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Original Article

Astragaloside IV attenuates pressure overload-induced cardiac hypertrophy by regulating PGC-1α signaling mediated energy biosynthesis

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Abstract: We previously reported that Astragaloside IV (AsIV) extracted from Chinese medicine Astragalus membranaceus (Fisch) Bge, could attenuate hypertrophy induced by isoproterenol. The present study was designed to investigate the effects and the possible mechanism of AsIV on pressure overload induced-hypertrophy in rats with focus on peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) signaling mediated energy biosynthesis. Pressure overload induced-cardiac hypertrophy model was established by abdominal aortic constriction (AAC), and AsIV was administrated one day before surgical procedure and continued for 15 days post surgery. The results showed that administration of AsIV attenuated the pathological changes, reduced the ratios of heart weight/body weight (HW/BW) and left ventricular weight/body weight (LVW/BW), improved the cardiac hemodynamics, down-regulated mRNA expressions of ANP and BNP, increased the total content of ATP+ADP+AMP, decreased the content of free fatty acid (FFA) and lactic acid (LA), and increased the mitochondrial membrane potential (MMP) in heart tissue. In addition, pretreatment with AsIV significantly increased the protein expressions of PGC-1α, pyruvate dehydrogenase kinase 4 (PDK4) and carnitine palmityl transferase 1 (CPT-1). The results suggested that AsIV attenuates pressure overload-induced cardiac hypertrophy through regulating PGC-1α signaling mediated energy biosynthesis.

Keywords: Cardiac hypertrophy, energy metabolism, astragaloside IV, pressure overload

Introduction

Cardiac hypertrophy is an adaptive response of the heart to chronic mechanical overload and can lead to functional deterioration and heart failure [1, 2]. Except for the changes in cardiac structure, energy metabolic substrate switching from fatty acids (FA) to glucose has been an early characteristic of cardiac hypertrophy and can precede the development of these pathologic processes [3]. Pathological cardiac hypertrophy and heart failure results in myocardium shifting away from FA utilization, relying predominantly on glucose and lactic acid (LA) as the chief energy substrate [4, 5].

Cardiac expression of most nuclear genes encoding mitochondrial fatty acid β-oxidation (FAO) enzymes and glycolysis is controlled by peroxisome proliferator-activated receptor coactivator 1α (PGC-1α) which is highly expressed in heart tissues and is indispensable for the heart to match the increased demand for adenosine triphosphate and work output in response to various physiologic stimuli [6]. In the myocardium PGC-1α interacts with various members of transcription factors to regulate the expression of several genes involved in the electron transport chain, FAO, glucose oxidative and mitochondrial biogenesis [7, 8]. Particularly, decreased expression and activity of PGC-1α and its target genes are involved in cardiac hypertrophy [9]. For instance, repression of PGC-1α and its transcriptional partners contributed to shift away from FAO

towards glucose oxidation and impaired ATP production in pressure overload hypertrophy, and PGC-1α deficient hearts also exhibited increased oxidative stress in response to pressure overload hypertrophy [5, 10], which accelerated heart failure process [11]. In accordance with these studies, we recently reported that expressions of PGC-1α and its downstream gene PDK4 were decreased in isoproterenol-induced cardiac hypertrophy, which lead to the decrease in ATP production and the cardiac dysfunction [4, 12].

Astragaloside IV (AsIV) is the major active ingredient extracted from the root of Astragalus membranaceus (Fisch) Bge, which has been widely used for traditional Chinese medicine. Recently, more and more studies have focused on the cardioprotective effect of AsIV [13, 14]. Our previous studies demonstrated that AsIV exhibited cardioprotection in response to myocardial ischemia reperfusion injury and lipopolysaccharide induced cardiac hypertrophy [15, 16]. Beside, several studies including our reports have demonstrated the potential regulative effect of AsIV on energy metabolism in cardiovascular diseases [17]. We recently reported cardiac protective role of AsIV in isoproterenol-induced cardiac hypertrophy, which is at least partly attributed to regulation of the PGC-1α signaling pathway and energy biosynthesis [12]. However, whether AsIV alleviates cardiac hypertrophy induced by pressure overload remains unknown. The present study was designed to investigate the effects of AsIV on pressure overload-induced hypertrophy, fo-cusing on the energy mechanism involved in PGC-1α signaling pathway and cardiac energy biosynthesis.

Material and methods

Materials

AsIV was obtained from Nanjin Jingzhu Biotechnology Company (purity > 98%; Nanjing, China). The Enzyme-linked immunosorbent assay (ELISA) kits for free fatty acids (FFA), and LA were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against PDK4, PGC-1α, CPT-1 and β-actin were from abcam (Cambridge, MA, USA). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) kit was obtained from Beyotime Institute of Biotechnology (China). ANP and BNP primers were obtained from TaKaRa Biotechnology Co. (Dalian, China). The ANP forward primer: GGGCTCCTTCTCCATCAC, reverse primer: CCCTCAGTTTGCTTTTCA; BNP forward primer: TTTTCCTTAATCTGTCGCCG, reverse primer: CACTGTGGCAAGTTTGTGCT; the GAPDH forward primer: AATGCATCCTGCCACCACCAACTGC, reverse primer: GGAGGCCATGTAGTAGGCCATGAGGTC.

Animal model of AAC and experimental protocols

The experimental protocols were approved by Committee of Liaoning Medical University for the Use of Experimental Animals for Research and Teaching. 36 Male Sprague-Dawley rats, weighing 280 to 300 g, were purchased from the Animal Center of Liaoning Medical University (SCXK 2009-0004). The rats were housed at 12:12 light/dark cycle, temperature of 22±2°C, humidity of 65%-69%, and received standard diet and water ad libitum. 36 rats were randomly divided into 3 groups (n=10): (1) Sham group; (2) AAC group; (3) AsIV group. Cardiac hypertrophy model was established by constriction of the abdominal aorta as described previously [18]. Rats were anaesthetized with 20% urethane (0.5 ml/100 g, i.p.). Then a laparotomy was performed and the aorta exposed at the level of the renal arteries. A reproducible band was placed using a 0.7 mm OD blunted needle and 3.0 mm suture where the needle was withdrawn leaving a ligature in place. For the age-matched sham operation, the identical procedure was performed without the ligation. Rats in AsIV group were gavaged with 80 mg/kg/d of AsIV which suspended in 0.5% sodium carboxymethylcellulose (CMC) one day before surgical procedure and continued for 15 days post-surgery. Rats in sham and AAC group were gavaged with equal volume of CMC. The criteria for selection of the doses of AsIV was based on our preliminary experiment and report [12].

Assessment of hemodynamics and heart weight index

All animals were anaesthetized with a 20% urethane (0.5 ml/100 g, i.p.) at the end of experiment. The right carotid were cannulated with a polyethylene catheter and then inserted into the left ventricle cavity, and the left ventricular systolic pressure (LVSP), left ventricular

end-diastolic pressure (LVEDP), the maximal rate of left ventricular systolic and diastolic pressure ($\pm dp/dt_{max}$) were recorded by using BL-420S polygraph (Chengdu TaiMeng Technology Corp., LTD). Then all animals were sacrificed by complete collection of blood and the hearts were immediately harvested, rinsed in ice-cold 0.9% NaCl solution, dissected and weighed. The heart-weight index (HW/BW) and the left ventricle-weight index (LVW/BW) were calculated separately. Then heart tissues were weighed on a balance and then immediately put into liquid nitrogen or 4% formaldehyde for the next experiments.

Histological analysis

Hearts were fixed in 4% formaldehyde for 24 hours, then were embedded with paraffin, cut into 5 μm sections, and stained with Hematoxylin-eosin (HE) and Masson trichrome staining to evaluate morphological changes.

Real-time RT-PCR analysis

The mRNA expression levels were analyzed by quantitative real time RT-PCR using the BioRad iQ5 Real Time PCR system (BioRad Company). Total RNA from tissues was extracted with TRIzol reagent (Invitrogen). The first strand cDNA was synthesized using AMV reverse transcriptase (TaKaRa, Dalian, China). Amplification was performed according to the manufacturer's instructions using the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China). The cDNA was denatured at 95°C for 5 seconds followed by 40 PCR cycles (95°C, 5 s; 60°C, 30 s). All results were repeated in four independent experiments. The relative level of mRNA was calculated by the comparative CT method with GAPDH mRNA as the invariant control.

Isolation of the cardiomyocytes and assessment of MMP

The mitochondrial membrane potential (MMP) was determined by JC-1 assay. After anaesthetized with 20% urethane, the heart was quickly excised, mounted on a perfusion system, and perfused via the aorta with the Tyrode solution under constant flow conditions (10 ml/min) for 5 min, then tissue digestion was initiated by type II collagenase solution. After 10 min, the type II collagenase was washed out by 5 min perfusion with Ca^{2+}-free Tyrode solution. The right ventricle was cut off first, and then the interventricular septum followed by the left ventricle. Separate ventricular parts were dispersed mechanically, and cardiomyocyte solutions were adjusted to the same cell density and transferred to culture medium.

Cardiomyocytes were incubated with 2 μM JC-1 at 37°C for 20 minutes, and centrifuged at 600 g for 4 min. After the supernatant was aspirated, the cells with JC-1 buffer solution were resuspended. The above centrifugation, aspiration, and resuspension were repeated. Thereafter, labeled cells were analyzed and quantified by fluorescence microscope (Leica. DMI3000 B, Germany).

Determination of adenosine phosphates

Heart tissue was added with precooled 0.4 mol/L $HClO_4$ (5 mL/g) in the mortar followed by homogenization in an iced bath, then the homogenate was centrifuged at 4,000 rpm for 10 minutes. The supernatant was collected and mixed with equal volume of 1 mol/L K_2HPO_3 solution followed by adjusting the pH to 6.5. The supernatant was then stored in a -20°C freezer. Compounds were separated by Shimpack ODS HYPERSIL C18 column (5 μm, 250 mml×4.6 mm) eluted with 100% of 50 mM potassium phosphate buffer (pH 6.5) at a flow rate of 0.5 mL/min. The column temperature was set at 25°C. The UV detection wave length was 254 nm and sample injection volume was 20 μL.

Western blot

The protein concentration was determined by the Bradford method. After boiling the samples for 5 min, the protein samples were fractionated by SDS-PAGE (10%-12% polyacrylamide gels) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were blocked with milk powder at room temperature for 2 h. The membranes were incubated with primary antibodies of PGC-1α, PDK4 and CPT-1 at room temperature for 2 h, following primary antibody incubations membranes were incubated with horseradish peroxidase-linked secondary antibodies (anti-rabbit, anti-mouse, or anti-goat IgG) mouse, or anti-goat IgG.

Statistic analysis

All data are expressed as mean ± standard errors (SEM). Statistical analysis was performed

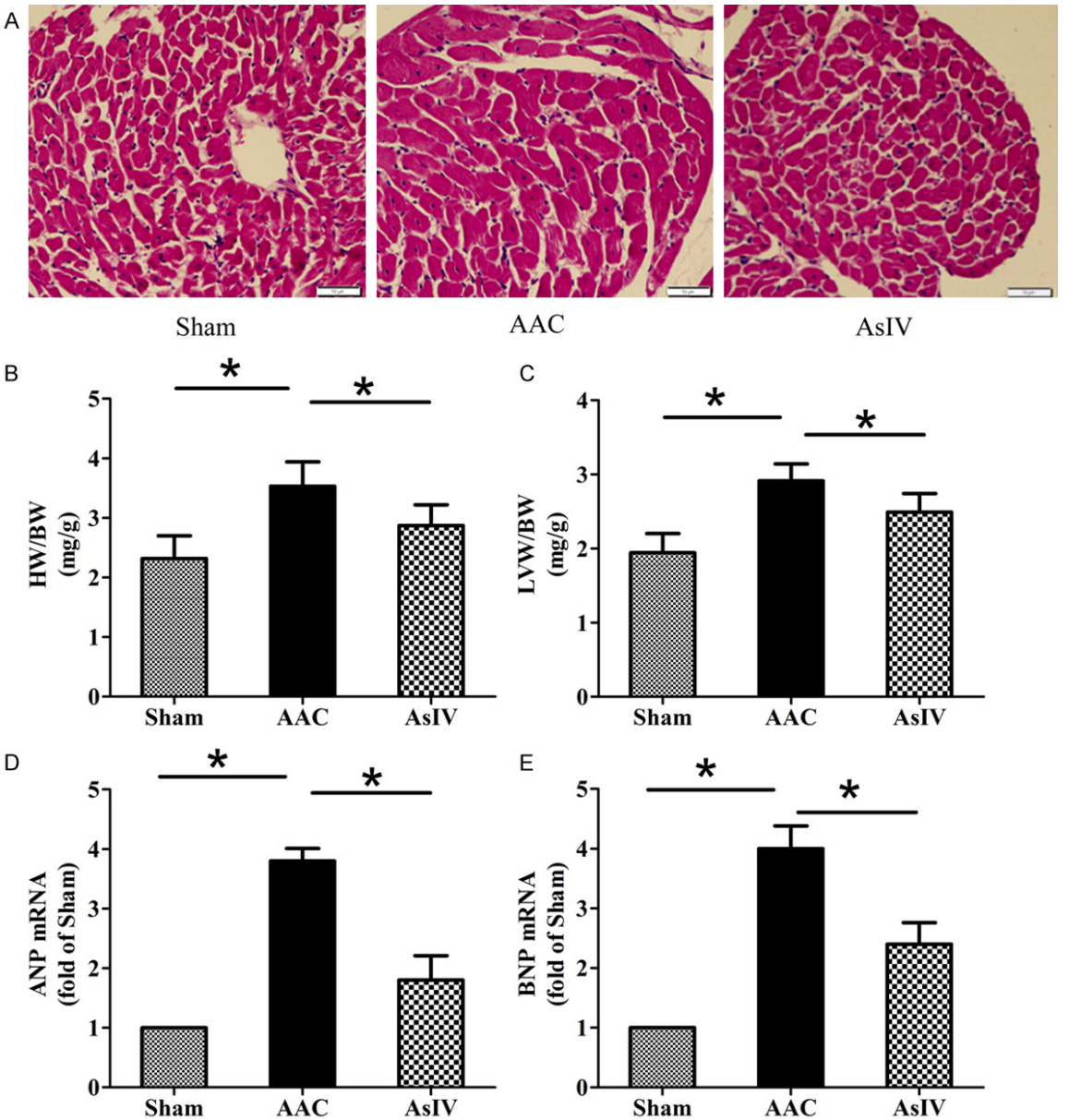

Figure 1. AsIV attenuates cardiac hypertrophy induced by pressure overload. A: Left ventricular tissue section stained with H&E; B and C: Ratios of heart weight/body weight (HW/BW) and left ventricular weight/body weight (LVW/BW) respectively; D and E: mRNA expression of ANP and BMP respectively. Data are presented as mean ± SEM. n=12 for A, B, and C; n=4 for D and E. *: $P < 0.05$ is considered statistical significance.

using one-way ANOVA followed by Bonferroni's test. Differences were considered as statistically significant if $P < 0.05$.

Results

AsIV attenuates cardiac hypertrophy induced by pressure overload

We previously reported that AsIV could attenuate cardiac hypertrophy induced by isoproterenol. To further investigate the cardioprotective effects of AsIV, we established pressure overload-induced cardiac hypertrophy by AAC. Results show that the ratios of both HW/BW and LVW/BW, the mRNA expressions of ANP and BNP, and relative cell surface area were increased in AAC group. However, treatment with AsIV significantly reduced cell surface area (**Figure 1A**), decreased the ratios of HW/BW (**Figure 1B**) and LVW/BW (**Figure 1C**) and down-regulated the mRNA expressions of ANP (**Figure 1D**) and BNP (**Figure 1E**), suggesting that AsIV

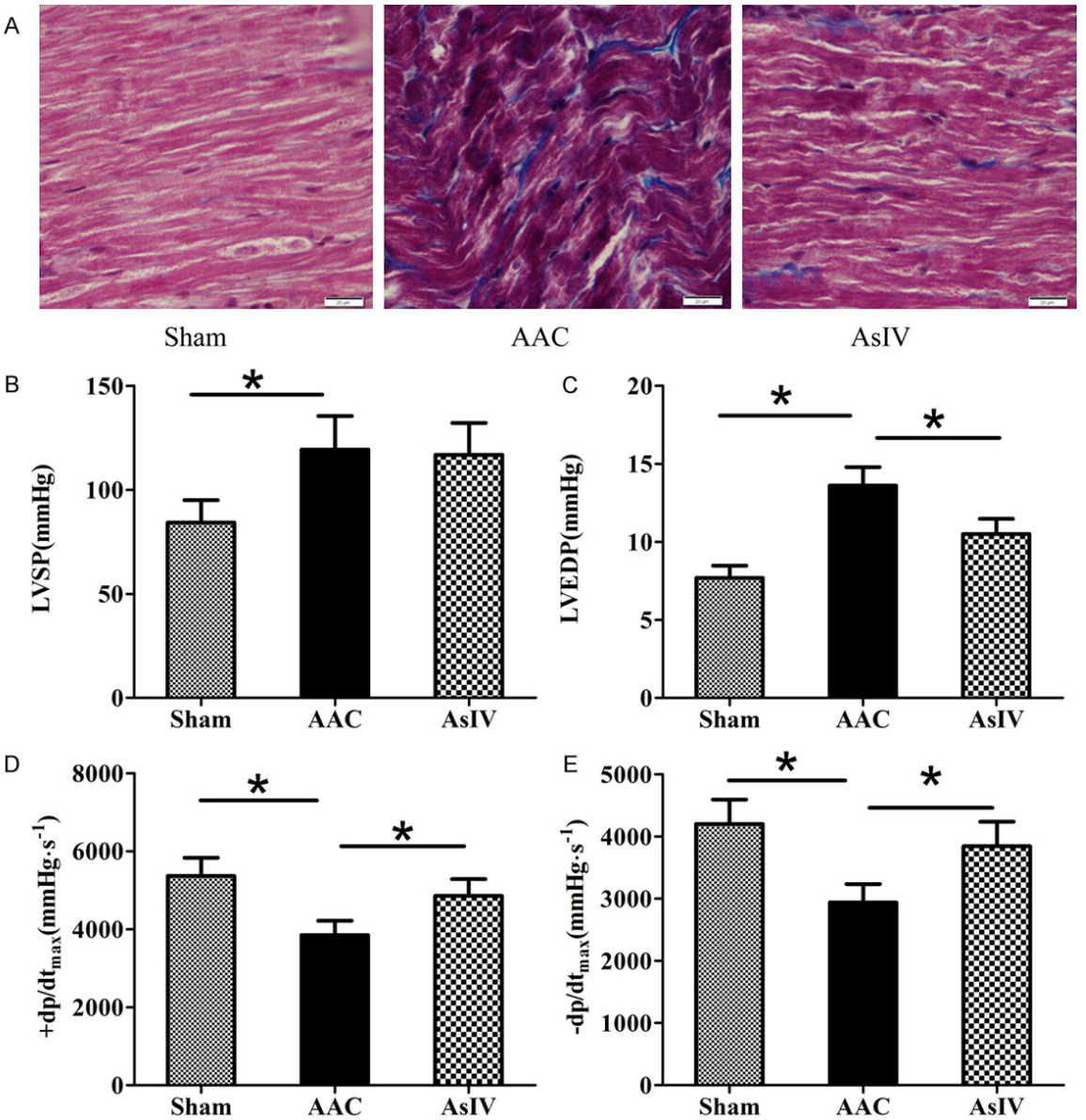

Figure 2. AsIV improves cardiac function of hypertrophic rats. A: Masson trichrome staining of heart tissue; B: LVSP, Left ventricular systolic pressure; C: LVEDP, Left ventricular end-diastolic pressure; D: +dp/dt, Maximal positive time derivative of developed pressure; E: -dp/dt, Maximal negative time derivative of developed pressure. Data are presented as mean ±SEM. n=12. *: $P < 0.05$ is considered statistical significance.

could attenuate the cardiac hypertrophy induced by AAC.

AsIV improves cardiac function of hypertrophic rats

Cardiac fibrosis from pressure overload affects myocardial compliance resulting in increased myocardial stiffness, which is a major determinant of diastolic dysfunction. In the present we examined cardiac fibrosis and cardiac hemodynamics to evaluate the effect of AsIV on cardiac function. Masson trichrome staining showed that collagen was dramatically increased in heart tissue from rat with AAC, and the degree of cardiac fibrosis was reduced by AsIV administration (**Figure 2A**). In addition, pressure overload resulted in increase in LVSP (**Figure 2B**) and LVEDP (**Figure 2C**) and decrease in +dP/dt_{max} (**Figure 2D**) and -dP/dt_{max} (**Figure 2E**), whereas these parameters were conversely recovered by AsIV administration except for LVSP. These findings suggest that the ability of

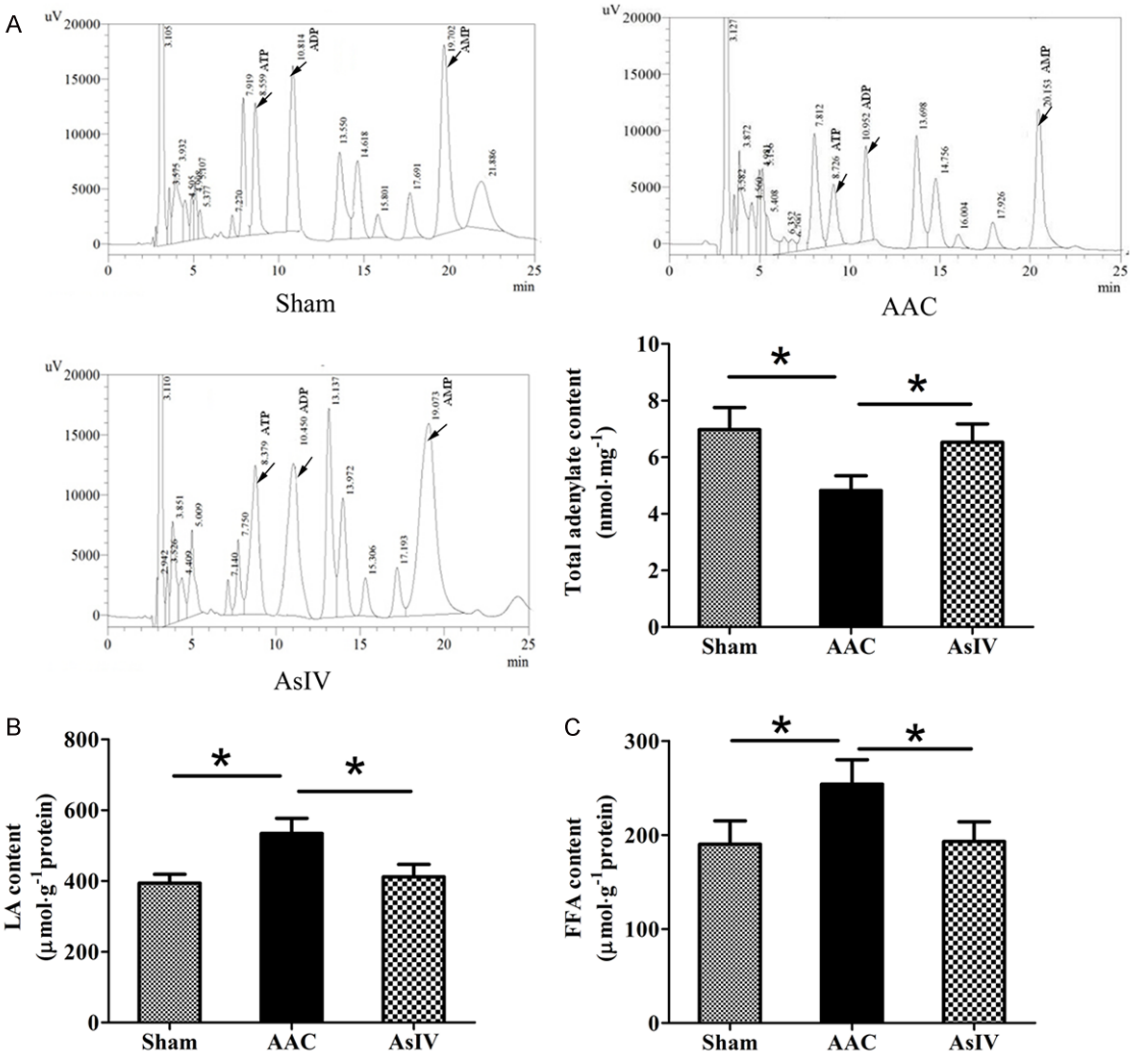

Figure 3. AsIV corrects the dysfunction of cardiac energy biosynthesis. A: Representative of HPLC graph of ATP, ADP and AMP as well as statistical data; B: LA content; C: FFA content. Data are presented as mean ± SEM. n=12. *: P < 0.05 is considered statistical significance.

AsIV to lessen cardiac fibrosis and improve hemodynamics may at least partly contribute to attenuate cardiac hypertrophy.

AsIV corrects the dysfunction of cardiac energy biosynthesis

Dysfunction of energy biosynthesis in cardiomycytes contributes to cardiac hypertrophy. To further explore the mechanisms underlying the improvement of AsIV on cardiac hypertrophy, we examined the effects of AsIV on AAC induced dysfunction of cardiac energy biosynthesis of rats. The results showed that total content of ATP+ADP+AMP was decreased and FFA and LA increased in the AAC group. However, treatment with AsIV significantly increased total content of ATP+ADP+AMP (**Figure 3A**, **3B**) and reduced the LA (**Figure 3C**) and FFA (**Figure 3D**) content. The results suggest that AsIV prevent cardiac hypertrophy at least partly through improvement of cardiac energy biosynthesis.

AsIV improves MMP of hypertrophic rats

Energy metabolism in cardiac myocytes is closely related to mitochondrial function status. In the present study, we examined the effect of AsIV on MMP of hypertrophic rats. MMP was determined by JC-1 assay, a decrease in red fluorescence intensity represents mitochondrial swelling, dominance of red fluorescence over

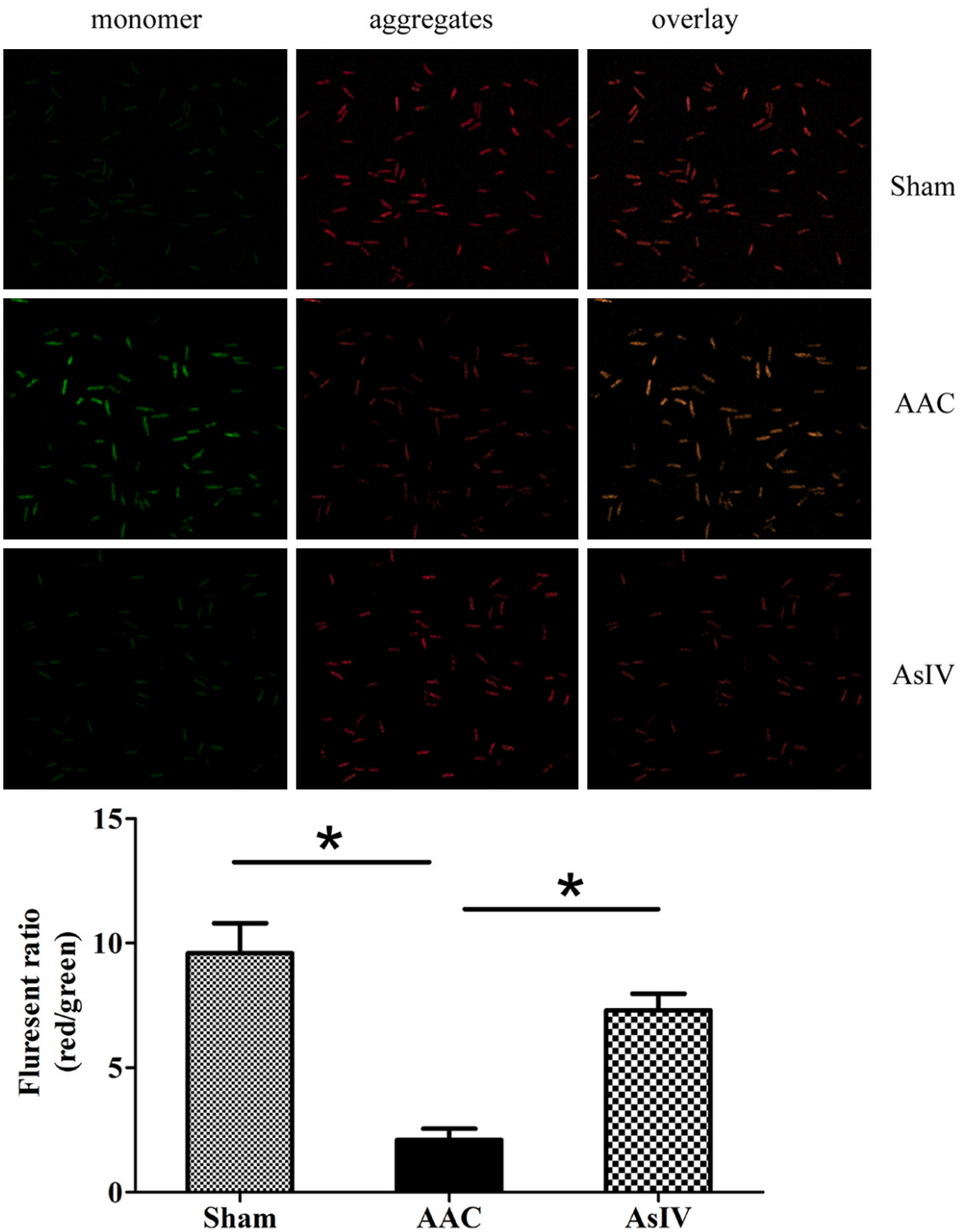

Figure 4. AsIV improves MMP of hypertrophic rats. Representative of MMP (upper panel) of cells and statistical data (lower panel). MMP was determined by JC-1 assay, a decrease in red fluorescence intensity represents mitochondrial swelling, dominance of red fluorescence over green fluorescence was a feature of normal myocardial cells, suggesting preservation of functional integrity of mitochondria. Data are presented as mean ± SEM. n=4. *: $P < 0.05$ is considered statistical significance.

green fluorescence was a feature of normal myocardial cells, suggesting preservation of functional integrity of mitochondria. The results showed that a significant decreased ratio of

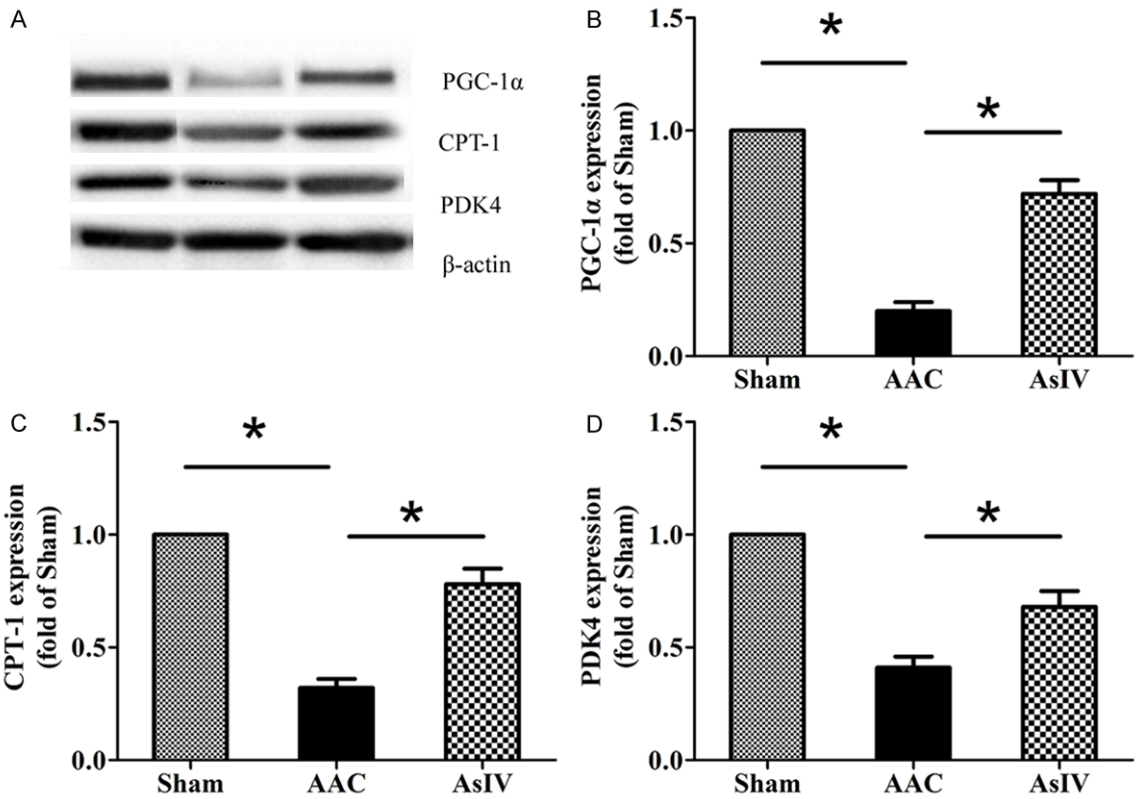

Figure 5. AsIV increases the protein expressions of PGC-1α, CPT-1 and PDK4. A: Representatives of western blot graphs of PGC-1α, CPT-1 and PDK4 respectively; B-D: Statistical data of PGC-1α, CPT-1 and PDK4 respectively. Data are presented as mean ± SEM. n=4. *: $P < 0.05$ is considered statistical significance.

red fluorescence to green fluorescence was observed in the AAC group compared with the sham group. Whereas the ratio was markedly increased in the AsIV treated group compared with AAC group (**Figure 4**), indicating that AsIV restore the mitochondrial function could be at least partly contribute to improvement of cardiac energy biosynthesis.

AsIV increases the protein expressions of PGC-1α, PDK4 and CPT-1

In the present study, we examined expressions of PGC-1α, PDK4 and CPT-1, all of which are involved in FAO and glycolysis, to explore the signaling mechanism by which AsIV improves cardiac energy biosynthesis. The results showed that AAC resulted in decrease in expressions of PGC-1α (**Figure 5A**, **5B**), CPT-1 (**Figure 5A**, **5C**) and PDK4 (**Figure 5A**, **5D**), where as these protein expressions were conversely increased by AsIV administration. The results suggested that AsIV exhibits the improvement of dysfunction in cardiac energy biosynthesis at least partly through up-regulation of PGC-1α, PDK4 and CPT-1 involved in FAO and glycolysis.

Discussion

Astragaloside IV, one of the major and active components of *Astragalus membranaceus*, has been shown diverse pharmacological activities including anti-inflammation, antioxidation, antivirus and anti-apoptosis [19-21]. We have recently shown that AsIV is cardioprotective in model of cardiac hypertrophy induced by isoproterenol [12]. However, it is unknown whether AsIV can be effective to attenuate cardiac hypertrophy induced by pressure overload. Using AAC model, we showed that treatment with AsIV not only significantly reduced cell surface area, decreased the ratios of HW/BW and LVW/BW and down-regulated the mRNA expression of ANP and BNP, but also lessen cardiac fibrosis and improve hemodynamics. These results suggested that administration of AsIV attenuate cardiac hypertrophy, improve cardiac

dysfunction induced pressure overload, which further verify the cardioprotective effects of AsIV on cardiac hypertrophy.

Cardiac hypertrophy is companied with the increase in glucose utilization and decrease in fatty acid oxidation, which contribute to increase in lactic acid and accumulation of FFA levels within the cells, leading to intracellular acidosis and increasing cardiac myocytes injury [22]. What is more, though the shift towards glucose utilization in the hypertrophied heart results in lower oxygen consumption cost per mole of ATP generated compared with FAO, it will cost the diminished energy reserves and exhaust the adenylate pool due to its lower efficiency of ATP generation [5]. And these were consistent with the experimental results from our present study. However administration of AsIV decreased the accumulation of FFA and lactic acid, which was accompanied by significant increase in total adenylate content. Energy metabolism in cardiac myocytes is closely related to mitochondrial function status. When the membrane potential decreases uncoupling of oxidative phosphorylation, ATP depletion and increase in oxygen radicals take place, there by inducing cardiac myocytes into the irreversible process of apoptosis [23]. To investigate the improvement of AsIV on energy dysfunction, we examined the MMP in the present study. Results showed that MMP was decreased in the AAC group compared with Sham group, whereas, AsIV administration increased the MMP in paralleled with the improvement of cardiac function and energy dysfunction. Taken together, those data indicate that the effects of AsIV on protecting mitochondrial membrane integrity and improving energy metabolism may contribute to the attenuation of cardiac hypertrophy and improvement of cardiac dysfunction induced by pressure overload.

The constant generation of ATP plays a vital role for the heart to perform its contractile function. Cardiac metabolism at the transcriptional level is mostly regulated by PGC-1α and its target transcription factors. In the myocardium PGC-1α interacts with myocyte enhancer factor 2 and activates the expression of CPT-1, a rate-controlling enzyme in the FAO. PGC-1 is also involved in the regulation of PDK4, which is a negative regulator of glucose oxidation, by interacting with ERRα. Together, the PGC-1α network serves to coordinately regulate the expression of numerous genes involved in mitochondrial pathways such as FAO, oxidative phosphorylation, and ATP synthesis [24, 25], thus getting involved in cardiac hypertrophy and heart failure. Coincident with this view, recent studies reported that PGC-1α and its downstream target genes were decreased in pressure overload and ISO induced cardiac hypertropy [4, 5], and PGC-1α null mice were found to develop early heart failure. Therefore, to further explore the mechanism by which AsIV improves cardiac energy biosynthesis, we examined the protein expression of PGC-1α, PDK4 and CPT-1. The results showed that AsIV increased the protein expressions of PGC-1α, PDK4 and CPT-1, suggesting that AsIV improves cardiac energy metabolic disturbance at the transcriptional level through up-regulation of PGC-1α, PDK4 and CPT-1 expressions.

In conclusion, the present study demonstrated for the first time that AsIV could attenuate pressure overload-induced hypertrophy and alleviate energy metabolic disturbance, partly through PGC-1α/PDK4/CPT-1 pathway. These findings further strengthen the therapeutic rationale for AsIV in the cardiovascular disease.

Acknowledgements

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Disclosure of conflict of interest

None.

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