Original Paper

Astragaloside-IV Protects Against Heat-induced Apoptosis by Inhibiting Excessive Activation of Mitochondrial Ca^{2+} Uniporter

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Key Words

Astragaloside-IV • Heat injury • MCU • Ca^{2+} overload • ROS • Apoptosis

Abstract

Background: Heat causes airway damage during inhalation injury because of bronchial epithelial cell damage. Accumulating evidence shows that mitochondrial uniporter (MCU) is involved in cell damage. We investigated the MCU activity after heat treatment and assessed whether Astragaloside-IV (AS-IV) suppresses heat-induced apoptosis in bronchial epithelial cells by inhibiting the activation of the mitochondrial Ca^{2+} uniporter (MCU), mitochondrial depolarisation and reactive oxygen species (ROS) production. **Methods:** The bronchial epithelial cell line 16HBE14o- was heat treated, and cell apoptosis was induced *in vitro* and *in vivo*. AS-IV was inorganically administered to Wistar rats twice a day after thermal inhalation injury, and 16HBE14o- cells were treated with AS-IV after incubation at 47°C for 5 min. Protein expression was determined using Western blotting and commercial kits, apoptosis with TUNEL staining, mitochondrial channel activity by patch clamp, reactive oxygen species by MitoSOXTM fluorescence, ATP levels and enzyme activities by commercial kits as well as mitochondrial respiration and calcium by fluorescence. **Results:** AS-IV markedly inhibited heat-induced apoptosis, as indicated by the increased expression of the pro-apoptotic genes Bak, Bik and Bmf and increased expression of the apoptosis markers Bax, cleaved parp, cleaved caspase3 and cytochrome C. We found that MCU activation promoted mitochondrial Ca^{2+} overload, ATP depletion, mitochondrial ROS production and cytochrome c release and rapidly induced apoptosis. However, AS-IV treatment reduced excessive MCU activation and led to resistance against mitochondrial Ca^{2+} overload and excessive cytochrome C release; these effects were blocked by the MCU activator spermine. AS-IV treatment elevated ATP production and decreased ROS activity. **Conclusions:** MCU plays crucial roles in heat-induced mitochondrial apoptosis in 16HBE14o- cells, suggesting a potential target for AS-IV treatment.

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Introduction

Damage from heat exposure can result in various pathological changes in multiple tissues and organs, and it primarily affects bronchial epithelial cells and lung cells [1]. The main factors underlying this injury include reduced enzyme activity and changes in cell signalling. Heat exposure is known to cause degradation and mortification of cells, and the functional inhibition of organelles and induction of oxidative stress cause apoptosis [2, 3]. Although the precise mechanisms are not well understood, mitochondrial dysfunction plays a key role in heat-induced apoptosis.

Mitochondria play critical roles in energy production and metabolic functions and are highly susceptible to oxidative stress [4, 5]. Moreover, mitochondrial damage depletes ATP and induces cytochrome c cascades of activated caspases, eventually leading to apoptosis [6]. Hence, the elimination of heat-induced mitochondrial damage is essential for continued cell survival. Studies of molecular mechanisms underlying mitochondrial damage following heat exposure are therefore crucial for understanding the ensuing pathology and for developing novel therapeutic targets.

Mitochondrial Ca^{2+} homeostasis strongly influences mitochondrial functions, including the production of ATP, and exacerbates oxidative stress and apoptosis [7]. MCU is the primary mediator of Ca^{2+} influx into mitochondria and sets the threshold for mitochondrial Ca^{2+} uptake. Accordingly, MCU dysfunction leads to Ca^{2+} overload and disruption of mitochondrial membrane potential and mitochondrial ROS production. Recent studies have shown that MCU silencing protects against toxic cellular reactions and oxidative stress [8, 9]. It is generally accepted that MCU responds acutely to mitochondrial Ca^{2+} levels, with very high uptake rates in the presence of Ca^{2+} levels that exceed the threshold value [10]. It is also well established that MCU has a high capacity for Ca^{2+} uptake into mitochondria and can thus induce mitochondrial Ca^{2+} overload [11, 12]. In addition, unregulated MCU is insensitive to matrix Ca^{2+} concentrations, and the ensuing excessive Ca^{2+} uptake promotes the opening of permeability transition pores (PTP) and a series of detrimental consequences that are associated with disorders of Ca^{2+} homeostasis and ATP depletion [13]. Furthermore, Ca^{2+} is a critical sensitizing molecule that activates pro-apoptotic processes [7]. Accordingly, mitochondrial Ca^{2+} overload induces mitochondrial swelling and promotes the release of apoptotic factors. Increased mitochondrial Ca^{2+} levels have also been associated with increased ROS production and increased mitochondrial cytochrome c release, leading to mitochondrial dysfunction and cell death [14, 15]. However, the detailed mechanisms by which MCU and MCU-mediated Ca^{2+} overload lead to apoptosis following heat exposure remain unclear.

Thermal inhalation injury is a common injury in burn patients which is associated with significant lung damage, resulting into the pulmonary dysfunction and mortality. [16]. The main cause of inhalation injury is hot smoke air inhaled from the superhot environment. Heat is widely considered as a major extracellular stimulus to cause cellular oxidative stress and apoptosis [17]. The temperature of the air from conflagration environment is about 800°C [18]. Surprisingly, the temperature of the air can be dramatically cooled to about 48.3 °C when it is passed into the bronchi airway because nearly 90% of the heat is dissipated in the upper airway [19]. What's more, high temperature above 43°C *in vitro* can also cause pathological changes and cell disfunctions [3]. The temperature 47°C which was consist with the pathological change in thermal injury to elucidate the pathogenesis of heat induced bronchial epithelia apoptosis. Previous studies have suggested that 16HBE14o- cells, a kind of bronchial epithelial cells, are the mainly and early targets of thermal inhalation injury [20].

Astragaloside-IV (AS-IV) is a small molecular saponin that is purified from Astragaloside membranaceous and has multiple beneficial effects, including anti-inflammatory, anti-apoptotic and anti ROS activities *in vitro* and *in vivo* [21, 22]. A recent study suggested that AS-IV also protects against mitochondrial dysfunction, although the related mechanisms have not been characterised [23]. In this study, we investigated the protective effects of AS-IV

against heat-induced mitochondrial apoptosis and demonstrated a mechanism that involves MCU-mediated mitochondrial injury in 16HBE140- cells.

Materials and Methods

Cell culture and treatment

16HBE140- cells were cultured in Dulbecco's modified Eagle medium containing 10% foetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cells were incubated at 47°C for 5 mins and were then harvested one day later. Cultured cells were treated with 20, 40 or 60 µmol/L AS-IV (Sigma-Aldrich, Shanghai, China) from 6 h after heat treatment. Cells were also treated with Ru360 (500 nM), spermine (100 µM) or MitoTEMPO (10 µM).

Animals and thermal inhalation injury

Thermal inhalation injury was induced in adult male Wistar rats. All procedures were approved by the Animal Ethics Committee of Shanghai University and Traditional Chinese Medicine and were performed in accordance with the approved guidelines. The animals were anaesthetised with 2.5% isoflurane in 95% oxygen and 5% CO₂ via a face mask. They were then placed on controlled heating pads, and the core temperature was maintained at 37°C. Tracheotomy was performed, and 1-cm catheters were gently inserted into the trachea. Subsequently, a heat gun (TAK-3316E, Shenzhen Takgiko Technology, Shenzhen, China) was used to produce heated air at 47°C, and the air was blown into mouse tracheae at a flow rate of 190 L/min for a total of 10 min, with 1-min intervals between each minute. Wounds were then sutured, and intraperitoneal injections of acetylpromazine (0.75 mg/kg body weight) were administered to relieve pain. Tracheotomy was also performed in the sham-operated group, and rats of the treatment group were administered AS-IV by gavage two times every day. Control and sham-operated groups were given gavages of distilled water at the same volume as drugs.

Western blot analysis

Whole cells were lysed in RIPA (Radioimmunoprecipitation) buffer containing 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 7.5), 0.1% sodium dodecylsulfate and 1 mM PMSF supplemented with a mixture of protease inhibitors (Beyotime, Shanghai, China) (cocktail 1, 1 mmol/L phenylmethyl sulfonyl fluoride, 20 mmol/L β-glycerophosphate, 40 mmol/L NaF and 3 mmol/L Na₃VO₄). Protein concentrations were measured using BCA protein assays (Beyotime, Shanghai, China), and equal amounts of protein were loaded onto SDS-polyacrylamide gels and then transferred to PVDF membranes (Bio-Rad, Richmond, CA.) Membranes were then blocked with 10% nonfat milk for 1 h at room temperature and incubated with primary antibody overnight at 4°C.

TUNEL staining

Apoptosis was determined in rat bronchial epithelial cells using TUNEL staining with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. TUNEL-positive cells were observed using a fluorescence microscope (ZEISS, Jena, Germany) and were quantified by randomly counting in 30 areas from three independent experiments.

IMCU recording

Mitoplast patch-clamp recordings were performed at 30°C, as described previously [24, 25], and currents were recorded using an Axon200B patch-clamp amplifier with a Digidata 1320A acquisition board (pCLAMP 10.0 software; Axon Instruments, CA, USA). In brief, freshly prepared mitoplasts were isolated using the protocol of the mitochondrial isolation kit, placed on Cell-Tak-coated coverslips and mounted on a microscope. Mitoplasts were bathed in a standard solution containing sodium gluconate (150 mM), KCl (5.4 mM) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 10 mM; pH 7.2). After the formation of GΩ seals (20 to 35 MΩ), mitoplasts were ruptured with 2–6-ms pulses of 200 mV, and mitoplast capacitance was measured (2.5 to 3.8 pF). External and bath solutions (5 mM Ca²⁺) were chosen on the basis of previous measurements. After capacitance compensation, mitoplasts were held at 0 mV, and IMCU was elicited with a voltage ramp from –160 to 80 mV at 120 mV/s.

Determination of mitochondrial ROS

Mitochondrial ROS levels were determined using the MitoSOXTM Red mitochondrial superoxide indicator (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. In brief, the cells were collected and incubated with 5 $\mu\text{mol/L}$ MitoSOXTM reagent working solution in the dark for 10 min. Fluorescence intensities were then determined using confocal microscopy (ZEISS, Jena, Germany).

Cellular ATP Measurement

ATP levels in 16HBE140- cells were determined using the colorimetric ATP Assay Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Data were normalised to protein levels.

Measurements of mitochondrial respiration

Mitochondrial oxygen consumption was measured using microplates (type XF 24) and an extracellular analyser (Seahorse Bioscience) (Agilent Technologies, Santa Clara, CA). In brief, 16HBE140- cells were cultured for 12 h and were then washed in XF base medium. XF base medium was then removed and replaced with assay medium, which was pre-equilibrated at 37°C without CO₂ for 1 h. Oxygen consumption rates (OCR) were measured under basal conditions and after sequential injections of oligomycin (0.5 μM), FCCP (1 μM) and finally rotenone and antimycin A (1 μM), and basal and maximal respiration rates were calculated.

Real-time fluorescence analysis and confocal microscopy

To determine mitochondrial Ca²⁺ levels, 16HBE140- cells were plated on six-well plates and were incubated with 2.5 μM Rhod-2 AM (Thermo Fisher Scientific, Waltham, MA, USA) for 40 min. Changes in opening states of mitochondrial permeability transition (MPT) pores were determined using Calcein. In these experiments, cells were stained with calcein and cocl2 (Sigma-Aldrich, St. Louis, MI, USA) for 20 min at room temperature. The mitochondrial membrane potential was then measured using the TMRE Mitochondrial Membrane Potential Assay Kit (Molecular Probes, Eugene, OR, USA), according to the manufacturer's protocol. Fluorescence images were obtained using a Zeiss LSM510-META laser scanning confocal microscope (ZEISS, Jena, Germany).

Mitochondrial Ca²⁺ dynamics

16HBE140- cells were placed on 25 mm glass coverslips for 24 h and stained with 2 μM rhod-2/AM (50 min). After 20 min, the stained cells were used for confocal analysis. After 1 min of baseline recording, an agonist (histamine, 100 μM ; Sigma-Aldrich) was added, and confocal images were recorded every 3 s (510 Meta; ZEISS, Jena, Germany) at 488- and 561-nm excitation using a 40 $\times$ oil objective to simultaneously monitor cytoplasmic and mitochondrial Ca²⁺ dynamics. The images were analysed and quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Estimation of antioxidant enzyme activities

Activities of superoxide dismutase (SOD) and catalase (CAT) were photometrically determined using kits, according to the manufacturer's instructions (Cayman Chemicals Company, Ann Arbor, MI, USA). In brief, SOD activities in samples or standards (10 μL) were assayed in 96-well plates. Xanthine oxidase (20 μL) was added to each well to initiate the reaction, and absorbance was determined at 450 nm using a microplate reader after 30-min incubation at RT. To determine CAT activities, samples or standards (20 μL) were added to assay buffer (100 μL) and methanol (30 μL) in 96-well plates, and H₂O₂ (20 μL) was then added to initiate the reaction. After 20 min at room temperature, potassium hydroxide (20 μL), catalase purpald (20 μL) and catalase potassium periodate (10 μL) (Sigma-Aldrich St. Louis, MI, USA) were added to terminate the reaction, and absorbance was determined at 540 nm using a microplate reader. SOD activity was expressed as units/mg protein, and CAT activity was expressed as nmol/min/mg protein.

Ca²⁺ uptake in the cell permeability system

After collection and washing in phosphate-buffered saline without Ca²⁺, 4 $\times$ 10⁶ cells were permeabilised using 40 $\mu\text{g/ml}$ digitonin in 1.5 mL of intracellular medium (ICM) containing 120 mM KCl, 10 mM NaCl, 1 mM KH₂PO₄, 20 mM HEPES-Tris (pH 7.2), 2 μM thapsigargin (Sigma-Aldrich St. Louis, MI,

USA), 5 mM succinate (Sigma-Aldrich St. Louis, MI, USA) and 0.5 μ M Fura-FF, as described previously (Life Technologies, Grand Island, NY). Thapsigargin, succinate and Fura-2FF were used to block the SERCA pump, energise mitochondria and mark the cytosolic Ca^{2+} separately. After 20 s of data recording, Ca^{2+} (20 μ M) was added at 500 s and CCCP (2 mM) (Sigma-Aldrich St. Louis, MI, USA) was added at 800 s. To determine total mitochondrial Ca^{2+} concentrations, The cells from treatment groups were permeabilised with 40 μ g/mL digitonin (Sigma-Aldrich St. Louis, MI, USA) in 1.5 mL of ICM with SERCA blocker, thapsigargin (2 μ M), Ru360 (1.5 μ M; Calbiochem, San Diego, CA, USA) and CGP37157 (10 μ M)(Sigma-Aldrich St. Louis, MI, USA)). At 500 s, carbonyl cyanide m-chlorophenylhydrazone (CCCP)(2 mM)(Sigma-Aldrich, St. Louis, MI, USA) was added to depolarise mitochondrial membranes and trigger complete release of mitochondrial Ca^{2+} . Fluorescence was measured using a multiwavelength excitation dual-wavelength emission fluorimeter (DeltaRAM, Photon Technology International, Edison, NJ, USA).

Statistical analysis

Statistical analyses were performed using one-way ANOVA and t-tests. Data are presented as means $\pm$ standard deviations (s.d.s) of three independent experiments for each group and were analysed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). All tests were two-tailed, and differences were considered significant at $P < 0.05$.

Results

AS-IV treatment protects against apoptosis following heat exposure in vitro and in vivo

Consistent with the findings of a previous study [1], *in vitro* heat exposure was induced by incubation of 16HBE140- cells at 47°C for 5 mins. In these experiments, remarkable increased expression of apoptosis-related proteins and increased number of TUNEL-positive cells were observed with obvious cell death after exposure to 47°C, as detected in western blotting analyses (Fig. 1A) and TUNEL staining assays (Fig. 1B). To investigate the potential protective effects of AS-IV against damage from heat exposure, 16HBE140- cells were treated with AS-IV at 20, 40 or 60 μ mol/L. As shown in Fig. 1A, the expression of the apoptosis-related proteins Bax, cleaved PARP and cleaved caspase3 and the pro-apoptotic proteins Bak, Bik and Bmf was significantly decreased in cells treated with the highest dose of AS-IV, and the ratio of Bcl2/Bax was greater than that in the heat control ($P < 0.05$). Moreover, compared with the heat control, AS-IV (60 μ mol/L) treatment significantly decreased numbers of TUNEL-positive cells in TUNEL staining assays after incubation at 47°C (Fig. 1B).

In vivo experiments, heated air (47°C) was blown into the tracheae of rats to induce thermal inhalation injury. Subsequently, AS-IV was administered to rats by gavage at 5, 10 or 20 mg/kg body weight/day each day. As shown in Fig. 1C, compared with the sham-operated group, expression levels of Bax, cleaved PARP, cleaved caspase3, Bak, Bik and Bmf were significantly increased following heat inhalation and numbers of TUNEL-positive cells were also significantly increased (Fig. 1D). Moreover, treatments with AS-IV at high doses decreased the expression of apoptotic-related proteins in western blotting experiments and decreased the numbers of TUNEL-positive cells in TUNEL staining assays. Taken together, these results suggest that AS-IV prevents heat-induced apoptosis and markedly alleviates heat-induced damage *in vitro* and *in vivo*.

AS-IV treatment inhibits MCU activation and mitochondrial shape changes following heat exposure damage

To determine the effects of heat exposure on mitochondrial function *in vitro*, we monitored the expression of MCU and various respiratory chain enzymes in 16HBE140- cells. Western blotting analyses showed significant increases in the expression of the respiratory enzymes succinyl dehydrogenase A (SDHA) and cytochrome c oxidase (COX) IV following heat exposure (Fig. 2A). Conversely, treatment with AS-IV at 60 μ mol/L significantly decreased SDHA and COX IV expression, whereas no significant changes were observed after AS-IV

Fig. 1. AS-IV treatment protects against apoptosis in *in vitro* and *in vivo* heat exposure models. 16HBE140-cells were cultured to 70%–85% confluence at 37°C and were then heat treated at 47°C for 5 mins, followed by recovery at 37°C. (A) Western blotting analyse and corresponding quantification of the apoptosis related proteins Bcl2, Bax, cleaved PARP and cleaved caspase3 and the pro-apoptotic proteins Bak, Bik and Bmf *in vitro*; Values are expressed as means $\pm$ standard deviations (s.d.) from at least three independent experiments; ns, no significant; ***P < 0.001 vs.heat control; n = 3. (B) 16HBE140-cells were incubated at 47°C and were then treated with AS-IV at 20, 40, or 60 μ mol/L; Representative images and corresponding TUNEL staining determinations. (C) Western blots are presented with corresponding *in vivo* quantifications of the apoptosis related proteins Bcl2, Bax, cleaved PARP and cleaved caspase3 and the pro-apoptotic proteins Bak, Bik and Bmf. (D) Representative images and corresponding TUNEL staining determinations. Values are expressed as means $\pm$ s.d. from at least three independent experiments; ns, no significant; ***P < 0.001 vs.heat control.n = 3.

treatments at 40 or 20 μ mol/L; MCU protein expression did not differ in the with treatment and without treatment groups (Fig. 2A). We also measured expression levels of mitochondrial shape-related proteins such as the optic atrophy 1 (OPA1) and metal response element binding transcription factor 2 (Mtf2) using the reported outer mitochondrial membrane (OMM) marker translocase of outer membrane 20 (TOM20). The results revealed significant increases in OPA1 and Mtf2 expression levels following heat injury. In contrast, treatment

Fig. 2. AS-IV treatment inhibits MCU activation following heat exposure damage. (A) Western blotting analysis of MCU, SDHA and COX IV respiratory chain enzyme expression *in vitro*; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$ ($n = 4$). (B) Western blotting analysis of OPA1 and Mtf2 expression *in vivo*; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$. (C) Confocal images of mitochondrial morphologies under various experimental conditions. (D) Mitoplast currents (IMCU) from 16HBE140- cells were recorded before and after the application of 5 mM Ca^{2+} to the bath medium. Currents were measured during a voltage ramp, as indicated. Traces are representative single recordings of IMCU; $n = 6$. (E) Western blotting analysis of MCU, SDHA and COX IV respiratory chain enzyme expression *in vivo*; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$ ($n = 3$). (F) Western blotting analysis of OPA1 and Mtf2 expression *in vivo*; * $P < 0.05$ ($n = 3$).

with 60 $\mu\text{mol/L}$ AS-IV significantly downregulated OPA1 protein expression, and Mtf2 was simultaneously downregulated (Fig. 2B). Subsequent examinations of the mitochondrial morphology (Fig. 2C) indicated higher percentages of mitochondrial fragmentation in heat-

Fig. 3. AS-IV alleviates mitochondrial Ca^{2+} overload by inhibiting excessive MCU activation in heat-injured 16HBE140- cells. (A) Calcium uptake into isolated myocardial cells was determined using the fluorescent Ca^{2+} sensor Rhod-2 AM and confocal microscopy. (B) The capacity for calcium uptake in 16HBE140- cells was determined from mitochondrial Ca^{2+} (Rhod-2 AM) dynamics after stimulation with histamine (100 μM). Data were assayed using confocal microscopy. (C) 16HBE140- cells were permeabilised with 40- $\mu\text{g}/\text{mL}$ digitonin in 1.5 mL of intracellular medium containing thapsigargin, Ru360 and CGP37157. Accumulation of Ca^{2+} in basal mitochondrial matrixes was determined using a multiwavelength excitation dual-wavelength emission fluorimeter (PTI). (D) 16HBE140- cells were pulsed with 10 μM Ca^{2+} , and mitochondrial Ca^{2+} uptake rate was measured using PTI; ** $P < 0.01$ and *** $P < 0.0001$ ($n = 3$). Data are presented as means $\pm$ standard deviations.

treated groups than in controls, and these increases were accompanied by visible changes in the mitochondrial morphology. Accordingly, mitochondrial fragmentation was lower in the high-dose AS-IV-treated group than in the heat-treated group (Fig. 4C). Thus, to further determine whether the effects of AS-IV following deleterious heat exposure reflected the regulation of MCU currents, mitochondria were isolated from 16HBE140- cells and were subjected to IMCU current recordings. In patch-clamp experiments with whole mitochondrial configuration, the addition of 5 mM Ca^{2+} to the bath triggered an inwardly rectifying Ca^{2+} current. Moreover, remarkable increases in IMCU currents following heat exposure were reduced by AS-IV treatments at 60 $\mu\text{mol}/\text{L}$ (Fig. 2D).

In subsequent *in vivo* experiments, SDHA and COX IV expression were significantly decreased following AS-IV treatment at the high dose, and no significant changes were observed at lower doses in comparison with sham operated animals (Fig. 2E). We also measured expression levels of mitochondrial shape related proteins such as the mitochondrial fusion proteins OPA1 and Mtf2 *in vivo*. These experiments revealed significant increases in OPA1 and Mtf2 expression levels following heat inhalation. However, treatment with 20mg/Kg AS-IV significantly downregulated OPA1 protein expression, and Mtf2 was simultaneously downregulated (Fig. 2F). Collectively, these results provide evidence that AS-IV efficiently inhibits excessive activation of the MCU and overexpression of respiratory chain enzymes and alters gene expression to counteract changes in mitochondrial shapes.

AS-IV alleviates mitochondrial Ca^{2+} overload by inhibiting excessive MCU activation following heat injury in 16HBE140- cells

Ca^{2+} overload is a key feature of cell apoptosis and death. Thus, to determine whether heat exposure causes mitochondrial Ca^{2+} overload in 16HBE140- cells, we loaded cells with

Fig. 4. AS-IV alleviates ATP depletion and oxygen consumption following heat injury in 16HBE140- cells. (A) ATP levels are expressed relative to the control and as ADP/ATP ratios for each treatment group ($n = 4$); $*P < 0.05$, $**P < 0.01$ and $***P < 0.0001$ ($n = 3$). (B) Mitochondrial function was assessed in terms of basal and maximal cellular oxygen consumption rates (OCRs), and representative time course data for OCR and aggregated data are shown.

Fig. 5. AS-IV inhibits heat-induced mitochondrial ROS generation in 16HBE140- cells. Mitochondrial ROS production was detected using MitoSOXTM (A). 4-hydroxynonenal (4-HNE) and nitrotyrosine (NT) test (B); $P < 0.05$, $**P < 0.01$ and $***P < 0.0001$ ($n = 5$). (C) Superoxide dismutase (SOD) and catalase (CAT) activities; $**P < 0.01$ and $***P < 0.0001$ ($n = 3$). Data are presented as means $\pm$ standard deviations.

the mitochondrial Ca^{2+} indicator Rhod-2 AM+ and monitored the resulting fluorescence following heat and AS-IV treatments. Compared with the control group, Rhod-2 fluorescence intensities were enhanced after heat exposure and concomitant AS-IV treatment moderated these increases. Thus, to investigate whether the effects of AS-IV on mitochondrial Ca^{2+} levels are mediated by MCU following heat injury, 16HBE140- cells were treated with the MCU activator spermine. In these experiments, the effects of AS-IV on mitochondrial Ca^{2+} overload were completely blocked by spermine (100 μM) treatment, and no significant AS-IV-mediated changes were observed in the presence of the alternative MCU inhibitor Ru360.

Fig. 6. AS-IV inhibits MPT and exerts protective effects against mitochondria-mediated apoptosis. (A) Calcein staining of open mitochondrial permeability transition pores was performed as described in the Methods; **P < 0.01 and ***P < 0.0001 (n = 3); Scale bars, 10 μ m. (B) Tetramethylrhodamine ethyl ester (TMRE) staining was performed to detect mitochondrial inner membrane permeability in 16HBE140-cells; **P < 0.01 and ***P < 0.0001 (n = 3). Data are presented as means $\pm$ standard deviations; Scale bars, 10 μ m. (C) Western blotting analyses of cytochrome c, AIF, procaspase-9 and Endo G expression; *P < 0.05, **P < 0.01 and ***P < 0.0001 (n = 3). (D) Western blotting and corresponding quantification of apoptosis-related proteins Bcl2, Bax, cleaved parp and cleaved caspase3 and pro-apoptotic proteins Bak, Bik and Bmf; *P < 0.05, **P < 0.01 and ***P < 0.0001 (n = 3).

These data show that AS-IV alleviates heat-induced mitochondrial Ca^{2+} overload by inhibiting MCU (Fig. 3A). We also measured the maximal mitochondrial Ca^{2+} dynamic change after exposure to 10 μ M histamine (a G-protein-coupled receptor agonist). Compared with the control group, we observed a significant increase in the amplitude of mitochondrial Ca^{2+} after heat exposure. In contrast, AS-IV treatment inhibited the enhancement in mitochondrial Ca^{2+} response to histamine in 16HBE140-cells (Fig. 3B). To confirm these results, we investigated the release of total mitochondrial Ca^{2+} using CCCP to depolarise mitochondrial membranes and trigger mitochondrial Ca^{2+} release (Fig. 3C). In agreement with the findings of previous experiments, we observed increased Ca^{2+} accumulation in the basal mitochondrial matrix after heat exposure, and this was significantly attenuated by AS-IV treatment but only in the absence of spermine. In further experiments, we pulsed 16HBE140-cells with 10 μ M Ca^{2+} and determined mitochondrial Ca^{2+} uptake. As shown in Fig. 3D, heat exposure enhanced Ca^{2+} uptake and AS-IV treatment significantly reduced heat-induced Ca^{2+} uptake. Taken together, these results suggest that AS-IV inhibits mitochondrial Ca^{2+} overload after heat exposure injury and confirm that MCU is a potential target for AS-IV treatment.

AS-IV alleviates ATP depletion and oxygen consumption in heat-injured 16HBE140- cells

Previous reports indicate that mitochondrial Ca^{2+} overload leads to ATP depletion and high oxygen consumption in multiple cell types [12]. Accordingly, we determined whether heat-induced Ca^{2+} overload leads to ATP depletion in 16HBE140- cells. As shown in Fig. 4A, ATP levels were significantly reduced and ADP/ATP ratios were significantly increased after heat exposure. However, the reduction of ATP was significantly attenuated by AS-IV and was prevented by Ru360 treatment. To determine the effects of AS-IV on mitochondrial respiration following heat injury, we determined basal and maximal mitochondrial respiration rates and showed that decreased basal and maximal respiration rates following heat exposure were partially reversed by AS-IV and Ru360 treatments but were unchanged in the presence of spermine and AS-IV (Fig. 4B). Collectively, these data demonstrate that AS-IV prevents mitochondrial ATP depletion after heat exposure by inhibiting MCU.

AS-IV inhibits heat-induced mitochondrial ROS generation in 16HBE140 cells

It is accepted that mitochondrial dysfunction is associated with elevated mitochondria-derived ROS, which subsequently promote apoptosis. Thus, to determine whether AS-IV affects mitochondrial ROS production following heat exposure in 16HBE140- cells, we measured 4-hydroxynonenal and nitrotyrosine contents, which are oxidative modifications of lipids and proteins, respectively, and performed experiments using the specific mitochondrial ROS probe MitoSOXTM. The ensuing data showed significantly increased 4-hydroxynonenal, nitrotyrosine and mitochondrial ROS generation following heat exposure, and AS-IV or Ru360 treatments attenuated ROS levels under these conditions (Fig. 5A and B). Conversely, compared with the control group, intracellular ROS levels were markedly increased in the spermine-treated group, suggesting predominant involvement of MCU in mitochondrial ROS generation. Numerous previous studies have confirmed the crucial roles of SOD and CAT in the maintenance of cellular redox homeostasis. Therefore, we determined the effects of AS-IV on enzyme activities of SOD and CAT in heat-exposed cells. As shown in Fig. 5C, compared with the heat control, SOD and CAT activities were significantly improved after AS-IV treatment, whereas no significant changes were observed in the presence of both spermine and AS-IV. These observations demonstrate that AS-IV protects against heat-induced oxidative damage in 16HBE140- cells in an MCU-dependent manner.

AS-IV inhibits MPT and protects against mitochondria-mediated apoptosis

It is accepted that mitochondrial Ca^{2+} overload and ROS accumulation cause mitochondrial membrane depolarisation. Recently, it has been demonstrated that OMM plays a crucial role in the release of pro-apoptotic factors that lead to OMM rupture and subsequent MPT [26]. To examine the effects of AS-IV on these processes, we used tetramethylrhodamine ethyl ester (TMRE) to indicate mitochondrial inner membrane permeability and measured the activities of MPT pores using calcein AM. These experiments showed marked enhancement of TMRE and calcein fluorescence intensities following heat exposure, suggesting the rupture of OMM and increased prevalence of MPT. Moreover, these heat-induced consequences were significantly reduced in the presence of AS-IV, and treatments with mitoTEMPO (mitochondrial ROS inhibitor) and Ru360 resulted in remarkable reductions of TMRE and calcein fluorescence intensities. In contrast, these parameters were significantly enhanced in the spermine-treated group compared with the control group (Fig. 6A and B). In further experiments, we determined expression levels of the mitochondrial pro-apoptotic factors cytochrome c, apoptosis-inducing factor (AIF), procaspase-9 and endonuclease G (Endo G) using western blotting analyses and observed significant increases after heat exposure. In accordance with previous experiments, AS-IV treatment attenuated these effects of heat treatment (Fig. 6C); while MitoTEMPO and Ru360 treatments inhibited the expression of pro-apoptotic factors, spermine treatment abolished these beneficial effects. Finally, expression levels of Bax, compared with the heat controls, cleaved PARP, cleaved caspase3,

Bak, Bik and Bmf were significantly decreased following MitoTEMPO or Ru360 treatments (Fig. 6D). These data suggest that AS-IV inhibits mitochondria-mediated apoptosis and that this effect is dependent on the inhibition of MCU and mitochondrial ROS.

Discussion

In the present study, we used *in vivo* and *in vitro* models of heat exposure to confirm the protective effects of AS-IV against mitochondria-mediated apoptosis. MCU activity was significantly enhanced by heat-induced thermal inhalation injury in rats and by heat exposure of cultured 16HBE140- cells and was accompanied by mitochondrial Ca^{2+} overload, ATP depletion, elevated ROS levels and mitochondria-mediated apoptosis. However, AS-IV restored MCU activity, alleviated mitochondrial Ca^{2+} overload and ATP depletion, reduced ROS generation and reversed mitochondria-mediated apoptotic processes. Moreover, the pronounced protective effects of AS-IV against mitochondria-mediated apoptosis were abolished by the MCU activator spermine, suggesting that the protective effects of AS-IV on heat exposure injury reflect the inhibition of MCU activation, alleviating mitochondrial Ca^{2+} overload, ROS overproduction and mitochondria-mediated apoptosis.

MCU has been characterised as a highly selective channel that mediates Ca^{2+} entry into mitochondria and maintains mitochondrial Ca^{2+} homeostasis. Previous studies have shown that Ca^{2+} accumulation via MCU triggers cell death, particularly under stress conditions [27]. Our results demonstrated that heat exposure could enhance the activation of MCU and induce Ca^{2+} accumulation into mitochondria. Moreover, MCU-mediated Ca^{2+} overload has been associated with various apoptotic stimuli, and the relationship between MCU dysfunction and mitochondria-mediated apoptosis is well established. We hypothesised that heat-induced apoptosis could be due to MCU-mediated Ca^{2+} overload. It has already been demonstrated that the MCU activator spermine enhances mitochondrial Ca^{2+} uptake and leads to ATP deficiency. At lower levels, mitochondrial Ca^{2+} can actually promote ATP synthesis by stimulating the activity of metabolic mitochondrial enzymes. However, excessive MCU activation results in continuous Ca^{2+} overload and further causes ATP depletion. The present data show that heat exposure significantly enhanced MCU activity and subsequent AS-IV treatments markedly decreased MCU activation and inhibited mitochondrial Ca^{2+} overload and ATP depletion. Accordingly, the MCU activator spermine completely blocked these effects of AS-IV following heat exposure, indicating that AS-IV protects against heat-induced mitochondrial Ca^{2+} by inhibiting MCU.

Mitochondrial Ca^{2+} homeostasis plays critical roles in maintaining normal cellular physiology, and excessive activation of MCU causes mitochondrial Ca^{2+} overload followed by overproduction of mitochondrial ROS [28]. Several studies have shown that the consequence of mitochondrial Ca^{2+} -driven increases in $\Delta\psi$ is the oxidation of mitochondria and production of ROS [29]. In addition, some studies have suggested that Ca^{2+} dysregulations are largely responsible for ROS overproduction [30]. The resulting oxidative stress leads to protein, lipid and DNA damage and can induce cellular apoptosis and necrosis. Conversely, low ROS levels have been associated with moderate enhancements of cell viability [31]. Mitochondrial ROS are released from mitochondrial complex I and III, and although some studies have suggested complex II to be a main source of ROS under special conditions, the increased production of mitochondrial ROS has been proved to be a relevant process in many pathological conditions and participates in apoptosis and necrosis [32, 33]. Under stress conditions, different antioxidant systems are triggered, such as SOD and CAT, to prevent the production of mitochondrial ROS [34]. Here we provide important evidence about the efficacy of AS-IV in reducing the production of ROS. Accordingly, SOD and CAT activities were enhanced by AS-IV treatment and this effect was inhibited by spermine treatment, indicating that AS-IV protects against heat-induced oxidative damage, partly by inhibiting MCU.

Elevated ROS levels can cause mitochondrial swelling and promote permeability of the mitochondrial membrane, which induces the release of pro-apoptotic factors [35]. In particular, ruptured mitochondria release large amounts of cytochrome c, AIF and Endo G and subsequently form apoptosomes, which initiate caspase and cascades and induce apoptosis [36, 37]. The key finding of the present study is that heat-induced mitochondrial dysfunction and apoptosis follows excessive production of mitochondrial ROS and enhanced mitochondrial inner membrane permeability, leading to MPT. These conditions were largely reversed in the presence of AS-IV or mitoTEMPO, and AS-IV-attenuated expression of cytochrome c, AIF, procaspase-9 and Endo G was blocked by spermine treatment. Taken together, these data further confirm that the protective effects of AS-IV are related to MCU inhibition and subsequent prevention of mitochondrial Ca^{2+} overload, mitochondrial ROS production and release of pro-apoptotic factors.

Astragalus membranaceus is one of the most common traditional Chinese medicines used in clinical treatment by the oral administration to treat a wide variety of diseases and body disorders [38-40]. As-IV is a monomer located in an extract of Astragalus membranaceus [41]. Herein, our study is the first time to report that AS-IV prevents against heat induced apoptosis in heat-induced thermal inhalation injury and elucidated the mechanisms responsible for this protective effect.

In conclusion, AS-IV prevents excessive activation of MCU following heat exposure, mitochondrial Ca^{2+} overload, excessive ROS production, MPT open and the induction of heat-induced apoptosis. These studies warrant further consideration of the therapeutic applications of this natural compound from A. membranaceus in the thermal inhalation injury.

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Disclosure Statement

The authors declare no competing financial interests.

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