Cbl-regulated Akt and ERK signals are involved in β-elemene-induced cell apoptosis in lung cancer cells

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Abstract. β-elemene is an active component of a natural plant-derived antineoplastic agent. Although the anticancer activity of β-elemene has been reported, its antitumor mechanism has not been elucidated. The aim of the present study was to investigate how Cbl-regulated Akt and ERK affect β-elemene-induced cell apoptosis in lung cancer cells. In the present study, β-elemene induced cell apoptosis in a dose-dependent manner in A549 cells. Moreover, β-elemene decreased bcl-2 expression, increased bax expression and caused the cleavage of PARP. However, β-elemene also induced the phosphorylation of Akt and ERK in a short period of time. The inhibition of Akt and ERK activation rapidly enhanced β-elemene-induced apoptosis, and the up-regulation of c-Cbl and Cbl-b expression was also detected. These results suggest that Cbl-regulated Akt and ERK signals are involved in β-elemene-induced cell apoptosis in lung cancer cells.

Introduction

Lung cancer is one of the leading causes of cancer-related death in China, and the 5-year relative survival rate of lung cancer patients is only 15% (1). Chemotherapy achieves a response rate of 20-50% in advanced non-small cell lung cancer (NSCLC) cases. Although it provides useful palliation, advanced lung cancer remains incurable. Resistance to standard chemotherapy is a common clinical problem that requires novel strategies to enhance the antitumorigenic effects of

chemotherapeutic agents. Many anticancer agents, including bufalin and Taxol, have been isolated from natural products and are used for the treatment of cancer (2,3). Elemene (1-methyl-1-vinyl-2,4-diisopropenyl-cyclohexane), the active component of the traditional Chinese medicinal herb *Rhizoma zedoariae*, contains α-, β- and δ-elemene. It has been reported that β-elemene effectively treats various carcinomas, such as glioblastoma, and lung and ovarian carcinomas, and recent studies have shown that β-elemene triggers apoptosis through the down-regulation of Bcl-2 and Bcl-XL (4-7). Whether another signaling pathway is involved in β-elemene-induced apoptosis in lung cancer cells has yet to be determined.

Several drugs have been reported to stimulate a variety of tyrosine kinase receptors, leading to a rapid elevation in the enzymatic activity of serine-threonine kinases, known as MAP kinases. Extracellular signal-regulated kinases (ERKs) are members of the mitogen-activated protein kinase superfamily that functions in response to DNA damage (8,9). Additionally, the constitutively active Akt, the central mediator of the PI3K pathway, contributes to cell survival and therapeutic resistance in cancer cells (10,11). However, whether β-elemene-induced apoptosis is accompanied by an alteration in the survival pathway has yet to be determined.

c-Cbl and Cbl-b are members of the Cbl protein family and negatively regulate cellular viability by interacting with Fyn, Grb2, Lck or p85 (12,13). Studies have shown that Cbl protein binding to the p85 subunit of PI3K induces the ubiquitination of the p85 subunit and the inactivation of the PI3K/Akt pathway (14,15). Our previous study showed that the up-regulation of Cbl-b reduced ERK activition in gastric cancer and leukemia cells (16). However, whether c-Cbl and Cbl-b are involved in β-elemene-induced apoptosis in lung cancer cells has not been elucidated.

In the present study, β-elemene induced cell apoptosis together with transient activation of Akt and ERK. The up-regulation of c-Cbl and Cbl-b was also detected. The inhibition of Akt and ERK signals enhanced β-elemene-induced apoptosis in lung cancer cells.

Materials and methods

Reagents and antibodies. β-elemene was purchased from Holley Kingkong Pharmaceutical Co., Ltd. (Dalian, China).

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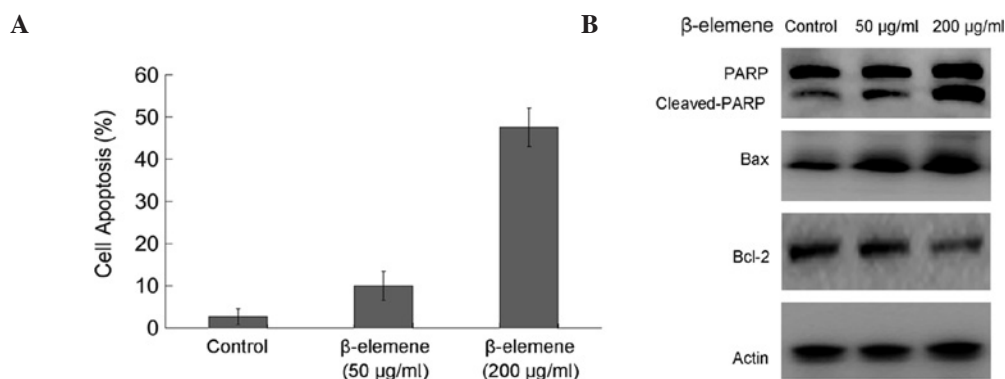
*Contributed equally

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B

Figure 1. β-elemene induces cell apoptosis in A549 cells. (A) A549 cells were treated with 50 and 200 μg/ml β-elemene for 24 h. Cell apoptosis was quantified using flow cytometry. (B) The expression of PARP, bax and bcl-2 was detected by Western blotting.

Anti-bax, anti-bcl-2 and anti-Cbl-b antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Akt, anti-phospho-ERK, anti-Akt and anti-ERK antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-c-Cbl antibody was purchased from Transduction Laboratories (Lexington, KY, USA). LY294002 was from Sigma-Aldrich (St. Louis, MO, USA). PD98059 was purchased from Promega (Madison, WI, USA).

Cell culture. A549 human NSCLC cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences. Cell culture was carried out in RPMI-1640 medium containing 10% fetal bovine serum (FBS), penicillin (5 U/ml) and streptomycin (50 mg/ml) in a 95% air/5% CO_2 atmosphere.

MTT assay. A549 cells were plated in 96-well flat bottom plates and treated with different concentrations of β-elemene for the indicated times. The cells were then incubated with 20 μl of MTT (5 mg/ml) for 4 h at 37°C and 200 μl of DMSO were added to solubilize the crystals for 20 min at room temperature. The optical density was determined with a spectrophotometer (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm. Four replicate wells were measured for each group and each experiment was repeated three times. The median inhibitory concentration (IC_{50}) values were determined using the CurveExpert 1.3 software and plotted in dose-response curves.

Flow cytometric analysis. A549 cells were treated with different concentrations of β-elemene for the indicated times. Then, cells were harvested and fixed with 70% ethanol at 4°C overnight. The cells were incubated in 0.5 ml phosphate-buffered saline (PBS) containing 10 μl RNaseA (0.2 mg/ml) at 37°C for 30 min and then stained with propidium iodide (PI) dye. Finally, the samples were evaluated by flow cytometry and the data were analyzed using WinMDI software.

Western blot analysis. A549 cells were washed twice with ice-cold PBS and solubilized in 1% Triton lysis buffer [1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 2 μg/ml aprotinin] on ice. The amount of protein was quantified using the Lowry method. Total proteins (40 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel eletrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes
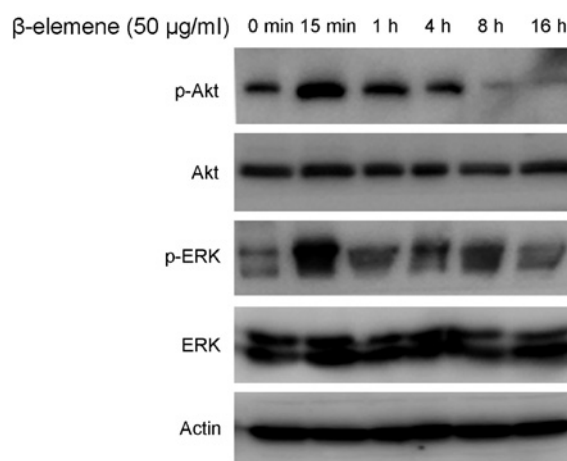

Figure 2. β-elemene induces the activation of Akt and ERK. A549 cells were treated with 50 μg/ml β-elemene for 15 min, 1, 4, 8 and 16 h. The phosphorylation of Akt and ERK was detected by Western blotting.

(Immoblin-P, Millipore, Bedford, MA, USA). Membranes were blocked with 5% skimmed milk in TBST (10 mM Tris, pH 7.4, 150 mM NaCl and 0.1% Tween-20) at room temperature for 2 h and incubated with the indicated primary antibodies at 4°C overnight. After washing with TBST, the membrane was reacted with the appropriate horseradish peroxidase-conjugated secondary antibody, as indicated, for 30 min at room temperature. Finally, proteins were visualized using the enhanced chemiluminescence reagent (SuperSignal Western Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA).

Statistical analysis. Data are expressed as the means ± SD. Differences between the two groups were evaluated by the Student's t-test. $P<0.05$ was considered to be statistically significant. Analyses were conducted using the SPSS 13.0 software.

Results

β-elemene induces cell apoptosis in A549 cells. Lung cancer A549 cells were treated with different concentrations of β-elemene for 24 h; the IC_{50} value was 50 μg/ml. Hence, A549 cells were treated with 50 and 200 μg/ml for 24 h, and the cell apoptosis rate increased to 10.01±3.43 and 47.56±4.57%,
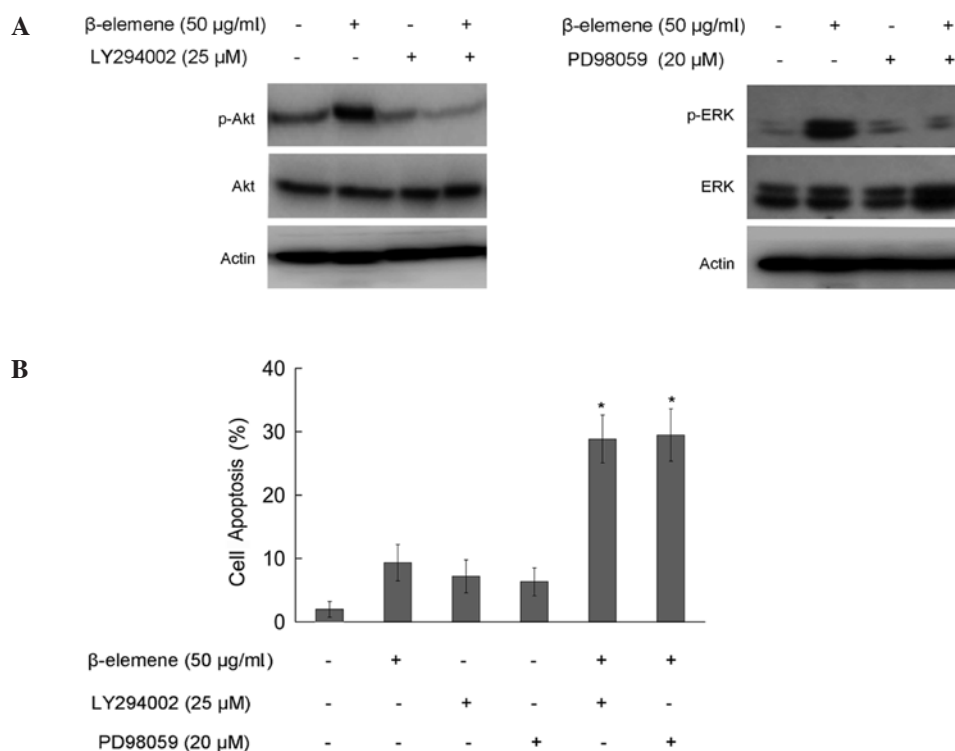
Figure 3. Inhibition of Akt and ERK enhances β-elemene-induced apoptosis. (A) A549 cells were pre-treated with 25 μM LY294002 or 20 μM PD98059 for 1 h followed by 50 μg/ml β-elemene for 15 min. The phosphorylation of Akt or ERK was detected by Western blotting. (B) A549 cells were pre-treated with 25 μM LY294002 or 20 μM PD98059 for 1 h followed by 50 μg/ml β-elemene for 24 h. Cell apoptosis was quantified using flow cytometry. *Compared to β-elemene alone, P<0.05.

respectively (Fig. 1A). With the increase in the concentration of β-elemene, bcl-2 expression was down-regulated, whereas bax expression was significantly up-regulated at 24 h, and the cleavage of PARP was detected (Fig. 1B). These results suggest that β-elemene-induced apoptosis may be through the mitochondrion-dependent apoptotic pathway in A549 cells.

β-elemene induces transient activation of Akt and ERK. To determine whether the Akt and ERK signaling pathway is involved in β-elemene-induced apoptosis, A549 cells were treated with 50 μg/ml β-elemene for 15 min, at 1, 4, 8 and 16 h. As shown in Fig. 2, 50 μg/ml β-elemene induced a rapid and transient phosphorylation of both Akt and ERK at 15 min. Then, Akt and ERK activation started to decrease and declined to lower levels at 16 h. These results indicate that β-elemene induces transient activation of Akt and ERK in A549 cells.

Inhibition of Akt and ERK enhances β-elemene-induced apoptosis. To further confirm that Akt and ERK activation is involved in β-elemene-induced apoptosis, 25 μM LY294002 (the specific inhibitor of PI3K) and 20 μM PD98059 (the specific inhibitor of ERK) were used for further experimentation. Pre-treatment with LY294002 or PD98059 for 1 h reversed the phosphorylation of Akt and ERK induced by 50 μg/ml of β-elemene at 15 min (Fig. 3A). LY294002 or PD98059 did not affect the cell apoptosis of A549 cells. Compared to β-elemene alone, pre-treatment with LY294002 or PD98059 followed by 50 μg/ml of β-elemene for 24 h the rate of cell apoptosis reached 28.83±3.78 and 29.47±4.13%, respectively (P<0.05) (Fig. 3B). Our results showed that the inhibition of Akt and ERK enhanced β-elemene-induced apoptosis in A549 cells.
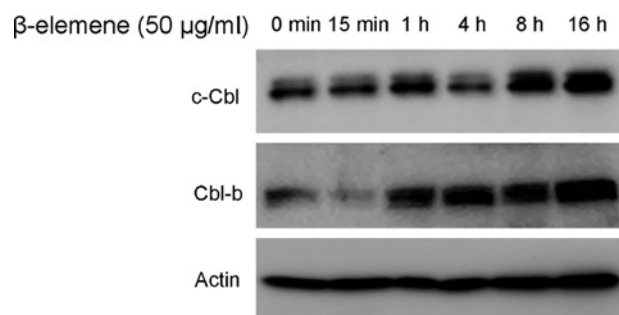

Figure 4. β-elemene up-regulates the expression of c-Cbl and Cbl-b. A549 cells were treated with 50 μg/ml β-elemene for 15 min, 1, 4, 8 and 16 h. The expression of c-Cbl and Cbl-b was detected by Western blotting.

β-elemene up-regulates the expression of c-Cbl and Cbl-b. To investigate the effect of c-Cbl and Cbl-b in β-elemene-induced apoptosis, A549 cells were treated with 50 μg/ml β-elemene for 15 min, at 1, 4, 8 and 16 h. As shown in Fig. 4, β-elemene up-regulated the expression of c-Cbl and Cbl-b at 1 h. Both c-Cbl and Cbl-b protein reached the peak at 16 h. With the up-regulation of c-Cbl and Cbl-b, the phosphorylation of Akt and ERK was reduced (Fig. 2). These results indicate that the Cbl protein family may be involved in Akt and ERK inactivation in β-elemene-induced apoptosis of A549 cells.

Discussion

β-elemene was found to inhibit cell viability and induce apoptosis in several types of cancer cells (5-7). However, the signaling pathways involved have not been elucidated. It has

been reported that β-elemene induces apoptosis by promoting pro-apoptotic-related proteins and down-regulating anti-apoptotic-related proteins, such as Bcl-2 (4). In the present study, β-elemene induced cell apoptosis in a dose-dependent manner in A549 cells. At the same time, β-elemene increased bax expression and decreased bcl-2 expression, and finally induced the cleavage of PARP. These results indicate that β-elemene induces cell apoptosis through the mitochondrion-dependent apoptotic pathway in lung cancer cells.

Recent studies have shown that the activation of Akt and ERK saves cancer cells from drug-induced DNA damage resulting in drug resistance (16-18). In the present study, Akt and ERK were transiently activated at 15 min after treatment and then depressed by 50 μg/ml of β-elemene. In addition, the PI3K inhibitor LY294002 or ERK inhibitor PD98059 reversed the activation of Akt or ERK induced by β-elemene, and finally increased the sensitivity of β-elemene in the A549 cells. Our results suggest that the inhibition of the survival pathway increased β-elemene sensitivity in lung cancer cells.

It is evident that the Cbl protein family is crucial to the ubiquitination and degradation of many proteins. Thus, certain drugs that enhance the activity of the Cbl protein family may provide new strategies for cancer therapy (19-21). Our previous studies found that up-regulation of Cbl-b inhibited Akt and ERK activation, and thus increased the sensitivity of different drugs in gastric cancer and leukemia cells (22,23). In the present study, β-elemene up-regulated the expression of c-Cbl and Cbl-b. During this process, the activation of Akt and ERK was also inhibited. These results indicate that the Cbl protein family is involved in β-elemene-induced apoptosis probably via regulation of Akt and ERK activation.

Taken together, our results show that β-elemene induces apoptosis by delivering apoptotic signals, and Cbl-regulated Akt and ERK signals are involved in β-elemene-induced apoptosis of lung cancer cells. Our study is important for understanding the clinical applications of β-elemene in lung cancer.

Acknowledgements

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