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European Journal of Pharmacology 511 (2005) 89–97

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Induction of human leukemia HL-60 cell differentiation via a PKC/ERK pathway by helenalin, a pseudoguainolide sesquiterpene lactone

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Received 16 September 2004; received in revised form 2 February 2005; accepted 9 February 2005
Available online 16 March 2005

Abstract

Helenalin, a cell-permeable pseudoguainolide sesquiterpene lactone, is a potent anti-inflammatory agent that inhibits nuclear factor-kappaB (NF-κB) DNA binding activity. In this report, we investigated the effect of helenalin on cellular differentiation in the human promyelocytic leukemia HL-60 cell culture system. Helenalin by itself markedly induced HL-60 cell differentiation in a concentration-dependent manner. Cytofluorometric analysis and cell morphologic studies indicated that helenalin induced cell differentiation predominantly into granulocytes. Protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) inhibitors significantly inhibited HL-60 cell differentiation induced by helenalin, while p38 mitogen-activated protein kinase (MAPK) inhibitors did not. Moreover, helenalin enhanced PKC activity and protein level of PKCβI and PKCβII isoforms, and also increased the level of pERK in a concentration-dependent manner. In addition, the enhanced levels of cell differentiation closely correlated with the decreased levels of NF-κB binding activity by helenalin. These results indicate that PKC, ERK, and NF-κB may be involved in HL-60 cell differentiation induced by helenalin.

Keywords: Helenalin; Cell differentiation; Protein kinase C; Extracellular signal-regulated kinase; Nuclear factor-κB

1. Introduction

Most cancer cells exhibit a defect in their capacity to mature into non-replicating adult cells, thereby existing in a highly proliferating state, which results in outgrowing their normal cellular counterparts. The induction of terminal differentiation represents an alternative approach to the treatment of cancer by conventional anti-neoplastic agents since cells exposed to chemical or biological inducers of differentiation do not undergo the cytodestruction produced by cytotoxic agents. Instead they acquire the phenotypic characteristics of end-stage adult cell forms with no replicative capacity and ultimately undergo programmed cell death. Leukemia cells can be induced to undergo terminal differentiation by a variety of chemical and biological agents, indicating that the malignant state is not an irreversible process. Certain cancers may eventually be treated with agents that induce terminal differentiation, presumably with less morbidity than that produced by cytodestructive agents (Beere and Hickman, 1993).

Several sesquiterpene lactones have received considerable attention in pharmacological research due to their potent anti-neoplastic and anti-inflammatory activity (Hehner et al., 1999; Ohnishi et al., 1997). Cytostatic and cytocidal effects of sesquiterpenes against tumor cells have also been reported (Hall et al., 1988; Ross et al., 1999). Helenalin, which can be isolated from several plant species of the Asteraceae family, is a potent anti-inflammatory and anti-neoplastic agent. Helenalin and its derivatives are of potential medicinal interest, since they are potent anti-inflammatory agents in vitro as well as in vivo. Furthermore, helenalin inhibited human neutrophil migration and chemotaxis (Lee et al., 1977; Schmidt, 1997).

Protein kinase C (PKC) was identified as a possible target for the development of novel anti-cancer therapeutic

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doi:10.1016/j.ejphar.2005.02.008

agents. PKC plays a key role in regulating the response of hematopoietic cells to both physiological and pathological inducers of proliferation and differentiation (Caponigro et al., 1997; Nishikawa and Shirakawa, 1992). The 12 PKC isoforms identified so far are classified into three distinct groups on the basis of the presence of functional domains and subsequent differences in their regulation. The conventional isozymes (cPKC: α, βI, βII, and γ) are calcium- and diacylglycerol-dependent; the novel isozymes (nPKC: δ, σ, η, θ, and μ) are diacylglycerol-dependent and calcium-independent; and the atypical PKC isozymes (aPKCs: ζ, ι, and λ) are diacylglycerol- and calcium-independent (Quest, 1996). Of the PKC isoforms, the calcium-dependent PKCs are the most abundantly expressed in leukemic cells and have been implicated in HL-60 cell differentiation (Komada et al., 1991).

The MAPK kinase (MEK)/ERK signal transduction pathway is also known to regulate cellular proliferation, survival, and differentiation. Previous studies clearly demonstrate the involvement of the MEK/ERK/MAP kinas pathway in phorbol 12-myristate 13-acetate (PMA)-, RA-, and G-CSF-induced myeloid differentiation (Marcinkowska, 2001; Woessmann and Mivechi, 2001). Another study showed the involvement of ERKs in both the growth and the erythroid-like differentiation of K562 cells (Miranda et al., 2002). NF-κB is a ubiquitous transcription factor complex that is involved in the activation of a large number of cellular and viral genes. A large number of reports have provided evidence that NF-κB is involved in the development of cancer (Sharma and Narayanan, 1996). The interference with the activation of NF-κB appears to be a common feature for agents that regulate the growth and enhance the differentiation (Kopp and Ghosh, 1994; Suzuki and Packer, 1993).

Human promyelocytic leukemia HL-60 cell line is frequently used as a model system for investigation of the mechanism of cell differentiation, since dimethyl sulfoxide (DMSO) or RA lead this cell to differentiate to a granulocyte, whereas PMA, 1,25-dihydroxy vitamin D_3, or sodium butyrate promote the cell to differentiate to a macrophage (Breitman et al., 1980; Collins et al., 1978; Rovera et al., 1979; Tanaka et al., 1983).

In this report, we investigated the effect of helenalin on cellular differentiation in the human promyelocytic leukemia HL-60 cell culture system. We also investigated signaling pathways on the HL-60 cell differentiation induced by helenalin.

2. Materials and methods

2.1. Materials

HL-60 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). Helenalin, SB 202474, and SB 203580 were purchased form the Calbiochem (San Diego, CA, USA). PMA, 2-[4-Morpholinyl]-8phenyl-1[4*H*]-benzopyran-4-one (LY 294002), wortmannin, chelerythrine, giemsa staining solution, methanol-free paraformaldehyde, and all other reagents were purchased from the Sigma Chemical (St. Louis, MO, USA). Bisindolylmaleimide (GF 109203X), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H 7), and 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD 98059) were purchased from the Tocris Cookson (UK). Helenalin was dissolved in dimethylsulfoxide to make a stock solution of 20 mM. The solutions were diluted at least 1000-fold in the growth medium such that the final concentration of ethanol or DMSO had no effect on the differentiation and proliferation of HL-60 cells. All manipulations were performed in subdued light.

2.2. Determination of cell viability and proliferation

Cell viability was determined by the trypan blue exclusion assay as previously described (Coligan et al., 1995). Viability was calculated as the percentage of live cells in the total cell population. Cell proliferation was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay. In brief, after each treatment, 10 μl of MTT (5 mg/ml) was added to each well in 96-well plates. After incubation for 4 h at 37 °C, the crystals of viable cells were dissolved with 100 μl of 0.04 N HCl in isopropanol. The absorbance of each well was then read at 540 nm using a kinetic microplate reader.

2.3. Determination of cell differentiation

HL-60 cell differentiation was assessed by the nitroblue tetrazolium (NBT) reduction assay as previously described (Collins et al., 1978). This assay is based on the ability of phagocytic cells to produce superoxide upon stimulation with PMA. For this assay, 2×10^5 cells were harvested by centrifugation and incubated with an equal volume of 1% NBT dissolved in PBS containing 200 ng/ml of freshly diluted PMA at 37 °C for 30 min in the dark. Cytospin slides were prepared and were examined for blue-black nitroblue diformazan deposits, indicative of a PMA-stimulated respiratory burst. At least 200 cells were assessed for each experiment.

2.4. Morphologic studies

Single-cell suspensions were prepared and 2×10^5 cells were loaded into a cyto-funnel and spun at 500 rpm in a cytospin centrifuge. The slides were fixed with methanol and dried. The slides were stained with Giemsa staining solution for 20 min and rinsed in deionized water, air-dried,

and observed under a microscope with a camera. The stained cells were assessed for size, regularity of the cell margin, and morphological characteristics of the nuclei.

2.5. Immunofluorescent staining and cytofluorometric measurements

Quantitative immunofluorescence measurements were performed in an Epic XL flow cytofluorograph equipped with a multi-parameter data acquisition and display system as previously described (Kim et al., 2001). Briefly, a single-cell suspension was collected from the various cultures and washed twice with ice-cold phosphate buffered saline (PBS, pH 7.4). Afterwards, PE-conjugated anti-human CD11b or fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 monoclonal antibodies (Becton Dickinson, San Jose, CA, USA) were added, followed by incubation at 4 °C for 1 h. After incubation, the cells were washed with PBS and were fixed in PBS containing 1% paraformaldehyde, and cytofluorometric analysis was performed. Background staining was determined by staining the cells with PE- or FITC-conjugated isotype control monoclonal antibodies. One parameter fluorescence histograms were generated by analyzing at least 1×10^4 cells.

2.6. Preparation of cell lysates and Western blot analysis

Cells were lysed in lysis buffer [50 mM Tris buffer, pH 7.5 containing 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 50 μg/ml phenylmethanesulfonyl fluoride (PMSF)] by incubation on ice for 30 min. Lysates were then centrifuged at 13,000×*g* at 4 °C for 10 min. The proteins in 15 μg of the supernatants were separated using a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to the nitrocellulose (NC) membrane. The blots were probed with rabbit anti-human PKC isoforms, mouse anti-pERK and rabbit anti-ERK2 antibodies, washed and exposed to horseradish peroxidase-conjugated anti-mouse IgG2a or rabbit IgG antibodies. Immunoreactive bands were visualized by the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

2.7. Protein kinase C activity assay

HL-60 cells were lyzed in lysis buffer containing 50 mM Tris (pH 7.5), 2 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, 1% Triton X-100, 150 mM NaCl, 1 μM dithiothreitol, 1 mM PMSF, 50 mM NaF, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, and 50 μg/ml aprotinin by incubation on ice for 30 min. Lysates were then centrifuged at 14,000×*g* at 4 °C for 20 min. The proteins in 200 μg of the supernatants were incubated with PKC antibody at 4 °C for 2 h. After protein A was added, the mixture was shaken at 4 °C for 1 h and washed with lysis buffer. The antibody-coupled proteins were centrifuged at 5000×*g* for 1 min and reacted with 5 μg myelin basic protein and 0.5 μl [$\gamma-^{32}$P]ATP in reaction buffer (0.5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, 10 mM $MgCl_2$, 20 mM HEPES (pH 7.4), 50 mM ATP, 2 mM dithiothreitol, 2 mM NaF, and 2 mM sodium orthovanadate) at room temperature for 30 min. The reaction mixture was analyzed by electrophoresis on a 15% SDS-PAGE.

2.8. Electrophoretic mobility shift assay (EMSA)

The nuclear extracts were prepared from the cells, as previously described (Chung et al., 2000). An oligonucleotide containing an NF-κB-binding site within the Ig κ-chain (5′ CCG GTT AAC AGA GGG GGC TTT CCG AG 3′) was used as a probe. Labeled oligonucleotides (10,000 cpm) were incubated for 30 min at room temperature, along with 10 μg of nuclear extracts, in 20 μl of binding buffer (10 mM Tris.HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly dI–dC, and 1 mM dithiothreitol). The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5× Tris–borate buffer. Specific binding was confirmed by competition experiments with a 50-fold excess of unlabeled, identical oligonucleotides or cAMP response element-containing oligonucleotides.

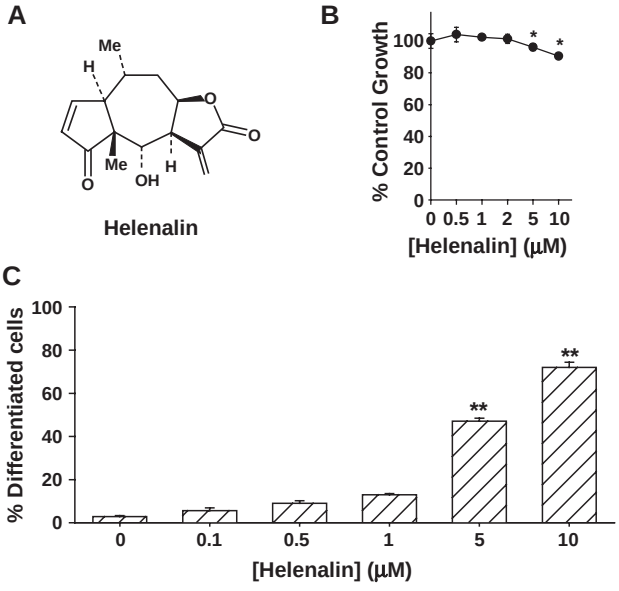

Fig. 1. Chemical structure and effect of helenalin on HL-60 cell proliferation and differentiation. (A) Chemical structure of helenalin. HL-60 leukemia cells were treated with various concentrations of helenalin for 72 h. The cell proliferation was determined by the MTT assay (B) and the cell differentiation was assessed by the NBT reduction assay (C). Each value represents the mean±S.E.M. of triplicate determinations from one representative experiment. The experiment was repeated more than three times with similar results. *P<0.01, relative to an untreated group. **P<0.005, relative to an untreated group.

2.9. Statistical analysis

Student's *t*-test and one-way analysis of variance were used to determine the statistical significance of differences between values for various experimental and control groups. *P* values<0.05 were considered significant.

3. Results

3.1. Effect of helenalin on HL-60 cell proliferation and differentiation

To determine the effect of helenalin on HL-60 cell differentiation, the HL-60 cells were treated with various concentrations of helenalin, and the numbers of differentiated cells, as measured by nitroblue tetrazolium (NBT) positivity, were determined. As a control, the cells were treated with DMSO. As shown in Fig. 1C, helenalin by itself strongly enhanced the degree of cell differentiation in a concentration-dependant manner. The effects were maximal at 10 μM of helenalin, with greater than 71% of the treated cells attaining a differentiated state.

The cell proliferation and viability for each treatment group were determined. As shown in Fig. 1B, treatment with 10 μM helenalin inhibited cell proliferation by 10%, as determined by the MTT assay. For all treatment, cells' viability was greater than 98% throughout the incubation period, as demonstrated by the trypan blue exclusion assay (data not shown).

To further confirm the cell differentiation induced by helenalin, the morphologic phenotypes and the expression of cell surface antigens on HL-60 cells were analyzed. As shown in Fig. 2A, Giemsa-stained undifferentiated control HL-60 cells were predominantly promyelocytes with round and regular cell margins, and large nuclei, suggesting that the cells were highly active in DNA synthesis and were rapidly proliferating. Helenalin-treated cells showed a marked increase in cellular size, cytoplasmic content, and nuclear complexity.

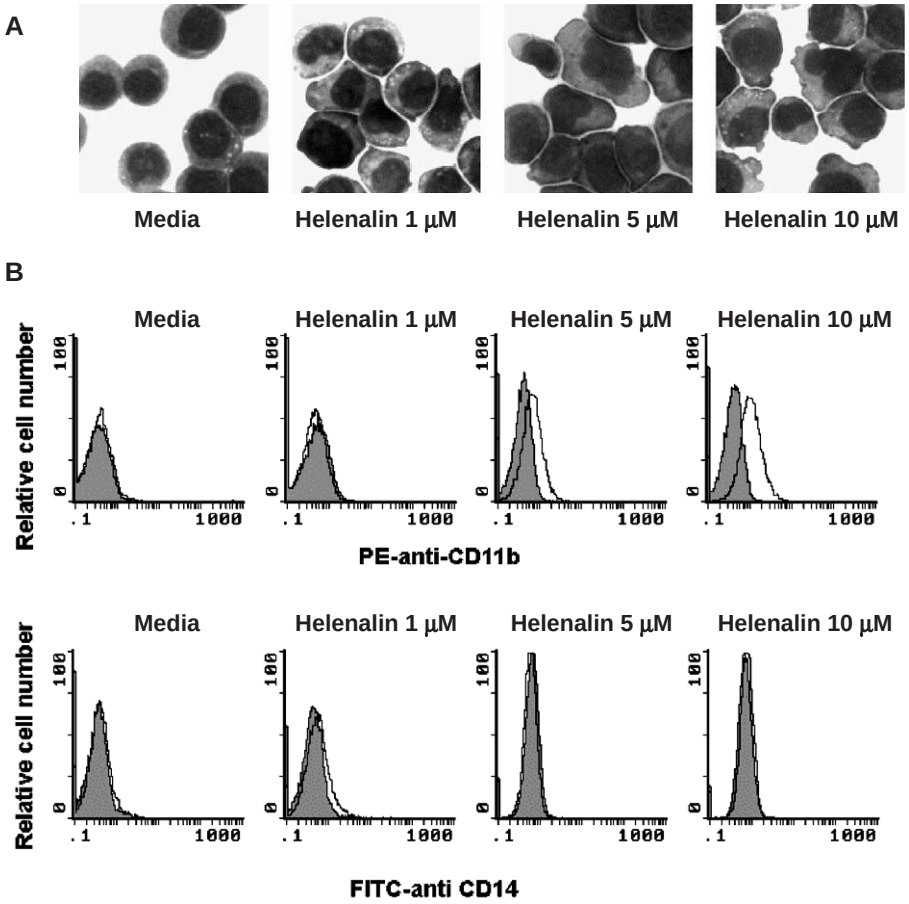

Fig. 2. Morphologic and cytofluorometric analysis of HL-60 leukemia cells treated with helenalin. (A) HL-60 cells were treated for 72 h with various concentrations (0–10 μM) of helenalin. The cells were assessed by morphologic analysis using Giemsa stain. (B) The cells were treated with helenalin as shown in panel (A), and the expression of cell surface markers was assessed by cytofluorometric analysis using PE-conjugated anti-CD11 mAb (upper panel) or FITC-conjugated anti-CD14 mAb (bottom panel) (unshaded area), or isotype control mAb (shaded area). The data are representative of three separate experiments.

3.2. Effects of helenalin on differentiation pathways of HL-60 leukemia cells

To determine the differentiation pathway that HL-60 cells have followed after treatment with helenalin, HL-60 cells were treated with various concentrations of helenalin, and flow cytometric analysis using monoclonal antibody for the monocytic surface antigen CD14 was determined. CD14 antibody reacts with a glycosyl phosphatidyl inositol-anchored single chain glycoprotein expressed at high levels on monocytes (Wright et al., 1990). The CD14 antigen is expressed exclusively when HL-60 leukemia cells are differentiated into monocytes. As shown in Fig. 2B, HL-60 cells treated with helenalin did not react with anti-CD14 monoclonal antibody. In contrast, HL-60 cells treated with helenalin strongly stained with a monoclonal antibody against HL-60 cell differentiation marker CD11b, indicating that helenalin stimulated HL-60 cell differentiation along the granulocytic pathway.

3.3. Effect of inhibitors for p38 MAPK, PKC, and ERK on HL-60 cell differentiation induced by helenalin

The activation of PKC is necessary for the differentiation of HL-60 cells (Wu et al., 1989). To determine any relationship between the effect of helenalin on cellular differentiation and PKC activation, HL-60 cells were treated with chemical PKC inhibitors, GF 109203X, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), or chelerythrine, or a peptide PKC inhibitor in the presence of helenalin. Afterwards, the degree of cellular differentiation was assessed by the nitroblue tetrazolium reduction assay. As

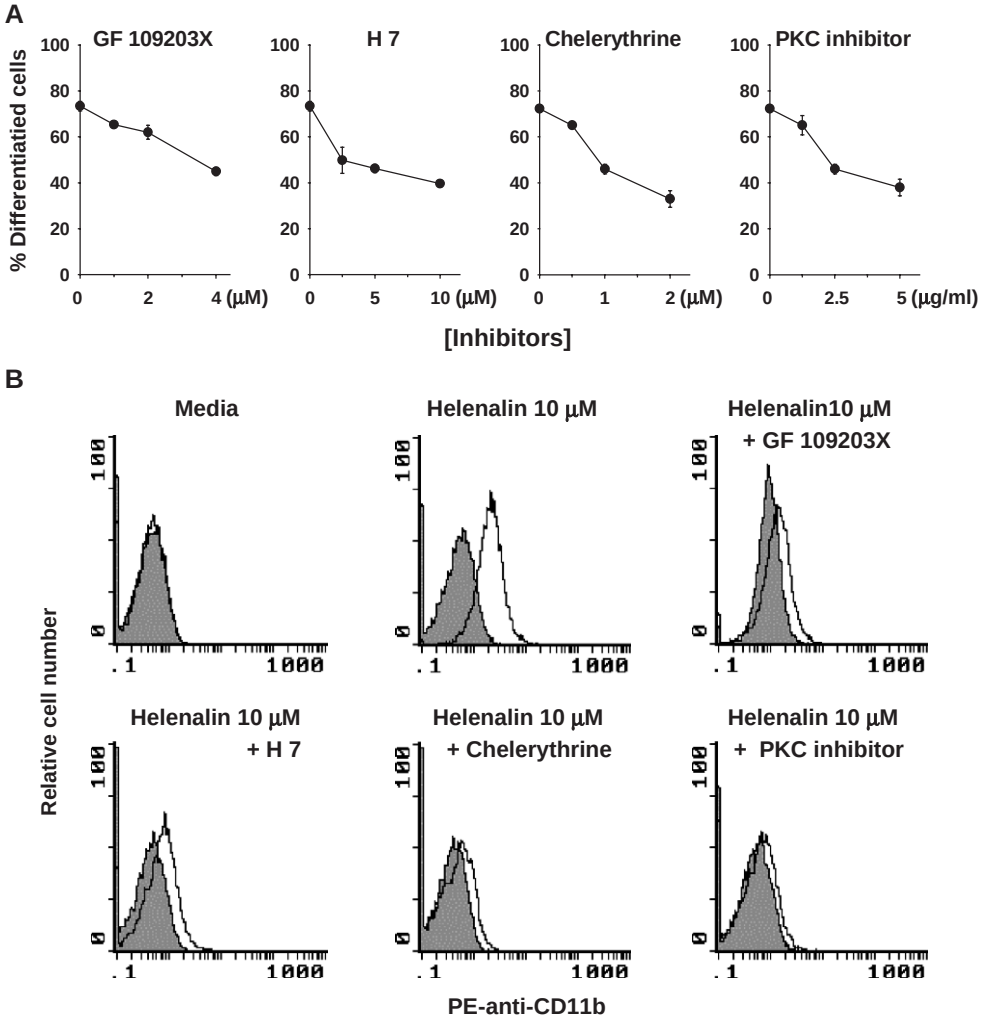

Fig. 3. Effect of PKC inhibitors on HL-60 cell differentiation induced by helenalin. (A) HL-60 cells were treated with various concentrations of chemical PKC inhibitors (GF 109203X, H7, or chelerythrine) or a peptide PKC inhibitor for 40 min, followed by incubation with 10 μM helenalin. The cellular differentiation was assessed by the NBT reduction assay. The data are presented as a percentage of differentiated cells with the mean±S.E.M. (n=3). (B) HL-60 cells were treated with 4μM GF 109203X, 10 μM H 7, 2 μM chelerythrine, or 5μg/ml a PKC peptide inhibitor for 40 min, followed by incubation with 10 μM of helenalin for 72 h. The cells were assessed by cytofluorometric analysis using PE-conjugated anti-CD11 mAb (unshaded area), or isotype control mAb (shaded area). The data are representative of three separate experiments.

shown in Fig. 3A, all of PKC inhibitors significantly inhibited HL-60 cell differentiation induced by helenalin. The PKC inhibitors were not toxic to the HL-60 cells at concentrations used in the experiments, as demonstrated by the trypan blue exclusion assay (data not shown). To further confirm the effect of PKC inhibitors on the helenalin-induced cell differentiation, we added each of PKC inhibitors to the cell culture, followed by the treatment with 10 μM helenalin for 72 h, and the expression of the differentiation surface antigen CD11b was determined by a cytofluorometric analysis. As shown in Fig. 3B, all of PKC inhibitors decreased the population of HL-60 cell population stained with anti-CD11b, indicating that those PKC inhibitors inhibited the cell differentiation induced by helenalin.

Previous studies have provided evidence that phosphatidylinositol 3-kinase (PI3-K) activity also plays an essential role in differentiation of HL-60 cells (Bertagnolo et al., 1999; Zakaria et al., 1999). To determine any role of PI3-K in the HL-60 cell differentiation induced by helenalin, HL-60 cells were treated with specific PI3-K inhibitors, 3H-furo[4,3,2-de]indeno[4,5-h]-2-benzopyran-3,6,9-trione (wortmannin) or 2-(4-Morpholinyl)-8-phenyl-1(4*H*)-1-benzopyran-4-one (LY 294002), in the presence of helenalin. Both PI3-K inhibitors did not inhibit HL-60 cell differentiation after treatment with helenalin (data not shown).

Next, to investigate the involvement of PKC in the HL-60 cell differentiation enhanced by helenalin, HL-60 cells were treated with various concentrations of helenalin, and PKC activity in the treated cells was determined. In this report, we focused on conventional PKC isoforms such as α, βI, βII, and γ, since these PKC forms are the most abundantly expressed in leukemia cells and the expression levels have been closely correlated with the cell differentiation in HL-60 cell system (Komada et al., 1991). As shown in Fig. 4A and B, helenalin significantly increased PKC activity in a concentration-dependent manner, and the increased activity was prolonged approximately for 48 h. In addition, to determine conventional PKC isoforms induced by helenalin, HL-60 cells were treated with helenalin, and the protein levels of PKC isoforms were determined by Western blot analysis using mAbs for each PKC isoform. As shown in Fig. 4C and D, helenalin increased the protein levels of PKC βI and PKC βII in HL-60 leukemia cells, but the protein levels of PKC α and PKC γ were constant.

Mitogen-activated protein kinases (MAPKs) are downstream elements in the PKC signaling pathway of HL-60 cells (Marcinkowska et al., 1997). To determine the involvement of extracellular signal-regulated kinase (ERK), which is a MAPK, in the helenalin-induced cell differentiation, HL-60 cells were treated with 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD 98059), a specific ERK inhibitor, in the presence of helenlin. The synthetic compound, PD 98059, inhibits the ERK pathway by preventing the activation of ERK kinase by c-Raf

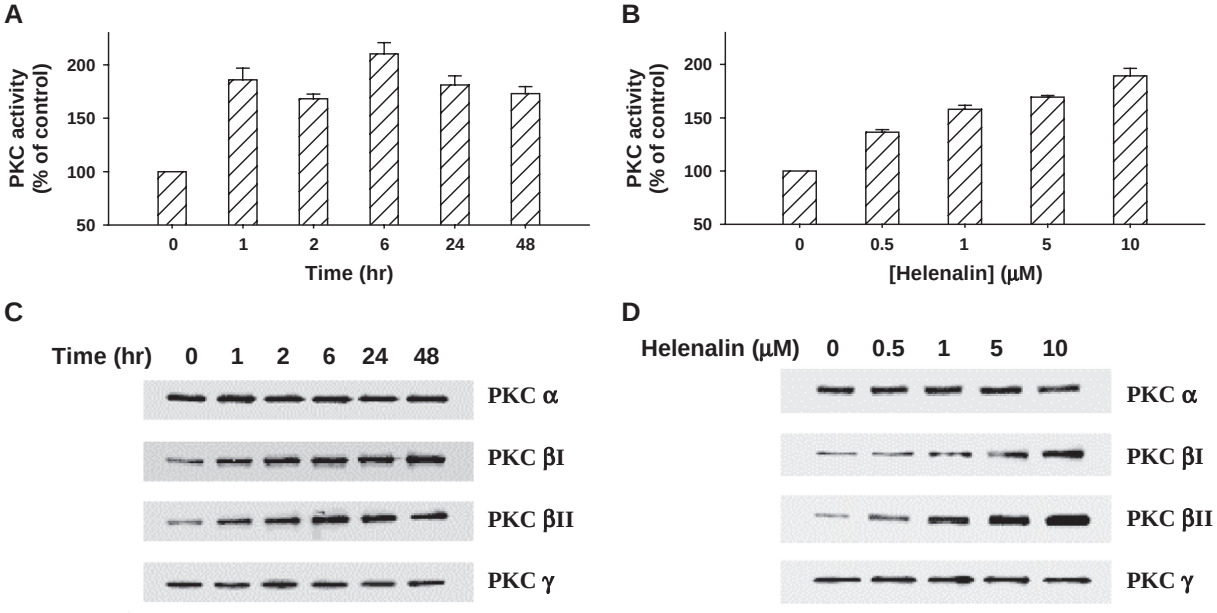

Fig. 4. Effect of helenalin on PKC activity and protein levels of conventional PKC isoforms in HL-60 cells. HL-60 cells were treated with 10 μM helenalin for the indicated times (0–48 h; A) or treated with various concentrations of helenalin (0–10 μM) for 2 h (B), and total PKC activity in the treated cells was determined. PKC activity represents the percentage of PKC activity of each treated group relative to the untreated control group. The values represent the means±S.E.M. (n=3). HL-60 cells were treated with 10 μM helenalin for the indicated times (0–48 h; C) or with various concentrations of helenalin (0.5–10 μM) for 48 h (D), and the conventional PKC isoforms were determined by Western blot analysis. The experiment was repeated twice with similar results.

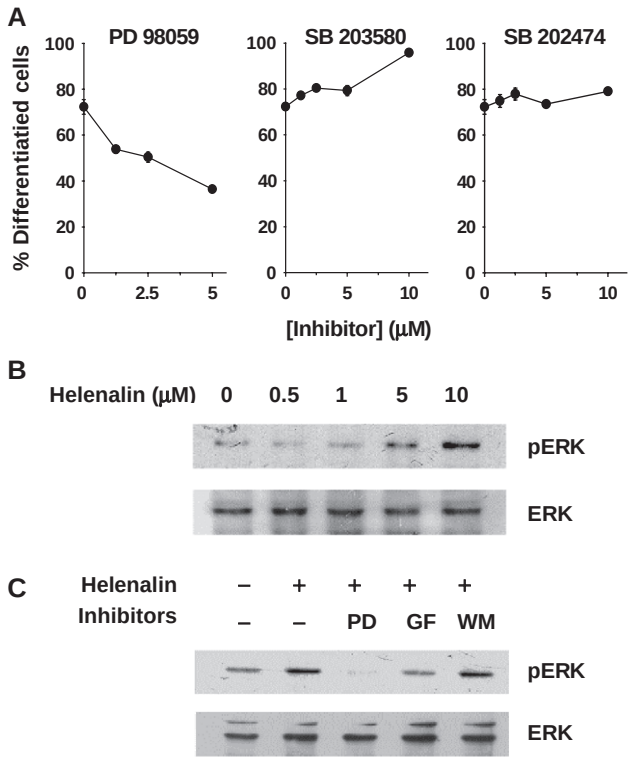

Fig. 5. Involvement of ERK in HL-60 cell differentiation induced by helenalin. (A) HL-60 cells were treated for 40 min with varying concentrations of ERK inhibitor (PD 98059), p38 MAPK inhibitor (SB 203580), or its analogue (SB202474), followed by incubation with 10μM helenalin for 72 h. The cellular differentiation was assessed by the NBT reduction assay. The results are presented as a percentage of differentiated cells with the mean±S.E.M. (n=3). (B) HL-60 cells were treated with varying concentrations of helenalin for 1 h and the levels of ERK were determined by Western blot analysis. (C) HL-60 cells were treated with 5 μM PD 98059 (PD), 4 μM GF 109203X (GF), or 100 nM wortmannin (WM) for 40 min. The levels of ERK were determined by Western blot analysis at 1 h after treatment with 10 μM helenalin. The band intensity of each treatment group was measured by densitometric analysis and represented as relative intensity to that of an untreated control HL-60 cells. The experiment was repeated twice with similar results.

(Alessi et al., 1995). As shown in Fig. 5A, PD 98059 inhibited HL-60 cell differentiation induced by helenalin. In contrast, SB 203580, a selective and potent inhibitor of p38 MAPK, did not inhibit the cell differentiation, but increased HL-60 cell differentiation induced by helenalin. SB202474, an inactive analogue of SB 203580, also had no effect. Helenalin also increased the ERK activation in a concentration dependent-manner. Moreover, inhibitor for PKC inhibited the ERK activation stimulated by helenalin but inhibitor for PI3-K did not (Fig. 5C).

Therefore, helenalin may induce HL-60 cell differentiation, and PKC and ERK may be involved in the cell differentiation induced by helenalin.

3.4. NF-κB is active in HL-60 leukemia cells and the inhibition of NF-κB activity by helenalin may be involved in the induction of HL-60 cell differentiation

Helenalin selectively inhibits the activation of NF-κB transcription factor by targeting the p65 component and disturbing the formation of DNA-binding NF-κB complex (Lyss et al., 1998). To ascertain whether the levels of helenalin employed were capable of causing the inhibition of NF-κB in unstimulated HL-60 leukemia cells, we analyzed nuclear extracts of HL-60 cells for NF-κB activity by an electrophoretic mobility shift assay (EMSA), using a probe specific for the κB DNA-binding motif of NF-κB. As shown in Fig. 6A, HL-60 cells showed relatively high levels of NF-κB DNA binding activity in an untreated control condition. The binding was specific since it was competed with an unlabeled, identical oligonucleotide, but not with unrelated, non-specific oligonucleotide. A 24 hr exposure to helenalin caused a marked reduction in the NF-κB DNA binding activity in a concentration-dependent manner.

To determine the effects of inhibitors for PKC and ERK on NF-κB activation in the helenalin-treated HL-60 cells, HL-60 cells were treated with helenalin, followed by

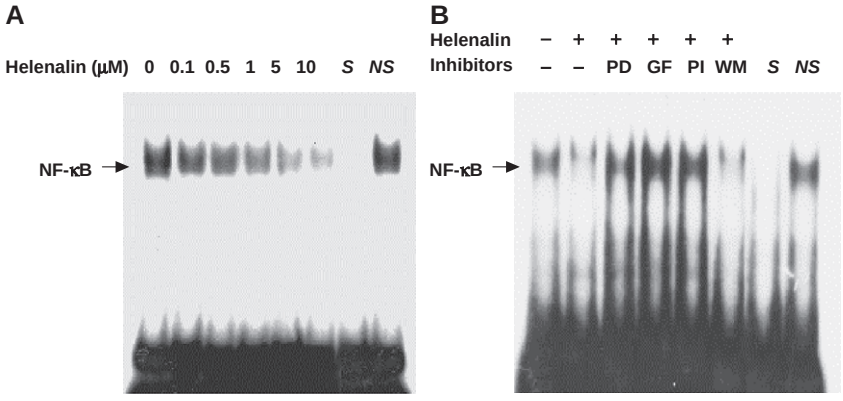

Fig. 6. Effect of inhibitors for PI3-K, PKC and ERK on NF-κB activation in HL-60 cell differentiation induced by helenalin. (A) HL-60 cells were treated with varying concentrations of helenalin for 48 h, and the NF-κB binding activity was analyzed by EMSA. (B) HL-60 cells were pretreated with 5 μM PD 98059 (PD), 4 μM GF 109203X (GF), 5 μg/ml PKC peptide inhibitor (PI), or 100 nM wortmannin (WM) for 40 min, followed by incubation with 10 μM helenalin for 48 h, and analyzed by the EMSA for NF-κB DNA binding activity. S and NS indicate the presence of an unlabeled, identical oligonucleotide and non-specific oligonucleotide, respectively. Data are representative of two independent experiments.

treatment with each inhibitor, and the levels of NF-κB DNA binding activity were determined by the NF-κB EMSA. As shown in Fig. 6B, helenalin inhibited NF-κB binding activity in HL-60 cells. The inhibitors for PKC and ERK recovered the NF-κB binding activity inhibited by helenalin.

4. Discussion

In this study, we have demonstrated that helenalin, a sesquiterpene lactone, induced differentiation of HL-60 promyelocytic leukemia cells into granulocytes via a PKC β1,β2/ERK pathway. HL-60 cells represent a convenient model to study biochemical factors regulating myeloid differentiation, as these cells can be induced to differentiate along either monocytic or granulocytic lineages. Treatment of HL-60 cells with DMSO or RA leads to granulocytic differentiation, while monocytic differentiation can be induced by agents such as PMA, 1,25-dihydroxy vitamin D_3, or sodium butyrate (Breitman et al., 1980; Collins et al., 1978; Rovera et al., 1979; Tanaka et al., 1983).

The mechanism by which helenalin induces HL-60 cell differentiation is not clear. Induction of HL-60 cells differentiation requires the activation of variety of signal transduction pathways, such as the phosphatidylinositol 3-kinase (PI3-K; Bertagnolo et al., 1999), PKC (Caponigro et al., 1997; Nishikawa and Shirakawa, 1992), and MAPK pathways (Woessmann and Mivechi, 2001). Previous report demonstrates the involvement of the MEK/ERK/MAP kinase pathway in PMA-, RA-, and G-CSF-induced myeloid differentiation. Additionally, another study has indicated that PMA-induced MAPK activation is dependent on PKC signaling in myeloid differentiation. In our study, inhibitors for PKC and ERK significantly decreased HL-60 cell differentiation induced by helenalin, while inhibitors of PI3-K did not. These results suggest that PKC and ERK may be, at least in part, involved in the induction of HL-60 cell differentiation by helenalin. Importantly, inhibitors for PKC inhibited ERK activation stimulated by helenalin (Fig. 5C), indicating that PKC may be upstream components of ERK activation induced by helenalin. In contrast, a p38 MAPK inhibitor, SB 203580, increased HL-60 cell differentiation induced by helenalin, consistent with a previous report (Wang et al., 2000).

Many previous studies have demonstrated that interference with the activation of NF-κB appears to be a common feature for agents that enhance the differentiation of HL-60 cells. Our NF-κB EMSA indicated that HL-60 leukemia cells showed relatively high levels of NF-κB activity, in consistent with other's report (Sokoloski et al., 1998). Constitutively high levels of NF-κB activity have been detected in many tumor cells because the aberrant expression of NF-κB was associated with oncogenesis and carcinogenesis (Sharma and Narayanan, 1996). Furthermore, we previously reported that inhibition of this NF-κB activity sensitized the HL-60 cells to differentiation-inducing agents such as dihydroxy vitamin D_3 (Kang et al., 2002). Helenalin is known as a potent NF-κB inhibitor, which has previously been shown to completely inhibit NF-κB DNA-binding at 10 μM in Jurkat T cells. The inductive levels of cellular differentiation by helenalin closely correlated with the inhibitory levels of NF-κB binding activity (Fig. 6), suggesting possible involvement of NF-κB inhibition in the HL-60 cell differentiation induced by helenalin. However, the role of NF-κB inhibition in the helenalin-mediated enhancement of cell differentiation should be further investigated. Furthermore, the inhibitors for PKC and ERK increased the NF-κB binding activity, suggesting both PKC and ERK may be involved in NF-κB inhibition of HL-60 cell differentiation by helenalin (Fig. 7).

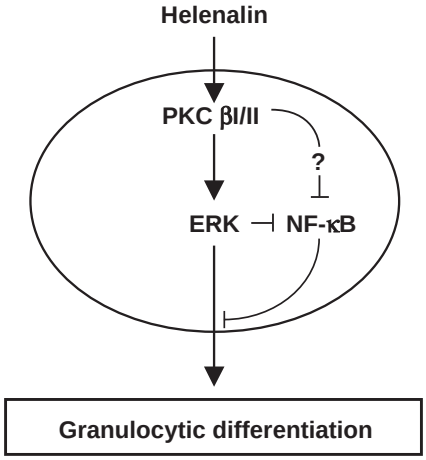

Fig. 7. A proposed mechanism of helenalin-induced HL-60 cell differentiation.

Another possibility of the helenalin-mediated inducing effect on cell differentiation is that helenalin may enhance cell differentiation via inhibition of cytochrome *P*450 enzymes. Helenalin is known to inhibit cytochrome *P*450 enzymes and isoforms of cytochrome *P*450 families are expressed in human myeloid leukemia cell lines (Nagai et al., 2002). The inhibition of cytochrome *P*450 enzymes may be mediated via inhibitory effect of NF-κB activity since many compounds induced cytochrome *P*450 enzymes by enhancing NF-κB activation (Puga et al., 2000).

In summary, we have shown that helenalin induces HL-60 cell differentiation via the PKC/ERK signaling pathway and inhibition of NF-κB activity. These results may explain some known activities of helenalin, including its anti-carcinogenic effects, and suggest a possible use of helenalin in the treatment of neoplastic diseases.

Acknowledgments

This work was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (01-PJ10-PG6-01GN16-0005).

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