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Original Paper

Astragaloside-IV Alleviates Heat-Induced Inflammation by Inhibiting Endoplasmic Reticulum Stress and Autophagy

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Key Words
Astragaloside-IV • Endoplasmic reticulum stress • Autophagy • Inflammation • ROS

Abstract
***Background*:** Thermal injury is the main cause of pulmonary disease in stroke after burn and can be life threatening. Heat-induced inflammation is an important factor that triggers a series ofinduces pathological changes. However, this mechanism underlying heat-induced inflammation in thermal inhalation injury remains unclear. Studies have revealed that astragaloside-IV (AS-IV), a natural compound extracted from *Astragalus membranaceus*, has protective effects in inflammatory diseases. Here, we investigated whether the protective effects of AS-IV occur because of the suppression of heat-induced endoplasmic reticulum (ER) stress and excessive autophagy. ***Methods*:** AS-IV was administered to Wistar rats after thermal inhalation injury and 16HBE140-cells were treated with AS-IV. TNF-α, IL-6, and IL-8 levels were determined by ELISA and real-time PCR. ER stress and autophagy were determined by western blot. Autophagic flux was measured by recording the fluorescence emission of the fusion protein mRFP-GFP-LC3 by dynamic live-cell imaging. ***Results*:** AS-IV had protective effects against heat-induced reactive oxygen species production and attenuated ER stress. AS-IV alleviated heat-induced excessive autophagy *in vitro* and *in vivo*. Excessive autophagy was attenuated by the PERK inhibitor GSK2656157 and eIF2α siRNA, suggesting that heat stress-induced autophagy can activate the PERK-eIF2α pathway. Beclin 1 and Atg5 siRNAs inhibited the upregulation of the inflammatory cytokines TNF-α, IL-6, and IL-8 after heat exposure.
***Conclusions*:** Thus, AS-IV may attenuate inflammatory responses by disrupting the crosstalk between autophagy and the PERK-eIF2α pathway and may be an ideal agent for treating inflammatory pulmonary diseases.

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Introduction

Thermal inhalation injury is quite common in fire victims and is the leading cause of mortality in heat-related injuries. Thermal inhalation injury induces an abnormal inflammatory response in the small airways and alveoli, contributing to pneumonia, SARS, and sepsis and resulting in multiple organ dysfunction and even death [1, 2]. The bronchial epithelium acts as the main barrier for protecting the lungs from endogenous and exogenous damage. In addition, they are a main source of various cytokines and chemokines, which can initiate a series of inflammatory responses [3]. Heat stress is a common factor that induces inflammation [4]; however, its underlying mechanism remains obscure. Therefore, clarifying the molecular mechanisms involved in heat-induced alterations in bronchial epithelial cells may offer clues regarding thermal inhalation lung injury pathogenesis and treatment.

Astragalosides IV (AS-IV), a major active compound extracted from the *Astragalus* root, possesses comprehensive pharmacological activities such as anti-inflammatory [5], anti-oxidative [6], and anti-cancer functions [7]. AS-IV also increases T and B lymphocyte proliferation and antibody production and inhibits IL-1 and TNF-α production from peritoneal macrophages. Furthermore, TNF-α- and LPS-induced inflammatory reactions may be inhibited by AS-IV treatment via the NF-kB pathway in endothelial cells [8]. Evidence from pharmacological research and clinical practice suggests that AS-IV possesses effective anti-inflammatory properties and the ability to treat infectious diseases [9, 10].

Reactive oxygen species (ROS) induces cell apoptosis, cell death, and inflammation. The endoplasmic reticulum (ER) is an important organelle in cells that is highly responsive to the cell changes and plays an important role in the folding of newly synthesized proteins [11]. Under hypoxic conditions, the increased ROS production leads to oxidative damage, possibly affecting ER homeostasis and causing ER stress [12]. ER stress elicits the unfolded protein response (UPR) to facilitate repair toward reestablishing normal ER function through the mediation of three ER-resident transducers: activating the pancreatic ER stress kinase (PERK), transcription factor 6 (ATF6), and IRE1 [13, 14]. However, the role of ER stress in heat exposure has not been completely defined. ER stress is involved in regulating various cell processes, including apoptosis and autophagy. A recent study suggested that GRP78 and PERK /eukaryotic initiation factor 2 alpha (eIF2α) pathway are closely involved in activating autophagy.

Under normal conditions, basal autophagy is a key cellular mechanism that provides a protective role for maintaining cell homeostasis. In mammalian cells, autophagy can eliminate damaged proteins and organelles by lysosome degradation and recycle the resulting amino acids, free sugars, and fatty acids for the energy needs of the cell [15]. Under pathological conditions, autophagy is induced in response to stress conditions including cell starvation, hypoxia, oxidant injury, and heat [13]. In these cases, elevated autophagy degrades damaged material to produce metabolic substrates and to meet the bioenergy needs of the cell [16]. However, excessive and impaired autophagy can result in cell damage. Several studies have focused on the functional link between autophagy and inflammation-associated disease; findings suggest a critical role of autophagy in inflammation regulation in diseases caused by acute lung injury (ALI) [17]. Autophagy has been demonstrated to induce inflammation, such as the activation of NF-kB, the polarization of immune cells, and the regulation of cytokine production [18, 19]. Emerging investigations have demonstrated that autophagy can be activated by heat in lung cells, acting as a deleterious process during lung injury [20]. Moreover, some reports have indicated that ER stress is responsible for the subsequent induction of autophagy, with a loss of the functional link between autophagy and the UPR. Recent studies have shown that AS-IV protects cells from injury via the inhibition of ER stress and the production of ROS. In the present study, we hypothesized that the crosstalk between ER stress and various autophagy pathways is one of the primary mechanisms underlying the pathogenesis and progression of inflammation in heat-induced lung inflammation. In particular, we investigated whether AS-IV could attenuate heat-induced inflammatory

responses by affecting the activation of the crosstalk between ER stress and autophagy cellular pathways.

Materials and Methods

Cell culture and treatment

The 16HBE140 cells were cultured at 37°C under a 5% CO_2 humidified-atmosphere in Dulbeccos' modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin–streptomycin. Cells were incubated at 47°C for 5 min to as the *in vitro* heat exposure model. Cultured cells were treated with AS-IV (2.5, 5, or 10 μmol/L) (Sigma-Aldrich, Shanghai, China) 24 h after heat treatment.

Animals and thermal inhalation injury

Adult male Wistar rats were used to induce thermal inhalation injury. All procedures were approved by the Animal Ethical Committee of Shanghai University and Traditional Chinese Medicine, and were conducted according to the approved guidelines. The animals were anesthetized with 2.5% isoflurane in 95% oxygen and 5% CO_2 with a face mask. Animals were placed on controlled heating pads, and the core temperature was maintained at 37°C. Tracheotomy was performed and a 1 cm catheter was gentle inserted into the trachea. We used a heat gun (TAK-3316E, Shenzhen Takgiko Techonology, Shenzhen, China) to produce heated air at 47°C. Air was then blown into the trachea, which was maintained at a flow rate of 190 L/min for a total of 10 min, with 1 min intervals between each minute. After that, we sutured the wounds and intraperitoneally injected Acetylpromazine (0.75 mg/kg body weight) to relieve pain. The tracheotomy was also performed in a sham group. The AS-IV treatment group involved the intragastric administration of AS-IV two times every day (12.5, 25, or 50 mg/kg/day). The model group and the sham group were given a gavage of distilled water of the same volume as the drug.

Determination of ROS

To evaluate ROS generation, the dye 2′,7′-dichlorofluorescein diacetate (DCFDA) (Thermo fisher scientific, Waltham, MA, USA) was used to measure changes in ROS levels. Briefly, cells were cultured in glass bottom dishes and incubated with 5 μmol/L of H_2DCFDA in the dark for 30 min. Cells were then washed three times with pre-warmed phosphate buffered saline (PBS) and resuspended in PBS. The relative levels of fluorescence were determined by confocal microscopy at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Pictures were taken at 10 frames/s using a 4× objective at 510 nm.

Western blot analysis

Cells were lysed in RIPA buffer containing 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris pH 7.5, 0.1% sodium dodecylsulfate, 1 mM PMSF, and a mixture of protease inhibitors. The cell lysates were then centrifuged at 12,000× g for 15 min at 4°C. The protein concentrations of the supernatants were determined using the BCA protein assay (Thermo Fisher Scientific, Shanghai, China). Cell lysates were loaded onto SDS–polyacrylamide gels and transferred to PVDF membranes. Nonspecific binding sites were blocked with 10% fat-free milk for 1 h at room temperature. Membranes were then incubated with primary antibody. Antibodies against GRP78, CHOP, PERK, phosphate PERK, eIF2α, phosphate eIF2α, LC3, P62, IRE1, XBP1s ,ATF6, and Beclin 1 were all obtained from Abcam. Phosphate PERK, eIF2α, and phosphate eIF2α were purchased from Cell Signaling Technology.

mRFP-GFP-LC3 Assay

The 16HBE140 cells was plated on the cover slip of the 6-well plate and added with the mRFP-GFP-LC3 virus (MOI 15) (Hanbio. Co. LTD, Shanghai, China). New media was changed after 12 h. After 48 h of infection, confocal images were obtained at 561 and 488 nm excitation using a 63× oil objective (iXon Ultra 897 CCD). The puncta of each cell was counted and 10 pictures were taken for each sample. The GFP signal is sensitive to the acidic conditions of the lysosome lumen, whereas mRFP is more stable. Therefore, colocalization of both GFP and mRFP fluorescence indicates a compartment that has not fused with a lysosome. In contrast, an mRFP signal without GFP indicates a compartment fused with a lysosome. Thus, autophagy flux was then measured by confocal counting of GFP+/mRFP+(yellow) and GFP-/mRFP+(red) puncta.

siRNA transfection assay

eIF2α siRNA, CHOP siRNA, ATF4 siRNA, Beclin 1 siRNA, and ATG5 siRNA were purchased from Santa Cruz Biotechnology. They were used for transient transfection of 16HBE140 cells cells with Lipofectin 2000 (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA) to suppress the expression of targeted gene. After 48 h of the initial transfection and treatment, cell samples were collected and analyzed using Western blotting and Real-time PCR to confirm the expressions of proteins.

Real-time PCR

cDNA samples were mixed with primers and the SGExcel UltraSYBR Mixture (Thermo Scientific, Fremont, USA). PCR was conducted using the following parameters: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. Quantitative real-time PCR was performed for IL-1β, IL-6, IL-10 ,TGF- β and TNF-α, and normalized to the copies of GAPDH mRNA from the same sample. All PCR assays were performed in triplicate and results are represented as the mean values. The specificity of primers was confirmed by melt curve analysis. The sequences of primers used were as follows:

IL-6 forward primer sequence: ACAACCACGGCCTTCCCTACTT;
IL-6 reverse primer sequence: CACGATTTCCCAGAGAACATGTG;
IL-8 forward primer sequence: ATGACTTCCAAGCTGGCCGTGGCT;
IL-8 reverse primer sequence: TCTCAGCCCTCTTCAAAAACTTCTC;
TNF-α forward primer sequence: CAGAGGGAAGAGTTCCCCAG;
TNF-α reverse primer sequence: CCTTGGTCTGGTAGGAGACG;
IL-10 forward primer sequence: CTTAATGCAGGACTTTAAGGGTTA;
IL-10 reverse primer sequence: ATTCATGGCCTTGTAGACACC;
TGF- β forward primer sequence: AGAGACGTGGGGACTTCTTG;
TGF- β reverse primer sequence: GAATAGGGGCGTCTGAGGAA.

ELISA of IL-10 ,TGF- β ,IL-6, IL-8, and TNF-α Protein Production

IL-6, IL-8, IL-10 ,TGF-β and TNF-α secretion amounts in the culture medium and supernatant homogenated from the lung tissue were analyzed by an Immunoassay Kit (BioSource International, Inc., Camarillo, CA) and used according to manufacturer's instructions. The MPO activities in the lung tissue were assayed by MPO and MDA kits (Jiancheng Bioengineering Institute of Nanjing (Nanjing, China).), following the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed by using one-way ANOVA and t-tests. Data are presented as means ± SD of three independent experiments for each group and were analyzed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). All tests were two tailed. A P value of <0.05 was considered significant.

Results

AS-IV treatment exerted a protective effect by inhibiting the production of ROS and mitigating ER stress

The 16HBE140 cells were incubated at 47°C for 5 min as the heat exposure damage model. To examine whether damage from heat exposure could lead to ER stress and the overproduction of ROS, we assessed the expression of the ER stress molecular chaperones GRP78 and the ER stress-associated apoptosis protein CHOP, which are produced in part by ER stress. The intracellular ROS were labeled with H_2DCFDA fluorescence. As shown in Fig. 1A, 16HBE140 cells were incubated at 47°C and the expression of GRP78 and CHOP reached the highest levels at 12 h; levels returned to normal on day two. The trend of ROS production also peaked at 12 h and decreased on day two in comparison with the control group (Fig. 1B). Furthermore, given that heat exposure lead to the increased expression of GRP78 and CHOP, hence we further investigated all branches of ER stress induced by heat exposure including PERK / eIF2α ,IRE1/XBP1s and ATF6 axis. As shown in Fig. 1C, the expression of phosphorylated PERK and phosphorylated eIF2α reached the highest levels at 12 h; levels returned to normal on day two. However, the expression of IRE1, XBP1s and ATF6 had no

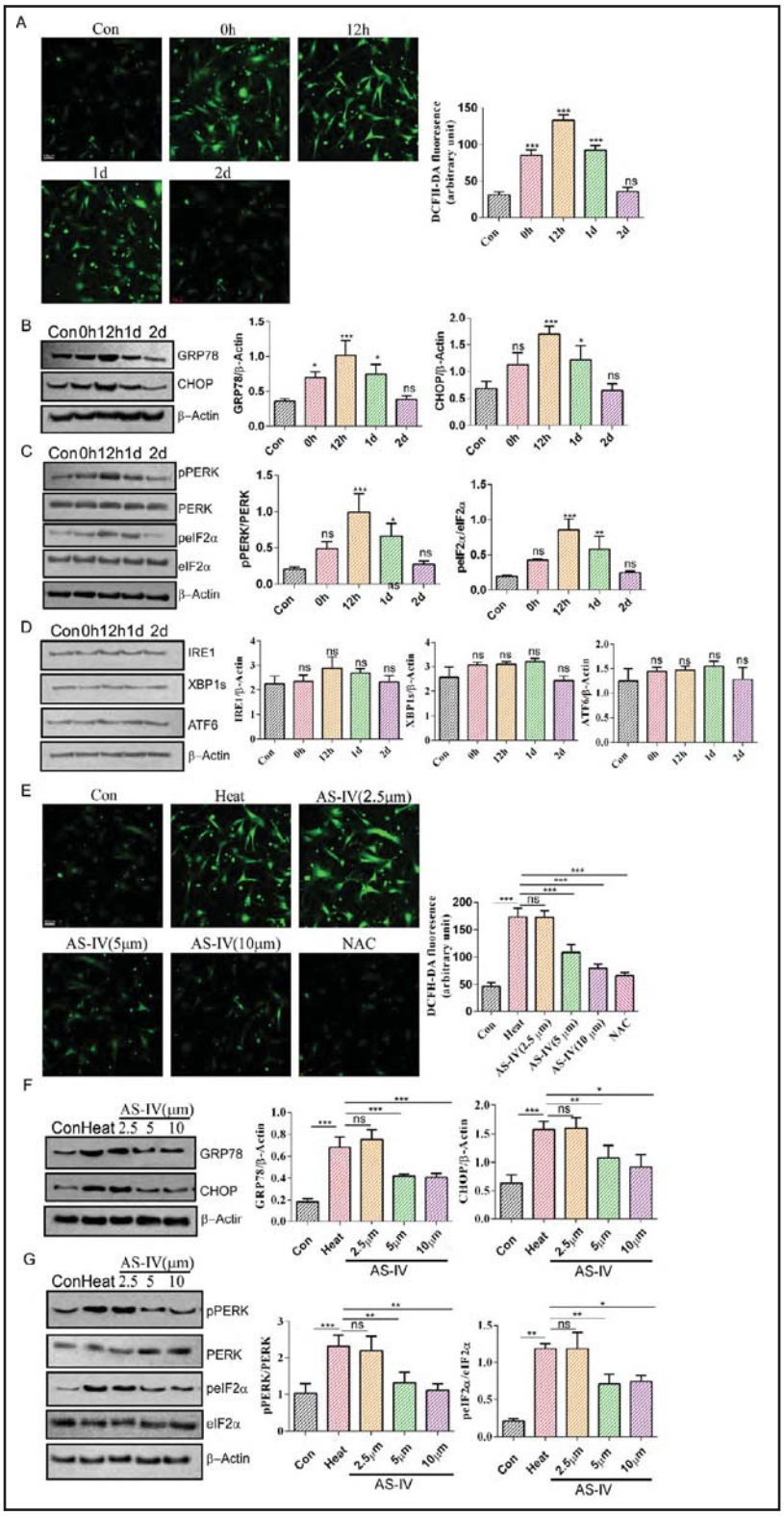

Fig. 1. AS-IV treatment exerted a protective effect by inhibiting the production of ROS and mitigating endoplasmic reticulum (ER) stress. (A) and (E) ROS production was detected by H_2DCFDA. ns,no significant difference ,***P<0.001 vs. Ctrl. n=3. ***P<0.001 vs. heat control. n=3. (B) and (F) Representative Western blot and quantification data of the ER stress markers GRP78 and CHOP. ns,no significant difference ,P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. control group (n ≥ 3).P* < 0.05, **P < 0.01, ***P<0.001 vs. heat control. (C) and (G) Representative Western blot and quantification data of PERK, phosphorylated PERK, eIF2α and phosphorylated eIF2α. P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. control group. P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. heat group (n ≥3).(D) Representative Western blot and quantification data of IRE1,XBP1s and ATF6. P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. control group. P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. heat group (n ≥3).

significant changes in heat exposure damage in comparison with control group(Fig. 1D). To confirm the effect of AS-IV in heat exposure damage, the cells were treated with AS-IV (2.5, 5, and 10 μmol/L). Compared with the heat group, the expression of GRP78 and CHOP was significantly reduced with both the middle and high dose AS-IV treatment, with no

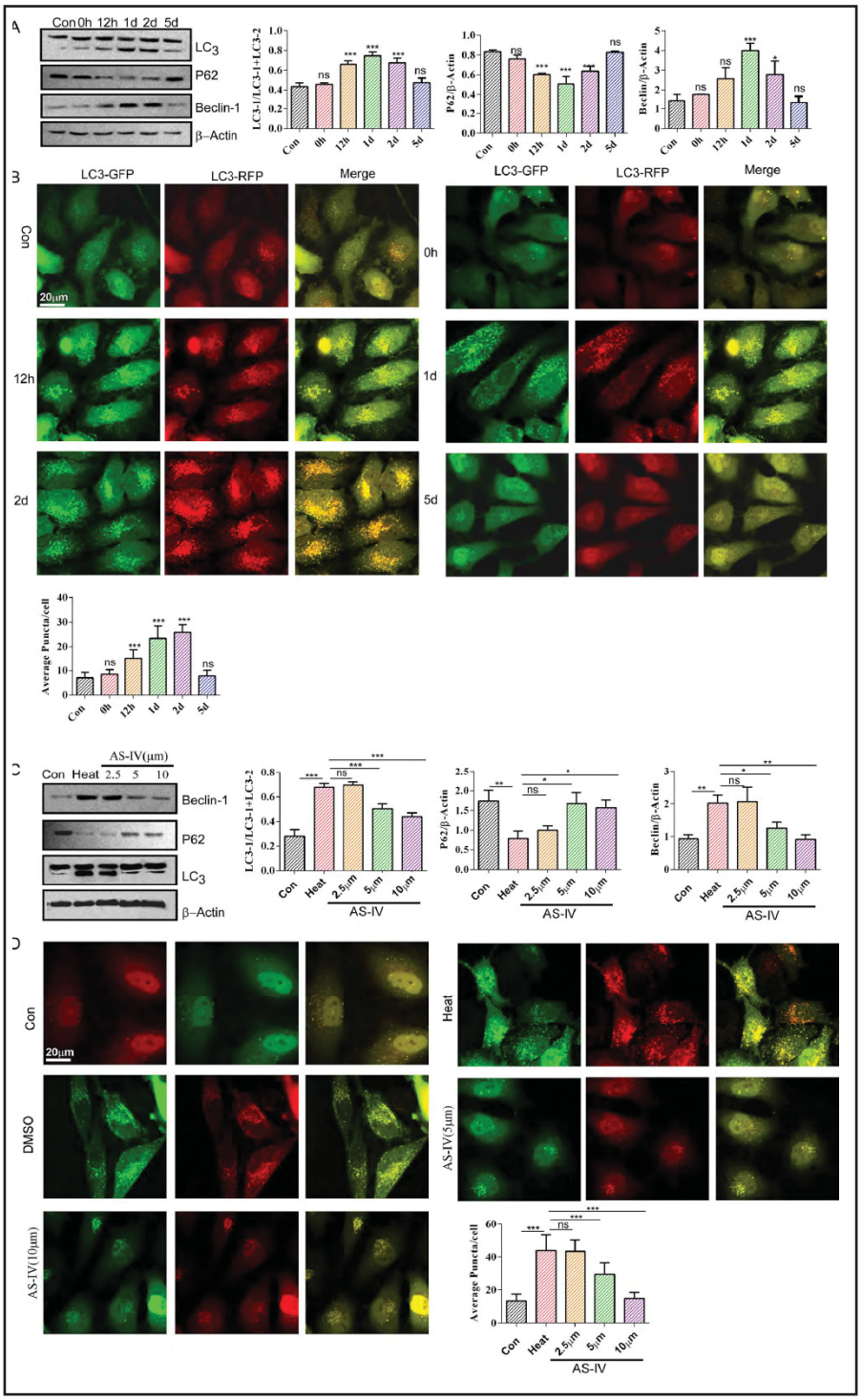

Fig. 2. AS-IV downregulated heat-induced excessive activation of autophagy in 16HBE140 cells. (A) and (C) Representative Western blots and quantification data of the autophagy markers, Beclin 1, P62, and LC3. P* < 0.05, ***P < 0.0001 vs. control group (n ≥ 3). P* < 0.05, **P < 0.01, and ***P < 0.0001 vs.heat group. (B) and (D) Representative confocal fluorescent images of autophagy flux; 16HBE140-cells were infected with Ad-LC3-GFP-RFP, and autolysosome (red) and autophagosome (yellow) puncta were quantified in each group of cells (original magnification 400 x).

changes observed with low dose AS-IV treatment. Decreased production of ROS provided further evidence of the protection of AST in heat-induced oxidative damage(Fig. 1E and F). To confirm that the effect of AS-IV on ER stress involved the PERK/ eIF2α pathway, we determined the expression of PERK and eIF2αInterestingly, AS-IV treatment remarkably downregulated the expression of phosphorylated PERK and phosphorylated eIF2α after heat exposure damage(Fig. 1G), indicating that PERK/eIF2α are closely involved in the protective mechanism of AS-IV in ER stress.

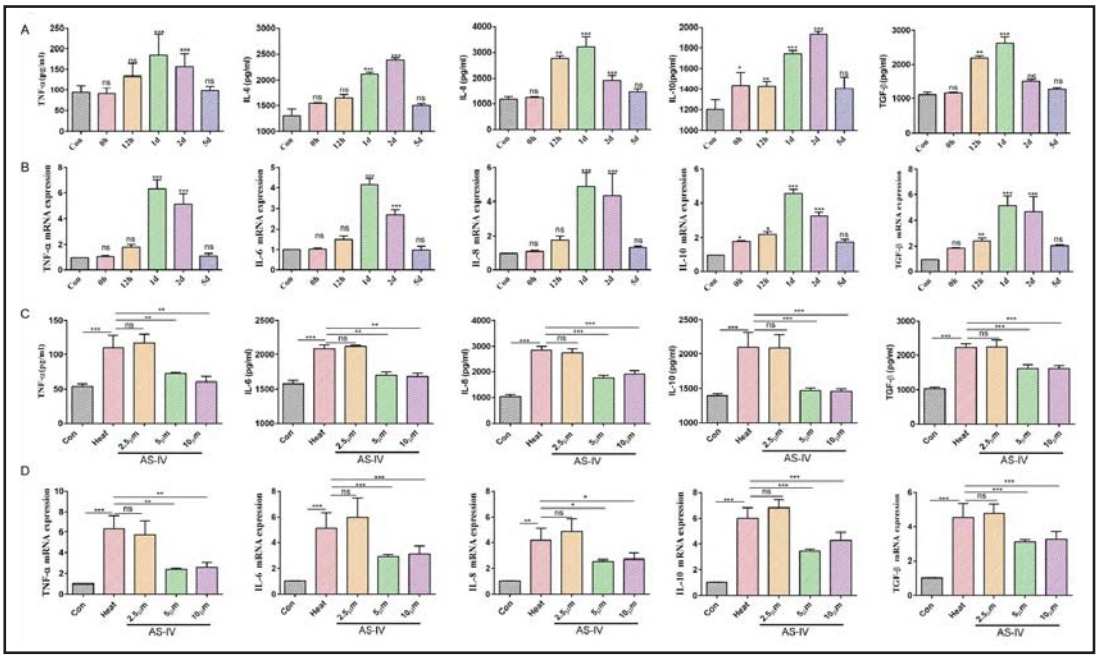

Fig. 3. AS-IV treatment alleviated the heat-induced release of inflammatory factors in 16HBE140 cells. (A) and (C) Real-time PCR of genes coding for TNF-α, IL-6, IL-10, TGF- β and IL-8 on 16HBE140 cells. Relative mRNA levels were normalized to the levels of 18s. Results are the average of three independent experiments, ns,no significant difference, **P < 0.01, and ***P < 0.0001 vs. control group. (B) and (D) TNF-α, IL-6,IL-10, TGF- β and IL-8 production in culture supernatants of 16HBE140 cells were measured by ELISA. Data are means ± SD of three independent experiments. ns,no significant difference, ***P < 0.0001 vs. control group. P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. heat group.

AS-IV downregulated heat-induced excessive activation of autophagy in 16HBE140 cells

Autophagy has been shown to play a crucial role in the progression of heat exposure damage and is closely associated with ER stress; excessive autophagy activation leads to autophagic death [17]. To determine the effects of AST on autophagy in heat exposure damage, we examined the expression of autophagy-associated proteins, including LC3, p62, and Beclin 1. Surprisingly, as shown in Fig. 2A, the expression of LC3 and Beclin 1 were significantly increased at 12 h and peaked on day one, while the expression of p62 was remarkably decreased on day one. However, AS-IV treatment exhibited significant downregulation of the expression of LC3 and Beclin 1, and upregulation of p62 after heat exposure (Fig. 2C). To further demonstrate these results, 16HBE140 cells were infected with Ad-LC3-GFP-RFP, and heat exposure exhibited elevated LC3 puncta and autophagy markers from 12 h to 2 days (Fig. 2B). This effect was remarkably inhibited by AS-IV treatment (5 and 10 μmol/L) (Fig. 2D), suggesting that AS-IV may exert protective effects against heat exposure damage through the inhibition of excessive autophagy.

AS-IV treatment alleviated the heat-induced release of inflammatory factors in 16HBE140 cells

It is accepted that, under stressed conditions, cells release danger signals to notify the immune system of urgent situations. The activation of innate immune receptors leads to inflammatory cytokine production. To determine whether heat exposure could induce inflammation, the expression of TNF-α, IL-6, IL-10 ,TGF- β and IL-8 were detected by ELISA and real-time PCR. As shown in Fig. 3A and B, compared with the heat group, the expression of TNF-α, IL-6, IL-10 ,TGF- β and IL-8 were significantly increased after heat exposure on day one, and returned to normal levels on day five. To determine whether AS-IV can

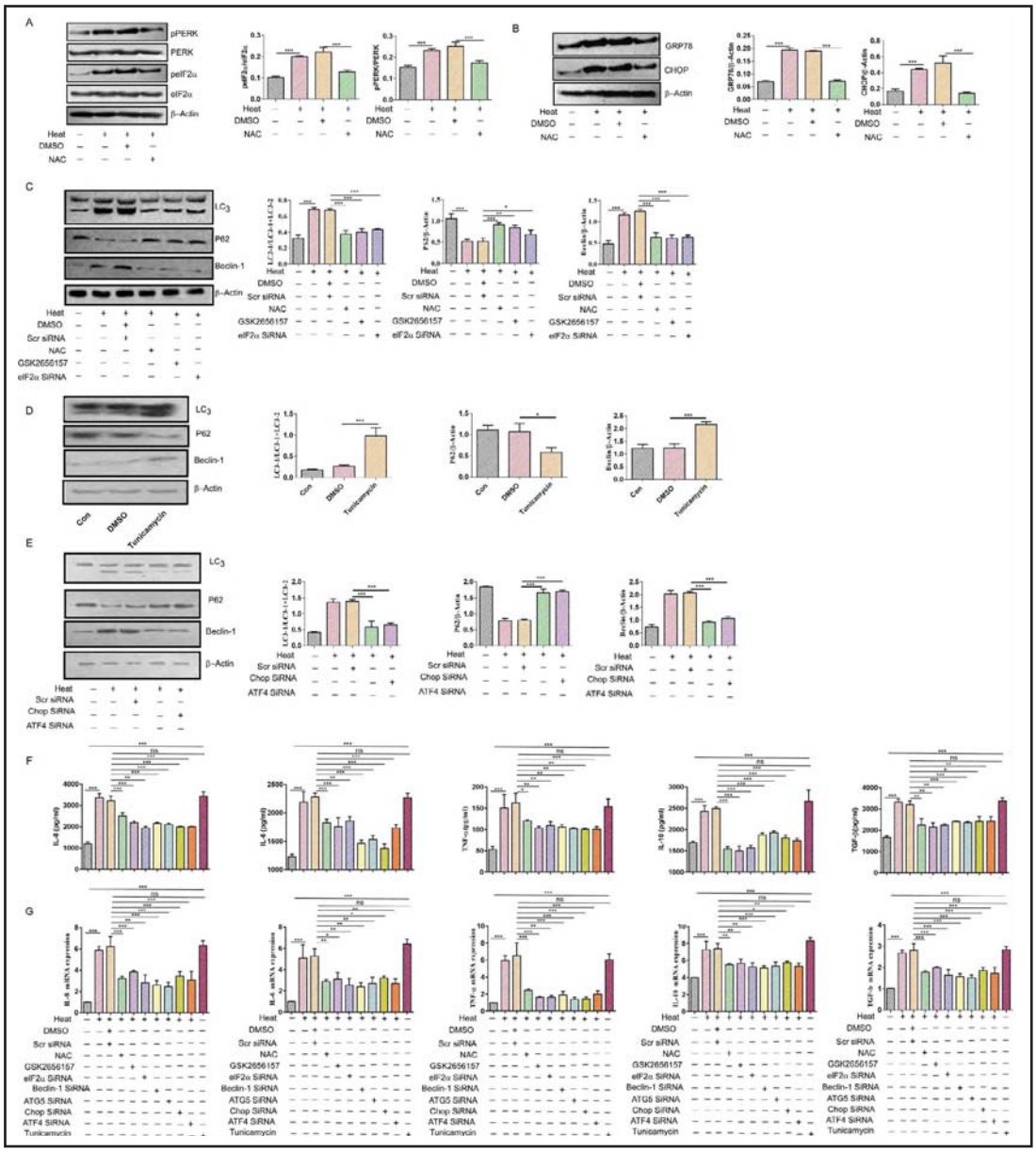

Fig. 4. Crosstalk between ER stress, autophagy, and inflammatory reactions after heat exposure. (A) Representative Western blot and quantification data of PERK, phosphorylated PERK, eIF2α, and phosphorylated eIF2α. ***P < 0.0001 vs. heat control (n ≥ 3). (B) Representative Western blot and quantification data of the ER stress markers GRP78 and CHOP. ***P < 0.0001 vs. heat control (n ≥ 3). (C) -(E) Representative Western blots and quantification data of the autophagy markers, Beclin 1, P62, and LC3. P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. heat control (n ≥ 3). (F) TNF-α, IL-6, IL-10, TGF- β and IL-8 production in culture supernatants of 16HBE140 cells were measured by ELISA. P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. heat group (n ≥ 3). (G) Real-time PCR of genes coding for TNF-α, IL-6, IL-10, TGF- β and IL-8 on 16HBE140 cells. Relative mRNA levels were normalized to the levels of 18s. Results are the average of three independent experiments. P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. heat group.

alleviate heat-induced inflammation, we examined the expression of inflammatory factors. Interestingly, AS-IV treatment also showed clear effective protection against the increased expression of TNF-α, IL-6, IL-10 ,TGF- β and IL-8, as determined by ELISA and real-time PCR (Fig. 3C and D), suggesting that AS-IV has a protective effect against heat exposure-induced inflammatory reactions.

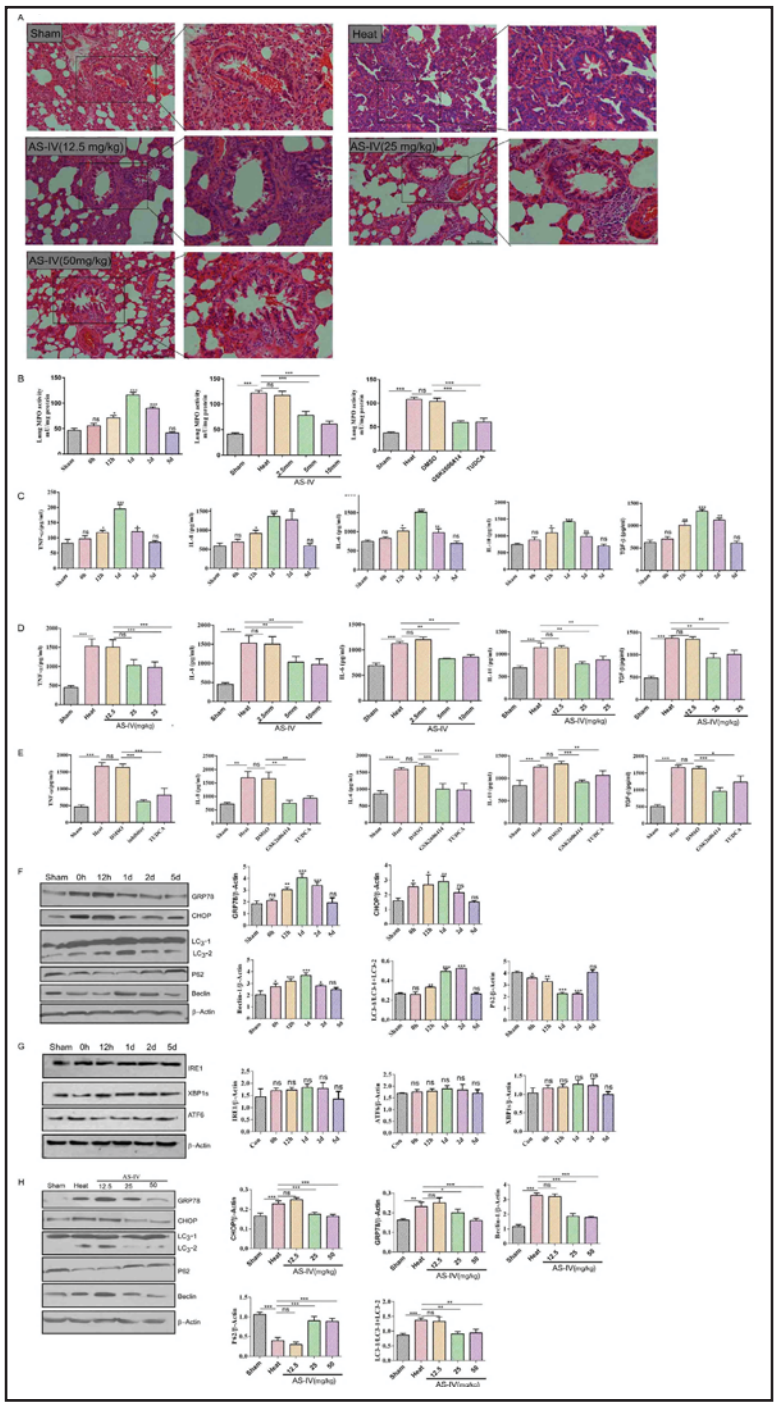

Fig. 5. AS-IV treatment ameliorates heat-induced inflammation through the inhibition of ER stress and by reducing autophagy activation in vivo. (A) Representative images of immunohistochemistry show the airway morphology of rats with heat-inhalation (heat) or without heat-inhalation (sham), and those treated with AS-IV (25 mg/kg and 50 mg/kg) or DMSO (blank control group). (B) Assay of myeloperoxidase (MPO) activity in rat lung tissues. ns,no significant difference , ***P < 0.0001 vs. control group (n ≥ 3). ***P < 0.0001, vs.heat group. (C) TNF-α, IL-6, IL-10, TGF-β and IL-8 production in rat lung tissues were measured by ELISA. ns,no significant difference ,P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. control group (n ≥ 3). **P < 0.01, and ***P < 0.0001 vs. heat control. (D) and (E)Real-time PCR of genes coding for TNF-α, IL-6, IL-10, TGF- β and IL-8 on rat lung tissues. Relative mRNA levels were normalized to the levels of 18s. Results are the average of three independent experiments. ns,no significant difference ,P* < 0.05, **P < 0.01, and ***P < 0.0001 vs.control group. **P < 0.01, and ***P < 0.0001 vs.heat group.(F) and (H) Representative Western blots and quantification data of GRP78, CHOP, Beclin 1, P62, and LC3. ns,no significant difference, P* < 0.05, **P < 0.01, and ***P < 0.0001 vs. control group .**P < 0.01, and ***P < 0.0001 vs.heat group (n ≥ 3).(G) Representative Western blot and quantification data of IRE1,XBP1s and ATF6. ns,no significant difference vs. control group. (n ≥3).

Crosstalk between ER stress, autophagy, and inflammatory reactions after heat exposure

To determine the role of crosstalk between ER stress and autophagy after heat exposure, the ROS scavenger NAC, the ER stress inhibitor GSK2656157, ER stress activator tunicamycin and eIF2α siRNA were administrated to 16HBE140 cells. As shown in Fig. 4A and B, the expression of phosphorylated PERK and eIF2α were significantly decreased in the NAC group as compared with the heat group, while the expression of GRP78 and CHOP were

also downregulated after NAC treatment, suggesting that ROS are involved in regulating ER stress in heat exposure damage. Next, we found that the expression of LC3 and Beclin 1 were further decreased with GSK2656157 treatment and eIF2α siRNA, while increased with tunicamycin treatment compared with the heat group, respectively (Fig. 4C and Fig. 4D). These data indicated that the inhibition of ER stress by GSK2656157 and eIF2α siRNA abolishes excessive autophagy induced by heat exposure. To determine if PERK is inducing autophagy through ATF4 and CHOP, we used CHOP siRNA and ATF4 siRNA to treat the cells, respectively. Interestingly, both of the CHOP siRNA and ATF4 siRNA also inhibited autophagy induced by heat exposure, as determined by the expression of LC3 and Beclin 1(Fig. 4E). In order to detect the role of ER stress and autophagy on inflammation after heat exposure, the ER stress inhibitor GSK2656157 and eIF2α siRNA, and the autophagy inhibitor Beclin 1 siRNA and ATG5 siRNA, were treated to 16HBE140 cells, respectively. Interestingly, as shown in Fig. 4F and 4G, compared with the heat group, the protein levels and transcriptional levels of IL-8, IL-6, IL-10, TGF- β and TNF-α were significantly inhibited in the GSK2656157, eIF2α siRNA, CHOP siRNA, ATF4 siRNA, Beclin 1 siRNA, and ATG5 siRNA groups, while remarkably activated in the tunicamycin group, respectively. Taken together, our results indicated that the inhibition of ER stress not only abolishes the activation of autophagy but also attenuates the release of inflammatory factors after heat exposure.

AS-IV treatment ameliorates heat-induced inflammation by inhibiting ER stress and reducing autophagy activation in vivo

To further confirm the effect of AS-IV on ER stress, autophagy, and inflammation after heat exposure *in vivo*, intragastric administration of AS-IV two times every day (12.5, 25, or 50 mg/kg/day) in heat-injured rats. First, we detected lung injury in each group by Hematoxylin and eosin (HE)staining and myeloperoxidase (MPO) activity (a marker of neutrophil infiltration and an indicator of ALI). As shown in Fig. 5A and B, the inflammatory exudation was obviously increased after heat injury, and MPO activity was significantly enhanced at 12 h, peaked on day one, and returned to normal levels on day five. In addition, the expression levels of TNF-α, IL-8, IL-10, TGF- β and IL-6 after heat injury were consistent with the MPO activity assay results. The activity of MPO and the expressions of TNFα, IL-8, IL-10, TGF- β and IL-6 were significantly decreased after treatment with AS-IV (25 mg/kg and 50 mg/kg) when compared with the heat injury group (Fig. 5C and D). Moreover, we investigated the effect of tauroursodeoxycholic acid (TUDCA), a ER stress inhibitor on heat induced inflammation. Unsurprisingly, TUDCA treatment obviously inhibited the activity of MPO and the expressions of TNFα, IL-8, IL-10, TGF- β and IL-6 after heat exposure(Fig. 5B and E).

Consistent with the results *in vitro*, the expressions of GRP78 and CHOP reached the highest levels at 12 h, and returned to normal levels on day two after heat injury(Fig. 5F). Compared with the heat group, the expressions of GRP78 and CHOP were significantly decreased in the AS-IV (25 mg/kg and 50 mg/kg) group (Fig. 5E and F). We also detected the expression levels of LC3, p62, and Beclin 1 by Western blot. As shown in Fig. 5F and Fig. 5H , the expression of LC3 and Beclin 1 were further increased in the heat injury group. In contrast, decreased expression of P62 was observed in the heat injury group, which was partly inhibited in the AS-IV (25 mg/kg and 50 mg/kg) group. In addition, we found that the expression of IRE1, XBP1s and ATF6 had no significant changes in heat exposure damage compared with sham group *in vivo*(Fig. 5G).These data confirmed that the protective effect of AS-IV on heat-induced inflammation is through the inhibition of ER stress and autophagy activation.

Discussion

In the clinic, heat treatment is beneficial for cancer therapy because heat can kill cancer cells. However, heat stress can also be harmful to normal tissues and cells. In this regard, heat stress is cytotoxic to cells and can induce many cellular changes, including a loss of cellular

homeostasis. While there have been some reports on the effects of long-time hyperthermia in cells [21, 22], additional research is needed in the area of cell damage and cell signaling pathway after heat treatment in short time, which may be useful for the prevention or treatment of .

In present study, we observed increased levels of ROS-dependent oxidants and the accumulation of ER stress, autophagy, and inflammation after heat treatment. To the best of our knowledge, the present study is the first report demonstrating that the crosstalk between ER stress and autophagy is involved in heat-induced inflammation. Our findings illustrate the potential protective effect of AS-IV on heat exposure-induced inflammatory response. The mechanism of action of AS-IV may be through the regulation of crosstalk between ER stress and autophagy. Oxidative stress is a leading cause in the development and genesis of multisystem disorders. The antioxidant capacity of AS-IV has been shown through its beneficial effects on the antioxidant system. The results of the present study indicate that, after AS-IV treatment (2.5, 5, and 10 μmol/L), the levels of ROS significantly decreased, with a more evident decrease following 10 μmol/L AS-IV treatment. AS-IV has been widely studied for its anti-inflammatory function *in vivo*. In the present study, three doses of AS-IV (12.5, 25, and 50 mg/kg body weight) were used. The data demonstrated that AS-IV (25 and 50 mg/kg body weight) had potential protective effects against heat-induced inflammation. However, the 50 mg/kg dose exhibited a marked response against the antioxidant and anti-inflammatory parameters. Furthermore, since a lower dose may induce less potential adverse events, more studies are required to investigate low doses of AS-IV.

Astragalus membranaceus is one of the most widely used Chinese medicinal herbs for the treatment of many diseases, and AS-IV administration has been used for various inflammatory conditions [5, 8, 23-25]. In addition, the therapeutic potential of AS-IV in several pulmonary disease models has recently attracted attention. Consistent with our previous study in thermal inhalation injury, which showed that AS-IV could dampen lung inflammatory responses and oxidative stress, the present study further demonstrated the anti-inflammatory effect of AS-IV and the possible underlying mechanisms in heat-treated 16HB140 cells.

Even the temperature of the air around the fire source in the conflagration can reach to as high as 800°C [26], the temperature of the air in the bronchi airway can be about 48.3°C because over 90% of the heat inhalation is released after passing the upper airway. High temperature(above 43°C) can also cause pathological changes , affecting cell biological function [27]. Accordingly, we implemented our research using 47°C in short time which was consist with the pathological change in inhalation injury after burn. A sensitive increase in temperature (40–46°C) can cause protein unfolding, entanglement, and aggregation, and further induce ER stress [27]. ER stress was induced with increased eIF2α phosphorylation and XBP1 splicing, resulting in the increased expression of GADD34, CHOP and BiP genes [14, 28]. We obtained similar ER stress responses in heat-treated 16HB140 cells. It was accepted that PERK/eIF2α, ATF6, and IRE1α/XBP1 can activate ER chaperone genes to increase the folding capacity of the ER by leading the expression of the X-Box binding protein 1 (XBP1). However, in our models, we didn't find the change of the ATF6 and IRE1α/XBP1 after the heat treatment. According to different kinds of cell types, treatment, animal models, it is possible that different branches of ER stress could be activated. Excessive ER stress may result in apoptosis by activating CHOP and other apoptotic signals. It was reported that hyperthermia will induce ER stress via the phosphorylation of PERK, eIF2α, and IRE1α in cancer cells [29]. Our study has also found that ER stress was significantly induced by heat exposure, and AST attenuated heat-induced ER stress in 16HB140 cells, as shown by reduced expression of GRP78, CHOP, PERK, and eIF2α.

Autophagy is a lysosome-dependent dynamic intracellular process that involves the delivery and degradation of misfolded or long-lived proteins and damaged organelles. It is activated by extracellular or intracellular stress such as starvation, heat, infection, and ER stress. There is some evidence that ER stress-induced autophagy can also keep cells from oxidants, ATP depletion, and IR injury by eliminating damaged organelles and protein

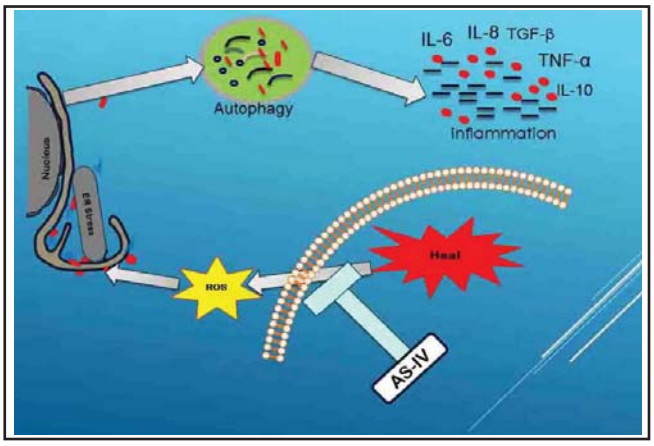

Fig. 6. AS-IV alleviates heat-induced inflammation by inhibiting endoplasmic reticulum stress and autophagy.

aggregates formed during the injury [30, 31]. However, in some conditions, excessive autophagy caused by stress may exceed the cell-bearing capacity and lead to inflammation, apoptosis, and cell death. Although autophagy has recently been demonstrated to play an important role on the regulation of inflammation, the role of autophagy in the process of lung inflammation has been controversial [32-34]. A decrease in the autophagosome has been shown to induce inflammatory responses and the rescue of autophagy may be used as a novel anti-inflammatory target [35]. In avian influenza A-infected lung epithelial cells and PM-induced airway inflammation, autophagy activation is required for the production of proinflammatory cytokines [15]. Whether autophagy is protective or detrimental in the process of lung inflammation seems to be different according to the specific circumstances and cell types. Consistent with the latter studies, our data implies that autophagy is deleterious in heat-induced inflammation, and the inhibition of autophagy resulted in the decreased release of inflammatory cytokines. Thus, targeting autophagy signals may be effective for heat-induced airway inflammation and the treatment of thermal lung diseases. A recent study indicates that the activity of autophagy is in response to ER stress and inflammation in adipocytes. In addition, ER stress inhibitors can inhibit the activation of autophagy and this might aggravate brain ischemia. In this study, we found that the PERK inhibitor and eIF2α siRNA significantly reduced the activation of autophagy in heat-treated 16HB140 cells. Collectively, the data highlight the effect of AS-IV on heat-induced autophagy and indicates that it is primarily dependent on the PERK-eIF2α pathway. Furthermore, we observed that induction of ROS by heat treatment rapidly activated ER stress, while the inhibition of ER stress can inhibit the activation of autophagy, which can attenuate heat-induced inflammation. AST treatment attenuated ER stress and inhibited excessive the activation of autophagy, providing protection against heat-induced inflammation both *in vivo* and i*n vitro*.

This study also has some limitations. The most important is that the experiments *in vitro* used an immortal cell line. However, it is not ethically possible to do an *in vivo* study on humans, and there is a controversial issue regarding whether autophagy detrimental or protective. Our present data provide novel evidence of a positive association between heat stroke upregulated autophagy to severe heat stress injury. Additionally, heat-induced cell injury can be attenuated with autophagy inhibitors. Our present results are, in part, supported by the findings of another study on heat-induced autophagy in several cell lines without nutrient depletion. Heat treatment increased the light chain 3 of the microtubule associated protein 1 (LC3-I) and LC3-II autophagy markers at the protein levels. These findings provide evidence that heat is a potent inducer of autophagy in mammalian cells. Furthermore, there should be more signaling pathway in crosstalk between ER stress, autophagy, and inflammatory reactions after heat exposure which we should focus in the furture. And the immune system was reported to be closely related with the inflammation and involved in the anti-inflammatory effect of AS-IV [36], which was needed the further research in the future.

In conclusion, the current study respectively determined the roles of ER stress and autophagy inheat exposure damage. Heat exposure-induced ROS and ER stress accelerates the excessive activity of autophagy and further leads to inflammation both *in vitro* and *in vivo*(Fig. 6). Thus, AS-IV may attenuate inflammatory responses through the intervention of the crosstalk between autophagy and the ER strees, which may provide a new therapeutic target for heat induced inflammatory pulmonary disease.

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Disclosure Statement

The authors of this article declare no conflict of interests.

References

1 Cheng CH, Yang FF, Liao SA, Miao YT, Ye CX, Wang AL, Tan JW, Chen XY: High temperature induces apoptosis and oxidative stress in pufferfish (takifugu obscurus) blood cells. J Therm Biol 2015;53:172–179.
2 Chang Y, Zhao XZ, Wang C, Ning FG, Zhang GA: Simulation of the velocity and temperature distribution of inhalation thermal injury in a human upper airway model by application of computational fluid dynamics. J Burn Care Res 2015;36:500–508.
3 Chimenti L, Morici G, Paterno A, Bonanno A, Vultaggio M, Bellia V, Bonsignore MR: Environmental conditions, air pollutants, and airway cells in runners: A longitudinal field study. J Sports Sci 2009;27:925–935.
4 Dong ZW, Chen J, Ruan YC, Zhou T, Chen Y, Chen Y, Tsang LL, Chan HC, Peng YZ: Cftr-regulated mapk/nf-kappab signaling in pulmonary inflammation in thermal inhalation injury. Sci Rep 2015;5:15946.
5 Li J, Huang L, Wang S, Yao Y, Zhang Z: Astragaloside iv attenuates inflammatory reaction via activating immune function of regulatory t-cells inhibited by hmgb1 in mice. Pharm Biol 2016;54:3217–3225.
6 Cai Z, Liu J, Bian H, Cai J: Astragaloside iv ameliorates necrotizing enterocolitis by attenuating oxidative stress and suppressing inflammation via the vitamin d3-upregulated protein 1/nf-kappab signaling pathway. Exp Ther Med 2016;12:2702–2708.
7 He CS, Liu YC, Xu ZP, Dai PC, Chen XW, Jin DH: Astragaloside iv enhances cisplatin chemosensitivity in non-small cell lung cancer cells through inhibition of b7-h3. Cell Physiol Biochem 2016;40:1221–1229.
8 Zhang WJ, Frei B: Astragaloside iv inhibits nf- kappa b activation and inflammatory gene expression in lps-treated mice. Mediators Inflamm 2015;2015:274314.
9 Bao KF, Yu X, Wei X, Gui LL, Liu HL, Wang XY, Tao Y, Jiang GR, Hong M: Astragaloside iv ameliorates allergic inflammation by inhibiting key initiating factors in the initial stage of sensitization. Sci Rep 2016;6:38241.
10 Zhang L, Li Z, He W, Xu L, Wang J, Shi J, Sheng M: Effects of astragaloside iv against the tgf-beta1-induced epithelial-to-mesenchymal transition in peritoneal mesothelial cells by promoting smad 7 expression. Cell Physiol Biochem 2015;37:43–54.
11 Li L, Tan H, Yang H, Li F, He X, Gu Z, Zhao M, Su L: Reactive oxygen species mediate heat stress-induced apoptosis via erk dephosphorylation and bcl-2 ubiquitination in human umbilical vein endothelial cells. Oncotarget 2016 DOI: 10.18632/oncotarget.14186
12 Zhang C, Lu T, Wang GD, Ma C, Zhou YF: Costunolide, an active sesquiterpene lactone, induced apoptosis via ros-mediated er stress and jnk pathway in human u2os cells. Biomed Pharmacother 2016;80:253–259.
13 Chandrika BB, Yang C, Ou Y, Feng X, Muhoza D, Holmes AF, Theus S, Deshmukh S, Haun RS, Kaushal GP: Endoplasmic reticulum stress-induced autophagy provides cytoprotection from chemical hypoxia and oxidant injury and ameliorates renal ischemia-reperfusion injury. PloS one 2015;10:e0140025.
14 Yin P, Xu J, He S, Liu F, Yin J, Wan C, Mei C, Yin Y, Xu X, Xia Z: Endoplasmic reticulum stress in heat- and shake-induced injury in the rat small intestine. PloS one 2015;10:e0143922.

15 Chen ZH, Wu YF, Wang PL, Wu YP, Li ZY, Zhao Y, Zhou JS, Zhu C, Cao C, Mao YY, Xu F, Wang BB, Cormier SA, Ying SM, Li W, Shen HH: Autophagy is essential for ultrafine particle-induced inflammation and mucus hyperproduction in airway epithelium. Autophagy 2016;12:297–311.
16 Netea-Maier RT, Plantinga TS, van de Veerdonk FL, Smit JW, Netea MG: Modulation of inflammation by autophagy: Consequences for human disease. Autophagy 2016;12:245–260.
17 Sun Y, Li C, Shu Y, Ju X, Zou Z, Wang H, Rao S, Guo F, Liu H, Nan W, Zhao Y, Yan Y, Tang J, Zhao C, Yang P, Liu K, Wang S, Lu H, Li X, Tan L, Gao R, Song J, Gao X, Tian X, Qin Y, Xu KF, Li D, Jin N, Jiang C: Inhibition of autophagy ameliorates acute lung injury caused by avian influenza a h5n1 infection. Sci Signal 2012;5:ra16.
18 Liu QP, Zhou DX, Lin P, Gao XL, Pan L, Jin FG: Participation of autophagy in acute lung injury induced by seawater. Exp Lung Res 2013;39:441–452.
19 Mayer ML, Blohmke CJ, Falsafi R, Fjell CD, Madera L, Turvey SE, Hancock RE: Rescue of dysfunctional autophagy attenuates hyperinflammatory responses from cystic fibrosis cells. J Immunol 2013;190:1227–1238.
20 Swanlund JM, Kregel KC, Oberley TD: Autophagy following heat stress: The role of aging and protein nitration. Autophagy 2008;4:936–939.
21 Fisher-Hubbard AO, Sung L, Hubbard SA, Hlavaty L: Hyperthermia, thermal injuries, and death from a forced convection heat source: A case report and experimental model. J Forensic Sci 2016 doi: 10.1111/1556-4029.13307.
22 El-Battrawy I, Lang S, Zhao Z, Akin I, Yucel G, Meister S, Patocskai B, Behnes M, Rudic B, Tulumen E, Liebe V, Tiburcy M, Dworacek J, Zimmermann WH, Utikal J, Wieland T, Borggrefe M, Zhou XB: Hyperthermia influences the effects of sodium channel blocking drugs in human-induced pluripotent stem cell-derived cardiomyocytes. PloS one 2016;11:e0166143.
23 Liu R, Jiang H, Tian Y, Zhao W, Wu X: Astragaloside iv protects against polymicrobial sepsis through inhibiting inflammatory response and apoptosis of lymphocytes. J Surg Res 2016;200:315–323.
24 Wang YP, Li XY, Song CQ, Hu ZB: Effect of astragaloside iv on t, b lymphocyte proliferation and peritoneal macrophage function in mice. Acta pharmacologica Sinica 2002;23:263–266.
25 Wang Z, Li Q, Xiang M, Zhang F, Wei D, Wen Z, Zhou Y: Astragaloside alleviates hepatic fibrosis function via par2 signaling pathway in diabetic rats. Cell Physiol Biochem 2017;41:1156–1166.
26 Lv YG, Liu J, Zhang J: Theoretical evaluation of burns to the human respiratory tract due to inhalation of hot gas in the early stage of fires. Burns 2006;32:436–446.
27 Roti Roti JL: Cellular responses to hyperthermia (40-46 degrees c): Cell killing and molecular events. Int J Hyperthermia 2008;24:3–15.
28 Bohnert KR, McMillan JD, Kumar A: Emerging roles of er stress and unfolded protein response pathways in skeletal muscle health and disease. J Cell Physiol 2017 doi: 10.1002/jcp. 25852.
29 Cubillos-Ruiz JR, Mohamed E, Rodriguez PC: Unfolding anti-tumor immunity: Er stress responses sculpt tolerogenic myeloid cells in cancer. J Immunother Cancer 2017;5:5. doi: 10.1186/s40425-016-0203-4.
30 Zhu Q, Wang H, Wang H, Luo Y, Yu Y, Du Q, Fei A, Pan S: Protective effects of ethyl pyruvate on lipopolysaccharideinduced acute lung injury through inhibition of autophagy in neutrophils. Mol Med Rep 2017 doi: 10.3892/mmr.
31 Esteves AR, Arduino DM, Silva DF, Viana SD, Pereira FC, Cardoso SM: Mitochondrial metabolism regulates microtubule acetylome and autophagy trough sirtuin-2: Impact for parkinson's disease. Mol Neurobiol 2017 doi: 10.1007/s12035-017-0420-y.
32 Zhou Y, Wu Y, Liu Y, He Z, Zou S, Wang Q, Li J, Zheng Z, Chen J, Wu F, Gong F, Zhang H, Xu H, Xiao J: The cross-talk between autophagy and endoplasmic reticulum stress in blood-spinal cord barrier disruption after spinal cord injury. Oncotarget 2017;8:1688–1702.
33 Baek A, Yoon S, Kim J, Baek YM, Park H, Lim D, Chung H, Kim DE: Autophagy and krt8/keratin 8 protect degeneration of retinal pigment epithelium under oxidative stress. Autophagy 2017:1–16.
34 Borodkina AV, Shatrova AN, Deryabin PI, Griukova AA, Abushik PA, Antonov SM, Nikolsky NN, Burova EB: Calcium alterations signal either to senescence or to autophagy induction in stem cells upon oxidative stress. Aging 2016;8:3400–3418.
35 Liu JN, Suh DH, Trinh HK, Chwae YJ, Park HS, Shin YS: The role of autophagy in allergic inflammation: A new target for severe asthma. Exp Mol Med 2016;48:e243.
36 Hazeldine J, Hampson P, Lord JM: The diagnostic and prognostic value of systems biology research in major traumatic and thermal injury: A review. Burns & Trauma 2016;4:33.