies in HL-60 cells, however neither PD (10 μM) nor SB (10 μM) does (Fig. 6(A)). Analysis of DNA ladders formation indicates that SP (2.5 and 5 μM), but not PD or SB (10 and 20 μM), blocks the preventive effect of TPA against BE-induced DNA ladder formation in HL-60 cells (Fig. 6(B), upper panel). Data of MTT assay supported that SP incubation suppresses TPA prevention of BE-reduced viability in HL-60 cells (Fig. 6(B), lower panel). An increase in the expression of phosphorylated ERKs and JNKs, but not p38, protein was detected in TPA-treated HL-60 cells in a time-dependent manner, and no change in the level of total forms of ERKs, p38, and JNKs protein was detected (Fig. 6(C)). A respective inhibition of TPA-induced ERKs and JNKs protein phosphorylation by PD and SP was identified in HL-60 cells (Fig. 6(D) and (E)). Addition of PKC inhibitor GF significantly reduced TPA-induced ERKs and JNKs protein phosphorylation, represented that activation of PKC was located at upstream of MAPKs in TPA-treated HL-60 cells (Fig. 6(F)). Furthermore, TPA reduction of BE-induced caspase 3 and D4-GDI protein cleavages was blocked by SP (Fig. 6(G)). These data indicate that activation of JNKs may participate in TPA prevention of BE-induced apoptosis, which locates at the downstream of PKC activation.

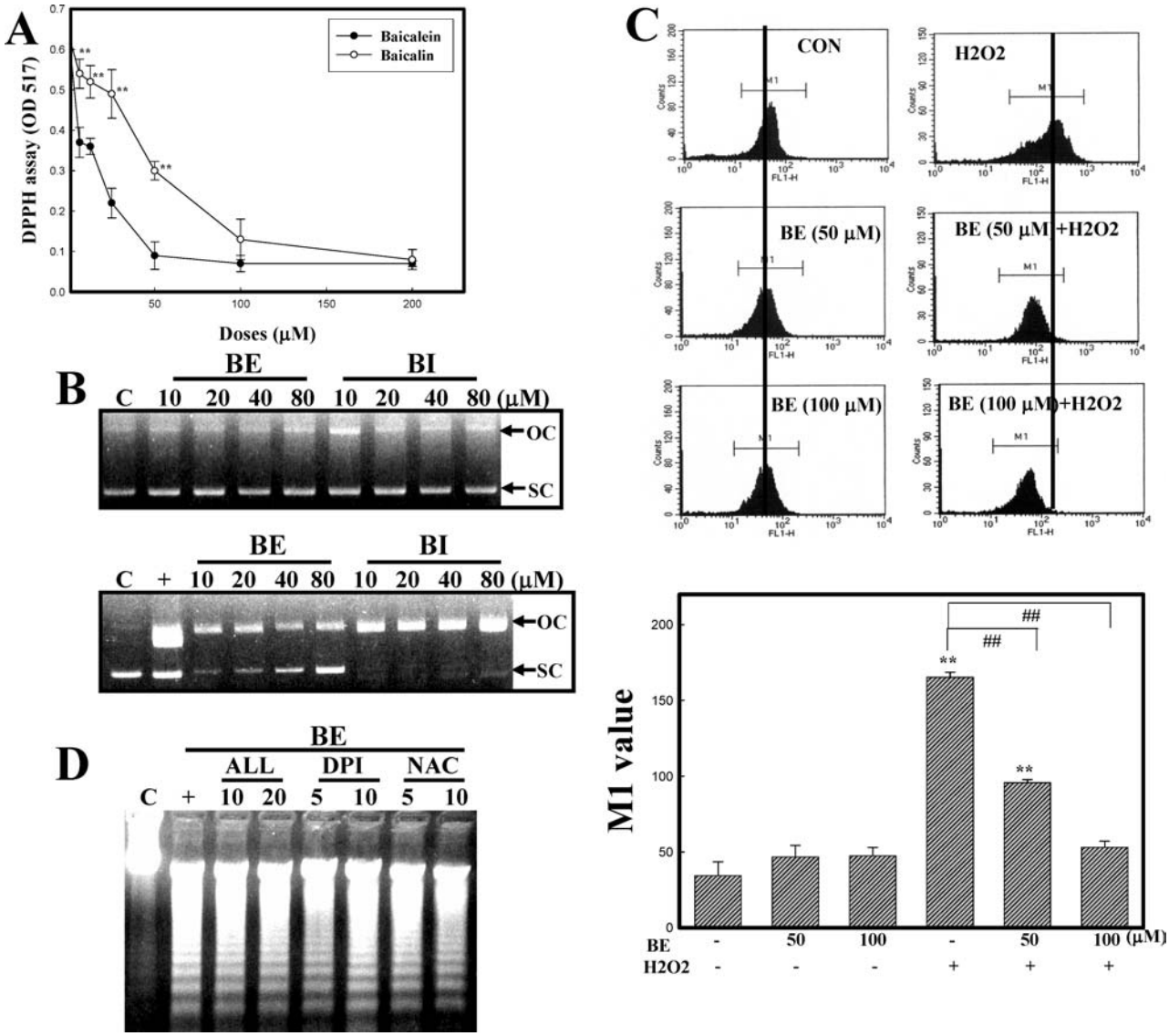

Fig. 4 Baicalein (BE)-mediated apoptosis depends on ROS production. (A) An anti-DPPH radical assay was performed to examine the effects of BE and baicalin (BI) on the production of DPPH radicals *in vitro*. Briefly, different concentrations (0, 12.5, 25, 50, 100, and 200 μM) of BE or BI were added to 10 mM Tris–HCl (pH 7.9) containing 500 μM DPPH in methanol for 20 min, and the value at 517 nm was determined. Each value is expressed as the mean ± SE of three independent experiments. (B) BE but not BI inhibited OH radical-induced DNA damage according to a plasmid digestion assay. The pBR322 plasmid was incubated with different concentrations of BE or BI in the presence (*lower panel*) or absence (*upper panel*) of an OH production system as described in "Materials and methods." Conformational changes in the plasmid under different treatments were analyzed by agarose electrophoresis. OC, open-circle form; SC, supercoiled form. (C) BE reduced the intracellular peroxide production induced by H_2O_2. HL-60 cells were treated with BE or BI (40 or 80 μM) for 30 min with or without H_2O_2 (200 μM) treatment for a further hour. At the end of the reaction, DCHF-DA was added to the medium for 30 min, and the fluorescence intensity in cells was detected by flow cytometric analysis. (*Upper panel*) A representative of the flow cytometric analysis is presented. (*Lower panel*) Quantification of the values from three independent experiments is shown, and each value is expressed as the mean ± SE. $^{**}p < 0.01$, significantly different from the control as analyzed by Student's *t*-test. $^{\#\#}p < 0.01$, significantly different from indicated groups. (D) Antioxidants including NAC, ALL, and DPI showed no protective effect against BE-induced DNA ladder formation in HL-60 cells. Cells were treated with different concentrations of NAC (5 and 10 mM), ALL (10 and 20 μM), and DPI (5 and 10 μM) for 30 min followed by BE (80 μM) treatment for a further 12 h. The integrity of DNA was analyzed by electrophoresis as described previously

TPA inhibition of BE-induced apoptosis occurs via blocking mitochondrial dysfunction in HL-60 cells

Since mitochondria are an important organelle in apoptosis induction, we investigated the role of mitochondria in TPA prevention of BE-induced apoptosis. DiOC6 is a fluorescent dye and is used to detect the mitochondrial membrane potential via flow cytometric analysis. Data in Fig. 7(A) show that a decrease in the mitochondrial membrane potential was detected in BE-treated HL-60 cells, which was prevented by

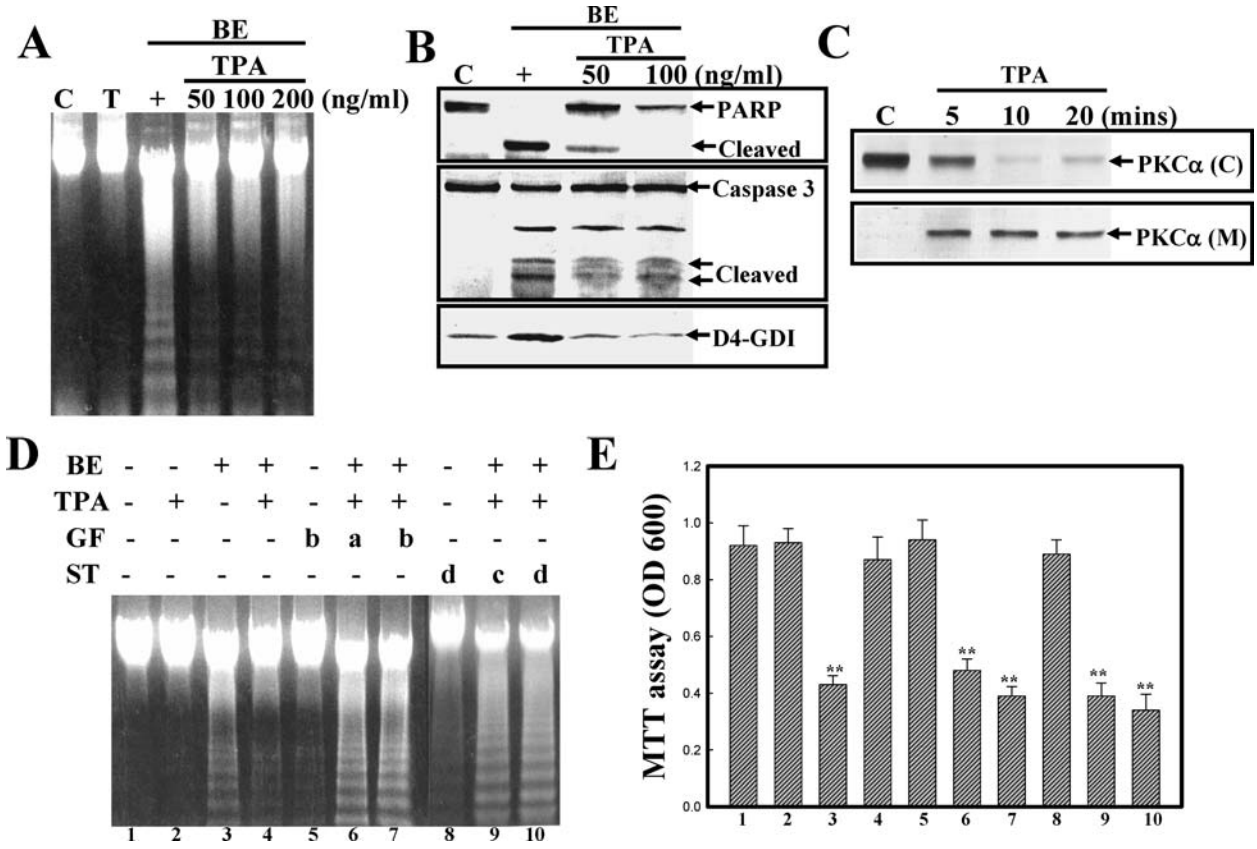

Fig. 5 TPA incubation inhibited baicalein (BE)-induced apoptosis in HL-60 cells. (A) TPA treatment inhibited BE-induced DNA ladder formation in HL-60 cells. Cells were treated with or without TPA (50, 100 or 200 ng/ml) for 30 min followed by BE (80 μM) treatment for a further 12 h. The integrity of DNA in cells under different treatments was analyzed by agarose electrophoresis. T, TPA (200 ng/ml)-treated HL-60 cells. (B) TPA inhibited BE-induced protein processing of caspase 3, PARP, and the D4-GDI protein. HL-60 cells were treated with TPA (50 and 100 ng/ml) for 30 min followed by BE (80 μM) treatment for a further 12 h. The expression of indicated proteins was analyzed by Western blotting. (C) Induction of PKCα protein translocation from cytosol to membrane was detected in TPA-treated HL-60 cells. Cells were treated with TPA (100 ng/ml) for different times (5, 10, and 20 min), and the expression of PKCα protein in both cytosolic and membrane fractions was detected by Western blotting. (D) The PKC inhibitors, ST (staurosporine; a broad-spectrum kinase inhibitor) and GF-109203X (GF; a highly selective PKC inhibitor), reversed the protective effect of TPA against BE-induced apoptosis. HL-60 cells were pre-treated with GF or ST for 30 min, followed by adding TPA for a further 30 min. At the end of incubation, cells were treated with BE for 12 h, and the viability and DNA ladders of cells were analyzed. HL-60 Cells were treated with GF (a, 2 μM; b, 4 μM) or ST (c, 25 nM; d, 50 nM) for 30 min followed by adding TPA (100 ng/ml) for a further 30 min. At the end of reaction, BE (80 μM) was added into each group for 12 h, and the integrity of DNA in HL-60 cells under different treatments was analyzed. (E) As described in (D), the viability of cells under different treatments was detected by the MTT assay. $^{**}p < 0.01$, significantly different from the control as analyzed by Student's *t*-test

TPA addition. Incubation of cells with the PKC inhibitor, GF, disrupted the protective effect of TPA on the membrane potential of mitochondria. A decrease in the antiapoptotic Bcl-2 (not Mcl-1) protein and an increase in the proapoptotic Bad protein via phosphorylation induction were detected in BE- but not BI-treated HL-60 cells (Fig. 7(B)). Translocation of Cyt *c* from mitochondria to the cytosol has been shown in mitochondria-dependent apoptosis. Data in Fig. 7(C) show that BE treatment induced the release of Cyt c protein from mitochondria to the cytosol in HL-60 cells. TPA treatment significantly attenuated the BE-induced cytoxolic Cyt *c* protein, which was interrupted by the PKC inhibitor, GF, and the JNK inhibitor, SP, respectively (Fig. 7D and E). Furthermore, TPA prevention of BE-induced caspase 9 enzyme activity was detected by adding a peptidyl substrate Ac-LEHD-pNA, which was blocked by GF and SP (Data not shown).

Discussion

In the current work, we demonstrate that BE, but not its glycoside, BI, stimulated apoptosis in human leukemia cells via an ROS-independent mitochondrial pathway. TPA addition significantly reduced the apoptotic events induced by BE in HL-60 cells through activation of JNK protein phosphorylation to block mitochondrial dysfunction induced by BE. Scientific evidence to support the preventive mechanism of TPA against BE-induced apoptosis via activation of PKC-JNKs is provided in the present study.

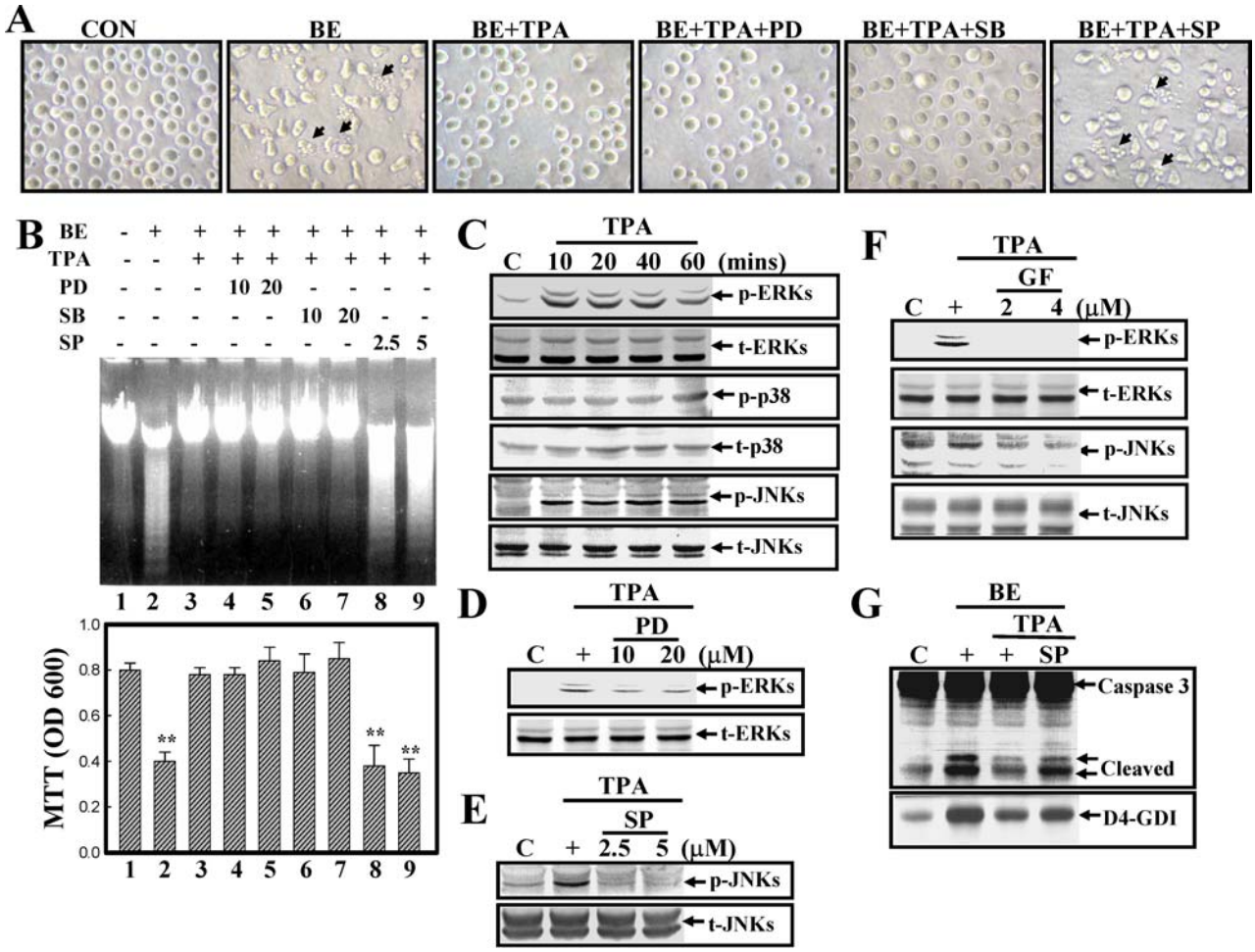

Fig. 6 Activation of JNKs is involved in TPA prevention of baicalein (BE)-induced apoptosis (A) The JNK inhibitor, SP600125 (SP), but not the ERK inhibitor, PD98059 (PD), or the p38 inhibitor, SB203580 (SB), inhibited the protective effect of TPA on BE-induced apoptotic bodies in HL-60 cells under microscopic observation. HL-60 cells were treated with PD (20 μM), SB (20 μM), and SP (5 μM) for 30 min followed by TPA (100 ng/ml) treatment for an additional 30 min. The cells were treated with BE (80 μM) for 12 h, and the morphology of cells under different treatments was examined via microscopic observation. Arrows indicate the apoptotic bodies. (B) The JNK inhibitor SP (2.5 and (5 μM), but not PD or SB (10 and 20 μM), inhibited TPA's protection against BE-induced DNA ladder formation in HL-60 cells. Cells were treated with PD, SB, and SP for 30 min, followed by incubation with TPA (100 ng/ml) for an additional 30 min. HL-60 cells under different treatments were treated with BE (80 μM) for 12 h, and the integrity of DNA was analyzed by agarose electrophoresis (*Upper panel*). As described in upper panel, the viability of cells under different treatments was evaluated by MTT assay (*Lower panel*). $^{**}p < 0.01$, significantly different from the control as analyzed by Student's *t*-test. (C) TPA addition induced ERKs and JNKs, but not p38, protein phosphorylation in HL-60 cells. Cells were treated with TPA (100 ng/ml) for different times (10, 20, 40, 60 min), and the expressions of total (*t*-ERKs, *t*-p38, *t*-JNKs) and phosphorylated (*p*-ERKs, *p*-p38, *p*-JNKs) ERKs, p38, and JNKs proteins were detected by Western blotting using specific antibodies. (D) PD addition inhibited TPA-induced ERKs protein phosphorylation in HL-60 cells. HL-60 cells were treated with PD (10 and 20 μM) for 30 min, followed by incubation with TPA for an additional 40 min. The expressions of total (*t*-ERKs) and phosphorylated (*p*-ERKs) ERKs proteins were detected as described in (E) As the condition described in (D), SP (2.5 and 5 μM) addition inhibits TPA-induced JNKs protein phosphorylation in HL-60 cells. (F) GF-109203X (GF; 2 and 4 μM) inhibits TPA-induced phosphorylated, but not total, ERKs and JNKs protein in TPA-treated HL-60 cells. As described in (E), the expression of phosphorylated and total ERKs and JNKs proteins is detected by Western blotting using specific antibodies. (G) SP (5 μM) addition prevents TPA-inhibited caspase 3 and D4-GDI protein cleavage induced by BE in HL-60 cells. As described previously, the expression of caspase 3 and D4-GDI protein was detected by Western blotting

The release of Cyt *c* from mitochondria to the cytosol in accordance with loss of the mitochondrial membrane potential has been shown in apoptosis, which is regulated by Bcl-2 family proteins. Activation of proapoptotic Bcl-2 family proteins such as Bax and Bad, and inactivation of antiapoptotic Bcl-2 family proteins such as Bcl-2 cause permeabilization of the outer mitochondrial membrane, facilitating the release of Cyt *c* from mitochondria to the cytosol in response to apoptotic stimuli [28–31]. Bad is regulated by a variety of kinases in vitro via phosphorylation of serine residues. Phosphorylation of Bad by AKT/PKB on Ser 112 and 135 transduces the survival signals in cells

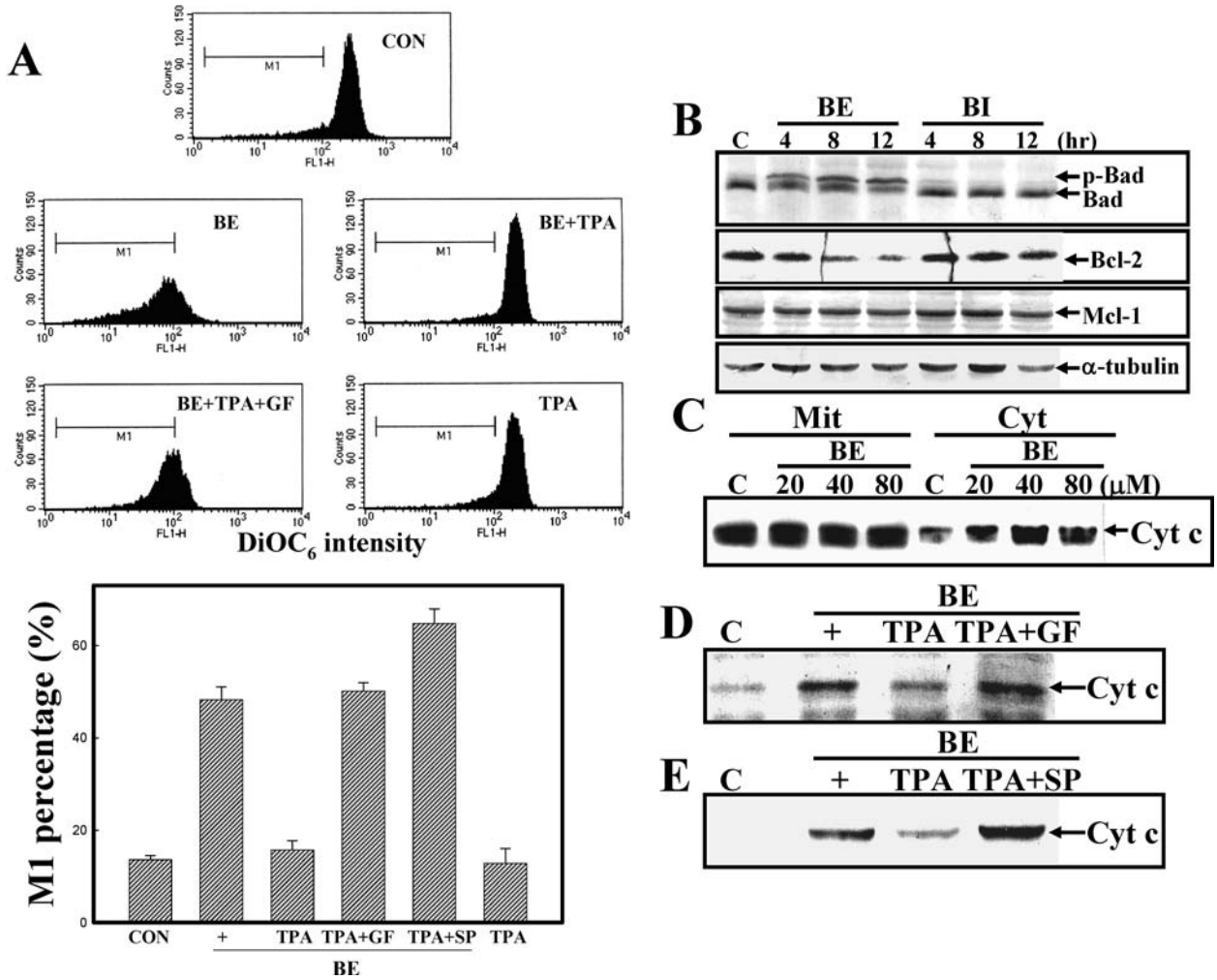

Fig. 7 Reduction of the mitochondrial membrane potential with the release of cytochrome (Cyt) *c* protein to the cytosol is involved in baicalein (BE)-induced apoptosis. (A) TPA protected against BE-induced loss of the mitochondrial membrane potential which was blocked by GF. Cells were treated with TPA (100 ng/ml) and TPA + GF (5 μM) with or without BE (80 μM) for 6 h, and $DiOC_6(3)$ was added to the culture medium for a further 30 min at the end of the reaction. The fluorescence intensity of $DiOC_6(3)$ in cells was analyzed by flow cytometry. (*Upper panel*) A representative example of the data is presented here. (*Lower panel*) Data derived from three independent experiments were quantitated, and are expressed as the mean ± SE. (B) BE, but not baicalin (BI), induced Bad protein phosphorylation and a decrease in the Bcl-2 protein. Cells were treated with BE or BI (80 μM) for different times (4, 8, and 12 h), and the expression of the indicated proteins was analyzed by Western blotting. (C) the release of the Cyt *c* protein from mitochondria to the cytosol was detected in BE-treated HL-60 cells. Cells were treated with different concentrations of BE for 12 h, and the expression of Cyt *c* in both mitochondrial (Mit) and cytosolic (Cyt) fractions was examined by Western blotting. (D, E) TPA inhibition of BE-induced Cyt *c* protein translocation was blocked by GF (D) and SP (E). Cells were treated with GF (2 μM) or SP (5 μM) for 30 min, followed by TPA (100 ng/ml) treatment for an additional 30 min. At the end of the reaction, BE (80 μM) was added for 12 h, and the expression of cytoxolic Cyt *c* protein was examined by Western blotting

[32], and PKA phosphorylates Bad protein at Ser 135 to attenuate the binding affinity of Bad with Bcl-2 [33]. Betito and Cuviillier indicated that sphingosine 1-phosphate inhibited Fas-induced apoptosis by stimulation of Bad protein phosphorylation in Jurkat cells [34]. In contrast to the antiapoptotic effect of phosphorylated Bad, Konishi et al. indicated that phosphorylation of the Bad protein by Cdc2 kinase was linked to apoptosis induction in cells [35]. In the present study, we found that the Bad protein seems to be phosphorylated in accordance with decreasing Bcl-2 protein expression in BE- but not BI-treated HL-60 cells. The mechanism of BE-induced protein phosphorylation in HL-60 cells is still unclear. Analysis of the mitochondrial membrane potential and Cyt c protein expression in both mitochondria and cytosol fractions showed that BE treatment induced a loss in the mitochondrial membrane potential, with release of Cyt c protein from mitochondria to the cytosol. These data support the existence of mitochondria-dependent apoptosis in BE-treated HL-60 cells.

ROS are generated by various agents and have been considered to contribute to the pathogenesis of such human diseases as diabetes, cancer, and arthritis. ROS may play the

role of second messengers in various signal transductions to elicit several responses such as growth arrest and apoptosis. Both the apoptotic and antiapoptotic properties of BE have previously been reported. Suk et al. indicated that BE inhibited lipopolysaccharide-induced apoptosis via reducing NO production [36]. Lee et al. suggested that BE addition inhibited cisplatin-induced apoptosis of human glioma cells [37]. Additionally, Lebeau et al. showed that BE possessed the ability to protect rat cortical cells from A β-induced apoptosis [7]. Although several biological effects of BE have been reported, the role of ROS in BE-induced apoptosis is still unclear. Data of the present study show that BE addition did not change the level of intracellular peroxide, and significantly reduced H_2O_2-induced peroxide production according to the DCHF-DA assay in HL-60 cells. Furthermore, BE prevented OH radical-induced DNA damage according to the plasmid digestion assay, and incubation of cells with the antioxidants, NAC, ALL, and DPI, produced no preventive effect on BE-induced apoptosis. These data support the supposition that BE-induced apoptosis is ROS-independent.

Mitogen-activated protein kinases including ERKs, JNKs, and p38 form a signal cascade to link several cell functions such as proliferation, differentiation, and apoptosis. JNKs play important roles in regulating cell viability, and targeted disruption of JNKs results in defects in apoptosis. In macrophages, suppression of JNKs results in cell cycle arrest at G2/M and apoptosis in response to CSF-1 treatment [38]. Several studies also indicated that activation of JNKs might contribute to apoptosis. Yin et al. indicated that JNK activation participated in apoptosis during spinal cord injury [39]. Jin et al. suggested that apoptosis induced by nitric oxide is mediated by sustained JNK activation which activates the proapoptotic Bcl-2 family proteins, Bim and Bax, in human glioblastoma cells [40]. SP600125 has been shown to have more than 300-fold selectivity for JNK activity over ERKs and p38. Data of the present study show that incubation of HL-60 cells with SP600125 (but not PD98059 or SB203580) induced DNA fragmentation, accompanied by the occurrence of PARP, caspase 3, and D4-GDI protein cleavage, in HL60 cells. TPA's protection against BE-induced cell death is by the induction of JNK protein phosphorylation, which was suppressed by the JNK inhibitor, SP600125. These data indicate that activation of JNKs is involved in TPA prevention of BE-induced apoptosis in HL-60 cells. Additionally, we found that the addition of the PKC inhibitor, GF, inhibited JNK protein phosphorylation induced by TPA (data not shown). This suggests that the protective effect of TPA against BE-induced apoptosis occurs through activation of the PKC-JNK pathway.

Our previous studies showed that glycoside addition attenuates the biological effects of flavonoids including the NO-inhibitory and apoptosis-inducing activities [41, 42]. Data of the present study also support glycoside addition reducing the apoptosis-inducing activity of BE in leukemia cells. In conclusion, we provide additional scientific evidence to confirm the apoptosis-inducing mechanism of BE in human leukemia cells, and a pharmacological study indicated that activation of PKC-JNKs participates in TPA protection of BE-induced apoptosis. This suggests that agents possessing the ability to inhibit PKC-JNK activation have good potential for further development.

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References

1. Chan FL, Choi HL, Chen ZY, Chan PS, Huang Y (2000) Induction of apoptosis in prostate cancer cell lines by a flavonoid, baicalin. Cancer Lett 160:219–228
2. Lee HJ, Noh YH, Lee Do Y, Kim YS, Kim KY, Chung YH, Lee WB, Kim SS (2005) Baicalein attenuates 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y cells. Eur J Cell Biol 84:897–905
3. Chen CJ, Raung SL, Liao SL, Chen SY (2004) Inhibition of inducible nitric oxide synthase expression by baicalein in endotoxin/cytokine-stimulated microglia. Biochem Pharmacol 67:957–965
4. Chen YC, Shen SC, Chen LG, Lee TJ, Yang LL (2001) Wogonin, baicalin, and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide. Biochem Pharmacol 61:1417–1427
5. Kimura Y, Matsushita N, Yokoi-Hayashi K, Okuda H (2001) Effects of baicalein isolated from *Scutellaria baicalensis* Radix on adhesion molecule expression induced by thrombin and thrombin receptor agonist peptide in cultured human umbilical vein endothelial cells. Planta Med 67:331–334
6. Gao Z, Huang K, Xu H (2001) Protective effects of flavonoids in the roots of *Scutellaria baicalensis* Georgi against hydrogen peroxide-induced oxidative stress in HS-SY5Y cells. Pharmacol Res 43:173–178
7. Lebeau A, Esclaire F, Rostene W, Pelaprat D (2001) Baicalein protects cortical neurons from beta-amyloid (25–35) induced toxicity. Neuroreport 12:2199–2202
8. Wong BC, Wang WP, Cho CH, Fan XM, Lin MC, Kung HF, Lam SK (2001) 12-Lipoxygenase inhibition induced apoptosis in human gastric cancer cells. Carcinogenesis 22:1349–1354
9. Pidgeon GP, Kandouz M, Meram A, Honn KV (2002) Mechanisms controlling cell cycle arrest and induction of apoptosis after 12-lipoxygenase inhibition in prostate cancer cells. Cancer Res 62:2721–2727
10. Tong WG, Ding XZ, Witt RC, Adrian TE (2002) Lipoxygenase inhibitors attenuate growth of human pancreatic cancer xenografts and induce apoptosis through the mitochondrial pathway. Mol Cancer Ther 1:929–935
11. Po LS, Chen ZY, Tsang DS, Leung LK (2002) Baicalein and genistein display differential actions on estrogen receptor (ER) transactivation and apoptosis in MCF-7 cells. Cancer Lett 187:33–40
12. Lee HZ, Leung HW, Lai MY, Wu CH (2005) Baicalein induced cell cycle arrest and apoptosis in human lung squamous carcinoma CH27 cells. Anticancer Res 25:959–964

13. Ko CH, Shen SC, Lin HY, Hou WC, Lee WR, Yang LL, Chen YC (2002) Flavanones' structure-related inhibition on TPA-induced tumor promotion through suppression of extracellular signal-regulated protein kinases: involvement of prostaglandin E2 in anti-promotive process. J Cell Physiol 193:93–102
14. Matsuzaki Y, Takaoka Y, Hitomi T, Nishino H, Sakai T (2004) Activation of protein kinase C promotes human cancer cell growth through downregulation of p18 (INK4c). Oncogene 23:5409–5414
15. Chen Y, Wu Q, Song SY, Su WJ (2002) Activation of JNK by TPA promotes apoptosis via PKC pathway in gastric cancer cells. World J Gastroenterol 8:1014–1018
16. Shim M, Eling TE (2005) Protein kinase C-dependent regulation of NAG-1/placental bone morphogenic protein/MIC-1 expression in LNCaP prostate carcinoma cells. J Biol Chem 280:18636–18642
17. Garzotto M, White-Jones M, Jiang Y, Ehleiter D, Liao WC, Haimovitz-Friedman A, Fuks Z, Kolesnick R (1998) 12-*O*-tetradecanoylphorbol-13-acetate-induced apoptosis in LNCaP cells is mediated through ceramide synthase. Cancer Res 58:2260–2264
18. Gonzalez-Guerrico AM, Kazanietz MG (2005) Phorbol ester-induced apoptosis in prostate cancer cells via autocrine activation of the extrinsic apoptotic cascade: a key role for protein kinase C delta. J Biol Chem 280:38982–38991
19. Schaar DG, Liu H, Sharma S, Ting Y, Martin J, Krier C, Ciardella M, Osman M, Goodell L, Notterman DA, Strair RK (2005) 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced dual-specificity phosphatase expression and AML cell survival. Leuk Res 29:1171–1179
20. Zhuang S, Demirs JT, Kochevar IE (2001) Protein kinase C inhibits singlet oxygen-induced apoptosis by decreasing caspase-8 activation. Oncogene 20:6764–6776
21. Shin HJ, Gye MH, Chung KH, Yoo BS (2002) Activity of protein kinase C modulates the apoptosis induced by polychlorinated biphenyls in human leukemic HL-60 cells. Toxicol Lett 135:25–31
22. Tanaka Y, Gavrielides MV, Mitsuuchi Y, Fujii T, Kazanietz MG (2003) Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. J Biol Chem 278:33753–33762
23. Shen SC, Ko CH, Hsu KC, Chen YC (2004) 3-OH flavone inhibition of epidermal growth factor-induced proliferation through blocking prostaglandin E2 production. Int J Cancer 108:502–510
24. Shen SC, Ko CH, Tseng SW, Tsai SH, Chen YC (2004) Structurally related antitumor effects of flavanones *in vitro* and *in vivo*: involvement of caspase 3 activation, p21 gene expression, and reactive oxygen species production. Toxicol Appl Pharmacol 197:84–95
25. Chen YC, Shen SC, Chow JM, Ko CH, Tseng SW (2004) Flavone inhibition of tumor growth via apoptosis *in vitro* and *in vivo*. Int J Oncol 25:661–670
26. Chen YC, Shen SC, Lin HY (2003) Rutinoside at C7 attenuates the apoptosis-inducing activity of flavonoids. Biochem Pharmacol 66:1139–1150
27. Ko CH, Shen SC, Chen YC (2004) Hydroxylation at C4′ or C6 is essential for apoptosis-inducing activity of flavanone through activation of the caspase-3 cascade and production of reactive oxygen species. Free Radic Biol Med 36:897–910
28. Jin HO, Park IC, An S, Lee HC, Woo SH, Hong YJ, Lee SJ, Park MJ, Yoo DH, Rhee CH, Hong SI (2006) Up-regulation of Bak and Bim via JNK downstream pathway in the response to nitric oxide in human glioblastoma cells. J Cell Physiol 206:477–486
29. Hetz C, Vitte PA, Bombrun A, Rostovtseva TK, Montessuit S, Hiver A, Schwarz MK, Church DJ, Korsmeyer SJ, Martinou JC, Antonsson B (2005) Bax channel inhibitors prevent mitochondrion-mediated apoptosis and protect neurons in a model of global brain ischemia. J Biol Chem 280:42960–42970
30. Gao Z, Shao Y, Jiang X (2005) Essential roles of the Bcl-2 family of proteins in caspase-2-induced apoptosis. J Biol Chem 280:38271–38275
31. Koc M, Nadóva Z, Truksa J, Ehrlichova M, Kovar J (2005) Iron deprivation induces apoptosis via mitochondrial changes related to Bax translocation. Apoptosis 10:381–393
32. She QB, Solit DB, Ye Q, O'Reilly KE, Lobo J, Rosen N (2005) The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. Cancer Cell 8:287–297
33. Yu F, Sugawara T, Maier CM, Hsieh LB, Chan PH (2005) Akt/Bad signaling and motor neuron survival after spinal cord injury. Neurobiol Dis 20:491–499
34. Betito S, Cuvillier O (2006) Regulation by sphingosine 1-phosphate of Bax and Bad activities during apoptosis in a MEK-dependent manner. Biochem Biophys Res Commun 340:1273–1277
35. Konishi Y, Lehtinen M, Donovan N, Bonni A (2002) Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. Mol Cell 9:1005–1016
36. Suk K, Lee H, Kang SS, Cho GJ, Choi WS (2003) Flavonoid baicalein attenuates activation-induced cell death of brain microglia. J Pharmacol Exp Ther 305:638–645
37. Lee SW, Song GS, Kwon CH, Kim YK (2005) Beneficial effect of flavonoid baicalein in cisplatin-induced cell death of human glioma cells. Neurosci Lett 382:71–75
38. Himes SR, Sester DP, Ravasi T, Cronau SL, Sasmono T, Hume DA (2006) The JNKs are important for development and survival of macrophages. J Immunol 176:2219–2228
39. Yin KJ, Kim GM, Lee JM, He YY, Xu J, Hsu CY (2005) JNK activation contributes to DP5 induction and apoptosis following traumatic spinal cord injury. Neurobiol Dis 20:881–889
40. Jin HO, Park IC, An S, Lee HC, Woo SH, Hong YJ, Lee SJ, Park MJ, Yoo DH, Rhee CH, Hong SI (2006) Up-regulation of Bak and Bim via JNK downstream pathway in the response to nitric oxide in human glioblastoma cells. J Cell Physiol 206:477–486
41. Chow JM, Shen SC, Huan SK, Lin HY, Chen YC (2005) Quercetin, but not rutin and quercitrin, prevention of H_2O_2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages. Biochem Pharmacol 69:1839–1851
42. Ko CH, Shen SC, Hsu CS, Chen YC (2005) Mitochondrial-dependent, reactive oxygen species-independent apoptosis by myricetin: roles of protein kinase C, cytochrome *c*, and caspase cascade. Biochem Pharmacol 69:913–927