Enhancement of the antimycobacterial activity of macrophages by ajoene

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Abstract

Ajoene, a garlic-derived sulfur-containing compound, has broad-spectrum antimicrobial activity. To assess the potential of ajoene for treating tuberculosis (TB), we determined whether it induces the stress response of the endoplasmic reticulum (ER), which plays an important role in TB. We showed that ajoene stimulation induced the production of ER stress sensor molecules and reactive oxygen species (ROS) levels. Ajoene-induced ROS production was dependent on c-Jun N-terminal kinase (JNK) activation. Interestingly, the inhibition of JNK activity and suppression of ROS production reduced ajoene-induced CHOP production in macrophages. Because ER stress activates autophagy, the activation of which suppresses the growth of mycobacteria, we investigated the ajoene-induced production of autophagy-related factors, including LC3-II, P62 and Beclin-1. As expected, ajoene treatment increased the levels of these factors in RAW 264.7 cells. Remarkably, the total amount of *Mycobacterium tuberculosis* (Mtb) H37Rv was significantly reduced in ajoene-treated RAW 264.7 cells. The treatment of macrophages with ajoene resulted in the activation of JNK, induction of ROS synthesis and accumulation of ROS, possibly leading to the activation of ER stress and autophagy. These results reveal the mechanism of the antimycobacterial effects of ajoene against Mtb H37Rv. Our findings might facilitate the development of novel therapies for patients with TB.

Introduction

Tuberculosis (TB) is an unresolved public health problem, particularly in developing countries.[1] *Mycobacterium tuberculosis* (Mtb) uses several strategies to avoid host defence mechanisms and survive and multiply within phagocytes.[2] Although combination therapy is usually effective against drug-susceptible Mtb strains, TB remained one of the top-10 causes of death worldwide in 2015.[1] Anti-TB drugs have several side effects, including hepatotoxicity, ototoxicity, hyperuricaemia, exanthema and arthritis.[3,4] The emergence of multidrug-resistant TB (MDR-TB) and extremely drug-resistant TB represent new challenges to global health. Therefore, new therapeutic tools, including natural products, are needed to prevent and treat TB. A number of herbal compounds, such as propolis and extracts of garlic, have antiviral and antibacterial effects. Ajoene, a garlic extract, has two isoforms, E-ajoene and Z-ajoene, and has anticancer, antioxidant, antimicrobial, antithrombosis, antiviral and

antiparasitic effects.[5,6] In addition, ajoene (10 mg/kg) has similar antifungal effects to the antifungal drugs sulfamethoxazole/trimethoprim in mice intratracheally infected with *Paracoccidioides brasiliensis*.[7] Naganawa et al. showed that ajoene (5 μg/ml) restricts the growth of Gram-positive bacteria, such as *Bacillus cereus*, *Bacillus*

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subtilis, *Mycobacterium smegmatis* and *Streptomyces griseus*. The disulfide bonds in ajoene are necessary for its antimicrobial activity.[8] Although ajoene exhibits antimycobacterial activity, little is known about the underlying molecular mechanism(s).

Recent studies have shown that host-directed therapy might be useful for the treatment of TB.[9–11] Our previous studies also suggested that the stress response of the endoplasmic reticulum (ER) is important for suppressing the intracellular survival of Mtb.[12–14] The ER is involved in protein synthesis, folding and secretion.[14] Upon infection or in the presence of stress, the production of unfolded protein response (UPR)-associated proteins is up-regulated. BIP, which is also known as the 78-kDa Glc-regulated protein (GRP78), participates in facilitation of protein folding in the ER and binding to misfolded ER proteins for quality control.[15] BIP is a master regulator of ER stress and controls the activation of UPR signalling.[16] ER stress is initiated by the disassociation of BIP from three major ER membrane-bound molecules, activating transcription factor 6, inositol-requiring enzyme 1 (IRE1), and protein kinase RNA-like endoplasmic reticulum kinase, when the balance between the folding capacity of the ER and the accumulation of misfolded and unfolded proteins is disrupted.[14,15] A transcription factor CCAAT-enhancer-binding protein homologous protein (CHOP) is critical not only in inflammatory responses, but also in ER stress-induced apoptosis.[17] BIP and CHOP are well-studied proteins that are induced by ER stress.

Another important protein induced by ER stress is IRE1α. IRE1α forms a complex with Tumour necrosis factor (TNF)-α-receptor-associated factor 2, which leads to c-Jun N-terminal kinase (JNK) activation.[18] This could result in cell death and autophagy via the suppression of Bcl-2 and inhibition of Beclin-1 by direct binding.[19,20] In this study, we investigated whether ajoene treatment induces the ER stress response in Mtb-infected macrophages.

Materials and methods

Animals

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Chungnam National University, South Korea (Permit Number: CNU-00425). All animal experiments were performed in accordance with Korean Food and Drug Adminitstration guidelines.

Mtb culture and intracellular survival analysis

Mtb strain H37Rv (ATCC 27294) was grown in Middlebrook 7H9 liquid medium supplemented with 10 % oleic acid, albumin, dextrose, catalase and 5 % glycerol. Mtb H37Rv was re-suspended in phosphate buffered saline (PBS) at a concentration of 1×10^8 CFU/ml. Aliquots were stored at $-70\,^{\circ}$C until required. Cells were infected with Mtb H37Rv at a multiplicity of infection (MOI) of 1. To remove extracellular bacteria, cells were washed with PBS and incubated with fresh medium without antibiotics for an additional 1 h. Mtb-infected cells were lysed in sterile distilled water and disrupted in a water bath sonicator to collect intracellular bacteria. The lysates were plated separately on 7H10 agar plates and then incubated for 14 days. Colony counts were performed in triplicate.

Preparation of ajoene and reagents

E/Z ajoene (CAS 92285-01-3) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). *Aralia cordata* and eapafolin were obtained from the Korea Institute of Oriental Medicine (Daejeon, Korea). SP600125, *N*-acetyl-L-cysteine (NAC) and tunicamycin were purchased from Calbiochem (San Diego, CA, USA). Lipopolysaccharide (LPS) was purchased from InvivoGen (San Diego, CA, USA). Staurosporine was purchased from Sigma-Aldrich (St Louis, MO, USA). RAW264.7 cells were pre-treated with indicated concentrations of SP600125 or NAC for 30 min before Mtb infection or ajoene treatment. Dimethyl sulfoxide (DMSO) alone was used as the negative control.

Cell culture and Mtb infection

A549 human alveolar epithelial cells (ATCC CCL-185) and RAW 264.7 murine macrophages (ATCC TIB-71) were maintained in DMEM (Lonza, Walkersville, MD, USA) supplemented with 10 % FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml) and 1 % L-glutamine. Bone marrow-derived macrophages (BMDMs) were isolated from the femurs and tibias of C57BL/6 mice (6–8-wk-old) and then differentiated by growth for 3–5 d in medium containing M-CSF (25 ng/ml; R&D, Minneapolis, MN, USA). The cells were infected for 3 h with Mtb H37Rv at an MOI of 1. Then the cells were washed to remove extracellular bacteria and cultured in fresh complete medium without antibiotics. Intracellular bacterial counts were determined at 24 h postinfection on Middlebrook 7H10 agar.

Flow cytometric analyses of ROS and cell cycle assays

Cells were harvested with PBS. For ROS staining, dihydroethidium [DHE; 2 μM (Molecular Probes, Eugene, OR, USA)] was added and cells were incubated at 37°C for 30 min. After being washed with PBS, the cells were fixed with 4 % paraformaldehyde. H_2O_2 (1 mM; InvivoGen, San Diego, CA, USA) was used as a positive control for ROS production. For cell-cycle analyses,

cells were fixed with 70 % ethanol at −20 °C for at least 12 h. Intracellular DNA was labelled with propidium iodide (PI; 10 µg/ml). Samples were analysed on an FACS Canto II cytometer (BD Biosciences, San Jose, CA, USA) and the data were processed using Flow Jo (Tree Star, Ashland, OR, USA).

Western blot analyses

Western blot analyses were performed as described previously.[13] Specific Abs against CHOP, LC3, Beclin-1, phospho (Ser-51)-eIF2α, total eIF2α, total JNK, phospho JNK (Thr183/Thy185) and GRP78/BIP were purchased from Cell Signaling (Danvers, MA, USA). Anti-β-actin, anti-IRE1α and anti-P62 Abs were purchased from Santa Cruz Biotechnology. Goat anti-rabbit-IgG-HRP (Cell Signaling) and rabbit anti-mouse-IgG-HRP (Calbiochem) were used as secondary Abs. Membranes were developed using a chemiluminescent reagent (ECL; Millipore, Billerica, MA, USA), and were subsequently exposed to chemiluminescence film to visualise the proteins.

Statistical analyses

Experiments were performed at least three times. Data were evaluated by Student's *t*-test or one-way ANOVA followed by Bonferroni's multiple comparison tests.

Results

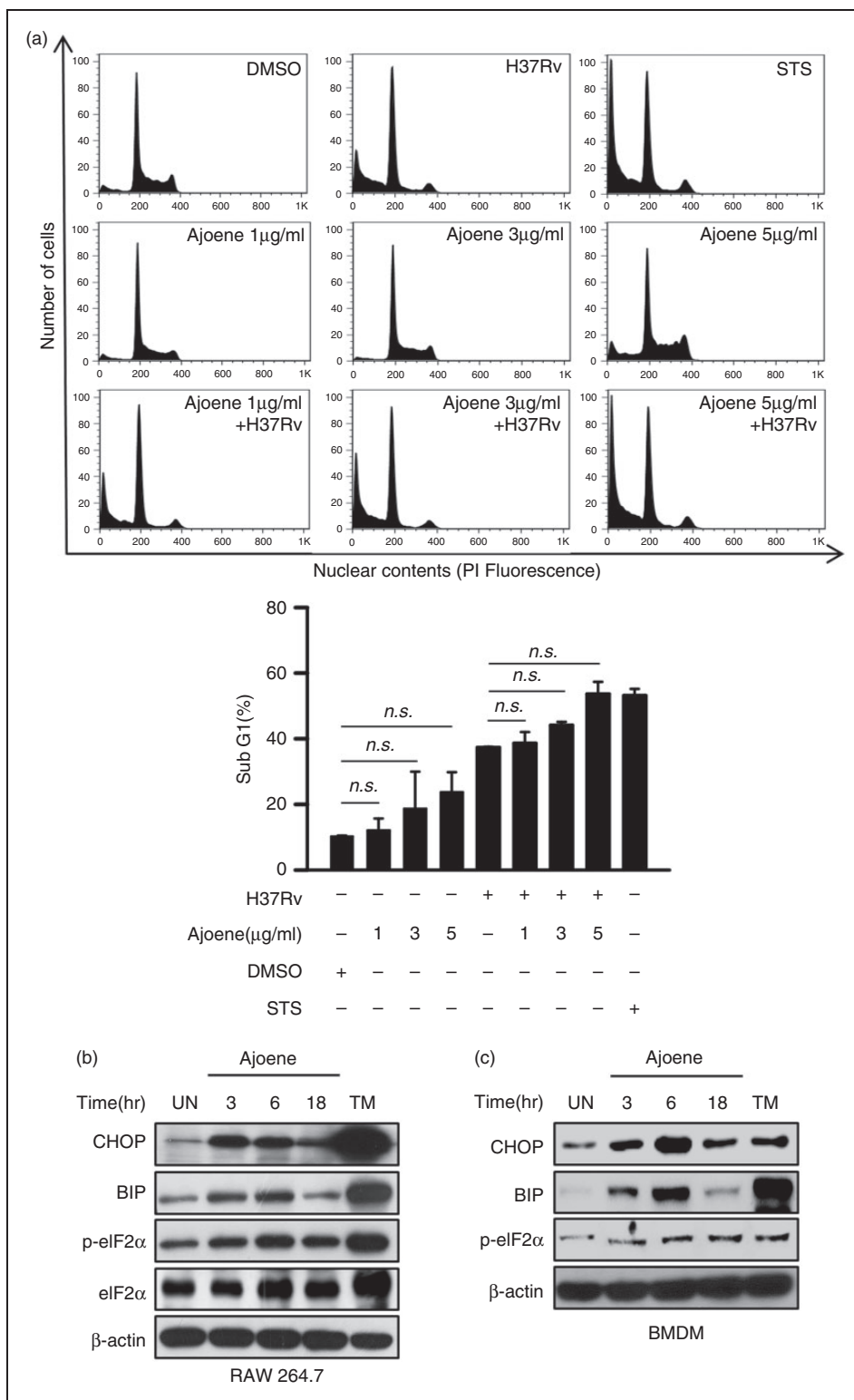### Ajoene induces the UPR/ER stress response

The antimycobacterial effects of several herbal compounds, such as propolis, have been reported.[21] We previously reported that the stress response of the ER is an important defence mechanism in hosts against mycobacterial infection.[12,13,22] To investigate whether herbal compounds induce this stress response, we examined the production levels of BIP and CHOP in A549 human epithelial cells after 24 h stimulation with herbal compounds (Figure S1a). Ajoene treatment strongly induced the production of BIP and CHOP; indeed, CHOP production increased in a dose-dependent manner (Figure S1b). To determine the sensitivity of RAW 264.7 cells to its cytotoxic effects, the cells were incubated with various concentrations of ajoene. The percentage of cells in the sub-G1 phase increased in a dose-dependent manner, and ajoene treatment was less toxic than H37Rv infection alone. In addition, ajoene treatment synergistically induced the sub-G1 phase in cells infected with H37Rv (Figure 1a). These data suggest that 3 µg/ml ajoene was not toxic to murine macrophages and induced the UPR and ER stress responses. Therefore, 3 µg/ml ajoene was used in this study. To confirm the induction of the UPR/ER stress response by ajoene, RAW 264.7 cells and BMDMs were stimulated with 3 µg/ml ajoene for various periods. Western blot analyses showed that ajoene stimulation induced the expression of stress-sensor molecules in the UPR and ER, such as CHOP and BIP, as well as the phosphorylation of eIF2α (Figure 1b). The levels of these proteins were increased at 3 h and 6 h after ajoene stimulation in RAW 264.7 cells and slightly increased at 6 h in BMDMs (Figure 1c). These results indicate that appropriate concentrations of ajoene can be administered to induce the UPR/ER stress response without cytotoxicity in murine macrophages.

Enhanced ROS production and JNK activation by ajoene is associated with the ER stress response

Ajoene may stimulate intracellular ROS production, which can activate the ER stress response.[12,23] To determine whether ajoene induces intracellular ROS production, macrophages were stained with DHE after ajoene treatment. The intensity of DHE fluorescence increased from 15 min to 1 h, and at 30 min it was 1.9-fold higher in ajoene-treated cells than in control cells (Figure 2a). Ajoene-induced increases in ROS levels were significantly reduced by pre-treatment with an ROS scavenger, NAC (Figure 2b). In addition, NAC pre-treatment significantly suppressed CHOP production in ajoene-stimulated RAW 264.7 cells (Figure 2c). These results suggest that ajoene-mediated ROS production is associated with CHOP production in RAW 264.7 cells.

ROS production may be involved in MAPK activation;[24,25] hence, we monitored the levels of MAPK-family proteins in RAW 264.7 cells treated with ajoene. Phosphorylation of JNK peaked from 30 min to 1 h (Figure 3a). Pre-treatment with 20 µM SP600125 decreased ajoene-mediated JNK activity in RAW 264.7 cells. However, no significant activation of p38 or ERK was detected (data not shown). These results suggest that the JNK pathway is important for the induction of ROS production during ajoene stimulation. Because JNK activation depends on IRE1α,[26] we investigated IRE1α induction in ajoene-stimulated RAW 264.7 cells. As expected, both IRE1α and JNK were strongly activated at 1 h after ajoene treatment (Figure 3a, b). Next, we explored the link between JNK signalling and ajoene-induced ROS generation. Cells were pre-treated with specific inhibitors of JNK (SP600125) for 30 min prior to exposure to ajoene. The inhibition of JNK dramatically reduced the ajoene-induced ROS production, as measured via Western blot and flow cytometry (Figure 3c, e). To investigate the effects of IRE1α on JNK activation, we pre-treated cells with the IRE1-specific inhibitor IREstatin (5 µM) for 30 min prior to stimulation with ajoene. The inhibition of IRE1α effectively attenuated ajoene-induced JNK phosphorylation (Figure 3d). These data suggest that ajoene-induced

Figure 1. Ajoene induces the ER stress response in macrophages. (a) RAW 264.7 cells were screened for induction of apoptosis using cell-cycle analyses. RAW 264.7 cells were incubated with the indicated concentrations of ajoene (1, 3 or 5 μg/ml) for 24 h after infection with H37Rv at an MOI of 1. The cells were stained with PI solution (10 μg/ml) for 5 min. The stained cells were analysed to determine the proportion at the sub-G1 stage using flow cytometry. Staurosporine (500 nM, 6 h) was used as the positive control for apoptosis. Data are representative of at least three independent experiments with similar results. There were no significant differences between the ajoene-treated and untreated groups. (b) RAW 264.7 cells and (c) mouse BMDMs were incubated with ajoene (3 μg/ml) for the indicated times. Western blot using Abs against CHOP, BIP, p-eIF2α and eIF2α was performed to analyse ER stress. TM (500 ng/ml, 6 h) was used as a positive control and β-actin was probed as a loading control. Data are representative of at least three independent experiments with similar results.
STS: Staurosporine; n.s.: nonsignificant; UN: unstimulated control; TM: Tunicamycine.
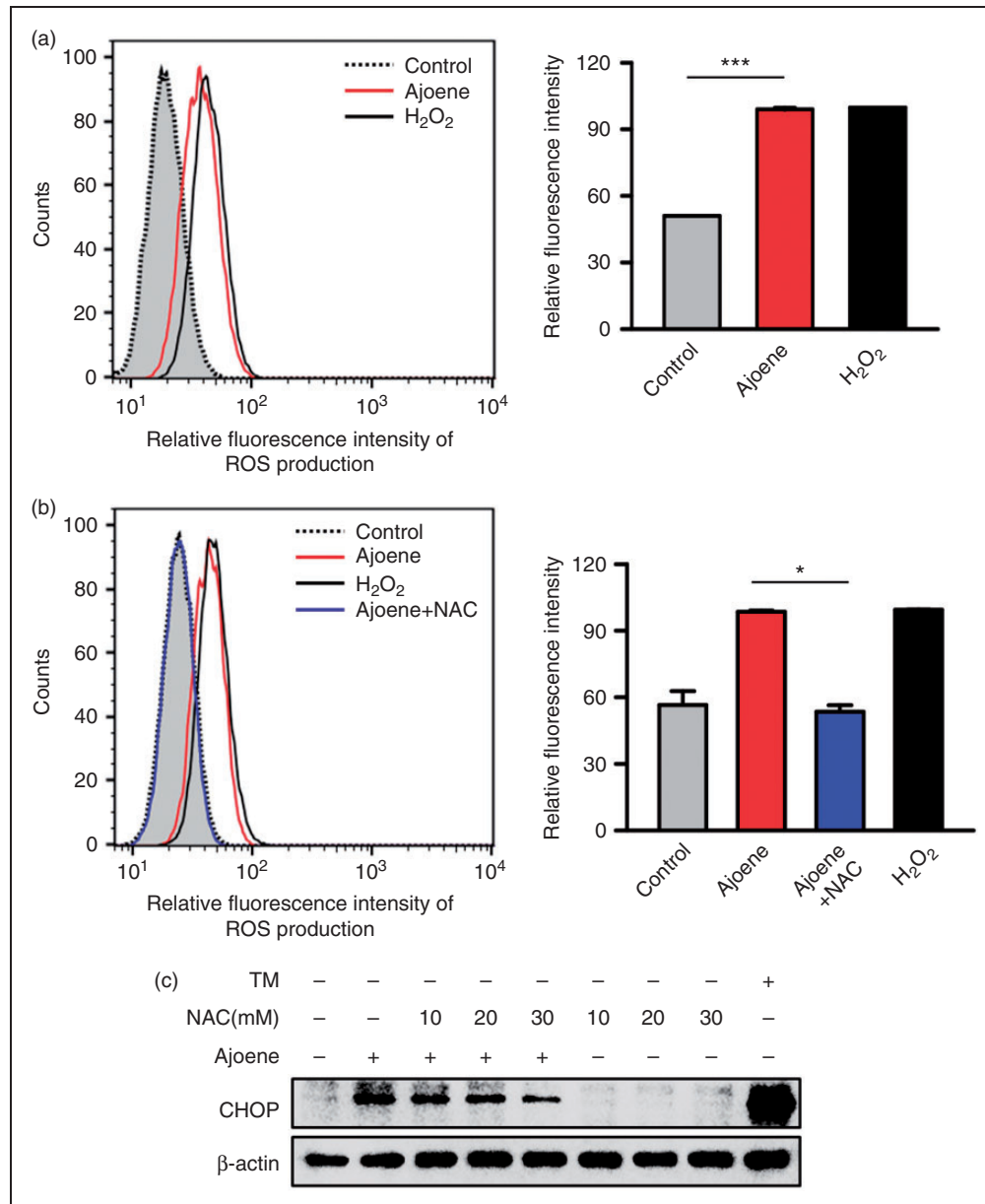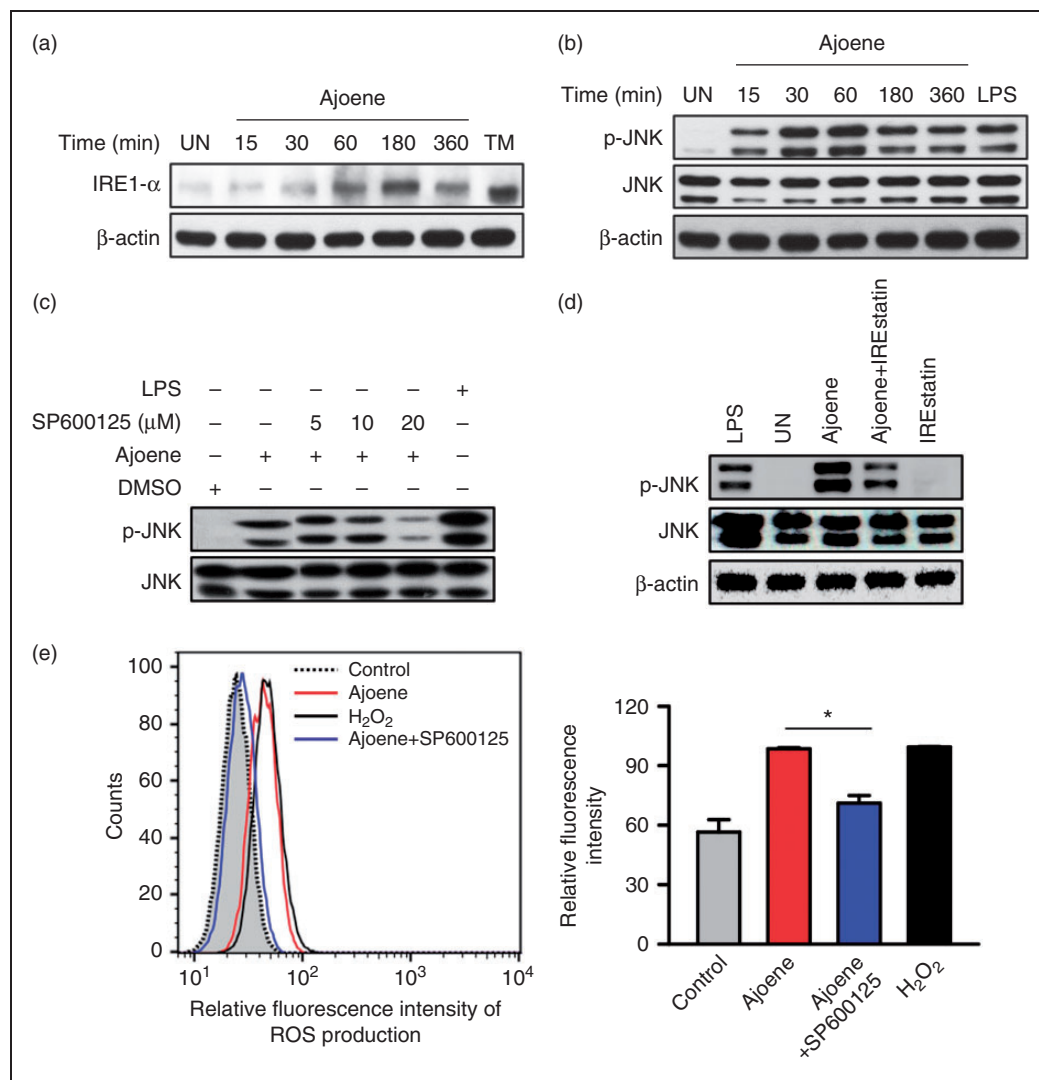
Figure 2. Ajoene induces ROS production in RAW 264.7 cells. (a) RAW 264.7 cells were stimulated with ajoene (3 μg/ml) for 30 min. (b) RAW 264.7 cells were pre-incubated with NAC (30 mM) for 30 min and then stimulated with ajoene (3 μg/ml) for 30 min. Cells were stained with DHE (2 μM) for 30 min at 37 °C and washed with PBS. Superoxide levels were evaluated using flow cytometry. H_2O_2 (1 mM, 30 min) was used as a positive control for the generation of intracellular superoxide. (c) RAW 264.7 cells were pre-incubated with NAC (10, 20 or 30 mM) for 30 min and then stimulated with ajoene (3 μg/ml) for 6 h. The cell lysates were subjected to Western blot analyses of CHOP. TM: Tunicamycin (500 ng/ml, 6 h) was used as a positive control and β-actin was probed as a loading control. Data are representative of at least three independent experiments with similar results. *$P < 0.05$, ***$P < 0.001$.

ROS production is dependent on the IRE1–JNK pathway and that increased intracellular ROS levels are associated with the induction of the ER stress response in RAW 264.7 cells following ajoene stimulation.

Ajoene stimulation induces autophagy in macrophages

The IRE1α–JNK pathway regulates autophagy activation in MEF cells.[27] Autophagy plays an important role in cellular homeostasis and defence against Mtb infection.[28] In addition, ER-stress-mediated autophagy results in cell death.[29] Accordingly, we assessed whether autophagy is activated by ajoene. The protein levels of LC3-II, P62 and Beclin-1, which are markers of autophagy, were elevated in macrophages, as determined by Western blot analyses (Figure 4a). Next, we examined whether JNK activation and ROS production are related to autophagy induction in RAW 264.7 cells stimulated with ajoene. Cells were pre-treated with

Figure 3. Ajoene-induced ROS production is dependent on JNK activation. RAW 264.7 cells were stimulated with ajoene (3 μg/ml) under various conditions, and the cell lysates were subjected to Western blot analyses using Abs against IRE1-α, JNK or p-JNK. TM: Tunicamycin (500 ng/ml, 6 h) and LPS (1 μg/ml, 1 h) were used as positive controls. The data shown are representative of at least three independent experiments with similar results. (a, b) RAW 264.7 cells were stimulated with ajoene for the indicated times. (c) RAW 264.7 cells were pre-incubated with SP600125 (5, 10 or 20 μM) for 30 min and then stimulated with ajoene for 1 h. (d) RAW 264.7 cells were pre-incubated with IREstatin (5 μM) for 30 min and then stimulated with ajoene for 1 h. (e) RAW 264.7 cells were pre-incubated with SP600125 (20 μM) for 30 min and then stimulated with ajoene (3 μg/ml) for 1 h. Cells were stained with DHE (2 μM) for 30 min at 37 °C and washed with PBS. Superoxide levels were evaluated by flow cytometry. H_2O_2 (1 mM, 30 min) was used as a positive control for the generation of intracellular superoxide. The data are means ± SD of three independent experiments. *$P < 0.05$. UN: unstimulated control.

SP600125 (JNK inhibitor) and NAC (ROS scavenger) for 1 h before the addition of ajoene. The inhibition of JNK or ROS production reduced the levels of the proteins LC3-II, P62 and Beclin-1 (Figure 4b). These data indicate that enhanced ROS production via the phosphorylation of JNK is required for autophagy activation in murine macrophages stimulated with ajoene. Next, we examined the effects of ajoene on autophagy activation during Mtb infection. Cells were infected with Mtb and then stimulated with ajoene for 18 h. Interestingly, molecular markers of autophagy such as LC3, P62 and beclin-1 were more highly activated in

Mtb-infected macrophages treated with ajoene than in Mtb-infected macrophages (Figure 4c). Next, we confirmed that ajoene-induced LC3 activation in Mtb-infected macrophages was significantly decreased by SP600125 or NAC, indicating that the ajoene-activated JNK-ROS pathway is involved in autophagy activation (Figure 4d). In addition, an augmentation of JNK phosphorylation (Figure 4e) and ROS production (Figure 4g) was observed in macrophages stimulated with ajoene after Mtb infection. The p-JNK augmented by Mtb and ajoene was decreased in RAW 264.7 cells pre-treated with an IRE1-specific inhibitor (Figure 4f).
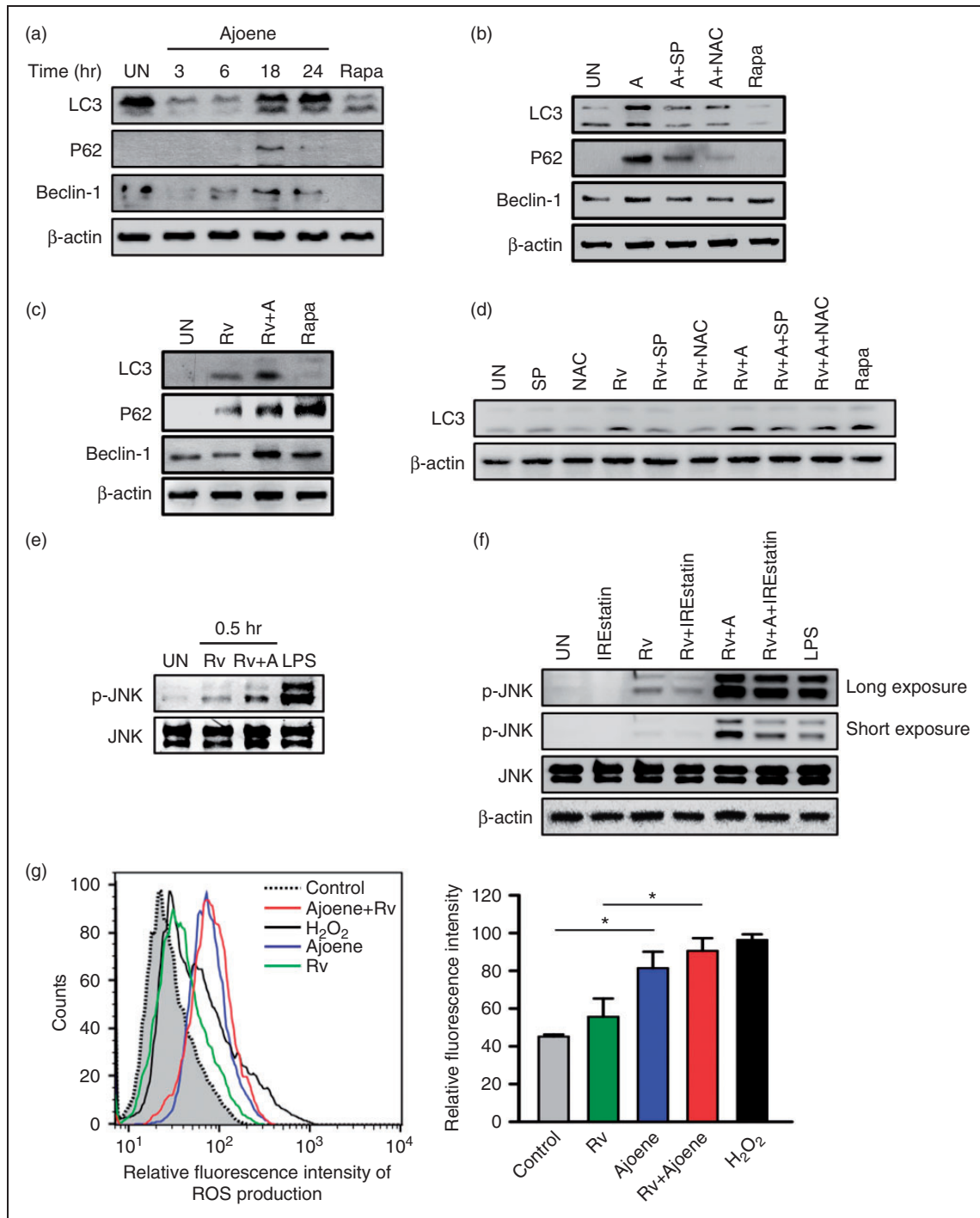
Figure 4. Ajoene-induced autophagy is suppressed by ROS or JNK inhibition. RAW 264.7 cells were stimulated with ajoene (3 μg/ml) under various conditions and the cell lysates were subjected to Western blot analyses with Abs against LC3, P62, Beclin-1, JNK or p-JNK. Rapamycin (Rapa; 400 nM, 4 h) and LPS (1 μg/ml, 1 h) were used as positive controls. The data shown are representative of at least three independent experiments with similar results. (a) RAW 264.7 cells were stimulated with ajoene for the indicated times. (b) RAW 264.7 cells were pre-incubated with SP600125 (20 μM) or NAC (30 mM) for 30 min and then stimulated with ajoene for 18 h. (c) RAW 264.7 cells were stimulated with ajoene for 18 h after infection with H37Rv at a MOI of 1. (d) RAW 264.7 cells were pre-incubated with SP600125 (20 μM) or NAC (30 mM) for 30 min and then stimulated with ajoene for 18 h after infection with H37Rv at a MOI of 1. (e) RAW 264.7 cells were stimulated with ajoene for 30 min after infection with H37Rv at a MOI of 1. (f) RAW 264.7 cells were pre-incubated with IREstatin (5 μM) for 30 min and then stimulated with ajoene for 30 min after infection with H37Rv at a MOI of 1. (g) RAW 264.7 cells were stimulated with ajoene for 30 min after infection with H37Rv at a MOI of 1. Cells were stained with DHE (2 μM) for 30 min at 37 °C and washed with PBS. H_2O_2 (1 mM, 30 min) was used as a positive control for generation of intracellular superoxide. Data are presented as means ± SD of three independent experiments. *$P < 0.05$. UN: unstimulated control; SP: SP600125.

Collectively, our results suggest that the ajoene-activated IRE1α-JNK-ROS pathway is involved in autophagy activation in macrophages during Mtb infection.

Ajoene suppresses intracellular survival of Mtb in murine macrophages

To evaluate the antimycobacterial activity of ajoene, we evaluated the *in vitro* intracellular growth of Mtb in RAW 264.7 cells using a CFU assay. The amount of intracellular viable bacteria was significantly decreased in response to ajoene treatment in a dose-dependent manner (Figure 5). These data indicate that ajoene modulates the intracellular survival of Mtb in macrophages by activating autophagy.

Discussion

TB treatment requires early diagnoses, shorter treatment durations and agents that are effective against drug-resistant strains. The prevention of the spread of MDR-TB is vital worldwide. Therapeutic regimens for TB are effective, but side effects have been reported for primary anti-TB drugs.[3,30] The development of new anti-TB agents for patients with MDR TB has been slow.

Natural compounds, such as polyphenols, alkaloids and saponins, target ER stress, which has been implicated in various diseases.[31] In this study, we showed that ajoene has a significant suppressive effect on mycobacteria infecting macrophages. Furthermore, ajoene stimulation induced the ER stress response in macrophages. Ajoene-mediated ROS production is associated with such stress induction. ROS production and oxidative stress are essential components of ER stress.[32] ROS-mediated ER stress might be important for cancer therapy, because some natural products promote the apoptosis of cancer cells, but not normal cells, through the induction of ER stress.[33,34] Our results indicate that ajoene-induced ROS is important for the induction of ER stress in macrophages. JNK signalling alters mitochondrial physiology, promoting ROS generation.[35] We found that ajoene activates ROS production in a JNK-dependent manner. Therefore, ajoene-mediated JNK activation might be important in inducing ROS production in macrophages, leading to the ER stress response.

Protein oxidation and protein folding are affected by ROS production, which leads to the induction of ER stress.[32] The production of ROS plays a role in host defence against invading pathogens. Although ROS are critical for controlling Mtb,[36] Mtb can survive within macrophages.[37] In several previous studies, we suggested that the stress response of the ER facilitates the removal of intracellular mycobacteria from macrophages.[12–14,38] Here, we showed that ajoene treatment significantly suppressed the intracellular survival of mycobacteria. These results suggest that ajoene-induced ROS modulates the intracellular survival of Mtb by inducing ER stress.

The induction of autophagy was enhanced by ajoene treatment during Mtb infection (Figure 4). Natural compound-induced autophagy or ER stress might be useful for the treatment of various human diseases.[29,31] In our study, the ajoene-activated JNK–ROS pathway was required for the induction of ER stress or autophagy in macrophages in Mtb infection. Because autophagy participates in host defence against Mtb infection,[28,39] our findings provide evidence that ajoene is capable to modulating autophagy, leading to the suppression of mycobacterial growth. Therefore, our results suggest that complex crosstalk between ER stress and ajoene-induced autophagy is important for host defence against mycobacteria. Therefore,
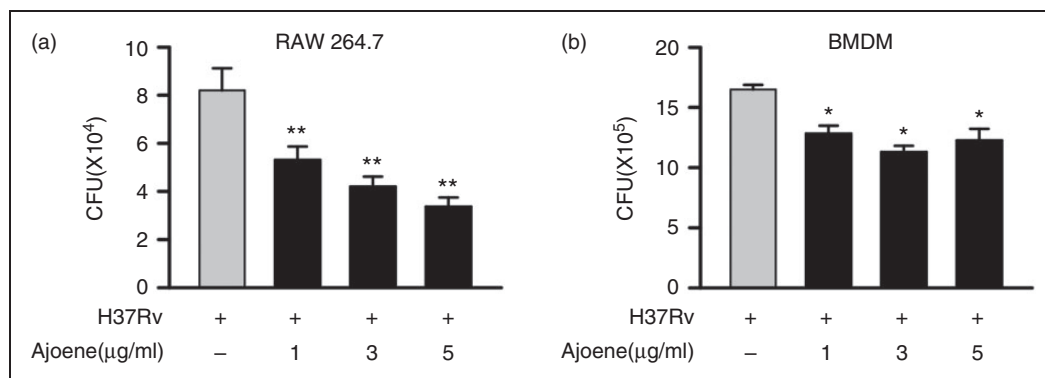
Figure 5. Suppression of intracellular survival of Mtb H37Rv by ajoene. (a) RAW 264.7 cells and (b) BMDMs were stimulated with the indicated concentrations of ajoene (1, 3 or 5 μg/ml) for 24 h after infection with H37Rv at a MOI of 1. The quantification of the intracellular survival of Mtb H37Rv in RAW 264.7 cells and BMDM cells is shown. The cells were collected at 24 h after treatment with ajoene and intracellular bacteria were enumerated using CFU assay. There was a significant difference between each ajoene-treated group and the untreated control (macrophages infected with only H37Rv) (*$P < 0.05$, **$P < 0.01$).

ajoene is not only useful for treating TB, but will also facilitate the development of novel antimycobacterial agents.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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