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Original article

β-elemene regulates endoplasmic reticulum stress to induce the apoptosis of NSCLC cells through PERK/IRE1α/ATF6 pathway

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ABSTRACT

Endoplasmic reticulum stress (ERs) has been regarded as an important cause for the pathogenesis of non-small-cell lung cancer (NSCLC). β-elemene is an active component in the essential oil extracted from a medicinal herb, *Curcuma wenyujin*, and has been reported to be effective against non-small-cell lung cancer (NSCLC). However, the potential effect and underlying mechanisms of β-elemene on regulating ERs to inhibit NSCLC are still unclear. In the present study, A549 cells and Lewis tumor-bearing C57BL/6J mice were established to evaluate this effect. Visualsonics Vevo 2100 Small Animal Dedicated High-frequency Color Ultrasound was performed to observe tumor volume *in vivo*. 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) was used to evaluate cell vitality of A549 cells. Furthermore, western blotting (WB), immunohistochemistry (IHC) and quantitative reverse transcription polymerase chain reaction (q-PCR) were applied to detect the ERs-related proteins. Flow cytometry was also applied to detect cell apoptosis and assay kit for reactive oxygen species (ROS) generation. Our results showed that β-elemene inhibited lung cancer tumor growth and cell vitality in a dose- and time-dependent manner. Not only that, β-elemene could up-regulate ERs-related proteins like PERK, IRE1α, ATF6, ATF4, CHOP and down-regulate the Bcl-2 expression. More importantly, ERs inhibitor 4-PBA, IRE1α inhibitor STF-083010, ATF6 inhibitor Anti-ATF6 and PERK inhibitor GSK2656157 can all reduce the amplitude of protein expression changes and apoptosis rates, then weaken the anti-tumor effect of β-elemene. Therefore, the present *in vivo* and *in vitro* study revealed that the anti-NSCLC effect of β-elemene is closely related to the activation of ERs through PERK/IRE1α/ATF6 pathway, and this might be beneficial for clinical therapy of NSCLC.

Abbreviations: NSCLC, non-small-cell lung cancer; ER, endoplasmic reticulum; ERs, endoplasmic reticulum stress; 4-PBA, 4-phenylbutyric acid; WB, western blotting; q-PCR, quantitative reverse transcription polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DCF, 2′, 7′-dichlorofluorescin; CTX, Cytoxan; IHC, immunohistochemistry; IRE1α, inositol-requiring enzyme-1α; PERK, PKR-like endoplasmic reticulum kinase; ATF6, activating transcription factor 6; ATF4, activating transcription factor 4; CHOP, C/EBP homologus protein; Bcl-2, B cell lymphoma/lewkmia-2; GADPH, glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; OD, optical density; SD, standard deviation; IC50, half-maximal inhibitory concentration; PBS, phosphate buffer saline; PVDF, polyvinylidene fluoride; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; UPR, unfolded-protein response; ECM, extracellular matrix.

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1. Introduction

Lung cancer is one of the leading causes of cancer death due to its high morbidity and mortality. In particular, NSCLC accounted for about 80% of all lung cancers [1]. Though endoplasmic reticulum (ER) with a strong system of homeostasis, there are many factors such as hypoxia, oxidative damage or viral infection and pathologic state in the occurrence and development of NSCLC cells. Of note that endoplasmic reticulum sress (ERs) plays an important role in the proliferation and cell vitality of cancer cells. ERs is crucial for maintaining cellular homeostasis environment and activates protein folding reaction (unfolded protein response, UPR) by reducing the synthesis of new proteins. It then could lead to the increase of the partner molecules and promote protein misfolding, which finally promoted cell apoptosis [2]. UPR in ERs have three pathways: PERK pathway, IRE1α pathway and ATF6 pathway. ATF4 is downstream of the PERK protein molecule and also plays an important role in regulating the expression of CHOP [3]. CHOP is expressed in response to ERs. When severe ERs condition appeared, apoptosis starts with the up-regulation of CHOP then activates Bcl-2 to promote apoptosis. The regulation of compounds on ERs of tumor cells are benifical to suppress growth and induce apoptosis of tumors.

Elemene (1-methyl-1-vinyl-2, 4-diisopropenyl-cyclohexane), a novel broad-spectrum antitumor molecule, extracted from traditional Chinese medicinal herb *Curcuma wenyujin* that includes α, β, γ and δ forms [4]. Its major terpene compounds β-elemene has been developed to a new drug which was approved by the State Food and Drug Administration of China for the treatment of malignant effusion and some solid tumors [5]. Previous studies have shown that β-elemene exhibited anti-cancer effects in many cancer cells, especially lung cancer cells by inducing apoptosis [6]. However, whether β-elemene inhibit proliferation or induce apoptosis of NSCLC cells by regulating ERs still need to be further elucidated.

In the present study, we explored the regulation of β-elemene on ERs of NSCLC *in vivo* and *in vitro*, and then reveal its mechanism on regulating ERs. This investigation may provides a new insight for the treatment of β-elemene to NSCLC.

2. Materials and methods

2.1. Reagents and antibodies

β-elemene (lot No.,515-13-9) (purity ≥ 99%) was purchased from Yuanda Pharmaceuticals Co., Ltd. (Dalian, China). 4-PBA (lot No., STBG2117 V) (purity ≥ 99%) was purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO, USA). STF-083010 (lot No., 4509-10), anti-ATF6 (lot No., 9H209) and GSK2656157 (lot No., S7033) were all ordered from Nanjing KeyGen Biotech. Co., Ltd. (Nanjing, China). DMEM medium (lot No., 160425), 0.25% trypsin (lot No., 20160317), 3-(4, 9 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) and dimethyl sulfoxide (DMSO) were provided by Nanjing Sunshine Biotechnology Co., Ltd. (Nanjing, China). Fetal bovine serum (FBS) (lot No., 20151130) was obtained from Invitrogen (Carlsbad, CA, USA). Antibody against CHOP (lot No., YB526) was obtained from Shanghai Yubo biological technology Co., Ltd. (Shanghai, China). Antibodies against PERK (lot No., 3192) and IRE1α (lot No., 3294) were purchased from Cell Signaling Technology, Inc. (ShangHai, China). Anti-ATF6 (lot No., 24169-1-AP) and anti-ATF4 (lot No., 10835-1-AP) were purchased from Proteintech Group, Inc. (Wuhan, China). Anti-Bcl-2 (lot No., ab48394) was obtained from Shanghai Maiyueer biological technology Co., Ltd. (Shanghai, China). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) antibody (lot No., Ab8226), HRP-labeled anti-rabbit (lot No.,Ab6721) and anti-mouse (lot No., Ab6789) immunoglobulin G (IgG) were all obtained from the Abcam Trading Company Ltd. (Shanghai, China). Allergic ECL chemiluminescence reagent kit (lot No., P0018) and Reactive Oxygen Species assay kit (lot No., S0033) were obtained from Beyotime Institute of Biotechnology (Shanghai, China).

2.2. Cell culture

Non-small-cell lung cancer adenocarcinoma cell lines A549 (human) and Lewis (mouse) were both ordered from Nanjing KeyGen Biotech. Co., Ltd. (Nanjing, China). Cells were all cultured in high-glucose DMEM containing 10% inactivated fetal bovine serum (FBS), 80 U/ml of penicillin and 0.08 mg/ml of streptomycin at 37 °C with 5% CO_2 in a humidified atmosphere and the medium should be replaced every day [7]. After having grown to 80%-85% confluence, cells were digested by 0.25% trypsin-0.02% EDTA solution for the passage or further experiments.

2.3. MTT assay

To evaluate inhibitory effects of different concentrations of β-elemene on A549 cells *in vitro*, MTT colorimetric method was performed in this study. Cells were seeded in 96-well plates at an approximate density of 1×10^5 and then divided into different groups. Cells were treated with blank medium without FBS as blank control group, and β-elemene at 1, 2.5, 5, 10, 20, 40, 80, 160, 320 μg/ml respectively as treated group. After 24 h, 48 h and 72 h incubation, MTT (5 mg/ml) of 10 μl was added to each well and then continued to incubate at 37 °C for 4 h. Sequentially, DMSO of 100 μl was added after the removal of culture medium. The optical density (OD) at 570 nm was determined using a 96-well plates reader (reference wavelength 630 nm) [8]. All measurements were repeated three times. The survival rates were calculated by the following formula: The cell survival rates (%) = [(the absorbance of treated group − absorbance of blank control group)/the absorbance of blank control group] × 100% [9]. The IC50 (concentration of drug that inhibits cell growth by 50%) values was calculated by linear regression [10].

2.4. Flow cytometry analysis

Flow cytometry was used to detect cells apoptosis by staining apoptotic cells with both Annexin V-fluorescein isothiocyanate (FITC) and Propidium iodide (PI) before and after administration. A549 cells were grown in 6-well plates (2×10^6) overnight and treated with blank medium, 1 μg/ml β-elemene, 1 μg/ml β-elemene plus 2 μg/ml 4-PBA, 5 μg/ml anti-IRE1α plus 1 μg/ml β-elemene, 5 μg/ml anti-ATF6 plus 1 μg/ml β-elemene, 5 μg/ml GSK2656157 plus 1 μg/ml β-elemene, respectively. At the end of incubation, cells were collected and washed twice with ice phosphate-buffered saline (PBS), then 400 μl of PBS binding buffer was added to each tube (12 mm × 75 mm, polystyrene round bottom) for suspension [11]. The following 5 μl of annexin V-FITC and 10 μl of PI were added and then incubated at room temperature in the dark for 15 min. Samples were detected by LSRIII flow cytometry and the data were analyzed with CellQuest Pro software (Becton-Dickinson) [12].

2.5. Western blotting analysis

A549 cells (2×10^6) in 6-well plates were exposed to vehicle and indicated doses of drugs for 24 h, then harvested by trypsinization for WB. After washing with ice-cold PBS, all samples were scraped in 200 μl of Thermo scientific RIPA buffer (50 mM Tris-HCl (pH 7.4), 1.0% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM aprotinin, 1 mg/ml PMSF, leupeptin and pepstatin) (Pierce,