



ARTICLE

Astragaloside IV attenuates myocardial ischemia/reperfusion injury in rats via inhibition of calcium-sensing receptor-mediated apoptotic signaling pathways

Bo Yin¹, Xu-wei Hou² and Mei-li Lu³

Astragaloside IV (AsIV) is an active saponin extracted from *Astragalus membranaceus*, which has shown cardioprotective effects in a number of experimental animals. In this study we investigated the molecular mechanisms by which AsIV attenuated the myocardial ischemia reperfusion (MI/R)-induced injury in vitro and in vivo by focusing on calcium-sensing receptor (CaSR) and extracellular signal-regulated kinase 1/2 (ERK1/2). Rat neonatal cardiac myocytes were subjected to a hypoxia/reoxygenation (H/R) procedure in vitro, which significantly decreased the cell viability, increased lactate dehydrogenase (LDH) release, induced cardiomyocyte apoptosis, and increased $[Ca^{2+}]_i$. H/R also increased the expression of CaSR and decreased ERK1/2 phosphorylation levels in H/R-exposed myocytes. Pretreatment with AsIV (60 μ mol/L) significantly improved the cell viability and decreased LDH release, attenuated myocyte apoptosis, decreased $[Ca^{2+}]_i$ and CaSR expression, and increased the ERK1/2 phosphorylation levels. The protective effects of AsIV against H/R injury were partially inhibited by co-treatment with a CaSR agonist, gadolinium chloride ($GdCl_3$) or with a specific ERK1/2 inhibitor U0126. For in vivo studies, a rat MI/R model was established. Pre-administration of AsIV (80 mg/kg every day, ig) significantly decreased the myocardium infarct size, creatine kinase-MB (CK-MB) production, serum cardiac troponin (cTnI) levels, and cardiomyocyte apoptosis in the rats with MI/R injury. The therapeutic effects of AsIV were associated with the downregulation of CaSR expression and upregulation of ERK1/2 phosphorylation in myocardial tissues. In summary, astragaloside IV attenuates myocardial I/R injury via inhibition of CaSR/ERK1/2 and the related apoptotic signaling pathways.

Keywords: astragaloside IV; myocardial ischemia/reperfusion; apoptosis; calcium-sensing receptor; ERK1/2; gadolinium chloride; U0126

Acta Pharmacologica Sinica (2019) 40:599–607; <https://doi.org/10.1038/s41401-018-0082-y>

INTRODUCTION

Myocardial ischemia reperfusion (MI/R) injury is a complex pathophysiological process that is associated with various factors and pathways, and the condition remains a major clinical problem in patients who receive thrombolysis, percutaneous coronary intervention, cardiac transplantation, and coronary artery bypass grafting therapies [1, 2]. For ischemic heart disease, reperfusion therapy can preserve myocardial viability and function by reversing myocardial ischemia, but the subsequent ischemia reperfusion injury may reduce the therapeutic benefit, induce cardiomyocyte death, and increase infarct size [3]. Cardiomyocyte apoptosis has been recognized as the main cause of ischemia reperfusion injury, and exploring anti-apoptotic agents represent a novel therapeutic opportunity for ischemia diseases.

Astragaloside IV (AsIV), a purified small molecular saponin, is one of the major and active components of *Astragalus membranaceus*. Studies on AsIV indicate that it has a wide spectrum of pharmacological effects, including anti-inflammation, antioxidation, regulating energy metabolism, and anti-apoptosis [4–7].

These beneficial pharmacological and biochemical activities suggest that AsIV has a potential therapeutic role in cardiovascular diseases. According to a previous study, AsIV not only inhibited inward K^+ currents to prolong action potential duration (APD) but also suppressed the peak of voltage-dependent Ca^{2+} channel current to prevent APD over-prolongation, protecting the heart against arrhythmias [8]. Consistent with this study, we previously demonstrated a cardiac protective role of AsIV on cardiac hypertrophy induced by isoprenaline, lipopolysaccharide, and pressure overload [9, 10]. In addition, some reports have indicated that AsIV could attenuate MI/R injury in animal models, and the mechanisms may be related to antioxidative damage, improvement of energy metabolic disturbance, and attenuation of calcium overload [11–13]. The present study aimed to further investigate the protective effect of AsIV against MI/R-induced myocardial damage by focusing on possible involvement of the calcium-sensing receptor (CaSR) and its related apoptotic pathways.

CaSR is a member of G protein-coupled receptors (GPCRs) superfamily, which is expressed in hearts and neonatal rat

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Received: 16 January 2018 Accepted: 20 May 2018

Published online: 20 July 2018

cardiomyocytes [14, 15] and is involved in systemic calcium homeostasis. Moreover, the CaSR in the cardiovascular system plays an important role in cardiac hypertrophy, atherosclerosis, and ischemia/reperfusion injury [16]. Further studies indicated that CaSR contributes to MI/R injury by activating mitogen-activated protein kinases (MAPKs), phospho-protein kinase C δ (p-PKC δ) translocation to the mitochondria, calcium overload, caspase-3, and Fas death receptor pathways [17–19]. The extracellular signal-regulated kinase 1/2 (ERK1/2) cascade plays a critical role in cell proliferation and apoptosis and is involved in $[Ca^{2+}]_i$ and cell apoptotic gene regulation in MI/R injury [20, 21]. However, the relationship between CaSR and ERK1/2 in MI/R remains unclear. Therefore, the present study was designed to further investigate the mechanism on MI/R by focusing on CaSR and ERK1/2. We tested the hypothesis that AsIV attenuates MI/R injury via inhibiting CaSR and its related apoptotic pathways. We demonstrated for the first time that the CaSR- and ERK1/2-mediated apoptotic pathway contributed to the protective effect of AsIV on MI/R, which further supports the use of AsIV as a promising therapeutic agent for cardiovascular disease.

MATERIALS AND METHODS

Materials

AsIV was obtained from Nanjing Jingzhu Biotechnology Company (purity >98%; Nanjing, China). In addition, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), gadolinium chloride ($GdCl_3$), and U0126 were from Sigma-Aldrich (USA). Nitroretetrazolium blue chloride (NBT) was purchased from Amresco. Antibodies against CaSR, ERK1/2, p-ERK1/2, bcl-2, bax, and cleaved caspase-3 were purchased from Proteintech Biotechnology. In Situ Cell Death Detection, creatine kinase-MB (CK-MB), and cardiac troponin (cTnI) content kits were purchased from Roche Molecular Biochemicals. The kit for determination of lactate dehydrogenase (LDH) content was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Neonatal cardiac myocyte culture and treatment protocol

The experimental protocols were approved by Committee of Jinzhou Medical University for the Use of Experimental Animals for Research and Teaching. Primary cardiomyocytes were isolated and cultured as previously described [22]. Hypoxia/reoxygenation (H/R) was performed based on the method previously described [23]. Briefly, cardiomyocytes were exposed to anoxia by replacing medium with anoxia buffer (in mM: NaCl 137, KCl 2l, $MgCl_2$ 0.49, $CaCl_2$ 1.0, HEPES 4, and sodium lactate 20, pH 6.8). Cells were incubated in the hypoxic/ischemic chamber at 37 °C for 2 h in a humidified atmosphere of 5% CO_2 and 95% N_2 . At the onset of reoxygenation, cardiomyocytes were randomly exposed to one of the following treatments: vehicle; AsIV (60 μM); AsIV plus $GdCl_3$ (30 μM), an agonist of CaSR; AsIV plus U0126 (20 μM), a specific inhibitor of ERK1/2; and AsIV plus NPS2143 (1 μM), a specific inhibitor of CaSR. The dosages of these drugs were selected based on previous studies [9, 24–26]. Then, cardiomyocytes were incubated again in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in 95% air and 5% CO_2 (reoxygenation) for 24 h.

Cell viability assay

Briefly, cells were seeded in 96-well plates at a density of 1.0×10^5 cells/mL. After different treatments, MTT solution was added to each well, and cells were incubated for an additional 4 h at 37 °C. Formazan crystals in viable cells were solubilized with 150 μL of DMSO, and the absorbance at 490 nm was measured using a microplate reader (BioTek Instruments, Inc).

Measurement of LDH release in culture supernatant

After reperfusion for 24 h, 200 μL culture supernatant of cardiomyocytes was taken and immediately assayed for LDH

release according to the manufacturer's instruction with a microplate reader.

Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was determined by JC-1 staining. Cardiomyocytes were cultured in six-well plates (1×10^5 /mL cardiomyocytes). After different treatments, cardiomyocytes were incubated 20 min in the presence of 2 μM of JC-1 at 37 °C. Then, the cells were washed twice with JC-1 buffer solution. Thereafter, labeled cells were analyzed and quantified using automatic image analysis software with a fluorescence microscope (Leica, DMI3000B).

Apoptosis assays

Apoptosis was determined using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit according to the manufacturer's instructions. Briefly, cardiomyocytes were seeded in 25-cm² flasks and incubated with indicated drugs for 24 h. The cells were harvested, washed twice with pre-chilled phosphate-buffered saline (PBS), centrifuged at 2000 r/min for 5 min, and stained with Annexin V-FITC using an assay kit. Data were analyzed using bioconsort software (USA).

Fluo-3/AM measurements of intracellular $[Ca^{2+}]_i$

Cardiomyocytes were cultured in confocal culture dishes at a density of 5×10^4 cells/mL and then exposed to 5 μM Fluo-3/AM for 30 min at 37 °C in the dark. Cardiomyocytes were washed twice with Ca^{2+} -free PBS to remove extracellular Fluo-3/AM and further incubated in DMEM. Changes in $[Ca^{2+}]_i$ were measured by the fluorescence intensity induced by Fluo-3 in cardiomyocytes recorded for 5 min using laser confocal scanning microscopy (Leica, TCS SP5II) with excitation and emission at 488 and 530 nm, respectively. Image Pro Plus 6.0 was used for analysis. In this experiment, the loaded cardiomyocytes were divided into control, H/R, $GdCl_3$, H/R+AsIV and $GdCl_3$ +AsIV groups.

Animal model of MI/R and experimental protocols

The MI/R model was established as described previously [27]. Briefly, male Sprague Dawley rats (280–300 g) were anesthetized with 20% urethane (0.3 mL/100 g, intraperitoneal). Ischemia was induced by exteriorizing the heart with a left thoracic incision followed by the placement of a slipknot (5-0 silk) around the left anterior descending coronary artery (LAD). After 30 min of ischemia, the slipknot was released, and the animal received 120 min of myocardial reperfusion. Experimental rats were randomly assigned to one of the following three groups: (1) sham group in which the silk was drilled underneath the LAD but the LAD was not ligated; (2) MI/R group in which LAD was ligated for 30 min followed by 120 min of reperfusion; (3) MI/R + 80 mg/kg of AsIV group. The dosage of AsIV was selected according to our previous study [28]. AsIV was dissolved in 0.5% sodium carboxymethylcellulose and was administered daily by gavage 7 days prior to the induction of ischemia until the animals were sacrificed.

Determination of the size of myocardial infarction and TUNEL staining

Myocardial infarction was determined by 0.5% NBT staining. The NBT-stained area (dark blue after staining, non-infarct area) and non-NBT-stained area (white area after staining, infarct area) were analyzed using a digital imaging system and a computer. The TUNEL assay was performed according to the manufacturer's protocol. The apoptosis index was expressed by the number of positively stained apoptotic cardiomyocytes/total number of cardiomyocytes counted $\times 100\%$.

Measurement of CK-MB and cTnI release in serum

Before the heart was moved, blood was collected, and serum was separated by centrifugation for the measurement of CK-MB and

cTnT levels according to the manufacturer's protocol, using a microplate reader (Thermo Scientific, USA).

Western blot analysis

Protein concentrations of the animal heart tissue and cardiomyocytes were determined using the Bradford method. After boiling the samples for 5 min, protein samples were fractionated by

SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with milk powder at room temperature for 2 h. The membranes were incubated with primary antibodies against CaSR, ERK, p-ERK, bcl-2, bax, and cleaved caspase-3 overnight. On the next day, membranes were incubated with horseradish peroxidase-linked secondary antibodies at room temperature for 1.5 h. Detection was performed with enhanced chemiluminescence reagents. The results were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules).

Statistics

All data are expressed as the mean \pm SD. SPSS 17.0 software was used to analyze all the data. Statistical analysis was performed using one-way analysis of variance followed by Bonferroni's test. $P < 0.05$ was considered statistically significant.

RESULTS

AsIV reduced cardiomyocyte injury induced by H/R

To study the therapeutic potential of AsIV, cell viability was determined by MTT assay, and cardiomyocytes injury was also assessed by determining the release of LDH in culture medium at the end of reoxygenation. The results revealed that H/R decreased cell viability and increased LDH release. AsIV treatment resulted in a significant increase in cell survival and decrease in LDH release. However, the effects of AsIV were abolished by the pretreatment with the CaSR agonist GdCl₃ or the ERK1/2 inhibitor U0126, separately (Fig. 1).

AsIV attenuated cardiomyocyte apoptosis induced by H/R

In this study, MMP was determined as an index of early myocardial apoptosis by JC-1 staining. In live cells, mitochondria appear red due to the aggregation of accumulated JC-1. In apoptotic and dead cells, the dye remains in its monomeric form, and mitochondria appear green. Therefore, a decreased ratio of red fluorescence to green fluorescence represents MMP dissipation. Annexin V-FITC/propidium iodide double staining was performed to examine the apoptotic rate of cardiomyocytes. Bcl-2, bax, and cleaved caspase-3 expression was also determined as indexes of cardiomyocyte apoptosis. The results showed that H/R induced

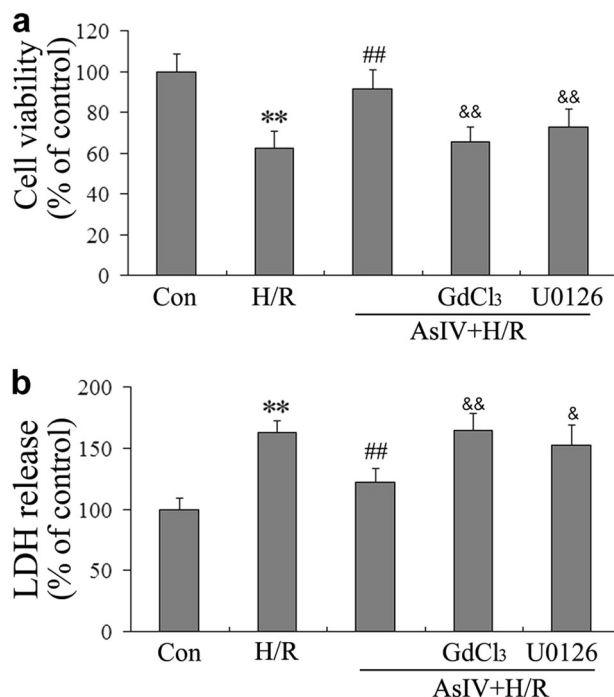


Fig. 1 AsIV decreased cardiomyocyte injury induced by H/R. **a** Cell viability was determined by MTT assay. **b** The release of LDH in culture medium at the end of reoxygenation was determined. Data represent the mean \pm SD of three independent experiments. ** $P < 0.01$ vs. Con group, ## $P < 0.01$ vs. H/R group, & $P < 0.05$, && $P < 0.01$ vs. AsIV group

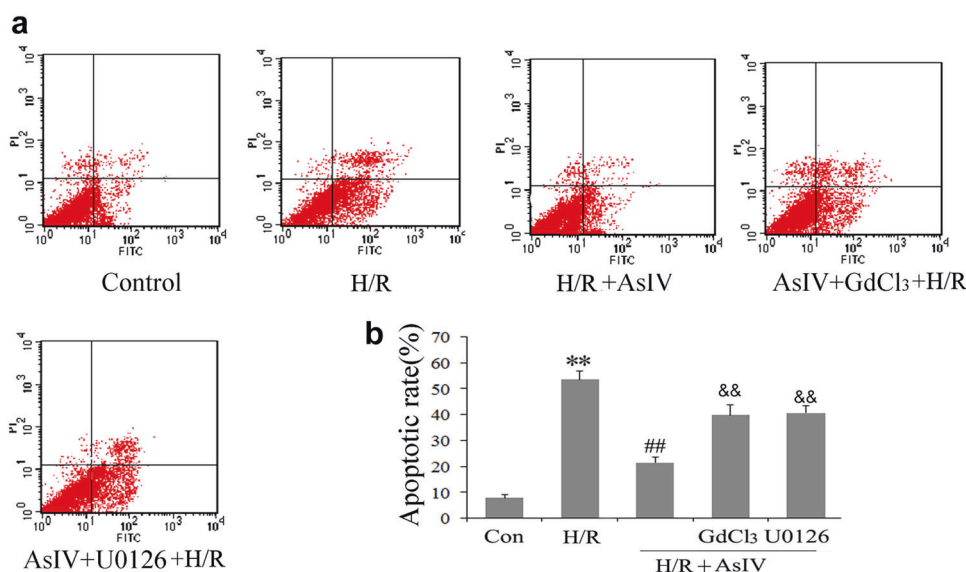


Fig. 2 AsIV attenuated cardiomyocyte apoptosis induced by H/R. **a** Apoptosis was detected in cardiomyocytes after different treatments by annexin V-FITC/PI double-staining assay. **b** Bar graphs show that the cell apoptotic rate decreased in cardiomyocytes after treatment with AsIV. Data represent the mean \pm SD of three independent experiments. ** $P < 0.01$ vs. Con group, ## $P < 0.01$ vs. H/R group, && $P < 0.01$ vs. AsIV group

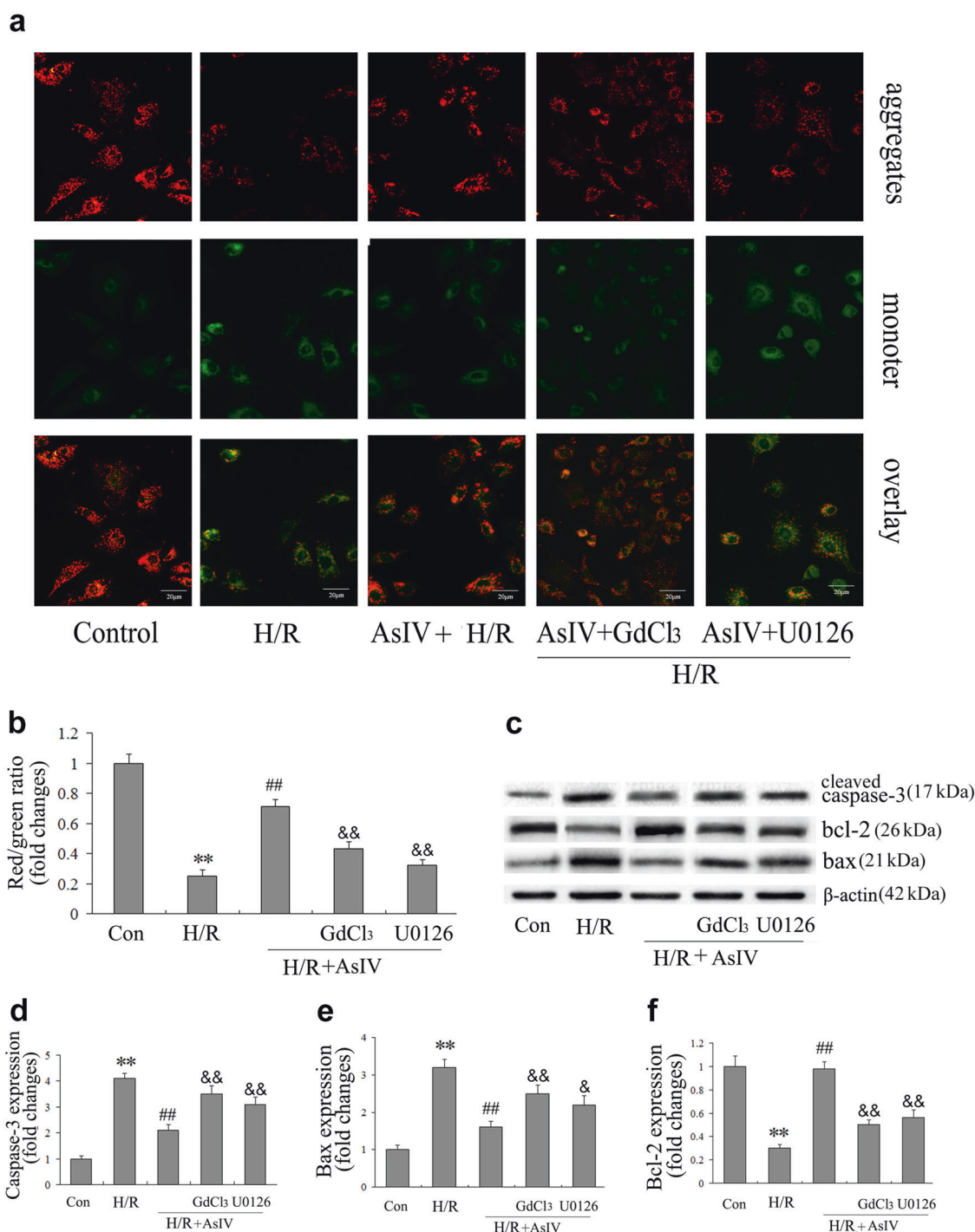


Fig. 3 AsIV increased MMP and decreased apoptosis-related proteins induced by H/R. **a, b** Cells were stained with JC-1 and MMP was assessed by fluorescence microscope. **c-f** Bcl-2, bax, and caspase-3 expressions were assessed by Western blot. Data represent the mean \pm SD of three independent experiments. ** $P < 0.01$ vs. Con group, ## $P < 0.01$ vs. H/R group, & $P < 0.05$, && $P < 0.01$ vs. AsIV group

cardiomyocyte apoptosis. Moreover, treatment with AsIV attenuated cardiomyocyte apoptosis as evidenced by the increased ratio of red fluorescence to green fluorescence and Bcl-2 expression and decreased apoptotic rate and bax and cleaved caspase-3 expression compared with the H/R group. However, the effects of AsIV were partly abolished by treatment with the CaSR agonist GdCl₃ or the ERK inhibitor U0126, separately (Figs. 2 and 3).

AsIV inhibited cardiomyocyte [Ca²⁺]_i induced by H/R and GdCl₃. To identify the effect of AsIV on [Ca²⁺]_i during H/R, we examined the change of [Ca²⁺]_i upon exposure to H/R or GdCl₃ by laser confocal scanning microscopy. The results showed that H/R or GdCl₃ increased [Ca²⁺]_i, whereas AsIV pretreatment reduced [Ca²⁺]_i compared with the H/R or GdCl₃ group (Fig. 4).

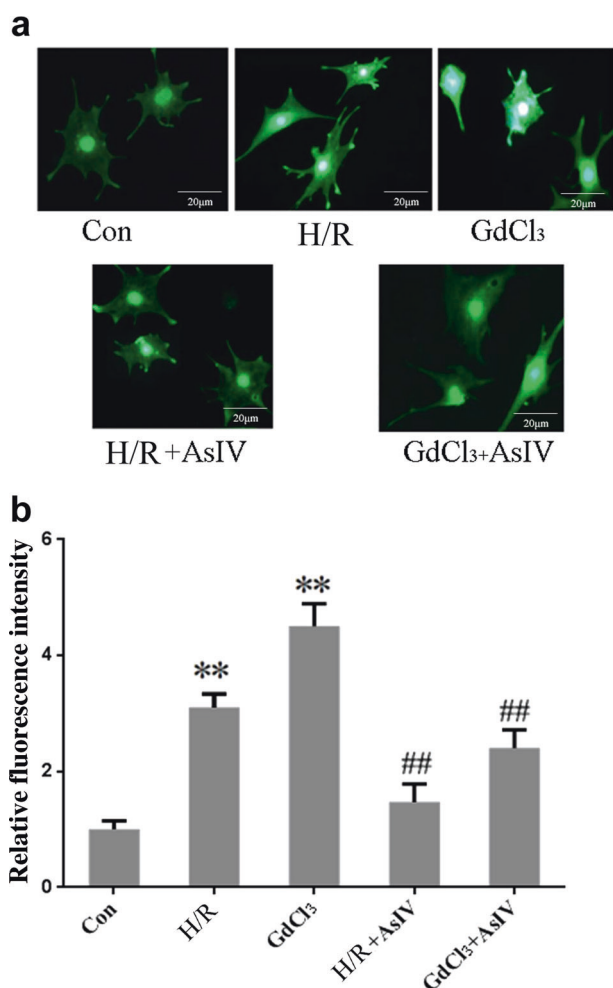


Fig. 4 AsIV inhibited cardiomyocytes $[Ca^{2+}]_i$ induced by H/R. Changes in the intensity of fluorescence of $[Ca^{2+}]_i$ were recorded with a laser scanning confocal microscope under different treatment conditions. Data represent the mean \pm SD of three independent experiments. ** $P < 0.01$ vs. Con group, ## $P < 0.01$ vs. H/R group or GdCl₃ group

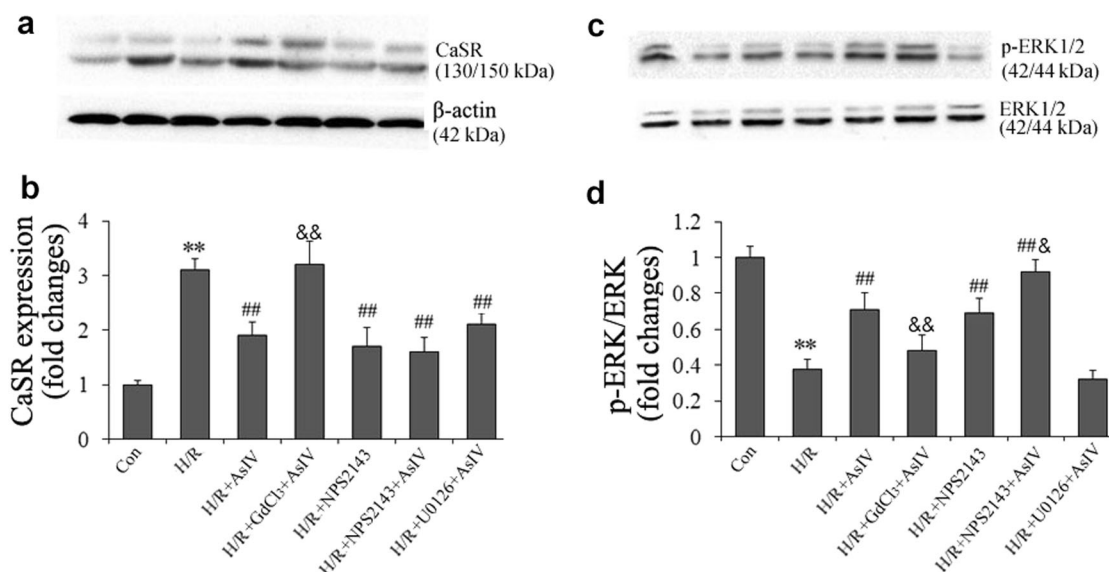


Fig. 5 AsIV decreased CaSR expression and increased phosphorylation level of ERK1/2 in cardiomyocytes. **a, b** CaSR protein expression in cardiomyocytes was determined by western blot. **c, d** ERK1/2 and p-ERK1/2 expression was determined by western blot. Data represent the mean \pm SD of three independent experiments. ** $P < 0.01$ vs. Con group, ## $P < 0.01$ vs. H/R group, & $P < 0.05$, && $P < 0.01$ vs. AsIV group

AsIV decreased CaSR protein expression and increased p-ERK1/2 protein expression. As we demonstrated that AsIV inhibited cardiomyocyte apoptosis and enhanced $[Ca^{2+}]_i$ induced by H/R, we then investigated the possible mechanisms involved. AsIV significantly decreased the CaSR expression and increased p-ERK1/2 expression in cardiomyocytes compared with the H/R group. Furthermore, the effect of AsIV on CaSR was completely offset by GdCl₃. To further investigate the relationship between CaSR and ERK1/2 involvement in the protective effect of AsIV, the CaSR inhibitor NPS2143 was used in this assay. Interestingly, AsIV in combination with the ERK1/2 inhibitor U0126 has no effect on CaSR expression, but AsIV in combination with the CaSR inhibitor NPS2143 further increased p-ERK expression compared with AsIV treatment alone. These results suggested that the CaSR-mediated p-ERK pathway may be involved in the protective effect of AsIV on H/R injury (Fig. 5).

AsIV reduced myocardial injury in MI/R rats. To examine how AsIV influences ischemia/reperfusion-induced myocardial injury, we measured infarct size and the release of CK-MB and cTnI in serum. The area with light white color represented the infarction region, and the area with the dark blue represented normal myocardial tissue. AsIV treatment significantly reduced infarct size and the release of CK-MB and cTnI in serum compared with the MI/R group (Fig. 6).

AsIV decreased myocardial cell apoptosis. In the present study, the TUNEL assay was performed using a commercial kit to investigate the anti-apoptotic effect of AsIV. The myocardium in MI/R animals exhibited extensive nuclear changes. In comparison, AsIV treatment resulted in a marked reduction in the number of TUNEL-positive cells. In addition, AsIV treatment also reduced bax and cleaved caspase-3 protein expression and increased bcl-2 protein expression (Fig. 7).

AsIV reduced CaSR protein expression and increased p-ERK1/2 protein expression in myocardial tissue. To further investigate the role of CaSR and ERK1/2 in the cardioprotective effect of AsIV against MI/R, CaSR and p-ERK1/2 protein expression in myocardial tissue was determined.

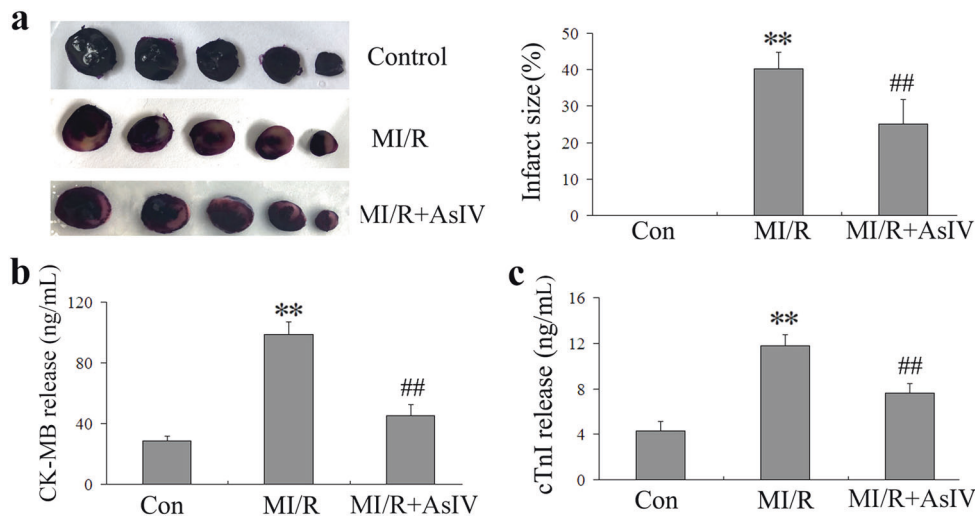


Fig. 6 AsIV decreased myocardial injury in MI/R rats. **a** Representative photomicrographs of heart sections stained with NBT. **b** Serum CK-MB activity. **c** Serum cTnI activity. Data represent the mean \pm SD for eight rats in each group. ** $P < 0.01$ vs. Con group, ## $P < 0.01$ vs. MI/R group

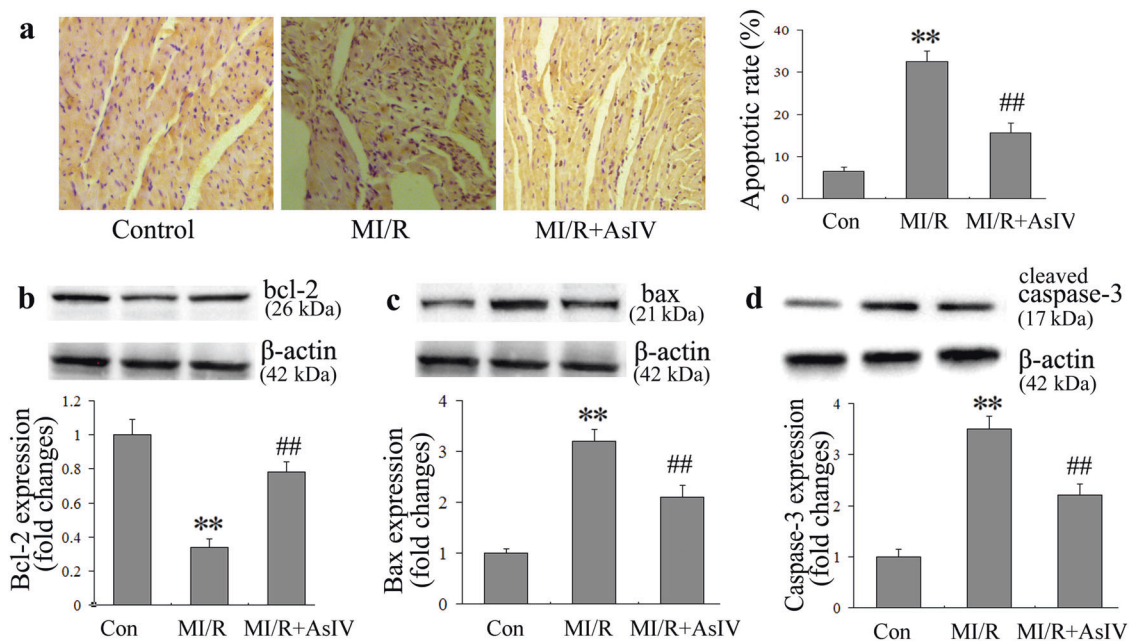


Fig. 7 AsIV decreased myocardial cells apoptosis. **a** Cell apoptosis assayed by TUNEL staining. TUNEL-positive cells were manifested as a marked appearance of dark brown apoptotic cell nuclei. **b–d** Bax, bcl-2, and cleaved-caspase-3 expressions assayed by Western blot. Data represent the mean \pm SD of three independent experiments. ** $P < 0.01$ vs. Con group, ## $P < 0.01$ vs. MI/R group

Consistent with in vitro results, AsIV treatment also reduced CaSR and increased p-ERK1/2 expression in myocardial tissue (Fig. 8).

DISCUSSION

Recently, a number of studies illustrated the benefits of AsIV treatment in MI/R injury. These actions were related to upregulation of hypoxia-inducible factor-1 α expression, antioxidation, anti-inflammation, energy metabolism regulation, and upregulation of several K_{ATP} channel subunits [29–31]. Cardiomyocyte apoptosis is one of the major pathogenic mechanisms underlying MI/R injury, which is initiated shortly after ischemia, accelerated by reperfusion, and partially contributes to overall cardiomyocyte death [32]. Timely and effective inhibition of apoptosis process could prevent the loss of cardiomyocytes, minimize cardiac injury induced by

both ischemia and reperfusion, and reduce or even prevent the occurrence of heart failure [33, 34]. In our previous study, we demonstrated that AsIV treatment attenuated MI/R injury via inhibition of Toll-like receptor 4- and nuclear factor- κ B-mediated inflammatory responses and subsequent myocardial apoptosis in a rat model [28]. The present study demonstrated that AsIV treatment attenuated myocardial injury, reduced cardiomyocyte apoptosis, decreased $[Ca^{2+}]_i$, inhibited CaSR expression, and increased ERK1/2 phosphorylation levels. These findings not only provided the underlying mechanisms of the cardioprotective effect of AsIV but also further demonstrated the pivotal role of CaSR in MI/R.

CaSR was first cloned in 1993 from bovine parathyroid gland [35] and discovered in rat cardiac tissue by Wang et al. [36]. It was subsequently reported that CaSR is expressed in rat neonatal ventricular cardiomyocytes, regulates systemic calcium

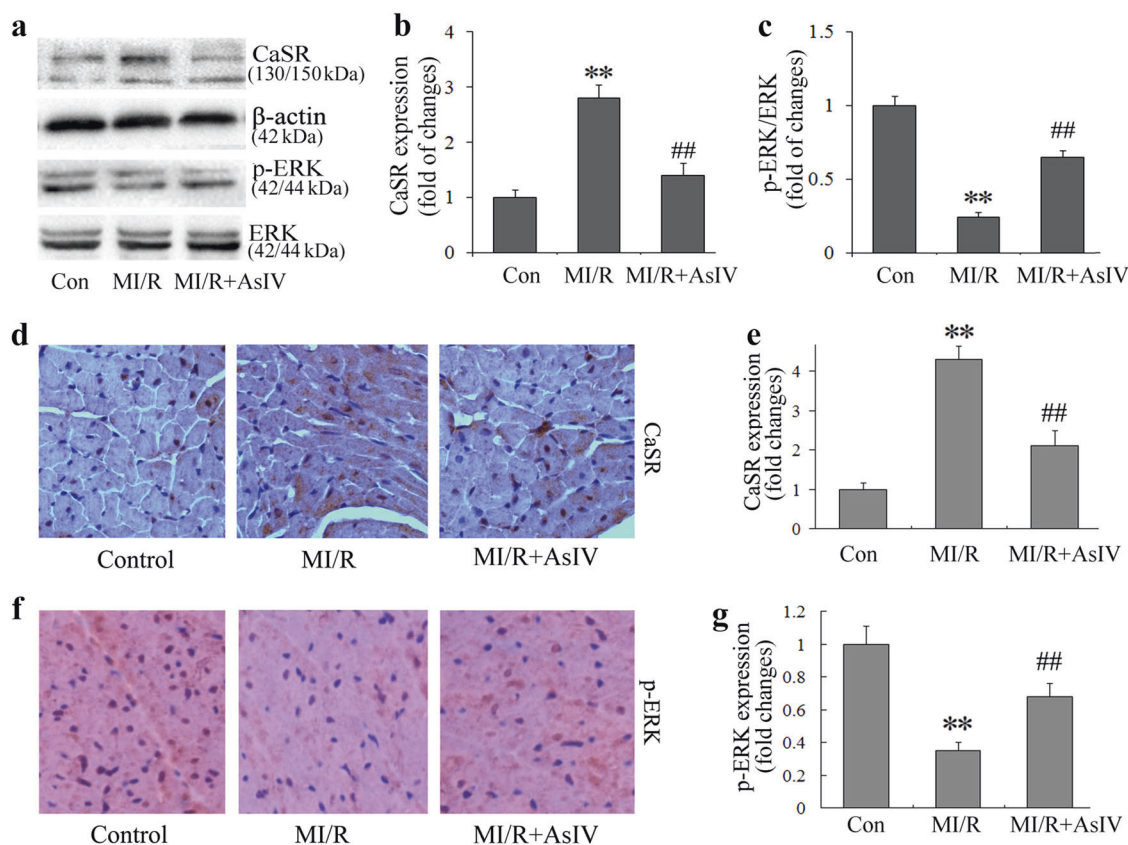


Fig. 8 AsIV decreased CaSR expression and increased ERK1/2 phosphorylation level in myocardial tissue. **a–c** CaSR and ERK1/2 protein expression in myocardial tissue was determined by Western blot. **d–g** CaSR and p-ERK1/2 expression in myocardial tissue was determined by immunohistochemistry. Data represent the mean \pm SD of eight independent photographs. ** $P < 0.01$ vs. Con group, ## $P < 0.01$ vs. MI/R group

homeostasis, and participates in cell proliferation, differentiation, and apoptosis [14]. As a GPCR, CaSR can be activated by extracellular Ca^{2+} (and Mg^{2+}), polyamines, amino acids, and neomycin, and increase $[\text{Ca}^{2+}]_i$ via the phospholipase C and inositol 1,4,5 triphosphate pathways. Increased $[\text{Ca}^{2+}]_i$ by CaSR contributes to cardiac hypertrophy induced by angiotensin II and MI/R, and increases the sensitivity of cardiomyocytes to acute myocardial infarction [24, 37]. In addition, CaSR also arguments mitochondrial Ca^{2+} via sarcoplasmic reticulum release and induces cardiomyocyte apoptosis through the sarcoplasmic reticulum and mitochondrial apoptotic pathway in heart failure [38]. The translocation of phosphorylated PKC δ to mitochondria was significantly increased when the heart was subject to ischemia reperfusion combined with CaSR agonist GdCl_3 . In addition, myocardial apoptosis, ERK1/2, protein kinase B (AKT), and glycogen synthase kinase-3 β contribute to the effect of CaSR on promoting MI/R [19, 39]. Our present study revealed that myocardial apoptosis was accompanied with increased $[\text{Ca}^{2+}]_i$ and upregulation of CaSR expression during H/R. These findings were consistent with previous studies. The novel finding of the present study is that AsIV treatment markedly decreased $[\text{Ca}^{2+}]_i$ and reduced CaSR expression in parallel with reduced cardiomyocyte apoptosis and myocardial injury. Moreover, the downregulation of CaSR and protective effect of AsIV were partly abolished upon pretreatment with the CaSR agonist GdCl_3 , indicating that downregulation of CaSR contributes to the protective effect of AsIV on MI/R injury.

To gain additional insight into the downstream signaling events following CaSR activation during MI/R, we analyzed the ERK1/2 signaling pathway *in vivo* and *in vitro*. As the primary pathway of the MAPK family, the ERK1/2 cascade determines cell proliferation, differentiation, cellular transcriptional activities, and

cell survival [40]. The ERK1/2 cascade plays a critical role in myocardial protection against MI/R injury and is involved in ischemic preconditioning and delayed pharmacological preconditioning [41]. Consistent with previous studies, ERK1/2 was inactivated during H/R injury in the present study. Of note, AsIV treatment not only reduced H/R injury but also activated ERK1/2, and the protective effects of AsIV on H/R was partly abolished by the ERK1/2 inhibitor U0126. In addition, AsIV in combination with the CaSR inhibitor NPS2143 could further increase p-ERK expression compared with AsIV treatment alone. These results indicated that activation of ERK1/2 might be involved in the cardioprotective effect of AsIV on MI/R. A previous study demonstrated that CaSR induced hippocampus apoptosis during H/R through ERK1/2 phosphorylation [42]. Enhanced porcine bone marrow mesenchymal stem cell proliferation in response to extracellular calcium is associated with CaSR and ERK1/2 signaling pathway activation [43]. With respect to myocardial tissue, increased CaSR expression promoted cardiomyocyte apoptosis and activated ERK1/2, which contributed to the acceleration of MI/R injury [17]. Treatment with the ERK1/2 inhibitor PD98059 or U0126 caused significant attenuation of ERK1/2 phosphorylation and enhancement of apoptosis in the context of MI/R. However, in the present study, U0126 in combination with AsIV had no effect on CaSR expression compared with AsIV alone, whereas the effect of AsIV on p-ERK1/2 expression was partially offset by the CaSR agonist GdCl_3 . These differences may due to the complexity and interaction between different signaling pathways, and further studies using signal specific inhibitors or small interfering RNAs are needed.

In summary, we previously demonstrated that AsIV protects myocardium from MI/R injury *in vivo*. Our present study extended these studies in both rat and cell models and demonstrated for

the first time that the protective effect of AsIV against MI/R injury was related with downregulation of CaSR, activation of ERK1/2, and a subsequent reduction in cell apoptosis. These findings might be meaningful to further understand the molecular mechanisms of AsIV cardioprotection.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No 81703739), the Natural Science Foundation of Liaoning Province (No 201602284), and the Innovative Training Program for College Students of Liaoning Province (No 201610160000040).

AUTHOR CONTRIBUTIONS

BY and MLL designed the research. BY, XWH, and MLL performed the research. BY analyzed the data. MLL wrote the paper.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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