

## Original Article

# Astragaloside IV prevents myocardial hypertrophy induced by mechanical stress by activating autophagy and reducing inflammation

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**Abstract:** Aims: The aim of the present study was to investigate the effects of astragaloside IV (As-IV) on mechanical stress-induced myocardial hypertrophy, with a focus on autophagy and inflammation. Methods: A rat cardiac hypertrophy model was established by narrowing the abdominal aorta, and a cell hypertrophy model was established by mechanically stretching primary cardiomyocytes. Cardiac function index and cardiac hypertrophy were measured by echocardiography, heart weight index (HWI) and left ventriculus weight index (LVWI) in vivo. Cell size was measured by phalloidin-tetramethyl treatment in vitro, while hematoxylin and eosin (HE) staining was used to observe the arrangement and morphology of myocardial cells. The expression of ANP, BNP, LC3II, p62, NLRP3, and IL-1 $\beta$  in both myocardial tissue and cardiomyocytes was assessed by Western blot, while TNF- $\alpha$  and IL-18 levels in serum and cell supernatants were measured by ELISA. Results: In the aortic banding model, the cardiac function index LVEF was decreased; the hypertrophy indexes LVPWd, LVPWs, IVSd and IVSs were significantly increased; cardiomyocytes were enlarged and disordered; the expression levels of ANP, BNP, NLRP3, IL-1 $\beta$  and p62 were increased; and LC3II expression was decreased in both myocardial tissue and cardiomyocytes. As-IV could significantly improve cardiac function and cardiomyocyte morphology and limit hypertrophy, thereby protecting damaged hearts, while rapamycin had a similar effect as As-IV. In addition, As-IV decreased the expression of NLRP3 and IL-1 $\beta$  and activated autophagy, as evidenced by increased LC3II expression and decreased p62 levels. Conclusion: As-IV prevents myocardial hypertrophy induced by mechanical stress by activating autophagy and reducing inflammation.

**Keywords:** Astragaloside IV, mechanical stress, myocardial hypertrophy, NLRP3, autophagy

## Introduction

Myocardial hypertrophy is an adaptive compensatory response that primarily occurs during long-term stress overload and can maintain adequate cardiac output at the early stage. If the stimulating factor persists, myocardial hypertrophy will gradually develop into irreversible myocardial systolic dysfunction, known as heart failure.

Autophagy is a highly conserved process in which waste proteins or organelles are delivered to lysosomes to achieve the metabolic needs of the cell and the renewal of specific organelles [1]. Increasing evidence shows that autophagy plays a crucial role in cardiac remodeling to maintain cardiac function and homeo-

stasis in heart cells, with autophagy-mediated degradation having been shown to be important for the development of cardiomyocyte hypertrophy [2, 3].

Recent studies have shown that the NLRP3 inflammatory complex is a key factor in initiating the inflammasome response, and it is well known that immune regulation and inflammation are involved in cardiac remodeling. Inflammasome assembly leads to the production of proinflammatory cytokines, such as IL-1 $\beta$  and IL-18, promoting their maturation, activation and secretion [4, 5] and leads to triggering of the inflammatory response [6, 7].

The NLRP3 and autophagy pathways are interregulated, and autophagy can downregulate

the secretion of IL-1 $\beta$  or directly control the activation of the inflammasome [8, 9]. Hypoxia activates autophagy to reduce inflammation in enteritis [6]. In the vascular endothelium, the autophagy inhibitor 3-methyladenine (3-MA) has been shown to aggravate the activation of the NLRP3 inflammasome [10], and an increase in autophagy activity decreases the expression of NLRP3 [11].

Under normal conditions, reactive oxygen species (ROS) levels in organisms are maintained in a stable range and play an active role in anti-inflammatory and antibacterial activities. However, if this balance is disturbed and ROS levels continue to rise, excessive ROS will activate NLRP3 [12, 13].

Astragalus is the dried root of the legume *Astragalus mongolicus* or *Astragalus membranaceus* and has been used in the treatment of cardiovascular diseases, hepatitis, nephropathy and skin diseases. Astragaloside IV (As-IV) is one of the primary active components of *Astragalus membranaceus* and has a wide range of pharmacological effects, including anti-inflammatory [14] and anti-cardiac hypertrophy effects [15]. However, the mechanism by which As-IV functions in myocardial hypertrophy is unclear. In particular, we investigated whether astragaloside IV prevents cardiac hypertrophy by regulating autophagy and inflammation.

Thus, in the present study, we assessed whether As-IV can reduce myocardial hypertrophy by activating autophagy to alleviate inflammation and to investigate its potential mechanism.

### Materials and methods

#### Reagents

As-IV was purchased from Nanjing Jingzhu Biotechnology Co., Ltd. (Nanjing, China). Rapamycin (Rapa) was purchased from Solarbio Co., Ltd. (Beijing, China). ELISA kits were purchased from Beijing Chenglin Biological Technology Co., Ltd. (Beijing, China). Antibodies against  $\beta$ -actin, BNP and ANP were purchased from ABclonal (Wuhan, China). Antibodies against p62 and LC3I/LC3II were purchased from Proteintech (Wuhan, China). Antibodies against NLRP3 and IL-1 $\beta$  were purchased from Abcam

(Cambridge, UK). BioFlex Collagen Type I culture plates were purchased from Flexcell® International Corporation.

#### Animal model

Healthy male Sprague-Dawley rats weighing 200-250 g were purchased from Liaoning Changsheng Biotechnology Incorporated Company (Benxi, China). Sixty rats were randomly divided into five groups, and myocardial hypertrophy was induced by ligation of the abdominal aorta with a 0.7 mm silver clip (aortic banding). The surgery treatment rats were divided into four groups: the aortic banding group (AB), the 40 and 80 mg/kg/d As-IV (intragastric administration) treatment groups and the 1 mg/kg/d Rapa (intraperitoneal injection) treatment group. The sham-operated group had a similar operation, except for the application of a silver clip. The rats received an intraperitoneal injection of gentamicin for a week after the surgery, after which the surviving rats were administered the treatments for 6 weeks.

#### Echocardiography

Rats were anesthetized with 7% chloral hydrate (0.5 ml/100 g), maintained in a supine position, and skin preparation (shaving) was performed, after which a Philips IE33 echocardiographic system was used to perform two-dimensional (2D)-guided M-mode echocardiography (Philips Medical Systems Nederland BV). The following parameters were measured: left ventricular ejection fraction (LVEF), left ventricular posterior wall end-diastolic thickness (LVPWd), left ventricular posterior wall end-systolic thickness (LVPWs), interventricular septum end-diastolic thickness (IVSd), and interventricular septum end-systolic thickness (IVSs).

#### Heart weights

Both the heart weight index (heart weight/body weight ratio, HWI = HW/BW) and left ventricular weight index (left ventricular weight/body weight ratio, LVWI = LVW/BW) were calculated from the results of the associated measurements.

#### HE staining and immunohistochemical analyses

Standard hematoxylin and eosin (HE) staining was performed, and the slides were sealed

with a neutral resin seal. Heart pathological changes were observed and imaged under a Leica DMI 3000B microscope. The expression of NLRP3 was observed by immunohistochemical assays, and images were obtained with a Leica DMI 3000B microscope.

## *Enzyme-linked immunosorbent assay (ELISA)*

The serum and cell supernatant levels of TNF- $\alpha$  and IL-18 were measured using commercial ELISA kits according to the manufacturer's instructions.

## *Cell culture and treatment*

SPF-grade one-day-old Sprague-Dawley rats were provided by the Animal Center of Jinzhou Medical University. Rat primary cardiomyocytes were isolated and cultured in DMEM with 10% fetal bovine serum (HyClone, USA), after which primary cardiomyocytes were placed into Bio-Flex Plate-Collagen Type. After 48 hours of cultivation, serum-free DMEM was added, and 100  $\mu$ M As-IV or 100 nM Rapa was administered in the corresponding treatment groups. The isolated primary cardiomyocytes were stretched for 24 hours using a Flexcell FX-5000 Tension System (1 Hz, 20% elongation). The control group was not stretched, and the inhibitor group was administered 3-MA without stretching. After the procedure, each group of cells was collected for subsequent experiments.

## *ROS production measurement by ethidium fluorescence analysis*

According to previous experimental reports, ROS production in AB rat heart tissues was determined by dihydroethidium (DHE) staining [16]. The detection of ROS levels in cells was performed using the same method.

## *Measurement of myocardial size by phalloidin-tetramethyl treatment*

Each group of cells was washed three times with PBS, fixed with 4% paraformaldehyde for 30 minutes, and then rinsed with PBS three times. Subsequently, phalloidin-tetramethyl was added in the dark at 37°C for 30 minutes. After three washes in PBS, images were acquired with a fluorescence microscope.

## *Western blot analysis*

Total protein was extracted from left ventricular myocardial homogenate and cell supernatant samples in each group, and the protein concentration was quantified using a BCA Protein Assay kit (Beyotime Biotechnology, China). After quantification, protein samples (25  $\mu$ g) were separated by SDS-PAGE (6-12%) and transferred onto PVDF membranes. Then, the PVDF membranes were immersed in a 1% bovine serum albumin solution and rocked slowly for 2 hours. The membranes were then incubated with primary antibodies against  $\beta$ -actin, ANP, BNP, NLRP3, IL-1 $\beta$ , LC3, or p62 at 4°C overnight, after which they were incubated with a rabbit secondary antibody at room temperature for 2 hours. The protein bands were subsequently detected using an ECL kit and scanned using a Bio-Rad imaging system (Bio-Rad).

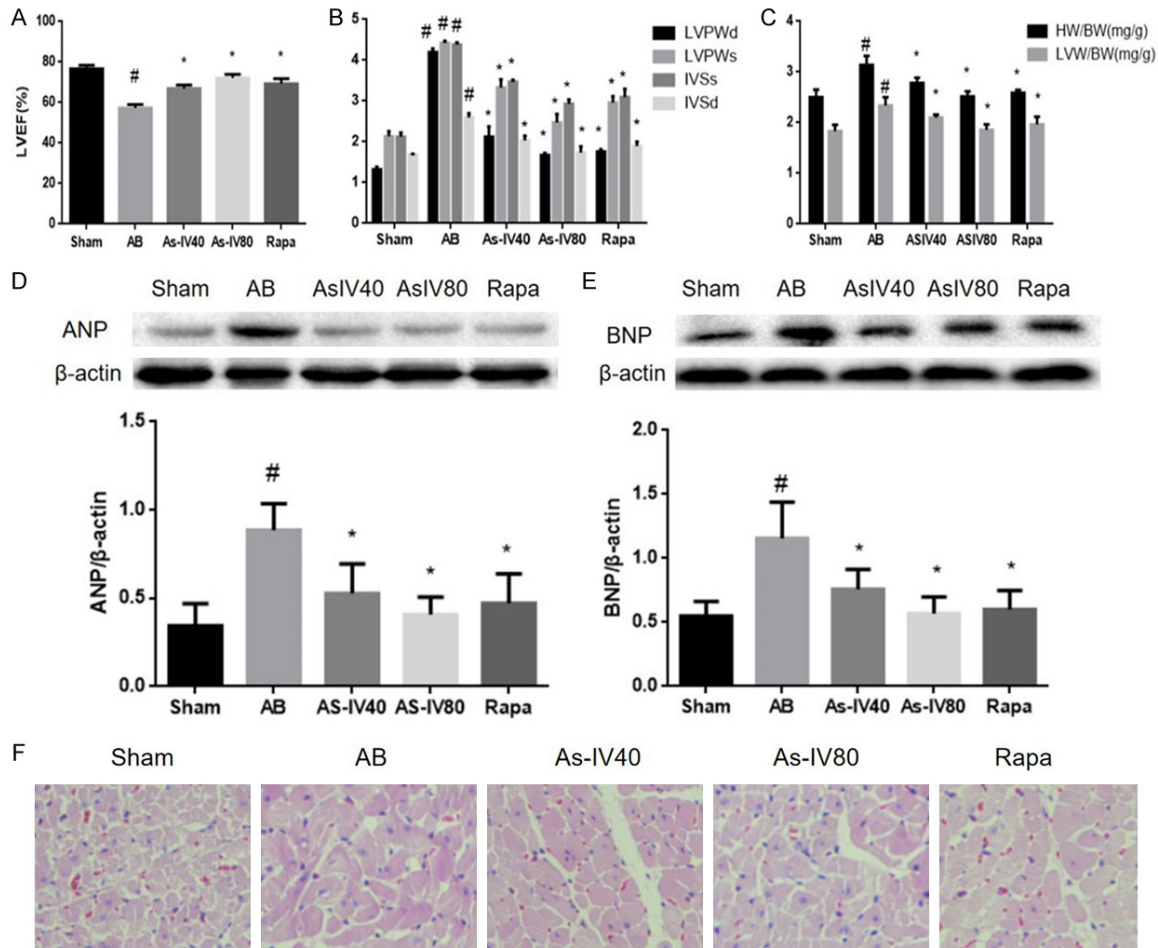
## *Statistical analysis*

The data are presented as the means  $\pm$  standard deviation (SD). Statistical analysis was performed using one-way analysis of variance followed by Tukey's test for all experimental analysis. The data were analyzed using SPSS 23.0, and *P* values < 0.05 were considered significant.

## **Results**

### *Astragaloside IV improves cardiac function and reduces myocardial hypertrophy in AB rats*

To determine whether As-IV can improve cardiac function and reduce myocardial hypertrophy, we measured cardiac function and hypertrophy-related indexes by echocardiography, heart weight, HE staining and Western blot analyses. Compared with that observed in the sham group, the LVEF (**Figure 1A**) decreased in the AB group. After 6 weeks of treatment with As-IV or Rapa, the LVEF significantly increased compared with that detected in AB rats. Compared with those observed in the sham group, the cardiac hypertrophy indexes LVPWd, LVPWs, IVSd, and IVSs (**Figure 1B**), HWI, and LVWI (**Figure 1C**) and the levels of hypertrophic marker proteins ANP (**Figure 1D**) and BNP (**Figure 1E**) were significantly increased in the AB rats. After 6 weeks of treatment with As-IV or Rapa, these hypertrophy indexes decreased



**Figure 1.** Evidence that astragaloside IV treatment can improve cardiac function and reduce the myocardial hypertrophy induced by AB at 6 weeks. (A) Left ventricular ejection fraction. (B) Hypertrophy indexes. (C) Heart weight index (HWI = heart weight/body weight) and left ventricular weight index (LVWI = left ventricular weight/body weight) measurements. (D and E) Expression of hypertrophic marker proteins ANP and BNP as detected by Western blot. (F) HE staining. Sham, sham operation group; AB, aortic banding group; As-IV40, 40 mg/kg astragaloside IV-treated aortic banding group; As-IV80, 80 mg/kg astragaloside IV-treated aortic banding group; Rapa, rapamycin-treated aortic banding (positive control) group. Values are presented as the means  $\pm$  SD,  $n = 4$  for (A and C),  $n = 5$  for (D-F).  $\#P < 0.05$  compared with the sham operation group;  $*P < 0.05$  compared with the model group.

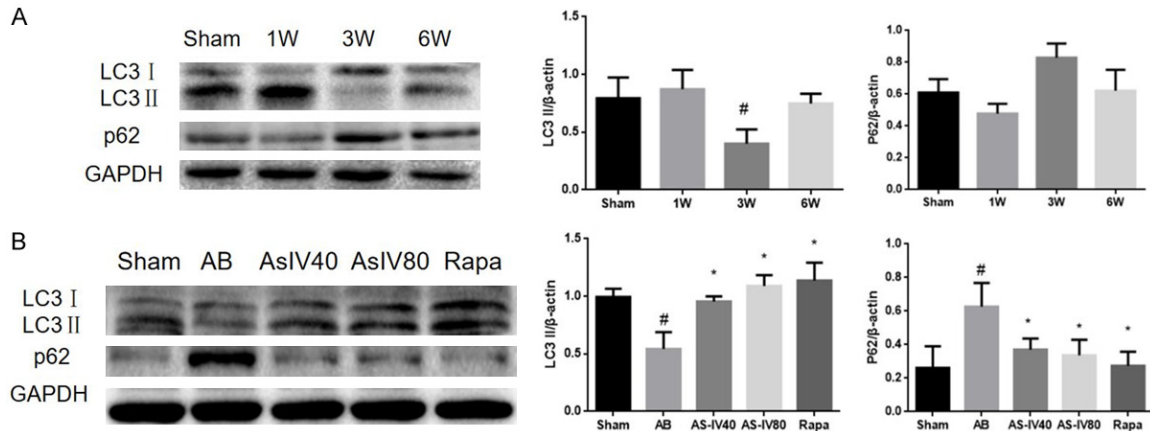
significantly compared with those observed in the AB rats. As shown in **Figure 1F**, HE staining results showed that the morphology of cardiac myocytes in the sham group was normal and that myocardial fibers were arranged neatly, while those in the AB rats were hypertrophied, and the myocardial fibers were disordered. However, after treatment with As-IV or Rapa, the cardiac myocyte morphology and fiber arrangement in the AB rats were obviously improved.

#### *Astragaloside IV can activate autophagy in the early stage of hypertrophy in AB rats*

To assess the temporal changes in autophagy in AB rats, a cardiac hypertrophy model was

generated by aortic banding, and the time-course expression of the autophagy marker proteins LC3II and p62 in the myocardial tissue of rats was measured by Western blot analysis 1, 3 and 6 weeks after surgery. As shown in **Figure 2A**, no significant differences in LC3II and p62 levels were observed between the AB and sham operation groups 1 week after surgery. At 3 weeks after surgery, LC3II was significantly downregulated and p62 was upregulated, suggesting that autophagy was inhibited in the early stage of hypertrophy. Compared with the levels observed at 3 weeks, LC3II was significantly upregulated and p62 was downregulated at 6 weeks after surgery, suggesting that autophagy was activated with the progression of the disease.





**Figure 2.** Astragaloside IV can activate autophagy in AB rats. A: Evidence of the temporal changes in autophagy in AB rats, autophagy is suppressed in the early stages of myocardial hypertrophy, and autophagy gradually increases as the disease progresses. The results demonstrate that excessive autophagy accelerates disease progression. B: Effect of As-IV on the autophagy-related proteins LC3II and p62 assessed by Western blot. Values are presented as the means  $\pm$  SD,  $n = 3$ . <sup>#</sup> $P < 0.05$  vs the sham group; <sup>\*</sup> $P < 0.05$  vs the AB group.

Taken together, the above results demonstrate that autophagy changes dynamically during the development of hypertrophy, where autophagy was inhibited in the early stage and suppressed with the aggravation of hypertrophy. Therefore, we chose the rats treated with drugs for 3 weeks to explore the effect of As-IV on autophagy. As shown in **Figure 2**, when the sham and AB groups were compared at 3 weeks after aortic banding, the levels of the autophagy marker protein LC3II decreased, while those of p62 increased significantly in AB rats, as demonstrated by Western blot analysis. Compared with those observed in the AB group, the levels of the autophagy marker protein LC3II were upregulated, while that of p62 decreased significantly in the groups treated with As-IV or Rapa.

#### *Astragaloside IV can reduce the expression of NLRP3 and that of its downstream inflammatory factors in AB rats*

We next investigated the effect of As-IV on inflammation in the aortic banding model, as shown in **Figure 3A**. The Western blot results showed that the expression of NLRP3 in AB rats was significantly higher than that observed in the sham operation group, while the expression of these proteins was significantly reduced in the rats in the treatment group. The immunohistochemistry results shown in **Figure 3B** demonstrated that the expression of NLRP3 in

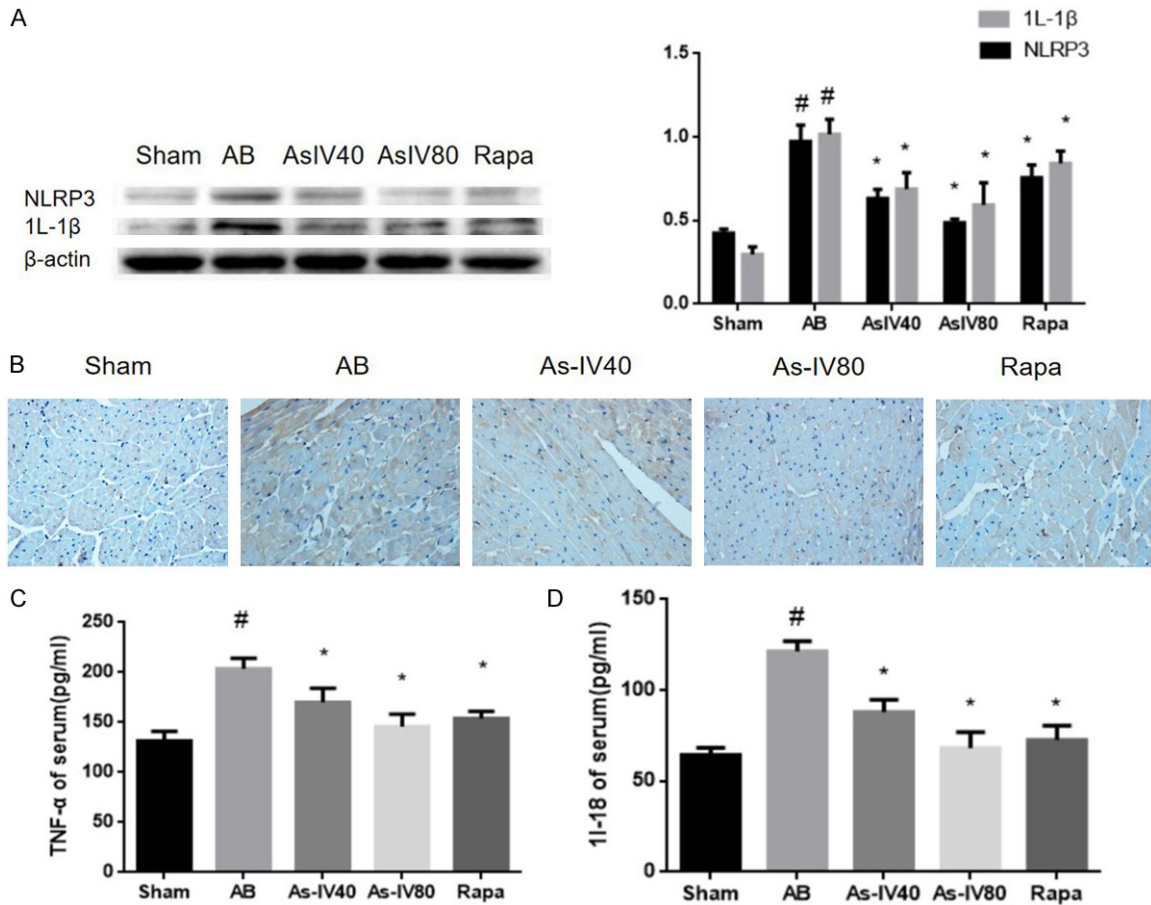
AB rats was significantly increased, while that observed in the treatment group was significantly decreased. Furthermore, the results of IL-1 $\beta$  and IL-18 (**Figure 3C** and **3D**) measurements by ELISA were similar to those observed by Western blot analysis, indicating that As-IV had a specific therapeutic effect on inflammation.

#### *Astragaloside IV can improve cardiomyocyte hypertrophy in vitro*

As-IV can improve cardiomyocyte hypertrophy by reducing the expression of ANP and BNP. In the phalloidin treatment experiment (**Figure 4A**), the primary cardiomyocytes in the stretch group were significantly enlarged, while those in the groups treated with As-IV or Rapa were reduced in size, where the protective effect of the As-IV treatment on hypertrophic cardiomyocytes was more obvious. Western blotting results showed that the cells in the stretch group exhibited markedly enhanced ANP and BNP expression (**Figure 4B**), while the administration of As-IV or Rapa significantly decreased the expression of these proteins.

#### *Astragaloside IV can activate autophagy in hypertrophic cardiomyocytes*

LC3II and p62 play major roles in autophagy. As shown by Western blot analysis (**Figure 5**), compared with that observed in the control



**Figure 3.** Evidence that astragaloside IV can reduce the expression of NLRP3 and that of its downstream inflammatory factors in AB rats. (A) Effect of As-IV on the NLRP3 inflammasome and IL-1 $\beta$  as assessed by Western blot. (B) Effect of As-IV on NLRP3 as assessed by immunohistochemistry assays. (C and D) Effect of As-IV on the serum inflammation-related factors TNF- $\alpha$  and IL-18. Values are presented as the means  $\pm$  SD,  $n = 3$  for (A),  $n = 8$  for (C and D). # $P < 0.05$  vs the sham group; \* $P < 0.05$  vs the AB group.

group, autophagy was significantly inhibited in the stretch group, while treatment with As-IV or Rapa could significantly activate autophagy.

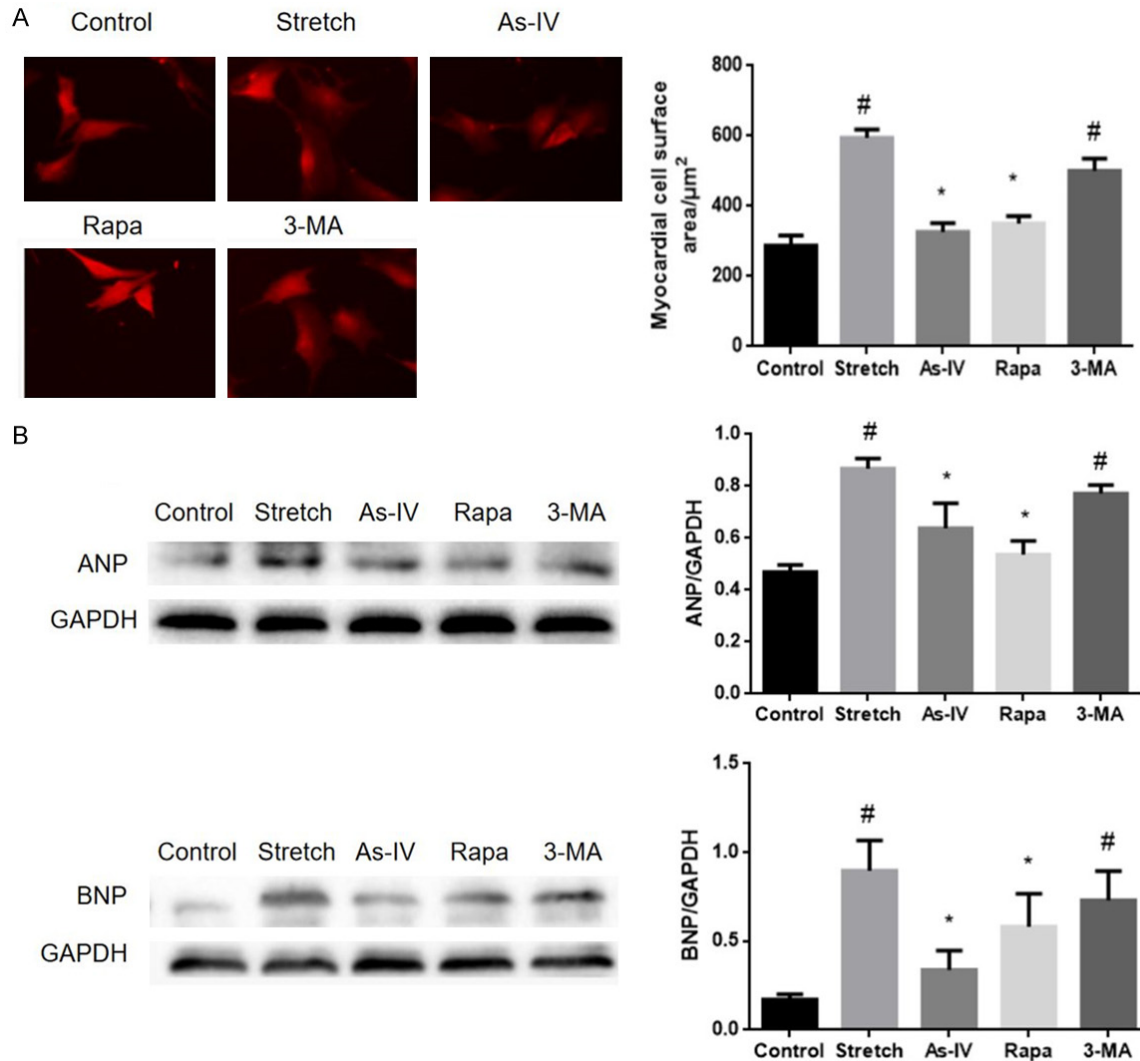
#### Astragaloside IV can reduce inflammatory response in hypertrophic cardiomyocytes

Western blotting results (**Figure 6A**) showed that the expression of NLRP3 and IL-1 $\beta$  in the stretch group was significantly increased compared with that observed in the primary myocardial cells in the control group. Interestingly, the inflammatory response in the groups treated with As-IV or Rapa decreased, while that observed in cells treated with the autophagy inhibitor 3-MA was increased. In addition, the experimental results from the TNF- $\alpha$  and IL-18

in the ELISA experiment were consistent with those of the Western blotting assay (**Figure 6B**).

#### Astragaloside IV can reduce ROS levels in the myocardial hypertrophy model

To investigate the effect of ROS in the myocardial hypertrophy model, ROS levels in rat myocardial tissue and primary cardiomyocytes were measured using DHE. As shown in in vivo and in vitro assays (**Figure 7A** and **7B**), ROS levels in the model group were significantly higher than those observed in the control group, while those detected in the groups treated with As-IV or Rapa decreased significantly, and those in the 3-MA group were increased. These results suggest that activat-



**Figure 4.** A: Phalloidin treatment experiment. B: Western blot analysis of ANP and BNP. When the autophagy of cardiomyocytes is inhibited, it will also be accompanied by cell hypertrophy. Astragaloside IV can improve cardiomyocyte hypertrophy in vitro. Values are presented as the means  $\pm$  SD,  $n = 3$ . <sup>#</sup> $P < 0.05$  vs the control group; <sup>\*</sup> $P < 0.05$  vs the stretch group.

ing autophagy at a specific level can reduce ROS and protect the hypertrophic heart tissue.

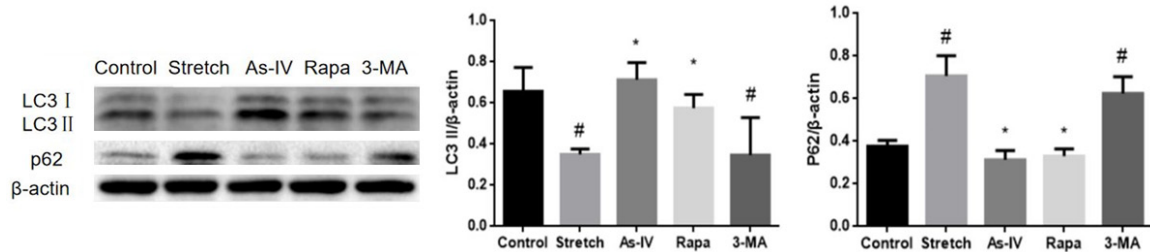
### Discussion

In the present study, we demonstrated that As-IV can attenuate cardiac hypertrophy and improve cardiac function in both AB rats and stretched cardiomyocytes. Moreover, As-IV-mediated cardioprotection was achieved by activating autophagy, decreasing inflammation, preventing the accumulation of ROS, and subsequently downregulating the NLRP3 signaling pathway.

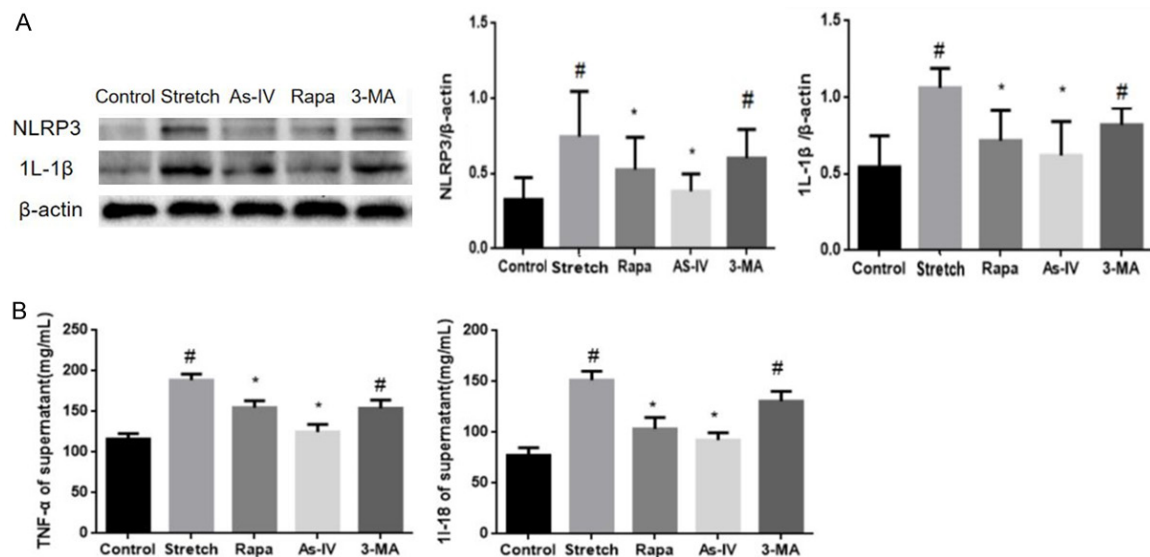
In the aortic banding model, the heart function of AB rats was significantly impaired, the size

and structure of the hearts were altered, and the expression of hypertrophy-related proteins was increased compared to that observed in the control group. A variety of data showed that myocardial hypertrophy had occurred in AB rats at this time. As-IV could reverse myocardial hypertrophy, which was consistent with the results of previous studies [14, 15], and could also reduce cell size.

Autophagy is a process of self-protection that maintaining cardiac function and cell morphology by eliminating damaged organelles and proteins. The loss of autophagy may disrupt protein homeostasis and increase intracellular oxidative stress, which is crucial in the development of cardiac hypertrophy and heart failure.



**Figure 5.** As-IV activates autophagy in stretched cardiomyocytes. Western blot results for LC3II and p62. Values are presented as the means  $\pm$  SD,  $n = 3$ . <sup>#</sup> $P < 0.05$  vs the control group; <sup>\*</sup> $P < 0.05$  vs the stretch group.



**Figure 6.** As-IV can reduce the inflammatory response in cardiomyocytes in vitro. (A) Effect of As-IV on the expression of the NLRP3 inflammasome and IL-1 $\beta$  as assessed by Western blot. (B) Effect of As-IV on TNF- $\alpha$  and IL-18 levels in cell supernatants as assessed by ELISA. Autophagy is inhibited with increased inflammation. Values are presented as the means  $\pm$  SD,  $n = 3$  for (A),  $n = 8$  for (B). <sup>#</sup> $P < 0.05$  vs the control group; <sup>\*</sup> $P < 0.05$  vs the stretch group.

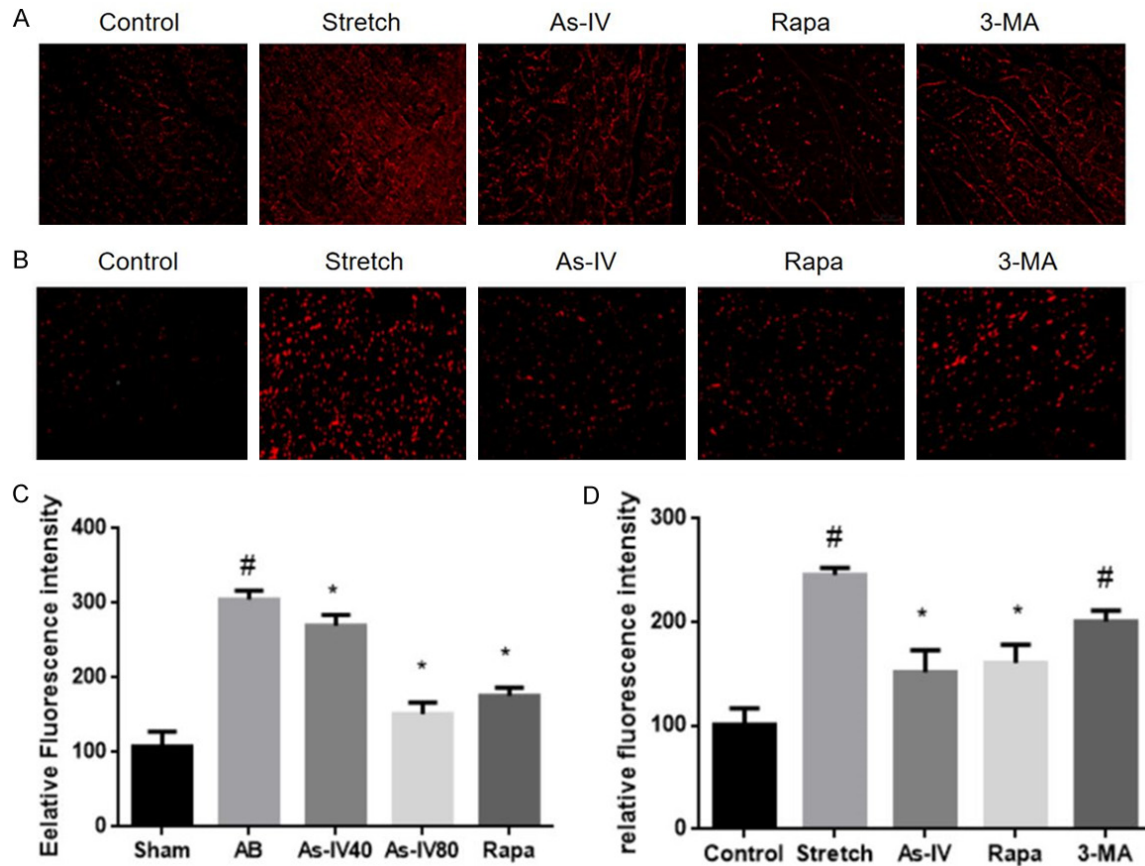
Previous studies have shown that cardiac myocyte autophagy is associated with a variety of heart diseases. Autophagy is reduced in the early stage of myocardial hypertrophy, thereby promoting further deterioration of the disease. Long-term pressure overload stress leads to excessive autophagy of cardiomyocytes, which ultimately leads to heart failure [17, 18]. In addition, sRAGE has been shown to attenuate angiotensin II-induced cardiomyocyte hypertrophy by inhibiting NLRP3 activation [19]. In a heart failure model, a selective NLRP3 inflammatory inhibitor was observed prevent the development of heart failure [20], and the inhibition of NLRP3 inflammasomes plays an important role in protection from hypertrophy and heart failure [21].

As an autophagy agonist, Rapa can prevent myocardial hypertrophy and even reverse stre-

ss-induced cardiac hypertrophy under thyroid hormone or isoproterenol treatment, thereby improving cardiac function [22, 23]. Rapa can reduce inflammation by activating autophagy in pulmonary fibrosis [24], inhibit IL-1 $\beta$  and IL-18 expression [25], and reduce cardiac remodeling and hypertrophy caused by aging [18]. As-IV is the primary pharmacological extract of *Astragalus membranaceus*, and a number of studies have shown that As-IV has a variety of pharmacological effects in the cardiovascular system, including antioxidative stress [26], antiapoptosis [27], regulation of calcium balance [15], anti-inflammatory effects [14], immunoregulation and cardiac protection activities [28].

The primary finding of this study is that As-IV increases autophagy activity, reduces inflammation and improves myocardial hypertrophy.





**Figure 7.** Astragaloside IV treatment can regulate ROS levels in the myocardial hypertrophy model. A and C: Effect of As-IV on ROS production induced by aortic banding in heart tissue. B and D: Effect of As-IV on ROS production induced by stretching in cardiomyocytes. Values are presented as the means  $\pm$  SD,  $n = 4$ . # $P < 0.05$  vs the control group or sham group; \* $P < 0.05$  vs the AB or stretch group.

As-IV was observed to inhibit the expression of LC3II, NLRP3, IL-1 $\beta$  and IL-18 in hypertrophic myocardium in a dose-dependent manner. These results demonstrate that the protective effect of As-IV on the heart may depend in part on activating autophagy to reduce inflammation, similar to Rapa.

In the in vitro experiments, the isolated primary cardiomyocytes were stretched by a Flexcell FX-5000 Tension System, with the results showing that As-IV could activate autophagy and reduce inflammation. After the addition of autophagy inhibitors in primary cardiomyocytes, autophagy was significantly inhibited, and inflammation was increased. Taken together, these results suggest a key role for autophagy in regulating inflammation.

So far, few studies have investigated the mechanism of improved myocardial hypertrophy by

the regulation of autophagy. As-IV protects against cisplatin-induced liver and kidney injury via autophagy-mediated inhibition of NLRP3 in rats [29]. In addition, As-IV regulates cardiac homeostasis and oxidative stress to prevent cardiac remodeling [30], and in a mouse model of abdominal aortic coarctation, As-IV can prevent pathological cardiac hypertrophy [31].

ROS are produced by damaged mitochondria, and the production of ROS can also activate NLRP3 [12]. Moreover, ROS are also signaling molecules that induce autophagy through a variety of signaling pathways, and autophagy can eliminate ROS by scavenging damaged mitochondria, thereby participating in the inhibition of NLRP3 expression. In our in vivo and in vitro DHE experiments, ROS levels were significantly increased in the myocardial hypertrophy model, while treatment with As-IV or Rapa could decrease ROS levels. Furthermore, the

ROS levels in primary cardiomyocytes were also increased in the presence of an autophagy inhibitor.

We further suggest that autophagy can regulate the assembly of NLRP3 inflammatory bodies by preventing the accumulation of ROS, thereby inhibiting the expression of NLRP3. In the present study, As-IV inhibited the development of hypertrophy by increasing autophagy, partly by inhibiting the increase in ROS, with subsequent inhibition of NLRP3 expression and downstream inflammatory factors to further improve myocardial hypertrophy.

Based on the above information, it is reasonable to investigate the relationship between inflammation and autophagy in myocardial hypertrophy, and the autophagy/ROS/NLRP3 inflammasome pathway may be a new target for drug development to prevent and treat myocardial hypertrophy.

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## Disclosure of conflict of interest

None.

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