

Original Article

Apigenin inhibits pressure overload-induced cardiac hypertrophy

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Received June 29, 2017; Accepted October 10, 2017; Epub April 15, 2018; Published April 30, 2018

Abstract: Apigenin (5,7,4'-trihydroxyflavone), a nontoxic citrus flavonoid, possesses comprehensive bioactive properties including anti-oxidation, anti-inflammatory, anticancer and antivirus functions. However, little is known about the role of apigenin on cardiac hypertrophy and fibrosis. The objective of our present study was to investigate the effect of apigenin on cardiac hypertrophy induced by aortic banding (AB) in mice and to elucidate the underlying molecular mechanisms. The extend of cardiac hypertrophy was quantitated by two-dimensional and M-mode echocardiography as well as by pathology and gene expression of hypertrophic markers analyses of the heart specimen. Our data demonstrated that apigenin significantly attenuated cardiac hypertrophy induced by AB through inhibiting Akt/GSK3 β pathway. Meanwhile, apigenin attenuated fibrosis and collagen synthesis. Taken together, these current findings indicated that apigenin, a feasible safe and natural treatment, has protective potential against cardiac hypertrophy.

Keywords: Apigenin, cardiomyopathy, hypertrophic, cystic fibrosis, glycogen synthase kinase 3, oncogene protein v-akt

Introduction

Cardiac hypertrophy is an adaptive response to a broad-spectrum of pathological stimulus such as mechanical overload, neuroendocrine stress. It is benefit for maintaining cardiac pump function at early stage. However, prolonged stresses are maladaptive and promote compensatory change into congestive heart failure and malignant arrhythmia or even sudden death, which is associated with an increased risk of cardiovascular morbidity and mortality worldwide [1, 2]. Pathological hypertrophy is characterized with myocytes hypertrophy and interstitial fibrosis at morphological level and re-expression of fetal gene program at gene level [3, 4]. A great deal of evidence verified several signaling pathways activation involved in cardiac hypertrophy. By using genetic and cellular models of cardiac hypertrophy it has proved pathological hypertrophy can be prevented or reversed [5]. Therefore, many new drugs aiming for special regulators and targets may become effective approach under this situation.

Apigenin (5,7,4'-trihydroxyflavone), a nontoxic citrus flavonoid, abundantly present in common vegetables and fruits have been showed to possess antioxidant, anti-inflammatory, anticancer and antivirus biological properties [6, 7]. Furthermore, it exhibits the function of inhibiting the cell cycle, diminishing oxidative stress, inducing apoptosis in vivo and in vitro. In human prostate cancer cells, apigenin induce cell cycle arrest accompanied with MAPK, PI3K-Akt change [8]. Apigenin inhibits HGF-promoted invasive growth and metastasis through blocking PI3K/Akt pathway in MDA-MB-231 breast cancer cells [9]. Apigenin inhibits HIF-1 and VEGF expression and suppress angiogenesis via PI3K/AKT/p70S6K1 and HDM2/p53 pathways in human ovarian cancer cells [10]. Apigenin induced apoptosis in human lymphoma B cells in vitro. Although it is demonstrated that apigenin could regulate many molecules, very little is known about whether it could regulate cardiac hypertrophy. Thus, in this study, we investigate the effects of apigenin on mice with cardiac hypertrophy by echocardiography, his-

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Table 1. Inhibits cardiac hypertrophy by apigenin in AB mice

Parameter	Vehicle-Sham	Apigenin-Sham	Vehicle-AB	Apigenin-AB
Number	n=10	n=10	n=10	n=10
BW (g)	29.3±0.3	29.9±0.4	29.9±0.2	30.7±0.4
HW/BW	4.24±0.10	4.01±0.10	7.58±0.18*	6.33±0.13*†
LW/BW	4.80±0.13	4.55±0.07	4.97±0.10*	4.64±0.10†
HW/TL	6.77±0.13	6.54±0.12	12.21±0.23*	10.55±0.22*†
LVEDD (mm)	3.66±0.04	3.53±0.05	4.95±0.07#	4.55±0.08*†
LVESD (mm)	2.06±0.04	2.12±0.04	3.76±0.08#	3.16±0.09*†
IVSd (mm)	0.70±0.011	0.69±0.007	0.82±0.009#	0.79±0.005*†
LVPWD	0.68±0.010	0.70±0.008	0.81±0.012#	0.79±0.017*
FS (%)	42±0.4	41±0.7	24±0.6*	32±0.9*†

BW, body weight; HW, heart weight; LW, lung weight; TL, tibia length; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; IVSd, left ventricular septum, diastolic; LVPWD, left ventricular posterior wall, diastolic; FS, fractional shortening. All values are mean ± SEM. #P<0.05 vs Vehicle-Sham, *P<0.01 vs Vehicle-Sham, †P<0.05 vs Vehicle-AB.

tological and molecular biologic technique and attempt to clarify the molecular mechanism.

Materials and methods

Materials

The polyclonal antibodies used to recognize total and phosphorylated Akt, GSK3β, IκBα were purchased from Cell Signaling Technology. The BCA protein assay kit was purchased from Thermo Scientific. Apigenin (> 98% purity as determined by high-performance liquid chromatographic analysis) was obtained from Shanghai Medical Technology Development Co. Ltd. Harmony.

Animal models of cardiac hypertrophy

Animal experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US national institute of health. Adult male C57BL/6 mice (8-10 weeks old, 23.5-27.5 g) were obtained from institute of laboratory animal sciences, Chinese academy of medical sciences and randomly assigned into four groups for surgery in double-blind fashion. Aortic banding (AB) was performed as described previously [11]. Mice received maintain feed or maintain feed containing 0.2% apigenin. Apigenin was administered from one week before AB or sham operation to the terminal point. After sacrificing mice, the hearts and lungs were dissected out and

weighed to compare heart weight/body weight (HW/BW, mg/g) and lung weight/body weight (LW/BW, mg/g) ratios in four groups. The length of tibia was measured for heart weight/tibia length (HW/TL, mg/mm).

Echocardiography and hemodynamic detection

The echocardiographic examination was performed by Mylab30CV (ESAOTE S.P.A) with 10 MHz linear array transducer after 8 weeks. Left ventricular end-diastolic diameter (LVEDD) and left

ventricular end-systolic diameter (LVESD) were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level. The indicators were assessed as measures of cardiac function. The 1.4F catheter (SPR-839, Millar Instruments, Houston, TX, USA) was inserted from mice right carotid artery into the left ventricular and acquired the invasive hemodynamic indexes.

Histological analysis

The hearts were excised and fixed in 10% buffered formalin and embedded in paraffin. Hearts were cut transversely to visualize the left and right ventricles. Several sections of heart (5 μm thick) were stained with hematoxylin and eosin (HE) for measuring myocyte cross-sectional area or Picrosirius red (PSR) for observing collagen deposition. The area of single myocytes and collagen percent (the area of collagen/total area of tissue) was calculated with an Image quantitative digital analysis system (Image Pro-plus 5.0 software). The outline of 100 cardiomyocytes was traced in each group.

Quantitative real-time PCR

The mRNA levels of hypertrophic and fibrotic markers were detected by real-time PCR. Total RNA of the hearts tissue was extracted by Trizol Reagent (Roche), and synthesized cDNA using oligo(dT) primer with the cDNA Synthesis Kit (Roche). Relative quantitation by real-time PCR utilized SYBR Green PCR Master Mix (Roche) to detect products of PCR in real time with the

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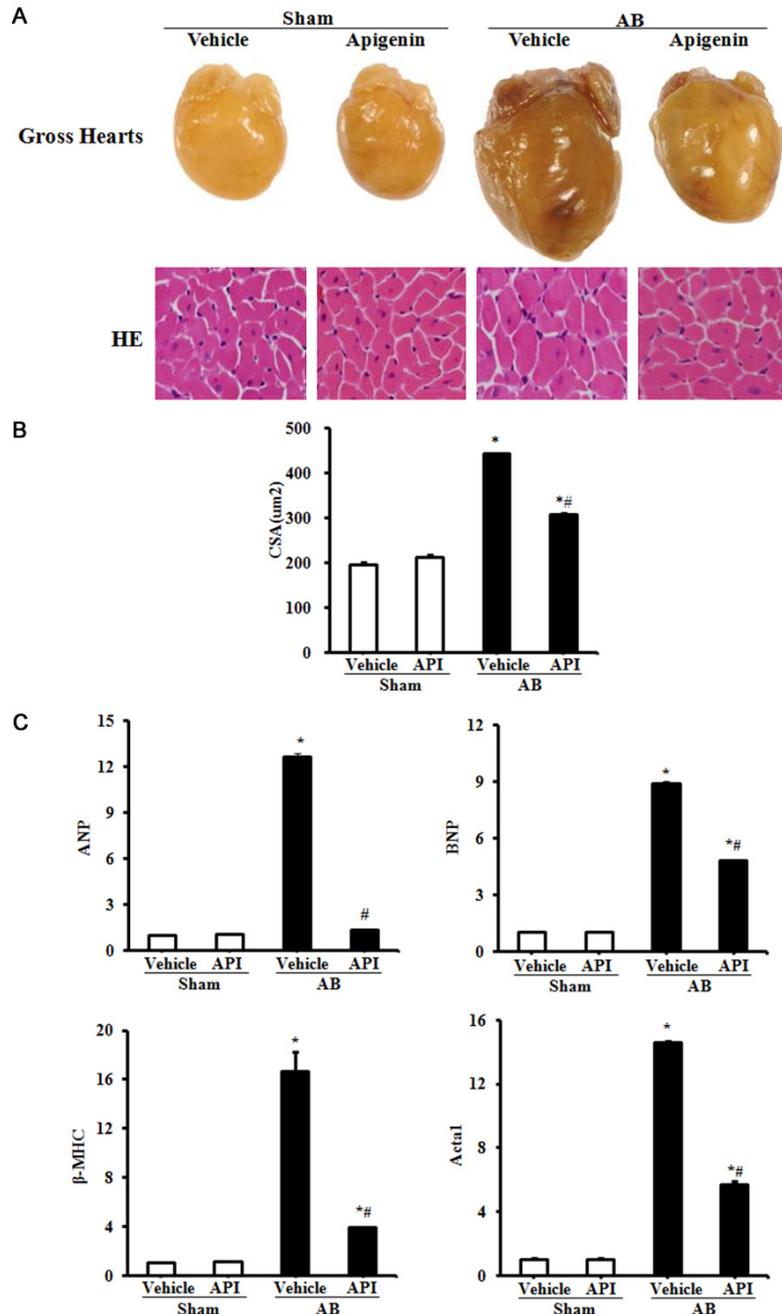


Figure 1. Apigenin attenuated cardiac hypertrophy phenotype induced by pressure-overload. A: Histology representative of gross hearts and H&E staining at 8 weeks post-AB or sham operations. B: Cardiomyocyte cross-sectional area of indicated groups. C: Analysis of hypertrophic markers (n=6). Total RNA was isolated from hearts of mice of the indicated groups, and expression of transcripts for ANP, BNP, Myh7 and Acat1 induced by AB were determined by RT-PCR analysis. * $P < 0.01$ was obtained for corresponding sham group. # $P < 0.01$ was obtained for vehicle-AB group.

LightCycle480 Software (Roche). The GAPDH RNA was amplified as a reference standard. Reactions were prepared in triplicate and heated to 95°C for 10 min, followed by 45 cycles of

95°C for 5 s, 60°C for 10 s, and 72°C for 20 s.

Western blotting

Cardiac tissues were lysed in RIPA lysis buffer containing protease inhibitors. Cell lysates were matched for protein concentration and equal amounts of total protein (50 μg) was separated in SDS/PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes subsequently were blocked in 5% nonfat milk and probed with corresponding primary antibodies overnight. After incubation with secondary fluorescent-labeled secondary antibodies IRdye 800, the blots including phosphoprotein and total protein were scanned and quantified by odyssey infrared imaging system (Li-Cor Biosciences). Every protein expression levels should be normalized to GAPDH protein. Three independent experiments were conducted at least.

Statistical analysis

All results are presented as mean ± standard error of the mean (SEM). Statistical analyses of the data were carried out with two-way ANOVA followed by unpaired Student's t-test. The possibility value $P < 0.05$ has significant differences.

Results

Apigenin inhibited cardiac hypertrophy and improved heart function

To investigate the effect of apigenin on pressure-overload heart, we performed aortic banding (AB) surgery on 8-10 weeks old C57BL/6 mice to induce cardiac hypertrophy. Cardiac

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Table 2. Improved cardiac function by apigenin in AB mice

Parameter	Vehicle-Sham	Apigenin-Sham	Vehicle-AB	Apigenin-AB
Number	n=6	n=6	n=6	n=6
HR (beats/min)	462±9	458±21	487±26	478±19
SBP (mmHg)	101±2	106±2	142±5*	162±3*
dPdt max (mmHg/sec)	9285±351	9957±453	6618±279*	8406±342†
dPdt min (mmHg/sec)	-8526±522	-8706±647	-5376±443*	-8265±583†
EF (%)	68±3	58±2	25±1*	37±3*†

HR, heart rate; SBP, End-systolic Pressure; EF, Ejection Fraction (%). All values are mean ± SEM. * $P < 0.05$ vs Vehicle-Sham, † $P < 0.01$ vs Vehicle-Sham, † $P < 0.05$ vs Vehicle-AB.

function was examined by echocardiography after 8 weeks of surgery. As shown in **Table 1** and **Figure 1A**, all vehicle-AB mice presented increase in heart size, left ventricle chamber dimensions and thickness. The increase of LVESD, LVEDD and IVSD induced by pressure overload were markedly reduced by apigenin administration. Fraction Shortening (FS) were declined in response to AB and accentuated with apigenin (**Table 1**). Apigenin administration significantly attenuated the increase of the heart weight/body weight (HW/BW), the lung weight/body weight (LW/BW) ratio and cardiomyocytes cross-sectional area in AB mice (**Table 1**). The significant decrease of the ejection fraction (EF), and the dP/dt max (mmHg/sec) in the vehicle- mice after 8 weeks of AB showed an amelioration of the LV function in the apigenin-administrated mice after 8 weeks of AB (**Table 2**). The result of gross hearts and HE staining further confirmed the inhibitory effect of apigenin on cardiac hypertrophy in AB hearts (**Figure 1A**). To determine whether apigenin affected the hypertrophic markers ANP, BNP, Myh7 and Acat1, we performed real-time PCR analysis. Apigenin attenuated the increase in hypertrophy maker expression caused by AB (**Figure 1B**). No significant differences were observed between the vehicle-sham group and apigenin-sham group.

Apigenin attenuated Akt/GSK3 β signaling

Both IGF-1 and IL6-like cytokines induce hypertrophic and antiapoptotic signals in cardiomyocytes through Akt/GSK3 β activation. To clarify whether apigenin inhibited cardiac hypertrophy through downregulating Akt/GSK3 β signaling, the phosphorylation of Akt and GSK3 β were detected. We demonstrate that Akt and GSK3 β phosphorylation induced by AB were significantly blocked after exposure to apigenin

(**Figure 2A, 2B**). Total levels of these kinases were unchanged among four groups. Our findings suggest that apigenin-mediated inhibitory effect partly depends on disrupting Akt/GSK3 β signalling pathway.

Apigenin attenuated

cardiac fibrosis

Besides cells hypertrophy, fibrosis is another characteristic of pathological cardiac hypertrophy. To further investigate whether apigenin attenuated fibrosis apart from hypertrophy, we examined LV collagen volume percent. The increase in LV collagen volume in the vehicle-AB mice by PSR staining was notably attenuated after administration of apigenin (**Figure 3A, 3B**). Analysis of mRNA expression levels of fibrosis-related mediator including TGF- β 1, TGF- β 2, CTGF, Collagen α , Collagen III and fibronectin revealed that the increases responses in vehicle-AB group were inhibited by apigenin administration (**Figure 3C**).

Discussion

Although pathological cardiac hypertrophy is initially an adaptive response to pathological stimulus, sustained hypertrophy is deleterious and long-term decompensation may lead to congestive heart failure, malignant arrhythmia or even sudden death, which is associated with an increased risk of cardiovascular morbidity and mortality [12-14]. It was reported that pathological hypertrophy can be prevented in models of cardiac hypertrophy recently [15, 16]. But there is no optimal therapeutic measure at present. Therefore, it is necessary to find the key drug as special regulators and targets for the effective treatment of pathological hypertrophy. In the present study, we for the first time found that apigenin not only preventing the progress of cardiac hypertrophy and fibrosis, but also attenuating the progression of LV dysfunction in response to pressure overload. And we further demonstrate that the protective role of apigenin on cardiac hypertrophy was mediated by reducing the expression of Akt/GSK3 β signaling pathway.

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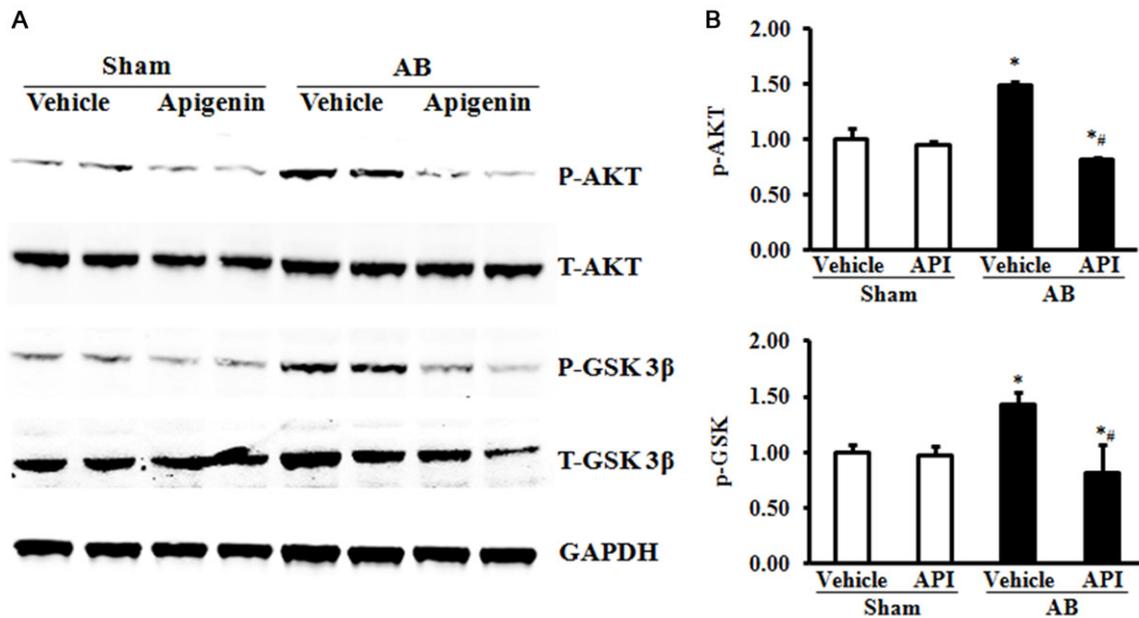


Figure 2. Apigenin inhibits Akt/GSK3 β signaling induced by pressure-overload. A: Representative blots of Akt, GSK3 β phosphorylation and their total protein expression at indicated group's mice. B: Quantification of Akt, GSK3 β protein expression in the heart of mice with 8 weeks AB or sham operation. The results were reproducible in three separate experiments as mean \pm SEM. * P <0.01 was obtained for vehicle-sham group. # P <0.01 was obtained for vehicle-AB group.

Apigenin (5,7,4'-trihydroxyflavone), a nontoxic citrus flavonoid, has been showed to possess antioxidant, anti-inflammatory, anticancer and antivirus biological properties [6, 7]. It is also reported that apigenin induced apoptosis in human lymphoma B cells *in vitro*. And apigenin could induce cell cycle arrest accompanied with the change of MAPK, PI3K/Akt in human prostate cancer cells [8]. However, the effects of apigenin on cardiac hypertrophy and the related molecular mechanisms are still unclear. Thus, in this study, we investigate the effects of apigenin on mice with cardiac hypertrophy by echocardiography, histological and molecular biologic detect including ANP, BNP, Myh7, Acat1, which are specially up-regulated during cardiac hypertrophy. We found that apigenin not only attenuated cardiac hypertrophy and fibrosis, but also improved cardiac performance. These novel findings suggest that apigenin is an effective regulator in protecting against cardiac hypertrophy induced by pressure overload.

The molecular mechanisms through which apigenin regulated the cardiac hypertrophic response still unclear. Plenty signaling mechanism leading to cardiac hypertrophy and the interaction between them have been researched [1,

4]. The activation of Akt/GSK3 β pathway in cardiac hypertrophy is well known [17]. Targeted overexpression of activated PI3-kinase in the heart increased the organ size, while expression of a dominant-negative mutant has the opposite outcome. PI3K activation inducing cardiac hypertrophy is associated with its downstream Akt, and the activation of Akt lead to cardiac hypertrophy and heart failure [18]. Two important downstream targets are regularly mentioned, glycogen synthase kinase 3 β (GSK-3 β) and mTOR. GSK3 β , which is a negative regulator of the cardiac hypertrophy, has been revealed disrupts cardiomyocyte hypertrophy [19]. Furthermore, apigenin inhibits HGF-promoted invasive growth and metastasis through blocking PI3K/Akt pathway in MDA-MB-231 breast cancer cells [9]. Apigenin inhibits HIF-1 and VEGF expression and suppress angiogenesis via PI3K/AKT/p70S6K1 and HDM2/p53 pathways in human ovarian cancer cells [10]. Therefore, we examined the effects of apigenin on Akt/GSK3 β signaling pathway. The present study showed that the phosphorylation levels of Akt and GSK3 β increased obviously under the pressure overload stimuli, and apigenin markedly reduced the phosphorylation of Akt and GSK3 β in banding mice. These findings

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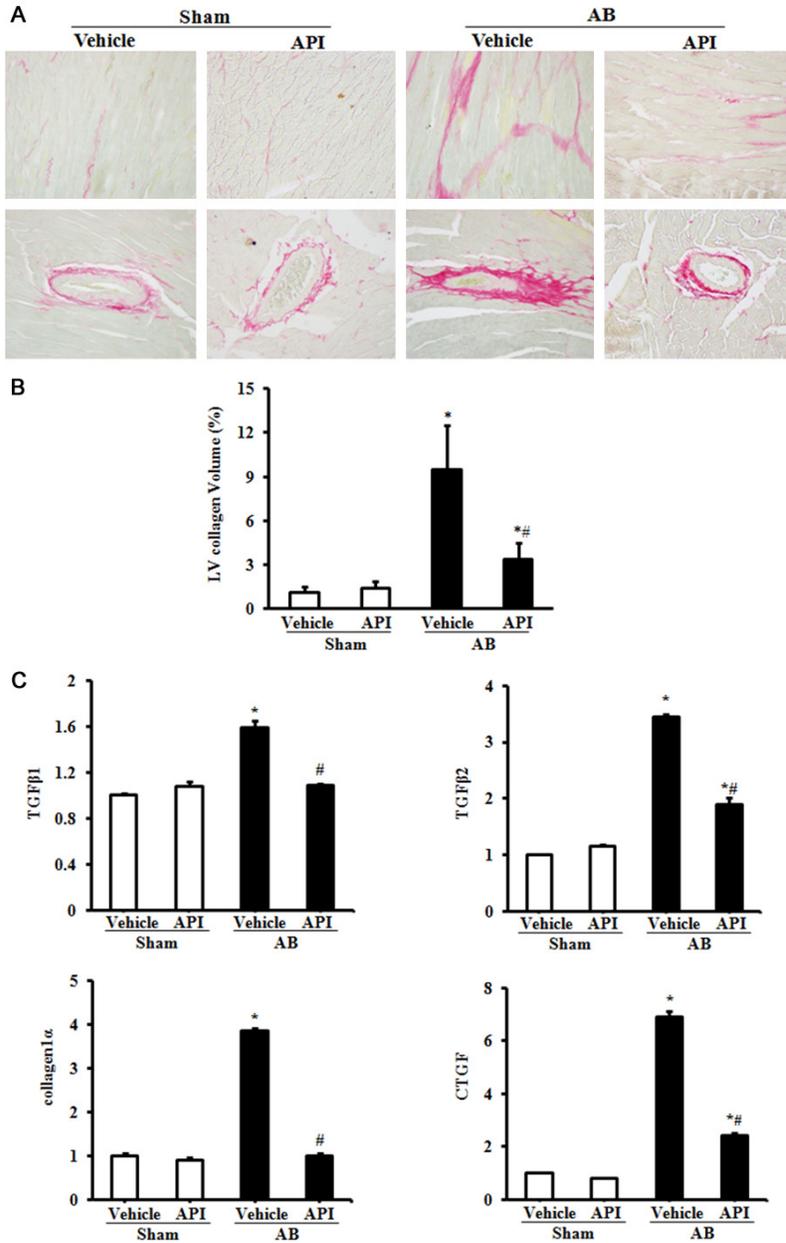


Figure 3. Apigenin blocks collagen synthesis induced by pressure-overload. A: PSR staining on histological sections of the LV was performed on each group 8 weeks after AB. B: Fibrotic areas from histological sections were quantified using an image-analyzing system (n=6). C: Apigenin was shown to inhibit the mRNA expression of TGF-β1, TGF-β2, CTGF, Collagen1α, Collagen III and Fibronectin in the myocardium obtained from indicated groups (n=6). The results were reproducible in three separate experiments. *P<0.01 was obtained for vehicle-sham group. #P<0.01 was obtained for vehicle-AB group.

indicate that anti-hypertrophy effect of apigenin can be in part ascribed to blocking Akt/GSK3β-dependent signaling pathway.

Cardiac fibrosis is another typical feature of hypertrophy and is characterized by increase of LV extracellular matrix attributed to the accu-

mulation volume and fibrosis mediator level [20]. Therefore, we further investigated whether apigenin attenuated fibrosis. This study demonstrates that the increase of LV collagen volume induced by AB was remarkably reduced after treatment with apigenin. The mRNA expression levels of known mediators of fibrosis including TGF-β, CTGF, Collagen α, Collagen III and fibronectin were notably decreased in AB model after administration of apigenin. In this study, we first found that apigenin attenuated the fibrosis.

Conclusions

In summary, our present study demonstrated for the first time that apigenin was effective in inhibiting cardiac hypertrophy induced by pressure overload and apigenin inhibits AB-induced cardiac hypertrophy in mice through blocking Akt/GSK3β signaling pathway. Our finding provided experimental evidence that apigenin may have a promising potential in effective therapy of cardiac hypertrophy and heart failure.

Acknowledgements

This research was supported by the University special fund for basic research business expenses of China (No. 4101002).

Disclosure of conflict of

interest

None.

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