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Emodin induces apoptosis in human promyeloleukemic HL-60 cells accompanied by activation of caspase 3 cascade but independent of reactive oxygen species production

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Abstract

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an active constituent of *Rheum palmatum*, and showed inhibitory activity on lipopolysaccharide-induced NO production in our previous study. However, the apoptosis-inducing activity of emodin has remained undefined. Among three structurally related anthraquinones, including emodin, physcion, and chrysophanol, emodin showed the most potent cytotoxic effects on HL-60 cells, accompanied by the dose- and time-dependent appearance of characteristics of apoptosis including an increase in DNA ladder intensity, morphological changes, appearance of apoptotic bodies, and an increase in hypodiploid cells. Emodin at apoptosis-inducing concentrations causes rapid and transient induction of caspase 3/CPP32 activity, but not caspase 1 activity, according to cleavage of caspase 3 substrates poly(ADP-ribose) polymerase and D4-GDI proteins, the appearance of cleaved caspase 3 fragments being detected in emodin- but not physcion- or chrysophanol-treated HL-60 cells. A decrease in the anti-apoptotic protein, Mcl-1, was detected in emodin-treated HL-60 cells, whereas other Bcl-2 family proteins including Bax, Bcl-2, Bcl-XL, and Bad remained unchanged. The caspase 3 inhibitor, Ac-DEVD-CHO, but not the caspase 1 inhibitor, Ac-YVAD-CHO, attenuated emodin-induced DNA ladders, associated with the blockage of PARP and D4-GDI cleavage. Free radical scavenging agents including NAC, catalase, SOD, ALL, DPI, L-NAME and PDTC showed no preventive effect on emodin-induced apoptotic responses, whereas NAC, CAT and PDTC prevented HL-60 cells from ROS (H_2O_2)-induced apoptosis through inhibition of caspase 3 cascades. Induction of catalase, but not SOD, activity was detected in emodin-treated HL-60 cells by in gel activity assays, and H_2O_2-induced intracellular peroxide level was significantly reduced by prior treatment of emodin in HL-60 cells. Our experiments provide evidence that emodin is an effective apoptosis inducer in HL-60 cells through activation of the caspase 3 cascade, but that it is independent of ROS production.

Keywords: Emodin; Apoptosis; Caspase 3; ROS; Catalase; SOD

1. Introduction

Apoptosis, or programmed cell death, is a highly regulated process that involves activation of a serious of molecular events, leading to cell death that is characterized by cellular morphological change, chromatin condensation, and apoptotic bodies which are associated with DNA cleavage into ladders [1,2]. Several pathways have been described to regulate apoptosis during development, tumorigenesis, and chemical treatments [3]. Previous studies indicated that cells from a variety of human

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Abbreviations: MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); PARP, poly(ADP-ribose) polymerase or poly(ADP-ribosyl) polymerase; Bcl-2, B-cell lymphoma 2; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; EMO, emodin; PHY, physcion; CHR, chrysophanol; SOD, superoxide dismutase; ALL, allopurinol; DPI, diphenylene iodonium; L-NAME, *N*-nitro-L-arginine methyl ester; PDTC, pyrrolidine dithiocarbamate; GSH, glutathione; GSSG, glutathione disulfide; DEVD, Asp-Glu-Val-Asp; YVAD, Tyr-Val-Ala-Asp.

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malignancies have a decreased ability to undergo apoptosis in response to some physiological stimuli [4,5]. Several genes' expressions have been demonstrated to be critical in the regulation of apoptosis such as caspase cascades and B-cell lymphoma 2 (Bcl-2) family proteins. Human caspases 1–10 have been described, and a previous study demonstrated that activation of the caspase cascade is involved in chemical- and agent-induced apoptosis [6]. Caspase 9 is an apoptosis initiator and is activated by binding with Apaf-1, a homologue of CED-4, to induce its oligomerization [7,8]. Activated caspase 9 then cleaves and activates executioner caspase 3, which exists as an inactive pro-caspase 3 in the cytoplasm and is proteolytically activated by multiple cleavages of pro-caspase 3 to generate the cleaved fragments in cells undergoing apoptosis [9,10]. After caspase 3 activation, some specific substrates for caspase 3 such as poly(ADP-ribose) polymerase or poly(ADP-ribosyl) polymerase (PARP) and D4-GDI proteins are cleaved which are important for the occurrence of apoptosis. PARP is required for DNA repair and activated caspase 3 cleaves PARP at Asp 216 to generate the 85 and 31 kDa apoptotic fragments in coordination with DNA fragmentation during apoptosis [11,12]. And, D4-GDI is a negative regulator of the ras-related Rho family of GTPases, and activation of Rho GTPases promoted cytoskeletal and membrane changes associated with apoptotic cells. Activated caspase 3 cleaves D4-GDI to 23- and 5-kDa fragments, and activates Rho GTPases to produce apoptotic morphological changes [13,14]. Bcl-2 family proteins modulate the occurrence of apoptosis and tumorigenesis [15]. Members of the Bcl-2 family proteins can be divided into two subfamilies, one is anti-apoptotic including Bcl-2, Mcl-1, and Bcl-XL proteins and the other is pro-apoptotic including Bax, Bcl-Xs and Bad. Induction of pro-apoptotic Bcl-2 family proteins and inhibition of anti-apoptotic family proteins have been detected in apoptosis induced by chemicals [16].

The maintenance of homeostasis in normal tissues reflects a balance between cell proliferation and apoptosis. However, apoptosis is often inhibited or attenuated in tumors with a higher cell proliferation rate. Therefore, one of the attractive ways in cancer chemoprevention or chemotherapy is dietary or pharmaceutical production to induce death of tumor cells through apoptosis. Several previous studies demonstrated that certain phytochemicals presented in medicinal herbs or dietary plants exert anti-tumorigenic activity by inducing apoptosis in cancer cells [17,18]. Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an active constituent of Chinese herbs including *Rheum officinale* and *Polygonum cuspidatum* [19,20]. Pharmacological studies have reported that emodin possesses anti-cancer, anti-bacterial, diuretic, immunosuppressive, and vasorelaxant activities [21,22]. In contrast to the above beneficial activities, emodin has also been found to be mutagenic [23]. The mutagenic effect of emodin was suspected to result from its pro-oxidant activity rather than the antioxidant activity of emodin [24]. A recent study reported that emodin is a potent inhibitor of the protein kinase, p56lck, and inhibits the transformation ability and growth rate of HER-2/neu-overexpressing breast cancer cells [25]. Further, emodin was shown to sensitize HER-2/neu-overexpressing lung cancer cells to chemotherapeutic agents such as etoposide and doxorubicin [26]. Our recent study also showed that emodin inhibited lipopolysaccharide-induced nitric oxide production in RAW264.7 cells [27]. These data indicated that emodin is a functional compound with multiple biological activities, however the apoptosis-inducing activity of emodin is still undefined. In order to evaluate an anti-tumorigenic potential of emodin, the apoptosis-inducing activity of emodin in cultured human promyeloleukemic cells HL-60 has been determined.

2. Materials and methods

2.1. Cell culture and chemicals

HL-60 human myelogenous leukemia cells were obtained from American Type Culture Collection (ATCC). HL-60 cells were grown at 37° in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS) in an atmosphere containing 5% CO_2. Exponentially HL-60 cells were exposed to drugs for the indicated time periods. The plates for HL-60 cells were purchased from Gibco (GIBCO/BRL). The colorigenic synthetic peptide substrates for caspase 3-like proteases (Ac-DEVD-pNA) and for caspase 1 (Ac-YVAD-pNA) were purchased from Calbiochem. The inhibitors for caspase-3-like proteases (Ac-DEVD-CHO) and for caspase 1 (Ac-YVAD-CHO) were purchased from Calbiochem. Propidium iodide was obtained from Sigma Chemical Co. Emodin, physcion and chrysophanol were isolated from the Chinese herbal plant Rhei Rhizoma, and the purities of them were more than 98% when analyzed by HPLC. Antibodies for PARP, caspase 3, and D4-GDI detection in western blotting were obtained from IMGENEX. And, antibodies for detecting Bcl-2 family proteins and α-tubulin were purchased from Santa Cruz. DCHF-DA was obtained from Molecular Probe.

2.2. Cell viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining as described by Mosmann [28]. Briefly, HL-60 cells were plated at a density of 10^6 cells/mL into 24-well plates and treated with different concentrations of indicated compounds for 12 hr. At the end of treatment, 20 μL of MTT (10 mg/mL) was added, and cells were incubated for further 4 hr. Cell viability was obtained by scanning

with an ELISA reader (Molecular Devices) with a 600-nm filter.

2.3. ROS production determination

The reactive oxygen species (ROS) production was monitored by flow cytometry using DCFH-DA [29]. This dye is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within cells. Hydrogen peroxide or low-molecular-weight peroxides produced by cells oxidizes DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. In the present study, HL-60 cells were treated with emodin (40 μM) for 2 or 4 hr, respectively. Then, emodin-treated cells were washed twice with PBS to remove the extracellular emodin, and addition of DCHF-DA (100 μM) with or without H_2O_2 (200 μM) for one more hour. Green fluorescence was excited by using an argon laser and was detected using a 525-nm band pass filter by flow cytometry analysis.

2.4. Western blots

Total cellular extracts (30 μg) were prepared and separated on 8% SDS-polyacrylamide minigels for PARP detection and 12% for SDS-polyacrylamide minigels for caspase 3, cleaved D4-GDI, Bcl-2 family and α-tubulin detection, and transferred to Immobilon polyvinylidenedifluoride membranes (Millipore). The membrane was incubated overnight at 4° with 1% bovine serum albumin (BSA) at room temperature for 1 hr and then incubated with indicated antibodies for a further 3 hr at room temperature followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG antibody for an hour. Protein was visualized by incubating with the colorimetric substrates nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as described in our previous paper [30].

2.5. DNA gel electrophoresis

Cells (10^6 mL^{-1}) under different treatments were collected, washed with PBS twice and then lysed in 100 mL of lysis buffer (50 mM Tris, pH 8.0; 10 mM ethylenediaminetetraacetic acid (EDTA); 0.5% sodium sarkosinate, and 1 mg/mL proteinase K) for 3 hr at 56° and treated with 0.5 mg/mL RNase A for another hour at 56°. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) before loading. Samples were mixed with loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting-point argarose, 0.025% (w/w) bromophenol blue) and loaded onto a pre-solidified 2% agarose gel containing 0.1 μg/mL ethidium bromide. The agarose gels were run at 50 V for 90 min in TBE buffer. The gels were observed and photographed under UV light.

2.6. Activities of caspase 3 and caspase 1 (ICE) proteases

After different treatments, cells (10^6 mL^{-1}) were collected and washed three times with PBS and resuspended in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 10 mM ethyleneglycoltetraacetic acid (EGTA). Cell lysates were clarified by centrifugation at 18,000 *g* for 3 min, and clear lysates containing 50 μg of protein were incubated with 100 μM of enzyme-specific colorimetric substrates including Ac-DEVD-pNA for caspase 3/CPP32 and Ac-YVAD-pNA for caspase 1 at 37° for 1 hr. Alternative activity of caspase 3 or caspase 1 was described as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm.

2.7. Flow cytometry analysis

Trypsinized cells (10^6 mL^{-1}) were washed with ice-cold PBS and fixed in 70% ethanol at −20° for at least 1 hr. After fixation, cells were washed twice, incubated in 0.5 mL of 0.5% Triton X-100/PBS at 37° for 30 min with 1 mg/mL of RNase A, and stained with 0.5 mL of 50 mg/mL propidium iodide for 10 min. Fluorescence emitted from the propidium–DNA complex was quantitated after excitation of the fluorescent dye by FACScan flow cytometry (Becton Dickenson).

2.8. Superoxide dismutase (SOD) activity assay in gels

HL-60 cells (10^6 mL^{-1}) were treated with emodin (40 μM) for different time periods, and cell lysates were collected as described in our previous paper [14]. Native gel analysis was performed to analyze the activity of SOD in the lysate. The gels (5% acrylamide stacking gel and 8% acrylamide resolving gel) were run in 15.0 mM Tris, 11.5 mM glycine buffer (pH 9.1), then stained for SOD activity using nitroblue tetrazolium [31].

2.9. Catalase activity assay in gels

HL-60 cells (10^6 mL^{-1}) were treated with emodin (40 μM) for different time periods, and cell lysates were collected as described in our previous paper [14]. Electrophoresis was performed in 7.5% polyacrylamide gels without 0.1% SDS. Catalase activity in lysates was analyzed by ferricyanide staining as described by Wayne and Diaz [32].

2.10. GSH and GSSG measurement

HL-60 cells (10^6 mL^{-1}) were treated with different compounds for 6 hr and washed twice with PBS, lysed with 3% perchloric acid for 15 min at 4°. The supernatants were then neutralized with 0.1 M NaH_2PO_4, and 5 mM EDTA, pH 7.5. Total glutathione (GSH) was assayed in the supernatant in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The rate of reduction of DTNB was

monitored at 412 nm. Glutathione disulfide (GSSG) was measured by means of the same method after derivatization with 2-vinylpyridine.

2.11. Statistics

Values are expressed as the mean ± SE. The significance of the difference from the respective controls for each experimental test condition was assayed by using Student's *t*-test for each paired experiment. A P value of <0.01 or <0.05 was regarded as indicating a significant difference.

3. Results

3.1. Induction of apoptosis by emodin

Our previous study demonstrated that emodin showed potent inhibitory activity on lipopolysaccharide-induced inflammatory responses [9]. However, the anti-tumor activity of emodin is still unclear. Three structurally related anthraquinones including emodin (EMO), physcion (PHY), and chrysophanol (CHR) were applied in this study. The chemical structures of EMO, PHY, and CHR are shown in Fig. 1. Arrows indicate the different groups among these three anthraquinones. When HL-60 cells were treated with various concentrations of each indicated compound (40 and 80 μM) for 12 hr, the viability of HL-60 cells was reduced significantly in the presence of EMO, but not in PHY- or CHR-treated HL-60 cells (Fig. 2A). DMSO, even at the highest dose of 0.5%, showed no effect on cellular viability of HL-60 cells. To characterize cell death induced by EMO, the integrity of genomic DNA, occurrence of apoptotic bodies, and the ratio of hypodiploid cells were examined. On analysis of DNA integrity by agarose electrophoresis, EMO treatment caused the digestion of genomic DNA into ladders in a concentration- and time-dependent way, associated with a decrease in intact DNA (Fig. 2B). However, with PHY and CHR, even at 80 μM, no significant DNA ladders were found. In the same part of the experiment, morphological changes and the ratio of hypodiploid cells were examined under microscopic observation and flow cytometric analysis, respectively. EMO, but not PHY or CHR, induced the occurrence of apoptotic bodies under microscopic observation, accompanied by an increased ratio of hypodiploid cells under flow cytometric analysis. (Fig. 3). No obvious DNA ladders, apoptotic bodies, or hypodiploid cells were detected in DMSO-treated HL-60 cells. These data demonstrate that emodin is an apoptosis inducer in HL-60 cells.

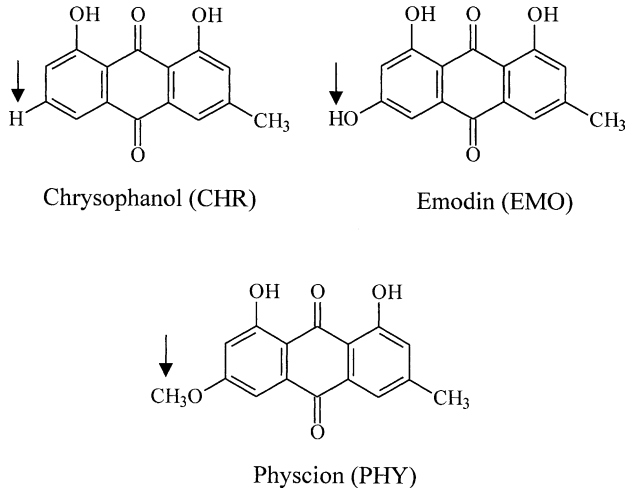

Fig. 1. Structure of the tested structurally related anthraquinones including emodin, physcion, and chrysophanol. Arrow indicted the difference between these compounds.

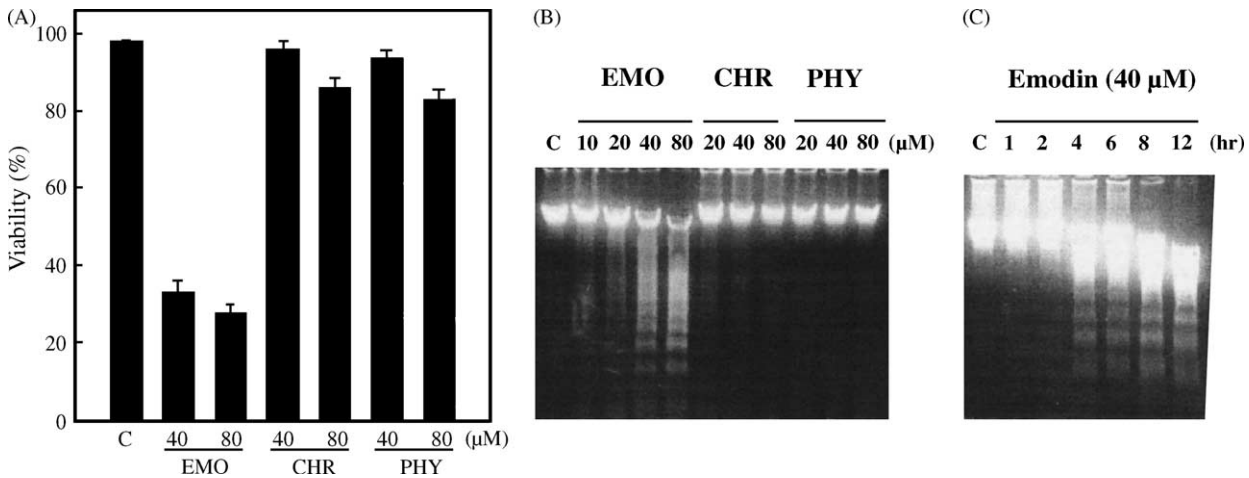

Fig. 2. Analysis of cell viability and DNA integrity in emodin (EMO), physcion (PHY) and chrysophanol (CHR)-treated HL-60 cells by MTT assay and agarose electrophoresis. (A) HL-60 cells were plated into 24-well plates for 24 hr and then treated with different concentrations of indicated compound (40 and 80 μM) for a further 12 hr. MTT was added into medium for an additional 4 hr. The viability of cells was detected by measuring the absorbance at a wavelength of 600 nm. (B) (Left panel) HL-60 cells were treated with different concentrations (10, 20, 40, 80 μM) of the EMO, PHY, and CHR for 12 hr. (Right panel) HL-60 cells were treated with emodin (40 μM) for 1, 2, 4, 6, 8 and 12 hr. Integrity of DNA in cells was extracted and electrophoresed through a 1.8% agarose gel and visualized by staining with ethidium bromide.

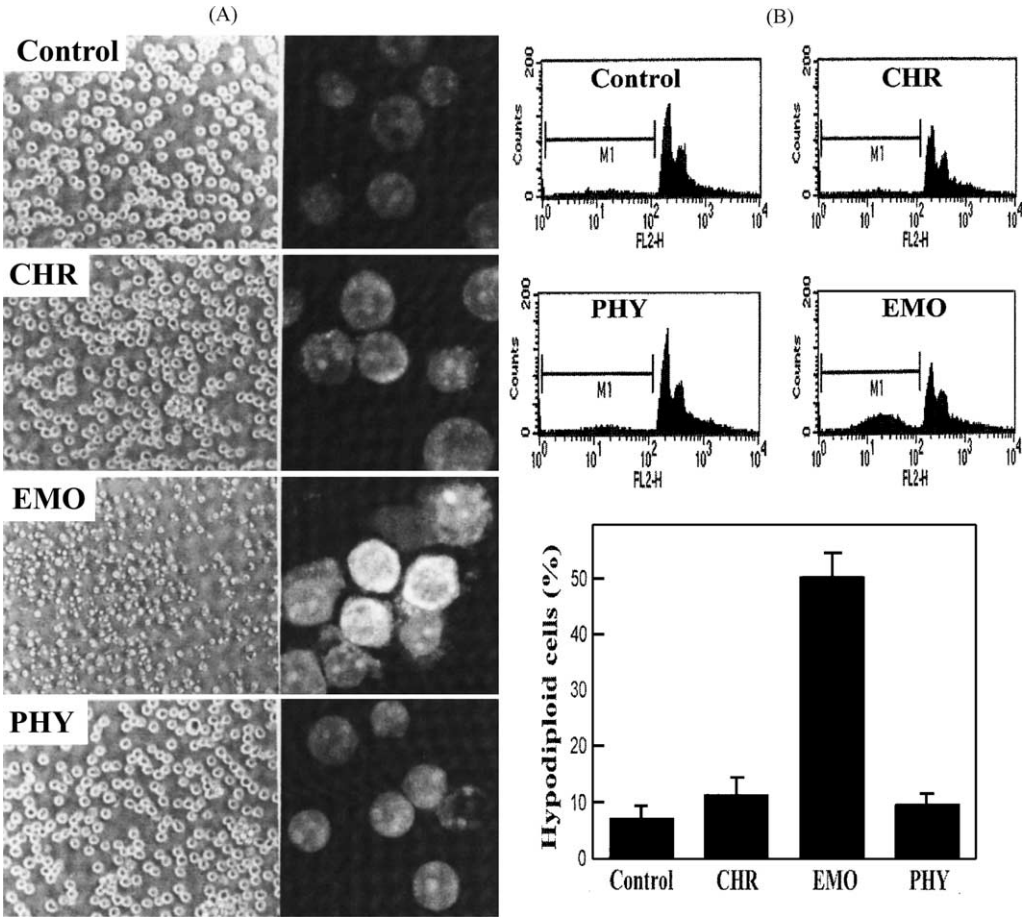

Fig. 3. Appearance of chromosomal condensation, hypodiploid cells and apoptotic bodies in EMO-treated HL-60 cells. (A) Occurrence of apoptotic bodies and chromosomal condensation in HL-60 cells was detected in EMO (but not PHY and CHR)-treated HL-60 cells under light microscopic observation (left panel) and fluorescent microscopic observations using acridine orange staining (right panel). (B) Induction of hypodiploid cells in EMO (but not PHY and CHR)-treated HL-60 cells. HL-60 cells were treated with or without EMO, PHY and CHR (40 μM) for 12 hr. Appearance of hypodiploid cells was detected by flow cytometry using PI staining. Upper panel is a representative of the result of flow cytometry analysis, and lower panel is the value presented as the mean ± SE of three independent experiments. EMO: emodin; PHY: physcion; CHR: chrysophanol.

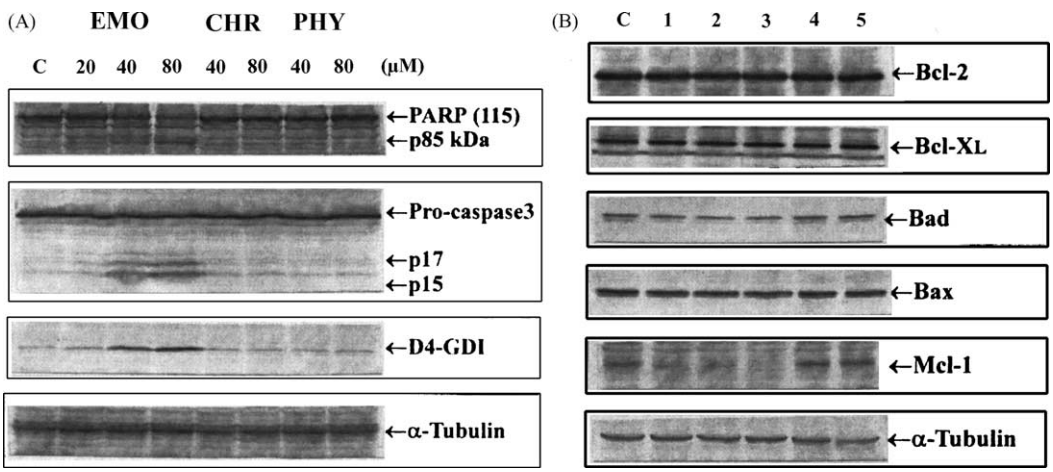

Fig. 4. Induction of caspase 3 protein processing, PARP and D4-GDI protein cleavage, and a decrease in Mcl-1 protein in EMO but not PHY and CHR–treated HL-60 cells. (A) HL-60 cells were treated with different concentrations (20, 40, and 80 μM) of EMO, PHY, CHR for 12 hr. PARP and D4-GDI cleavage and induction of caspase 3 protein procession were analyzed by Western blotting as described in Section 2. α-Tubulin presented here was used as an internal control. (B) Alternative expression of Bcl-2 family proteins including Bcl-2, Bcl-XL, Bad, Bax and Mcl-1 in EMO (but not PHY and CHR)-treated HL-60 cells. Cells were treated with different concentrations (20, 40, and 80 μM) of EMO or PHY and CHR (80 μM) for 12 hr, and the expression of the indicated protein was evaluated using specific antibodies for Western blot. C, control; (1) 20 μM EMO; (2) 40 μM EMO; (3) 80 μM EMO; (4) 80 μM CHR; (5) 80 μM PHY. EMO, emodin; PHY, physcion; CHR, chrysophanol.

3.2. Involvement of PARP and D4-GDI cleavage, caspase 3 protein procession, and a decrease in Mcl-1 protein in emodin-induced apoptosis

Activation of caspase 3 leads to the cleavage of a number of proteins, two of which are PARP and D4-GDI, another hallmark of apoptosis. Fig. 4A shows that exposure of HL-60 cells to EMO caused the degradation of 116-kDa PARP into 85-kDa fragments and the production of cleaved D4-GDI protein (23-kDa) in a concentration-dependent manner, associated with the protein procession of caspase 3 brought about by its cleavage, represented here as a decline in its pro-level and the appearance of cleaved fragments on the Western blot. However, PHY and CHR showed no obvious effects on PARP, D4-GDI, or caspase 3 cleavage in HL-60 cells. Bcl-2 family proteins act as important regulators of apoptosis and are located upstream of caspase activation. In EMO-treated HL-60 cells, a decrease in the Mcl-1 protein was detected in a dose-dependent manner (lane 1: 20 μM; lane 2: 40 μM; lane 3: 80 μM). In contrast to the alternative expression of Mcl-1 proteins, Bcl-2, Bcl-XL, Bax, and Bad proteins remained unchanged in EMO-treated HL-60 cells (Fig. 4B). No obvious change was detected in the protein expression of Bcl-2 family proteins in CHR (lane 4) and PHY (lane 5)-treated HL-60 cells at a dose of 80 μM.

3.3. Free radical scavengers showed no preventive effect on EMO-induced apoptotic responses

Free radical-producing and -scavenging activities have been described as biological effects of EMO. In order to demonstrate if ROS are involved in EMO-induced apoptosis, free radical-scavenging agents, *N*-acetyl-L-cysteine

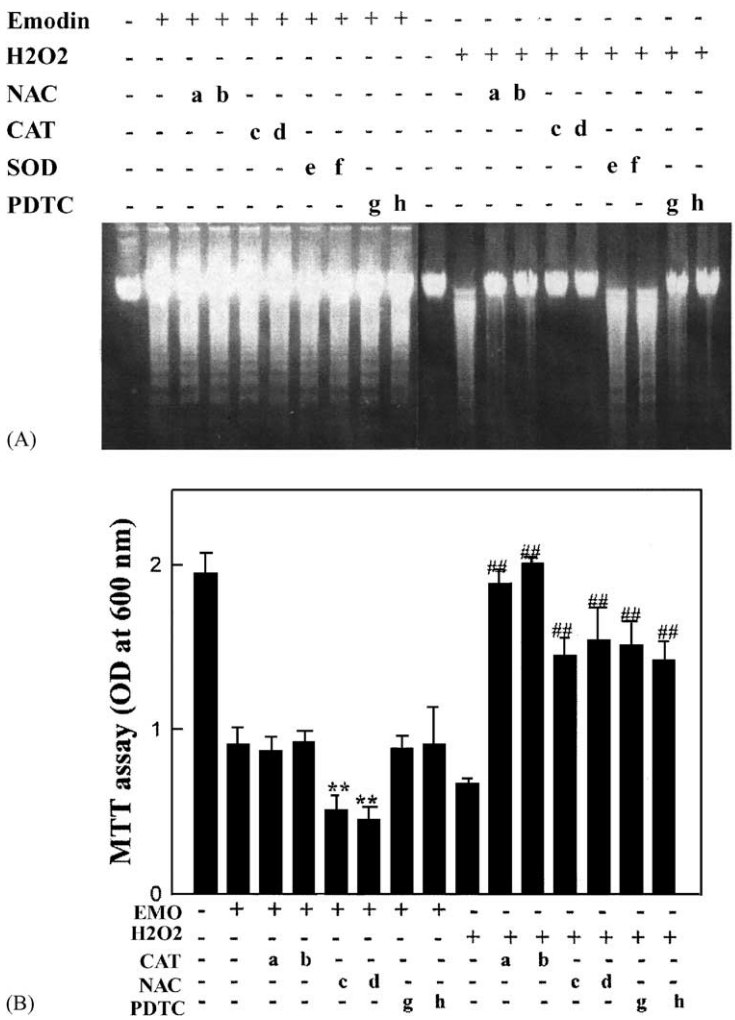

Fig. 5. Effects of NAC, CAT, DPI, PDTC, SOD, ALL and L-NAME on emodin-induced apoptosis. (A) HL-60 cells were treated with NAC ((a) 10 mM; (b) 20 mM), CAT ((c) 200 U/mL; (d) 400 U/mL), SOD ((e) 200 U/mL; (f) 400 U/mL), PDTC ((g) 20 μM; (h) 40 μM) for 1 hr followed by emodin (40 μM), or H_2O_2 (200 μM) treatment for a further 12 hr. Integrity of DNA was analyzed by agarose electrophoresis. (B) HL-60 cells were treated with CAT, NAC or PDTC for 1 hr followed by emodin (40 μM) treatment for a further 12 hr. MTT was added into medium for an additional 4 hr. The viability of cells was detected by measuring the absorbance at 600 nm. Each value is presented as the mean ± SE of three independent experiments. $^{\#\#}P < 0.01$ significantly different from the control, as analyzed by Student's *t*-test. $^{**}P < 0.01$ significantly different from emodin-treated group as analyzed by Student's *t*-test. NAC, *N*-acetyl-cysteine; CAT, catalase, SOD, superoxide dismutase, PDTC, pyrrolidine dithiocarbamate, ALL, allopurinol, DPI, diphenylene iodonium and L-NAME, *N*-nitro-L-arginine methyl ester.

(NAC), catalase (CAT), superoxide dismutase (SOD), pyrrolidine dithiocarbamate (PDTC), a xanthine oxidase inhibitor (allopurinol; ALL), a NADPH oxidase inhibitor (diphenylene iodonium; DPI) and a NOS inhibitor (*N*-nitro-L-arginine methyl ester; L-NAME) were used in this study. In the absence of EMO, NAC and DPI at dose of 40 μM showed cytotoxicity to HL-60 cells, represented here as the loss of DNA integrity by agarose electrophoresis, whereas, CAT, ALL, SOD, and L-NAME at the highest tested dose showed no obvious cytotoxic effect on HL-60 cells (data not shown). Therefore, prior treatment of cells with NAC ((a) 10 mM; (b) 20 mM), CAT ((c) 200 U/mL; (d): 400 U/mL), SOD ((e) 200 U/mL; (f) 400 U/mL), PDTC ((g) 20 μM; (h) 40 μM), DPI (10 μM; 20 μM), or L-NAME (200 μM; 400 μM) for 30 min followed by EMO (40 μM) or H_2O_2 (200 μM) treatment for further 12 hr was performed. Results in Fig. 5A show that none of the agents listed above showed a preventive effect on EMO-induced DNA fragmentation, whereas NAC, CAT, and PDTC significantly inhibited H_2O_2-induced DNA fragmentation. Results of the MTT assay in Fig. 5B show that a decrease in cell viability was evident in EMO- or H_2O_2-treated HL-60 cells, and CAT, NAC, and PDTC could recover the decreased viability induced by H_2O_2, but not by EMO. Under NAC and EMO co-treatment, it is appeared that NAC showed the significant potentiation on EMO-induced cytotoxicity by MTT assay. NAC is a free radical scavenger and glutathione precursor. Therefore, intracellular GSH and glutathione disulfide (GSSG) were measured in HL-60 cells under different circumstances with or without EMO. As shown in Table 1, the intracellular levels of GSH (not GSSG) in NAC-treated cells were significantly elevated in the presence or absence of EMO. However, EMO, CAT, SOD and L-NAME showed no effect on the intracellular levels of GSH and GSSG, compared with control group. Upon Western blot analysis, neither NAC nor CAT showed inhibition of EMO-induced PARP or D4-GDI cleavage, and caspase 3 protein processing, however PARP cleavage, D4-GDI cleavage and caspase 3 processing induced by H_2O_2 were obviously attenuated by CAT in cells (Fig. 6A). Furthermore, decreases in Bcl-2 and Mcl-1 proteins were detected in H_2O_2-treated cells and prior CAT treatment prevented their decline. However, CAT showed no effect on EMO-inhibited Mcl-1 protein levels (Fig. 7B). These data indicated that the apoptosis-inducing effect of EMO does not parallel that of H_2O_2, and that pro-oxidant activity might not be involved in EMO-induced apoptosis.

3.4. Stimulation of caspase 3-like activities, not caspase 1-like activities, in emodin-induced apoptosis

Previous data indicated that induction of the caspase 3 protein procession participated in EMO-induced apoptosis. To detect the enzymatic activity of caspases during EMO-induced apoptosis, two colorimetric substrates, Ac-DEVD-pNA for caspase 3-related activities and Ac-YVAD-pNA for caspase 1-related activities, were used in this study. As illustrated in Fig. 7, EMO induced a dramatic increase in DEVD-specific, but not YVAD-specific, caspase activity in HL-60 cells. Adding the caspase 3 inhibitory peptide Ac-DEVD-CHO, but not the caspase 1 inhibitory peptide Ac-YVAD-CHO, into the reaction mixture containing cell lysates from EMO-treated HL-60 cells inhibited the increase in caspase 3-like activities. A prior treatment of HL-60 cells with CAT or NAC showed no effect on EMO-induced caspase 3 activity. Similarly, H_2O_2 treatment significantly induced DEVD-specific, but not YVAD-specific, caspase activity in HL-60 cells, while CAT, NAC, and PDTC (but not ALL) attenuated its induction. These data demonstrate that activation of caspase 3-like activity is involved in EMO-induced apoptosis.

Table 1
Glutathione (GSH) and glutathione disulfide (GSSG) levels of human promyeloleukemic cells HL-60 treated with NAC, CAT, SOD, and L-NAME in the presence or absence of EMO (40 μM)

Treatment[a]	GSH (μg/mg protein)	GSSG (ng/mg protein)
Control	2.9 ± 0.3	193.8 ± 20.2
DMSO	2.6 ± 0.8	162.5 ± 50.2
NAC (10 mM)	15.8 ± 0.9**	147.6 ± 34.4
NAC (20 mM)	17.9 ± 1.1**	152.8 ± 27.9
CAT (200 U/mL)	3.1 ± 0.4	206.7 ± 24.3
CAT (400 U/mL)	2.9 ± 1.2	170.6 ± 25.1
SOD (200 U/mL)	2.5 ± 0.4	167.8 ± 19.6
SOD (400 U/mL)	3.6 ± 0.6	173.3 ± 22.7
L-NAME (200 μM)	3.2 ± 0.7	213.3 ± 17.9
L-NAME (400 μM)	3.0 ± 0.9	214.3 ± 19.0
EMO (40 μM)	2.1 ± 0.8	161.6 ± 21.5
EMO (40 μM) + NAC (10 mM)	13.2 ± 0.4**	162.4 ± 31.2
EMO (40 μM) + NAC (20 mM)	12.3 ± 0.5**	158.9 ± 17.6
EMO (40 μM) + CAT (200 U/mL)	2.2 ± 0.5	169.6 ± 18.2
EMO (40 μM) + CAT (400 U/mL)	2.4 ± 0.4	184.5 ± 11.9
EMO (40 μM) + SOD (200 U/mL)	2.0 ± 0.7	203.4 ± 20.3
EMO (40 μM) + SOD (400 U/mL)	2.6 ± 0.6	197.3 ± 15.9
EMO (40 μM) + L-NAME (200 μM)	2.2 ± 0.3	185.4 ± 24.3
EMO (40 μM) + L-NAME (400 μM)	2.3 ± 0.3	188.4 ± 20.4

[a] HL-60 cells were treated with indicated compounds for 6 hr, and the GSH (μg/mg protein)and GSSG (ng/mg protein)levels were measured as described in Section 2. The data are expressed as the mean ± SE of three independent experiments.

** $P < 0.01$ significantly different from the DMSO-treated group, as analyzed by Student's *t*-test.

3.5. A caspase 3-like protease inhibitor, Ac-DEVD-CHO, attenuates emodin-induced apoptotic responses

To determine if the activation of caspase 3-like protease is necessary for apoptosis induced by EMO, caspase inhibitors including the caspase 3-like protease inhibitor, Ac-DEVD-CHO, and the caspase 1-like protease inhibitor, Ac-YVAD-CHO, were used to block intracellular protease; emodin-induced DNA ladders and PARP and D4-GDI cleavage were analyzed by agarose electrophoresis and Western blotting. Results in Fig. 8A and B show that the caspase 3-like

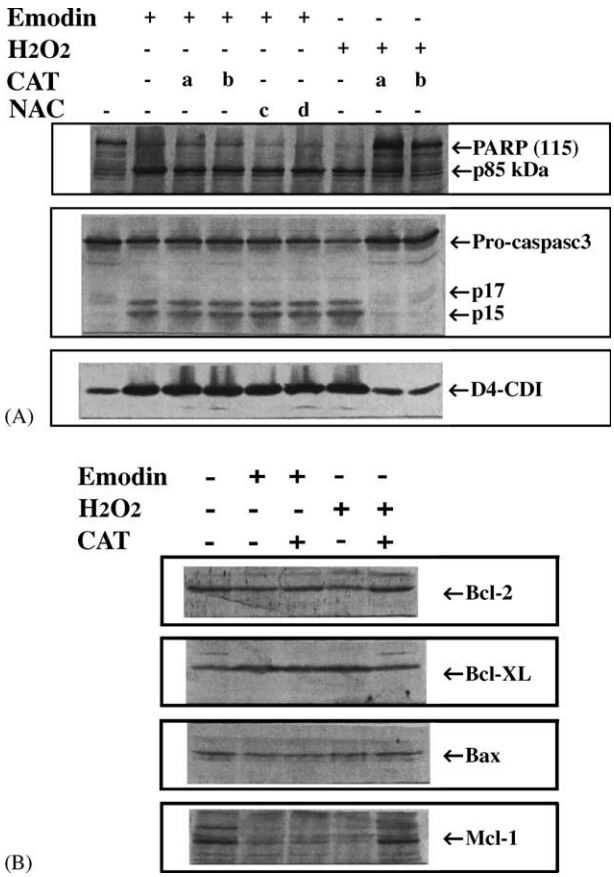

Fig. 6. Effects of CAT and NAC on emodin- and H_2O_2-induced PARP and D4-GDI cleavage and caspase 3 protein processing. (A) HL-60 cells were treated with NAC ((a) 10 μM; (b) 20 μM) or CAT ((c) 200 U/mL; (d) 400 U/mL) for 1 hr followed by emodin (40 μM) or H_2O_2 (200 μM) treatment for a further 12 hr. PARP and D4-GDI cleavage and caspase 3 protein processing were analyzed by Western blotting. (B) HL-60 cells were treated with CAT (400 U/mL) for 1 hr followed by emodin (40 μM) or H_2O_2 (200 μM) treatment for a further 12 hr. Expression of Bcl-2, Bcl-XL, Bax and Mcl-1 proteins was analyzed by Western blotting.

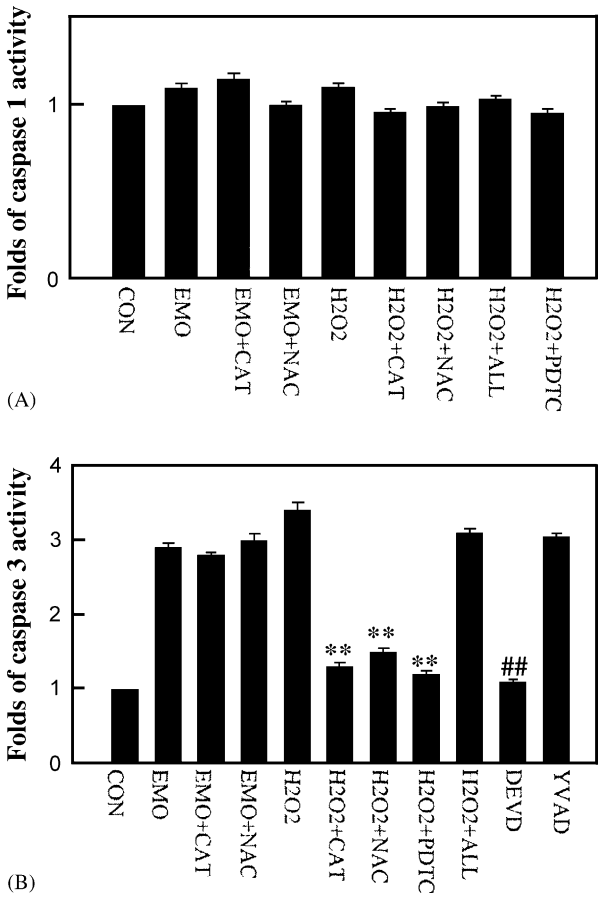

Fig. 7. Activation of caspase 3 but not caspase 1 activity in emodin (40 μM)- or H_2O_2 (200 μM)-treated HL-60 cells. HL-60 cells were treated with or without NAC (20 μM) or CAT (400 U/mL) for 1 hr followed by emodin (40 μM) or H_2O_2 (200 μM) treatment for 12 hr. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase 1 (A) and caspase 3 (B) like proteases were determined by incubation with specific colorigenic substrates, Ac-DEVD-pNA or Ac-YVAD-pNA, as described in Section 2. DEVD and YVAD indicate that the addition of 200 μM of Ac-YVAD-CHO or Ac-DEVD-CHO in the caspase 3 activity assay reaction containing the cell lysates from emodin-treated HL-60 cells.

inhibitor, Ac-DEVD-CHO (200 and 400 μM), was able to inhibit the occurrence of DNA ladders induced by EMO, accompanied by blockage of PARP and D4-GDI protein cleavage. However, Ac-YVAD-CHO, an inhibitor of caspase-1-like activity, showed no obvious effect at similar concentrations. Further, the MTT assay was used to analyze the viability of HL-60 cells under different treatments. Results in Fig. 8C show that a decrease in cellular viability was detected in EMO-treated cells, and that Ac-DEVD-CHO (but not Ac-YVAD-CHO) prevented cells from experiencing the EMO-induced cytotoxic effect. The data of Ac-DEVD-CHO-inhibited caspase 3 cleavage were according to the results of previous papers [33,34].

3.6. Elevation of catalase, but not SOD, activity, and decrease in intracellular ROS associated with emodin-induced apoptosis

Results described above suggest that apoptosis induced by EMO might not be mediated by its pro-oxidant activity. In order to determine if activation of anti-oxidative enzymes such as CAT and SOD is involved in EMO-induced apoptosis, in gel activity assays for CAT and SOD were performed in this study. As illustrated in Fig. 9, a time-dependent induction of CAT activity was detected in EMO-treated HL-60 cells, however SOD activity remained unchanged. In order to further demonstrate if CAT activation by EMO was able to block ROS production in HL-60 cells, the intracellular peroxide level was determined by the fluorescence intensity of DCF, and changes in intracellular peroxide levels were determined by flow cytometric analysis. The results in Fig. 10 show that EMO produced the slight inhibition on endogenous intracellular peroxide levels in the absence of H_2O_2 treatment. Adding H_2O_2 (200 μM) into the medium with DCHF-DA elevated the intracellular fluorescence intensity to 1090.7 ± 19.7 and is described as a positive control here. Interestingly, prior treatment of EMO (40 μM) for 2 or 4 hr followed by H_2O_2 (200 μM) treatment significantly attenuated H_2O_2-induced peroxide in

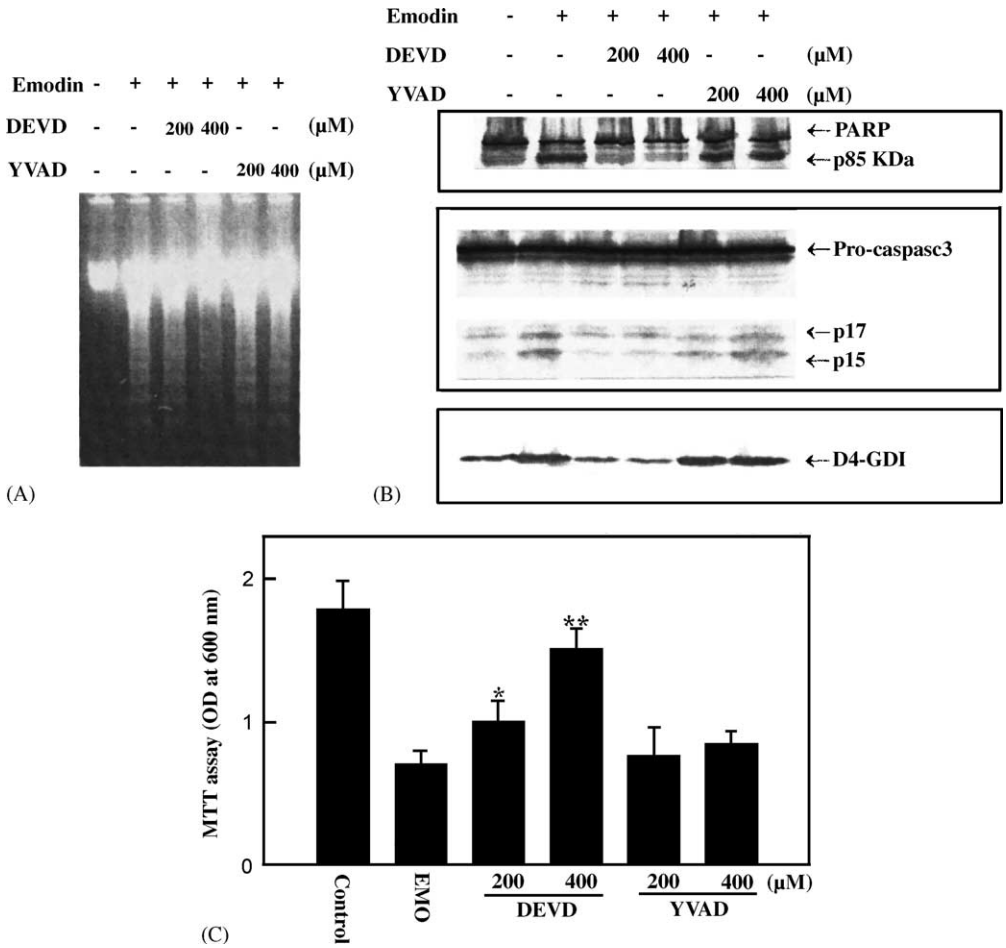

Fig. 8. Effects of caspase 1 and 3 peptidyl inhibitors on emodin-induced apoptotic responses in HL-60 cells. HL-60 cells were pre-treated with either inhibitor, Ac-DEVD-CHO or Ac-YVAD-CHO (200 or 400 μM), for 3 hr followed by emodin (40 μM) or H_2O_2 (200 μM) treatment for a further 12 hr. (A) The integrity of genomic DNA in each treatment was detected by electrophoresis on a 1.8% agarose. (B) Expressions of PARP and D4-GDI protein cleavage, and caspase 3 protein processing were analyzed by Western blotting. (C) MTT assay was performed to examine the effect of Ac-DEVD-CHO and Ac-YVAD-CHO on emodin-induced cell death. HL-60 cells were treated with emodin (40 μM) in the presence of Ac-DEVD-CHO or AC-YVAD-CHO for 12 hr. MTT was added into medium for an additional 4 hr. The viability of cells was detected by measuring the absorbance at 600 nm.

HL-60 cells ($^{*}P < 0.05$; $^{**}P < 0.01$). These data demonstrate that EMO is able to block ROS production induced by H_2O_2, accompanied by induction of CAT activity.

4. Discussion

A variety of stimuli has been described to induce cell death. In the present study, we demonstrate that EMO is able to induce apoptosis in the human leukemia cell line HL-60. The induction of apoptosis by EMO is consistent with the activation of apoptotic machinery. Treatment with EMO caused a rapid induction of caspase 3-like activity, and the degradation of PARP and D4-GDI preceded the onset of apoptosis. Pretreatment of cells with a caspase 3-like inhibitor, Ac-DEVD-CHO, inhibited EMO-induced caspase activity and apoptosis, suggesting that apoptosis induced by EMO involves a caspase 3-dependent mechanism. Caspase 1/ICE-like activity was found to increase prior to the activation of caspase 3-like activity in previous studies [35,36], and an *in vitro* study indicated that caspase 1/ICE can directly activate caspase 3/CPP32 [37]. In EMO-treated cells, we were unable to detect any significant change in the activity of caspase 1-like proteases, and a specific caspase 1/ICE inhibitor Ac-YVAD-CHO, had no preventive effect on EMO-induced apoptosis. This suggests that proteases other than caspase 1/ICE located upstream of caspase 3 are involved in the mechanism of EMO-induced apoptosis.

Free radicals are a family of molecules, which modulate several important physiological functions including proliferation and apoptosis, and a high level of ROS in malignant tumor cells than that in normal cells. Previous studies reported that ROS participated in chemical-induced apoptosis through inducing DNA damage [29,38]. However, an anti-oxidant vitamin E showed to induce apoptosis in malignant cell lines, but not in normal cells [39]. And, Senturker *et al.* also suggested that apoptosis induced by anti-cancer agent etoposide is independent of ROS generation [40]. Natural products have exhibited several biological activities

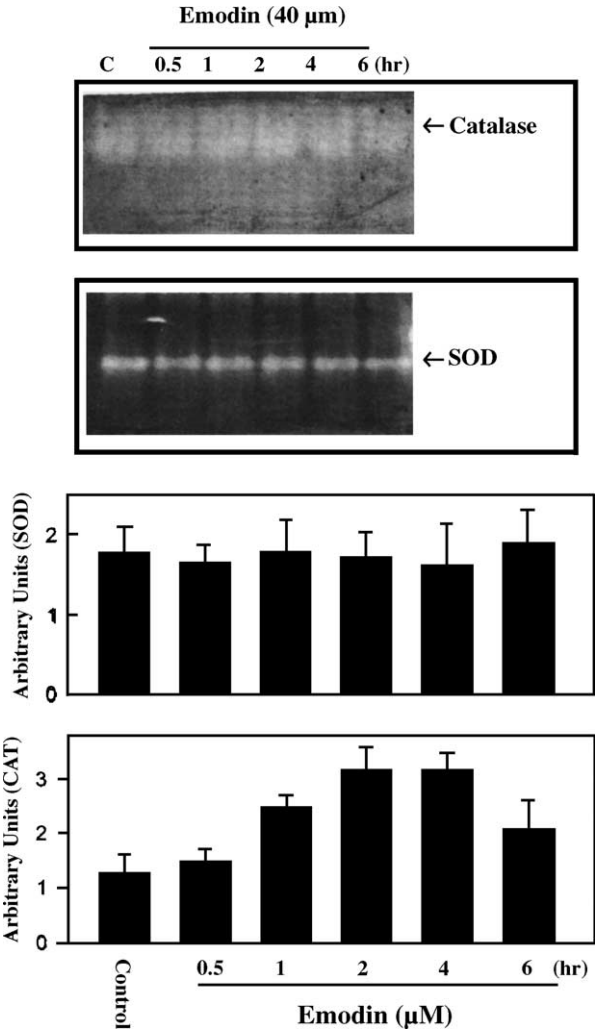

Fig. 9. Induction of catalase but not SOD activity was detected in emodin-treated HL-60 cells. HL-60 cells were treated with emodin (40 μM) for 0.5, 1, 2, 4, 6 hr. Activities of catalase and SOD were analyzed as described in Section 2. Band intensity was measured by densitometry analysis and described as the mean ± SE from three independent experiments. SOD, superoxide dismutase.

such as free radical scavenging, anti-inflammatory, and anti-cancer activities, and a great number of hydroxyl groups means more free radical scavenging and producing activities [41]. Our recent study demonstrated that a decrease in ROS production was occurred in apoptosis induction by wogonin and fisetin [34]. In the present study, free radical scavengers including CAT, SOD, DPI, PDTC, and L-NAME showed no preventive effect on EMO-induced apoptosis; however NAC, CAT, and PDTC inhibited ROS (H_2O_2)-induced apoptotic responses. It is suggested that ROS production might not be involved in EMO-induced apoptosis.

Catalase is an anti-oxidative enzyme, which converts H_2O_2 to H_2O and O_2, and protects cells from ROS-mediated damages. In the present study, EMO induced catalase activity in HL-60 cells, and suppressed the intracellular peroxide stimulated by H_2O_2. Furthermore, NAC pretreatment increased the intracellular GSH level potentiated emodin-induced cytotoxicity in HL-60 cells by the MTT assay (Fig. 5B). These data provide evidence to support that apoptosis induced by EMO is independent of ROS production. SOD is a well-known anti-oxidative enzyme with the activity to convert superoxide to hydrogen peroxide, and at least two types of SOD have been identified, one is mitochondrial Mn-SOD, and the other is cytosolic Cu/Zn-SOD, which can be inhibited by cyanide. Results of the present study indicated that total cellular SOD activity did not change in HL-60 cells in the presence or absence of EMO. Addition of cyanide (1 mM) exhibits $68 \pm 5.6\%$ maximal inhibition on SOD activity in HL-60 cells, and similar inhibitory percentage of cyanide on SOD activity was observed in EMO-treated HL-60 cells. It is suggested that both Cu/Zn and Mn SODs occurred in HL-60 cells, and EMO-induced apoptosis is irrelevant to SOD activation (data not shown).

Bcl-2 family proteins have been demonstrated to be involved in the process of apoptosis. Two different types of Bcl-2 family proteins have been identified, one type is pro-apoptotic and includes Bax, Bak, and Bcl-Xs, while the other type is anti-apoptotic and includes Bcl-2, Bcl-XL, and Mcl-1. Previous studies indicated that an increase in pro-apoptotic Bcl-2 family proteins and a decrease in anti-apoptotic Bcl-2 family proteins participated in the process of apoptosis [42,43]. Further, over-expression of anti-apoptotic Bcl-2 proteins protected cells from apoptosis induced by stimulants [44]. This study showed a decrease in Mcl-1 protein in EMO-treated HL60 cells; however other Bcl-2 family proteins including Bcl-2, Bcl-XL, Bax and Bad remained unchanged. We propose that a decrease in the Mcl-1 protein participates in EMO-induced apoptosis in HL-60 cells.

EMO is an active constituent of many widely used Chinese herbs, e.g. the rhizome of *R. officinale*, *P. cuspidatum* and *Polygonum multiflorum*, and it possesses antibacterial, anticancer, diuretic, immunosuppressive, and vasorelaxant activities. The immunosuppressive effect of EMO might be mediated through hydrogen peroxide generated from semiquinone and regulated by arachidonic acid metabolites [45]. Chang *et al.* reported that EMO was able to increase unscheduled DNA synthesis and reduce DNA adducts in cisplatin-treated cells [46]. Zhang *et al.* reported that EMO and its derivatives were potent tyrosine kinase inhibitors and repressed cellular transformation and metastasis-associated properties. A structurally related activity study showed that the CH_3 group at C_3 and an OH group at C_6 are critical for the activity of EMO in suppressing the tyrosine phosphorylation in HER-2/neu-overexpressing breast cells [7]. In the present study, three structurally related anthraquinones, including EMO, PHY, and CHR, were used to study their apoptotic effects on HL-60 cells. EMO, PHY and CHR have different structural substitutes at C_6. The substitutes at C_6 for EMO, PHY and CHR are OH, CH_3O, and H, respectively. Results of the present study demonstrated that EMO, but not PHY and CHR, showed potent apoptosis-inducing activity in HL-60 cells.

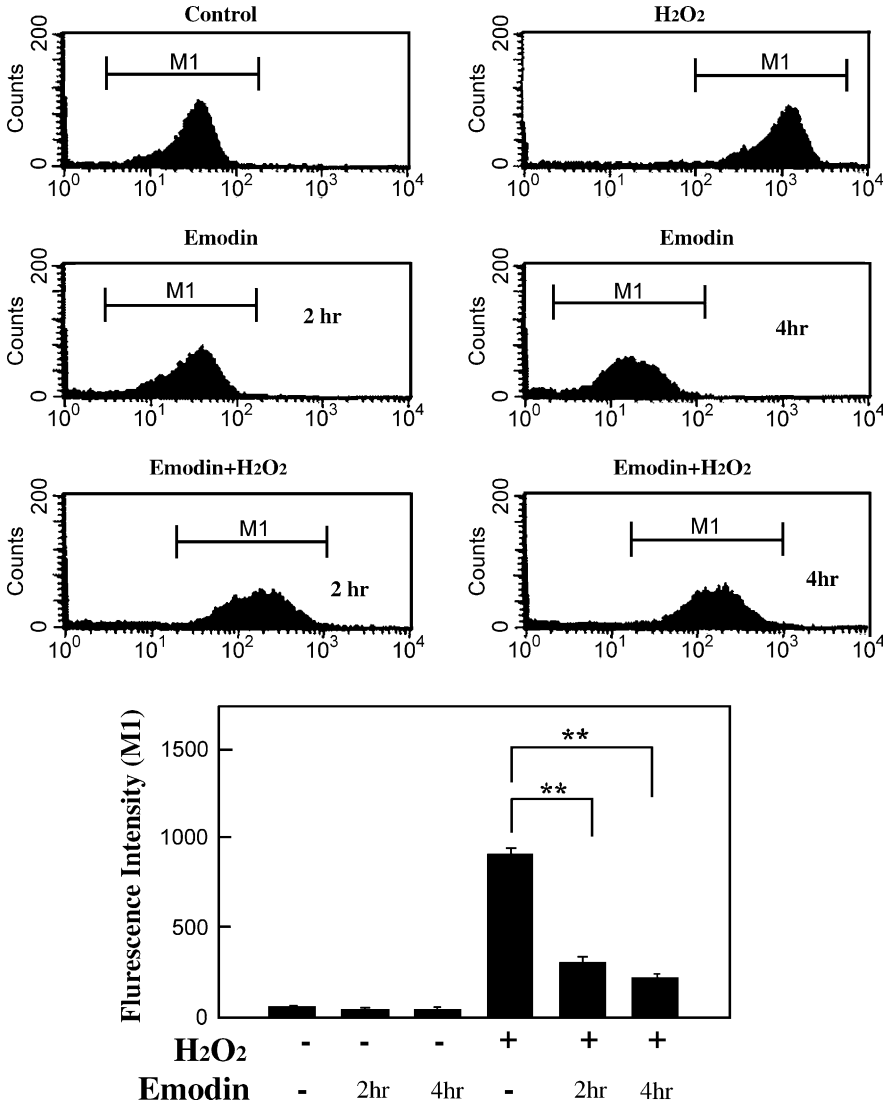

Fig. 10. Inhibition of H_2O_2 (200 μM)-induced intracellular peroxide by emodin in the DCHF-DA assay. HL-60 cells were treated with emodin (40 μM) for 2 or 4 hr, respectively. After emodin treatment, HL-60 cells were washed twice with PBS to remove the extracellular emodin, and addition of DCHF-DA (100 μM) with or without H_2O_2 (200 μM) for one more hour. The fluorescence intensity of cells was measured by flow cytometry analysis. Each value is presented as the mean ± SE of three independent experiments. $^{**}P < 0.01$ significantly different from the control, as analyzed by Student's *t*-test. Upper panel is the representative of the result of flow cytometry analysis.

We proposed that the OH at C_6 is important for the apoptosis-inducing activity of EMO.

Acknowledgments

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