Apoptotic effects of *Antrodia cinnamomea*¹ fruiting bodies extract are mediated through calcium and calpain-dependent pathways in Hep 3B cells

Po-Lin Kuo ^a, Ya-Ling Hsu ^a, Chien-Yu Cho ^b, Lean-Teik Ng ^c,
Yueh-Hsiung Kuo ^d, Chun-Ching Lin ^{b,*}

^a Department of Biotechnology, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan

^b Graduate Institute of Natural Products, Kaohsiung Medical University, 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan

^c Department of Food Science and Technology, Ta-Jen Institute of Technology, Ping-Tung, Taiwan

^d Department of Chemistry, National Taiwan University, Taipei, Taiwan

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Abstract

Antrodia cinnamomea is well known in Taiwan as a traditional medicine for treating cancer and inflammation. The purpose of this study was to evaluate the apoptotic effects of ethylacetate extract from *A. cinnamomea* (EAC) fruiting bodies in Hep 3B, a liver cancer cell line. EAC decreased cell proliferation of Hep 3B cells by inducing apoptotic cell death. EAC treatment increased the level of calcium (Ca^{2+}) in the cytoplasm and triggered the subsequent activation of calpain and caspase-12. EAC also initiated the mitochondrial apoptotic pathway through regulation of Bcl-2 family proteins expression, release of cytochrome *c*, and activation of caspase-9 in Hep 3B cells. Furthermore, the mitochondrial apoptotic pathway amplified the calpain pathway by Bid and Bax interaction and Ca^{2+} translocation. We have therefore concluded that the molecular mechanisms during EAC-mediated proliferation inhibition in Hep 3B cells were due to: (1) apoptosis induction, (2) triggering of Ca^{2+} /calpain pathway, (3) disruption of mitochondrial function, and (4) apoptotic signaling being amplified by cross-talk between the calpain/Bid/Bax and Ca^{2+} /mitochondrial apoptotic pathways.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal and one of the four most prevalent malignancies in adults in China, Taiwan, Korea, and sub-Africa (Kern et al., 2002; Seow et al., 2001). Several etiologic factors, including exposure to aflatoxin B1, and infection with hepatitis B virus and hepatitis C virus, have been classified as high-risk factors in association with HCC (Kern et al., 2002; Okuda, 1992; Seow

et al., 2001). Apoptosis has been characterized as a fundamental cellular activity to maintain the physiological balance of the organism. It is also involved in the immune system (Schuchmann and Galle, 2004) and plays a necessary role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells which have proliferated owing to various chemical agents' induction (Schuchmann and Galle, 2004). Emerging evidence has demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which is regarded as the preferred way to manage cancer (Ma and Hendershot, 2004; Schuchmann and Galle, 2004).

The endoplasmic reticulum (ER) is the cytoplasmic compartment where proteins and lipids are synthesized and modified (Ma and Hendershot, 2004; Orrenius et al.,

¹ The generic name of *Antrodia camphorata* is currently corrected by Chang and Chou (2004).

* Corresponding author. Tel.: +886 7 3121101x2122; fax: +886 7 3135215.

E-mail address: aalin@ms24.hinet.net (C.-C. Lin).

2003). Dysfunctions of calcium homeostasis, protein misfolding, or oxidative stress can cause ER stress and cell death (Ma and Hendershot, 2004; Orrenius et al., 2003). ER-induced apoptosis is associated with the activation of caspase-12 and calpain (Chiang et al., 2005; Orrenius et al., 2003). Caspase-12 is specifically localized on the cytoplasmic side of the ER and connects ER stress to the caspase activation cascade (Yoneda et al., 2001). Calpains are calcium-dependent proteases that have been proposed to participate in apoptosis by activation of caspases and cleavage of proapoptotic factors. Recent studies indicated that calpain can cleave the proapoptotic Bcl-2 family proteins Bid (p22) and Bax (p21). Interaction of truncated Bid (t-bid, p15) and Bax (p18) with the mitochondrial permeability transition pore causes mitochondrial membrane potential ($\Delta\Psi_m$) loss and cytochrome *c* release, leading to cell death (Altnauer et al., 2004; Chen et al., 2002; Mandic et al., 2002; Orrenius et al., 2003).

Antrodia cinnamomea is one of the most important traditional Chinese crude drugs for treating diarrhea, hypertension, and liver cancer. Previous studies have shown that it possesses a wide range of biological functions, such as antioxidative, anti-inflammatory, hepatoprotective, vasorelaxative and anti-hepatitis B virus effects (Hsiao et al., 2003; Lee et al., 2002; Shen et al., 2004; Song and Yen, 2002; Wang et al., 2003). Recent studies reported that the mycelium of *A. cinnamomea* showed appreciable cytotoxic activity against tumor cells (Liu et al., 2004; Nakamura et al., 2004). In this study, we determined the apoptotic activity of ethylacetate extract from *A. cinnamomea* (EAC) in a human liver cancer cell line, Hep 3B. Furthermore, to establish the anticancer mechanism of EAC, we assayed the apoptosis-related molecules, including calcium (Ca^{2+}), calpain, cytochrome *c*, caspase-9, and Bcl-2 family proteins which are strongly associated with the signal transduction pathway of apoptosis and affect the chemosensitivity of tumor cells to anticancer agents.

2. Materials and methods

2.1. Reagents

Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), penicillin G, streptomycin, and amphotericin B were obtained from GIBCO BRL (Gaithersburg, MD). Dimethyl sulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO). XTT was obtained from Roche Diagnostics GmbH (Germany). Calpeptin and PD15060 was purchased from Calbiochem (Cambridge, MA). Bak and Bcl-X_L antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bax antiserum was purchased from BD PharMingen, (San Diego, CA). The m-calpain antibody was obtained from Chemicon International Inc., (Temecula, CA). Bid antibody was obtained from Cell Signaling Technology (Beverly, MA).

2.2. Preparation of *A. cinnamomea* extract

Powdered fruiting bodies of cultivated *A. cinnamomea* (about 250 g) were soaked in 500 ml methanol for three days. The sample was filtered with filter paper (Advantec No. 1, Japan) while the residue was further

extracted twice more under the same conditions. The filtrates collected from three separate extractions were combined and evaporated to dryness under vacuum. Four grams of concentrated methanol extract was partitioned between water and ethylacetate (1:3 v/v) three times to produce an ethylacetate (EA) fraction of 300 ml, which was then evaporated to dryness under vacuum to a yield of about 600 mg.

The extract was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C . For all experiments, final concentrations of the tested compound were prepared by diluting the stock with DMEM. Control cultures received the carrier solvent (0.1% DMSO).

2.3. Cell line and culture

Human hepatocellular carcinoma cell line, Hep 3B (ATCC HB 80640) was maintained in monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS, 10 U/mL of penicillin, 10 $\mu\text{g}/\text{mL}$ of streptomycin, and 0.25 $\mu\text{g}/\text{mL}$ of amphotericin B.

2.4. Assay for cell proliferation inhibition

To measure the effect of EAC in cell proliferation, the Hep 3B cells (1×10^4 cells/well) were seeded into a 96-well plate. After 24 h of incubation, the cells were treated with vehicle (0.1% DMSO), 30, 60, 90, and 120 $\mu\text{g}/\text{mL}$ EAC for 48 h. At the end of the assay time period, cell proliferation was measured by sodium 3-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitrobenzene-sulfonic acid hydrate) (XTT) assay. In this study, the drug concentration required to inhibit cell proliferation by 50% (IC_{50}) was determined by interpolation from dose to response curves.

2.5. Apoptosis assay

Cells (1×10^6) were treated with vehicle alone (0.1% DMSO) and various concentrations of EAC for 48 h, and then collected by centrifugation. Pellets were lysed by DNA lysis buffer (10 mM Tris, pH 7.5, 400 mM EDTA, and 1% Triton X-100) and then centrifuged. The supernatant obtained was incubated overnight with proteinase K (0.1 mg/mL) and then with RNase (0.2 mg/mL) for 2 h at 37°C . After extraction with phenol-chloroform (1:1), the DNA was separated in 2% agarose gel and visualized by UV after staining with ethidium bromide.

Quantitative assessment of apoptosis was analyzed by an Annexin V-FITC assay kit (BD Biosciences, San Jose, CA). Briefly, cells grown in 10 cm Petri dishes were harvested with trypsin and washed in PBS. Cells were then resuspended in binding buffer (10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl_2) and stained with Annexin V-FITC and PI at room temperature for 15 min in the dark. Cells were then analyzed in an EPICS flow cytometer (Coulter Electronics) within 1 h after staining. Data from 10,000 cells were collected for each data file. Apoptotic cells were defined as Annexin V-FITC-positive and PI-negative cells.

2.6. Calcium measurement

Cells were treated as indicated and harvested with cell dissociation solution (Sigma Aldrich), and the suspended cells were then incubated with the Ca^{2+} indicator FLUO-3 (20 mM) (Molecular Probes, Inc.) for 60 min. The intracellular Ca^{2+} levels, seen as a fluorescent signal, were then assessed by flow cytometry with the FL1 channel. Data are presented as fold increases in fluorescence compared to that in untreated cells (Mandic et al., 2002). Cells were also examined by a fluorescence microscope.

2.7. Mitochondrial membrane potential assay

We used the mitochondrial-specific cationic dye (JC-1) (Molecular Probes, Inc.) that undergoes potential-dependent accumulation in the mitochondria. It is a monomer when the membrane potential ($\Delta\Psi$) is

lower than 120 mV and emits green light (540 nm) following excitation by blue light (490 nm). At higher membrane potentials, JC-1 monomers convert to J-aggregates that emit red light (590 nm) following excitation with green light (540 nm). Cells were seeded in a 96-well plate. At various times following treatment with EAC, cells were stained with 25 μ M JC-1 for 30 min at 37 °C. Fluorescence was monitored with the fluorescence plate reader at wavelengths of 490 nm (excitation)/540 nm (emission) and 540 nm (excitation)/590 nm (emission) pairs. Changes in the ratio between the measurements at test wavelengths of 590 nm (red) and 540 nm (green) fluorescence intensities are indicative of changes in the mitochondrial membrane potential (Martin and Forkert, 2004).

2.8. Assay for caspase-3, caspase-9, caspase-12 and calpain activity

The assay is based on the ability of the active enzyme to cleave the chromophore from the specific enzyme substrate, LEHD-pNA (for caspase-9), DEVD-pNA (for caspase-3), ATAD-AFC (for caspase-12) and Ac-LLY-AFC (for calpain) (BioVision, California). The cell lysates were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4 for caspase-3, -9, and -12; 10 mM HEPES, 1% Triton X-100, 100 mM CaCl₂, pH 7.4 for calpain) for 2 h at 37 °C. The release of *p*-nitroaniline was monitored at 405 nm. The release of AFC was measured in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. Results are represented as the percent change of the activity compared to the untreated control.

2.9. Western blotting

Cells (8×10^6 /dish) were seeded in a 10 cm dish. After 24 h of incubation, cells were treated with 100 μ g/ml of EAC for the indicated times. The mitochondrial fraction and cytoplasmic fraction was separated using Cytochrome *c* Releasing Apoptosis Assay Kit (BioVision, California, USA). Total cell extracts were prepared in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na₃VO₄, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 5 μ g/ml Aprotinin, 5 μ g/ml Leupetin). Equivalent amounts of protein were resolved by SDS-PAGE (10–12%) and transferred to PVDF membranes. After the membranes were blocked in Tris-buffer saline (TBST) containing 0.05% Tween 20 and 5% nonfat powdered milk, the membranes were incubated with primary antibodies specific to cytochrome *c*, calpain, Bid, Bax, Bak, and Bcl-X_L at 4 °C for 16 h. After washing three times with TBST for 10 min each, the membranes were incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection was performed using the enhanced chemiluminescence blotting detection system (Amersham, USA) (Hsu et al., 2005a,b).

2.10. Statistical analysis

Data were expressed as means $\pm$ standard errors. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the means of control and EAC-treated cells were analyzed by Dunnett's test.

3. Results

3.1. Cell proliferation inhibition effect of EAC in Hep 3B through apoptosis

Cell proliferation inhibition was determined by XTT assay. As shown in Fig. 1A, EAC treatment induced proliferation inhibition in Hep 3B. At 48 h, the maximum effect on proliferation inhibition was observed with 120 μ g/ml EAC, which inhibited cell proliferation in 89.23% of Hep

3B, and the IC₅₀ value was 78.3 μ g/ml (Fig. 1A). Next, we assessed the effect of EAC on the induction of apoptosis in Hep 3B cells by DNA fragmentation assay. Agarose gel electrophoresis at 48 h showed that EAC treatment results in the formation of DNA fragments in Hep 3B cells (Fig. 1B). Additionally, a quantitative evaluation was sought using Annexin V-FITC dye to detect the translocation of phosphatidylserine from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface). Compared with vehicle-treated cells, 100 μ g/ml EAC induced 32.5% and 38.5% of apoptotic cells in Hep 3B at 24 and 48 h, respectively (Fig. 1C).

3.2. EAC increases intracellular Ca²⁺ levels, calpain and caspase-12 activation

Using FLUO-3-AM, a cell-permeable Ca²⁺ indicator, we assessed the effect of EAC treatment on intracellular Ca²⁺. The level of cytosolic Ca²⁺ induced by 100 μ g/ml EAC started to increase at 1 h, and maximum induction was observed at 3 h. As expected, the increase in Ca²⁺ was abrogated by Ca²⁺ chelator BAPTA-AM (10 μ M) (Fig. 2A). The release of calcium in EAC-treated Hep 3B cell was also revealed by fluorescence microscope (Fig. 2B). In addition, treatment with Ca²⁺ ionophore ionomycin (1 μ M, 20 min) similarly induced a 1.7-fold increase in Ca²⁺ level (Fig. 2A).

Activation of calcium-dependent proteases such as calpains is thought to play an important role in apoptotic cell death (Chiang et al., 2005; Orrenius et al., 2003). In addition, several studies have shown that calpains can contribute to tumor death, and calpain inhibitors have been used to block apoptosis (Kobayashi et al., 2002; Orrenius et al., 2003). The calpain activity of EAC-treated Hep 3B was assessed in cell lysates using fluorogenic calpain substrates and immunoblot assay. Moreover, we also analyzed the common small regulatory subunit of calpains, which is dissociated in response to increased levels of cytosolic calcium (Pal et al., 2001). EAC treatment for 1 h did not yield calpain activation, while more than 2-fold induction was observed at 3 and 6 h. Furthermore, the activation of calpain by EAC was blocked by pre-treatment with Ca²⁺ chelator BAPTA-AM (Fig. 2C). Similarly, immunoblotting analysis detecting the autolysis of large subunits of calpain has shown that EAC-treated Hep 3B cells contained the autolysed form of m-calpain (78 kDa) after 3 h treatment. Consistent with the results of large subunits of the calpain, a decrease in the intensity of the band corresponding to the calpain small subunit (30 kDa) was observed after 3 h of treatment, whereas after 6 h, the band was no longer detectable (Fig. 2D). Autolysis of the 30-kDa calpain small subunit is associated with calpain activation during apoptosis (Daniel et al., 2003). These data strongly indicated that the activation of calpain is in a Ca²⁺-dependent manner in EAC-treated Hep 3B cells.

To examine whether ER is involved in EAC-induced apoptosis, ER-specific effectors are examined. We assessed

Fig. 1. Effect of EAC on cell proliferation inhibition and apoptosis induction in Hep 3B cells. (A) Cell proliferation inhibition effect of EAC. The induction of apoptosis in EAC-treated cells assessed by electrophoresis assay (B) and Annexin-V-FITC/PI (C) in Hep 3B. For (A), cell proliferation inhibition of EAC was assessed by XTT. For (B), cells were treated with vehicle and EAC for 48 h, and then DNA fragmentation was assessed by agarose gel electrophoresis. For (C), the phosphatidylserine translocation of EAC-treated cells was estimated by Annexin V assay kits. Results are expressed as percentage of cell proliferation relative to the proliferation of control. Each value is the mean $\pm$ SD of three determinations. The asterisk indicates a significant difference between control and EAC-treated cells (* $p < 0.05$).

the activation of caspase-12 since it has been indicated that activation of these two enzymes is apparent in ER-mediated apoptosis (Chiang et al., 2005; Goll et al., 2003). Caspase-12 that locates on the cytoplasmic side of ER is proteolytically activated following ER stress and calpain activation (Goll et al., 2003). Activation of caspase-12 was observed at 6 h after EAC treatment, and increased progressively for up to 12 h (Fig. 2E). The activation of caspase-12 by EAC was blocked by pretreatment with BAPTA-AM, suggesting caspase-12 was activated in a Ca^{2+} -dependent manner. Furthermore, Both Ca^{2+} translocation and calpain activation were observed before caspase-12 activation, revealing that both effectors were possibly attributed to caspase-12 activation.

3.3. Inhibitors of calpain inhibit EAC-induced apoptosis in Hep 3B cells

To confirm that activation of calpain plays a key role in EAC-induced apoptosis, the effect of two different calpain inhibitors, calpeptin and PD150606, on EAC-induced apoptosis was then studied in Hep 3B cells. Calpeptin acts on the active site of calpain, whereas PD150606 blocks its calcium-binding site (Junoy et al., 2002; Mandic et al., 2002). As shown in Fig. 3A, the EAC-mediated calpain activation was effectively inhibited by 10 μM calpeptin and 20 μM PD150606. At 24 h treatment, apoptosis, seen as phosphatidylserine translocation, was inhibited by calpeptin pretreatment (Fig. 3B). Pretreatment of cells with PD150606 was

Fig. 2. Effect of EAC on Ca^{2+} release, calpain and caspase-12 activation in Hep 3B cells. EAC increases cytosolic Ca^{2+} concentrations assessed by flow cytometry (A) and fluorescence microscope (B). The activation of calpain in EAC-treated Hep 3B assay by specific substrate (C) and immunoblot assay (D). The activation of caspase-12 (E). Cells were pretreated with or without BAPTA-AM (10 μM) for 1 h before the addition of 100 $\mu\text{g}/\text{ml}$ EAC or 1 μM ionophore ionomycin (20 min exposure for Ca^{2+} assay, 6 h exposure for calpain and caspase-12 activation) for the indicated times. For (A) and (B), the levels of Ca^{2+} was assessed by Ca^{2+} indicator FLUO-3 and flowcytometry. For (C) and (E), that activation of calpain and caspase-12 was determined by Caspase-12 and calpain Fluorometric Assay Kit (BioVision, California, USA). For (D), the cleavage of calpain was assessed by immunoblot assay. Each value is the mean $\pm$ SD of three determinations. The asterisk indicates a significant difference between control and EAC-treated cells (* $p < 0.05$).

also found to inhibit EAC-induced apoptosis in a manner similar to calpeptin (Fig. 3B), showing that calcium/calpain is required in the apoptotic process induced by EAC.

3.4. Effect of EAC on the mitochondrial apoptotic pathway in Hep 3B cells

To investigate the mitochondrial apoptotic events involved in EAC-induced apoptosis, we first analyzed the mitochondrial membrane potential ($\Delta\Psi_{\text{m}}$). As shown in Fig. 4A, the $\Delta\Psi_{\text{m}}$ of EAC-treated Hep 3B cell was decreased at 12 h and continued to decrease for up to 24 h.

We next assessed the release of cytochrome *c* from mitochondria into the cytoplasm. As shown in Fig. 4B, EAC-

induced apoptosis was associated with both decreased mitochondrial cytochrome *c* content and increased cytochrome *c* release into the cytosol in Hep 3B cells. In addition, biochemical analysis showed that treatment with EAC increased caspase-9 activity in Hep 3B cells (Fig. 4C), which was consistent with the release of cytochrome *c* into the cytosol. Furthermore, EAC also increased effector caspase-3 activity in Hep 3B (Fig. 4D).

3.5. EAC induces calpain-mediated Bid cleavage and initiates mitochondrial apoptotic pathway

Because Bcl-2 family proteins play an important regulatory role in the induction of mitochondrial apoptosis (Hen-

Fig. 2 (continued)

gartner, 2000), we next studied the effects of EAC on the expressions of Bax, Bid, Bak, Bcl-Xs, and Bcl-X_L in Hep 3B cells. As shown in Fig. 5A, EAC treatment induced an increase in the levels of Bax, Bak and Bcl-Xs, and a decrease of Bcl-X_L in Hep 3B. The results also showed that full size Bid and Bax (22 and 21 kDa) proteins were cleaved

to yield 15 kDa and 18 kDa fragments, which exhibit a stronger proapoptotic activity than that of full-size proteins (Eskes et al., 2000).

Since EAC-mediated apoptosis involved initiation of Ca²⁺/calpain pathway and mitochondrial signaling, it is possible that EAC activates the mitochondrial apoptotic

Fig. 3. Specific inhibition of calpain decreased EAC-mediated apoptosis in Hep 3B cells. (A) Calpeptin and PD150606 inhibit EAC-mediated calpain activation. (B) Calpeptin and PD150606 inhibit EAC-mediated apoptosis. Cells were pretreated with calpeptin (10 μ M) or PD150606 (20 μ M) for 1 h before the addition of 100 μ g/ml EAC for the indicated times. The activation of calpain was measured using Calpain Fluorometric Assay Kit. The induction of apoptosis induced by EAC after 24 h treatment was assessed by Annexin V-FITC/PI kit. Each value is the mean $\pm$ SD of three determinations. The asterisk indicates a significant difference between control and EAC-treated cells (* p < 0.05).

pathway through calpain-mediated Bid and Bax cleavage, which then results in cytochrome *c* release and caspase-9 activation. To test this idea, we sought to determine whether proteolytic cleavage by calpain is really responsible for the truncation of Bid and Bax. We investigated the effects of various calpain inhibitors on Bid cleavage. When cells were pretreated with the specific calpain inhibitors calpeptin and PD150606 before EAC treatment, the

cleavage of Bid and Bax protein recovered in EAC-treated cells was near that of the untreated control cells (Fig. 5B). These results indicated that Bid and Bax proteins are cleaved by calpain, which is activated by EAC treatment.

3.6. The cross-talk of calcium between ER and the mitochondria is required in EAC-induced apoptotic cell death

To experimentally verify the role of ER Ca^{2+} in EAC-induced apoptosis, Hep 3B cells were pretreated for 1 h with thapsigargin, a specific inhibitor for SECRA (an ER outer membrane pump whose function is to maintain the levels of Ca^{2+} above that of cytosol), to deplete ER Ca^{2+} (Chandra et al., 2004). Thapsigargin causes the passive release of Ca^{2+} from ER stores and an increase in cytosolic Ca^{2+} (Scorrano et al., 2003). Subsequently, the inhibitor-treated cells were exposed to EAC, and apoptosis was determined. As shown in Fig. 6A, pretreatment of Hep 3B cells with 50 nM thapsigargin significantly decreased EAC-mediated apoptosis.

The release of Ca^{2+} is rapidly taken up by the mitochondria resulting in enhanced cytochrome *c* release (Orrenius et al., 2003). To assess whether the mitochondrial uptake of Ca^{2+} was associated with EAC-mediated apoptosis, we used Ru360, an oxygen-bridged dinuclear ruthenium amine complex that specifically blocks mitochondrial Ca^{2+} uptake (Matlib et al., 1998). As shown in Fig. 6B, pretreatment of Hep 3B cells with Ru360 significantly inhibited EAC-induced apoptosis.

4. Discussion

A. cinnamomea, a traditional Chinese herb found locally in Taiwan, has been widely used to treat cancer and inflammation. Previous studies revealed that an activated steroid acid of *A. cinnamomea* fruiting bodies, zhankuic acid, exhibits a significant cytotoxic effect against P338 murine leukemia (Chen et al., 1995). Furthermore, Hseu et al. have reported that aqueous extract of *A. cinnamomea* mycelia possesses cytotoxic effect against HL-60 leukemia cells (Hseu et al., 2002). The maleic and succinic acid derivatives of the mycelium of *A. cinnamomea* have also shown appreciable cytotoxicity against LLC cells (Nakamura et al., 2004). Our previous study also reported that ethylacetate extract of *A. cinnamomea* fruiting bodies (EAC) induces apoptosis in liver cancer cell lines Hep G2 and PLC/PRF/5 by inhibiting the NF- κ B pathway (Hsu et al., 2005a,b). In this study, we report the inhibition of cell proliferation and apoptosis inducing effect of EAC in another human liver cancer cell line, Hep 3B. Our results first demonstrated that EAC inhibits the proliferation of human liver Hep 3B cancer cells. Treatment of Hep 3B cells with EAC caused the cells to undergo apoptotic cell death by way of the Ca^{2+} -calpain-mitochondria signaling pathway.

The ER is the main intracellular storage compartment for Ca^{2+} , an important secondary messenger required for

Fig. 4. Effect of EAC on mitochondrial membrane potential ($\Delta\Psi_m$), cytochrome *c* release, and activation of caspase-9 and -3. (A) The loss of $\Delta\Psi_m$. (B) The release of cytochrome *c*. The activation of caspase-9 (C) and caspase-3 (D) in EAC-treated Hep 3B cells. Cells were treated with 100 $\mu\text{g/ml}$ EAC for the indicated times. For (A) the $\Delta\Psi_m$ was measured by JC-1 and flow cytometry. For (B), the cytoplasm and mitochondria were extracted from cell pellets by lysis buffer and centrifugation. Western blotting analysis assessed the protein expressions. For (C) and (D), the activity of caspase-9 and caspase-3 was assessed by Caspase-9 and Caspase-3 Activity Assay Kit. Each value is the mean $\pm$ SD of three determinations. The asterisk indicates a significant difference between control and EAC-treated cells (* $p < 0.05$).

numerous cellular functions. Apoptosis occurs upon the perturbation of cellular Ca^{2+} homeostasis, such as cytosolic Ca^{2+} overload, ER Ca^{2+} depletion, and mitochondrial Ca^{2+} increase (Ma and Hendershot, 2004; Orrenius et al., 2003). Calpains are calcium-dependent proteases that have been hypothesized to participate in apoptosis (Goll et al., 2003). Our results found that EAC treatment causes a rapid increase in concentrations of cytosolic Ca^{2+} in Hep 3B cells within 1 h treatment. The elevation of Ca^{2+} may depend on the type of cells and stimulation. The onset of Ca^{2+} influx is range from several minutes to several hours. For example, incubation of neuron cells with glutamate

caused a significant increase in intracellular free Ca^{2+} after 24 h treatment (Das et al., 2005). Genistein (50 μM) caused a sustained increase in Ca^{2+} levels in MCF-7 after 24 h incubation (Sergeev, 2004). In addition, SNP (0.3 mM) increased Ca^{2+} levels in 661 W photoreceptor cells after 20 h treatment (Sanvicens et al., 2004). In contrast, peroxynitrite generator SIN-1 increased cytosolic Ca^{2+} in human articular chondrocytes only after 10 min exposure. (Whiteman et al., 2004). In addition, EAC treatment also enhances the activation of calpain. the activation of calpain was abolished by Ca^{2+} chelator BAPTA-AM, suggesting that the elevation of Ca^{2+} induced by EAC is necessary

Fig. 5. The inhibitor effect of calpain inhibitor on EAC-mediated cleavage of Bid and Bax. (A) The effect of EAC on Bcl-2 family protein expression. (B) The effect of calpain inhibitor on the cleavage of Bid and Bax. Cells were treated with 100 $\mu\text{g/ml}$ EAC for the indicated times, and then the levels of Bcl-2 family protein was assessed by Western blotting analysis. For blocking experiments, cells were preincubated with calpeptin (10 μM) or PD150606 (20 μM) for 1 h before the addition of 100 $\mu\text{g/ml}$ EAC for another 24 h. The cleavage of Bid and Bax was assessed by Western blotting analysis.

for calpain activation. Thus, it is reasonable to postulate that EAC treatment may cause apoptotic cell death by enhancing the activity of calpain due to the elevation of cytosolic Ca^{2+} levels. Furthermore, the importance of this pathway was further confirmed by the partial protection on cell survival conferred by the calpain inhibition.

Evidence is accumulating that the mitochondrial apoptotic pathway plays a central and amplifying role in various cell death pathways (Gottlieb, 2000; Bouchier-Hayes et al., 2005). A number of pro- and anti-apoptotic members of the Bcl-2 family proteins regulate the release of cytochrome *c* and apoptosis inducing factor (AIF) from the mitochondrial intermembrane space into the cytosol (Hengartner, 2000). Cytochrome *c* interacts with pro-caspase-9 and Apaf-1 to activate caspase-9, then switches on caspase-3, -6, and -7, leading to apoptosis (Hengartner, 2000). In our studies, we observed that EAC treatments showed alterations of the expression of antiapoptotic (Bcl-X_L) and proapoptotic (Bax, Bak, and Bcl-Xs) proteins, resulting in apoptosis in Hep 3B cells. This effect is correlated with the release of cytochrome *c* from the mitochondria into the cytoplasm and the activation of caspase-9.

We studied the interaction of ER and the mitochondrial pathway in our ongoing efforts to determine the apoptotic mechanism of EAC against Hep 3B cells. Release of ER Ca^{2+} to elevate cytosolic Ca^{2+} levels has been reported to be a key event in apoptosis (Orrenius et al., 2003). Ca^{2+} overload can induce mitochondrial depolarization,

Fig. 6. Depletion of ER Ca^{2+} (A) or inhibition of mitochondria uptake of Ca^{2+} (B) decreased EAC-mediated apoptosis in Hep 3B cells. Cells were pretreated with 50 nM thapsigargin or 50 μM Ru360 for 1 h before the addition of 100 $\mu\text{g/ml}$ EAC for another 24 h. The induction of apoptosis by EAC after 24 h treatment was then measured by Annexin V-FITC/PI kit. Each value is the mean $\pm$ SD of three determinations. The asterisk indicates a significant difference between control and EAC-treated cells (* $p < 0.05$).

and subsequently, the release of apoptosis-inducing factors (such as cytochrome *c*) which lead to the sequential activation of caspase-9 and caspase-3 (Chandra et al., 2004; Breckenridge et al., 2003). Several proapoptotic Bcl-2 family proteins, including Bax and Bid, are cleaved by calpain (Chiang et al., 2005; Mandic et al., 2002; Orrenius et al., 2003). In both cases, this cleavage of fragments enhances

rather than inhibits their proapoptotic activity (Chiang et al., 2005; Mandic et al., 2002; Orrenius et al., 2003). Our results demonstrated that cross-talk between calpain and the mitochondrial pathway is provided by Bid and Bax in EAC-treated Hep 3B cells. The cleavage of Bid by calpain supports the hypothesis that the proteolysis event of Bid is suppressed by calpain inhibitor pretreatment. Moreover, the inhibition of ER Ca^{2+} release or mitochondrial Ca^{2+} uptake blocks EAC-mediated apoptotic cell death. These results are consistent with recent evidence of close ER-mitochondrion cross-talk and the important role for ER in apoptotic signaling (Chandra et al., 2004; Breckenridge et al., 2003).

In this study, we concluded that the molecular mechanisms during EAC-mediated proliferation inhibition in Hep 3B involved: (1) induction of apoptosis, (2) initiation of Ca^{2+} /calpain pathway in Hep 3B cells, (3) triggering of the mitochondrial pathway, and (4) modulation of Bcl-2 family proteins. These results demonstrated that extract of *A. cinnamomea* fruiting bodies appears to be a promising chemopreventive agent for treating liver cancer.

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