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Original research article

Dioscin Ameliorates Cardiac Hypertrophy Through Inhibition of the MAPK and Akt/ GSK3β/mTOR Pathways

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Abstract: Cardiac hypertrophy occurs in response to multiple stimuli and develops into congestive heart failure with morbidity and mortality. Dioscin exerts protective effects against tumor growth and ischemia/reperfusion injury. However, whether and how dioscin attenuates angiotensin II (AngII)-induced cardiac hypertrophy is still unknown. In the current study, we found that dioscin attenuated cardiac hypertrophy and restored the impaired cardiac function induced by AngII infusion *in vivo*. In addition, dioscin blocked the activation of the MAPK and Akt/GSK3β/mTOR pathways and nuclear accumulation of p-Akt1 in AngII-infused mice. *In vitro*, dioscin inhibited the activation of the MAPK and Akt/GSK3β/mTOR pathways and nuclear translocation of p-Akt1 and thus alleviated the hypertrophic growth. Our study demonstrated dioscin protects against AngII-induced cardiac hypertrophy via inhibition of the MAPK and Akt/GSK3β/mTOR pathways and is a potential therapeutic candidate.

Keywords: Cardiac hypertrophy; dioscin; angiotensin II; MAPK; Akt1

1. Introduction

Cardiac hypertrophy is a compensatory response to various stimuli such as volume or pressure overload, valvular insufficiency, hypertension, ischemic heart diseases and familial cardiomyopathy[1]. It is characterized by increased cardiomyocyte size and reorganization of cytoskeleton and associated with activation of transcription factors, enhanced protein synthesis, and re-expression of fetal cardiac genes such as natriuretic peptides and β-myosin heavy chain[2, 3]. However, the sustained hypertrophy leads to contractile dysfunction and interstitial fibrosis and increases the risk of congestive heart failure, arrhythmia, and sudden death[4]. In spite of extensive study and advanced treatment strategies, pathological cardiac hypertrophy continues to be a global public health problem with high morbidity and mortality[5]. Previous studies have demonstrated that a suite of signaling pathways play important roles in the process of cardiac hypertrophy, including AMPK/mTOR and PI3K/Akt pathways[2, 6]. Such studies may provide potential targets for pharmacological candidates for the treatment of cardiac hypertrophy.

Dioscin (molecular formula: C45H72O16) is a typical steroid saponin and isolated from medicinal herbs such as *Dioscorea nipponica Makino* and *Dioscorea zingiberensis Wright*[7, 8]. Pharmacological investigations have demonstrated that dioscin has anti-tumor, anti-inflammation and anti-hyperlipidemic activities. Dioscin inhibits the growth of malignant tumors such as pancreatic cancer, prostate cancer, colon carcinoma and osteosarcoma [9-11]. In addition, dioscin has a protective effect against ischemia/reperfusion injury in hepatic, cerebral and renal tissues via inhibiting inflammation[12-14]. Recently it has been revealed that dioscin attenuates mitochondrial apoptosis in H9C2 cells and alleviated the cardiotoxicity induced by doxorubicin[15, 16]. However, it remains unknown whether dioscin protected heart from cardiac hypertrophy.

The present study aimed to investigate the effect of dioscin on AngII-induced cardiac hypertrophy and the underlying mechanism.

2. Materials and Methods

2.1. Animals and Treatments

All experiments conformed to the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No.85-23, revised 1996). All protocols were in compliance with the principles of the Institutional Animal Research Committee of Tongji Medical

college and complied with the ARRIVE Guidelines. Eight-week-old male C57BL/6 mice were purchased and housed in the Animal Center of Tongji Medical College (Wuhan, China) in a specific pathogen-free environment.

Forty-two male mice were divided into seven groups: Sham group, AngII Day 3 group, AngII Day 7 group, AngII Day 14 group, AngII+Dioscin Day 3 group, AngII+Dioscin Day 7 group and AngII+Dioscin Day14 group (n=6 in each group). Mice were treated with the gavage of PBS or Dioscin daily (80mg/kg/d) (Selleck, USA) at the same time as AngII-infusion (1.5 μg/kg/min) (Sigma, USA) via subcutaneous osmotic pump (Model 1002; Alzet, Cupertino, CA, USA). The mice at day 3, day 7 and day14 after AngII-infusion were sacrificed and subjected to subsequent assays.

Another animal experiment was performed to determine the effect of pretreatment of dioscin on AngII-induced cardiac remodeling. All mice were divided into four groups: vehicle group (n=10), with a gavage of 50 μl PBS once daily for seven consecutive days; Dioscin group (n=10), with a gavage of dioscin at the dosage of 80 mg/kg/d once daily for seven consecutive days; AngII+vehicle group (n=10), with a gavage of PBS once daily for seven consecutive days and followed by AngII infusion (1.5 μg/kg/min); AngII+Dioscin group (n=10), with a gavage of dioscin at a dosage of 80 mg/kg/day once daily for seven consecutive days and then supplemented with AngII infusion. After two weeks' infusion, all mice were anesthetized and subjected to transthoracic echocardiography and cardiac catheterization.

2.2. Echocardiography and hemodynamic measurement

Transthoracic echocardiography was performed at day 14 after AngII infusion. Mice were anesthetized by isoflurane inhalation. A comprehensive echocardiographic study was performed via an echocardiography system with 30-MHz high frequency scanhead[17] (VisualSonics 770, Toronto, Canada). For hemodynamic measurement, a 1.4 F pressure catheter (SPR 671, Millar Instruments, USA) was inserted into the left ventricle through the right common carotid artery after anesthesia. dP/dt max and -dP/dt min were measured as described previously[18]. Data were analyzed by three operators blinded to the treatment assignment.

2.3. Histological Analysis

Hearts were harvested, weighed, fixed in 4% paraformaldehyde overnight, embedded in paraffin and cut into 4 μm cross-sections. Morphology of cardiac myocytes was determined by hematoxylin and eosin staining and cardiomycyte size was determined by wheat germ agglutinin (WGA) staining. Collagen deposition was detected and quantified by picrosirius red staining. The intracellular location of p-Akt1 was identified by immunofluorescence histochemistry and observed under an Olympus microscope. Cross sectional area of cardiomyocytes was analyzed by using Image-Pro Plus 6.0 software (Media Cybemetics, Bethesda, USA). Analyses were performed by three experienced staff.

2.4. Isolation, Culture and Treatment of Primary Rat Cardiomyocytes

Primary cardiomyocytes were isolated and cultured as described previously natriuretic peptides[18]. Newborn Sprague Dawley rats within two days were selected and sacrificed. Hearts were minced and digested in Hanks' buffer containing 0.05 % trypsin, 0.05 % collagenase type II and 1% BSA by a serial digestion. Then, the cells were centrifuged and resuspended in DMEM with 20% fetal calf serum and 100 U/mL penicillin/streptomycin. Cardiac fibroblasts were allowed to attach to the flask and removed by differential adhesion. The suspended cells were cultured for 48 h in 6-well or 24-well plates.

Primary cardiomyocytes were incubated with PBS or dioscin at different concentrations (50 ng/mL, 100 ng/mL and 200 ng/mL) for 1 h and then treated with AngII (1 μmol/L) for another 24 h. Cells were harvested for western blotting or stained with FITC-Phalloidin (100 nmol/L). In addition, primary cardiomyocytes were incubated with dioscin (200 ng/mL) and AngII (1 μmol/L) or PE (100 μmol/L) simultaneously for 12h or 24 h. The cells were harvested for subsequent assays.

2.5. Extract of nuclear and cytoplasmic proteins

Lysates of primary cardiomyocytes with different treatments were subjected to extraction of nuclear and cytoplasmic proteins. Briefly, 50 μL Cytoplasmic Protein Extraction Reagent was added into each well. Cells were collected, vortexed, resuspended and lysated on the ice for 30 min. After the centrifuge of 12,000 g for 15 min, the supernatant were collected as lysates of cytoplasmic proteins and the precipitations were washed and added with 20 μL Nuclear Protein

Extraction Reagent. The precipitations were lysated on the ice for 1 h and vortexed 15 second every 15 min. After centrifuge, supernatants were collected as lysates of nuclear proteins. Lysates of nuclear and cytoplasmic proteins were subjected to western blotting.

2.6. Western Blotting

Cell lysates or animal tissues homogenates were prepared. Protein concentration was measured by the BCA method. Protein samples (15μg/lane) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% BSA for 2 hours, incubated with corresponding primary antibody at 4℃ overnight and HRP-conjugated secondary antibody at room temperature for 2 hours. The results were analyzed and quantified by using Gel-Pro Analyzer 4.0 (Media Cybemetics, Bethesda, MD, US). GAPDH antibody was purchased from Boster Biotech (1:1000 dilution, Boster, Wuhan, China) and other antibodies were purchased from Cell Signaling Technology (1:1000 dilution, Cell Signaling Technology, Beverly, MA, US).

2.7. Quantitative PCR

Total RNA was extracted from hearts or primary cardiomyocytes with TRIzol Reagent Kit (Invitrogen, Life technology) and reverse transcribed to cDNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, US). The mRNA levels of hypertrophic biomarkers were semi-quantified by qPCR assay (GeneAMP 7900, Applied Bio systems, CA, US). All samples underwent 40 cycles of amplifications in triplicates. GAPDH was used as an internal standard to normalize the mRNA levels. Sequence of specific primers used for qPCR was listed in **Table S1**.

2.8. Immunofluorescence

Primary cardiomyocytes were incubated with dioscin (200 ng/mL) and then treated with AngII (1 μmol/L) for 24 h. The cells were washed, fixed in 4% paraformaldehyde and treated with 0.5% Triton X-100. Subsequently the cells were blocked with 5% BSA, incubated with p-Akt1 (Cell Signaling Technology，Beverly, MA, US) at 4 ℃ overnight and finally with PE-conjugated goat anti-Rabbit secondary antibody for 2 h at room temperature. The cells were observed under an Olympus microscope and images were captured to calculate the percentage of nuclear p-Akt1

positive cells.

2.9. Detection of tissue Akt kinase activity

Hearts from different groups were weighed, homogenated and centrifuged. Supernatants were collected, diluted and subjected to the Akt kinase assay using the Akt Kinase Activity Assay Kit (ab139436, Abcam, USA) according to instruction. Wavelength at 450 nm was set and absorbance was measured to calculate the activated Akt in different tissues.

2.10. Statistical Analysis

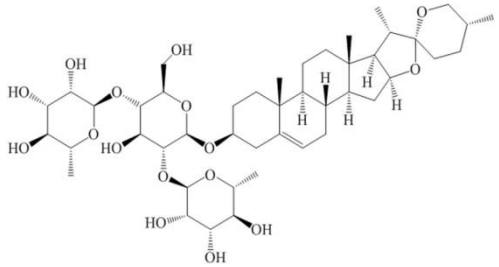
Quantitative data were presented as mean ± SD. One-way analysis of variance (ANOVA) were used to compare the means after identification of a normal distribution. All statistical calculations were performed by SPSS 17.0 (SPSS Inc, Chicago, IL, USA) and $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Dioscin Mitigates the Development of Cardiac Remodeling Induced by AngII-Infusion *in Vivo*

To investigate the role of dioscin in the pathogenesis of cardiac hypertrophy in mice (Figure 1), we administrated the gavage of dioscin (80 mg/kg/day, for seven days) in priority and induced the cardiac remodeling via AngII infusion (1.5 μg/kg/min, for two weeks). The heart mass measured by the ratio of heart weight/body weight, heart weight/lung weight and heart weight/tibia length revealed that administration of dioscin alleviated AngII infusion-induced increase in heart mass (**Figure 2A-D**). Morphological analysis showed that AngII infusion significantly induced gross cardiomegaly and increased cell size as compared with saline and these effects were ameliorated by the addition of dioscin (**Figure 2E**). In addition, the mRNA levels of hallmarks of cardiac hypertrophy such as atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC) substantially elevated in mice with AngII infusion as compared with saline control and were attenuated by dioscin administration (**Figure 2F-H**). Generally, AngII infusion induces significant fibrosis. We observed that administration of dioscin ablated AngII infusion-induced cardiac fibrosis, including interstitial and

perivascular fibrosis (**Figure 2I-J**). Moreover, the pretreatment of dioscin ameliorated the elevated mRNA levels of Collagen I, Collagen III and connective tissue growth factor (CTGF) in the cardiac tissues with AngII infusion. (**Figure 2K-M**). The results above indicated that pretreatment of dioscin alleviated AngII-induced cardiac hypertrophy and fibrosis.



Chemical structure of dioscin

Figure 1. Chemical structure of dioscin

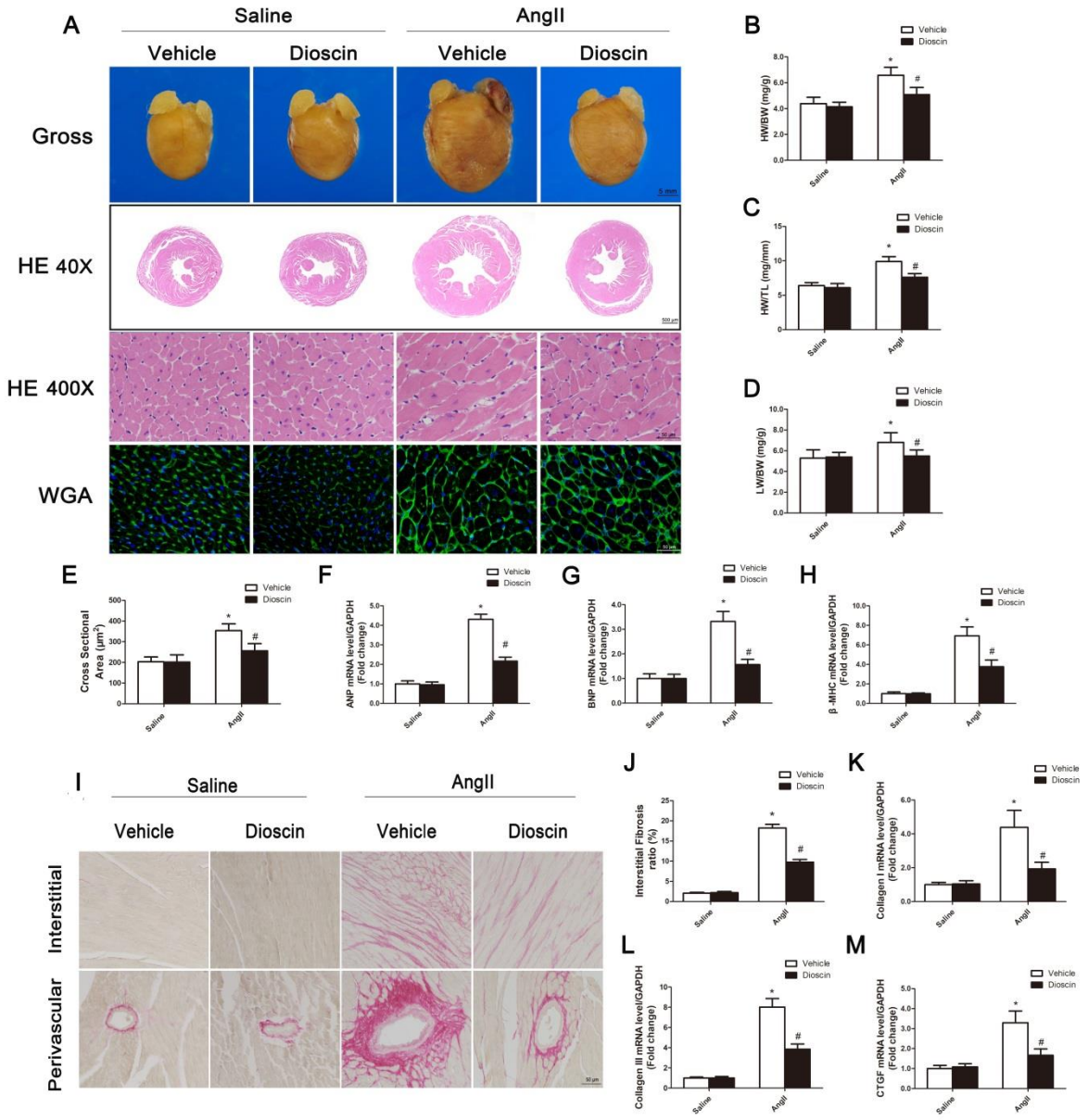


Figure 2. Pretreatment of dioscin mitigates the development of cardiac hypertrophy and fibrosis induced by AngII-infusion *in vivo*. **(A)** The representative images of hearts in indicated groups with gross morphological analysis (first panel, scar bar: 5 mm), hematoxylin and eosin (HE) staining (second panel, scar bar: 500 μm. third panel, scar bar: 50 μm) and WGA staining (forth panel, magnification: 400x and scar bar: 50 μm). The ratio of **(B)** heart weight/body weight, **(C)** heart weight/tibia length and **(D)** lung weight/body weight presenting the mass of heart and lung in different groups. **(E)** The calculation of cardiomyocyte cross-sectional area in the indicated groups. **(F-H)** The mRNA levels of ANP, BNP and β-MHC in the heart of mice from indicated groups by Realtime PCR. **(I)** The representative images of fibrosis and **(J)** respective quantitation of fibrosis area in indicated groups. The magnification: 200x and scar bar: 50 μm. **(K-M)** The mRNA levels of Col I, Col III and CTGF in the heart of mice from indicated groups by Realtime PCR. n=6-8, *, P <0.05, AngII+vehicle group vs saline+vehicle group; #, P <0.05, AngII+dioscin group vs AngII+vehicle group.

3.2 Dioscin Ameliorates the Cardiac Dysfunction Induced by AngII-Infusion *in Vivo*

Parameters of echocardiography revealed that AngII infusion impaired the systolic function of heart with increases in the thickness of interventricular septum and left ventricular posterior wall and decreases in ejection fraction and fractional shortening in comparison with control. The increased thickness of ventricular wall and decreased ejection fraction and fractional shortening were restored by the addition of dioscin prior to AngII infusion (**Figure 3A-G** and **Table S2**). Hemodynamic analysis demonstrated that dp/dt max (mmHg/s) and dp/dt min (mmHg/s) were decreased in mice subjected to AngII infusion, while gavage of dioscin substantially alleviated the decreases of dp/dt max (mmHg/s) and dp/dt min (mmHg/s) (**Figure 3H, I** and **Table S3**). Cardiac dysfunction induced by AngII-infusion was suppressed by gavage of dioscin.

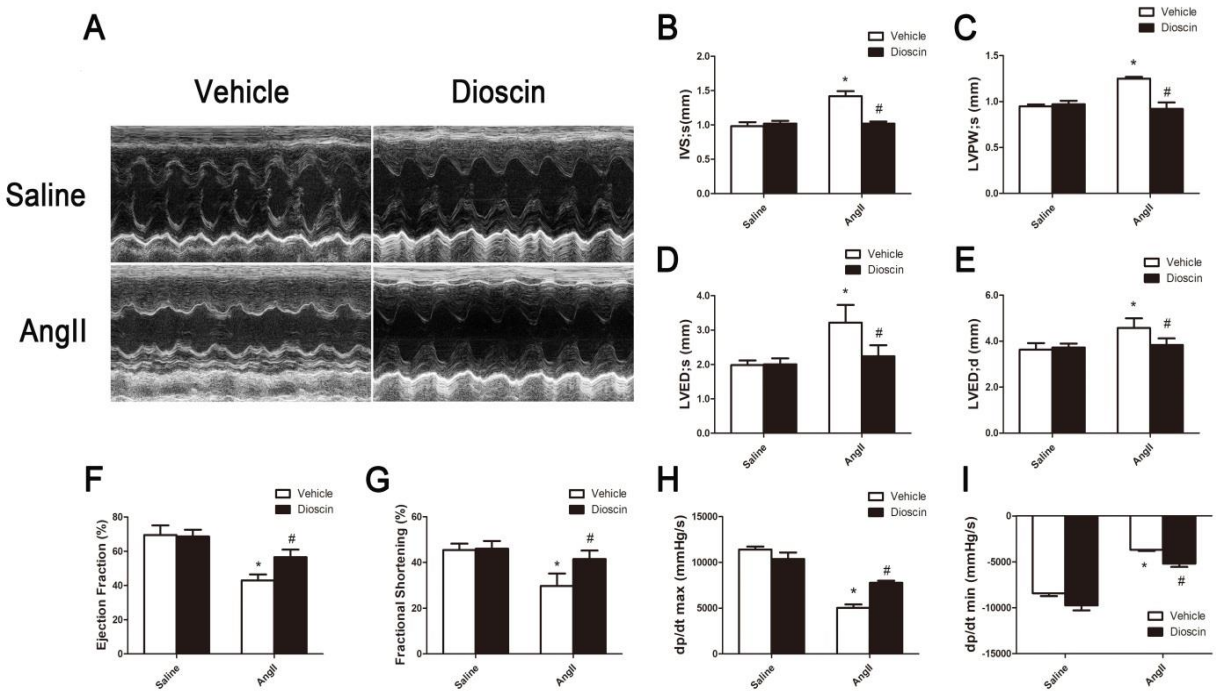


Figure 3. Dioscin restores cardiac dysfunction induced by AngII-infusion *in vivo*. **(A)** Representative images of echocardiograms of hearts from indicated groups. **(B-G)** Parameters of echocardiography in mice with indicated administration. **(H-I)** Measurements of dp/dt max (mmHg/s) and dp/dt min (mmHg/s) of hearts in mice from indicated groups. n=6-8, *, P <0.05, AngII+vehicle group vs saline+vehicle group; #, P <0.05, AngII+dioscin group vs AngII+vehicle group.

3.3 Dioscin Blocks AngII-induced Cardiac Remodeling by Inhibiting the Activation of the MAPK and Akt/GSK3β/mTOR pathways *in Vivo*

Previous studies have demonstrated that the MAPK and Akt/GSK3β/mTOR pathways are

activated in cardiac hypertrophy [19, 20]. In parallel with previous reports, we found that dioscin ablated the up-regulation of p-JNK1/2, p-ERK1/2, p-p38, p-Akt1, p-GSK3β and p-mTOR induced by AngII infusion (**Figure 4A-H**). Immunofluorescence staining revealed that enhanced accumulation of nuclear p-Akt1 in cardiomyocytes after AngII infusion, which was reversed by the administration of dioscin in vivo (**Figure 4I**).

In order to study the changes of pathways above were secondary to the cardiac hypertrophy or not, we performed another animal experiment that we treated the mice with the gavage of dioscin and AngII infusion at the same time. Then we investigated the pathogenesis of cardiac hypertrophy and changes of relevant pathways at three time points (day 3, day 7 and day 14). The significant changes of cardiac hypertrophy and fibrosis was not found at the early time point at day 3 via morphological testing, qPCR and Western Blotting, but the changes were apparent at day 14. The treatment of dioscin attenuated the increased sizes of cardiomyocytes and elevated mRNA levels of hypertrophic hallmarks (ANP, BNP and β-MHC) induced by AngII-infusion at three time points, but significantly at day 7 and 14 (**Figure 5A-H**). The expressions of p-Akt1, p-JNK1/2, p-ERK1/2 and p-p38 increased gradually from day 3 to day 14 after AngII-infusion by Western Blotting, which was attenuated by dioscin (**Figure 5I-M**). The changes of Akt1 kinase activity in the mice's hearts from different time points were consistent with the changes above (**Figure 5N**).

These results suggested that morphological features of cardiac hypertrophy and fibrosis were obvious at day 14 after AngII-infusion. However, the changes of MAPK and Akt/GSK3β/mTOR pathways were generated from day 3 and enhanced gradually at day 7 and day 14 after AngII-infusion, which were all suppressed by dioscin. The fact that the activation of these pathways and absence of significant cardiac hypertrophy at the early time point (day 3) indicated the activation of these pathways contributed to, not secondary to, cardiac hypertrophy. Dioscin effectively inhibited the activation of MAPK and Akt/GSK3β/mTOR pathways induced by AnII-infusion and thus blocked the hypertrophic growth of cardiomyocytes.

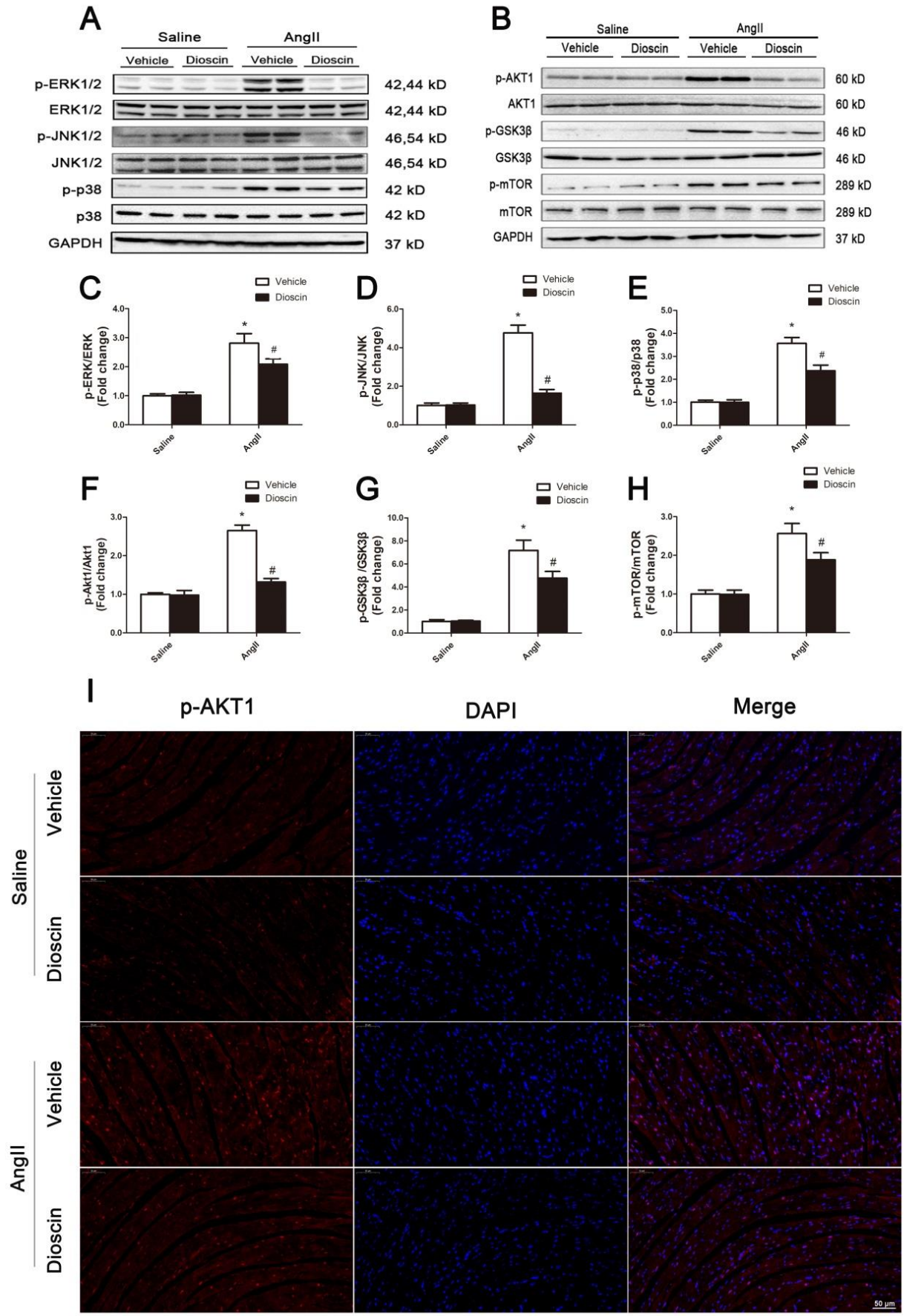

A
Saline
AngII
Vehicle
Dioscin
Vehicle
Dioscin
p-ERK1/2
ERK1/2
p-JNK1/2
JNK1/2
p-p38
p38
GAPDH
42,44 kD
42,44 kD
46,54 kD
46,54 kD
42 kD
42 kD
37 kD
B
Saline
AngII
Vehicle
Dioscin
Vehicle
Dioscin
p-AKT1
AKT1
p-GSK3β
GSK3β
p-mTOR
mTOR
GAPDH
60 kD
60 kD
46 kD
46 kD
289 kD
289 kD
37 kD
C
Vehicle
Dioscin
p-ERK/ERK (Fold change)
Saline
AngII
D
Vehicle
Dioscin
p-JNK/JNK (Fold change)
Saline
AngII
E
Vehicle
Dioscin
p-p38/p38 (Fold change)
Saline
AngII
F
Vehicle
Dioscin
p-Akt1/Akt1 (Fold change)
Saline
AngII
G
Vehicle
Dioscin
p-GSK3β /GSK3β (Fold change)
Saline
AngII
H
Vehicle
Dioscin
p-mTOR/mTOR (Fold change)
Saline
AngII
I
p-AKT1
DAPI
Merge
Saline
Vehicle
Dioscin
AngII
Vehicle
Dioscin
50 μm

Figure 4. Pretreatment of dioscin blocks the activation of MAPK and Akt/GSK3β/mTOR pathways induced by AngII-infusion *in vivo*. **(A)** Results of Western Blotting showing the expressions of p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2, p-p38 and p38 in the hearts from indicated groups. **(B)** Results of Western Blotting showing the expressions of p-Akt1, AKT1, p-GSK3β, GSK3β, p-mTOR and mTOR in the hearts from indicated groups. **(C-H)** Quantitation of the expressions of hallmarks in MAPK and Akt/GSK3β/mTOR pathway in the hearts from indicated groups. (**I**) Representative images of immunofluorescence showing the location and expression of p-Akt1 in the cardiac tissues from indicated groups with p-Akt1 antibody (red) and DAPI (blue) (magnification: 400x and scar bar: 50 μm). *, P <0.05, AngII+vehicle group vs saline+vehicle group; #, P <0.05, AngII+dioscin group vs AngII+vehicle group. The data of Western Blotting represent the mean±SD from at least three independent experiments.

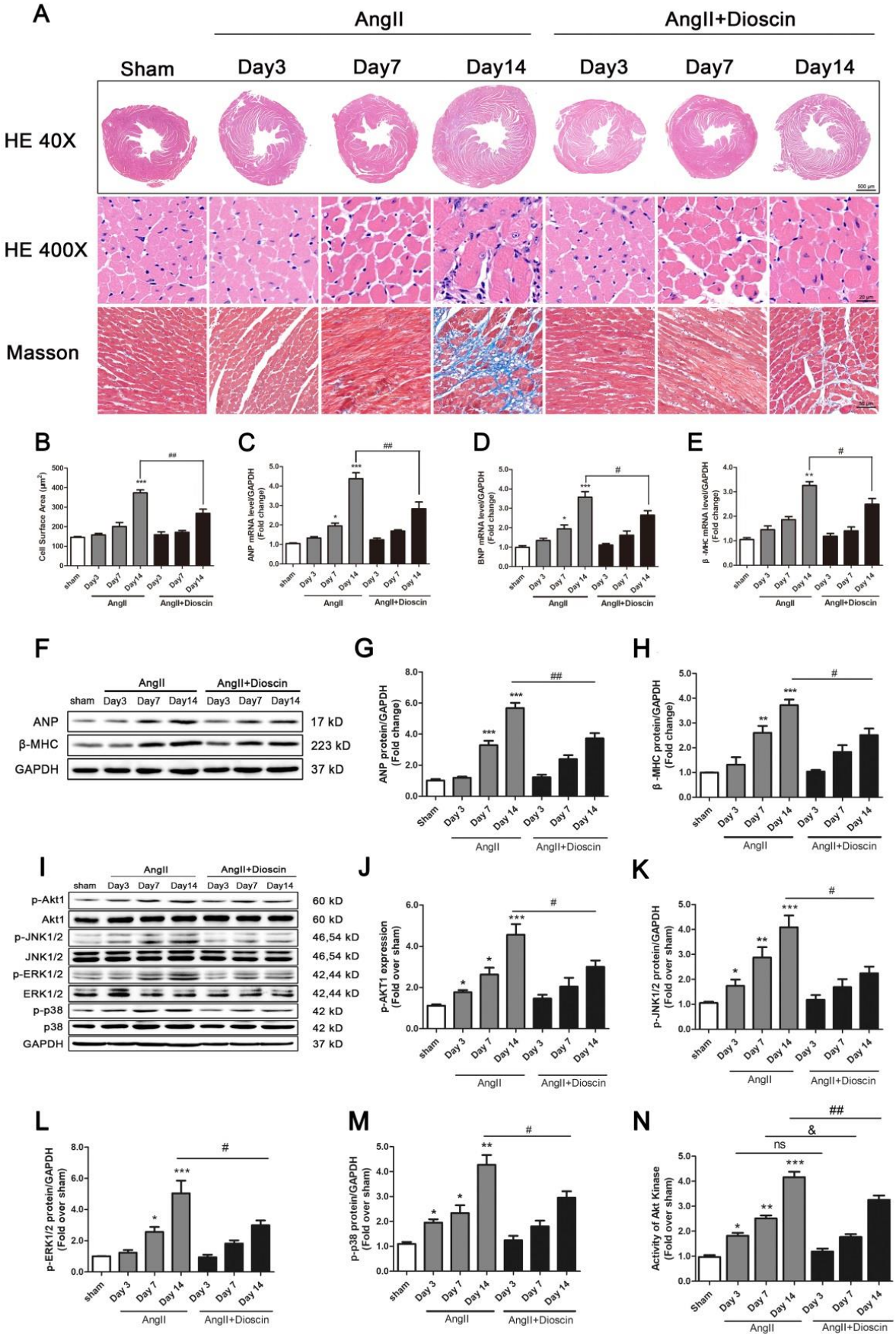


Figure 5. Dioscin inhibited the activation of MAPK and Akt1 pathways induced by AngII-infusion from the early time point and consequently suppressed the formation of cardiac hypertrophy in vivo. Mice in each group were treated with gavage of dioscin and AngII-Infusion at the same time and hearts were harvested at three time points. (**A**) Morphological analysis of hearts from different groups at day 3, day 7 and day 14 after AngII-infusion by HE and Masson staining (The first panel, scar bar: 500 μm. Second panel, scar bar: 20 μm. Third panel, magnification: 200x and scar bar: 50 μm). (**B**) Calculation of cellular area of cardiac myocytes in different groups at day 3, day 7 and day 14 after AngII-infusion. (**C-E**) The mRNA levels of ANP, BNP, β-MHC in cardiac tissues from different groups at three time points by realtime PCR. (**F-H**) Western Blotting showing the expressions of ANP and β-MHC proteins in the hearts from indicated groups. (**I-M**) Western Blotting showing the expressions of p-Akt1, Akt1, p-JNK, JNK, p-ERK, ERK, p-p38 and p38 in the heart from indicated groups. (**N**) The determination of Akt kinase activity in different hearts from indicated groups. *, $P< 0.05$, corresponding group vs sham group; **, $P <0.01$, corresponding group vs sham group; ***, $P< 0.001$, corresponding group vs sham group. #, $P <0.05$, AngII+dioscin day14 group vs AngII day 14 group; ##, $P <0.01$, AngII+dioscin day14 group vs AngII day 14 group. &, $P <0.01$, AngII+dioscin day 7 group vs AngII day 7 group. The data of Western Blotting represent the mean±SD from at least three independent experiments.

3.4 Dioscin Attenuated AngII-induced Hypertrophic Response of Primary Cardiomyocytes by Blocking the Activation of MAPK and Akt/GSK3β/mTOR pathways *in Vitro*

As we found that dioscin protected the heart from cardiac hypertrophy in vivo, we next determined whether and how dioscin exerts a protective role in the progression of cardiomyocytic hypertrophy in response to AngII in vitro. Through identifying the cellular sizes of cardiomyocytes by phalloidin staining, we found that exogenous addition of dioscin could alleviate AngII-induced cardiomyocyte hypertrophy in a dose-dependent manner (**FigureS1 A-B**). Results of Realtime PCR indicated that the mRNA levels of ANP and β-MHC elevated in response to AngII as compared with the control and the addition of dioscin prevented the increase in ANP and β-MHC mRNA (**FigureS1 C-D**). Based on our findings, we selected the optimal concentration of dioscin (200 ng/mL) for subsequent assays.

Primary cardiomyocytes were incubated with dioscin and AngII (1 μmol/L) or PE (100 μmol/L) simultaneously for 24 h. Administration of AngII or PE successfully induced the hypertrophy of primary cardiomyocytes via phalloidin staining (AngII group, about 2.5 fold over PBS), which was inhibited by the dioscin (**Figure 6A-B**). In accordance with the experiment in vivo, we chose AngII for subsequent assays. Dioscin inhibited AngII-induced upregulation of ANP and β-MHC in both mRNA and protein levels in primary cardiomyocytes (**Figure 6C-G**). Then we found the

expressions of critical hallmarks in the MAPK and Akt pathways were up-regulated from 1 h and predominant at 12 h after incubation with AngII (**Figure S2**). Results of Western blotting indicated that the addition of dioscin inhibited the up-regulation of hallmarks of the MAPK and Akt/GSK3β/mTOR pathways at 12 h after treatment of AngII in primary cardiomyocytes (**Figure 6H-K**). All the data above suggested that the activation of MAPK and Akt/GSK3β/mTOR pathways exerted a critical role in the pathogenesis of AngII-induced hypertrophy in primary cardiomyocytes and could be blocked by dioscin.

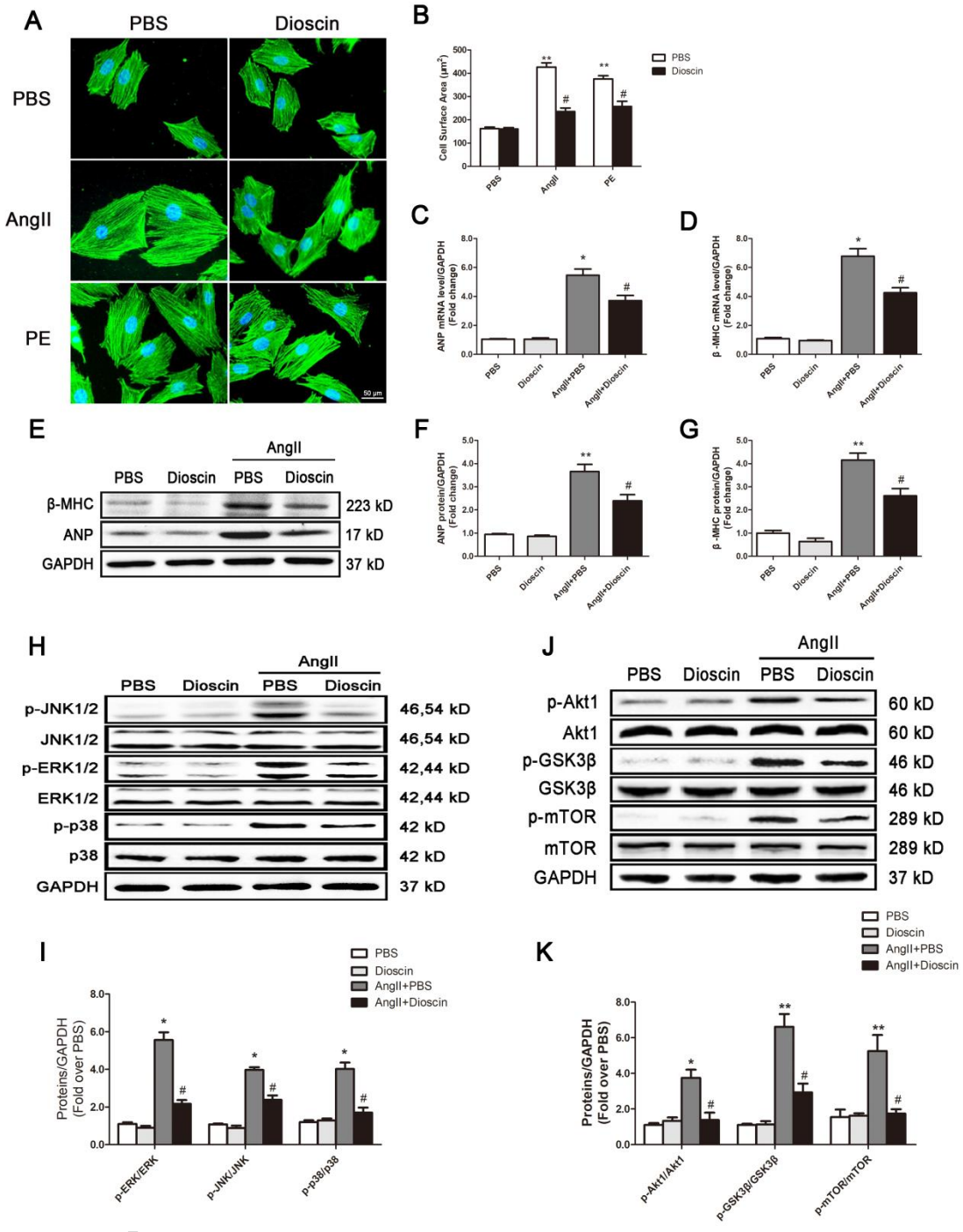


Figure 6. Dioscin Attenuated AngII-induced hypertrophy of primary cardiomyocytes by blocking the activation of MAPK and Akt/GSK3β/mTOR pathways. Primary cardiomyocytes were incubated with dioscin (200ng/mL) and AngII (1 μmol/L) or PE (100 μmol/L) simultaneously for 24 h. **(A)** Representative images of primary cardiomyocytes in different groups at 24 h via Phalloidin staining (magnification: 400x and scar bar: 50 μm). **(B)** Calculation of cellular size of primary cardiomyocytes in different groups. **(C-D)** The detection of β-MHC and ANP mRNA levels in the primary cardiomyocytes in indicated groups by realtime PCR. **(E-G)** Result and quantitation of Western Blotting showing the expressions of β-MHC and ANP in indicated groups. **(H-K)** Result and quantitation of Western Blotting showing the expressions of markers in the MAPK and Akt/GSK3β/mTOR

pathways at 12 h after incubation with AngII in primary cardiomyocytes. *, $P< 0.05$, AngII+PBS group vs PBS group; **, $P <0.01$, AngII+PBS group vs PBS group. #, $P <0.05$, AngII+dioscin group vs AngII+PBS group. The data of Western Blotting represent the mean±SD from at least three independent experiments.

3.5 Dioscin Suppressed AngII-induced Hypertrophic Response of Primary Cardiomyocytes via Reduced Nuclear Translocation of p-Akt1

We found that dioscin alleviated the nuclear translocation of p-Akt1 in primary cardiomyocytes challenged with AngII through analysis of nuclear and cytoplasmic extracts by western blotting (**Figure 7A-B**). Immunofluorescence staining revealed that the majority of p-Akt1 was located in the cytoplasm without stimulation. Challenge with AngII promoted the nuclear translocation of p-Akt1, which was suppressed by dioscin (**Figure 7C**).

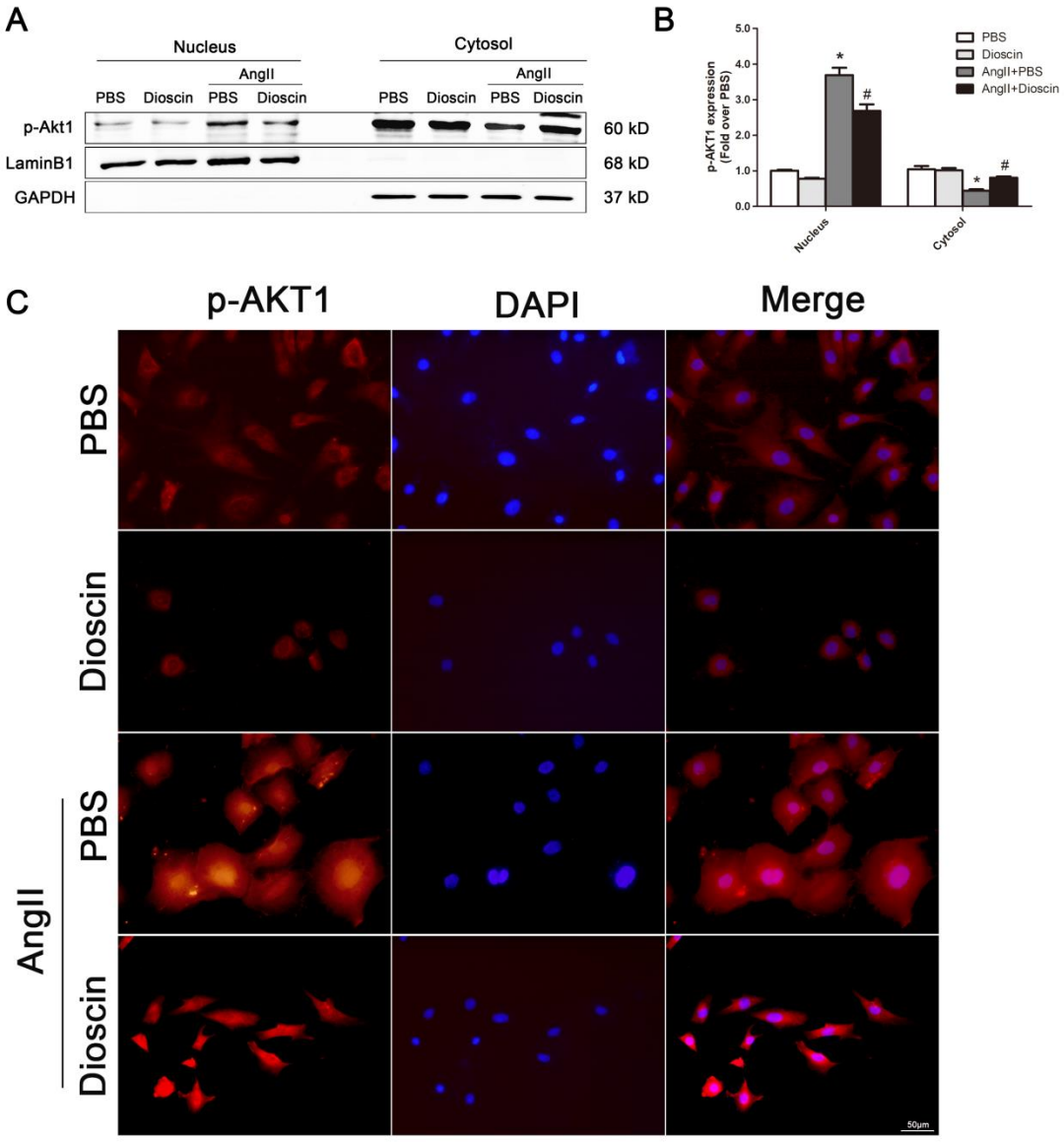


Figure 7. Dioscin inhibited AngII-induced. nuclear accumulation of p-Akt1 in the primary cardiomyocytes Primary cardiomyocytes were incubated with dioscin (200ng/mL) and AngII (1 μmol/L) for 24 h. **(A-B)** Analysis and quantitation of nuclear and cytoplasmic p-Akt1 by Western blotting. **(C)** Immunofluorescence staining with p-Akt1 in the primary cardiomyocytes (magnification: 400x and scar bar: 50 μm). *, *P* <0.05, AngII+PBS group vs PBS group; #, *P* <0.05, AngII+dioscin group vs AngII+PBS group. The data represent the mean±SD from at least three independent experiments.

4. Discussion

In this study, we first demonstrated that dioscin ameliorated the cardiac remolding induced by AngII. *In vivo*, significant cardiac hypertrophy and fibrosis were induced in response to AngII

infusion and alleviated by oral administration of dioscin. We elucidated that dioscin exerted the protective effect against cardiac hypertrophy by suppressing the MAPK and Akt/GSK3β/mTOR pathways. Additionally, dioscin attenuated AngII infusion-induced nuclear accumulation of p-Akt1 in cardiac myocytes. *In vitro*, we observed that dioscin inhibited AngII-induced cardiac hypertrophy in a dose-dependent manner. Besides, dioscin blocked the activation of Akt1 and inhibited the nuclear translocation of p-Akt1 in cardiomyocytes treated with AngII. In summary, dioscin can ablate the activation and nuclear translocation of p-Akt1 and block the activation of the MAPK and GSK3β/mTOR pathways, thus alleviating the pathogenesis of cardiac hypertrophy.

Cardiac hypertrophy is initially a compensatory mechanism in response to various stimuli in order to maintain cardiac function[21]. However, under detrimental circumstances, such as hypertension or aortic valve stenosis, cardiac hypertrophy proceeds into a non-compensatory state with cardiac remodeling and finally lead to congestive heart failure, arrhythmia and sudden death[21, 22]. Dioscin, a natural product, shows potent protective effects against tumor growth, liver injury and kidney injury by various mechanisms[23-27]. Further, dioscin is also identified to have cardioprotective effects against doxorubicin-induced cardiotoxicity[16]. In our work, we observed that cardiac hypertrophy induced by AngII was characterized by increased cardiomyocyte sizes and elevated expressions of hypertrophy related genes such as ANP, BNP and β-MHC *in vivo* and *in vitro*. In addition, AngII infusion promoted cardiac fibrosis with tremendous collagen deposition in the interstitial and perivascular tissues together with increased mRNA levels of collagen I, collagen III and CTGF. The cardiac hypertrophy and fibrosis induced by AngII infusion were alleviated by dioscin *in vivo* and *in vitro*.

Multiple mechanisms are involved in cardiac hypertrophy. Previous studies have demonstrated that the activation of the GPCR and CaMKII/MAPK pathways leads to pathological cardiac hypertrophy[3]. Sirtuins play a critical role in regulating Akt signaling and affecting the pathogenesis of cardiac hypertrophy[28]. Li H *et al.* found that the activation of the TBK1/AKT/GSK3β/mTOR and TRAF6/TAK1/MAPK pathways are involved in the process of cardiac hypertrophy[19, 20]. Consistently, we found that dioscin ameliorated AngII-induced activation of hallmarks of the MAPK and AKT/GSK3β/mTOR pathways from the early time point at day3 *in vivo* and 1 h *in vitro* after treatment of AngII, even though no significant hypertrophy was found. It suggested that the activation of MAPK and AKT/GSK3β/mTOR pathways were not secondary to

the formation of hypertrophy.

Akt is involved in a variety of cellular functions and contributes to cell survival[29, 30]. Although Akt has three isoforms (Akt1, Akt2 and Akt3), Akt1 is the major form[31]. Activation of Akt1 promotes the growth of cardiac myocytes and increase of protein synthesis[32]. Cardiomyocyte-specific overexpression of Akt1 induced cardiac hypertrophy [33, 34]. However, Akt1-KO mice developed an exacerbated state of cardiac hypertrophy when subjected to pressure overload[35, 36]. However, a controversial study demonstrated that CYP2J2 attenuated cardiac hypertrophy by enhancing the nuclear translocation of Akt1[6]. Short-term activation of Akt1 involves in the physiological hypertrophy but long-term activation of Akt1 causes the pathological hypertrophy and heart failure[37]. In our study, we found that dioscin reduced the nuclear accumulation of p-Akt1 induced by AngII infusion *in vivo*. Dioscin decreased the up-regulation of p-Akt1 and inhibited the nuclear translocation of p-Akt1 induced by AngII in cardiac myocytes *in vitro*. To further elucidate the protective role of dioscin in AngII-induced hypertrophy, we need to overexpress the full-length or truncated mutation of Akt1 protein in the primary cardiomyocytes by adenovirus. However, the construction, package and purification of adenovirus are beyond our ability.

In summary, our results indicated that dioscin alleviates AngII-induced cardiac hypertrophy by suppressing the activation of the MAPK pathway, blocking the nuclear translocation of p-Akt1 and subsequently inhibiting the activation of GSK3β/mTOR pathway.

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