Int J Clin Exp Med 2017;10(7):10202-10213
www.ijcem.com /ISSN:1940-5901/IJCEM0015904

Original Article

Evodiamine attenuates pressure overload-induced cardiac hypertrophy

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Received September 9, 2015; Accepted May 17, 2016; Epub July 15, 2017; Published July 30, 2017

Abstract: Evodiamine, a traditional Chinese herb Evodia rutaecarpa, has been shown its anti-inflammatory and anti-bacterial properties. However, the role of evodiamine in cardiac hypertrophy and its possible molecular mechanisms remain unclear. Therefore, this study aimed to investigate the effect of evodiamine on cardiac hypertrophy and explore the possible mechanisms. Aortic banding (AB) on mice was performed to induce the models of cardiac hypertrophy. Evodiamine was administered to mice for 7 weeks begining at one week after AB or sham surgery. Cardiac function was evaluate by Echocardiography and hemodynamics; The makers of cardiac hypertrophy were measured with real-time polymerase chain reaction (RT-PCR) and Western blot, and cardiomyocyte apoptosis were investigated by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL). We found that evodiamine could attenuate pressure overload-induced cardiac hypertrophy and cardiac fibrosis through inhibiting phosphorylated extracellular regulated protein kinases 1/2 (Erk1/2) and Akt protein expressions. Furthmore, evodiamine reduced the positive percentage of cardiomyocyte apoptosis in response to AB surgery. Therefore, evodiamine was a potential drug to inhibit cardiac hypertrophy via Erk1/2 and Akt signal pathways.

Keywords: Evodiamine, cardiac hypertrophy, apoptosis, Erk1/2, Akt

Introduction

Cardiac hypertrophy is a response to pathological stress containing neurohumoral activation and hemodynamic overload [1]. It is usually coupled with many other diseases, such as myocardial infarction, hypertension and heart failure. Heart failure becomes a major cause of morbidity and mortality worldwide. Cardiac hypertrophy leads to cardiomyocyte enlargement, which is characterized by an increase in protein synthesis and changes in the organization of sarcomeres [2]. In the compensatory stage of pathological hypertrophy, in order to meet work overload, the heart has been through some pathological changes, which do not affect cardic function. However, in the subsequent decompensatory phase of pathological hypertrophy, it would lead to cardiac dysfunction [3]. Numerous studies have reported some molecular mechanisms are involved in hypertrophic responses, and inhibition of the pathways and factors involved in the pathological hypertrophy is the target to ameliorate cardiac hypertrophy. Therefore, finding new pharmacological agents which can regulate the molecular mechanism of cardiac hypertrophy can prevent or reverse the development of heart failure induced by pressure overload [4].

Evodiamine, a major component derived from Chinese herb evodia rutaecarpa, has been shown to possess anti-tumor property which could suppress tumor growth, tumor metastasis and angiogenesis [5, 6]. A previous study showed that evodiamine could induce intracellular calcium/JNK-mediated autophagy and apoptosis in glioma cells [7]. However, the effect of evodiamine on cardiac hypertrophy and its potential molecular mechanisms have not been explored. Therefore, in this study, we established the models of cardiac hypertrophy on mice induced with aortic banding (AB) to observe the role of evodiamine on cardiac hypertrophy and discuss its possible molecular mechanisms.

Materials and methods

Chemicals

Evodiamine (99% purity by high-performance liquid chromatography analysis) was purchased from Shanghai Winherb Medical S&T Development Co., Ltd. (Shanghai, China).

Animals

All animal experiments were performed in accordance with the Animal Care and Use Committee of Renmin Hospital of Wuhan University. Male C57BL/6 mice, weighing 23.5-27.5 g and 8 to 10 weeks old, were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). The mice were feed in the Cardiovascular Research Institute of Wuhan University (Wuhan, China) under proper temperature and humidity [8]. The mice were randomly divided into 5 groups and each group was 16 mice. Three of the groups were subjected to AB and the two other groups were subjected to sham surgery (sham). Three of the treatment groups were orally administered evodiamine at doses of 50 mg/kg body weight (Low dose-evodiamine) or 100 mg/kg body weight (High dose-evodiamine) for 7 weeks [9], and the remaining groups were given oral doses of normal saline (vehicle) for 7 weeks as control groups. The five groups were divided as follows: sham + High dose-evodiamine, sham + vehicle, AB + vehicle, AB + Low dose-evodiamine, and AB + High dose-evodiamine. Cardiac function of all mice were evaluated by echocardiography and catheter-based measurements of hemodynamic parameters at 8 weeks after AB surgery before the heart was taken.

Echocardiography and hemodynamics

Mice were anesthetized with 1.5% isoflurane before performing echocardiography using MyLab™ 30CV (Esaote S. p. A., Genoa, Switzerland) with a 10MHz linear array ultrasound transducer [10]. Left ventricular end-diastolic diameter (LVEDd), left ventricular end-systolic diameter (LVESd), interventricular septum depth (IVSd) and left ventricular posterior wall thickness (LVPWd) were measured by the left ventricle (LV) M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level [11].

Mice were anesthetized with 1.5% isoflurane before hemodynamic measurements were pergormed. A microtip catheter transducer (SPR-839, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the left ventricle. Data were recorded with a Millar Pressure-Volume System (MPVS-400, Millar Instruments, Houston, TX, USA), and analyzed by PVAN Analysis Software [12].

Histological analysis

To investigate the cardiac changes in histological level, hearts were quickly excised, washed with phosphate-buffered saline, weighed, and then immersed by perfusion with 10% neutral buffered formalin. Some sections of mouse heart were prepared for hematoxylin and eosin (HE) staining for morphological evaluation and picrosirius red (PSR) staining for collagen deposition evaluation. cardiac slides were observed with light microscopy. Other sections were evaluate the cross-sectional area of cardiomyocytes by staining with fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA) (Invitrogen,Carlsbad, CA, USA) for membranes and 4',6-diamidino-2-phenylindole (DAPI) for nuclei. The areas of cardiomyocytes were calculated by Image Pro-Plus 6.0, a quantitative digital image analysis system [8].

In vitro experiment

The H9c2 rat cardiomyocytes were acquired from The Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM/F12 (GIBCO, Grand Island, NY, USA), supplemented with 10% calf serum (GIBCO, Grand Island, NY, USA), 1% penicillin (100U/ml) and streptomycin (100 mg/ml) (GIBCO, Grand Island, NY, USA). Evodiamine was dissolved in dimethyl sulfoxide (DMSO). Ang II (1 μM) was incubated with these cells for 24 h in the presence or absence of different concentrations of evodiamine (1 μM, 5 μM, 10 μM). Then total RNA was extracted from the H9c2 cells. The mRNA expression levels of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were examined by quantitative real-time PCR.

Quantitative real-time PCR (RT-PCR)

The mRNA levels of hypertrophic and fibrotic markers atrial natriuretic peptide (ANP), B-type

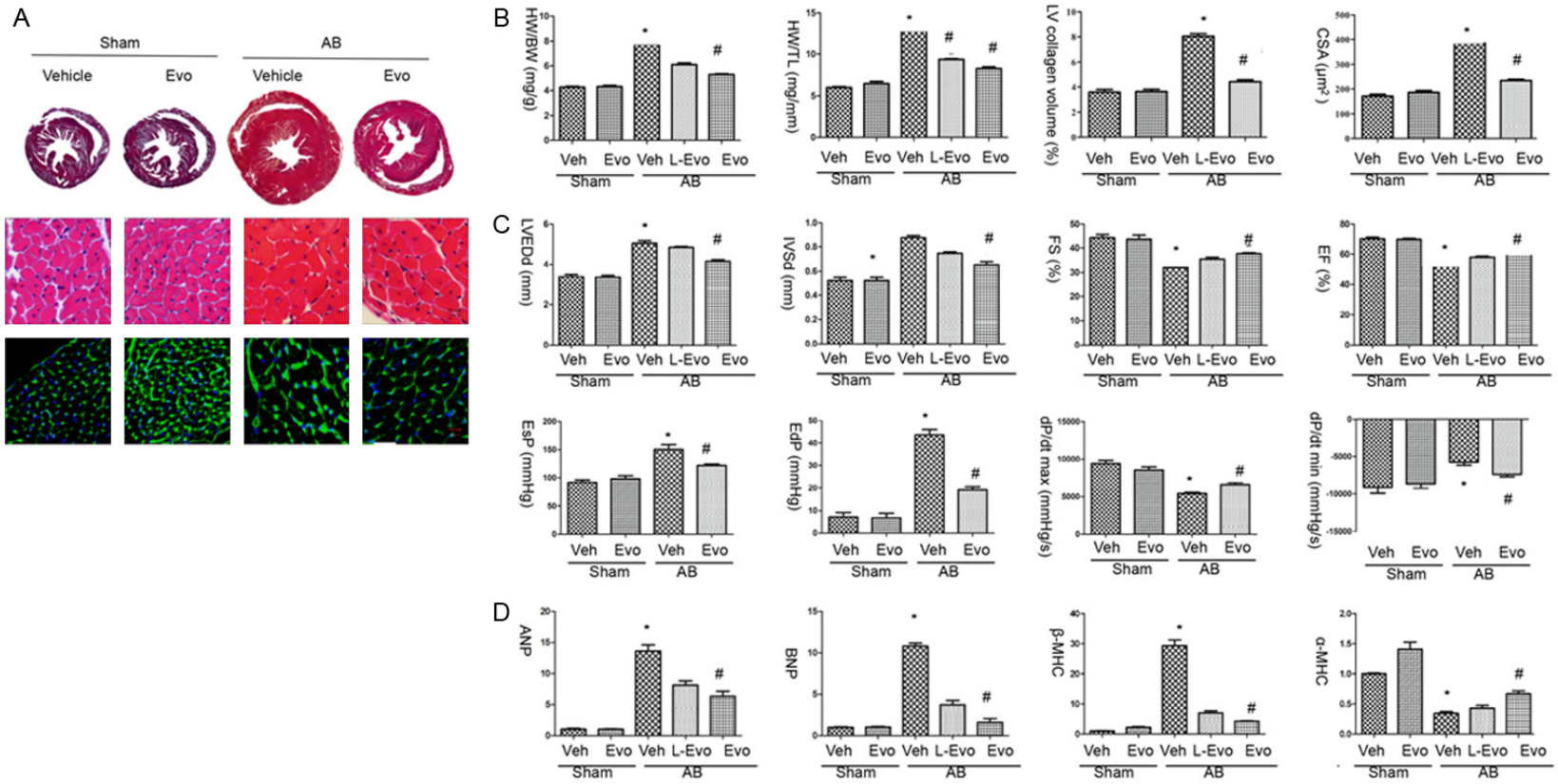


Figure 1. Evodiamine protects against cardiac hypertrophy. A. Hematoxylin and eosin staining and fluorescein isothiocyanate-labeled wheat germ agglutinin staining of sham and aortic-banded (AB) mice at 8 weeks post-surgery. B. Statistical results of the heart weight/body weight (HW/BW) ratio and heart weight/tibial length (HW/TL) ratio. Fibrotic areas from histological sections were quantified using an image-analysis system and cardiomyocyte cross-sectional areas of indicated groups. C. Evodiamine amoliorates hemodynamic parameters induced by pressure overload. D. mRNA expression levels of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), β-myosin heavy polypeptide (MHC), and α-MHC induced by AB were detected by reverse transcription-polymerase chain reaction analysis (n=6). Veh: vehicle. AB: aortic banding. EVO: Evodiamine 100 mg/kg. L-EVO: 50 mg/kg. *P<0.05 compared with the corresponding sham group. $^{\#}$P<0.05 vs. the AB + vehicle group.

natriuretic peptide (BNP), α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), connective tissue growth factor (CTGF), transforming growth factor β-1 (TGFβ-1), collagen I, collagen III and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected by quantitative real-time RT-PCR. Total RNA was extracted from hearts or H9c2 cells with TRIzol reagent following the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The RNA purities were evaluated on the OD260/OD280 ratios with the SmartSpec Plus Spectrophotometer (Bio-Rad, Hercules, CA, USA). The mRNA was reversely transcribed into cDNA using oligo(dT) primers and the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). PCR amplifications were quantified using the LightCycler 480 SYBR Green I Master kit (Roche), and the results were normalized against GAPDH expression [13].

Western blot

Ice-cold radioimmunoprecipitation assay buffer was used to extract protein from hearts as described in our previous study [8]. The protein concentrations were measured by the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The extracted protein samples were separated on SDS-PAGE, transferred to Immobilon-FL Transfer Membranes (Merck Millipore, Billerica, MA, USA) and incubated with primary antibodies against total Erk1/2, phosphorylated Erk1/2, total JNK, phosphorylated JNK, total P38, phosphorylated p38, total GSK3β, phosphorylated GSK3β, total AKT, phosphorylated AKT, total PI3K, phosphorylated PI3K, total FOXO3a, phosphorylated FOXO3a and GAPDH for 24 h in 4°C [14]. After removal of the primary antibody, the blots were incubated with the corresponding peroxidase-conjugated secondary antibody for 2 h. Finally the bands were then scanned by a two-color infrared imaging system (Odyssey, Danvers, MA, USA). Target protein expression levels were normalized to GAPDH protein.

Assessment of apoptosis

To evaluate the postive percentage of cardiomyocyte apoptosis, the cardiomyocytes were detected with an *in situ* terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) kit (ApopTag® Plus Fluorescein In Situ Apoptosis Detection Kit, Chemicon, Temecula, CA, USA). Mouse hearts from all groups were divided into 5 μm slices. Some Sections were stained with TUNEL reagents and DAPI. The α-actin primary antibody (Millipore) was diluted in 1% goat serum at 1:100 dilution. The numbers of positive apoptosis and total cells were calculated with immunofluorescence microscopy.

Statistical analysis

All Data were analyzed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The differences between two groups were used the Independent-Samples T test. One-way ANOVA test with a post hoc of Tukey's test was used to compare the difference among groups and $P < 0.05$ was considered a statistically significant difference.

Results

Evodiamine attenuated pressure overload-induced cardiac hypertrophy in wild-type mice

In order to determine whether evodiamine can ameliorate the hypertrophic response to pressure overload, Evodiamine administration began 7 days after AB or sham surgery, After 7 weeks of evodiamine treatment, evodiamine could attenuate pressure overload-induced increases in heart weight/body weight ratio (HW/BW), heart weight/tibial length ratio (HW/TL) and cross-sectional areas (CSA) of cardiomyocytes. In accordance to morphological changes including HE staining and WGA staining (**Figure 1**). The results of echocardiographic and pressure-volume loop analyses of the mice showed that pressure overload significantly exacerbated cardiac hypertrophy in the vehicle-treated group, However, evodiamine could attenuate increased LVEDd and IVSd, and improve decreased fractional shortening and ejection fraction induced by pressure overload. Moreover, Evodiamine-treated mice exhibited inhibition of cardiac dilation and increased LV function. Vehicle-treated mice showed ventricular dysfunction at 8 weeks after AB surgery, increases in diastolic blood pressure and decreases in systolic and diastolic function. It suggested that the hemodynamic parameters dP/dt max and dP/dt min in mice with evodiamine treatment were ameliorated. In addition, the expression levels of hypertrophic markers, including ANP, BNP, and β-MHC significantly were increased after AB surgery. However, the

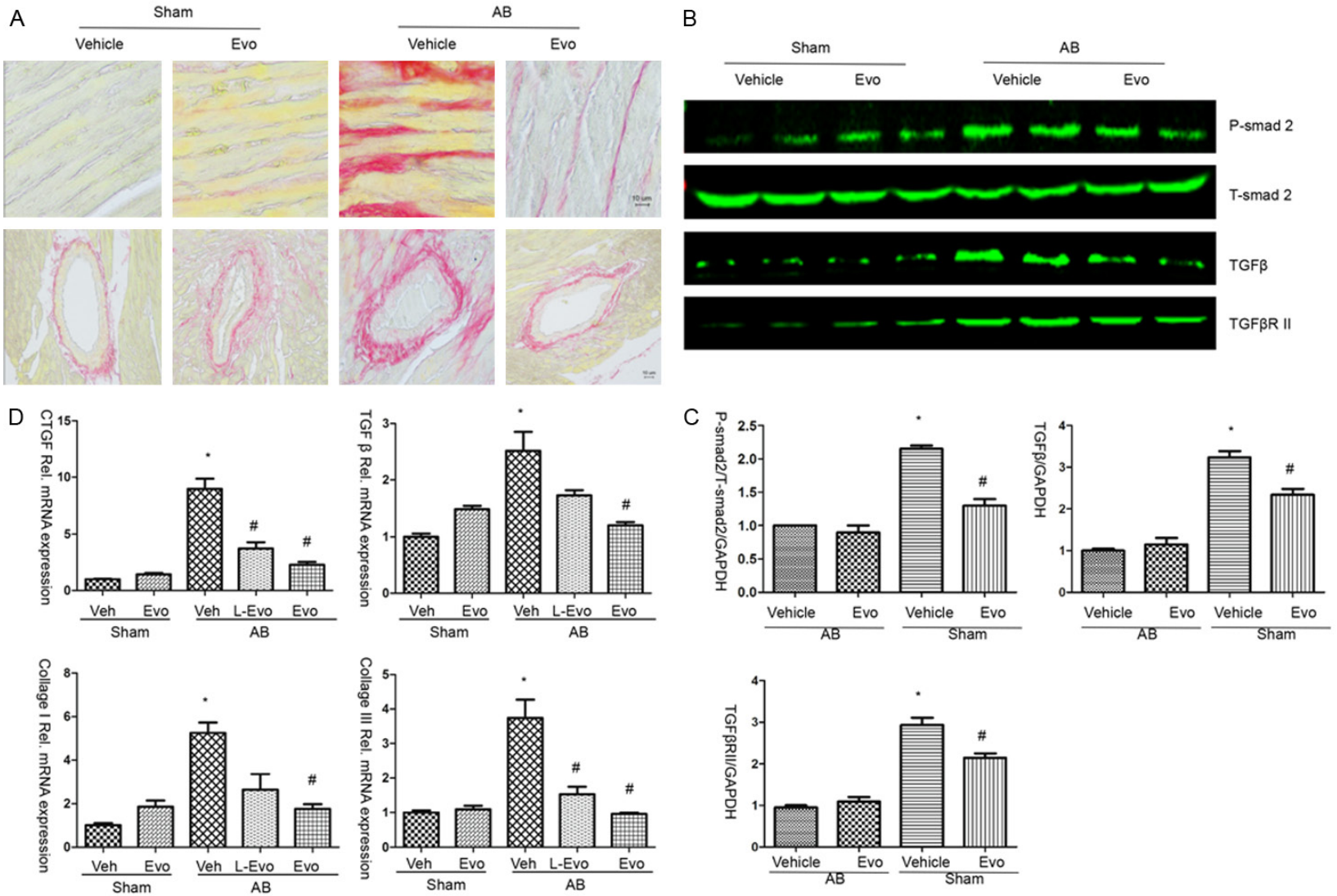


Figure 2. Evodiamine attenuates fibrosis in pressure-overloaded-induced mouse hearts. A. Histological sections of the left ventricle were stained with picrosirius red. B. Representative blots of P-smad2, TGF-β1, and TGF-βRII, in the heart tissues of mice (*n*=6). C. Quantitative analysis of expression levels. D. Expression levels of CTGF, collagen Iα, collagen III, and TGF-β1 in the heart were determined by RT-PCR. Veh: vehicle. AB: aortic banding. EVO: Evodiamine 100 mg/kg. L-EVO: 50 mg/kg. *P<0.05 compared with the corresponding sham group. #P<0.05 vs. the AB + vehicle group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

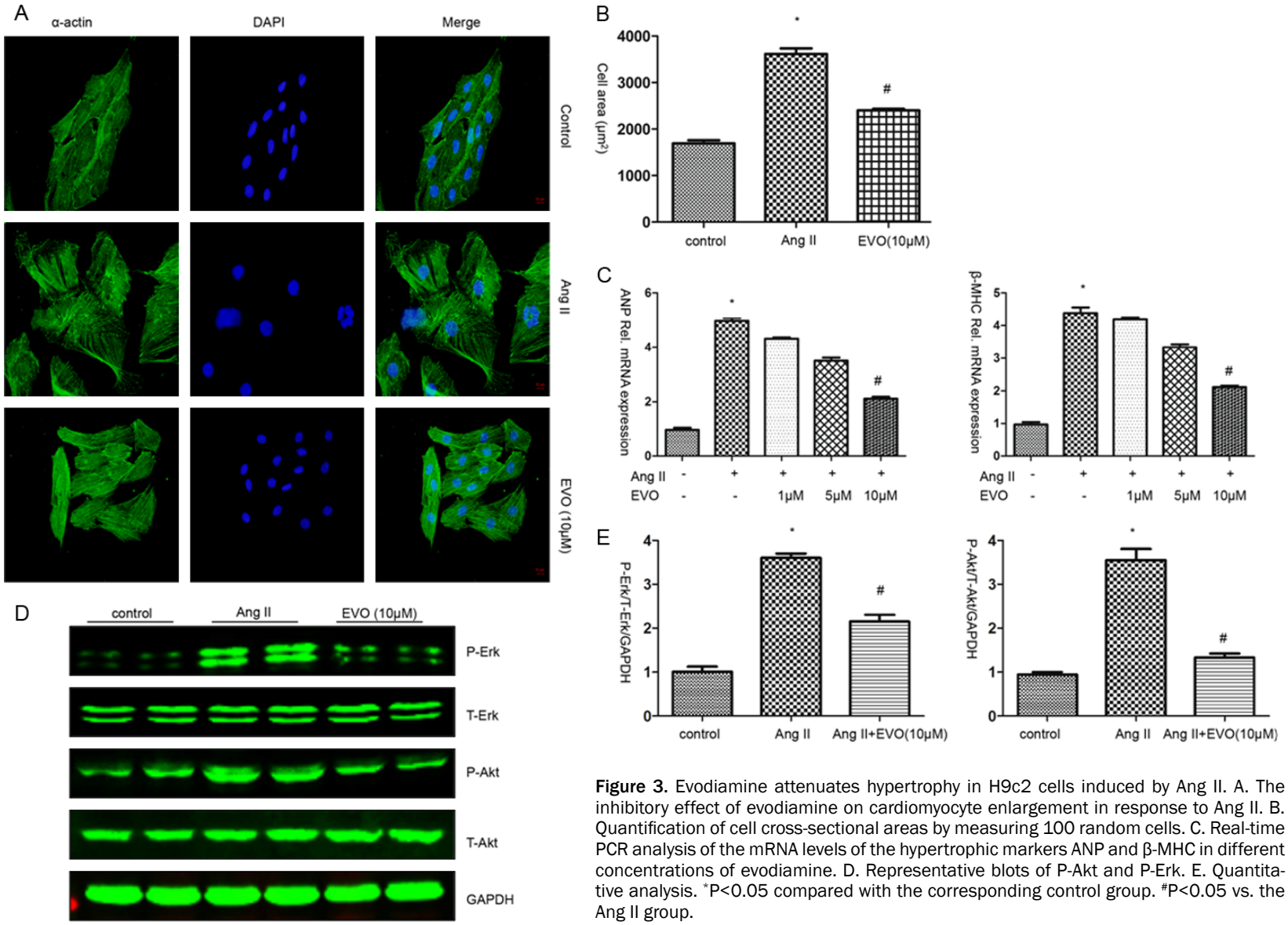
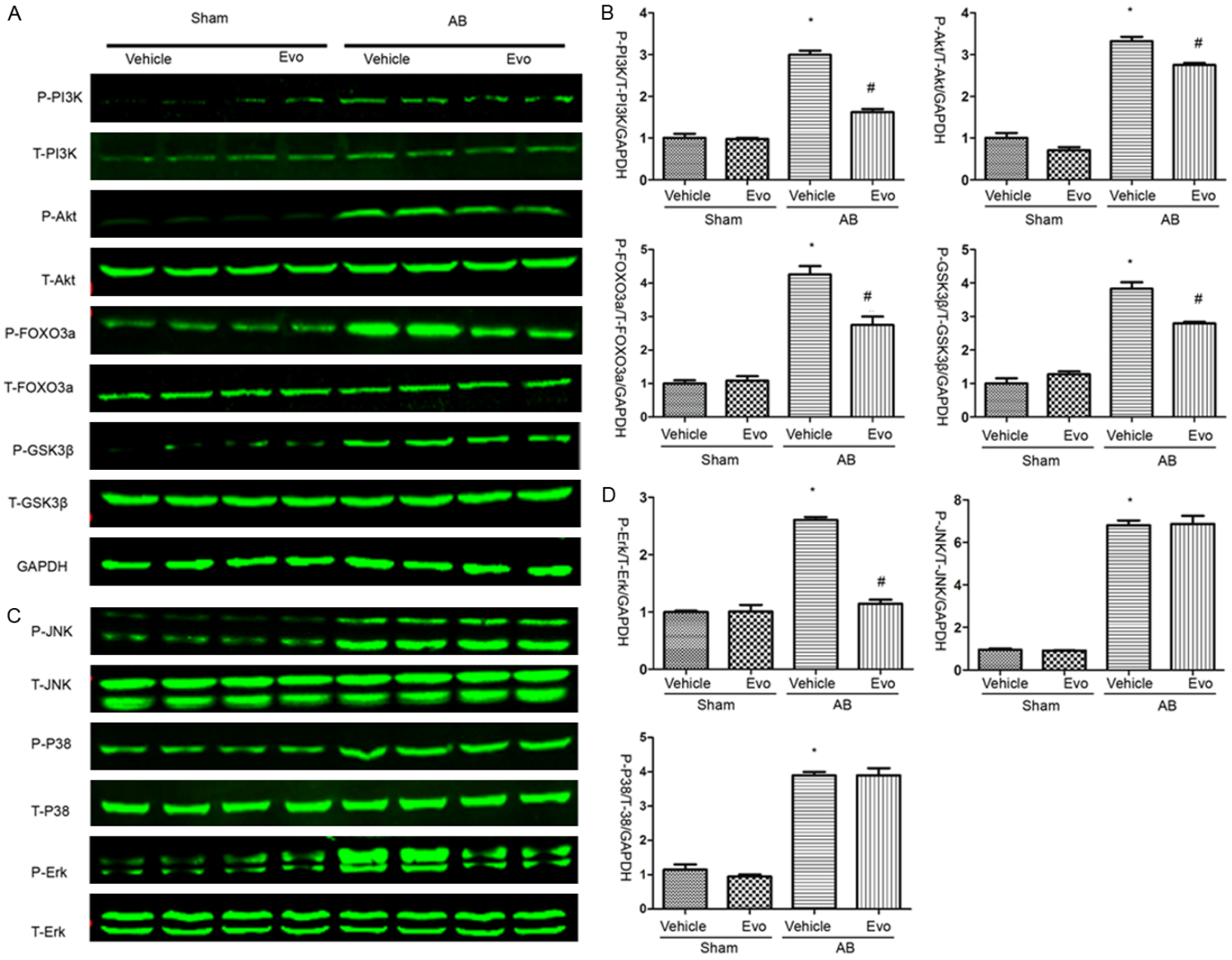


Figure 3. Evodiamine attenuates hypertrophy in H9c2 cells induced by Ang II. A. The inhibitory effect of evodiamine on cardiomyocyte enlargement in response to Ang II. B. Quantification of cell cross-sectional areas by measuring 100 random cells. C. Real-time PCR analysis of the mRNA levels of the hypertrophic markers ANP and β-MHC in different concentrations of evodiamine. D. Representative blots of P-Akt and P-Erk. E. Quantitative analysis. *P<0.05 compared with the corresponding control group. #P<0.05 vs. the Ang II group.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T-GSK3β
GAPDH
C
P-JNK
T-JNK
P-P38
T-P38
P-Erk
T-Erk
B
P-PI3K/T-PI3K/GAPDH
P-Akt/T-Akt/GAPDH
P-FOXO3a/T-FOXO3a/GAPDH
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P-P38/T-38/GAPDH
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Figure 4. The effect of evodiamine on the PI3K/Akt and Erk signaling pathways. A. Representative blots of P-PI3K, P-Akt, P- GSK3β, and P-FoxO3a in the heart tissues of mice (*n*=6). B. Quantitiative analysis of protein expression levels. C. Representative blots of P- Erk1/2, P-p38, and P-JNK1/2 in the cardiac tissues of mice (*n*=6). D. Quantitative results. Veh: vehicle. AB: aortic banding. EVO: Evodiamine 100 mg/kg. L-EVO: 50 mg/kg. *P<0.05 compared with the corresponding sham group. #P<0.05 vs. the AB + vehicle group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

increased expression levels of hypertrophic markers were attenuated after evodiamine administration.

Evodiamine amelorated pressure overload-induced cardiac fibrosis

Left ventricular interstitial fibrosis was evaluated with PSR staining. Interstitial fibrosis was observed in vehicle-treated mice after AB. To investigate the effect of evodiamine on collagen synthesis and excessive deposition, we examined the mRNA expression of genes involved in cardiac fibrosis, such as CTGF, TGF-β1, collagen I and collagen III. Our results showed that evodiamine administration attenuated the mRNA expression of cardiac fibrosic marker induced by AB (**Figure 2**).

Evodiamine attenuated cardiac hypertrophy in vitro

The mRNA expression of hypertrophic markers including ANP and BNP were elevated after Ang II treatment (1 μM, 24 h). The mRNA expressions of ANP and BNP were significantly increased in Ang II group. After evodiamine (1 μM, 5 μM, and 10 μM) Treatment, the increased mRNA expressions of of hypertrophic markers were reduced in a concentration-dependent manner. To evaluate single cardiomyocyte hypertrophy, we stained cells with immunocytochemistry for primary antibodies of cardiac α-actin, as shown in **Figure 3**.

Evodiamine attenuated fibrosis by inhibiting Erk1/2 and Akt signaling pathways

To further explore the molecular mechanisms , we tested whether the activation of mitogen activated protein kinase (MAPK) and Akt pathways were involved in the effects of evodiamine on cardiac hypertrophy and cardiac fibrosis. We found that Erk1/2, c-Jun N-terminal kinase (JNK), and p38 were significantly activated in AB group, and evodiamine administration could decrease increased phosphorylation of Erk1/2 and Akt. We also examined the protein expression levels of GSK3β and FoxO3a at 8 weeks after surgery. The phosphorylated expression levels of GSK3β and FoxO3a was remarked increased in AB group. However, Evodiamine significantly attenuated increased phosphorylated expression levels of GSK3β and FoxO3a. As shown in **Figure 4**. To discuss the molecular mechanisms which was responsible for the anti-fibrosis effect of evodiamine, we also detected the phosphorylation of Smad 2 and transforming growth factor β receptor (TGFβR) II (**Figure 2**). Evodiamine administration significantly could decrease these protein expression induced by pressure overload. In addition, the experiments in vitro were consistent with animal experiments.

Evodiamine alleviated pressure overload-induced cardiomycyte apoptosis

To explore the role of evodiamine in cardiomyocyte apoptosis, we stained hearts with TUNEL assay. TUNEL-positive cells in all groups were assessed, and the increased percentage of apoptotic cardiomyocytes induced by pressure overload was significantly decreased after evodiamine administration (**Figure 5**). We detected the protein expression levels of cleaved caspase-3, cleaved caspase-9, Bax and Bcl2 proteins with Western blotting. Compared to the vehicle-treated group, the expression level of Bax was decreased and the expression level of Bcl2 was increased. Moreover, levels of cleaved caspase-3 and cleaved caspase-9 were reduced in evodiamine-treated mice in response to AB. Therefore, it indicated that evodiamine alleviated pressure overload-induced cardiomyocyte apoptosis.

Discussion

Evodiamine is the major active component of Evodia rutaecarpa and possesses several biological properties, including antitumor, vasorelaxant and antinociceptive effects [15]. However, the effect of evodiamine on cardiac hypertrophy and fibrosis remains unclear. In this study, we found that evodiamine could

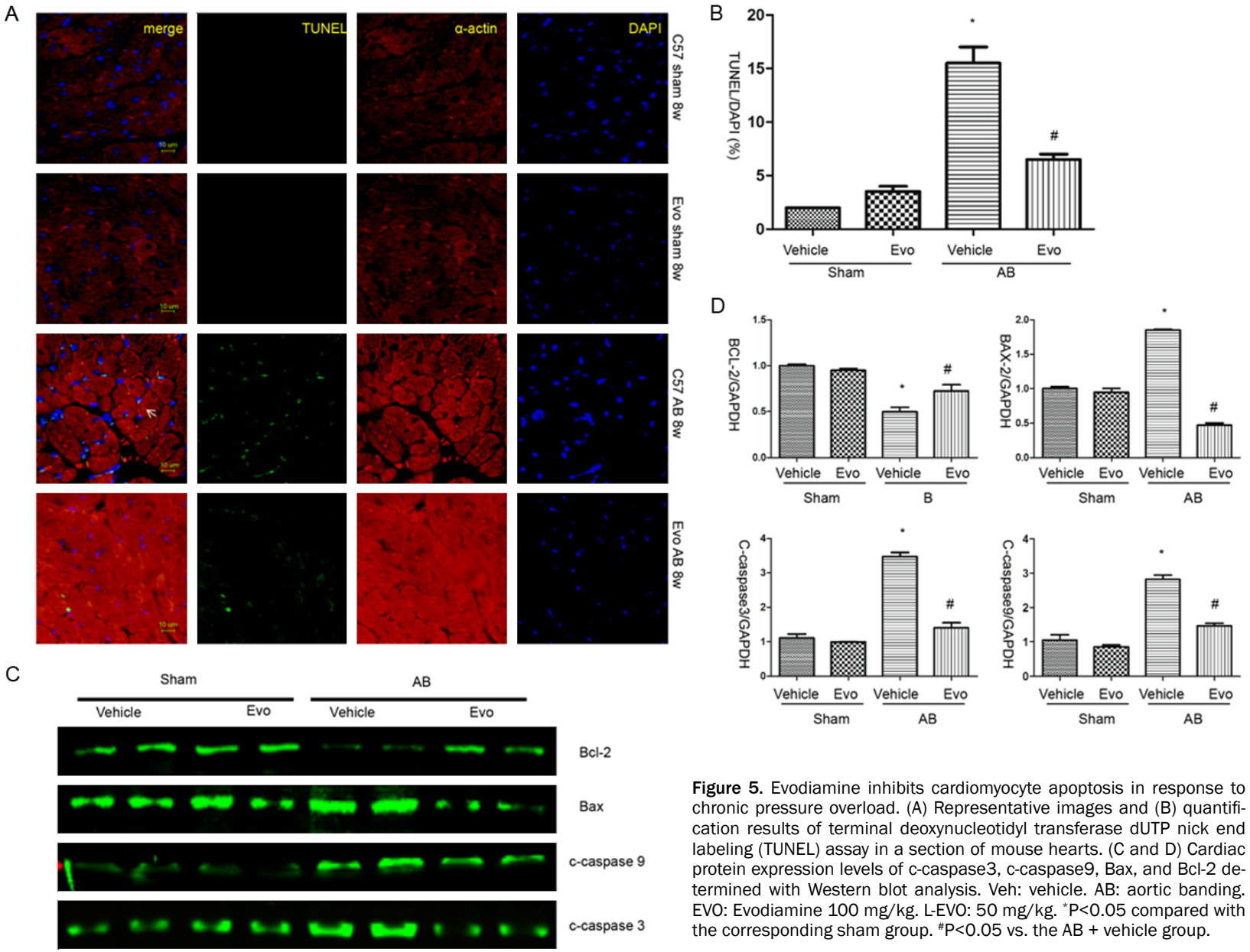

Figure 5. Evodiamine inhibits cardiomyocyte apoptosis in response to chronic pressure overload. (A) Representative images and (B) quantification results of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in a section of mouse hearts. (C and D) Cardiac protein expression levels of c-caspase3, c-caspase9, Bax, and Bcl-2 determined with Western blot analysis. Veh: vehicle. AB: aortic banding. EVO: Evodiamine 100 mg/kg. L-EVO: 50 mg/kg. *P<0.05 compared with the corresponding sham group. #P<0.05 vs. the AB + vehicle group.

attenuate cardiac remodeling induced by pressure overload via regulating the Erk1/2 and Akt signaling pathways.

Due to the treatment of some human diseases, Evodiamine becomes one of the most popular traditional Chinese medicine [16]. Many studies have demonstrated that evodiamine showed some biological activities, such as anti-inflammatory [17], anti-obesity [18, 19], and antitumor effects. The possible molecular mechanisms may be involved in the MAPK [15, 20], PI3K/Akt [21], and NF-κb signaling pathways [22]. The biological mechanisms of evodiamine antagonism toward human cancer cells and hindrance of their invasion and metastasis have especially attracted researchers' attention [15, 16, 23-25]. Wang and his colleagues showed that evodiamine inhibited activation of mTOR-S6K and IRS1 serine phosphorylation induced by insulin and increased glucose tolerance in adipocytes of adiabetic mice model [19]. Other previous studies also proved that evodiamine could up-regulate the caspase-dependent apoptotic pathway and was closely associated with the Akt pathway [24]. Our study demonstrated that evodiamine could attenuate pressure overload-induced cardiac hypertrophy and improve cardiac systolic function.

To investigate the molecular mechanisms which was responsible for the effect of evodiamine on cardiac hypertrophy, we examined whether the MAPK and Akt signaling pathways in response to stress stimuli were activated. Intracellular MAPK signaling cascades participated in the pathogenesis of cardiac hypertrophy.

The increased protein expression levels of phosphorylated PI3K, Akt, GSK3β, FoxO3a and Erk in response to pressure overload was reduced after evodiamine treatment. Our findings indicated that the role of evodiamine in cardiac remodeling may be mediated by PI3K/Akt and Erk signaling pathways. Erk1/2 is a central mediator of cardiac hypertrophy and the potential therapeutic target. Many studies have examined the potential function of Erk1/2 on cardiac hypertrophy [26]. A study by Ruppert and colleagues showed that interference with Erk1/2 phosphorylation impaired pathological but not physiological hypertrophy [27]. Vidal and his colleagues showed that phosphorylation of Erk1/2 was involved in cardiac hypertrophy through activation of β-adrenergic receptor and depended on a complex mechanism, including activation of Erk1/2 and G proteins [28]. In our study, increased expression levels of the phosphorylated Erk1/2, JNK, and p38 could be induced by pressure overload. We also found that the phosphorylated level of Mek-Erk1/2 was increased after AB surgery and the phosphorylated levels of these proteins could be reduced by evodiamine. Evodiamine exhibited the similar effect on Akt phosphorylation. Akt, also protein kinase B, which is a serine-threonine kinase participating in regulation of a large number of cellular processes [29]. It was reported that Akt was important in the development of cardiac hypertrophy induced by pressure overload, chronic exercise training, and normal cardiac growth [30]. Our data showed that evodiamine attenuated the phosphorylated protein levels of PI3K and Akt.

FoxO3a belongs to the forkhead transcription factor family and is an important substrate of Akt. FoxO3a has been proven to induce apoptosis in previous studies. In this study, evodiamine could decrease cardiomyocyte apoptosis. In addition, phosphorylation of FoxO3a was decreased in evodiamine-treated group. A study by Yang et al. found that Erk could down-regulate FOXO3a by directly phosphorylating FOXO3a at Ser 294, Ser 344, and Ser 425, and then affected cell proliferation [31]. In the process of cardiac hypertrophy, FoxO3a was activated to promote mitochondrial-induced apoptosis. it was consistent with our experiments.

Fibrosis is an important feature of cardiac hypertrophy characterized by disproportionate expression of the extracellular matrix and excessive accumulation of fibrillar collagen. The major types of collagen in heart, including collagen I and collagen III, are main contributors to interstitial fibrosis and accelerate progression of cardiac remodeling. Previous studies have shown that chronic fibrosis-related diastolic dysfunction was caused by TGF-β. Smad proteins are transcription factors activated by the TGF-β superfamily. In our study, evodiamine decreased the phosphorylated level of Smad 2. Cardiomyocyte apoptosis played a significant role in the hypertrophic heart, especially in progression from cardiac injury to heart failure [8]. Thus, cardiomyocyte apoptosis was closely associated with chamber dilation and cardiac systolic performance dete-

rioration [32, 33]. In this study, cardiomyocyte apoptosis induced by pressure overload was ameliorated by evodiamine treatment. We found that evodiamine downregulated Bax protein expression and upregulated Bcl-2 protein expression in the mitochondrial apoptotic pathway. Evodiamine also diminished the levels of cleaved caspase-3 and caspase-9. Interestingly, the pharmacological effect of evodiamine on anti-fibrotic and anti-apoptotic protein expressions showed that evodiamine could protect against extracellular remodeling in pressure overload. Our results provided a novel pharmacotherapeutic strategy for treating cardiac hypertrophy in pressure overload and retarding progression of heart failure.

In conclusion, long-term oral administration of evodiamine could attenuate the development of cardiac hypertrophy induced by pressure overload and fibrosis and improve cardiac function. The protective effect of evodiamine against cardiac hypertrophy and fibrosis may be involved in the PI3K/Akt and Erk signaling pathways.

Acknowledgements

This work was supported by the Fundamental Research Fund for the Central Universities, Ministry of Education of China (Grant No. 2014302020202) and Hubei Province's Outstanding Medical Academic Leader program.

Disclosure of conflict of interest

None.

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