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β-Elemene induces apoptosis of human rheumatoid arthritis fibroblast-like synoviocytes *via* reactive oxygen species-dependent activation of p38 mitogen-activated protein kinase

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ABSTRACT

Background: β-Elemene is a natural anticancer compound extracted from the Chinese medicinal herb *Curcuma Wenyujin*. This study was done to determine the effect of β-elemene on the apoptosis of rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) and associated molecular mechanisms.
Methods: RA-FLS were treated for 72 h with β-elemene at 10–200 μg/ml and cell viability and apoptotic changes were examined. The involvement of reactive oxygen species (ROS) and mitogen-activated protein kinases (MAPKs) was checked.
Results: We found that β-elemene significantly inhibited the viability and promoted apoptosis of RA-FLS in a concentration-dependent fashion. β-Elemene-treated FLS showed a significant decline in mitochondrial membrane potential, an accumulation of cytochrome c in the cytosol, and increased activities of caspase-9 and caspase-3. β-Elemene treatment caused an enhancement of p38 MAPK phosphorylation and ROS production. The pro-apoptotic activity of β-elemene was significantly reversed by pretreatment with the p38 inhibitor SB203580 or ROS inhibitor N-acetyl-L-cysteine.
Conclusions: Taken together, β-elemene is effective in inducing mitochondrial apoptosis of RA-FLS, which is mediated through induction of ROS formation and p38 MAPK activation. β-Elemene may thus have therapeutic benefits for RA.

Introduction

Rheumatoid arthritis (RA) is a chronic disorder characterized by synovial hyperplasia and progressive joint destruction [1]. Fibroblast-like synoviocytes (FLS) are the principal cells implicated in the pathogenesis of RA [2]. RA-FLS display several tumor-like features, including uncontrolled growth, apoptosis resistance, and high invasiveness [3]. The aggressive expansion of FLS contributes to destruction of the articular tissue. Several lines of evidence suggest that defects in apoptosis induction accounts for FLS hyperplasia in RA [4,5]. Therefore, promotion of apoptosis in FLS may represent a potential therapeutic approach for RA.

In general, apoptosis is induced through two main pathways: the extrinsic pathway initiated by ligand binding to cell death receptors and the intrinsic pathway initiated by the release of cytochrome c from the mitochondria [6]. Reactive oxygen species (ROS), which can oxidize or nitrify proteins, lipids and DNA by direct chemical modification, are involved in triggering and mediating apoptosis under physiological and pathophysiological conditions [7,8]. It has been documented that release of a large amount of ROS is causally linked to apoptosis of RA-FLS induced by apigenin, a dietary plant-flavonoid [9]. ROS-induced apoptosis is associated with activation of mitogen-activated protein kinases (MAPKs) [9,10], which comprise the extracellular signal-regulated kinases (ERKs), p38, and the c-Jun N-terminal kinases (JNKs). These studies suggest that activation of ROS/MAPK pathways is an important mechanism for apoptosis induction in RA-FLS.

β-Elemene is a natural anticancer agent extracted from the Chinese medicinal herb *Curcuma Wenyujin* [11]. It can trigger apoptotic death in a variety of cancer cells [11,12]. For instance, β-elemene is effective in inducing apoptosis in both cisplatin-sensitive and -resistant ovarian cancer cells *via* activation of caspase-3, -8 and -9 [12]. In prostate cancer cells, β-elemene shows the ability to cause apoptotic death *via* downregulation of Bcl-2, promotion of cytochrome c release, and activation of

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caspase-3, -7, -9, and -10 [13]. Increased intracellular ROS generation is causally linked to β-elemene-induced apoptosis in cisplatin-resistant human lung adenocarcinoma A549/DDP cells [14]. β-Elemene has been reported to activate p38 MAPK in glioblastoma cells, leading to growth suppression [15]. However, the effect of β-elemene on the survival of RA-FLS has not been determined yet.

In this study, we aimed to check whether β-elemene treatment promotes apoptosis of RA-FLS and if so, to examine the molecular mechanisms involved.

Materials and methods

Materials

β-Elemene (98% in purity) was obtained from Yuanda Pharmaceuticals, Ltd., Inc. (Dalian, China). Dimethyl sulfioxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetyl-L-cysteine (NAC), and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). JC-1 Mitochondrial Membrane Potential Assay Kit was purchased from Biotium (Hayward, CA, USA) and Annexin V-FITC Apoptosis Detection Kit from Becton Dickinson Biosciences (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Radioimmunoprecipitation assay (RIPA) buffer and protease and phosphatase inhibitors were purchased from Cell Signaling Technology (Beverly, MA, USA). Cell Fractionation Kit Standard was purchased from Abcam (Cambridge, MA, USA). Primary antibodies used in this study are listed in Table 1. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) Kit was purchased from Pierce Biotechnology (Rockford, IL, USA) and Caspase-3 or Caspase-9 Colorimetric Assay kit from R&D Systems (Minneapolis, MN, USA).

Cells and treatment

Primary human FLS cells isolated from synovial tissues of healthy subjects and patients with RA were obtained from Cell Application, Inc. (San Diego, CA, USA). Cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were treated with different concentrations (10–200 μg/ml) of β-elemene for 72 h and tested for cell viability, apoptosis, and gene expression changes. For inhibitor experiments, cells were pretreated with the p38 inhibitor SB203580 (1 μM) [16] 1 h or NAC (5 mM) 2 h before exposure to β-elemene.

Table 1
Primary antibodies used in this study.

Antigens	Species	Dilution	Sources
phospho-p38	Rabbit	1:500	Cell Signaling Technology (Beverly, MA, USA)
p38	Rabbit	1:1000	Cell Signaling Technology
phospho-ERK1/2	Mouse	1:500	Cell Signaling Technology
ERK1/2	Mouse	1:1000	Cell Signaling Technology
phospho-JNK	Mouse	1:500	Cell Signaling Technology
JNK	Mouse	1:1000	Cell Signaling Technology
Cytochrome c	Rabbit	1:500	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
β-Aactin	Rabbit	1:3000	Santa Cruz Biotechnology

Cell viability assay

Cells were seeded in 96-well plates at 5×10^3 cells/well and incubated overnight. The cells were then treated with various concentrations of β-elemene or 0.1% DMSO (used as vehicle) for 72 h. MTT at a final concentration of 5 mg/ml was added to each well and incubated for another 4 h. The resulting formazan crystals were dissolved by dimethyl sulfoxide and absorbance was measured at a wavelength of 570 nm.

Apoptosis detection by flow cytometry

Apoptotic cells were differentiated from viable or necrotic ones by combined application of annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). In brief, cells were seeded at a density of 2×10^5 cells in 6-well plates. The following day, cells were incubated with indicated concentrations of β-elemene or 0.1% DMSO. After 72 h, cells were suspended in binding buffer and stained with FITC-conjugated annexin V and PI for 20 min in the dark. Stained cells were analyzed by a FACSCalibur flow cytometer (Becton Dickenson, San Jose, CA, USA).

Mitochondrial membrane potential ($\Delta\Psi m$) assay

Mitochondrial membrane potential was measured with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide) dye, as described previously [17]. Cells (2×10^5 cells in 6-well plates) were grown to 60% confluence and treated with 100 μg/ml β-elemene or 0.1% DMSO. After 72 h, cells were harvested, washed, and stained with 10 mM JC-1 at 37 °C for 15 min in the dark. At a low $\Delta\Psi$m, JC-1 localizes in the cytoplasm in a green fluorescent monomeric form, while at a relatively high $\Delta\Psi$m, the JC-1 dye aggregates and yields red fluorescence. Both red and green fluorescence emissions were analyzed by flow cytometry. A decline in the ratio of red/green fluorescence indicates loss of $\Delta\Psi$m.

Western blot analysis

Cells were seeded in 6-well plates at a density of 2×10^5 cells/well. The next day, the cells were treated with 100 μg/ml β-elemene or 0.1% DMSO for 72 h. To prepare whole cell lysates, cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors. Cytoplasmic fractions were isolated using the Cell Fractionation Kit Standard according to manufacturer's protocol. Protein concentrations were quantified using the Bradford assay. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking with 5% fat-free milk, the membranes were incubated separately with the primary antibodies. After washing, the membranes were incubated with appropriate secondary antibodies. Signals were visualized using ECL.

Caspase activity

Cells (2×10^5 cells/well in 6-well plates) were treated with 100 μg/ml β-elemene or 0.1% DMSO for 72 h. Caspase-3 and -9 activities were measured using the colorimetric assay kits. In brief, cells were collected and lysed in caspase assay buffer for 30 min on ice. After centrifugation at $1000 \times g$ for 10 min, supernatant was collected and incubated for 90 min at 37 °C with colorimetric substrates of caspase-9 (Ac-LEHD-pNA) or caspase-3 (Ac-DEVD-pNA). The color intensity, which is proportional to caspase enzymatic activity, was measured spectrophotometrically at 405 nm.

ROS measurement

Cells (2×10^5 cells/well in 6-well plates) were treated with 100 μg/ml β-elemene or 0.1% DMSO. After 72 h, cells were collected. Intracellular ROS levels were determined by measuring oxidative conversion of cell-permeable DCFH-DA to fluorescent dichlorofluorescein (DCF), as described previously [18]. After treatment, cells were washed and incubated with DCFH-DA at 37 °C for 30 min. The levels of ROS were analyzed by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed with one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. The p values less than 0.05 were considered statistically significant.

Results

β-Elemene suppresses the viability and induces apoptosis in RA-FLS

We first investigated the effects of β-elemene (Fig. 1A) on the survival and apoptosis of RA-FLS. MTT assay showed that β-elemene at the concentration up to 100 μg/ml caused a significant and concentration-dependent inhibition of the viability of RA-FLS, compared to untreated cells (Fig. 1B). Apoptosis analysis (Fig. 1C) demonstrated that β-elemene treatment triggered a significant apoptosis in RA-FLS, increasing the apoptosis percentage from 3.5 ± 0.8% to 26.8 ± 2.2% (for 100 μg/ml β-elemene). However, β-elemene at 100 and 200 μg/ml yielded similar cytotoxic effects on RA-FLS. Therefore, if not stated otherwise, 100 μg/ml β-elemene was used in the following experiments.

We also examined the effect of β-elemene on the viability and apoptosis of normal FLS from healthy individuals. The results showed that β-elemene at 10–200 μg/ml did not alter the viability (Fig. 1D) or promote apoptosis (Fig. 1E) in normal FLS, compared to untreated cells.

β-Elemene activates the mitochondrial apoptotic cascade

To determine whether β-elemene caused apoptotic death through the mitochondria-dependent pathway, we examined changes in $\Delta\Psi$m and cytochrome c release from the mitochondria. Compared to untreated cells, β-elemene-treated RA-FLS showed a >65% reduction in $\Delta\Psi$m ($p < 0.05$; Fig. 2A). Western blot analysis showed that the cytosol of untreated cells contained few cytochrome c molecules, whereas β-elemene-treated RA-FLS had a marked accumulation of cytochrome c in the cytosol (Fig. 2B). To determine whether activation of caspase-9 and -3 plays a role in β-elemene-induced apoptosis, RA-FLS were incubated with 100 μg/ml β-elemene and caspase-9 and -3 activities were measured *via* cleavage of specific fluorogenic peptide substrates. As shown in Fig. 2C, there was a significant ($p < 0.05$) increase in the activities of caspase-9 and -3 in RA-FLS treated with β-elemene.

β-Elemene-induced apoptosis is linked to activation of p38 MAPK signaling

Next, we checked the involvement of MAPKs in β-elemene-induced apoptosis. Western blot analysis demonstrated that β-elemene displayed a strong inductive effect on p38 MAPK signaling. The phosphorylation of p38 was enhanced after β-elemene treatment (Fig. 3A). However, β-elemene did not alter the phosphorylation status of ERK or JNK. To determine the role of p38

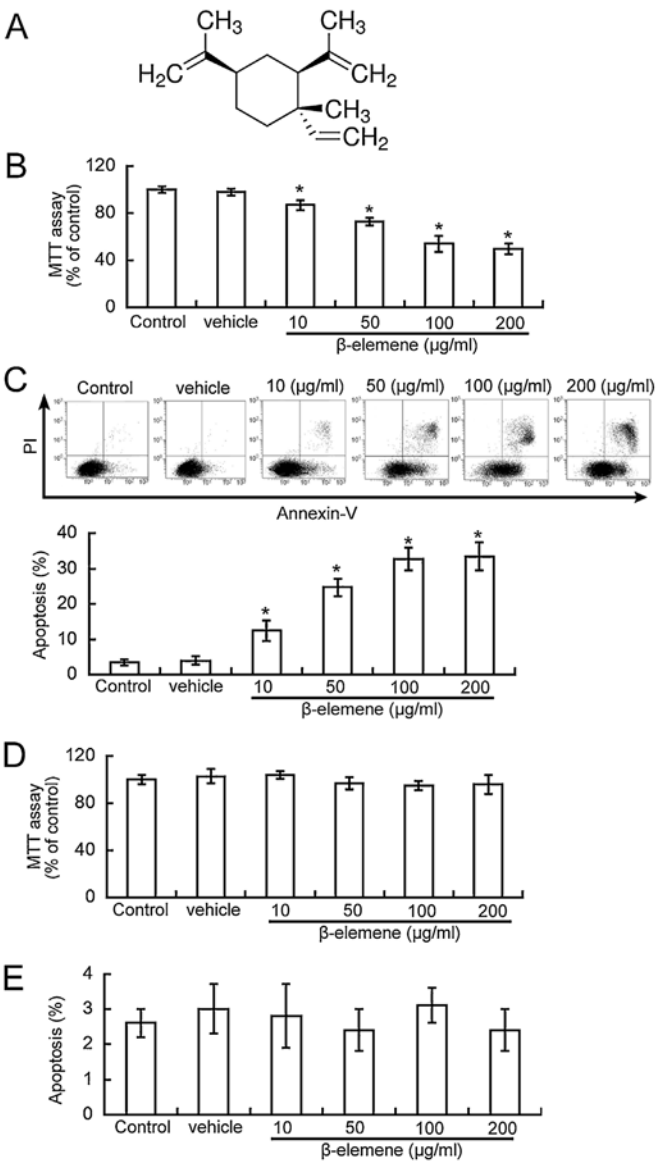

Fig. 1. β-elemene suppresses the viability and induces apoptosis in RA-FLS. (A) Chemical structure of β-elemene. (B and C) RA-FLS were treated with indicated concentrations of β-elemene or 0.1% DMSO (as vehicle) for 72 h and tested for cell viability and apoptosis. (B) Cell viability detected by the MTT assay. Data are expressed as percentage of control (untreated cells). (C) Apoptosis detection by annexin-V/PI staining. Representative dot plots of flow cytometry analysis are shown in upper panels. Bar graphs depict mean percentages of annexin-V-positive apoptotic cells from three independent experiments. $^*p < 0.05$ *vs.* control. (D and E) Assessment of the effect of β-elemene on the viability and apoptosis of normal FLS from healthy individuals. No significant difference was noted between β-elemene-treated and untreated cells.

MAPK signaling in β-elemene-induced apoptosis, RA-FLS were pretreated with the p38 inhibitor SB203580 for 1 h before β-elemene exposure and apoptosis was examined using annexin-V/PI staining. As shown in Fig. 3B, β-elemene-induced apoptotic death was significantly ($p < 0.05$) prevented by SB203580.

ROS generation is involved in β-elemene-induced p38 MAPK activation and apoptosis

Next, we examined the effect of β-elemene on the cellular ROS level in RA-FLS. Treatment with β-elemene caused an approximately 9-fold increase in intracellular ROS levels, compared to

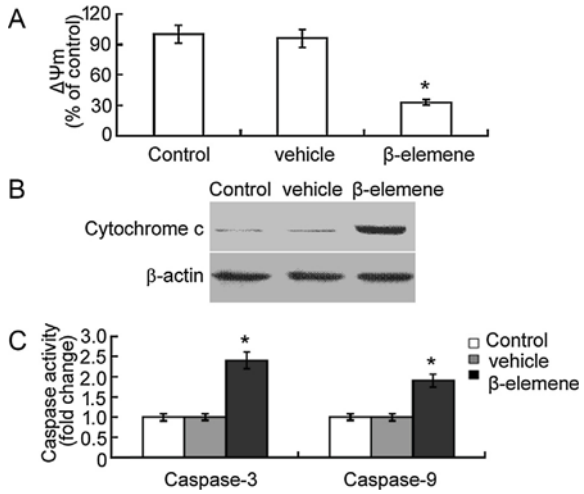

Fig. 2. β-Elemene activates the mitochondrial apoptotic cascade in RA-FLS. Cells were treated with 100 (g/ml β-elemene or 0.1% DMSO (as vehicle) for 72 h and activation of the mitochondrial apoptotic cascade was assessed. (A) Measurement of changes in $\Delta\Psi$m. Data are expressed as percentage of control (untreated cells). (B) Western blot analysis of cytochrome c protein levels in the cytoplasmic extracts. Representative blots of three independent experiments are shown. (C) Measurement of caspase activity using colorimetric assays. Data are expressed as fold change relative to control. $^{*}p < 0.05$ *vs.* control.

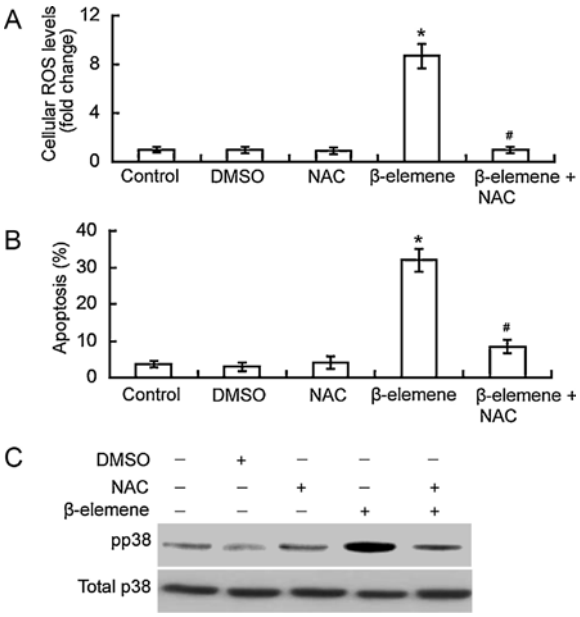

Fig. 4. ROS generation is involved in β-elemene-induced p38 MAPK activation and apoptosis. (A) Measurement of cellular ROS levels in RA-FLS treated with or without NAC before β-elemene treatment. 0.1% DMSO was used as vehicle. Data are shown as a fold change relative to control (untreated cells). (B) Apoptosis detection by annexin-V/PI staining and flow cytometry. $^{*}p < 0.05$ *vs.* control; $^{\#}p < 0.05$ *vs.* β-elemene alone. (C) Western blot analysis of phosphorylated and total p38 MAPK. Representative blots of three independent experiments are shown.

untreated cells ($p < 0.05$; Fig. 4A). Finally, we checked whether or not the inductive effect of β-elemene on RA-FLS apoptosis and p38 MAPK activation was ROS dependent. Pretreatment with NAC (5 mM) for 2 h almost completely blocked the ROS generation induced by β-elemene (Fig. 4A). Most importantly, NAC pretreatment significantly ($p < 0.05$) attenuated β-elemene-induced apoptosis (Fig. 4B) and p38 phosphorylation (Fig. 4C).

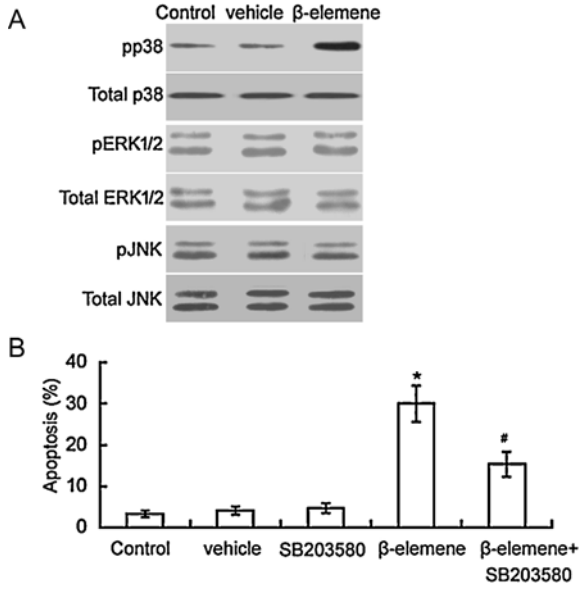

Fig. 3. p38 MAPK signaling is involved in β-elemene-induced apoptosis of RA-FLS. (A) Western blot analysis of indicated proteins in RA-FLS treated with 100 (g/ml β-elemene or 0.1% DMSO (as vehicle) for 72 h. Representative blots of three independent experiments are shown. (B) Cells were pretreated with or without SB203580 before exposure to β-elemene and apoptosis was detected by annexin-V/PI staining and flow cytometry. Data are representative of three independent experiments and shown as mean ± SD. $^{*}p < 0.05$ *vs.* control; $^{\#}p < 0.05$ *vs.* β-elemene alone.

Discussion

Mounting evidence points toward the anticancer activity of β-elemene [19,20]. For instance, β-elemene has been reported to suppress the *in vitro* growth of human breast cancer cells by inducing apoptosis [19]. Given similar malignant features observed between tumor cells and RA-FLS [3], we tested the possibility that β-elemene also exerted cytotoxic effects against RA-FLS. As expected, we found that β-elemene at 10–100 μg/ml significantly inhibited the viability of RA-FLS in a concentration-dependent fashion. Induction of apoptosis is an important mechanism for suppression of cell viability. We also checked the effect of β-elemene on the apoptosis of RA-FLS. Notably, our results showed that β-elemene treatment significantly induced apoptotic death in RA-FLS. However, β-elemene at the same concentration range did not either suppress the viability or induce apoptosis of normal FLS. A previous study also revealed little cytotoxicity of β-elemene in normal human ovary cells [21]. These findings suggest that β-elemene can effectively and selectively target RA-FLS, causing no cytotoxicity to normal healthy FLS.

Our data demonstrated that β-elemene-treated RA-FLS had a significant decline in $\Delta\Psi$m and accumulation of cytochrome c in the cytosol. These findings suggest that β-elemene treatment triggers the mitochondrial apoptotic cascade. Depolarization of mitochondria is usually associated with activation of caspase-9 and -3, consequently leading to apoptosis [22]. Our results confirmed the involvement of caspase-9 and -3 activity in the induction of apoptosis by β-elemene, as evidenced by a significant elevation in the activities of caspase-9 and -3 in β-elemene-treated RA-FLS. Taken together, β-elemene is capable of inducing apoptosis in RA-FLS, which is associated with initiation of the mitochondrial apoptotic pathway. β-Elemene-induced mitochondrial apoptosis has also been documented in cisplatin-resistant A549 lung cancer cells [14].

Activation of MAPKs can lead to either cell survival or apoptosis depending on cellular contexts and stimuli [23,24]. Liagre et al. [25] reported that diosgenin caused apoptosis of RA-FLS *via* activation of p38 and JNK and inhibition of ERK phosphorylation. They found that inhibition of p38 and JNK activity reduced diosgenin-induced apoptosis whereas inhibition of ERK amplified the proapoptotic effect of diosgenin. Another study by the research group showed that hecogenin- and tigogenin-induced apoptosis of RA-FLS is mediated through activation of p38 without affecting the JNK and ERK pathways [26]. Activation of the p38 MAPK pathway has been found to stimulate the expression of Cyr61, which protects RA-FLS from apoptosis [27]. Our data revealed that β-elemene treatment significantly enhanced the phosphorylation of p38 MAPK but not ERK or JNK. Moreover, pretreatment with the p38 inhibitor SB203580 significantly attenuated the apoptotic response in β-elemene-treated RA-FLS. Taken together, the proapoptotic activity of β-elemene in RA-FLS is associated with activation of p38 MAPK. In agreement with our findings, a previous study has shown that activation of p38 MAPK contributes to the anti-proliferation effect of β-elemene in glioblastoma cells [15].

Substantial ROS has been found to activate MAPK signaling cascades [28]. Consistent with activation of p38 MAPK, we found that β-elemene treatment caused a significant increase in the production of ROS. Similarly, β-elemene piperazine derivatives have been shown to induce the generation of ROS in human leukemia cells [29]. To check the role of ROS production in MAPK activation, RA-FLS were pretreated with the ROS inhibitor NAC before exposure to β-elemene. As expected, pretreatment with NAC almost completely impaired β-elemene-induced ROS formation in RA-FLS. Production of a large amount of intracellular ROS is causally linked to induction of apoptosis in RA FLS [9,30]. Shin et al. [9] reported that apigenin, a dietary plant-flavonoid, triggered apoptotic death in RA-FLS *via* promotion of ROS and activation of ERK1/2 signaling. Our data revealed that NAC pretreatment significantly reversed the apoptosis and p38 activation induced by β-elemene. Taken together, these results suggest that the pro-apoptotic activity of β-elemene is mediated through ROS-dependent activation of p38 MAPK.

In conclusion, β-elemene shows the ability to induce mitochondrial apoptosis of RA-FLS, which is associated with promotion of ROS formation and p38 MAPK activation. Further experiments may be required to validate the protective activity of β-elemene in experimental arthritis. This study also suggests that β-elemene may have potential therapeutic implications in RA.

Conflict of interest

We declare no conflict of interests.

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