Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 2281–2289
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Involvement of tumor suppressor protein p53 and p38 MAPK in caffeic acid phenethyl ester-induced apoptosis of C6 glioma cells

Yean-Jang Lee[a], Hsing-Chun Kuo[b], Chia-Yih Chu[c], Chau-Jong Wang[b], Wan-Chyi Lin[b], Tsui-Hwa Tseng[c,*]

[a]*Department of Chemistry, National Changhua University of Education, Changhua, Taiwan, ROC*
[b]*Institute of Biochemistry, Chung Shan Medical University, Taichung, Taiwan, ROC*
[c]*Department of Applied Chemistry, Chung Shan Medical University, No. 110, Section 1, Chien Kuo N. Road, Taichung, Taiwan, ROC*

Received 14 February 2003; accepted 30 July 2003

Abstract

Caffeic acid phenethyl ester (CAPE), an active component of propolis, has many biological and pharmacological activities including antioxidant, anti-inflammation, antiviral action, and anticancer effect. Our previous studies showed that CAPE exhibited significant cytotoxicity in oral cancer cells. Herein we further investigated the cytotoxicity potential of CAPE and the mechanism of its action in C6 glioma cells. The data exhibited that C6 glioma cells underwent internucleosomal DNA fragmentation 24 hr after the treatment of CAPE (50 μM). The proportion of C6 glioma cells with hypodiploid nuclei was increased to 24% at 36 hr after the exposure. Further results showed that CAPE induced the release of cytochrome *c* from mitochondria into cytosol, and the activation of CPP32. CAPE application also enhanced the expression of p53, Bax, and Bak. Finally, the potential signaling components underlying CAPE induction of apoptosis were elucidated. We found that CAPE activated extracellular signal-regulated kinase (ERKs) and p38 mitogen-activated protein kinase (p38 MAPK) in C6 glioma cells. More importantly, p38 kinase formed a complex with p53 after the treatment of CAPE for 0.5 hr. The expression of p53, phospho-serine 15 of p53, and Bax, and inactivate form of CPP32 was suppressed by a pretreatment of a specific p38 MAPK inhibitor, SB203580. The resultant data suggest that p38 MAPK mediated the CAPE-induced p53-dependent apoptosis in C6 glioma cells.

Keywords: Apoptosis; Caffeic acid phenethyl ester; C6 glioma cell; p44/42 extracellular signal-regulated kinase; p38 mitogen-activated protein kinase; p53 protein

1. Introduction

Caffeic acid phenethyl ester (CAPE), an active ingredient of propolis that is widely used as a folk medicine, has a broad spectrum of biological activities including antioxidant, anti-inflammatory, and antiviral properties [1–5]. It also inhibited the development of azoxymethane-induced aberrant crypts in the colon of rats [6], and blocked tumorigenesis in a two-stage model of phorbol ester-promoted skin cancer [7]. Furthermore, several groups reported that CAPE was cytotoxic to tumor or virally transformed cells [8–10], but not to normal cells. Recently, we synthesized and studied the constituents of natural honey-propolis and found that CAPE exhibited strong antitumor effects in oral cancer cells [11]. Therefore, it is worthy to investigate the chemopreventative or antitumorigenesis mechanisms of CAPE in other cancer cell line.

The tumor suppressor protein p53 is a nuclear phosphoprotein that can potently regulate the growth of mammalian cells [12]. Activation of p53 results in altered transcription of a wide variety of genes that are involved in many aspects of cell metabolism, cell cycle regulation and apoptosis. Although the mechanism of p53-mediated apoptosis after cellular stress remains unclear, current evidence showed that p53 induces cell death by a multitude of molecular pathways involving transactivation of target genes and direct signaling events [13–15]. Genes transcriptionally up-regulated by p53 that have been implicated in promoting

* Corresponding author. Tel.: +886-4-24730022; fax: +886-4-23248189.
E-mail address: tht@csmu.edu.tw (T.-H. Tseng).

Abbreviations: CAPE, caffeic acid phenethyl ester; CPP32, cysteine protease 32 kDa proenzyme; DMSO, dimethyl sulfoxide; ERK, p44/42 extracellular signal-regulated kinase; p38 MAPK, p38 mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly(ADP-ribose) polymerase.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.07.014

apoptosis include the Bcl-2 family members Bax, Bak, and Noxa. It is implied that Bax or Bak function is required for the release of cytochrome *c* from the mitochondria to the cytosol during apoptosis [16,17]. Cytochrome *c* is released from the mitochondria in the apoptotic signaling pathways implemented by p53 and TNF-α. This event is pivotal in the regulation of apoptosis because cytochrome *c* complexes with APAF-1 in the cytosol which, in turn, promotes caspase-9 and caspase-3 (CPP32) activation. The activation of caspase cascade is required for p53-dependent apoptosis, and results in cleavage of cellular proteins, such as poly(ADP-ribose) polymerase (PARP), as well as DNA fragmentation and cell death.

Intracelluar transmission of extracellular signals is mediated by several groups of sequentially activated protein kinases, some of which (ERK, JNK, and p38 MAPK) belong to the superfamily of mitogen-activated protein kinases (MAPK) [18]. MAPKs play an important role in regulating cell growth and survival, and are also involved in both mitogenic and stress responses of cells [19]. Each MAPK is activated through a specific phosphorylation cascade. In general, the ERK pathway plays a major role in regulating cell growth and differentiation, being highly induced in response to growth factors and cytokines [20]. On the other hand, ERK is activated by some conditions of stress, particularly oxidant injury, and is believed to confer a survival advantage on cells in such circumstances. In contrast, JNK and p38 are only weakly activated by growth factors, but are highly activated in response to a variety of stress signals including tumor necrosis factor, hyperosmotic stress, and ultraviolet irradiation. Finally, their activation is most frequently associated with induction of apoptosis [21]. Herein the present study sought to examine the role of p53 and MAPKs signal pathway in regulating the CAPE-induced apoptosis in C6 glioma cells.

2. Materials and methods

2.1. Cell culture

The rat C6 glioma cell line was originally derived from a *N*-nitrosomethylurea-induced rat brain tumor [22]. Cells were cultured with minimal essential medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) at 37° in a humidified atmosphere of 5% CO_2 and 95% air. All experiments were performed in plastic tissue culture flasks, dish or in microplates (Nunc, Naperville, Dennmark).

2.2. Assessment of cell viability

The cytotoxicity assay was performed according to the MTT colorimetric assay [23]. The cells were seeded at 2×10^4 cells/mL density and incubated with CAPE at various concentrations (0, 10, 50, 100, 200, and 400 μM) for 24 hr. Thereafter the medium was changed and incubated with MTT (0.5 mg/mL) for 4 hr. The viable cell number is directly proportional to the production of formazan, which was then solublized with isopropanol, and measured spectrophotometrically at 563 nm.

2.3. Analysis of cellular DNA content

Flow cytometric analysis of the CAPE-treated C6 glioma cells was performed using a FACScan [23] (Becton Dickinson Immunocytometry Systems). The cells were washed twice with buffer solution, and the cell suspension was centrifuged at 400 *g* for 5 min at room temperature. After removed all the supernatant, trypsin buffer (250 μL) was added to the pellet. After incubation for 10 min at room temperature, trypsin inhibitor and RNase buffer were added at room temperature. Prior to the analysis by the flow cytometry, cold propidium iodide (PI) stain solution (200 μL) was added to the mixture and incubated in the darkness for 10 min on ice. The DNA content of the stained nuclei was analyzed by flow cytometry. The distribution of DNA content was expressed as G_1, S, and G_2/M phases. The cells with DNA content less than G_1 were distributed in pre-G_1 (hypodiploid cells) and expressed as the apoptotic phase.

2.4. Determination of DNA fragmentation

Both detached and attached cells were harvested by scraping and centrifugation. After washing with PBS (with 1 mM $ZnCl_2$), the cells were resuspended in 0.5 mL lysis buffer (0.5% Triton X-100, 20 mM EDTA, and 5 mM Tris; pH 8.0) for 45 min. Fragmented DNA in the supernatant fraction after centrifugation at 14,000 rpm was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and once with chloroform, and, then, precipitated with ethanol and 5 M NaCl overnight at −20°. The DNA pellet was washed once with 70% ethanol and resuspended in Tris–EDTA buffer (pH 8.0), and treated with 100 μg/mL RNase A for 2 hr at 56°. After quantitative analysis of DNA content by spectrophotometry (260 nm), an equal amount of DNA was electrophoresed in horizontal agarose gel (1.8%) performing at 1.5 V/cm for 3 hr. DNA in gel was visualized under UV light after staining with ethidium bromide (0.5 mg/mL).

2.5. Release of cytochrome c

The basic methodology for the preparation of mitochondria and cytosol fractions was described as Tang *et al.* [24]. Cells (3×10^6) were harvested and washed with ice-cold PBS at the end of treatment. The cell pellet was resuspended in 500 μL of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA,

1 mM leupeptin, 1 μg/mL pepstatin A, and 1 μg/mL chymostatin), and homogenized in the same buffer with a Pyrex glass homogenizer using a type B pestle (40 strokes). The homogenate was then centrifuged at 1000 *g* for 10 min at 4°. The resulting supernatant was subjected to another centrifugation at 10,000 *g* for 20 min at 4°. The supernatant was further centrifuged at 100,000 *g* (4° for 1 hr) to generate cytosol. The protein concentration was determined by a Bio-Rad protein assay kit, and 25 μg of each fraction was loaded onto a 15% SDS–PAGE. Proteins were then blotted onto NC membrane (Sartorious), and the membranes were reacted with the primary antibodies (anti-cytochrome *c* from Transduction Laboratories; anti-β-actin from Sigma as internal control). The secondary antibody was a peroxidase-conjugated goat anti-mouse antibody. After binding, the bands were revealed by enhanced chemiluminescence using the ECL commercial kit.

2.6. Preparation of total cell extracts and immunoblots analysis

Cells were plated onto 15 cm^2 dishes at a density of 2×10^5 cells/mL with or without CAPE (50 μM, 0–8 hr) and harvested. To prepare the whole-cell extract, cells were washed with PBS plus zinc ion (1 mM) and suspended in a lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/mL aprotinine, 170 μg/mL leupeptin, 100 μg/mL PMSF; pH 7.5). After mixing for 30 min at 4°, the mixtures were centrifuged (10,000 *g*) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined with Bio-Rad protein assay reagent using bovine serum albumin as a standard. The ECL western blotting was performed as follows. An equal protein content of total cell lysates from control and CAPE-treated samples was resolved on 10–12% SDS–PAGE gels along with prestained protein molecular weight standard (Bio-Rad). Proteins were then blotted onto NC membranes (Sartorious), and reacted with the primary antibodies (anti-CPP32, anti PARP, and anti-p53 from Transduction Laboratories; anti-Bcl-2, anti-Bax, anti-Bcl-Xs, anti-Bak, and anti-phospho-serine 15-p53 from New England Biolabs; anti-ERKs, anti-JNKs, anti-p38 MAPK, anti-phospho-serine, anti-phospho-ERKs (Thr^{183}/Tyr^{185}), anti-phospho-JNKs (Thr^{183}/Tyr^{185}), and anti-phospho-p38 (Thr^{180}/Tyr^{182}) from Promega Inc.; anti-β-actin from Sigma as internal control). The secondary antibody was a peroxidase-conjugated goat anti-mouse antibody. After binding, the bands were revealed by enhanced chemiluminescence using the ECL commercial kit.

2.7. In vitro caspase assay

The activity of CPP32/caspase-3 was determined in cell extracts using a colorimetric protease assay kit (Chemicon International Inc.). Cells were collected and protein concentration of cell lysates was determined. 100 μg of protein was incubated with reaction buffer containing 5 μL of peptide substrate (DEVD-*p*NA) for 2 hr at 37°. The cleavage of colorimetric peptide substrate was monitored by *p*NA (*p*-nitroanilide) liberation using a micotiter plate reader at 405 nm. The activity was expressed as fold change of OD value over 0 hr.

2.8. Immunoprecipitation assay

The effect of CAPE on the interaction of ERKs or p38 MAPK with p53 as well as serine phosphorylaiton of p53 was analyzed by immunoprecipitation assay. Briefly, C6 glioma cells were exposed to 50 μM of CAPE for 0–12 hr and then lysed with lysis buffer. The lysates were immunoprecipitated using monoclonal anti-p53 and protein A/G-agarose. The eluates from beads were analyzed by immunoblotting using specific antibodies against phosphorylated p38 MAPK or phosphorylated ERKs or phosphorylated serine.

2.9. Determination of the effects of MAPKs inhibitors

C6 glioma cells were pretreated with 50 μM of MEK inhibitor, PD98059, or 5 μM SB203580 for 1 hr, followed by treatment with 50 μM of CAPE for 12 hr. The cell extracts were analyzed by immunoblotting using specific antibodies against p53, phospho-serine 15-p53, Bax, or CPP32 as previously described.

2.10. Statistical analysis

Data were reported as mean ± SD of three independent experiments and evaluated by one-way ANOVA. Significant differences were established at $P < 0.05$.

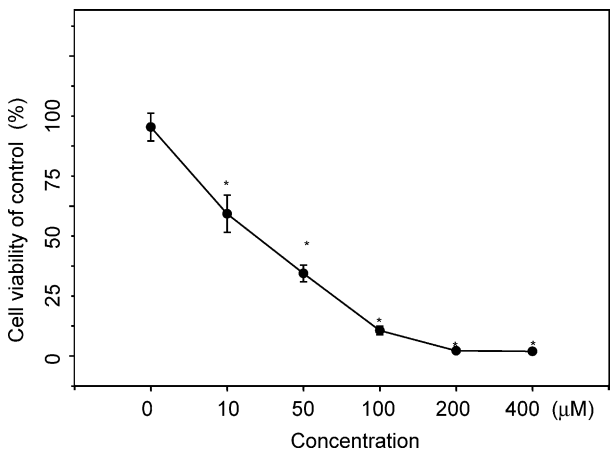

Fig. 1. Effect of CAPE on the cell viability of C6 glioma cells. C6 glioma cells were treated with either 0.2% DMSO (as control) or CAPE (10–400 μM) for 24 hr. The proportion of surviving cells was measured by the MTT assay as described in Section 2. The experiments were performed in triplicate. Data were presented as means ± SD of three independent experiments. $^*P < 0.05$, when compared with control group.

3. Results

3.1. Cytotoxicity of CAPE to C6 glioma cells

For most anticancer agents, cytotoxicity is measured by a standard MTT assay following a brief drug exposure. Figure 1 illustrates the results of MTT assays performed with the logarithmically growing C6 glioma cells treated with various concentrations of CAPE for 24 hr. The resulting survival curve shows that CAPE had a dose-dependent effect on the cytotoxicity of cells. The addition of 50 μM of CAPE reduced the viability to 42% of control that near IC_{50}, therefore, we use the concentration (50 μM) in the following investigation.

3.2. CAPE-induced apoptotic death

The DNA histogram of PI-stained cells in Fig. 2A resulted that the cells treated with 50 μM of CAPE for 24 and 36 hr had increased proportion with hypodiploid DNA, as indication of apoptosis. Within 36 hr the addition of CAPE caused an increase in the sub-G1 by 24%. In addition, the exposure of 50 μM CAPE for 24–36 hr also induced internucleosomal DNA fragmentation in C6

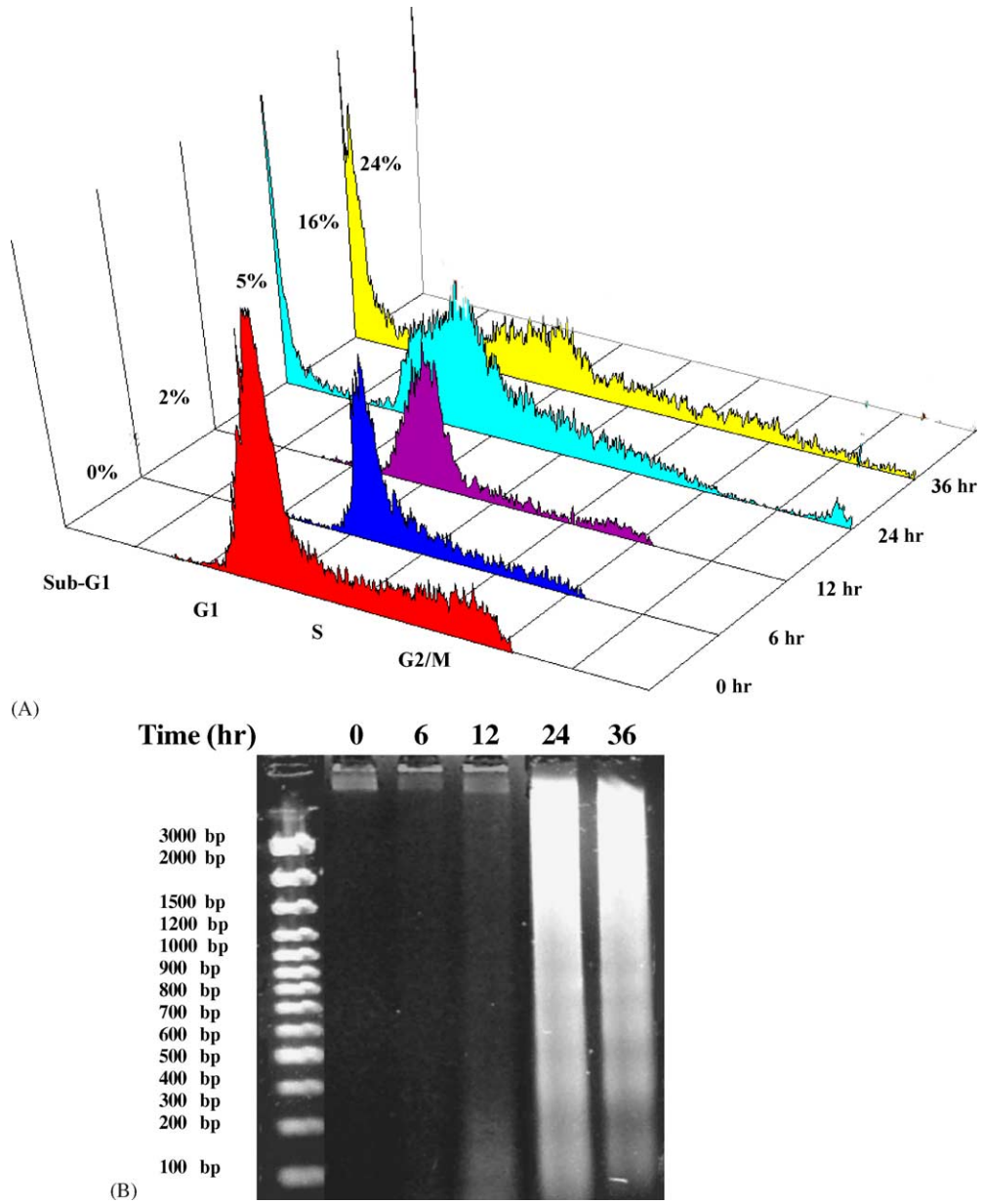

Fig. 2. CAPE induced apoptosis in C6 glioma cells. (A) Flow cytometric analysis of CAPE-treated C6 glioma cells. Cells were treated with 50 μM CAPE for 0–36 hr, stained with PI, and analyzed by flow cytometry to quantitate cells with a subdiploid DNA content. (B) Agarose gel electrophoresis of DNA from CAPE-treated C6 glioma cells. Cells were treated with 50 μM of CAPE for 0–36 hr, and assessed for DNA fragmentation assay as described in Section 2.

glioma cells as illustrated in Fig. 2B showing the characteristic ladder of oligonucleosomal DNA fragments.

3.3. Effect of CAPE on cytochrome c, CPP32, and PARP

Since cytochrome *c* is reported to be involved in the activation of the caspases that execute apoptosis, we first examined the level of cytochrome *c* in the cytosol by western blot analysis. This protein was detectable in the cytosol 3 hr after the treatment of CAPE (Fig. 3A). Because CPP32/caspase-3 (like) protease plays an essential role in executing apoptosis, we then examined its activity in C6 glioma cells cultured with the addition of CAPE for 0–48 hr. As shown in Fig. 3B, CAPE induced CPP32/caspase-3 (like) protease activity gradually from 3 to 48 hr. CPP32 is synthesized as a proenzyme, and is proteolytically cleaved into active heterodimers, when the cell undergoes apoptosis [25]. The results showed that cellular CPP32 was activated in response to CAPE treatment as assessed by the decline in the 32 kDa band from 3 to 36 hr after treatment (Fig. 3C). One of the substrates of CPP32 during apoptosis is PARP [26], an enzyme that appears to be involved in DNA repair, genome surveillance, and integrity, resulted from environmental stress. The proteolytic cleavage of PARP was used as an indicator of CPP32 activation. It is obvious that PARP was cleaved 12 hr after CAPE treatment (Fig. 3C).

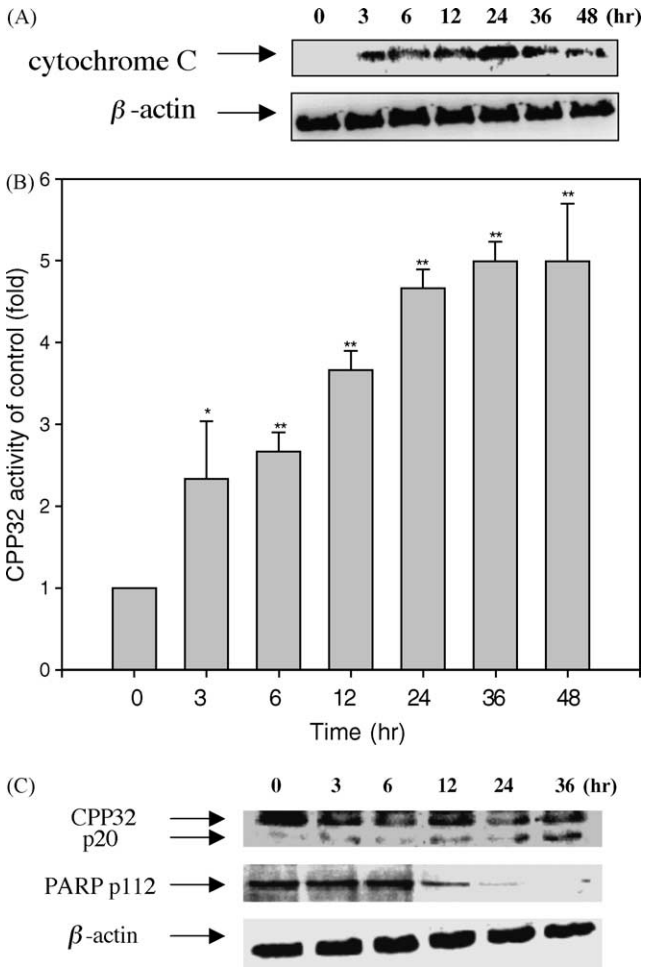

Fig. 3. Effect of CAPE on cytochrome *c*, CPP32, and PARP. (A) CAPE-induced translocation of cytochrome *c*. Equal amounts of protein from cytosolic fraction of C6 glioma cells which has been treated with 50 μM of CAPE for 0–48 hr were separated by a 15% SDS–PAGE, and, subsequently, immunoblotted with antibodies against cytochrome *c* and anti-β-actin. (B) Activation of caspase-3 (like) protease/CPP32 by CAPE. Cells were treated with 50 μM of CAPE for 0–48 hr, and determined photometrically (400 nm) by a colorimetric protease assay kit. Activity was expressed as fold change of OD value over 0 hr. The results were presented as means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, when compared with DMSO. (C) Effect of CAPE on CPP32 and PARP. Equal protein of total cell lysates for C6 glioma cells treated with 50 μM CAPE for the indicated times were analyzed by 12% SDS–PAGE, for CPP32 or 10% for PARP, and, subsequently, immunoblotted with antibody against CPP32, PARP, or β-actin which served as internal control.

3.4. Effect of CAPE on Bcl-2 family

Bcl-2 family proteins play a pivotal role in controlling cell life and death, with some members, such as Bcl-2 and Bcl-XL inhibiting apoptosis and others such as Bax, Bak, and Bcl-Xs, inducing cell death [27]. Therefore, we examined the cellular levels of Bcl-2 family proteins after the treatment of CAPE. Bax and Bak protein were increased after a 3-hr treatment, and Bcl-Xs level was also elevated after 12 hr (Fig. 4). On the other hand, Bcl-2 protein was obviously decreased after a 36-hr treatment (Fig. 4).

3.5. Effect of CAPE on the expression and serine phosphorylation of p53

Because there are p53-binding sites in the promoter of bax gene, we further studied the role of p53 in the CAPE-induced apoptosis. The regulation of p53 activity is through multiple mechanisms, one of which is phosphorylation. [28]. Therefore, we evaluated the effect of CAPE by determining the expression and serine phosphorylation of p53. The p53 protein level was augmented at 3 hr after treatment with CAPE (Fig. 5, upper panel), and the phosphorylation of p53 at serine was also induced by CAPE started from 0.5 hr after treatment (Fig. 5).

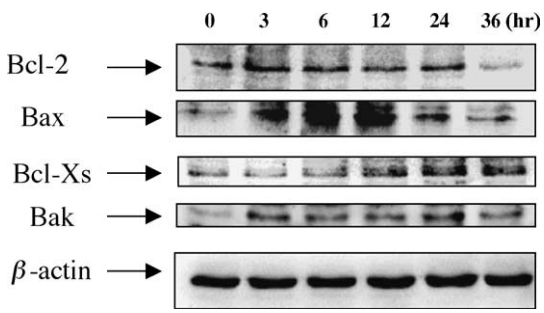

Fig. 4. Effect of CAPE on Bcl-2 family. Total cell lysates of C6 glioma cells treated with 50 μM of CAPE for indicated time were analyzed by 12% SDS–PAGE, and, subsequently, immunoblotted with antisera against Bcl-2, Bax, Bcl-Xs, Bak, and β-actin which served as an internal control.

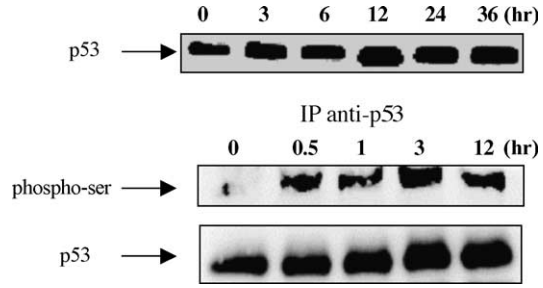

Fig. 5. Effect of CAPE on the expression and serine phosphorylation of p53. Total cell lysates of C6 glioma cells treated with 50 μM of CAPE for indicated time were analyzed by 10% SDS–PAGE, and, subsequently, immunoblotted with antisera against p53. In addition, lysates were immunoprecipitated using monoclonal antibody against p53 and phosphorylation at serine was detected as described in Section 2.

3.6. Activation MAPKs by CAPE

Because p53 is phosphorylated by various protein kinases, we next monitored the activation of three members of the MAPK family, p38 MAPK, JNK, and ERK, using the antibodies against the activated phosphorylated forms of the three kinases. As shown in Fig. 6A, we found that exposure to CAPE induced the phosphorylation of p38 MAPK and ERK but not JNK. We then tested the association of the activated p38 MAPK and ERKs with p53. It turned out that p38 MAPK was associated with p53 in the cells treated with CAPE from 0.5 to 12 hr after administration (Fig. 6B). On the other hand, ERKs appeared to be weakly interacted with p53.

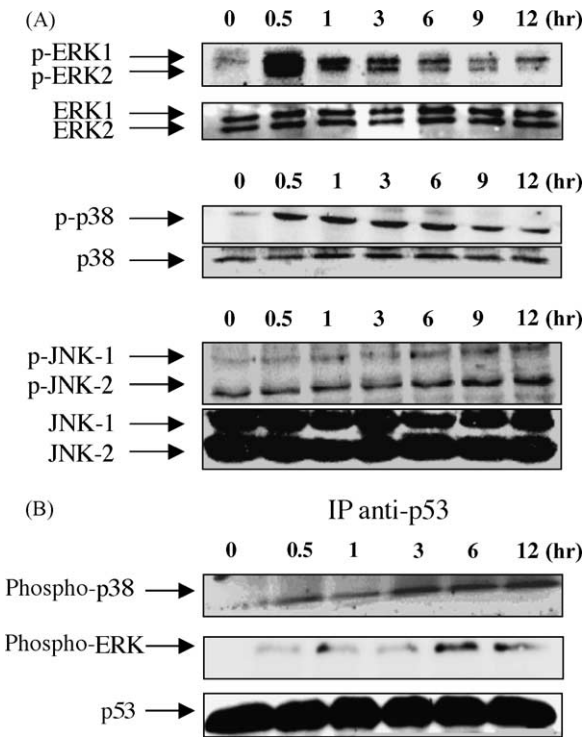

Fig. 6. Activation MAPKs by CAPE in C6 glioma cells. (A) Total cell lysates of C6 glioma cells treated with 50 μM of CAPE for indicated time were extracted, and the phosphorylated and total proteins of ERKs, p38 MAPK, and JNKs were immunodetected as described previously. (B) Association of p53 with p38 MAPK and ERKs. Total cell lysates were immunoprecipitated using monoclonal antibody against p53. The p53 immunoprecipitates were immunodetected with antibodies against phospho-p38 MAPK and phospho-ERKs.

3.7. Involvement of p38 MAPK in CAPE-induced activation of p53 and CPP32

To further test the role of p38 MAPK and ERK in p53 phosphorylation and CPP32 activation induced by CAPE, the inhibitors of MAPKs were used to block the enzymes in C6 glioma cells followed by CAPE treatment for 20 hr. As shown in Fig. 7, p38 MAPK inhibitor (5 μM), SB203580, inhibited the expression of p53, phospho-p53 (serine 15),

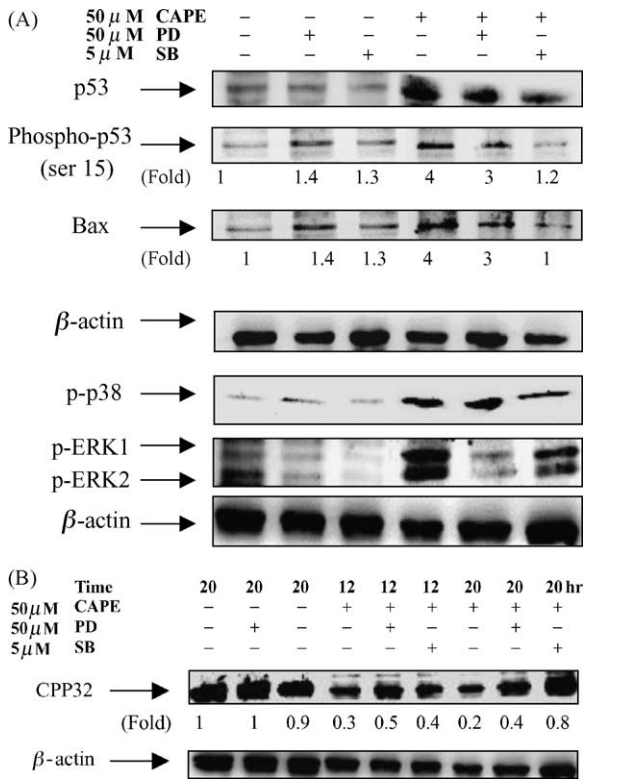

Fig. 7. Involvement of p38 MAPK in CAPE-induced activation of p53 and CPP32. (A) Inhibition of p53 accumulation and phosphorylation and Bax expression by p38 MAPK inhibitor, SB203580 and MEK inhibitor, PD98059. C6 glioma cells were preteated with or without 5 μM of SB203580 or 50 μM of PD98059 for 1 hr, followed by the treatment of 50 μM CAPE for 12 hr. Total cell lysates were analyzed by 10% SDS–PAGE and subsequently immunoblotted with antisera against p53, phospho-serine 15 p53, Bax, phospho-p38 MAPK, phospho-ERKs, and β-actin, which served as an internal control. (B) Inhibition of CPP32 activation by p38 MAPK inhibitor, SB203580 and MEK inhibitor, PD98059. C6 glioma cells were preteated with or without 5 μM of SB203580 or 50 μM of PD98059 for 1 hr, followed by the treatment of 50 μM CAPE for 12 hr and 20 hr. Total cell lysated were then analyzed by 12% SDS–PAGE with subsequent immunoblotting with antisera against p32 and β-actin which served as an internal control.

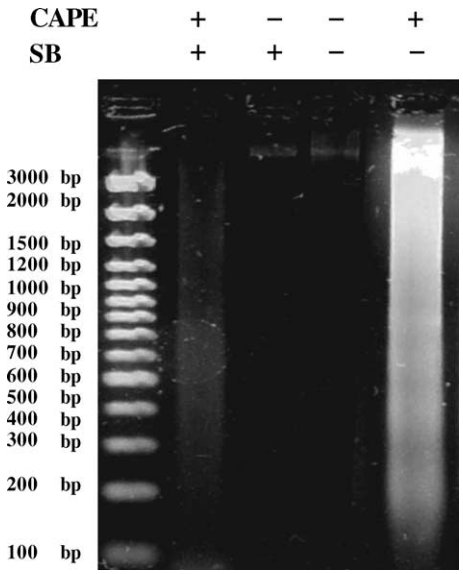

Fig. 8. Effects of the p38 kinase inhibitor in the CAPE-induced cell death. C6 glioma cells were pretreated with 5 μM SB203580 (or with solvent DMSO) for 1 hr, followed by the treatment of 50 μM CAPE for 24 hr. The cells were then assessed for DNA fragmentation assay as described in the text.

Bax (Fig. 7A) and the activation of CPP32 induced by CAPE, but abrogated only partially p38 MAPK phosphorylation. However, the inhibitor of MEK1, PD98059, abrogated almost totally the phosphorylation of ERK1/2 induced by CAPE, but inhibited only partially the expression of p53, phospho-p53 (serine 15), Bax (Fig. 7A) and the activation of CPP32. These results indicated that p38 MAPK involved in the CAPE-induced p53-dependent apoptosis.

3.8. Effect of SB203580 in CAPE-induced apoptosis of C6 glioma cells

To investigation the relation of CAPE-induced apoptosis and p38 MAPK, we pretreated cells with or without p38 MAPK inhibitor (5 μM), SB203580, then added CAPE and assessed for the DNA fragmentation assay by agarose gel electrophoresis. It showed that SB203580 blocked the CAPE-induced apoptosis of C6 glioma cells (Fig. 8).

4. Discussion

Many chemopreventive agents act through the induction apoptosis as a mechanism of antitumorigenesis. Therefore, induction of apoptosis by chemopreventive agents may be an effective strategy in antitumorigenesis. CAPE is a potent antioxidant and anti-inflammatory agent [1–4] as well as an apoptosis-inducing agent [8–10]. It has been suggested that apoptosis induced by CAPE is associated with mitochondria dysfunction, GSH depletion, and selective scavenging of hydrogen peroxide in human leukemia HL-60 cells. However, the precise mechanisms of its antitumorigenic activities remain largely unknown. The evidence from our study showed that CAPE treatment leaded to apoptosis preceded by activation of CPP32 in C6 glioma cells, and that tumor suppressor protein p53 is involved in the action. Although the mechanisms of p53-mediated apoptosis after cellular stress remain poorly understood, recent study on the mutation of p53 at serine 15 that impaired the apoptotic activity of p53 suggests a pivotal role for the phosphorylation at this site in the p53 activation and induction of apoptosis [29]. Further evidence indicated that this phosphorylation of p53 played a critical role in its stabilization, up-regulation, and functional activation [30]. In the present study, CAPE increased the phosphorylation of p53 and the expression of p53 and Bax, which can form heterodimers with Bcl-2 in mitochondria membrane and accelerates apoptosis. Miyashita and Reed found p53-binding sites in the promoter upstream of the bax gene [17]. Therefore, these data demonstrated that CAPE activated p53 to promote apoptotic activity in C6 glioma cells.

The phosphorylation of p53 is known to be mediated by multitude of protein kinases, including DNA-dependent protein kinase (DNA-PK), ATM, ATR, JNKs, ERKs, and p38 MAPK [31–34]. Herein CAPE treatment activated the MAPK cascade including ERKs and p38 MAPK. In addition, p38 MAPK was coimmunoprecipitated with p53 from the CAPE-treated C6 glioma cells, an observation consistent with previous report of direct interaction between p53 and p38 MAPK [35]. Finally, recent reports demonstrated that p38 MAPK phosphorylated p53 at serine 15 in response to UV irradiation and resververatrol treatment [36,37]. According to our study, pretreatment with a p38 MAPK specific inhibitor, SB203580 but not PD98059, significantly inhibited the phosphorylation of p53 at serine 15. However, other MAPK-mediated phosphorylation site of p53 that might be activated by CAPE and the effect of CAPE-activated ERKs in C6 glioma cells need further investigation.

Recently, it is reported that MAPK were differentially activated by dietary antioxidant compounds such as green tea polyphenols and involved in the transcriptional activation of antioxidant response element (ARE)-mediated reporter gene [38]. It is proposed that, at low concentration, these compounds activate MAPK pathway leading to activation ARE with subsequent induction of phase II enzymes thereby enhancing cell survival. At higher concentrations, these agents activate the caspase pathway leading to apoptosis in normal cells. CAPE is a phenolic antioxidant that showed stimulation human ARE-mediated expression of the NAD(P)H: quinone oxidoreductase gene [39]. Therefore, CAPE might activate MAPKs pathway. From our study, it showed that CAPE activated p38 MAPK and that involved in the induction of apoptosis of C6 glioma cells. However, the effect of CAPE-activated ERKs in C6 glioma cells need further study.

In conclusion, this study suggests that tumor suppressor protein p53 and p38 MAPK play a prominent role in the CAPE-induced apoptotic cell death that might contribute to the antitumor effects of CAPE.

Acknowledgments

This work was supported by the Grant of Chung Shan Medical University (CSMU 91-OM-A-0580) and National Science Council (NSC 91-2320-B-040-049), Republic of China.

References

[1] Bhimani RS, Troll W, Grunberger D, Frenkel K. Inhibition of oxidative stress in HeLa cells by chemopreventive agents. Cancer Res 1993;53:4528–33.
[2] Chen WK, Tsai CF, Liao PH, Kuo SC, Lee YJ. Synthesis of caffeic acid esters as antioxidants by esterification via acyl chlorides. Chin Pharm J 1999;1:271–8.
[3] Mirzoeva OK, Calder PC. The effect of propolis and its components on eicosanoid production during the inflammatory response. Prostaglandins Leukot Essent Fatty Acids 1996;55:441–9.
[4] Orban Z, Mitsiades N, Burke TR, Tsokos M, Chrousos GP. Caffeic acid phenethyl ester induces leukocyte apoptosis modulates nuclear factor-kappa B and suppresses acute inflammation. Neuroimmunomodulation 2000;7:99–105.
[5] Fesen MR, Pommier Y, Leteurtre E, Hiroguchi S, Yung J, Kohn KW. Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (CAPE) and related compounds. Biochem Pharmacol 1994;48:595–608.
[6] Rao CV, Desai D, Simi B, Kulkarni N, Amin S, Reddy BS. Inhibitory effect of caffeic acid esters on azoxymethane-induced biochemical changes and aberrant crypt foci formation in rat colon. Cancer Res 1993;53:4182–8.
[7] Frenkel K, Wei H, Bhimani R, Ye J, Zadunaisky JA, Huang MT, Ferraro T, Conney AH, Grunberger D. Inhibition of tumor promoter-mediated processes in mouse skin and bovine lens by caffeic acid phenethyl ester. Cancer Res 1993;53:1255–61.
[8] Grunberger D, Banerjee R, Eisinger K, Oltz EM, Efros L, Caldwell M, Estevez V, Nakanishi K. Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis. Experientia 1988;44:230–2.
[9] Su Z, Lin J, Grunberger D, Fisher PB. Growth suppression and toxicity induced by caffeic acid phenethyl ester (CAPE) in type 5 adenovirus-transformed rat embryo cells correlate directly with transformation progression. Cancer Res 1994;54:1865–70.
[10] Chen JH, Shao Y, Huang MT, Chin CK, Ho CT. Inhibitory effect of caffeic acid phenethyl ester on human leukemia HL-60 cells. Cancer Lett 1996;108:211–4.
[11] Lee YJ, Liao PH, Chen WK, Yang CC. Preferential cytotoxicity of caffeic acid phenethyl ester analogues on oral cancer cells. Cancer Lett 2000;153:51–6.
[12] Vogelstein B, Kinzler KW. P53 function and dysfunction. Cell 1992;70:523–6.
[13] Li PF, Dietz R, von Harsdorf R. p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome *c*-independent apoptosis blocked by Bcl-2. EMBO J 1999;18:6027–36.
[14] Lee SW, Fang L, Igarashi M, Ouchi T, Lu KP, Aaronson SA. Sustained activation of Ras/Raf/mitogen-activated protein kinase cascade by the tumor suppressor p53. Proc Natl Acad Sci USA 2000;97:8302–5.
[15] Yamaguchi A, Tamatani M, Matsuzaki H, Namikawa K, Kiyama H, Vitek MP, Mitsuda N, Tohyama M. Akt activation protects hippocampal neurons from apoptosis by inhibiting transcriptional activity of p53. J Biol Chem 1996;271:31929–36.
[16] Tsujimoto Y. Role of Bcl-2 family protein in apoptosis: apoptosomes or mitochondria? Genes Cells 1998;3:697–707.
[17] Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293–9.
[18] Talarmin H, Rescan C, Carious S, Glaise D, Zanninelli G, Bilodeau M, Loyer P, Guguen-Guillouzo C, Baffet G. The mitogen-activated protein kinase kinase/extracellular signal-regulated kinase cascade activation is a key signaling pathway involved in the regulation of G1 phase progression in proliferating hepatocytes. Mol Cell Biol 1999;19:6003–11.
[19] Petrache I, Choi ME, Otterbein LE, Chin BY, Mantell LL, Horowitz S, Choi AM. Mitogen-activated protein kinase pathway mediates hyperoxia induced apoptosis in cultured macrophage cells. Am J Physiol 1999;277:L589–595.
[20] Roulston A, Reinhard C, Amiri P, Williams LT. Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor. J Biol Chem 1998;273:10232–9.
[21] Nagata Y, Todokoro K. Requirement of activation of JNK and p38 for environmental stress-induced erythroid differentiation and apoptosis and of inhibition of ERK for apoptosis. Blood 1999;94:853–63.
[22] Benda P, Lightabody J, Satp G, Levine L, Sweet W. Differentiated rat glial cell strain in tissue culture. Science 1968;161:370–1.
[23] Mosmann T. Rapid colorimetric assay for growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[24] Tang DG, Li L, Zhu Z, Joshi B. Apoptosis in the absence of cytochrome *c* accumulation in the cytosol. Biochem Biophys Res 1998;242:380–4.
[25] Nicholson DW, Ali A, Thornberry NA, Vaillancout JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin TT, Yu VL, Miller DK. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 1995;376:37–43.
[26] Tewari M, Quan LT, O'Rourke, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM. Yamam/CPP32, a mammalian homologue of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 1995;81:801–9.
[27] Boise LH, Gottschalk AR, Quintans J, Thompson CB. Bcl-2 and Bcl-2-related proteins in apoptosis regulation. Curr Topics Microbiol Immunol 1995;200:107–21.
[28] Giaccia AJ, Kastan MB. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev 1998;12:2973–83.
[29] Unger T, Sionov RV, Moallem E, Yee CL, Howley PM, Oren M, Haupt Y. Mutations in serines 15 and 20 of human p53 impair its apoptotic activity. Oncogene 1999;18:3205–12.
[30] Meek DW. Multisite phosphorylation and the integration of stress signals at p53. Cell Signal 1998;10:159–66.
[31] Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell 1997;91:325–34.
[32] Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh SY, Taya Y, Prives C, Abraham RT. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev 1999;13:152–7.
[33] Hu MC, Wiu WR, Wang YP. JNK1, JNK2, JNK3 are p53 N-terminal serine 34 kinase. Oncogene 1997;15:2277–87.
[34] Huang C, Ma WY, Maxiner A, Sun Y, Nel A. p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. J Biol Chem 1999;274:12229–35.
[35] Kwon YW, Ueda S, Ueno M, Yodoi J, Masutani H. Mechanism of p53-dependent apoptosis induced by 3-methylcholanthrene. J Biol Chem 2002;277:1837–44.

[36] She QB, Chen NY, Dong Z. ERKs and p38 kinase phosphorylate p53 protein at serine 15 in response to UV radiation. J Biol Chem 2000;275:20444–9.

[37] Sje QB, Bode AM, Ma WY, Chen NY, Dong Z. Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinase and p38 kinase. Cancer Res 2001;61:1604–10.

[38] Owuor ED, Kong AT. Antioxidants and oxidants regulated signal transductin pathways. Biochem Pharmacol 2002;64:765–70.

[39] Jaiswal AK, Venugopal R, Mucha J, Carothers AM, Grunberger D. Caffeic acid phenethyl ester stimulates human antioxidant response element-mediated expression of the NAD(P)H: quinone oxidoreductase (*NQO1*) gene. Cancer Res 1997;57:440–7.