Original Article
Astragalus polysaccharide protects the heart from cardiac ischemic reperfusion mainly from regulating autophagy

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Abstract: Astragalus polysaccharide (APS) has been proved to be effective in the treatment of cardiac ischemia reperfusion (CIR)-induced injury. However, whether APS could improve CIR-induced cell autophagy remains largely unknown. To explore the effect of APS on reactive oxygen species (ROS) production, primary cardiomyocytes were treated with APS at a final concentration of 1 nM, 10 nM, 100 nM and 1 mM. MTT assay was applied to determine whether APS affected cardiomyocyte viability. The SD rats were randomly divided into three groups: Group I: sham group; Group II: ischemic reperfusion (IR) group; Group III: IR+APS (1.5 g/kg body weight). APS increased primary cardiomyocytes viability in a dose and time dependent manner. In vitro study found that treatment with APS significantly inhibited the activation of caspase3 and the expression of Bax. Furthermore, treatment with APS significantly enhanced the contents of SOD and reduced the level of MDA, suggesting the cardiotoxicity effect of APS. Compared with IR group, APS significantly enhanced the ejection fraction (EF)% and fraction shortening index (FS)%. Autophagy was significantly enhanced in the rat hearts of IR group, which was accompanied by enhanced Beclin1 expression. In comparison, APS treatment could decrease cardiomyocyte autophagy through repressing Beclin1 expression. To conclude, treatment with APS significantly improves cardiac function after IR-induced injury mainly through reducing cardiomyocytes autophagy.

Keywords: APS, cardiac ischemia reperfusion, autophagy, Beclin1, ROS

Introduction

Astragalus polysaccharide (APS) is a major component of Astragalus, which has been proved to be effective in the treatment of cardiac ischemia [1]. Specifically, this compound can protect the heart through improving coronary blood low, LPO content and superoxide dismutase activity [2-4]. However, whether APS could improve cardiac ischemic reperfusion-induced cell autophagy remains largely unknown.

Ischemic heart disease is a leading cause of mortality and disability since it is closely related to acute coronary artery blockage [5]. As the most common therapeutic strategies, thrombolysis or primary percutaneous coronary intervention are widely applied in the clinic [6]. It has been suggested that myocardial ischemia-reperfusion (IR) is an important step for the treatment of myocardial infarction [7]. However, subsequent reperfusion often causes further heart injury, such as myocardial stunning, cell apoptosis and cell death [8, 9]. Thus, how to reduce myocardial ischemia reperfusion injury is a key subject for the treatment of ischemic heart disease.

It is reported that cardioprotective interventions, including caloric restriction and exercise, could initiate autophagy [10]. Undoubtedly, housekeeping levels of autophagy plays a key role in maintaining cardiac function [11]. But abnormal activation of autophagy aggravated myocardial injury during the reperfusion period [12]. As a dynamic process, autophagy starts from the induction of autophagosome formation and stops with lysosome-mediated autophagosome degradation [13]. Recent studies

have indicated that abnormal accumulation of autophagosomes results in cell death during cardiac IR injury. Beclin1 and microtubule-associated protein 1 light chain 3-II (LC3II) play key roles in the formation of autophagososomes [14]. During the reperfusion period, they were reported to be widely upregulated, which then casued ongoing cell autophagy and injury [15].

In this study, we mainly aim to determine whether autophagy is involved in the cardiaprotective role of APS. We hypothesized that early reperfusion enhanced autophgic flux. By blocking autophagy, APS enhanced the clearance of autophagosomes thereby exerting a cardioprotective effect on infarct size and cell apoptosis.

Materials and methods

Primary cardiomyocyte culture

Primary cardiomyocytes from rat neonatal hearts were isolated as described [16]. Animal protocol was approved by the second clinical medical college of Inner Mongolia University for the Nationalities. In brief, hearts were isolated and digested with collagenase type II (Worthington) solution. After digestion, the cells were preplated for 2 hr to collect cardiomyocytes. Then, the attached cells were discarded and the unattached cells were primarily cardiomyocytes.

Study protocols-in vivo

Eight-week-old male Sprague-Dawley rats were purchased from Sibeifu Biotech Co. Then, the SD rats were randomly divided into three groups: Group I: sham group; Group II: IR group; Group III: IR+APS (1.5 g/kg body weight). After that, the hearts were excised and the proteins were extracted.

Dimethyl thiazolyl diphenyl tetrazolium (MTT) assay

Cell viability was determined by a colormetric, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, sigma). To determine the impacts of APS on cell viability, primary cardiomyocytes were cultured at approximately 70% confluency and starved in serum-free DMEM (SF-DMEM) (Life Technologies, Inc.) overnight. Then, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM

APS was preincubated with primary cardiomyocytes for 24 h. After drug treatment, the cells were cultured in fresh medium including 0.5 mg/mL MTT for 4 h. Then, DMSO was added into the wells to dissolve the blue formazan products and the density was determined spectrophotometrically at a wavelength of 550 nm. Besides, the cells were preincubated with 1 μM APS for 8, 16, 24, 48 h and cell viability was determined in the same method as described. Each experiment was independently performed at least 3 times.

Western blotting analysis

Total lysates were collected with Cell Lysis Buffer (Cell Signaling Technology) and protein concentrations were determined using the BCA Protein Assay (Millipore, Billerica, MA, USA). Equal amounts of proteins were separated on a 12% SDS-PAGE and transferred onto the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with 5% non-fat milk powder (w/v) for 2 h at room temperature. The membranes were incubated with primary antibody rabbit anti-Beclin1, Bcl-2, Bax and GAPDH (Cell Signaling). The antibody was diluted in 5% bovine serum albumin according to the manufacturer's instructions. Horseradish peroxidase-conjugated secondary antibodies were then added and the resulting signal detected through autoradiography using chemiluminescence (ECL, Amersham Biosciences). GAPDH was served as the internal control.

Apoptosis assay

To detect the effects of APS on primary cardiomyocytes apoptosis, the cells were treated with 1 μM APS for 24 h with or without preincubation of 1 μM APS. After APS treatment, the cells were washed with cold PBS for three times. Then, flow cytometry was used to determine cell apoptosis with an Annexin-V FITC-PI Apoptosis Kit (Invitrogen, Carlsbad, CA). In summary, cells were washed with 1 × PBS for three times and suspended at 2-3 × 10^6 cells/mL in 1 × Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin-V FITC and Propidium Iodide Buffer were added to the cells, which were then incubated at room temperature for 15 minutes in the dark. The cells without any treatment were used as internal control. After incubation, the
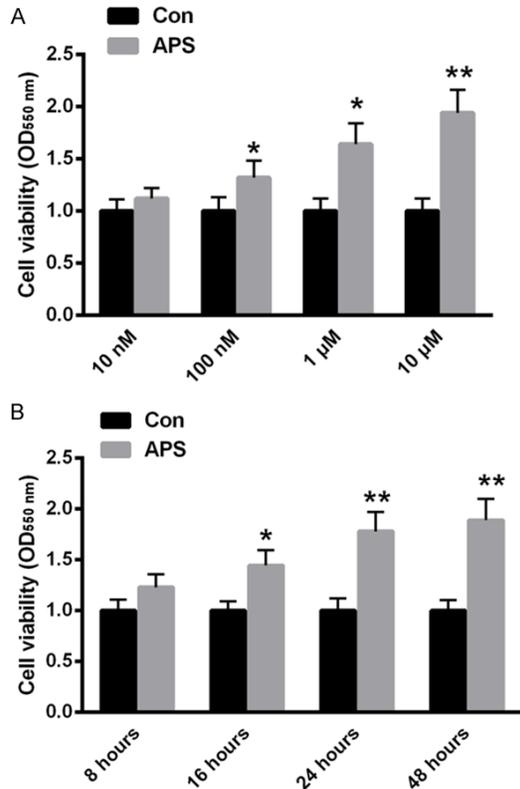
Figure 1. APS increases primary cardiomyocytes viability in a dose and time dependent manner. A. Incubation of primary caridiomyocytes with APS significantly increased cell viability at 100 nM, 1 μM, and 10 μM. B. T treatment with 100 nM APS enhanced cardiomyocyte viability by 34.5% and 42.3% at 48 h and 72 h, respectively Data represent the means ± SEM, n=3 independent experiments. **$P<0.01$, versus control.

cells were filtered by a filter screen and the cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) within 1 h of staining.

Autophagy measurement using GFP-LC3

Primary cardiomyocytes were transfected with a green fluorescent protein-microtubule-associated protein 1 light chain 3 (GFP-LC3) expression plasmid (Invitrogen Life Technologies, NE, USA). After 24 h, cells were treated with ad-p72 and the fluorescence of GFP-LC3 was observed under a fluorescence microscope. Then, the LC3 punctate spots were counted two days later. The percentage of cells undergoing autophagy was calculated from the ratio of autophagic cells to normal cells bearing GFP-LC3 fluorescence.

Enzyme activity assay

The tissue homogenate was centrifugated at 2000 rpm/min for 10 min. The contents of total protein, SOD, CAT and MDA were determined using the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instruction.

Echocardiography

Echocardiography was performed using Vevo 770 and Vevo 2100 (VisualSonics) instruments. Fraction shortening (FS), ejection fraction (EF), let ventric- ular internal diameter (LVID) during systole, LVID during diastole, endsystolic volume, and end-diastolic volume were calculated with Vevo Analysis sotware (version 2.2.3) as previously described [17].

Statistical analyses

The data were expressed as the means ± SEM. The statistical evaluation was performed using SPSS10.0 software. The statistical comparisons were performed using a one-way analysis of variance (ANOVA), and Dunn's method was used to discriminate the differences between different groups. $P<0.05$ was considered statistically significant.

Results

APS increases primary cardiomyocytes viability in a dose and time dependent manner

To explore the effect of APS on cell viability, MTT assay was applied. As shown in **Figure 1A**, incubation of primary caridiomyocytes with APS significantly increased cell viability at 100 nM, 1 μM, and 10 μM. Meanwhile, treatment with 100 nM and 1 μM APS enhanced cardiomyocyte viability by 34.5% and 42.3% at 48 h and 72 h, respectively (**Figure 1B**).

APS reduces cardiotoxicity and apoptosis in vivo

In vitro study found that treatment with APS significantly inhibited the activation of caspase3 (**Figure 2A**). Meanwhile, the protein level of Bax was significantly decreased (**Figure 2A**). We also detected the level of SOD and MDA when primary cardiomyocytes were treated with APS. The data showed that treatment with APS sig-
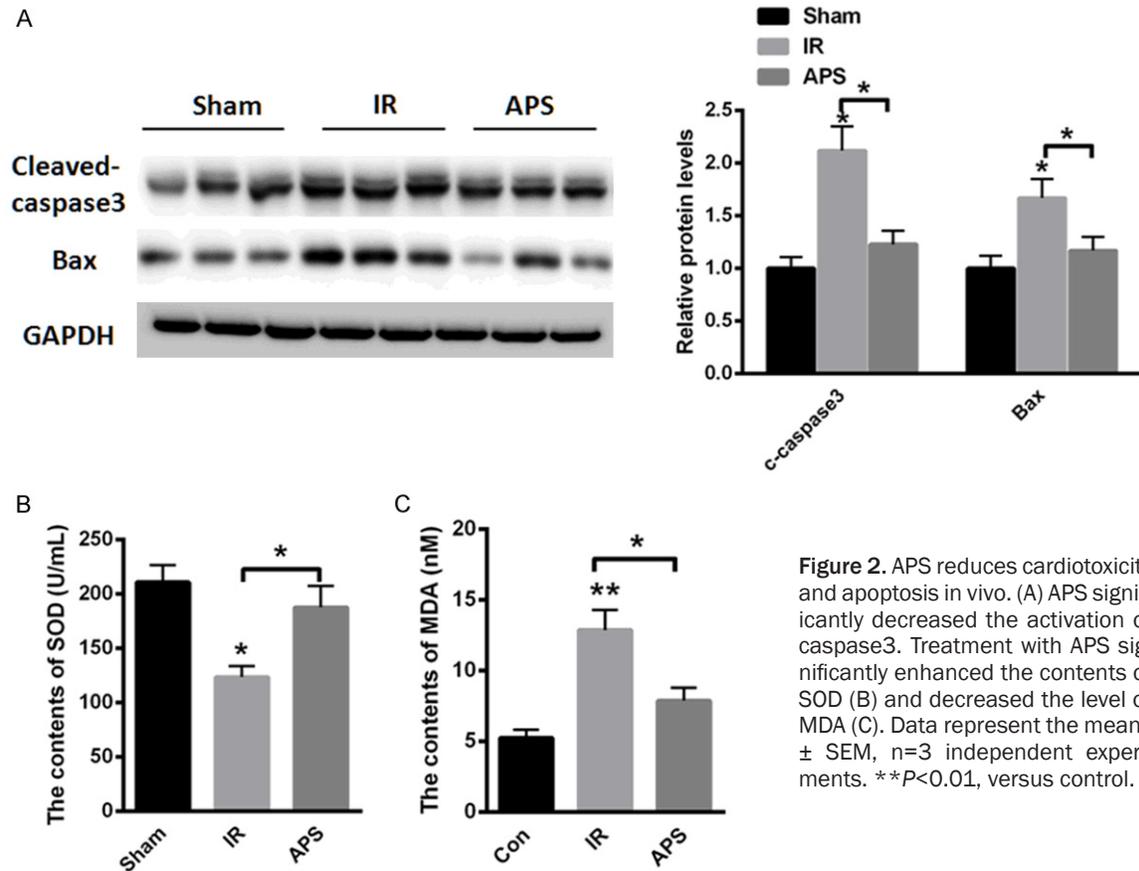
Figure 2. APS reduces cardiotoxicity and apoptosis in vivo. (A) APS significantly decreased the activation of caspase3. Treatment with APS significantly enhanced the contents of SOD (B) and decreased the level of MDA (C). Data represent the means ± SEM, n=3 independent experiments. **$P<0.01$, versus control.

nificantly enhanced the contents of SOD and reduced the level of MDA, suggesting the cardiotoxicity effect of APS (**Figure 2B** and **2C**).

APS ameliorates IR-induced cardiac injury in vivo

To explore the protective role of APS treatment on APS-induced cardiac injury, Echo analysis was conducted. Compared with the sham rats, the heart function was decreased by IR injury as measured by the ejection fraction (EF)% and fraction shortening index (FS)% (**Figure 3**). Compared with IR treatment, APS significantly enhanced the ejection fraction (EF)% (**Figure 3B**) and fraction shortening index (FS)% (**Figure 3C**).

Treatment with APS reduces cardiomyocytes autophagy

To explore the role of APS on IR injury, the circumstances of autophagy was explored in rat hearts with IR injury. As shown in **Figure 4A**, autophagy related protein, beclin1, was found

to be significantly enhanced with IR injury. Meanwhile, accumulation of GFP-LC3 dots was significant in primary cardiomyocytes of IR rats compared with sham group (**Figure 4B**). More importantly, we found that APS treatment significantly decreased the protein level of Beclin1 (**Figure 4C**). Furthermore, we found that APS treatment significantly decreased GFP-LC3 dots even in primary cardiomyocytes isolated from APS treated rats (**Figure 4D**). The data indicated that APS reduced primary cardiomyocytes autophagy and apoptosis partially through regulation of Beclin1 expression.

Discussion

APS has long been applied as an effective traditional medicine that can enhance immunity, reduce cancer cell growth and reduce inflammation [18]. Previous studies have indicated that APS may act as a potent protective medicine that can improve heart injury, such as myocardial hypertrophy and heart failure [19]. However, few studies have explored whether APS could protect cardiac injury induced cell
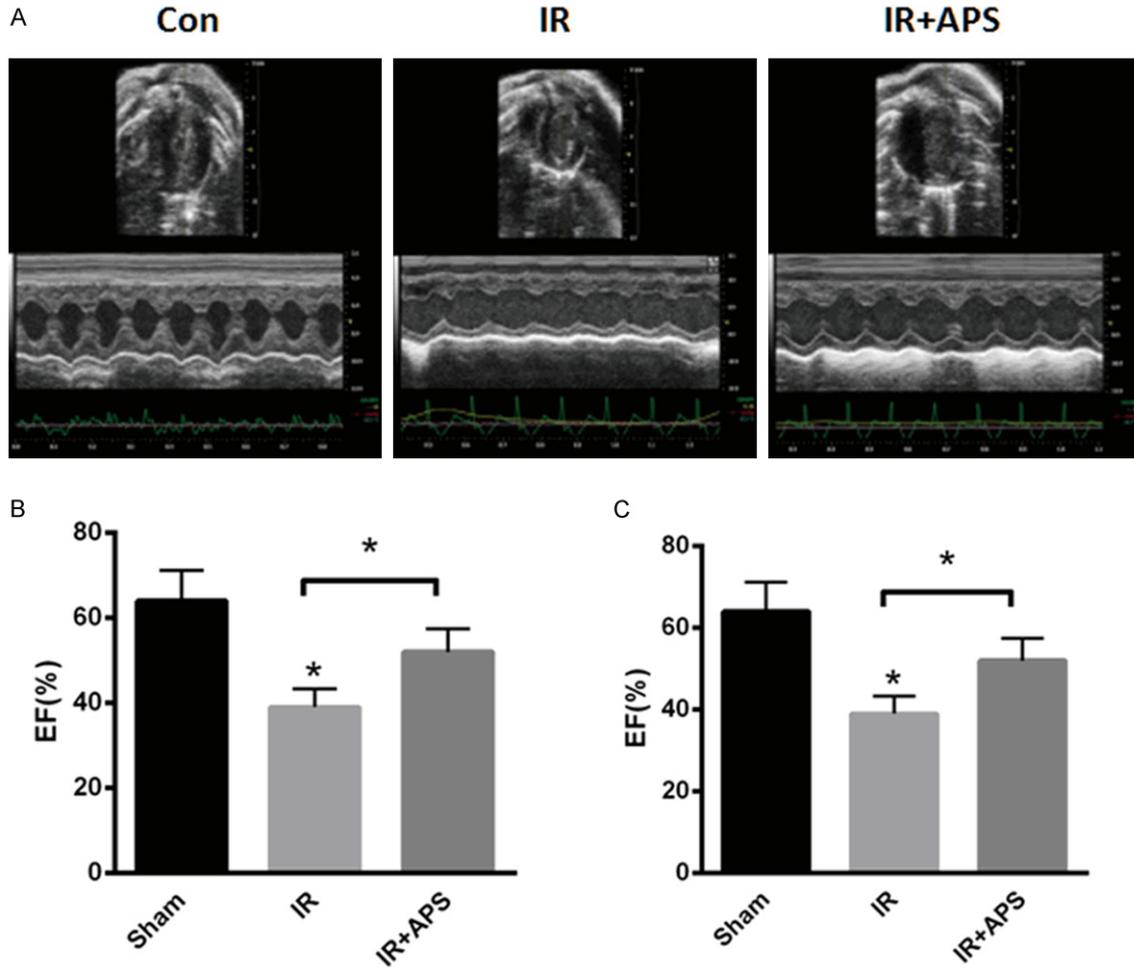
Figure 3. APS ameliorates IR-induced cardiac injury in vivo. (A) Representative echo figures for rat hearts from sham group, IR group and APS treatment group. Compared with IR treatment, APS significantly enhanced the ejection fraction (EF)% (B) and fraction shortening index (FS)% (C). Data represent the means ± SEM, n=3 independent experiments. **$P<0.01$, versus control.

autophagy. In this study, we first explored the role APS on cardiomyocytes autophagy.

Autophagy has been indicated to be involved in tumor progression and suppression [20]. It is reported that autophagy can maintain cellular homeostasis, genomic stability and metabolism by suppressing malignant transformation of normal cells [21]. It is reported that the actual function of autophagy on tumorgenesis is largely dependent on the microenvironment [22]. According to different tissue types and genetic context, autophagy exerts different functions on cell growth [23]. Beclin1 is suggested to interact with the class III phophoinositide 3-kinase complex, which is necessary for the autophagy initiation [24].

Moreover, autophagy appears in the heart with acute and chronic ischemia [25, 26]. It is reported that autophagy was significantly enhanced in ischemic-reperfusion hearts [27, 28]. In this study, we found that autophagy was significantly increased in hearts with IR injury. Previous study has suggested that excessive autophagy obviously enhanced the upregulation of Beclin1 during reperfusion [29]. In line with previous study, Beclin1 was increased after IR injury. More importantly, we explored the role of APS on autophagy after IR. We found that APS treatment significantly reduced IR-induced autophagy with reduced Beclin1 expression. We first reported the protective role of APS on cardiomyocyte autophagy after IR.
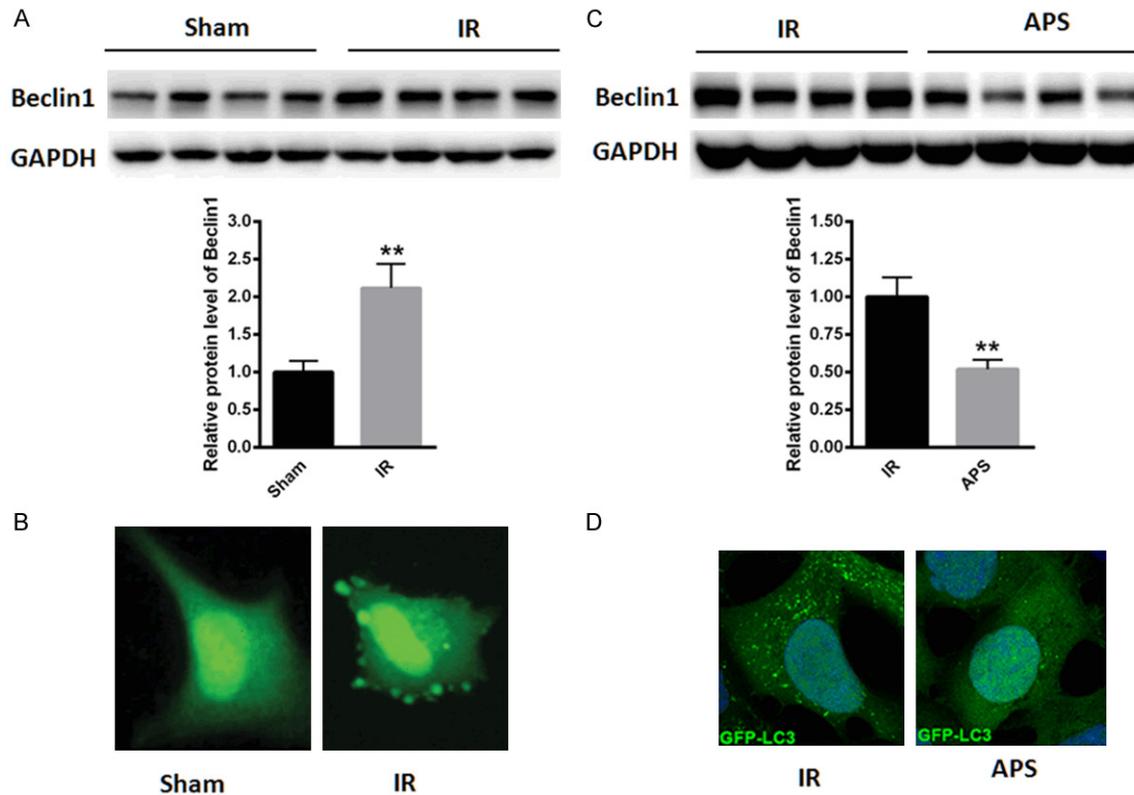
Figure 4. Treatment with APS reduces cardiomyocytes autophagy. A. Autophagy related protein, beclin1, was found to be significantly enhanced with IR injury. B. Accumulation of GFP-LC3 dots was significant in primary cardiomyocytes of IR rats compared with sham group. C. APS treatment significantly decreased the protein level of Beclin1. D. APS treatment significantly decreased GFP-LC3 dots even in primary cardiomyocytes. Data represent the means ± SEM, n=3 independent experiments. **$P<0.01$, versus control.

To conclude, treatment with astragalus polysaccharide significantly improves cardiac function after IR-induced injury mainly through reducing cardiomyocytes autophagy.

Disclosure of conflict of interest

None.

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References

[1] Lecour S, Baouali AB, Maupoil V, Chahine R, Abadie C, Javouhey-Donzel A, Rochette L and Nadeau R. Demonstration of the production of oxygen-centered free radicals during electrolysis using E.S.R. spin-trapping techniques: effects on cardiac function in the isolated rat heart. Free Rad Biol Med 1998; 24: 573-579.

[2] Wang XH and Huang WM. Astragalus polysaccharides exert protective effects in newborn rats with bronchopulmonary dysplasia by upregulating the expression of EGFL7 in lung tissue. Int J Mol Med 2014; 34: 1529-1536.

[3] Zhang Q, Gao WY, Zhang Y, Chen BY, Chen Z, Zhang WS and Man SL. Protective effects of astragalus extract against intermittent hypoxia-induced hippocampal neurons impairment in rats. Chin Med J 2013; 126: 1551-1554.

[4] Vitcheva V, Simeonova R, Krasteva I, Nikolov S and Mitcheva M. Protective effects of a purified saponin mixture from Astragalus corniculatus Bieb., in vivo hepatotoxicity models. Phytother Res 2013; 27: 731-736.

[5] Finegold JA, Asaria P and Francis DP. Mortality from ischaemic heart disease by country, region, and age: statistics from World Health Organisation and United Nations. Int J Cardiol 2013; 168: 934-945.

[6] Hausenloy DJ and Yellon DM. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. J Clin Investig 2013; 123: 92-100.

[7] Przyklenk K, Dong Y, Undyala VV and Whittaker P. Autophagy as a therapeutic target for ischaemia/reperfusion injury? Concepts, controversies, and challenges. Cardiovasc Res 2012; 94: 197-205.

[8] Dong Y, Undyala VV, Gottlieb RA, Mentzer RM Jr and Przyklenk K. Autophagy: definition, molecular machinery, and potential role in myocardial ischemia-reperfusion injury. J Cardiovasc Pharmacol Therapeut 2010; 15: 220-230.

[9] Przyklenk K, Undyala VV, Wider J, Sala-Mercado JA, Gottlieb RA and Mentzer RM Jr. Acute induction of autophagy as a novel strategy for cardioprotection: getting to the heart of the matter. Autophagy 2011; 7: 432-433.

[10] Choi AM, Ryter SW and Levine B. Autophagy in human health and disease. N Engl J Med 2013; 368: 1845-1846.

[11] Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B and Sadoshima J. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. Circ Res 2007; 100: 914-922.

[12] Gottlieb RA, Finley KD and Mentzer RM Jr. Cardioprotection requires taking out the trash. Basic Res Cardiol 2009; 104: 169-180.

[13] Yu L, McPhee CK, Zheng L, Mardones GA, Rong Y, Peng J, Mi N, Zhao Y, Liu Z, Wan F, Hailey DW, Oorschot V, Klumperman J, Baehrecke EH and Lenardo MJ. Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature 2010; 465: 942-946.

[14] Kang R, Zeh HJ, Lotze MT and Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Different 2011; 18: 571-580.

[15] Yao H, Han X and Han X. The cardioprotection of the insulin-mediated PI3K/Akt/mTOR signaling pathway. Am J Cardiovasc Drugs 2014; 14: 433-442.

[16] Ieda M, Tsuchihashi T, Ivey KN, Ross RS, Hong TT, Shaw RM and Srivastava D. Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. Dev Cell 2009; 16: 233-244.

[17] Shen T, Aneas I, Sakabe N, Dirschinger RJ, Wang G, Smemo S, Westlund JM, Cheng H, Dalton N, Gu Y, Boogerd CJ, Cai CL, Peterson K, Chen J, Nobrega MA and Evans SM. Tbx20 regulates a genetic program essential to adult mouse cardiomyocyte function. J Clin Investig 2011; 121: 4640-4654.

[18] Liu M, Qin J, Hao Y, Liu M, Luo J, Luo T and Wei L. Astragalus polysaccharide suppresses skeletal muscle myostatin expression in diabetes: involvement of ROS-ERK and NF-kappaB pathways. Oxidat Med Cell Longev 2013; 2013: 782497.

[19] Zou F, Mao XQ, Wang N, Liu J and Ou-Yang JP. Astragalus polysaccharides alleviates glucose toxicity and restores glucose homeostasis in diabetic states via activation of AMPK. Acta Pharmacol Sinica 2009; 30: 1607-1615.

[20] Galluzzi L, Pietrocola F, Bravo-San Pedro JM, Amaravadi RK, Baehrecke EH, Cecconi F, Codogno P, Debnath J, Gewirtz DA, Karantza V, Kimmelman A, Kumar S, Levine B, Maiuri MC, Martin SJ, Penninger J, Piacentini M, Rubinsztein DC, Simon HU, Simonsen A, Thorburn AM, Velasco G, Ryan KM and Kroemer G. Autophagy in malignant transformation and cancer progression. EMBO J 2015; 34: 856-880.

[21] Guo JY, Xia B and White E. Autophagy-mediated tumor promotion. Cell 2013; 155: 1216-1219.

[22] Kenific CM and Debnath J. Cellular and metabolic functions for autophagy in cancer cells. Trends Cell Biol 2015; 25: 37-45.

[23] Perez E, Das G, Bergmann A and Baehrecke EH. Autophagy regulates tissue overgrowth in a context-dependent manner. Oncogene 2015; 34: 3369-3376.

[24] Kihara A, Kabeya Y, Ohsumi Y and Yoshimori T. Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. EMBO Rep 2001; 2: 330-335.

[25] Costa R, Morrison A, Wang J, Manithody C, Li J and Rezaie AR. Activated protein C modulates cardiac metabolism and augments autophagy in the ischemic heart. J Thrombos Haemostas 2012; 10: 1736-1744.

[26] Xie Z, He C and Zou MH. AMP-activated protein kinase modulates cardiac autophagy in diabetic cardiomyopathy. Autophagy 2011; 7: 1254-1255.

[27] Sabe AA, Elmadhun NY, Dalal RS, Robich MP and Sellke FW. Resveratrol regulates autophagy signaling in chronically ischemic myocardium. J Thorac Cardiovasc Surg 2014; 147: 792-798; Discussion 798-799.

[28] Yan L, Vatner DE, Kim SJ, Ge H, Masurekar M, Massover WH, Yang G, Matsui Y, Sadoshima J and Vatner SF. Autophagy in chronically ischemic myocardium. Proc Natl Acad Sci U S A 2005; 102: 13807-13812.

[29] Hamacher-Brady A, Brady NR and Gottlieb RA. Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. J Biol Chem 2006; 281: 29776-29787.