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Herba houttuyniae extract induces apoptotic death of human promyelocytic leukemia cells *via* caspase activation accompanied by dissipation of mitochondrial membrane potential and cytochrome c release

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Abbreviations: Ac-DEVD-AFC, N-acetyl-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethyl-coumaine); Ac-DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp-CHO (aldehyde); Ac-LEHD-AFC, N-acetyl-Leu-Glu-His-Asp-AFC (7-amino-4-trifluoromethyl-coumaine); CsA, cyclosporin A; $DiOC_6(3)$, 3,3'-dihexyloxacarbocyanine; HH, *Herba houttuyniae*; PARP, poly(ADP-ribose) polymerase

Abstract

Herba houttuyniae **has been used as a constituent of herval medicine prescriptions for the treatment of inflammation, cancer, and other diseases. In the present study, we investigated the cellular effects of *herba houttuyniae* extract (HHE) and the signal pathways of HHE-induced apoptosis in HL-60 human promyelocytic leukemia cell line. HHE treatment caused apoptosis of cells as evidenced by discontinuous fragmentation of DNA, the loss of mitochondrial membrane potential, release of mitochondrial cytochrome *c* into the cytosol, activation of procaspase-9 and caspase-3, and proteolytic cleavage of poly(ADP-ribose) polymerase. Pretreatment of Ac-DEVD-CHO, caspase-3 specific inhibitor, or cyclosporin A, a mitochondrial permeability transition inhibitor, completely abolished HHE-induced DNA fragmentation. Together, these results suggest that HHE possibly causes mitochondrial damage leading to cytochrome *c* release into cytosol and activation of caspases resulting in PARP cleavage and execution of apoptotic cell death in HL-60 cells.**

Keywords: apoptosis; cancer; caspase; medicine, herbal; mitochondria; phytotherapy

Introduction

Herba houttuyniae, belongs to *Houttuynia cordata* THUNB (*Saururaceae*), is one of herbs that have been widely used in oriental medicine for treating allergy and cancer (Rho, 1998; Bae *et al.*, 2000). *Herba houttuyniae* extracts (HHE) administered on the acupuncture point prevented the increase of mass weight of melanoma BBL16 tumor cells inoculated into mice (Bae *et al.*, 2000). HHE treatment caused lowering of cell viability in various human cancer cell lines (Jung *et al.*, 1996). But the mechanism of tumorcidal affect of *Herba houttuyniae* is not well understood.

Apoptosis is a specific process that leads to programmed cell death through the activation of an evolutionarily conserved intracellular pathway (Bold *et al.*, 1997; Hall, 1999). Recently, many chemotherapeutic agents were reported to exert their anti-tumor effects by inducing apoptosis of cancer cells (Kamesaki, 1998). For example, PC-SPES, an eight-herb mixture, inhibited the proliferation of prostate cancer cells via apoptosis (Ikezoe *et al.*, 2001) and has been used for prostate cancer therapy in USA (Marks *et al.*, 2002). The molecular mechanisms of apoptosis have been extensively investigated in various models. Many investigators have demonstrated that mitochondria is a key regulator of apoptosis (Green and Reed, 1998; Susin *et al.*, 1998). It seems that mitochondrial changes including transition of mitochondrial membrane potential, cytochrome *c* release from mitochondria to cytosol provoke activation of series of caspases and finally cell death (Marchetti *et al.*, 1996; Kroemer and Reed, 2000). Caspases involved in apoptosis are divided into two groups, the initiator caspases, which include caspase-2, -8, -9, and -10, and the effector caspases, which include caspase-3, -6, and -7 (Shi, 2002). The activation of effector caspases by initiator caspases are responsible for the

proteolytic cleavage of cellular substrates including actin, lamin, poly(ADP-ribose) polymerase (PARP) and inhibitors of deoxyribonuclease (such as DFF45 or ICAD) (Kaufmann *et al.*, 1993; Shi, 2002). Cleavage of those substrates degrades the chromosomes into nucleosomal fragments during apoptosis (Enari *et al.*, 1998; Liu *et al.*, 1998).

In the present study, we investigated the molecular mechanism of HHE effects on HL-60 human promyelocytic leukemia cell line. HHE-induced apoptosis was accompanied by loss of mitochondrial membrane potential, release of cytochrome *c* into cytosol, procaspase-9 and caspase-3 activation and PARP cleavage in HL-60 cells. Finally we confirmed discontinuous ladder pattern of DNA, one of apoptotic signs, by treatment with HHE.

Materials and Methods

Culture conditions

The human leukemia cell line HL-60 was purchased from the American Type Culture Collection (Rockille, MD). Cells were placed into 75 cm^2 tissue culture flasks and grown at 37°C under a humidified, 5% CO_2 atmosphere in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 10% FBS and 2 mM glutamine, 10,000 units/ml of penicillin, 10 mg/ml of streptomycin, and 2.5 μg/ml of amphotericin B.

Preparation of herbal extract

The plant was identified as *Herba houttuyniae* by Do-Gon Ryu, keeper of Herbarium. Voucher samples were preserved for reference in the Herbarium of Department of Physiology, School of Oriental Medicine, Wonkwang Univ. (Omcphy, 2001-22). For extraction, 200 g of *Herba houttuyniae* were added to 1800 ml of water and boiled for 2 h, filtered and then concentrated to 200 ml. The sterile extract (58.55 g) was stored at -70°C.

MTT assay for cell viability

The viability of cultured cells was determined by assaying for the reduction of MTT to formazan (Oez *et al.*, 1990). In brief, after incubation with HHE, cells (10^4/well) in 96-well plates were washed twice with PBS and MTT (100 μg/0.1 ml of PBS) was added to each well. Cells were incubated at 37°C for 1 h, and DMSO (100 μl) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a spectrophotometer (model E-MAX, Molecular Devices).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was determined as the retention of the dye 3,3'-dihexyloxacarbocyanine ($DiOC_6(3)$; Molecular Probes Inc, Eugene, OR). Cells ($5×10^5$ in 500 μl of complete RPMI 1640 medium) were loaded with 100 nM $DiOC_6(3)$ during the last 30 min of treatment. The cells were then pelleted at 700 *g* for 10 min. The supernatant was removed, and the pellet was resuspended and washed twice in PBS. The pallet was then lysed by the addition of 600 μl of deionized water followed by homogenization. The concentration of retained $DiOC_6(3)$ was read on a spectrofluorometer (F-2500, Hitachi, Japan) with an excitation wavelength of 488 nm and an emission wavelength of 500 nm.

Detection of cytochrome c release

The release of mitochondrial cytochrome *c* was determined by Western blot (Kwon *et al.*, 2001). Briefly, at the end of various designated treatments, cells (1.5 $×10^7$ cells) were washed with PBS, and resuspended in ice-cold homogenizing buffer (250 mM sucrose, 20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). After 30 min incubation on ice, cells were homogenized with a glass Dounce homogenizer (30 strokes). The homogenate was subject to a series of centrifugations at 100,000 *g* for 60 min for the collection of mitochondria pellets and cytosolic fraction. Mitochondria pellets were resuspended in lysis buffer and frozen at -70°C for subsequent analysis. Thirty μg protein was loaded on 15% SDS polyacrylamide gel. After electrophoretic separation, the proteins were transferred to nitrocellulose membrane (Millipore, Bedford, MA) using a semi-dry blotting apparatus (Bio-Rad, Munich, Germany), and the blot was incubated with mouse anti-cytochrome *c* antibody (Pharmingen, San Diego, CA), followed by reaction with alkaline phosphatase conjugated secondary antibody.

Caspase activity assay

After treatment with HHE, cells were washed with ice-cold PBS and lysed in Triton X-100 buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.5) for 30 min on ice. Cell lysates were mixed with caspase assay buffer (10% glycerol, 2 mM DTT, and 20 mM HEPES, pH 7.5) containing 20 μM Ac-DEVD-AFC (specific for caspase-3) and 50 μM Ac-LEHD-AFC (specific for caspase-9), caspase substrates and incubated for 1 h at 37°C. Enzyme catalyzed release of AFC was monitored using a spectrofluorometer (F-2500, Hitachi, Japan) with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Western blot analysis of procaspases and PARP cleavage

Cell extract proteins were separated by SDS-PAGE. Subsequently the proteins were transferred onto a nitrocellulose membrane (Millipore) using a semi-dry blotting apparatus (Phamacia Biotech). Prior to incubation with antibodies against caspase-3, -9 and PARP (Transduction Lab.), membranes were blocked with 2% BSA for 30 min. After washing the membranes, an alkaline-phosphatase coupled secondary antibody was added. The target proteins became visible following the addition of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), a substrate of alkaline phosphatase.

Detection of DNA fragmentation by gel electrophoresis

Cell pellets ($3{\times}10^6$) were resuspended in 500 μl of lysis buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl, pH 8.0) at room temperature for 15 min and centrifuged at 16,000×g for 10 min. DNA was then extracted twice with phenol/chloroform (1:1), precipitated with ethanol, and resuspended in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNA was analyzed after separation by gel electrophoresis (2% agarose).

Protein determination

The cytosolic protein concentration in HL-60 cells was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard. All of the samples were assayed in triplicate.

Statistical analysis

Statistical analysis of the data was performed with Student's t-test and ANOVA. Differences with $P < 0.05$ were considered statistically significant.

Results

MTT conversion assay was used to determine the viability of HL-60 cells exposed to HHE. As shown in Figure 1A and 1B, treatment with HHE decreased cell viability in a dose- and time-dependent fashion. The IC_{50} of HHE for cell viability was 0.5 mg/ml.

To characterize the cell death of HL-60 cells, we further observed DNA fragmentation using agarose gel electrophoresis. DNA fragmentation was generally used as a marker of apoptotic cell death (Wyllie *et al.*, 1980). DNA extracts from HL-60 cells treated with HHE (1.0 mg/ml) for 24 h displayed a characteristic ladder pattern of discontinuous DNA fragments (Figure 2, lane 2). Figure 3A demonstrates HHE-induced loss of mitochondrial membrane potential (MMP). HHE reduced a retention time of $DiOC_6(3)$ in a time-related manner. Pretreatment of cyclosporin A (CsA), a mitochondrial permeability transition inhibitor, for 30 min completely protected against MMP disruption by HHE (Figure 3B).

Since release of cytochrome *c* from mitochondria into cytoplasm is considered to trigger a series of events leading to the activation of caspases, we determined whether HHE treatment could cause the translocation of mitochondrial cytochrome *c* into cytoplasm. In untreated cells, most of cytochrome c was localized in the mitochondria. However, the levels of

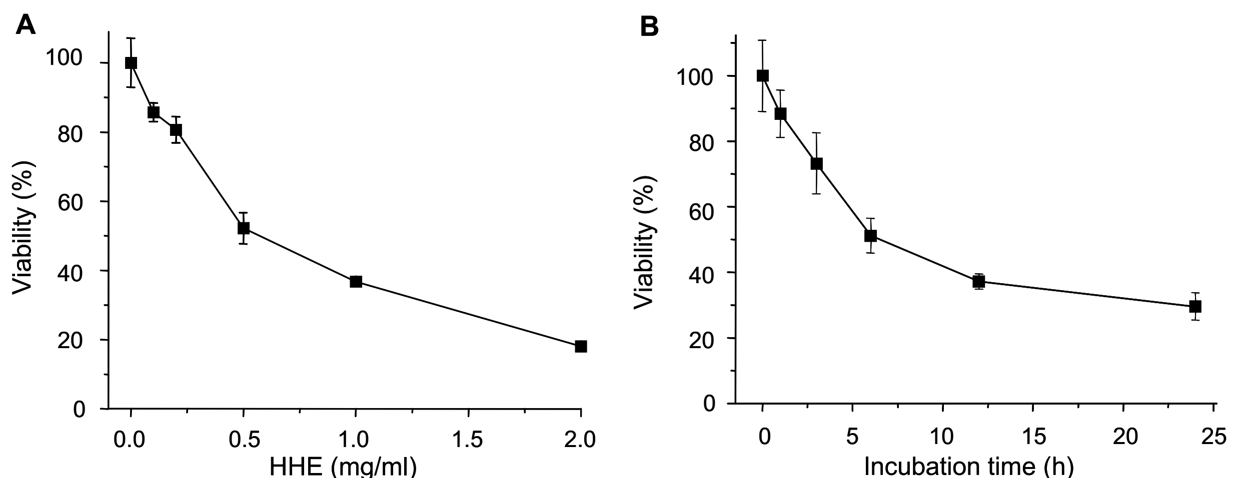


Figure 1. Effects of HHE on cell viability. (A) HL-60 cells ($1{\times}10^5$) were treated with various concentrations of HHE for 24 h. (B) Cells were treated with 1.0 mg/ml HHE for the indicated time periods. Their viability was determined by MTT assay. The percentage of viable cells was calculated as a ratio of A_{570} of treated- to control cells (treated with 0.05% DMSO vehicle). Each value is the mean±SEM of four independent experiments.

cytosolic cytochrome c increased in a time dependent manner after HHE treatment (Figure 4A) and this translocation was inhibited by the pretreatment of CsA (Figure 4B).

To asses whether MMP alteration and cytochrome *c* release are accompanied by activation of caspases that play a major role in execution of apoptotic events, the caspase enzyme activity was measured using specific fluorescence dyes, Ac-DEVD-AFC (specific for caspase-3) and Ac-LEHD-AFC (specific for caspase-9). As shown in Figure 5A, activation of caspase-3 occurred in a time-dependent manner. The cleavage of procaspase-9 in the cells treated with HHE was observed at 6 h, prior to the cleavage of procaspase-3 that started at 8 h (Figure 5B and C). PARP, a

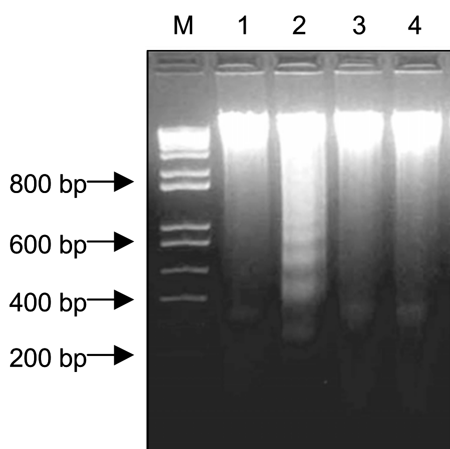


Figure 2. Inhibition of HHE-induced DNA fragmentation by CsA or caspase-3 inhibitor. HL-60 cells were treated with HHE for 24 h in the presence of CsA or Ac-DEVD-CHO. DNA was extracted and analyzed by 2% agarose gel electrophoresis as described in "Materials and Methods". Lane 1, control; Lane 2, 1.0 mg/ml HHE; Lane 3, 1.0 mg/ml HHE with 5 μM CsA, and Lane 4, 1.0 mg/ml HHE with 25 μM Ac-DEVD-CHO.

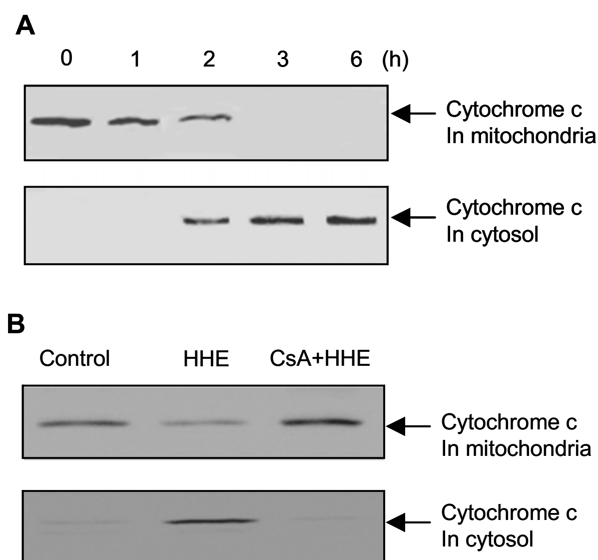
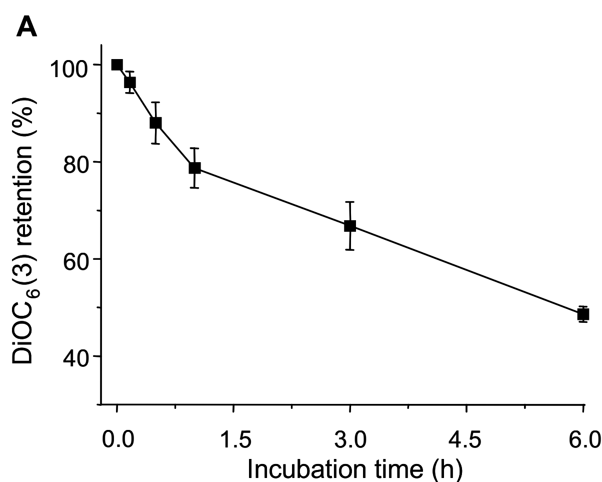


Figure 4. Effects of HHE on cytochrome c release from mitochondria to the cytosol. (A) HL-60 cells (1.5×10^7) were treated with 1.0 mg/ml HHE for the indicated time periods. (B) Cells were pretreated for 30 min with 5 μM CsA and then treated with HHE (1.0 mg/ml) for 6 h. Cytosolic and mitochondrial fractions were prepared and analyzed by western blotting as described in "Materials and Methods". Experiments were repeated three times with similar results and typical data were presented.



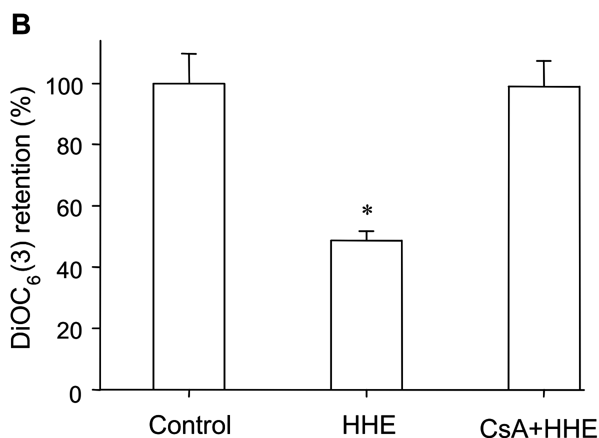


Figure 3. Effects of HHE on mitochondrial membrane potential in HL-60 cells. (A) Cells were treated with 1.0 mg/ml HHE for the indicated time periods. (B) Cells were pretreated for 30 min with 5 M CsA and then treated with HHE for 6 h. During the last 30 min of treatment, $DiOC_6(3)$ was added. An aliquot of the cells was used for the determination of cell-associated $DiOC_6(3)$ fluorescence. Each value is the mean± SEM of six independent experiments. *$P < 0.05$ compared with control.

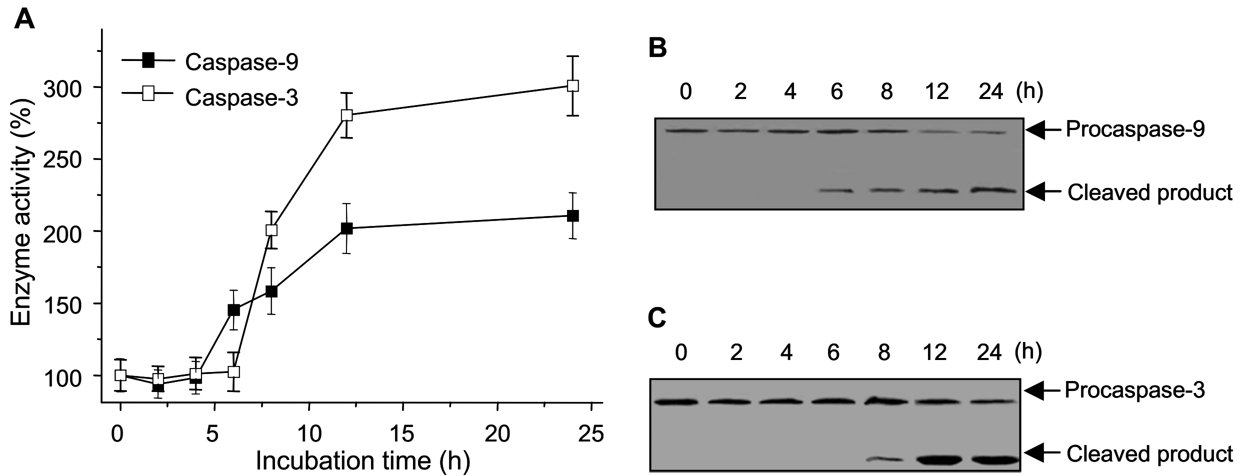


Figure 5. Induction of caspases activity and cleavage of procaspases by HHE. HL-60 cells (5×10^6) were treated with 1.0 mg/ml HHE for the indicated time periods. Cytosolic extracts were assayed for caspase-3 and 9 activities (A) and used for Western blotting against procaspase-9 (B), procaspase-3 (C) as described in "Materials and Methods". Values represent mean±SEM of six separate experiments. Experiments were repeated three times with similar results and typical data were presented.

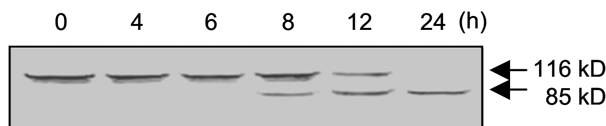


Figure 6. Time-related cleavage of PARP by HHE. HL-60 cells were treated with 1.0 mg/ml HHE for the indicated time periods and the cleavage of PARP was analyzed by western blotting as described in "Materials and Methods". Experiments were repeated three times with similar results and a typical data was presented.

DNA repair enzyme, recognized as one of the downstream substrates of caspase-3 and an excellent marker of apoptosis, was next examined as possible target substrates for HHE-treatment. Indeed HHE-induced activation of caspase-3 caused PARP cleavage which separates N-terminal DNA-binding domain from its C-terminal catalytic domain (85 kDa) (Kaufmann *et al.*, 1993). Figure 6 illustrated the gradual increase in the proportion of the 85 kDa cleavage product in a time-dependent manner. These results correspond to activation and cleavage of caspase-3 as shown in Figure 5A and C.

To further confirm the involvement of MMP disruption and of caspases in HHE-induced apoptosis of HL-60 cells, general inhibitors such as CsA and Ac-DEVD-CHO (specific inhibitor of caspase-3) were tested. When HL-60 cells were pretreated with 25 μM Ac-DEVD-CHO or 5 μM CsA, both caspase-3 activation and PARP cleavage by HHE treatment were completely blocked (Figure 7). Furthermore, the discontinuous ladder pattern of DNA induced by HHE-treatment was also prevented by the inhibitors (Figure 2, lane 3 and 4).

These observations suggest that MMP alteration, cytochrome *c* release to cytosol, activation of caspase enzyme activity and PARP cleavage are involved in HHE-induced apoptosis of HL-60 cells.

Discussion

The present finding demonstrated that a herb medicine, HHE induces apoptosis of human promyelocytic leukemia cells through caspases activation involving dissipation of mitochondrial membrane potential and cytochrome *c* release from mitochondria into the cytosol.

Cancer chemoprevention utilizing chemical compounds or natural products revert or inhibit malignant cell transformation and prevent invasion and metastasis would be a less painful, more economical and rational approach for cancer control. The use of natural herb medicines or dietary agents are being increasingly utilized as an effective way for the management of many cancer treatment (Mukhtar and Ahmad, 1999; Kelloff *et al.*, 2000; Sporn and Suh, 2000; Setiawan *et al.*, 2001). However their exact mechanisms involved in cancer prevention effects are not well understood.

Apoptosis is a biomarker in chemoprevention trials (Riss, 2001) and a complex process that involves many different signaling pathways and results in a multitude of changes in the dying cell. It is characterized by chromatin condensation, membrane blebbing, cell shrinkage, and DNA fragmentation (Wyllie *et al.*, 1980). In this experiment, we found that cell death

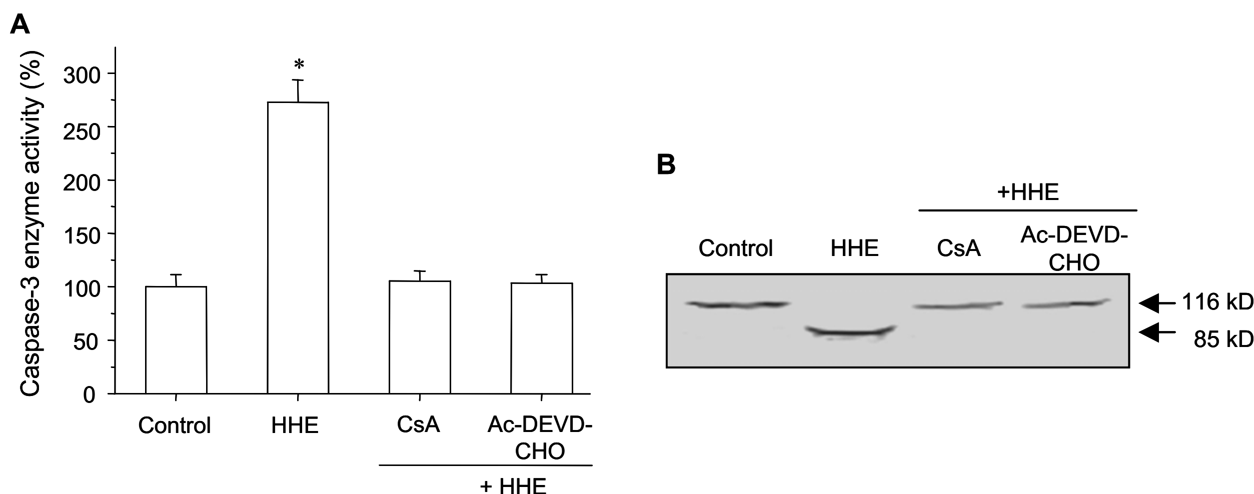


Figure 7. Inhibition of HHE-induced caspase-3 activation and PARP cleavage by CsA or Ac-DEVD-CHO. HL-60 cells (5×10^6) were pretreated with Ac-DEVD-CHO (25 μM) or CsA (5 μM) for 30 min followed by treatment with 2.0 mg/ml HHE for further 24 h. Cytosolic extracts were prepared and assayed for caspase-3 activity (A) and PARP cleavage (B) as described in Materials and Methods. Values represents means±SEM of six separate experiments. $^*P < 0.05$ compared with control.

by HHE represents an apoptotic pattern as proved by DNA fragmentation in Figure 2. In the course of apoptosis, mitochondria have shown to be involved in integrating different pro-apoptotic pathways via release of cytochrome *c* into the cytosol (Marchetti *et al.*, 1996; Green and Reed, 1998). Also, it has been suggested that disruption of MMP plays a pivotal role in initiation of apoptotic induction and is related to the release of cytochrome *c* (Marchetti *et al.*, 1996; Li *et al.*, 1997; Green and Reed, 1998). Once translocated into the cytosol, cytochrome *c* interacts with Apaf-1, which permits recruitment of procaspase-9 to form apoptosome, a critical activator of effector caspases including caspase-3 (Li *et al.*, 1997). In HL-60 cells, HHE produced a time-dependent loss of $DiOC_6(3)$, a result demonstrating the specificity of CsA in preventing the loss of MMP. The fluorescent dye $DiOC_6(3)$ localizes to mitochondria as a consequence of MMP, and the membrane permeability transition (MPT) reduces the accumulation of $DiOC_6(3)$ as a consequence of the loss of MMP (Vayssiere *et al.*, 1994; Krippner *et al.*, 1996). The loss of MMP by HHE induced the redistribution of cytochrome *c* from mitochondria into cytosol and the release of cytochrome *c* into cytosol was inhibited by pretreatment of CsA in HHE-treated HL-60 cells. These results suggest that mitochondrial changes are participated in HHE-induced death of HL-60 cells. The released cytochrome *c* activated the pro-caspase 9 in a dATP-dependent manner to form the apoptosome from which the release of activated caspase-9 further initiates the activation of caspase cascade leading to biochemical and morphological changes associated with apoptosis (Li *et al.*, 1997). Consistent with this report, HHE induced the activation and cleavage of procaspase-9 from 6 h of HHE treatment, but activation and cleavage of procaspase-3 was started from 8 h and sustained until 24 h. Indicating that caspase-9 is upstream of caspase 3-like caspase, however there may be other mechanism(s) for caspase-3 activation apart from the cascade mediated through activation of caspase-9. Further studies are needed to investigate those additional pathways of caspase-3 activaton by HHE. And involvement of other effector caspases (-1, -6, -7) (Shi, 2002) in HHE-induced apoptosis of HL-60 cells cannot be excluded. The increase in caspase-3 activity by HHE was accompanied by a PARP cleavage in a time-dependent fashion. Pretreating cells with the caspase-3 specific inhibitor, Ac-DEVD-CHO, and inhibitor of MMP dissipation, CsA, inhibited HHE-induced caspase-3 activation, PARP cleavage and DNA fragmentation. These results suggested the involvement of MMP damage, release of cytochrome *c*, activations of pro-caspase 9 and caspase-3, PARP cleavage in HHE-caused apoptotic death of HL-60 cells. However, in the present study, we did not notice any significant alterations in the level of Bcl-2 family of proteins localized at the outer mitochondrial membranes (data not shown). Bcl-2 and related protein, Bcl-xL exerts anti-apoptotic functions by preventing release of cytochrome *c* from mitochondria into cytosol (Bossy-Wetzel and Green, 1999).

Consequently, the studies of these signaling pathways will advance our knowledge and understanding of the efficacy of many chemopreventive compounds some of which may become important therapeutic drugs of the future.

Acknowledgement

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