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Astragaloside IV and cycloastragenol are equally effective in inhibition of endoplasmic reticulum stress-associated TXNIP/NLRP3 inflammasome activation in the endothelium

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ABSTRACT

Ethnopharmacological relevance: Astragaloside IV and cycloastragenol are present together in *Astragalus membranaceus* Moench (Fabaceae) and this study aims to simultaneously investigate their regulation of endothelial homeostasis in the setting of endoplasmic reticulum stress (ER stress).

Material and methods: We stimulated endothelial cells with palmitate (PA 100 μM) to evoked ROS-associated ER stress and observed the effects of astragaloside IV and cycloastragenol on thioredoxin-interacting protein (TXNIP) expression, NLRP3 inflammasome activation and mitochondrion-dependent apoptosis.

Results: Astragaloside IV and cycloastragenol inhibited ROS generation and attenuated ER stress inducer IRE1α phosphorylation, indicating the inhibition of ROS-associated ER stress. In response to ER stress, TXNIP expression increased, accompanied with NLRP3 induction and increased IL-1β and IL-6 production, but these alternations were reversed by treatment with astragaloside IV and cycloastragenol, demonstrating the inhibitory effects of astragaloside IV and cycloastragenol on TXNIP/NLRP3 inflammasome activation. Inflammasome activation led to mitochondrial cell death in endothelial cells, whereas astragaloside IV and cycloastragenol restored the loss of the mitochondrial membrane potential with inhibition of caspase-3 activity, and thereby protected cells from ER stress-induced apoptosis. Astragaloside IV and cycloastragenol enhanced AMPK phosphorylation and AMPK inhibitor compound C diminished their beneficial effects, indicative of the potential role of AMPK in their regulation.

Conclusions: Astragaloside IV and cycloastragenol suppressed ROS-associated ER stress and then inhibited TXNIP/NLRP3 inflammasome activation with regulation of AMPK activity, and thereby ameliorated endothelial dysfunction by inhibiting inflammation and reducing cell apoptosis. Simultaneous investigations further showed that astragaloside IV and cycloastragenol were equally effective in regulation of endothelial homeostasis.

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1. Introduction

The endoplasmic reticulum (ER) is a cellular organelle that functions as a center for lipid synthesis and protein folding as well as calcium store regulation (Lin et al., 2008). In addition, it is also a major signal-transducing organelle that responds to alternations in cellular homeostasis (Xu et al., 2005). In fact, endoplasmic reticulum stress (ER stress) is an adaptive response to the accumulation of unfolded protein, and therefore, it is also termed as the unfolded protein response (UPR). When ER homeostasis is disrupted, the accumulation of unfolded protein aggregates lead to the activation of transmembrane sensors, including IRE-1α, which result in changes in gene expression and protein synthesis for the resolve of unfolded protein (Cox et al., 1993). Failure to adapt to increased unfolded proteins or aberrant ER stress is proposed to trigger inflammation and cell death (Xu et al., 2005; Hetz, 2012). In response to ER stress, the increased protein-folding promotes ROS generation and the accumulation of unfolded protein in ER leads to IRE-1α induction, which is important for integrating ER-stress signaling with inflammatory-response signaling (Zhang and Kaufman, 2008). ER stress-associated oxidative stress and inflammation are now thought to be fundamental in the pathogenesis

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of metabolic disorders, such as diabetes mellitus, obesity, and atherosclerosis (Zhang and Kaufman, 2008; Ozcan et al., 2004).

Oxidative stress and inflammation are implicated in ER stress, however thioredoxin-interacting protein (TXNIP)/NLRP3 inflammasome activation emerges as a key link among ER stress and inflammation and cell apoptosis (Zhou et al., 2010; Oslowski et al., 2012). TXNIP is a binding partner of reduced thioredoxin (TRX) and functions as a negative regulator of the TRX function (Nishiyama et al., 1999), while NLRP3 inflammasome is a functional complex responsible for immune responses through the maturation of pro-inflammatory cytokine IL-1β (Schroder and Tschopp, 2010). In response to ER stress, TXNIP dissociates from the binding to TRX and then induces NLRP3 inflammasome activation, which mediates inflammation and cell death through the processing of mature IL-1β in a manner caspase-1 dependent (Choi and Ryter, 2014), and the resultant production of IL-1β is required for inflammation and apoptosis in specialized cells (Oslowski et al., 2012).

Endothelial dysfunction is an early manifestation of cardiovascular diseases and accumulating evidence demonstrates the implication of ER stress in endothelial dysfunction. Though oxidative stress, inflammation and apoptosis occur simultaneously in endothelial dysfunction, ER stress emerges as a potential cause for these events. TXNIP is also expressed in the endothelium and its induction has been demonstrated to be involved in the initiation of inflammation (Perrone et al., 2009; Wang et al., 2012). A recent study further showed that TXNIP/NLRP3 inflammasome activation is required for endothelial inflammation and cell death in rats fed with high-fat diet (Mohamed et al., 2014). Although the link between ER stress and NLRP3 inflammasome activation is not fully elucidated in the endothelium, above-mentioned evidence suggests that TXNIP/NLRP3 inflammasome activation plays an important role in endothelial dysfunction.

Astragaloside IV is a saponin and serves as the predominant constituent of *Astragalus membranaceus* (Kwon and Park, 2012). In China, *A. membranaceus* has been considered to be a health-enhancing herbal used for the treatment of metabolic and cardiovascular disorders, including diabetes and atherosclerosis. Although cycloastragenol is also present in the flowers and roots of *A. membranaceus* (Verotta et al., 2002), it can be generated from parent astragaloside IV through hydrolysis by intestinal bacteria (Zhou et al., 2012) (Fig. 1). Astragaloside IV inhibits inflammatory and oxidative responses (Li et al., 2013; Gui et al., 2013a), and these actions have been shown to be implicated in the protection of cells from apoptosis (Gui et al., 2012). Moreover, clinical application and published studies demonstrate its beneficial effects on regulation of endothelial functions (Zhang et al., 2006, 2007, 2011). Despite these studies show the actions of astragaloside IV in inhibition of inflammation and regulation of vasorelaxation in the endothelium, the potential molecular targets or pathways remain to be elucidated. The bioactivity of astragaloside IV has been well documented, but little is known about the action of cycloastragenol. Because cycloastragenol is a microbial transformation of astragaloside IV and both astragaloside IV and cycloastragenol are present together in the blood after oral administration of *A. membranaceus* (Zhou et al., 2012), it is tempting to know whether cycloastragenol demonstrates a similar or different action with astragaloside IV in regulation of the endothelial function, especially in the setting of ER stress. To address these issues, this study aims to investigate the effects of astragaloside IV and cycloastragenol simultaneously on the endothelial homeostasis under ER stress conditions. Our work demonstrated that astragaloside IV and cycloastragenol ameliorated endothelial dysfunction by suppression of TXNIP/NLRP3 inflammasome activation with regulation of AMPK activity and further showed that their actions were equally effective. Our findings provide novel mechanistic insights regarding the protective effects of astragaloside IV and cycloastragenol on endothelial homeostasis. Because astragaloside IV as well as cycloastragenol are the main active ingredients of *A. membranaceus* in regulating endothelial homeostasis and responsible for the action of *A. membranaceus* in the clinic, we expect this new knowledge would contribute to designing novel strategies for their application in the management of diabetes and cardiovascular diseases.

2. Materials and methods

2.1. Reagents

Astragaloside IV (purity > 98%) was purchased from Shanghai Forever Biotech Co., Ltd. (Shanghai, China). Product ID: E-0146. Cycloastragenol (purity > 97%) was obtained from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). Product ID: E-0646. AICA riboside (AICAR) and mitoquinone mesylate (Mito Q) were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Compound C was provided by Sigma (St. Louis, MO, USA). These agents were dissolved in dimethyl sulfoxide (DMSO) and the final concentration was less than 0.01% (v/v). Palmitate (PA) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and dissolved in ethyl alcohol as a stock solution and then was further diluted with medium containing 10% of bovine serum albumin before use. Enhanced chemiluminescence (ECL) was obtained from Beyotime Institute of Biotechnology (Shanghai, China). The following items were purchased from the cited commercial sources: anti-phospho-AMPKα (T172) (2531s) and anti-AMPKα (2532s), Cell Signaling Technology (Beverly, MA, USA); anti-phospho-IRE1α

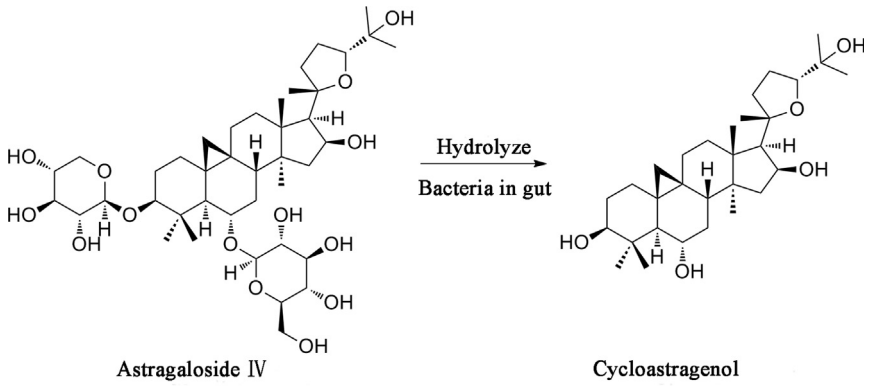

Fig. 1. Structures of astragaloside IV and cycloastragenol. As an aglycone, cycloastragenol can be generated from parent astragaloside IV through hydrolysis by intestinal bacteria after oral administration.

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(S724) (ab104157) and anti-IRE1α (ab37073), Abcam (Cambridge, MA, USA); TXNIP (NBP1-54578) and NLRP3 (NBP2-12446), Novus Biologicals (Littleton, CO, USA); Goat Anti-Rabbit IgG (H+L) HRP (BS13278), GAPDH (AP0063), Goat Anti-mouse IgG (H+L) and HRP (BS12478), Bioworld Technology (St. Paul, MN, USA).

2.2. Cell culture

EA.hy926 cells (a human umbilical vein cell line, established by fusing primary human umbilical vein cells with a thioguanine-resistant clone of A549) were from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China; CRL-2922.). Cells were cultured in Dulbecco's Minimum Essential Medium containing 10% (v/v) fetal bovine serum (FBS), supplemented with antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate), at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Assay for intracellular ROS

EA.hy926 cells were seeded in a 48-well culture plate (2×10^5 cells/well) and reached to 80% confluency. Cells were treated with astragaloside IV (10 μM), cycloastragenol (10 μM), AICAR (500 μM) or Mito Q (0.1 μM) with or without the presence of compound C (25 μM), respectively, and then incubated with PA (100 μM) for 24 h. According to the manufacturer's instructions, ROS specific fluorescent probe dye DCFH-DA (10 μM, Beyotime Institute of Biotechnology, Shanghai, China) was added and incubated at 37 °C for 30 min, and then intracellular ROS production was examined by fluorescence microscope. The degree of ROS was quantified by densitometry with Image-Pro Plus 6.0 software.

2.4. Mitochondrial membrane potential ($\Delta\psi m$) assay

Cells were treated with agents as same as mentioned above and then stimulated with PA (100 μM) for 24 h. After the treatment, cells were incubated with $\Delta\Psi m$-specific fluorescent dye JC-1 (Beyotime Institute of Biotechnology, Shanghai, China) at 37 °C for 20 min. Mitochondrial membrane potential was examined by fluorescence microscope with filters. According to the manufacturer's instructions, JC-1-aggregates produce red fluorescence assessed using excitation/emission wavelengths of 585/590 nm when the mitochondrial membrane potential is higher; otherwise, JC-1 is in the monomer form which can produce green fluorescence at 514/529 nm. The relative proportions of red and green fluorescence are used to measure the ratio of mitochondrial depolarization. The degree of fluorescence was quantified by densitometry with Image-Pro Plus 6.0 (IPP 6.0) software.

2.5. ELISA assay

Cells were cultured in 48-well plates with the mentioned agents at given concentrations in the presence of PA (100 μM) for 24 h. After the medium was collected, the supernatant was obtained by centrifugation (3000*g* for 10 min). The levels of IL-1β and IL-6 in the supernatant were assayed with commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D, USA).

2.6. Assay of caspase-3 activity

Cells were cultured in 6-well plates with the mentioned agents at given concentrations in the presence of PA (100 μM) for 24 h, then collected and lysed in ice-cold lysis buffer (Tris–HCl 50 mM, pH 7.2, containing 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% NP-40, NaCl 0.15 M, sodium orthovanadate 1 mM) and protein was collected by centrifugation at 12,000*g* for 15 min. Active caspase-3 activity was determined using the Caspase-3 Activity Assay Kit (Beyotime, China) based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide.

2.7. Apoptosis analysis

Endothelial cells were seeded in 48-well plates, and cultured until grown to confluency. Then cells were pretreated with astragaloside IV (10 μM), cycloastragenol (10 μM) or AICAR (500 μM) in the presence or absence of compound C, and then incubated with or without PA (100 μM) for 24 h. After incubation, apoptotic cells were assayed with a two color analysis of FITC-labeled Annexin V binding and PI uptake using the Annexin V-FITC Apoptosis Detection Kit (KeyGEN Biotech Co., Ltd. Nanjing, China) by fluorescence microscope with filters (494 nm, green; 528 nm, red). Annexin V-FITC staining green fluorescence represents early apoptosis and PI staining red fluorescence expresses late apoptosis, according to the manufacturer's instructions.

2.8. Western blot assay

For protein analysis, cells grown to confluency were treated with mentioned agents at given concentrations. Then cells were washed two times with ice-cold PBS and lysed in ice-cold lysis buffer (the same as the ones used to assay of caspase-3 activity), supplemented with 1 mM PMSF, to extract the protein. The protein concentration of each sample was determined using a Bicinchoninic Acid Protein Assay kit (Biosky Biotechnology Corporation, Nanjing, China). An equal amount of protein was separated by SDS-PAGE and transferred to PVDF membranes (0.45 μm, Millipore Co., Ltd.). The PVDF membranes were blocked with 5% skim milk in TBST buffer (containing 5 mmol/L Tris–HCl, pH 7.6, 136 mmol/L NaCl, 0.05% Tween-20) and the membrane was incubated with HRP-conjugated secondary antibody after overnight primary antibody incubation at 4 °C. Antibody–antigen complexes visualized with ECL and analyzed quantitatively by densitometry with IPP 6.0 software.

2.9. Statistics

Results were expressed as means ± SD (standard deviation). Data were firstly analyzed by using one-way ANOVA test, and secondly using Student–Newman–Keuls test for comparison of two groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Astragaloside IV and cycloastragenol inhibited IRE1α phosphorylation

To investigate the effects of astragaloside IV and cycloastragenol on ER stress, we first observed the change of inositol-requiring protein 1α (IRE1α) phosphorylation in endothelial cells. As an ER-localized protein sensor, IRE1α senses the change of unfolded protein in the ER. Upon palmitate (PA) stimulation, IRE1α phosphorylation increased by 3 fold in comparison with the untreated cells, indicating the initiation of ER stress. As shown in Fig. 2, astragaloside IV and cycloastragenol treatment significantly inhibited PA-induced IRE1α activation, as the phosphorylations of IRE1α in astragaloside IV and cycloastragenol treatment were reduced by 42% and 67%, respectively. As an AMPK activator, AICAR also inhibited IRE1α phosphorylation.

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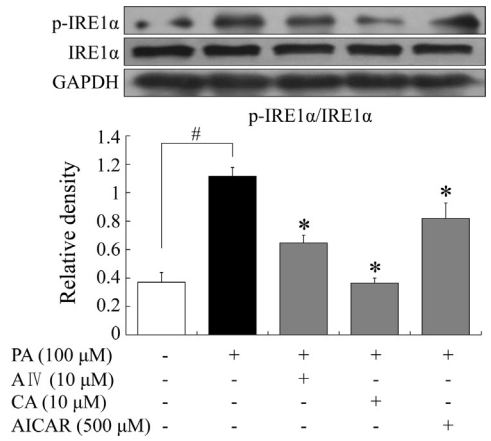

Fig. 2. Astragaloside IV and cycloastragenol normalized IRE1α phosphorylation. Cells were cultured with astragaloside IV (AIV,10 μM), cycloastragenol (CA, 10 μM), or AICAR (500 μM) followed by stimulation with PA (100 μM). IRE1α phosphorylation was determined by western blot. Data were expressed as the mean ± SD from three independent experiments. * $p < 0.05$ vs Control, # $p < 0.05$ vs indicated group.

3.2. Astragaloside IV and cycloastragenol suppressed ROS generation in endothelial cells

ROS generates and accumulates in the ER in response to the increased unfolded protein (Zhang and Kaufman, 2008). PA evoked ER stress, and as a result, we found an increase in intracellular ROS production indicated by the increased green fluorescence in the cells. Treating cells with astragaloside IV and cycloastragenol effectively inhibited ROS production in response to PA challenge (Fig. 3A and B). Similarly, AMPK activator AICAR also suppressed ROS generation. AMPK inhibitor compound C diminished the inhibitory effects of astragaloside IV and cycloastragenol on ROS production, indicative of the potential involvement of AMPK.

3.3. Astragaloside IV and cycloastragenol prevented TXNIP induction

In view of the important role of TXNIP in linking ER stress to inflammation, we further observed the influence of astragaloside IV and cycloastragenol on TXNIP expression in endothelial cells. PA challenge induced increased TXNIP expression, whereas this alternation was reversed by treating cells with astragaloside IV and cycloastragenol (Fig. 4), as we observed the expressions in astragaloside IV and cycloastragenol-treated cells were reduced by 50% and 42%, respectively. Co-incubation of compound C blocked the action of astragaloside IV and cycloastragenol and brought the attenuated TXNIP expression back to the PA-stimulated level, indicating the role of AMPK in the regulation of TXNIP.

3.4. Astragaloside IV and cycloastragenol suppressed NLRP3 inflammasome activation

To ask whether ER stress induces inflammasome activation, we observed the changes of NLRP3 expression and IL-1β secretion. In response to PA stimulation, NLRP3 expression increased by about 2.7 fold. In inflammasome complex, NLRP3 can promote IL-1β maturation by caspase-1 cleavage. Both astragaloside IV and cycloastragenol effectively reduced NLRP3 expression by about 42% and 40%, respectively, and thereby inhibited IL-1β production, demonstrating their inhibitory effects on NLRP3 inflammasome activation (Fig. 5A and B). Pro-inflammatory cytokine IL-6 is a major downstream target of IL-β. Compared with PA stimulation, astragaloside IV and cycloastragenol inhibited IL-6 production, indicative of their anti-inflammatory activity (Fig. 5C).

3.5. Astragaloside IV and cycloastragenol restored the loss of mitochondrial membrane potential ($\Delta\Psi m$)

As ER stress mediates cell death through mitochondrial pathway, we observed the change of mitochondrial function when cells exposed to PA insult. The fluorescent dye JC-1 accumulates in coupled mitochondria as red aggregates. When collapse of $\Delta\Psi m$ occurs, JC-1 produces green fluorescence. As shown in Fig. 6, cells exposed to PA exhibited attenuated red fluorescence with enhanced green fluorescence, indicating the collapse of $\Delta\Psi m$. Astragaloside IV and cycloastragenol effectively restored the loss of mitochondrial membrane potential, as evidenced by stronger red fluorescence accompanied by weaker green fluorescence (Fig. 6A and B). CCCP, an inhibitor of mitochondrial electron transport chain, also induced the collapse of $\Delta\Psi m$. AMPK inhibitor compound C blocked the protective action of astragaloside IV and cycloastragenol in the restoration of $\Delta\Psi m$.

3.6. Astragaloside IV and cycloastragenol inhibited caspase-3 activity and reduced apoptosis in endothelial cells

PA stimulation increased caspase-3 activity, which is a key molecule in the regulation of the process of cell apoptosis. As shown in Fig. 7A, treating cells with astragaloside IV or cycloastragenol successfully inhibited caspase-3 activity. We further observed the effects of astragaloside IV and cycloastragenol on cell survival. As expected, astragaloside IV as well as cycloastragenol effectively reduced cell apoptosis when cells were exposed to PA challenge (Fig. 7B and C). AMPK inhibitor compound C blocked the action of astragaloside IV or cycloastragenol in protection of cell survival, indicating the role of AMPK in their action.

3.7. Astragaloside IV and cycloastragenol enhanced AMPK phosphorylation in endothelial cells

AMPK inhibitor compound C attenuated the beneficial effects of astragaloside IV and cycloastragenol on PA-induced endothelial dysfunction, indicating the potential involvement of AMPA activity in their action. Therefore we observed their regulation of AMPK phosphorylation in endothelial cells exposed to PA. PA stimulation inhibited AMPK activity, evidenced by attenuated AMPK phosphorylation, whereas this alternation was prevented by treatment with astragaloside IV. Similar to the regulation by astragaloside IV, cycloastragenol also effectively restored the loss of AMPK phosphorylation against PA insult. The result was shown in Fig. 8.

4. Discussion

Although ER stress is an adaptive response to maintain cellular homeostasis, aberrant ER stress is involved in disease states because it can lead to inflammation and cell death in special cells and tissues. Because excessive circulating free fatty acids (FFAs) in dyslipidemia lead to abnormal endothelial function (Cersosimo and DeFronzo, 2006), and aberrant lipid can pose major challenges to ER homeostasis (Fu et al., 2011), in the current study, we stimulated endothelial cells with PA to induce ER stress and demonstrated that astragaloside IV and cycloastragenol protected endothelial homeostasis by suppression of ER stress-associated TXNIP/NLRP3 inflammasome activation.

ROS is manifested in ER stress because increased protein folding leads to the accumulation of ROS in the ER (Ozcan et al.,

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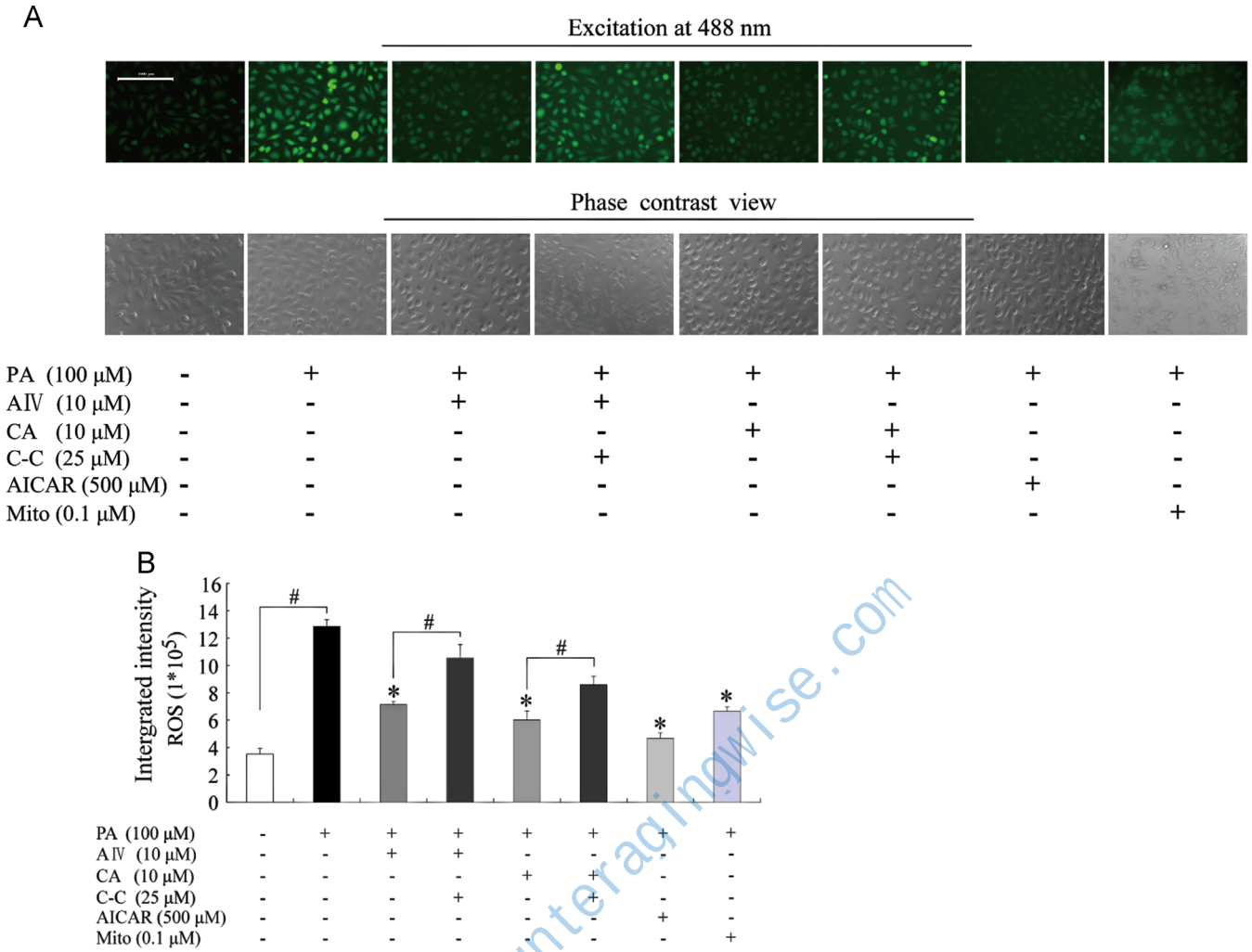

Fig. 3. Astragaloside IV and cycloastragenol reduced ROS production in endothelial cells. Cells were pretreated with astragaloside IV (AIV,10 μM), cycloastragenol (CA, 10 μM), AICAR (500 μM) or Mito Q (0.1 μM) in the presence of absence of compound C (25 μM) and then incubated with PA (100 μM) for 24 h. Intracellular ROS production was detected by DCFH- DA labeling with fluorescence microscopy (A), and the degree of ROS was quantified (B). Data were expressed as the mean ± SD from three independent experiments. Bar 1000 μm; * $p < 0.05$ vs Control, # $p < 0.05$ vs indicated group.

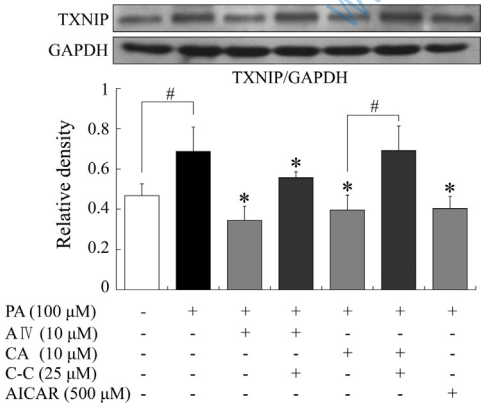

Fig. 4. Astragaloside IV and cycloastragenol prevented TXNIP expression. Cells were treated with astragaloside IV (AIV), cycloastragenol (CA), or AICAR with or without compound C at given concentrations and then incubated with PA (100 μM) for 24 h. NLRP3 expression was determined by western blot and data were expressed as the mean ± SD from three independent experiments. $p < 0.05$ vs Control, # $p < 0.05$ vs indicated group.

2004). PA evokes ER stress through several pathways in which oxidative stress is included (Listenberger et al., 2001). Among ER-localized transmembrane sensors, inositol-requiring protein 1α (IRE1α) regulates call fate (Han et al., 2009) and hyperphosphorylation of IRE1α can promote ER stress-mediated cell death under irremediable ER stress (Lerner et al., 2012). PA challenge led to hyperphosphorylation of IRE1α accompanied with increased ROS production, indicative of ER stress-associated oxidative stress in the endothelium. Consistent with the published studies which demonstrates that astragaloside IV suppress oxidative response in hepatic stellate cell activation (Li et al., 2013) and kidney injury (Gui et al., 2013b), our work further showed that astragaloside IV and cycloastragenol suppressed ER stress and thereby attenuated oxidative stress in the endothelium. Differently from the reported studies which shows that astragaloside IV prevented cerebral infarction and ameliorated endothelial dysfunction by enhancing Nrf2 expression and increasing the activities of antioxidant enzymes (Cao et al., 2014; Qiu et al., 2010), our work suggested that the action of astragaloside IV and cycloastragenol on suppression of oxidative stress was a result from its inhibition of ER stress.

TXNIP is a negative regulator of the TRX function (Nishiyama et al., 1999), and its overexpression has been demonstrated to be involved in the pathology of diabetes, endothelial dysfunction and ischemia-reperfusion injury in the heart (Parikh et al., 2007; Wang et al., 2012; Yoshioka et al., 2012). In response to oxidative stress, TXNIP dissociates from TRX and then activates NLRP3 inflammasome (Zhou et al., 2010; Oslowski et al., 2012). Given the central

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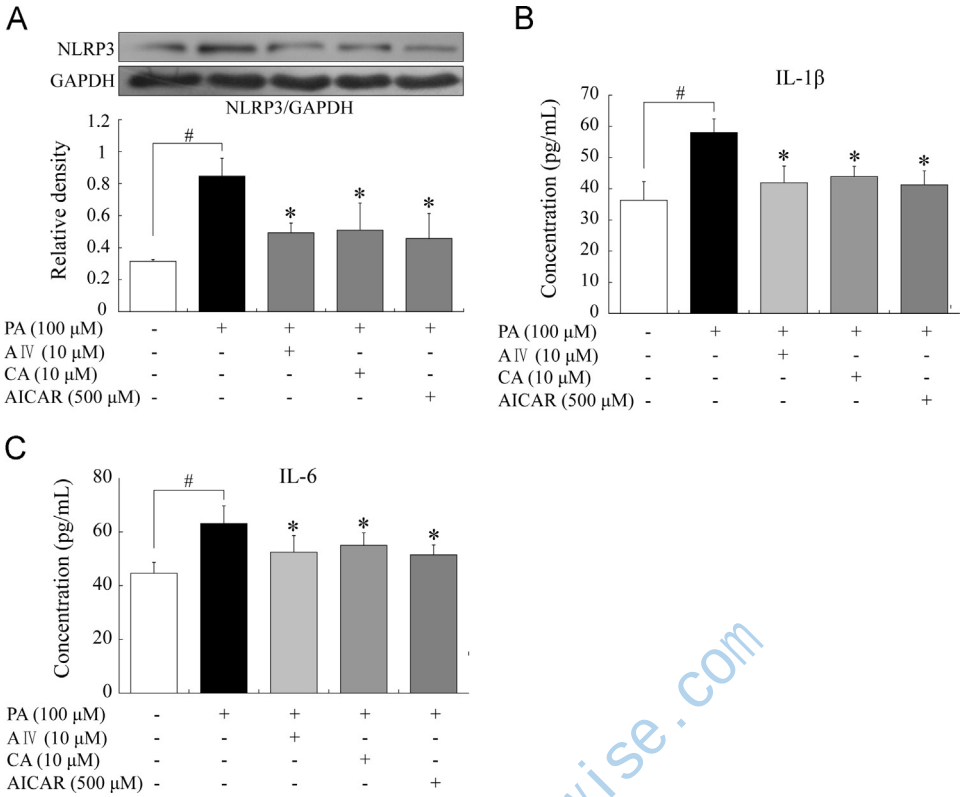

Fig. 5. Astragaloside IV and cycloastragenol suppressed NLRP3 inflammasome activation in endothelial cells. Cells were incubated with astragaloside IV (AIV, 10 μM), cycloastragenol (CA, 10 μM) or AICAR (500 μM) in the presence of PA (10 μM) for 24 h. (A):NLRP3 expression was determined by western blot and data were expressed as the mean ± SD from three independent experiments; (B, C): IL-1β and IL-6 in the supernatant were determined with ELISA Kit and data were expressed as the mean ± SD (n=4). *p < 0.05 vs Control, # p < 0.05 vs indicated group.

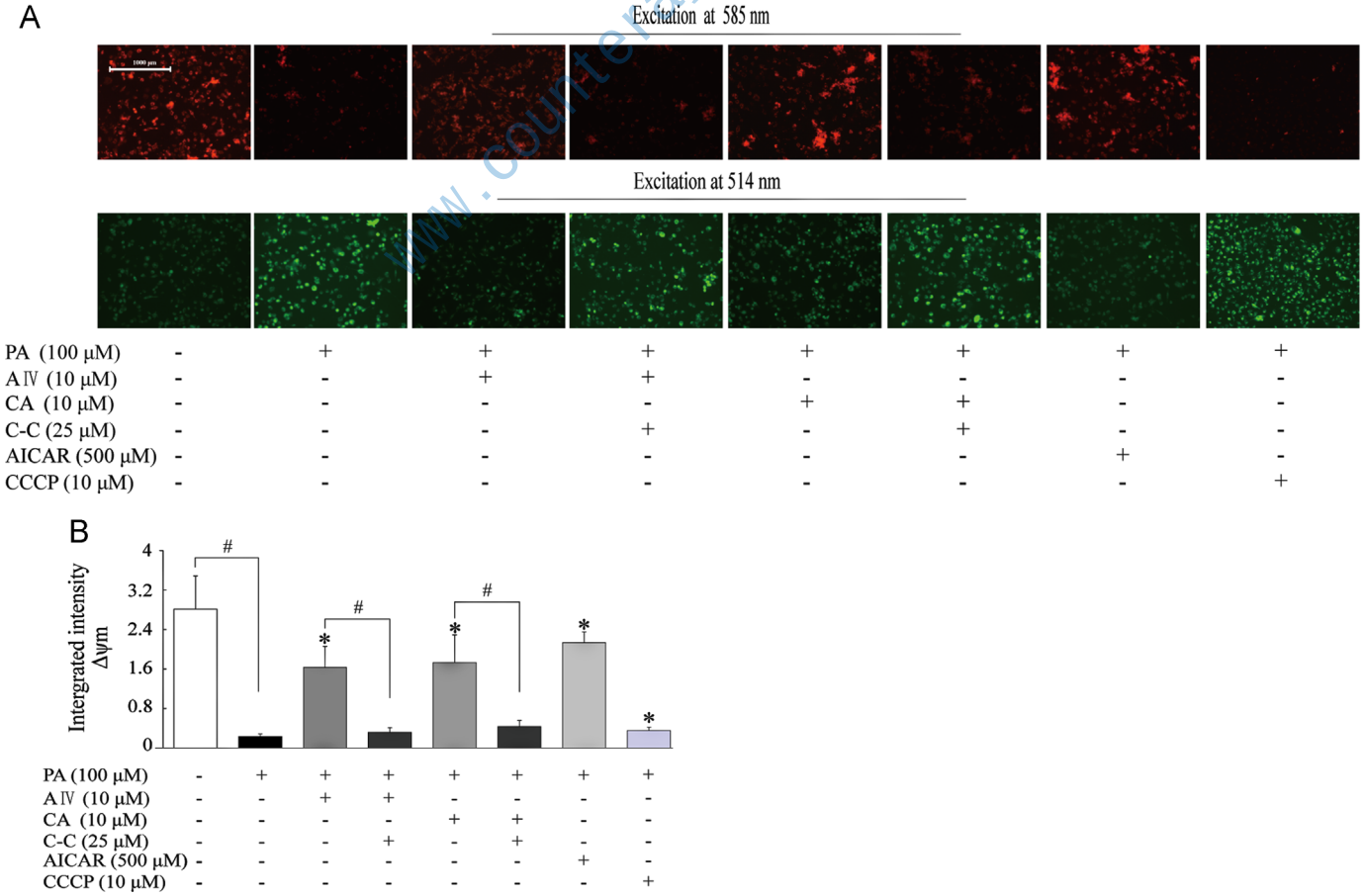

Fig. 6. Astragaloside IV and cycloastragenol reversed the collapse of mitochondrial membrane potential ($\Delta\psi m$) in endothelial cells. Cells were pretreated with astragaloside IV (AIV, 10 μM), cycloastragenol (CA, 10 μM) , AICAR (500 μM) or CCCP (10 μM) with or without compound C (25 μM), and then incubated with PA (100 μM) for 24 h. The Δψm was viewed by JC-1 labeling with fluorescence microscopy (A), and the degree of Δψm was quantified (B). Data were expressed as the mean ± SD from three independent experiments. Bar 1000 μm; * p < 0.05 vs Control, #p < 0.05 indicated group.

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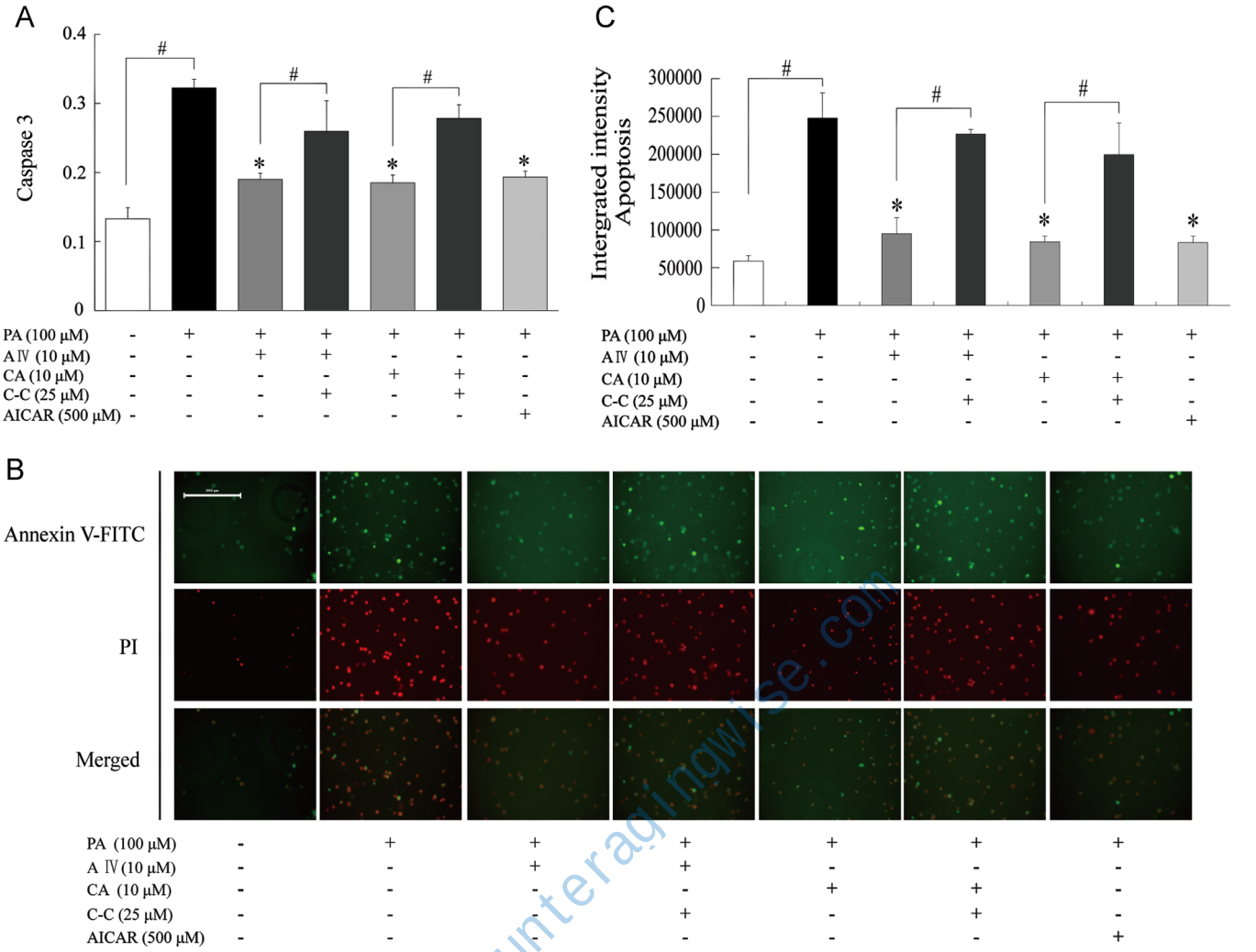

Fig. 7. Astragaloside IV and cycloastragenol inhibited caspase-3 activity and apoptosis in endothelial cells. Cells were pretreated with astragaloside IV (AIV, 10 μM), cycloastragenol (CA, 10 μM) or AICAR (500 μM) in the presence of absence of compound C (25 μM) for 24 h. (A) Caspase-3 activity was measured with ELISA kit and data were expressed as the mean ± SD (n=4); (B, C) The apoptosis assay was performed with the Annexin V-FITC Apoptosis Detection Kit and detected with fluorescence microscopy. Bar 1000 μm; Data were expressed as the mean ± SD from three independent experiments. *p < 0.05 vs Control; # p < 0.05 vs indicated group.

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role of TXNIP in the link between oxidative stress and NLRP3 inflammasome activation, we observed the effects of astragaloside IV and cycloastragenol on TXNIP and NLRP3 induction in endothelial cells. PA stimulation induced TXNIP overexpression, and as expected, NLRP3 induction occurred. These changes indicated that NLRP3 activation was a result from upstream ER stress. Astragaloside IV, as well as cycloastragenol, inhibited TXNIP expression and thereby suppressed the activation of NLRP3. NLRP3 inflammation is a platform for caspase-1 activation and IL-1β maturation. Upon NLRP3 activation, mature IL-1β is secreted alongside caspase-1 cleavage-dependent pathway (Schroder and Tschopp, 2010). In this context, astragaloside IV and cycloastragenol treatment led to a decrease in IL-1β production, indicative of their inhibitory role in NLRP3 inflammasome activation. In view of their inhibitory effects on ER stress-associated ROS oxidative stress, it was reasonable to believe that the inhibitory effects on TXNIP/NLRP3 activation should be a result derived from their suppression of upstream ER stress.

Pro-inflammatory cytokine IL-6 is a major target of IL-1β. Astragaloside IV and cycloastragenol inhibited IL-1β secretion, and as an expected result, we observed that IL-6 production was also inhibited. This result indicated that their action in suppression of NLRP3 inflammasome activation contributed to the inhibition of inflammation. In addition to evoking inflammation, IL-1β is responsible for mitochondrial apoptosis. It is well established that mitochondrial membrane potential is essential for the maintenance of mitochondrial function. For this, we observed the effects of astragaloside IV and cycloastragenol on mitochondrial function and found that both astragaloside IV and cycloastragenol effectively restored the loss of mitochondrial membrane potential, demonstrating their beneficial effects on mitochondrial function against ER stress. Mitochondrial apoptosis is characteristic of the initiation of caspase cascades, in which caspase-3 serves as an executioner of apoptosis. In response to the collapse of mitochondrial membrane potential, caspase-3 activity increased, and as a downstream result, we observed increased apoptosis cells when cells were exposed to PA. Astragaloside IV and cycloastragenol inhibited caspase-3 activity, and thereby reduced cell apoptosis, well demonstrating their action in protecting cell survival against ER stress. Some studies have demonstrated that astragaloside IV protected neuronal cells and cardiomyocytes from death, and some molecular mechanisms, such as anti-oxidative and PI3K/Akt pathways, have been proposed (Zhang et al., 2012; Hu et al., 2009; Jia et al., 2014), our findings further showed that the protective role of astragaloside IV on cell survival was relative with suppression of NLRP3 inflammasome activation.

As an energy sensor, AMPK regulates glucose and lipid metabolism. In addition, the anti-inflammatory activity of AMPK has

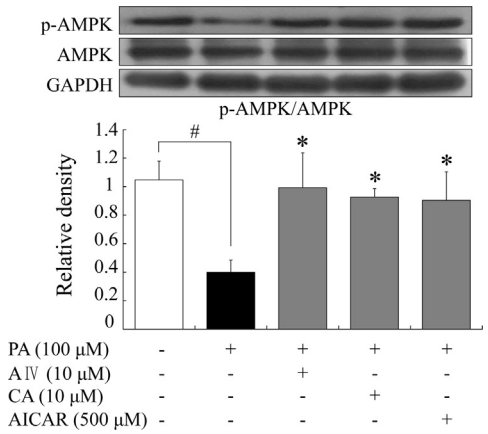

Fig. 8. Astragaloside IV and cycloastragenol restored AMPK phosphorylation in the presence of PA. Cells were incubated with astragaloside IV (AIV, 10 μM) and cycloastragenol (CA, 10 μM) with PA (100 μM) for 2 h. Phosphorylation of AMPK (p-AMPK) was determined by western blot. Data were expressed as the mean ± SD from three independent experiments. * $p < 0.05$ vs Control; # $p < 0.05$ vs indicated group.

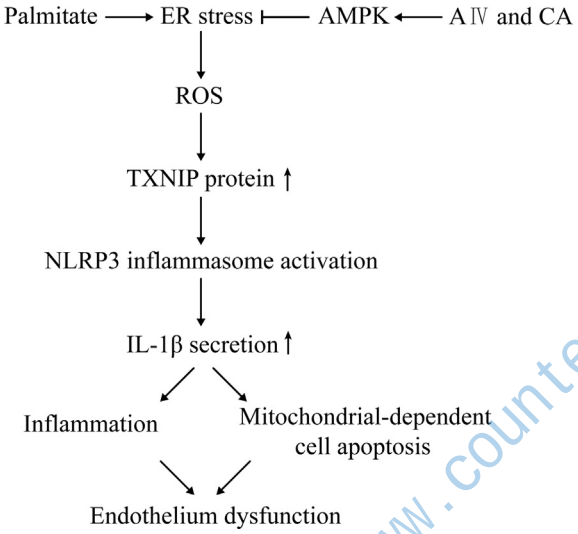

Fig. 9. The proposed pathway through which astragaloside IV and cycloastragenol protected endothelial function against ER stress. PA challenge induced ER stress and oxidative stress in endothelial cells evidenced by hyperphosphorylation of IRE1α and ROS production. In response to ER stress-associated oxidative stress, TXNIP induced NLRP3 activation, which promoted IL-1β secretion. The increased IL-1β secretion was responsible for the initiation of inflammation and apoptosis in endothelial cells. Astragaloside IV and cycloastragenol inhibited ER stress-associated oxidative stress via regulation of AMPK and effectively inhibited IL-1β induction by blocking TXNIP/NLRP3 inflammasome activation, and thereby inhibited inflammation and protected cell survival against ER stress.

been well documented. It is reported that AMPK suppresses TXNIP activity by enhancing TXNIP degradation (Wu et al., 2013). Based on the evidence, we observed the regulation of AMPK activity by astragaloside IV and cycloastragenol and found that both astragaloside IV and cycloastragenol effectively restored AMPK phosphorylation against PA insult, well demonstrating their positive regulation of AMPK activity in the endothelium. AMPK inhibitor compound C diminished the inhibitory effects of astragaloside IV and cycloastragenol on TXNIP expression, indicating the role of AMPK in their regulation. Compound C further attenuated the roles of astragaloside IV and cycloastragenol in the regulation of mitochondrial function, caspase-3 activity and apoptosis, suggesting that their regulation of AMPK contributed to protecting cell survival against TXNIP/NLRP3 inflammasome activation. Although compound C is not specific enough for inhibition of AMPK, these results raised the possibility that AMPK activity was involved in the action of astragaloside IV and cycloastragenol.

In the practice of the traditional Chinese medicine, the health-enhancing action of *A. membranaceus* is mainly dependent on its anti-stress effects. As a predominant constituent in *A. membranaceus*, astragaloside IV protects the brain from ischemic injury (Yang et al., 2012; Cao et al., 2014; Luo et al., 2004), well demonstrating its beneficial effects on suppression ER stress under hypoxic and nutrient-deprived conditions. Consistent with these, in the present study, we showed that astragaloside IV as well as cycloastragenol inhibited ROS-associated ER stress in the endothelium. This evidence raises the possibility that suppression of ER stress might be a potential mechanism for the action of astragaloside IV and cycloastragenol in the prevention of cardiovascular diseases.

Taken together, our work showed that astragaloside IV and cycloastragenol inhibited TXNIP/NLRP3 inflammasome activation by suppressing ER stress-associated oxidative stress, and thereby inhibited inflammation and protected cell survival against ER stress. The proposed molecular pathway was shown in Fig. 9. Although the anti-inflammatory and antioxidative actions of astragaloside IV have been documented in the published studies, our work suggested that inhibition of ER stress may be a common mechanism for these actions. As an aglycone, cycloastragenol is a microbial transformation of astragaloside IV, our work demonstrated that astragaloside IV and cycloastragenol are equally effective in amelioration of endothelial dysfunction by suppression of ER stress. These findings inform our better understanding of the functional constituents in medical herb *A. membranaceus* and therefore contribute to its further application in the protection of endothelial function.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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