Syringin prevents cardiac hypertrophy induced by pressure overload through the attenuation of autophagy

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Abstract. Syringin, extracted from *Eleutherococcus senticosus*, is a major biologically active component of Chinese herbs. Studies have certified the multiple pharmacological properties of syringin. However, the role of syringin in cardiac hypertrophy and the mechanisms involved remain unclear. In this study, aortic banding was performed on mice in order to induce cardiac hypertrophy, and the animals were then treated with syringin for 7 weeks. Echocardiography and catheter-based measurements of hemodynamic parameters were performed to evaluate cardiac function at 8 weeks following aortic banding. Morphological and pathological changes were also evaluated. Alterations in the expression levels of hypertrophy- and autophagy-related markers [atrial natriuretic peptide (ANP), β-myosin heavy chain MHC), α-MHC, B-type natriuretic peptide (BNP), autophagy-related gene (ATG)5, ATG7, beclin 1, light chain 3 (LC3) A/B] were measured by reverse transcription-quantitative PCR and western blot analysis. The effects of syringin on cardiomyocyte hypertrophy induced by angiotensin II in H9c2 cells were also investigated. The results revealed that syringin attenuated cardiac hypertrophy induced by aortic banding via the activation of AMP-activated protein kinase α (AMPKα) and autophagy-related signaling pathways. Thus, we our data suggest that syringin possesses therapeutic potential to attenuate the progression of cardiac hypertrophy.

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Abbreviations: AMPKα, AMP-activated protein kinase α; AB, aortic banding; LVEDd, left ventricular end-diastolic diameter; IVSd, interventricular septum depth; H&E, hematoxylin and eosin; WGA, wheat germ agglutinin; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; α-MHC, α-myosin heavy chain; β-MHC, β-myosin heavy chain; ATG, autophagy-related gene; LC3, light chain 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CSA, cross-sectional area; FS%, fractional shortening percentage; EF%, ejection fraction percentage; Ang II, angiotensin II

Key words: syringin, hypertrophy, autophagy, AMP-activated protein kinase α

Introduction

Cardiac hypertrophy is characterized by the enlargement of cardiomyocytes and interstitial fibrisis (1-4). Hypertension, myocardial infarction and cardiomyopathy are often associated with interstitial fibrosis, contractile and diastolic dysfunction, and the re-expression of fetal cardiac genes, such as atrial natriuretic peptides (ANPs), brain natriuretic peptides or B-type natriuretic peptides (BNPs) and the β-myosin heavy chain (MHC), which can finally result in heart failure (5-8). In the initial stage, myocardial hypertrophy is an adaptive and compensatory process which maintains cardiac output; however, sustained cardiac hypertrophy leads to detrimental cardiac remodeling and heart failure, which is the main reason of morbidity and mortality worldwide (1,2,5,6,8). New strategies to prevent or attenuate the enlargement of cardiomyocytes and cell apoptosis, and prevent the transition between adaptive hypertrophy and heart failure are necessary (1,9-11). Over the years, there have many efforts made to elucidate the molecular mechanisms of action of the intracellular signaling pathways involved in the progression of cardiac hypertrophy (1-8), in order to identify novel pharmacological agents with which to prevent heart failure.

Syringin ($C_{17}H_{24}O_9$, molecular weight, 372.37), also known as eleutheroside B, is the major biologically active component extracted from *Eleutherococcus senticosus* (12-14). Various studies have certified the multiple pharmacological properties of syringin. Cui *et al* demonstrated that syringin decreased sleep latency and prolonged sleep duration in mice; the molecular mechanisms involved may be associated with the nitric oxide synthase (NOS)/nitric oxide (NO) pathway (13). Furthermore, there is evidence proving that syringin exerts remarkable anti-inflammatory and antioxidant effects, and possesses immunomodulatory properties (15-17). However, the effects of syringin on cardiac hypertrophy and its potential mechanisms of action have not yet been elucidated. The results of our study demonstrated that syringin attenuates the progression of cardiac hypertrophy induced by hypertrophic stimuli via the AMP-activated protein kinase α (AMPKα) and autophagy-related signaling pathways.

The autophagy pathway is essential to myocardium homeostasis, and regulates cell survival and cell death pathways through the turnover of organelles and proteins (18-20). Nevertheless, under pathological conditions, the specific role of autophagy in cardiac geometry and function remains controversial. Zhu *et al* found that the autophagic response was activated by pressure overload, and they provided direct evidence that load triggered cardiac autophagy is a maladaptive response that contributes to the progression of heart failure (21). Another study demonstrated that multiple forms of stress, including pressure overload provoked an increase in autophagic activity in cardiomyocytes (22). On the contrary, other studies have suggested that autophagy activation may exert protective effects in cardiac hypertrophy and heart failure (23). Thus this study was designed to examine the role of syringin in the development of cardiac hypertrophy following pressure overload, and to elucidate the underlying mechanisms with a particular focus on autophagy.

Materials and methods

Chemicals. Syringin was obtained from Shanghai Winherb Medical S&T Development Co., Ltd. (Shanghai, China). High-performance liquid chromatography analysis was performed to confirm the purity of syringin (98%; data not shown).

Animals. All animal experiments were performed in accordance with the institutional guidelines of the Animal Care and Use Committee of Renmin Hospital of Wuhan University, Wuhan, China. Male C57BL/6 mice weighing 23.5-27.5 g and aged 8-10 weeks were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). The mice were housed in the Cardiovascular Research Institute of Wuhan University in an environment with controlled temperature and humidity. The mice were randomly divided into 5 groups of 20 mice each. Three of the groups were subjected to aortic banding (AB) and the two other groups were subjected to sham surgery (Sham group). The aortic arch branch was exposed with a chest expander upon opening of the second and third intercostals by an incision. The vessel was ligated using a 26G/27 G syringe needle placed parallel above the vessel. Subsequent to the rapid withdrawal of the needle to achieve aortic constriction, the chest was closed in layers and a total of 0.1 ml 0.5% bupivacaine (Sigma-Aldrich, St. Louis, MO, USA) was injected subcutaneously close to the edges of the skin incision to alleviate post-operative pain. The procedure for sham surgery was the same as that for AB but without vessel ligation. The mice in three of the treatment groups were gavaged with syringin at doses of 50 mg/kg body weight (L-syringin) or 100 mg/kg body weight (syringin) for 7 weeks. The mice in the remaining groups were gavaged with the same doses of normal saline (vehicle) for 7 weeks as a control. The 5 groups were designated as follows: sham + syringin, sham + vehicle, AB + vehicle, AB + L-syringin and AB + syringin. The mice were subjected to heart function determination by echocardiography and catheter-based measurements of hemodynamic parameters [heart weight/body weight (HW/BW; mg/g), lung weight/body weight (LW/BW; mg/g) and heart weight/tibia length (HW/TL; mg/ml) ratios; and the cross-sectional area of the cardiomyocytes (the areas

of individual myocytes were calculated using Image Pro-Plus 6.0 software)] before being sacrificed (by cervical dislocation) at 8 weeks following surgery.

The mice were also subjected to heart function determination by echocardiography and catheter-based measurements of hemodynamic parameters before being sacrificed at 8 weeks following surgery. The mice were anesthetized with 1.5% isoflurane before performing echocardiography using a MyLab™ 30CV (Esaote S.p.A., Genoa, Switzerland) and a 10 MHz linear array ultrasound transducer (10). End-systole and end-diastole pressure were measured at the end of the left ventricular systole and diastole, respectively. Left ventricular end-diastolic diameter (LVEDd), interventricular septum depth (IVSd), fractional shortening percentage (FS%) and ejection fraction percentage (EF%) were also measured.

Echocardiography and hemodynamics. The mice were anesthetized with 1.5% isoflurane before being subjected to echocardiography using a MyLab™ 30CV cardiovascular ultrasound system (Esaote S.p.A., Genoa, Switzerland) and a 10 MHz linear array ultrasound transducer. End-systolic and end-diastolic volume were measured at the end of the left ventricular systole and diastole, respectively. Left ventricular end-diastolic diameter (LVEDd), interventricular septum depth (IVSd) were measured from the left ventricle (LV) M-mode tracing with a sweep speed of 50 mm/sec at the mid-papillary muscle level. The mice were also anesthetized with 1.5% isoflurane before being subjected to hemodynamic measurements. A microtip catheter transducer (SPR-839; Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the left ventricle. The dPdt max and dPdt min end systolic volume and end-diastolic volume were determined. Data were continuously recorded with a Millar Pressure-Volume System (MPVS-400; Millar Instruments) and analyzed by PVAN analysis software.

Cell culture. The H9c2 cell line was acquired from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in standard DMEM-basic, supplemented with 10% calf serum, 1% penicillin (100 U/ml) and streptomycin (100 mg/ml) (all from Gibco, Grand Island, NY, USA). The cells were incubated in a CO_2 incubator (18 M; Sanyo, Osaka, Japan) with 5% CO2 at 37°C. Syringin was dissolved in dimethyl sulfoxide. The pharmaceuticals [angiotensin II (Ang II; Cat. no. A9525; Sigma-Aldrich), 2 μM; syringin, 15 μM and rapamycin (Cat. no. 53123-88-9; Sigma-Aldrich), 100 nM] were added to the medium and the cells were incubated for 24 h. Total RNA was then extracted from the H9c2 cells. The mRNA expression levels of ANP, β-MHC, α-MHC and BNP were examined by reverse transcription-quantitative PCR (RT-qPCR). We characterized the cells by immunocytochemistry for cardiac α-actin using α-actin antibody (Cat. no. 2207266; EMD Millipore, Billerica, MA, USA) to assess cardiomyocyte hypertrophy. The cells also were detected by primary antibody to light chain 3 (LC3) A/B (Cat. no. 12741, Cell Signaling Technology Inc., Danvers, MA, USA) to assess autophagy.

Histological analysis. After the 8-week treatment period described above, the mouse hearts were rapidly excised, washed with phosphate-buffered saline, weighed and then immersed by

Table I. Sequences of primers used for PCR.

mRNA	Forward primer	Reverse primer
ANP (rat)	5'-AAAGCAAACTGAGGGCTCTGCTCG-3'	5'-TTCGGTACCGGAAGCTGTTG CA-3'
β-MHC (rat)	5'-TCTGGACAGCTCCCCATTCT-3'	5'-CAAGGCTAACCTGGAGAAGATG-3'
α-MHC (rat)	5'-CAGAAAATGCACCATGAGGA-3'	5'-TCAAGCATTCATATTTATTGTGGC-3'
BNP (rat)	5'-CAGCAGCTTCTGCATCGTGGAT-3'	5'-TTCCTTAATCTGTCGCCGCTGG-3'
GAPDH (rat)	5'-GACATGCCGCCTGGAGAAAC-3'	5'-AGCCCAGGATGCCCTTTAGT-3'
ATG 5 (rat)	5'-GGGACCTCCTGAATCTCACA-3'	5'-CCAACAGGACAGCAGAGACA-3'
ATG 7 (rat)	5'-GTGTACGATCCCTGTAACCTAACCC-3'	5'-CGAAAGCAGAGAACTTCAACAGACT-3'
LC3 A/B (rat)	5'-GACCCTCTACGATGCTGGTG-3'	5'-TGCTGTCCTCAATGTCCTTCT-3'
Beclin 1(rat)	5'-GTGCTCCTGTGGAATGGAAT-3'	5'-GCTGCACACAGTCCAGAAAA-3'
ANP (mouse)	5'-ACCTGCTAGACCACCTGGAG-3'	5'-CCTTGGCTGTTATCTTCGGTACCGG-3'
BNP (mouse)	5'-GAGGTCACTCCTATCCTCTGG-3'	5'-GCCATTTCCTCCGACTTTTCTC-3'
α-MHC (mouse)	5'-GTCCAAGTTCCGCAAGGT-3'	5'-AGGGTCTGCTGGAGAGGTTA-3'
β-MHC (mouse)	5'-CCGAGTCCCAGGTCAACAA-3'	5'-CTTCACGGGCACCCTTGGA-3'
GAPDH (mouse)	5'-ACTCCACTCACGGCAAATTC-3'	5'-TCTCCATGGTGGTGAAGACA-3'
ATG 5 (mouse)	5'-ATATCAGACCACGACGGAGCG-3'	5'-CAGCATTGGCTCTATCCCGTG-3'
ATG 7 (mouse)	5'-CAGTTGGCTAGCTGTGTAA-3'	5'-GGCTATCGACCCACTTGGA-3'
LC3 A/B (mouse)	5'-GCCATGGATCAACTATGG-3'	5'-GGCACCTCACGGATCGGTA-3'
Beclin 1 (mouse)	5'-CTGGCCAGCAGCAGTTAGAC-3'	5'-TCACGCTCCTCCGACGTCAA-3'

ANP, atrial natriuretic peptide; MHC, myosin heavy chain; BNP, brain natriuretic peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ATG, autophagy-related protein; LC3, light chain 3.

perfusion with 10% neutral buffered formalin. To visualize the left and right ventricles, the hearts were cut transversely. Slides were prepared for hematoxylin and eosin (H&E) staining for morphological evaluation. The slides were then visualized and photographed under a light microscope (Olympus FSX100; Olympus Corp., Tokyo, Japan). The cross-sections of myocytes stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA; W849; Invitrogen, Carlsbad, CA, USA) to visualize membranes and DAPI to visualize nuclei were assessed. The areas of individual myocytes were calculated using Image Pro-Plus 6.0 software, a quantitative digital image analysis system.

RT-qPCR. The mRNA levels of the hypertrophic and autophagy markers, ANP, BNP, α-MHC, β-MHC, autophagy-related gene (ATG)5, ATG7, beclin 1, LC3 A/B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed by RT-qPCR. Total RNA was extracted from the cardiac tissues or H9c2 cells using TRIzol reagent following the manufacturer's instructions (Invitrogen). The RNA purities were evaluated based on the OD260/OD280 ratios detected with the SmartSpec Plus Spectrophotometer (Bio-Rad, Hercules, CA, USA). The mRNA was reverse transcribed into cDNA using oligo(dT) primers and the Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). The primers used are listed in Table I. PCR amplifications were quantified using the LightCycler 480 SYBR-Green I Master Mix (Roche) in the LightCycler® 480 Real-Time Quantitative PCR System (Roche). Briefy, subsequent to a 5-min initial denaturation at 95°C, a total of 42 primer-extension cycles were conducted. Each cycle consisted of a 10-sec denaturation step at 95°C, a 20-sec annealing step at 60°C and a 20-sec incubation at 72°C for extension. A final extension step was thene conducted at 72°C for 10 min. The double standard curve was used to quantify the PCR results. The results were normalized to the mRNA expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Western blot analysis. Ice-cold radioimmunoprecipitation assay buffer was used to extract protein from tissue homogenates or cell lysates. Protein quantification was carried out using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The extracted protein was subjected to SDS-PAGE, after which the proteins were transferred to Immobilon-FL Transfer Membranes (Merck Millipore, Billerica, MA, USA) and incubated with various primary antibodies for 24 h in 4°C. The primary antibodies were the following: GAPDH (Cat. no. sc-25778; Santa Cruz Biotechnology, Inc. Dallas, TX, USA), p-AMPKα (Cat. no. 2535; Cell Signaling Technology, Inc.), AMPKα (Cat. no. 2603P; Cell Signaling Technology, Inc. Danvers, MA, USA); ATG7 (Cat. no. 2631S; Cell Signaling Technology, Inc.), beclin 1 (Cat. no. ab55878; Abcam, Cambridge, UK), p62 (Cat. no. ab91526; Abcam), LC3 A/B (Cat. no. 12741; Cell Signaling Technology, Inc.). Following the removal of the primary antibody, the blots were incubated with the corresponding peroxidase-conjugated secondary antibodies [IRdye 800CW-conjugated goat anti-rabbit immunoglobulin (Ig)G (Cat. no. 926-32211; LI-COR Biosciences, Lincoln, NE, USA) and IRdye 800CW-conjugated goat anti-mouse IgG (Cat. no. 926-32210; LI-COR Biosciences)] for 2 h. The blots were then scanned using a two-color infrared imaging system (Odyssey, Danvers, MA, USA). Target protein expression levels were normalized to GAPDH protein.
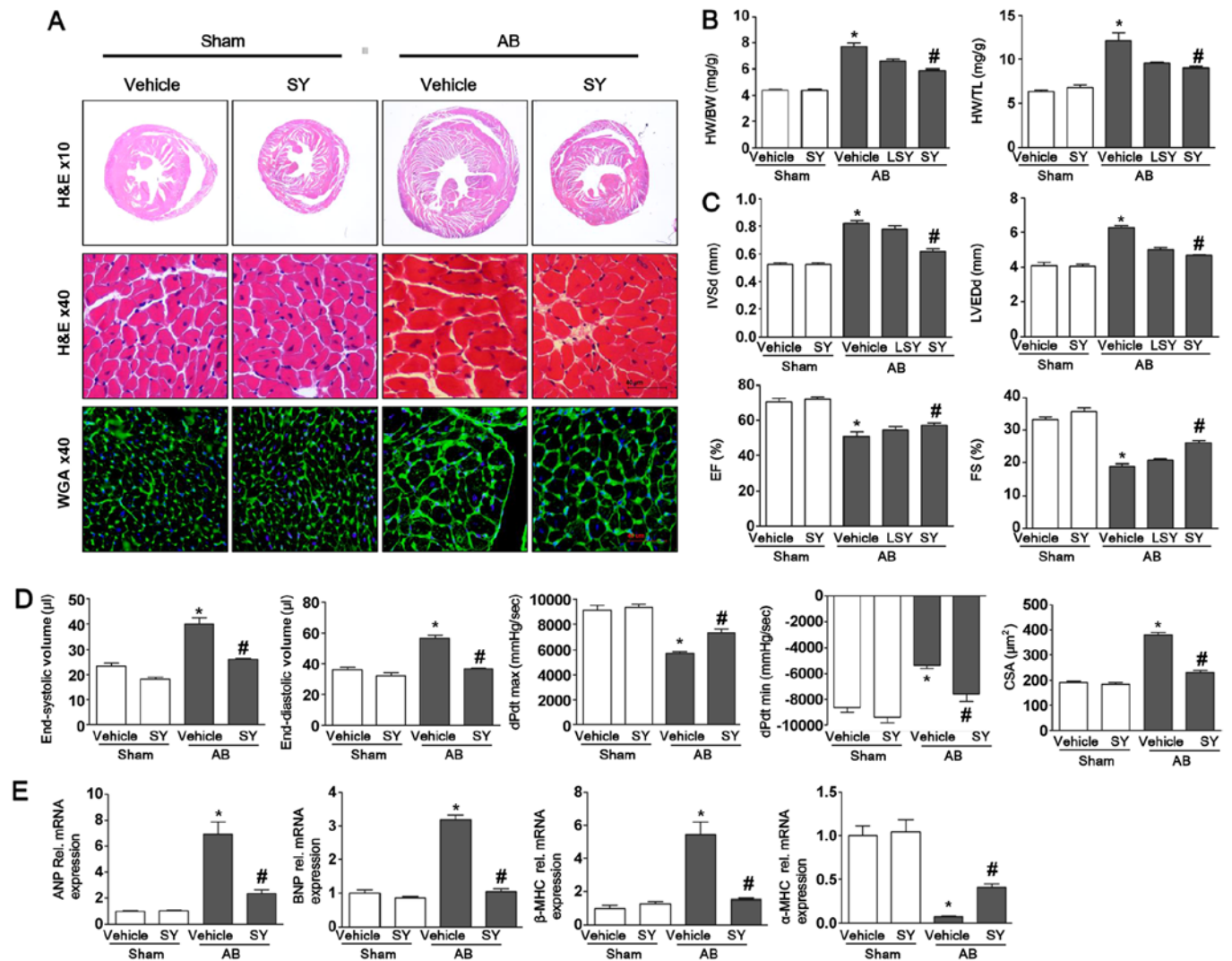
Figure 1. Syringin protects against cardiac hypertrophy. (A) Hematoxylin and eosin (H&E) staining and fluorescein isothiocyanate-labeled wheat germ agglutinin (WGA) staining of sham-operated mice (Sham) and mice subjected to aortic banding (AB) at 8 weeks post-surgery. (B) Statistical results of the heart weight/body weight (HW/BW) ratio and heart weight/tibial length (HW/TL) ratio. (C) Syringin ameliorated cardiac function as revealed by the parameter of left ventricular end-diastolic diameter (LVEDd), interventricular septum depth (IVSd), fractional shortening percentage (FS%) and ejection fraction percentage (EF%). (D) Syringin led to the hemodynamic parameters reverting back to normal levels and the myocyte cross-sectional areas decreasing. (E) Expression levels of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), β-myosin heavy chain (MHC), and α-MHC induced by AB were determined by RT-qPCR. LSY, syringin 50 mg/kg; SY, syringin 100 mg/kg. $^*p<0.05$ compared with the corresponding sham-operated group; $^\#p<0.05$ vs. the AB + vehicle group.

Statistical analysis. Data were analyzed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Differences among the groups were determined by one-way ANOVA, and a value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

Syringin attenuates cardiac hypertrophy induced by pressure overload in mice. To determine whether syringin attenuates the hypertrophic response to pressure overload, mice were administered syringin after 7 days of AB or sham surgery. The administration of syringin markedly decreased the heart weight/body weight ratio (HW/BW), heart weight/tibial length ratio (HW/TL) and the cross-sectional area (CSA) of the cardiomyocytes. As shown in Fig. 1, the protective effects of syringin on cardiac hypertrophy was confirmed by morphological anal-

ysis, H&E staining and WGA staining. The results of H&E and WGA staining revealed that syringin decreased cardiac mass and the myocyte cross-sectional area induced by pressure overload. The results of echocardiographic and pressure-volume loop analyses revealed that the LVEDd and IVSd increased, and the FS% and EF% decreased in the vehicle-treated mice following AB. Pressure overload significantly increased the LV mass and exacerbated cardiac function in the vehicle-treated group; however, treatment with syringin attenuated cardiac function and chamber dilation. The vehicle-treated mice exhibited ventricular dysfunction 8 weeks after AB, as well as an increase in diastolic blood pressure and a decrease in systolic and diastolic functions. Syringin attenuated the dysfunction of the hemodynamic parameters, dP/dt max and dP/dt min. In addition, the expression levels of hypertrophic markers, such as ANP, BNP and β-MHC significantly increased following AB.
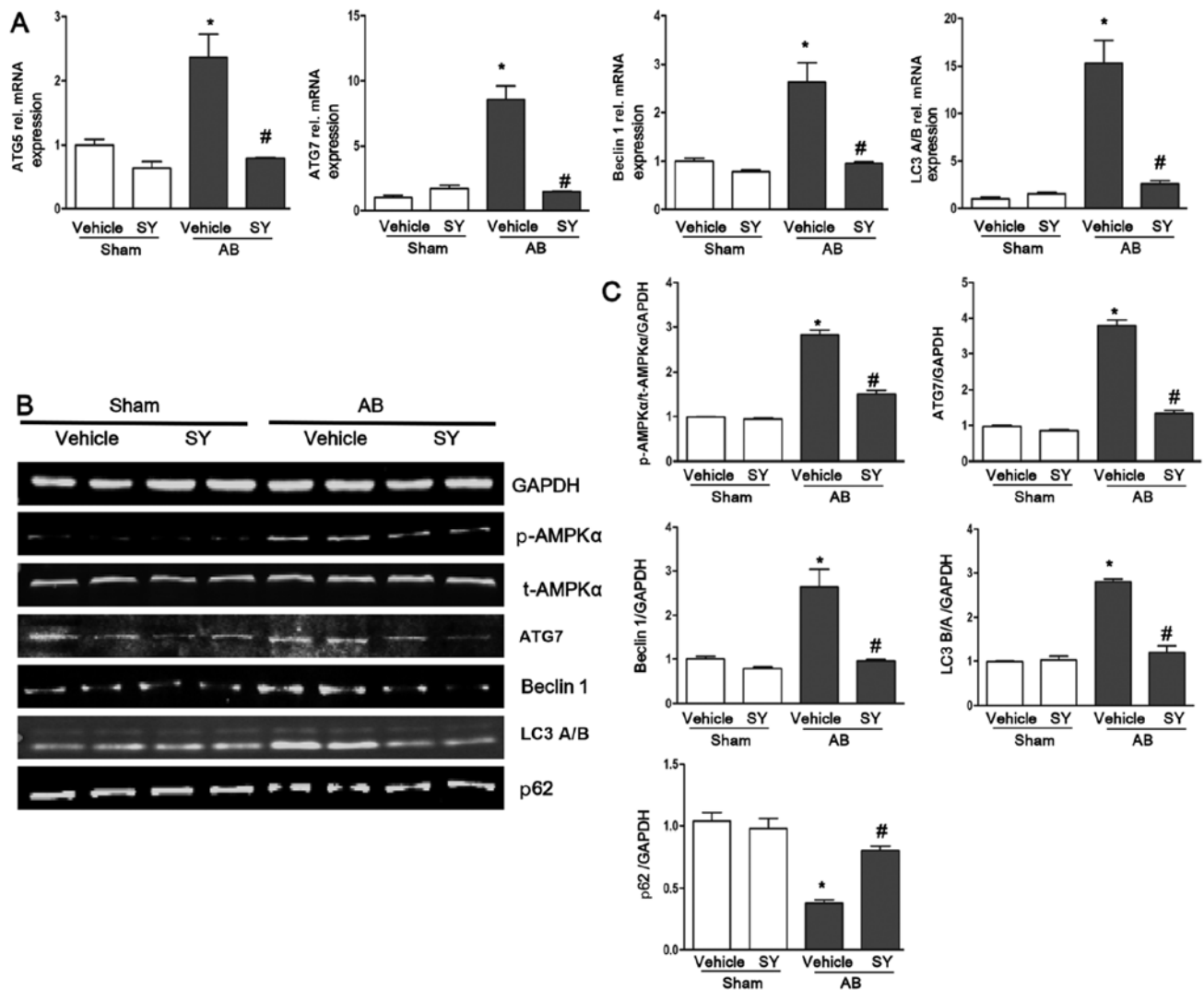
Figure 2. Syringin affects the AMP-activated protein kinase α (AMPKα) and autophagy-related signaling pathways. (A) Expression levels of autophagy-related gene (ATG)5, ATG7, beclin 1 and light chain 3 (LC3) A/B induced by AB were determined by reverse transcription-polymerase chain reaction analysis. (B) Representative blots of p-AMPKα, ATG7, beclin 1, p62 and LC3 A/B in the heart tissues of mice. (C) Quantitative results. *p<0.05 compared with the corresponding sham-operated (Sham) group; #p<0.05 vs. the AB + vehicle group. AB, aortic banding; p, phosphorylation; t, total; SY, syringin 100 mg/kg.

However, the administration of syringin decreased the expression levels of hypertrophic markers. In addition, the expression of α-MHC decreased following AB and was elevated by the administration of syringin.

Syringin alleviates cardiac autophagy induced by pressure overload. The expression levels of the autophagy markers, ATG5, ATG7, beclin 1 and LC3 A/B, were detected by RT-qPCR in the present study. The results revealed that the expression levels of ATG5, ATG7, beclin 1 and LC3 A/B significantly increased following AB, but were attenuated by the administration of syringin as shown in Fig. 2. We examined the protein expression levels of ATG7, beclin 1, p62 and LC3 A/B by western blot analysis. We found that the protein expression levels of ATG7 and beclin 1 were higher in the vehicle-treated mice than in the syringin-treated mice in response to AB. The ratio of LC3 B/A was elevated following AB and was attenuated by the administration of syringin. In addition, p62 was found to be in inverse proportion to the ATG7 and beclin 1 (Fig. 2). These findings illustrated that

syringin alleviated cardiac autophagy induced by pressure overload. To examine the molecular mechanisms responsible for the protective effects of syringin against the autophagy response, we examined the activation of AMPKα. We found that AMPKα was significantly activated in the mice subjected to AB. The administration of syringin decreasesd the phosphorylation levels of AMPKα (Fig. 2).

Protective effects of syringin on cardiomyocyte hypertrophy in H9C2 cells. In *in vitro* experiments, the mRNA expression levels of hypertrophic markers, including ANP, BNP and β-MHC, were increased following stimulation with Ang II (2 μM, 24 h). The levels of α-MHC were decreased following stimulation with Ang II. Treatment with syringin (15 μM) resulted in a marked decrease in the expression levels of hypertrophic markers and an inrease in the levels of α-MHC. The cardiomyocytes were examined by immunocytochemistry using primary antibodies to cardiac α-actin to assess cardiomyocyte hypertrophy. Thye H9c2 cells exposed to Ang II exhibited an enlarged cell surface area as compared with those treated with syringin (15 μM). As
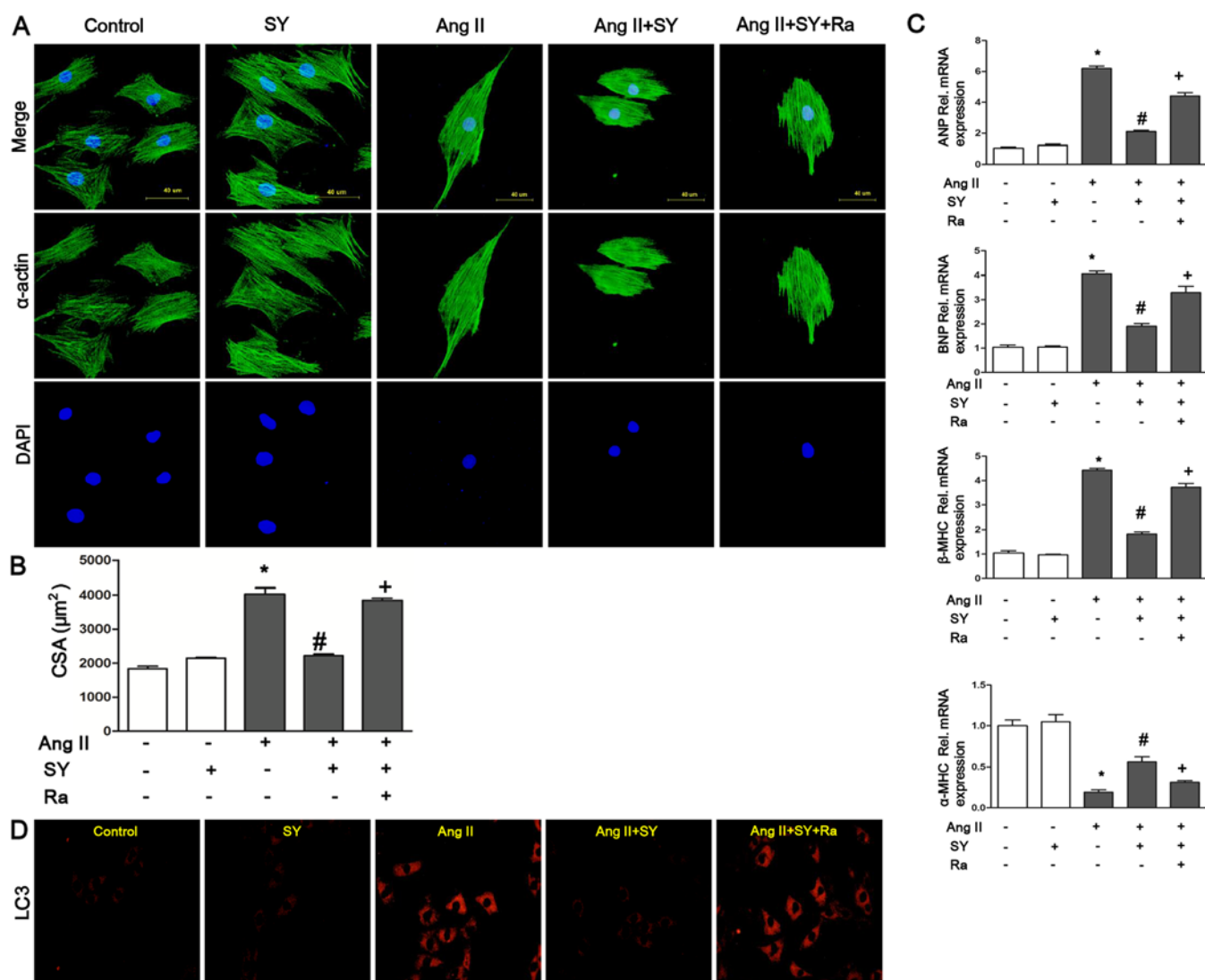
Figure 3. Syringin attenuates hypertrophy and autophagy in H9c2 cardiomyocytes induced by angiotensin II (Ang II). (A) Representative images of cardiomyocytes showing the inhibitory effect of syringin on cardiomyocyte enlargement in response to Ang II. (B) Quantification of cell cross-sectional areas by measuring 100 random cells. (C) RT-qPCR analysis of the mRNA levels of the hypertrophy markers, atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), β-myosin heavy chain (MHC) and α-MHC. (D) The cardiomyocytes stained by immunofluorescence for light chain 3 (LC3) in the different groups. $^*p<0.05$ compared with the corresponding control group; $^#p<0.05$ vs. the Ang II group; $^+p<0.05$ vs. the Ang II + SY group. SY, syringin 15 μM; Ra, rapamycin 100 nM.

shown in Fig. 3, syringin alleviated cardiomyocyte hypertrophy induced by Ang II. The quantification of the cell CSA (Fig. 3B), indicated that the enlarged cell surface area induced by Ang II was significantly reduced by treatment with syringin; however, when rapamycin was added to the medium, the effect of syringin was reversed. Furthermore, syringin reduced the protein expression levels of ATG7 and beclin 1 induced by Ang II and elevated the levels of p62 (Fig. 4). To explore the molecular mechanisms through which syringin exerts its anti-hypertrophy and anti-autophagy effects, rapamycin, an autophagy agonist, was used in further experiments. Treatment of the cells with rapamycin attenuated the protective effects of syringin on cardiomyocytes. Treatment with rapamycin increased the levels of ANP, BNP and β-MHC, and decreased the levels of α-MHC. We found that treatment of the H9c2 cells with syringin decreased the single cardiomyocyte area and the levels of hypertrophic markers. In order to examine the role of autophagy, the cells were examined by immunofluorescence

using primary antibodies to LC3. As shown in Fig. 3D, syringin decreased the expresstion of LC3. However, rapamycin attenuated this effect. Thus, our data prove that the protective effects of syringin against cardiac hypertrophy are closely related to autophagy.

Discussion

Syringin is a major biologically active component extracted from *Eleutherococcus senticosus* in Chinese herbs and possessed several biological functions, including anti-inflammatory, immunomodulatory actions and exerts antioxidant effects (13,15-17,24). However, whether syringin has an effect on cardiac hypertrophy remains unclear. In this study, we found that syringin attenuated cardiac remodeling induced by pressure overload. The beneficial effects of syringin may be mediated by inhibition of the AMPKα and autophagy-related signaling pathways.
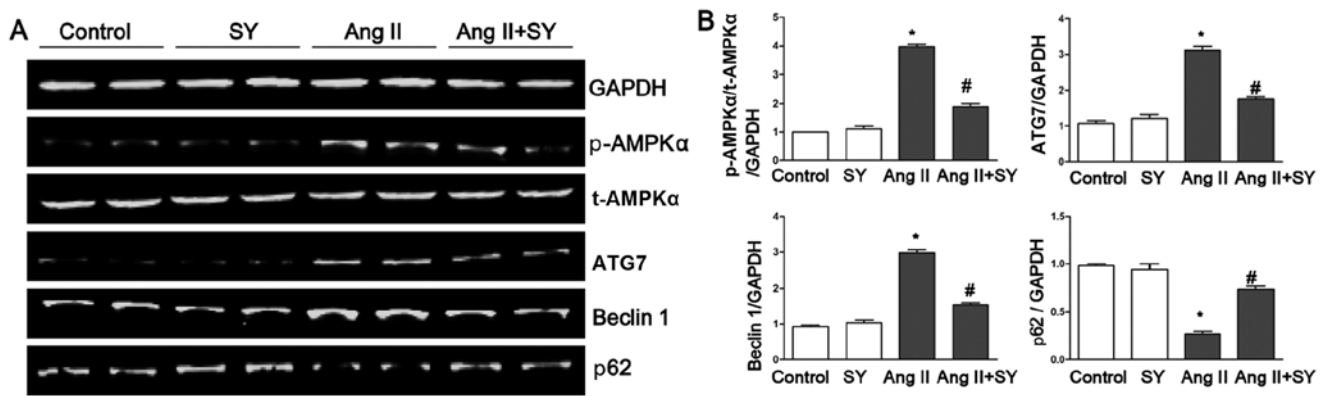
Figure 4. Syringin affects the AMP-activated protein kinase α (AMPKα) and autophagy-related signaling pathways in H9c2 cells induced by Ang II. (A) Representative blots of p-AMPKα, ATG7, beclin 1 and p62 in H9c2 cells. (B) Quantitative results. *p<0.05 compared with the control group; #p<0.05 vs. the Ang II group. p, phosphorylation; t, total; SY, syringin 15 µM; Ra, rapamycin 100 nM.

Syringin is widely regarded as the major constituent of eleutherosides (14,25). Over the years, this plant has been used extensively as a pharmacological agent to help the body to adapt to stress by supporting healthy adrenal gland function in many Asian countries (13,16). In recent years, a number of studies have demonstrated that syringin promotes a wide range of biological activities. A previous study indicated that syringin injection at the desired doses (100 g/kg) decreased plasma glucose and increased plasma insulin levels in fasted Wistar rats. This effect may be associated with the release of ACh from the nerve terminals, which in turn enhances insulin secretion (15). Another study by Niu *et al* indicated that in conscious rats with a regular sympathetic tone, the effect of syringin on plasma glucose regulation was impaired. Whereas in anesthetized animals, the decreased sympathetic tone may be helpful to the therapeutic benefits of syringin (16). Cui *et al* demonstrated that the administration of syringin decreased sleep latency and prolonged sleep duration, and that these effects may be mediated by the NOS/NO pathway (13). Another study indicated that syringin may alleviate the fulminant hepatic failure induced by lipopolysaccharide/D-galactosamine by attenuating NF-κB activation to reduce inflammatory factor production, such as TNF-α (12). In conclusion, syringin promotes a wide range of biological activities. In this study, syringin attenuated the cardiac hypertrophyhic response induced by pressure overload and Ang II, as evidenced by changes in HW/BW, HW/TL and CSA in cardiomyocytes. Furthermore, our data indicated that syringin ameliorated LVEDd, IVSd, FS%, EF%, dP/dt max and dP/dt min which had been affected by AB. Thus, syringin attenuated cardiac dilation and improved LV function. Syringin also attenuated the increase in diastolic blood pressure and the decrease in systolic and diastolic functions. ANP and BNP, belong to the natriuretic peptides family, and are synthetized mainly in the heart (26). The expression levels of ANP and BNP, as in parallel with the degree of LV dysfunction and hemodynamic stress, have become the main index for the measurement of conventional cardiovascular disease risk factors and in parallel with the degree of LV dysfunction and hemodynamic stress (26-28). The present study demonstrated that syringin decreased the expression levels of ANP and BNP which were increased by AB, suggesting that syringin attenuated cardiac hypertrophy induced by pressure overload.

To further investigate the molecular mechanisms through which syringin attenuates cardiac hypertrophy, we examined activation of the AMPKα signaling pathway in response to stress stimuli. Our data demonstrated that syringin attenuated the phosphorylation of AMPKα. Thus, we made the conjecture that syringing may play an important role in regulating the activation of AMPKα. AMPK mediates energy metabolism by modulating the activities of key enzymes or their transcription factors in metabolic pathways (29-32) and also plays an important role in protein synthesis, myocyte apoptosis and myocardial angiogenesis. AMPK is composed of α, β and γ subunits; each α subunit contains a phosphorylation site that plays a critical role in regulating AMPK function (30,31). The specific role that AMPKα plays in myocardial metabolism remains incompletely understood. The study by Dyck and Lopaschuk demonstrated that AMPKα activation can influence cardiac metabolism by regulating oxidative phosphorylation and the uptake of fatty acids (33). Another study indicated that AMPKα was highly expressed in the embryonic stages and reduced to the adult level after birth; interestingly, in heart failure, the expression of AMPKα is increased (30,34). Over the years, studies have demonstrated important roles of AMPKα in protecting the heart during ischemia/reperfusion injury, pathological hypertrophy and heart failure using both genetic and pharmacological approaches (35,36). In a study on a model of ischemia/reperfusion, it was shown that AMPK clearly plays a cardioprotective role during ischemic episodes, by increasing glycolytic ATP production. However, AMPK can also play a deleterious role in the reperfused heart (37). Indeed, during early reperfusion, the still existing AMPK activation helps fatty acid oxidation to predominate over glucose oxidation by phosphorylating and inactivating acetyl-CoA carboxylase. Fatty acid oxidation, occurring in parallel with the still present glycolytic stimulation, can promote a deleterious uncoupling of glucose oxidation and glycolysis (38). This activation of AMPK results in the stimulation of glucose uptake, glycolysis and fatty acid oxidation (32,34). These metabolic effects can be both beneficial and harmful during ischemia and during reperfusion following ischemia. In cardiac hypertrophy, AMPK activation can inhibit cardiac protein synthesis and the cardiac hypertrophic process (33,39,40); however, on the other hand, both activating and inactivating AMPK mutations have been

shown to contribute to cardiac hypertrophy (41-44). As our data demonstrated, it is possible that increased AMPK activity in the hypertrophic heart may actually be detrimental to cardiac function by accelerating fatty acid oxidation rates, such that they become the major oxidative substrate in the heart at the expense of glucose oxidation; syringin ameliorated the process. Furthermore, it has been proven that the anti-apoptotic and pro-apoptotic effects of AMPK, myocardial hypertrophy and ischemia can also induce apoptosis in the heart (33,45,46). The role of AMPK in apoptosis or the role of apoptosis in myocardial hypertrophy is still not clear. It is possible that AMPK activation may play dual roles in the development of cardiac hypertrophy (33). That is, the pharmacological activation of AMPK during the early phase of cardiac hypertrophy may be able to prevent hypertrophic growth, while AMPK activation during pathological hypertrophy may be an adaptive response to the metabolic stress (33,47,48). In addition, studies have elaborated that AMPKα plays an improtant role in cardiac hypertrophy by adjusting the process of autophagy (49-51). A previous study demonstrated that the elevation of autophagy during myocardial ischemia and glucose deprivation was accompanied by the activation of AMPKα, and the inhibition of AMPKα significantly attenuated the induction of autophagy (52). In this study, syringin decreased hypertrophy induced by pressure overload by attenuating the activation of AMPKα and autophagy. The role of autophagy is complex, but is indispensable to maintain cardiac homeostasis (22,49,53); it is difficult to confirm whether autophagy is protective or deleterious in the setting of cardiomyopathies (53,54). The variationd in the expression of LC3II reveal that the level of autophagy may change in the different periods of left ventricular hypertrophy induced by transverse aortic constriction. Autophagy has been shown to be reduced in hypertrophied hearts following this type of surgery for 1 week, and elevated for 4 weeks (55,56). Furthermore, our data indicated that cardiac hypertrophy induced by aortic banding involved AMPK-dependent autophagy. Another study indicated that autophagy was elevated in patient cardiac tissues affected by hypertrophic cardiomyopathy by detecting the expression of LC3II and beclin 1, which proved that miR-451 regulates cardiac hypertrophy and autophagy by targeting TSC1 (56). However, our data demonstrated that syringin reduced the autophagy level to alleviate cardiac hypertrophy. Our findings broaden our understanding of the protective role of syringing in cardiac hypertrophy, and provide a novel and potential pharmacotherapeutic strategy with which to mitigate cardiac hypertrophy induced by pressure overload and attenuate the progression of heart failure.

In conclusion, our data indicate that the long-term oral administration of syringin attenuates the development of cardiac hypertrophy induced by pressure overload and improves cardiac functions. The protective effects of syringin may potentially be attributed to the inhibition of the AMPKα and autophagy-related signaling pathways. Our results indicated the use of syringin may provide a potentially effective strategy with which to attenuate the progression of cardiac hypertrophy.

Acknowledgements

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