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Allicin protects against cardiac hypertrophy and fibrosis via attenuating reactive oxygen species-dependent signaling pathways

Chen Liu[a,1], Feng Cao[b,c,1], Qi-Zhu Tang[b,c], Ling Yan[b,c], Yu-Gang Dong[a], Li-Hua Zhu[b,c], Lang Wang[b,c], Zhou-Yan Bian[b,c], Hongliang Li[c,*]

[a]Department of Cardiology, The First Affiliated Hospital, Sun Yat-Sen University, 510080 Guangzhou, China
[b]Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan 430060, PR China
[c]Cardiovascular Research Institute of Wuhan University, Wuhan 430060, PR China

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Abstract

Increased oxidative stress has been associated with the pathogenesis of chronic cardiac hypertrophy and heart failure. Since allicin suppresses oxidative stress in vitro and in vivo, we hypothesized that allicin would inhibit cardiac hypertrophy through blocking oxidative stress-dependent signaling. We examined this hypothesis using primary cultured cardiac myocytes and fibroblasts and one well-established animal model of cardiac hypertrophy. Our results showed that allicin markedly inhibited hypertrophic responses induced by Ang II or pressure overload. The increased reactive oxygen species (ROS) generation and NADPH oxidase activity were significantly suppressed by allicin. Our further investigation revealed this inhibitory effect on cardiac hypertrophy was mediated by blocking the activation of ROS-dependent ERK1/2, JNK1/2 and AKT signaling pathways. Additional experiments demonstrated allicin abrogated inflammation and fibrosis by blocking the activation of nuclear factor-κB and Smad 2/3 signaling, respectively. The combination of these effects resulted in preserved cardiac function in response to cardiac stimuli. Consequently, these findings indicated that allicin protected cardiac function and prevented the development of cardiac hypertrophy through ROS-dependent mechanism involving multiple intracellular signaling.

Keywords: Allicin; Cardiac hypertrophy; MAPK; Reactive oxygen species

1. Introduction

Cardiac hypertrophy is the cellular response to increased biomechanical stress, such as arterial hypertension, valvular heart disease, and familial hypertrophic cardiomyopathy. It is characterized by increased size of cardiac myocytes, enhanced protein synthesis, sarcomeric assembly and organization and activation of fetal cardiac gene expression program [1]. Cardiac hypertrophy is a potent risk factor for the development of cardiac arrhythmias, diastolic dysfunction, congestive heart failure and death. [2,3]. Sustained pathological hypertrophy is deleterious and may lead to heart failure and death [4]. Therefore, it is very important to elucidate the underlying mechanism of cardiac hypertrophy. Increasing evidences showed that pathological hypertrophy and heart failure related to excessive production of reactive oxygen species (ROS)[5–8]. Cardiac ROS production can be triggered by a lot of factors including Angiotensin II (Ang II), phenylephrine (PE), endothelin-1 (ET-1) and tumor necrosis factor-α (TNF-α) as well as by mechanical stretch such as pressure overload [9–12]. Increasing studies demonstrated that ROS signaling plays critical role on cardiac hypertrophy [9–12]. The signaling pathways related to ROS included protein kinase C (PKC), the mitogen activated protein kinases (MAPKs) (extracellular signal-regulated kinase [ERK1/2], p38, Jun N-terminal kinase [JNK1/2]) and apoptosis-signaling kinase 1, AKT, calcineurin and nuclear factor κB (NF-κB) can be activated directly or indirectly by ROS [13,14]. Therefore, blocking the excess production of ROS can efficiently inhibit the progression of cardiac hypertrophy.

Garlic (*Allium sativum* L.) has a long history as being a food with many medicinal qualities. Modern scientific research has revealed that many beneficial health-related biological properties were attributed to garlic, including antioxidant, antibacterial and antiparasite activities, reducing serum lipid levels as well as inhibition of platelet aggregation [15–20]. One of the metabolites of garlic, allicin, which formed when the alliinase is brought into contact with the inert cysteine sulfoxide allicin, is responsible for most of functions of garlic. Previous reports have shown that allicin can prevent lipid perioxidation through scavenging OH molecules [21]. In endothelial cells, allicin decreased ROS generation and increased the level of glutathione [22]. The antioxidative activity of allicin could be mediated by inhibition of nitric oxide (NO) activity since it was shown to block the activity of inducible nitric oxide synthase (iNOS) in cardiomyocytes [23]. Recently, Sun and Ku [24] have reported that allicin could protect

* Corresponding author. JieFang Road 238, Wuhan 430060, PR China. Tel.: +86 27 88076990; fax: +86 27 88076990.
E-mail address: lihl@whu.edu.cn (H. Li).
[1] These authors contributed equally to this work.

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against coronary endothelial dysfunction and right heart hypertrophy in pulmonary hypertensive rats. However, the role of allicin on cardiac hypertrophy and related molecular mechanisms still remains unknown. In the present study, we investigated the effects of allicin on cardiac hypertrophy induced by Ang II or pressure overload. Our results showed that allicin significantly blocked cardiac hypertrophy, fibrosis and inflammation in vivo and in vitro through blocking ROS-dependent signaling pathways.

2. Materials and methods

2.1. Materials

The antibodies used to recognize ANP, BNP and β-myosin heavy chain (β-MHC) were bought from Santa Cruz Biotechnology. The total and phosphorylation of ERK1/2, P38, JNK1/2, AKT, GSK3β (glycogen synthase kinase) and p85 as well as Smad 2/3 were ordered from Cell Signaling. [^{3}H]-Leucine and [^{3}H]-proline were purchased from Amersham. connective tissue growth factor (CTGF), Collagen I and Collagen III antibodies were from Abcam. TGF-β1 was purchased from R&D Systems. Fetal calf serum (FCS) was obtained from Invitrogen. ANP-, NF-κB- and COL1A1-luc report constructs were described previously [25,26]. BNP promoter was made by polymerase chain reaction (PCR) of genomic mouse DNA from −1421 to +18 and was cloned into pGL3 basic vector using *KpnI* and *HindIII*. Allicin was ordered from LKT Laboratories, Inc. (St. Paul, MN, USA). Cell culture reagents and all other reagents were from Sigma. Allicin was dissolved in methanol for all in vitro studies.

2.2. Cultured neonatal rat cardiac myocytes and fibroblasts

Primary cultures of cardiac myocytes were prepared as described previously [26,27]. Cells from the hearts of 1–2-day-old Sprague-Dawley rats were seeded at a density of 1×10^6/well onto 6-well culture plates in plating medium consisting of F10 medium supplemented with 10% FCS and penicillin/streptomycin. After 48 h, the culture medium was replaced with F10 medium containing 0.1% FCS and BrdU (100 μM). After 24 h of serum starvation, allicin alone or allicin followed by angiogensin II (Ang II, 1 μM) were added to the medium and the cultures were incubated for the indicated time. Viability was determined by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay.

Cultures of neonatal rat cardiac fibroblasts were described previously [26]. Briefly, hearts obtained from neonatal rats were enzyme-digested as described above for myocytes. The adherent nonmyocyte fractions obtained during pre-plating were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% FCS until confluent and passaged with trypsin-EDTA. All experiments were performed on cells from the first or second passages which were placed in DMEM medium containing 0.1% FCS for 24 h before the experiment. The purity of these cultures was greater than 95% cardiac fibroblasts as determined by positive staining for vimentin and negative staining for smooth muscle actin and von Willebrand factor.

2.3. [^{3}H]-Leucine incorporation, surface area and collagen synthesis assay

[^{3}H]-Leucine incorporation was measured as described previously [26,27]. Briefly, cardiac myocytes were pretreated with allicin for 60 min and subsequently stimulated with Ang II (1 μM) and coincubated with [^{3}H]-leucine (2 μCi/ml) for the indicated time. At the end of the experiment, the cells were washed with Hanks' solution, scraped off the well, and then treated with 10% trichloroacetic acid at 4°C for 60 min. The precipitates were then dissolved in NaOH (1 N) and subsequently counted with a scintillation counter. For surface areas, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.1% Triton X-100 in PBS and stained with α-actinin (Sigma) at a dilution of 1:100 by standard immunocytochemical techniques. Collagen synthesis was evaluated by measuring [^{3}H]-proline incorporation as described previously [26]. In brief, cardiac fibroblasts were made quiescent by culturing in 0.1% FCS DMEM for 24 h, pretreating with allicin for 60 min and subsequently incubating with Ang II and 2 μCi/ml [^{3}H]-proline for the indicated time. Cells were washed with PBS twice, treated with ice-cold 5% trichloroacetic acid for 1 h and washed with distilled water twice. Cells were then lysed with 1 N NaOH solutions and counted in a liquid scintillation counter. The count representing the amount of newly synthesized collagen was normalized to the cell number.

2.4. Reporter assays, Western blotting and quantitative real-time PCR

Cardiac myocytes or fibroblasts were seeded in triplicate in six-well plates. Cells were transfected with 0.5 μg of luciferase reporter constructs and internal control plasmid DNA using 10 μl of LipofectAMINE reagent (Invitrogen), according to the manufacturer's instructions. After 6 h of exposure to the DNA-LipofectAMINE complex, cells were cultured in medium containing 10% serum for 24 h and then incubated with serum-free medium for 12 h. Cells were pretreated with allicin for 60 min and then treated with Ang II. Cells were harvested using passive lysis buffer (Promega) according to the manufacturer's protocol. The luciferase activity was normalized by control plasmid. All experiments were done in triplicate and repeated at least three times. For Western blot, cardiac tissue and cultured cardiac myocytes or fibroblasts were lysed in radioimmunoprecipitation (RIPA) lysis buffer. Fifty micrograms of cell lysate was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were then transferred to an Immobilon-P membrane (Millipore). Specific protein expression levels were normalized to both the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) protein for total cell lysate and cytosolic protein. For real time-PCR, total RNA was extracted from frozen, pulverized mouse tissues using TRIzol (Invitrogen) and synthesized cDNA using primers with the Advantage RT-for-PCR kit (BD Biosciences). We quantified PCR amplifications using SYBR Green PCR Master Mix (Applied Biosystems) and normalized results against GAPDH gene expression.

2.5. Electrophoretic mobility shift assay

To examine the DNA-binding activities of NF-κB, electrophoretic mobility shift assays (EMSA) were performed according to the manufacturer's instructions (Gel Shift Assay System E3300, Promega, Madison, WI). Nuclear proteins were isolated as described previously [28]. Protein concentrations were measured by BCA Protein Assay Reagents (PIERCE, Rockford, IL) using bovine serum albumin as a standard.

2.6. Animal models, echocardiography and blood pressure

All protocols were approved by institutional guidelines. All surgeries and subsequent analyses were performed in a blinded fashion for all groups. Adult male C57/B6 mice (8–10 weeks old) were used in the current study, which were purchased from the Institute of Laboratory Animal Science of Chinese Academic of Medicine Science and acclimatized for one week prior to experimental use. Aortic banding (AB) was performed as described previously [26]. Doppler analysis was performed to ensure that physiologic constriction of the aorta was induced. Allicin suspension was prepared using 0.5% carboxy methylcellulose solution for animal experiments. Suspensions were freshly prepared and administered at a constant volume of 1 ml/100 g body weight by oral gavage three times a day. The control group of these animal experiments was given the same volume of liquid but comprising solely of the vehicle solution (0.5% carboxy methylcellulose). The internal diameter and wall thickness of left ventricle (LV) were assessed by echocardiography in the indicated time after surgery or infusion. Hearts and lungs of the sacrificed mice were dissected and weighed to compare heart weight/body weight (HW/BW, mg/g) and lung weight/body weight (LW/BW, mg/g) ratios in allicin-treated and vehicle-treated mice. Echocardiography was performed by SONOS 5500 ultrasound (Philips Electronics, Amsterdam) with a 15-MHz linear array ultrasound transducer. The LV was assessed in both parasternal long-axis and short-axis views at a frame rate of 120 Hz. End-systole or end-diastole was defined as the phase in which the smallest or largest area of LV, respectively, was obtained. LV end-diastolic diameter (LVEDD) and LV 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. Blood pressure was recorded by microtip catheter transducer (SPR-839, Millar Instruments, Houston, TX, USA) inserted into the right carotid artery and advanced into the left ventricle for hemodynamic measurements.

2.7. Histological analysis

Hearts were excised, washed with saline solution, and placed in 10% formalin. Hearts were cut transversely close to the apex to visualize the left and right ventricles. Several sections of heart (4–5 μm thick) were prepared and stained with hematoxylin and eosin (H&E) for histopathology or PSR for collagen deposition and then visualized by light microscopy. For myocyte cross-sectional area, sections were stained HE. A single myocyte was measured with an image quantitative digital analysis system (Image Pro-Plus 4.5). The outline of 100 myocytes was traced in each section.

2.8. Measurements of ROS and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity

Cardiac myocytes were cultured on coverslips in 35-mm dishes and then pretreated with allicin and subsequently stimulated with 1 μM Ang II for indicated time. Intracellular generation of ROS was quantified using 2′,7′-dichlorofluorescein dilacerate (DCFH-DA). The cells were incubated with 5μM DCFH-DA in the dark for 60 min, and immunofluorescence was visualized using laser scanning confocal microscope (488 nm, 200 mW). ROS in the heart tissue were quantified using electron spin resonance (ESR) spectroscopy with 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (hydroxy-TEMPO) as described previously [28]. NADPH oxidase activity was measured in LV homogenates by luminescence assay using 5 μmol/l lucigenin.

2.9. Statistical analysis

Data are expressed as means±S.E.M. Differences among groups were tested by one-way analysis of variance. Comparisons between two groups were performed by unpaired Student's *t* test. A value of $P<.05$ was considered to be significantly different.

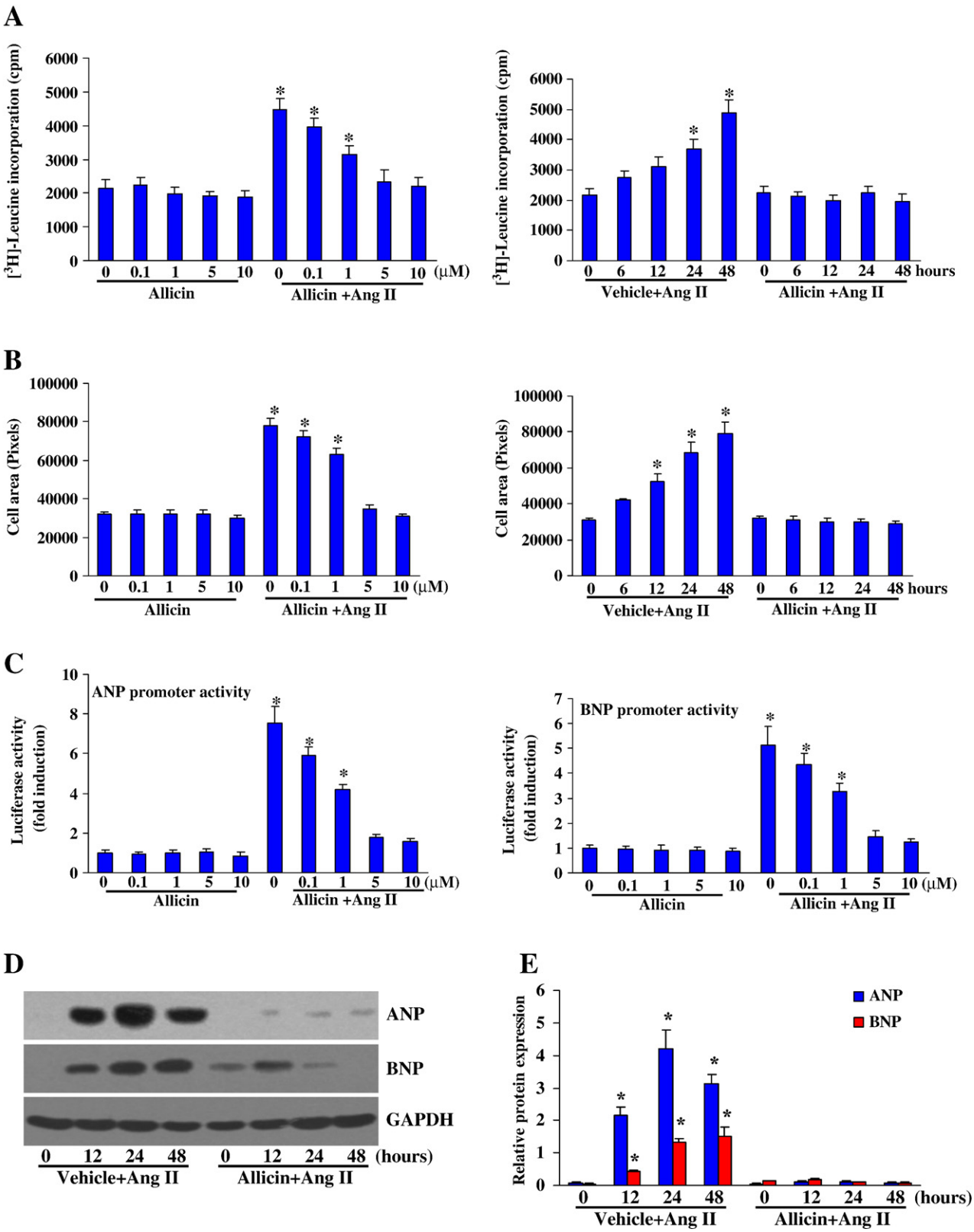

Fig. 1. Allicin inhibited cardiac hypertrophy in vitro. (A) Allicin inhibited Ang II induced [^{3}H]-leucine incorporation. (B) Quantification of cardiomyocyte surface area by measuring 60 random cells. (C) Allicin blocked ANP and BNP promoter activities induced by Ang II. (A–C) Cardiomyocyte were treated with different dose of allicin for 60 min and then incubated with 1 μM Ang II for 48 h. The results were reproducible in three separate experiments. (D and E) Allicin inhibited ANP and BNP protein level after Ang II stimulation. Cardiac myocytes were incubated with 1 μM Ang II or pretreated with 10 μM allicin for 60 min and then incubated with 1 μM Ang II for indicated time. *P<.05 vs. control.

3. Results

3.1. Allicin inhibited cardiac hypertrophy in vitro

To determine the possible cytotoxity of allicin in cardiomyocyte, we evaluated cell viability by MTT assay with different concentrations of allicin in myocytes. Our data showed that allicin was noncytotoxic for myocytes in all tested concentration, and no significant differences in cell viability were found between normal myocytes and myocytes treated with 10 μM allicin for 48 h (data not shown). To examine the effects of allicin on cardiac hypertrophy, we used Ang II to induce hypertrophy in vitro. Cardiac myocytes were pre-incubated with indicated doses of allicin for 60 min and then subsequently treated with Ang II

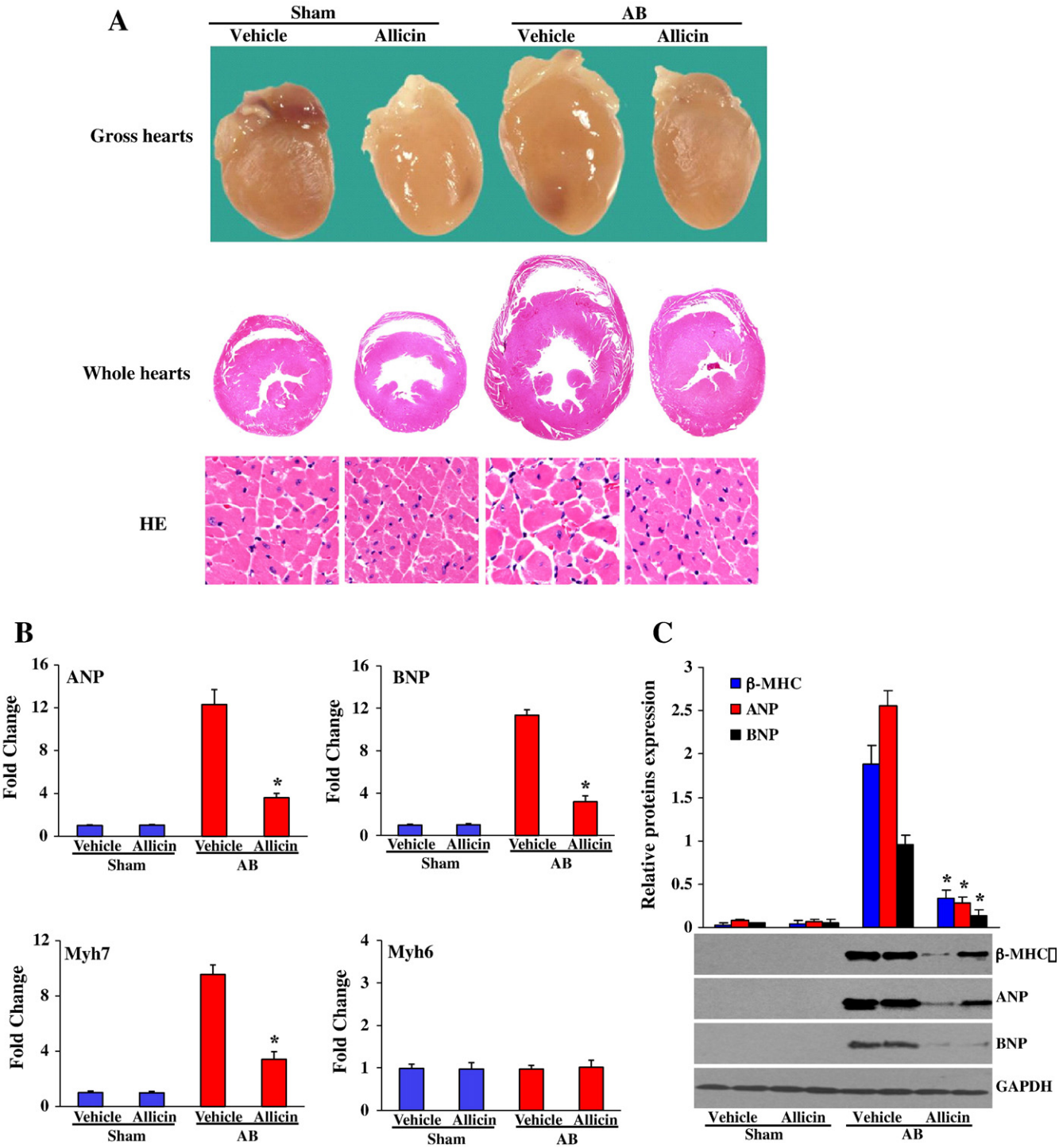

Fig. 2. Allicin inhibited cardiac hypertrophy in vivo. (A) Gross hearts (top), and whole hearts (middle), representative H&E staining (bottom) at 8 weeks after aortic banding or sham operation (n=6). (B) mRNA expression of hypertrophic markers, ANP, BNP, Myh7 and Myh6, evaluated by real-time PCR (n=3). (C) Bottom panel showed protein expressions of β-MHC, ANP and BNP determined by Western blot. Top bar graph is for corresponding statistic results (n=4). *P<.05 vs. vehicle treatment after AB.

Table 1
Echocardiographic data showed the effects of allicin on cardiac hypertrophy induced by AB

Parameter	Vehicle-sham	Allicin-sham	AB-vehicle	AB-allicin
Number	n=12	n=12	n=13	n=10
BW (g)	25.3±1.1	26.1±1.7	25.6±1.5	27.1±1.2
HW/BW (mg/g)	4.91±0.22	4.72±0.17	7.78±0.25 *	5.74±0.25**
LW/BW (mg/g)	4.56±0.26	4.77±0.13	8.23±0.28 *	5.66±0.17**
CSA (μm^2)	289±31	278±31	445±29 *	315±38**
SBP (mmHg)	112.8±3.2	115.4±2.5	157.0±5.5 *	148.4±2.8 *
HR (beats/min)	477±27	468±24	449±25	482±26
PWT (mm)	1.22±0.04	1.19±0.01	2.39±0. 03 *	1.51±0.02**
LVEDD (mm)	3.60±0.04	3.63±0.02	5.84±0.04 *	4.12±0.05**
LVESD (mm)	2.35±0.03	2.31±0.05	3.63±0.02 *	2.69±0.04**
IVSD (mm)	0.66±0.05	0.61±0.03	1.68±0.04 *	0.96±0.03**
LVPWD (mm)	0.65±0.01	0.62±0.02	1.32±0.04 *	0.78±0.03**
FS (%)	55.1±3.2	56.7±3.7	27.6±2.2 *	48.2±3.1***

CSA, cardiomyocyte cross-sectional area; SBP, systolic blood pressure; HR, heart rate; BW, body weight; HW, heart weight; LW, lung weight; PWT, posterior wall thickness; IVSD, left interventricular septum, diastolic; LVPWD, left ventricular posterior wall, diastolic; FS, fractional shortening. All values are mean±S.E.M.

* P<.01 was obtained for the vehicle-sham values.

** P<.01 was obtained for the vehicle-AB values after AB.

(1 μM) for 48 h. The results showed that allicin reduced the increase of [^{3}H]-leucine incorporation induced by Ang II in a dose-dependent manner, and the maximal inhibitory effect is 10 μM (Fig. 1A). Additionally, the inhibition of [^{3}H]-leucine incorporation by 10 μM allicin began at 6 h after Ang II treatment. Similar to the changes of [^{3}H]-leucine incorporation, the increased size of cardiomyocyte was also prevented by allicin (Fig. 1B). Further experiments showed that allicin inhibited the activities of atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) promoter(Fig. 1C), and Western blot showed the increased ANP and BNP protein expression were blocked by allicin treatment(Fig. 1D and E). Therefore, these data suggested that allicin could inhibit cardiac hypertrophy in vitro.

3.2. Allicin inhibited cardiac hypertrophy in vivo

To further confirm the inhibitory effect of allicin on cardiac hypertrophy, we used aortic banding (AB) model to induce hypertrophy in vivo. In order to further assess the effect of allicin on cardiac hypertrophy, mice were randomly assigned into four groups. Pretreatment with vehicle or 50 mg/kg per day of allicin for 1 week prior to AB surgery or sham operation allowed for critical evaluation. As expected, all vehicle-treated AB mice 8 weeks post surgery demonstrated the classical increase in heart size and dilatation of ventricular chambers as compared to the sham control group. After 8 weeks of pressure overload, the gross hearts were much smaller after allicin treatment than those of vehicle treated AB mice, the HW/BW and LW/BW ratios were also markedly decreased by allicin compared to the mice with vehicle after 8 weeks AB (Fig. 2A, Table 1). Gross hearts and H&E staining showed consistent results (Fig. 2A). Subsequent assessment of chamber size and wall thickness using M-mode echocardiography confirmed these findings. Allicin treatment prevented the development of adverse cardiac remodeling and ventricular dysfunction, as

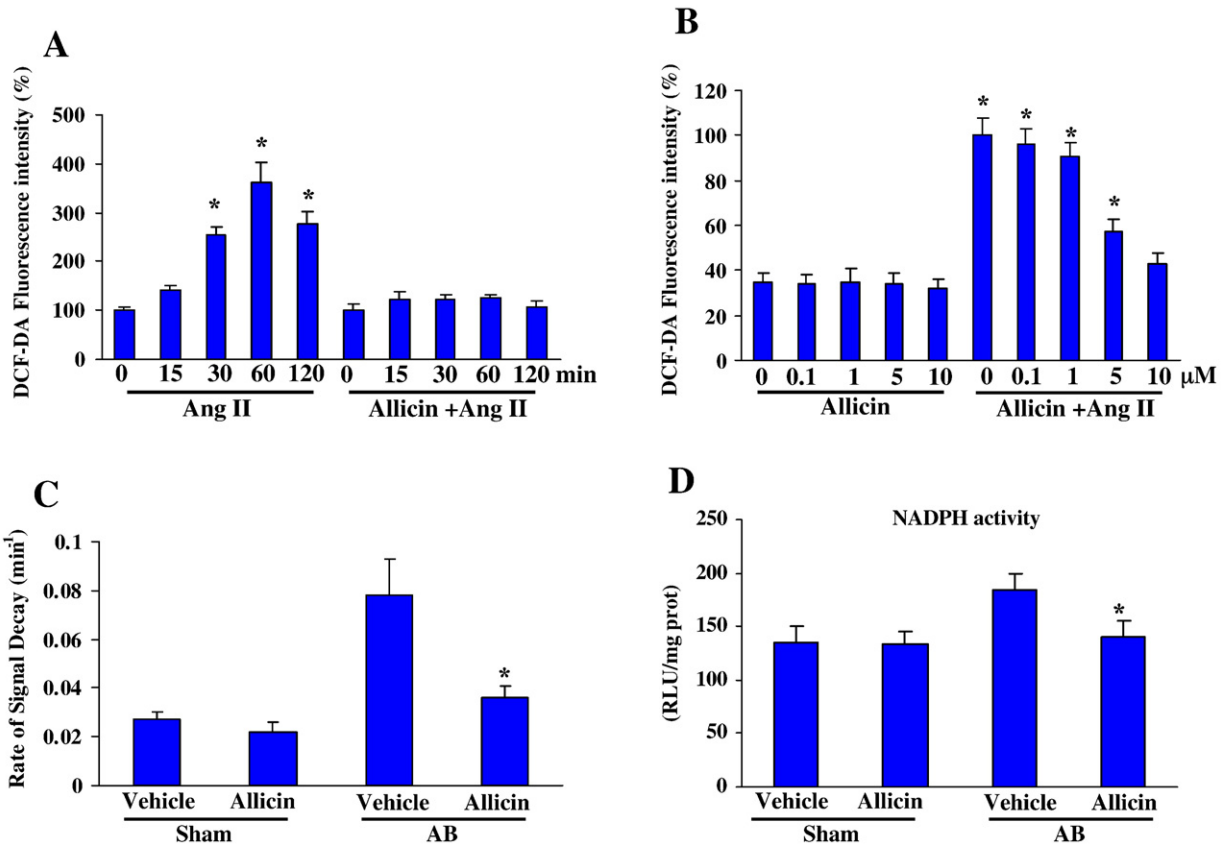

Fig. 3. Allicin blocked the generation of ROS induced by AB or Ang II. (A and B) Cardiac myocytes treated with different doses of allicin with/without Ang II for indicated times or incubated with Ang II with/without allicin (10 μM) for indicated times. ROS were quantified using DCFH-DA, and immunofluorescence was visualized using laser scanning confocal microscope (n=6). *P<.05 vs. corresponding control. (C) Allicin inhibited ROS in heart tissue at 8 weeks after AB. ROS were quantified by using ESR spectroscopy with hydroxy-TEMPO (n=6). (D) Allicin reduced NADPH oxidase activity at 8 weeks after aortic banding (n=6). *P<.05 vs. vehicle treatment after AB.

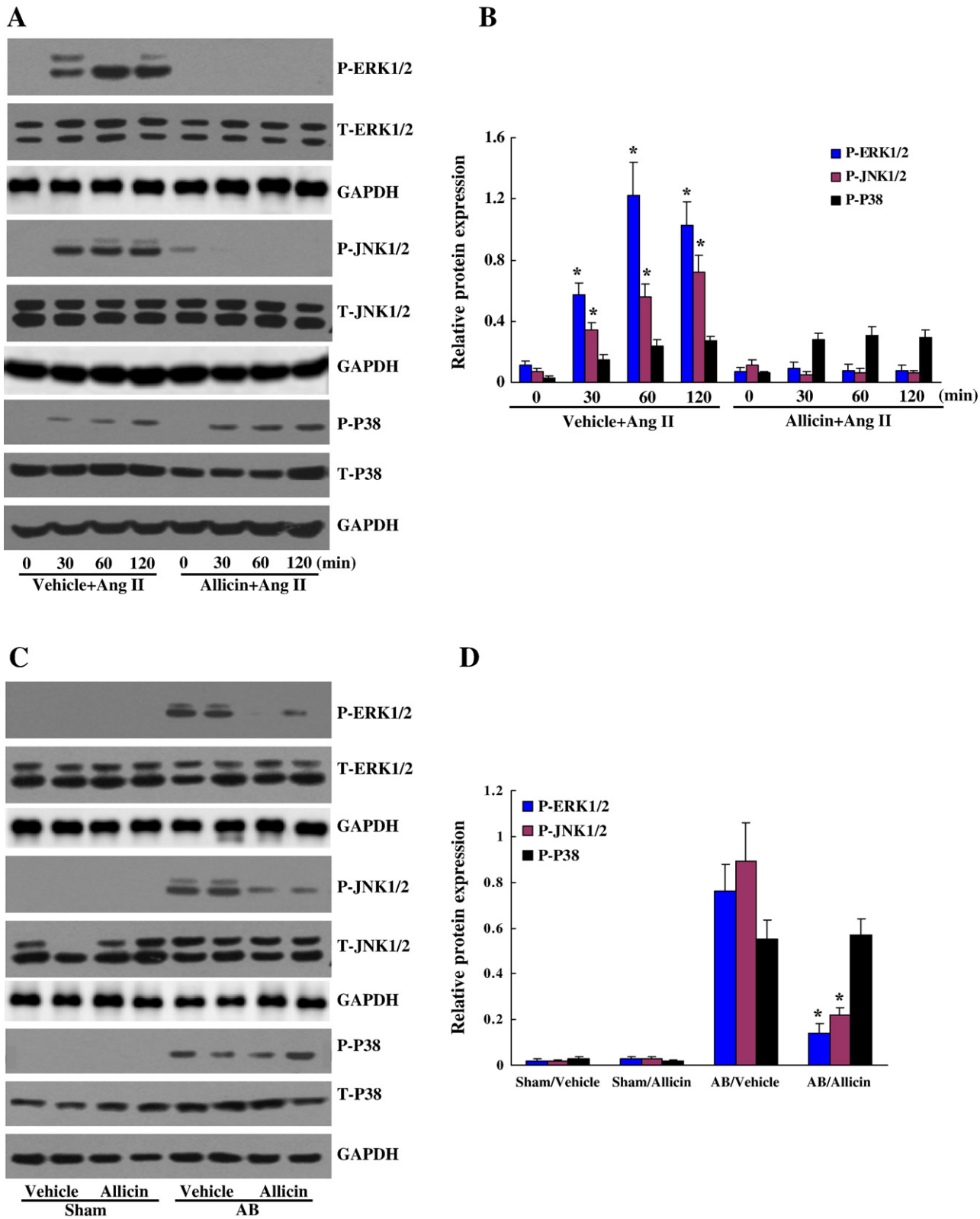

Fig. 4. Effects of allicin on MAPKs and PI3K/Akt/GSK3β signaling in vitro and in vivo (A and B). Time course of phosphorylated and total MAPKs and effects of Allicin on the phosphorylation of MAPKs induced by Ang II (n=4). *P<.05 vs. corresponding control. (C and D) Representative Western blot and quantification of ERK1/2, JNK1/2 and p38 phosphorylation and corresponding total protein expressions in the hearts of mice with 4 weeks AB or sham operation (n=4). *P<.05 vs. vehicle treatment after AB. (E and F) Time course of phosphorylated and total p85, Akt and GSK3β and effects of Allicin on the phosphorylation of them induced by Ang II (n=4). *P<.05 vs. corresponding control. (G and H) Representative western blot and quantification of p85, Akt and GSK3β phosphorylation and their total protein expressions in the hearts of mice with 4 weeks AB or sham operation (n=4). *P<.05 vs. vehicle treatment after AB.

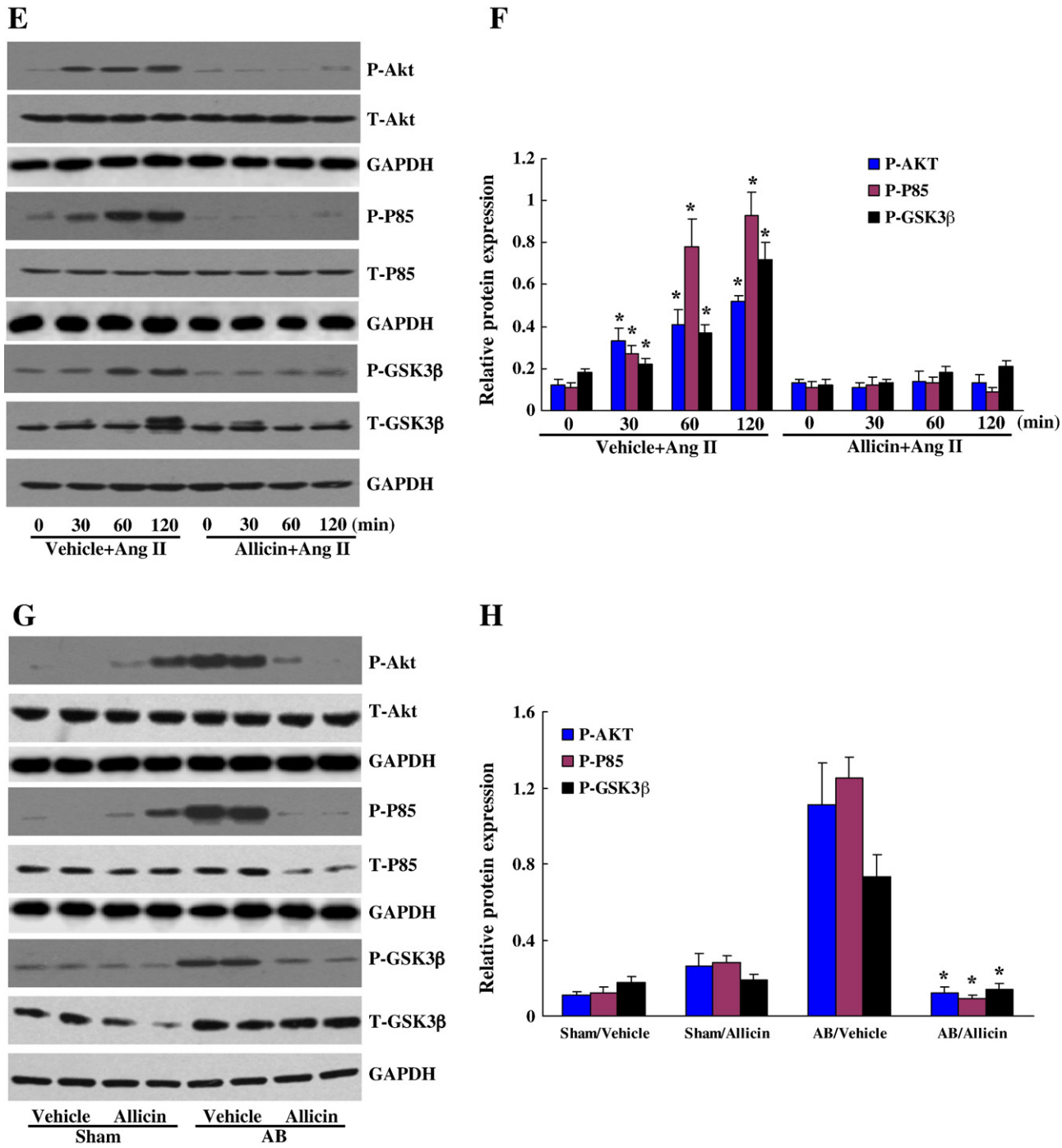

Fig. 4 (*continued*).

evidenced by decreased LVESD, LVEDD, diastolic left interventricular septum, diastolic left ventricular posterior wall and increased percent fractional shortening (%FS) (Table 1). To determine whether allicin affected the mRNA and protein expression levels of markers of cardiac hypertrophy, real-time PCR and Western blot analysis of ANP, BNP and myosin heavy chain 7 (Myh7) was performed. The results revealed a significant attenuation of the observed increase expression levels of ANP, BNP and Myh7 from the vehicle-treated AB group when these animals were treated with allicin (Fig. 2B and C). These results indicate that allicin inhibits the expression of cardiac hypertrophy markers ANP, BNP and Myh7 in the heart and results in an attenuated cardiac hypertrophic response induced by pressure overload. Collectively, these data suggested allicin impaired cardiac hypertrophy and preserved cardiac function after pressure overload.

3.3. Allicin blocked the excess production of ROS in vitro and in vivo

ROS play critical role in the development of cardiac hypertrophy. In order to detect the level of ROS, we used DCFH-DA to measure intracellular ROS. Cardiac myocytes were pretreated for 60 min with

different concentrations of allicin and then stimulated with 1 μM Ang II for 120 min. As shown in Fig. 3A, Ang II significantly increased the levels of ROS and such increase was markedly attenuated by allicin. The maximal inhibitory effect of allicin is 10 μM (Fig. 3B). To further validate these in vitro findings, we evaluated the levels of ROS in the murine heart receiving allicin treatment. Myocardial production of ROS was evaluated by ESR spectroscopy with hydroxy-TEMPO as a spin probe. The intensity of ESR signals declined more rapidly in banded mice than sham-operated controls and a linear relation was observed in the semilogarithmic plot of peak signal intensity vs. time (data not shown). The rate of signal decay has been shown to reflect the concentration of ROS in the reaction mixture. As shown in Fig. 3C, the rate of signal decay was significantly higher in banded mice than sham-operated animals, which was markedly reduced by treatment with allicin. Treatment by allicin alone had no significant effects. Since NADPH oxidase plays a pivotal role in the oxidative stress, to further understand the mechanism of allicin in ROS production, we tested the NADPH oxidase activity in sham or AB mice. Significant reduction of NADPH activity was found in allicin treated group after AB (Fig. 3D). Therefore, these data indicated that allicin inhibited the production of ROS following pressure overload through the blockage of NADPH oxidase activity.

3.4. Effects of allicin on MAPKs and PI3K/Akt/GSK3β signaling in vitro and in vivo

To investigate the molecular mechanism of allicin on cardiac hypertrophy, we examined the effects of allicin on MAPKs signaling pathway. Our data showed that ERK1/2, JNK1/2 and p38 were significantly phosphorylated after treatment with Ang II or AB, allicin treatment evidently blocked the activation of ERK1/2 and JNK1/2, but not p38 (Fig. 4A-D). Accordingly, ERK1/2 and JNK1/2 signaling were regulated by allicin in response to hypertrophic stress. PI3K/Akt/GSK3β signaling pathway is another important mediator in cardiac hypertrophy. In order to elucidate the relationship between allicin and this pathway, we evaluated the phosphorylation level of p85, which is the regulatory subunit of PI3K, Akt and GSK3β after Ang II or AB stimulation. Up-regulation of p-p85, p-Akt and p-GSK3β were observed after Ang II treatment or AB compared with vehicle or sham group, and allicin treatment

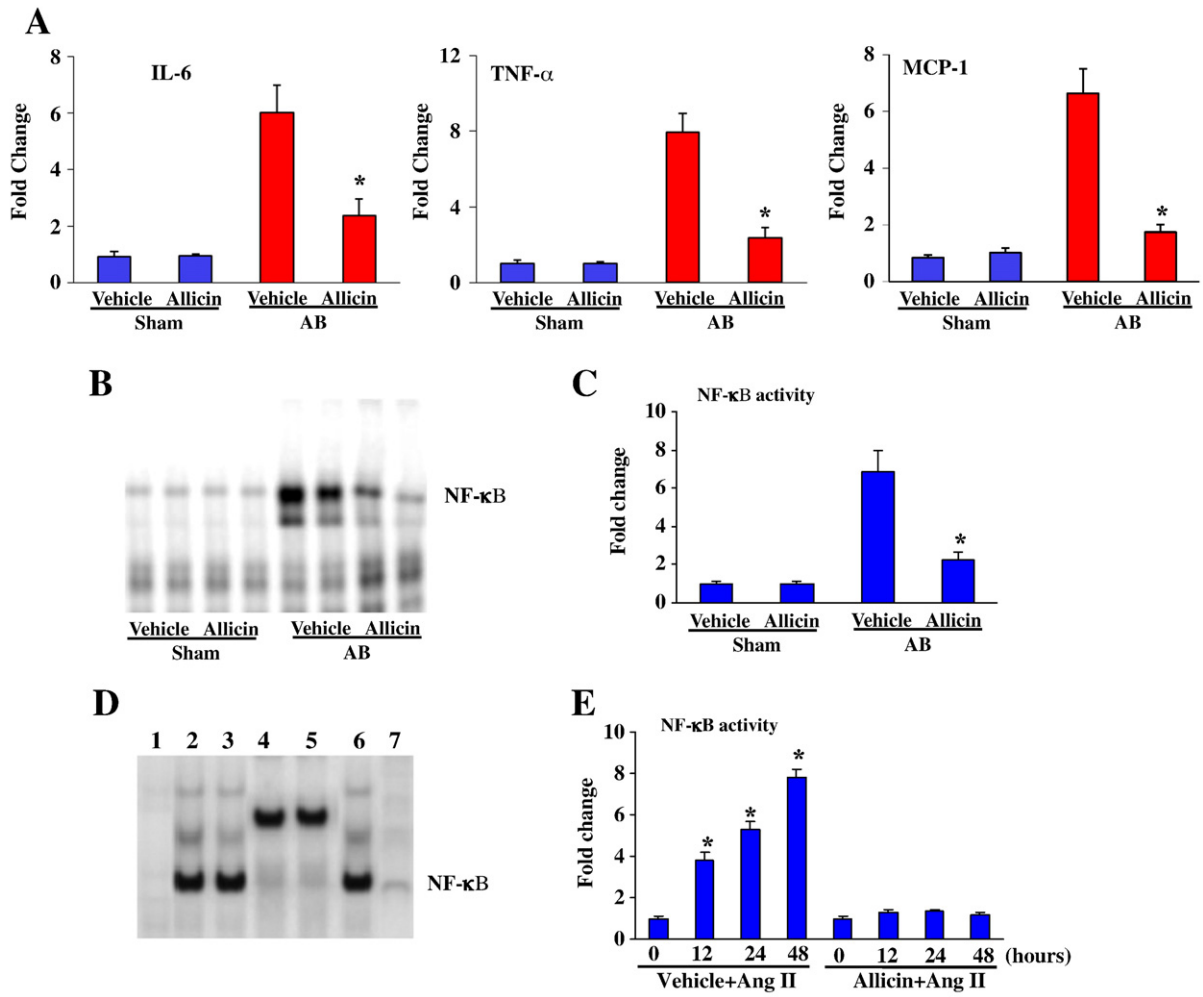

Fig. 5. Allicin inhibited inflammation induced by pressure overload. (A) Quantification of IL-6, TNF-α and MCP-1 mRNA levels by using of real-time PCR (n=4). *P<.05 vs. vehicle treatment after AB. (B and C) The DNA binding activity of NF-κB evaluated by EMSA in the hearts from indicated groups (n=6). *P<.05 vs. vehicle treatment after AB. (D) NF-κB induced by AB is composed of p65 and p50 subunits. Nuclear extracts from heart tissue in sham group or AB group were incubated with the indicated antibodies, an unlabeled NF-κB oligo probe, or a mutant oligo probe. They were then assayed for NF-κB activation by EMSA (1, sham; 2, AB/vehicle group; 3, Anti-p50; 4, anti-p65; 5, anti-p50/65; 6, mutant oligo; 7, competitor). (E) NF-κB activity was determined in vitro (n=6). *P<.05 vs. corresponding control.

significantly inhibited the levels of p-Akt, p-p85 and p-GSK3β in comparison to vehicle treated myocytes and AB mice, indicating PI3K/Akt/GSK3β signaling pathway was also blocked by allicin in response to hypertrophic stress (Fig. 4E-H).

3.5. Allicin inhibited inflammation in cardiac hypertrophy

More and more studies suggests inflammation promotes cardiac hypertrophy, and increased proinflammatory cytokines such as TNF-α, interleukin (IL)-1 and monocyte chemoattractant protein (MCP)-1 expression will lead to severe fibrosis and cardiac dilation. Therefore, it is important to investigate the role of allicin in inflammation. To determine whether allicin can suppress the inflammatory responses in the heart, we examined the expression of inflammatory mediators IL-6, TNF-α and MCP-1 in cardiac tissue at 8 weeks of AB or sham. Our data showed that allicin significantly decreased the levels of IL-6, TNF-α and MCP-1 mRNA expression compared with vehicle-treated AB mice (Fig. 5A). To evaluate the underlying mechanisms accounting for the potential etiologies of the inhibitory effects of allicin on inflammatory responses, we evaluated NF-κB signaling in the mice. Treatment with allicin abolished the increased DNA binding activity of NF-κB observed in the myocardium of vehicle-treated mice 8 weeks post-AB (Fig. 5B and C). In order to examine the specificity of the NF-κB band, the nuclear extracts from banded heart tissues were incubated with antibodies to the p50 (NF-κB) and the p65 (RelA) subunit of NF-κB; the resulting bands were shifted to higher molecular masses (Fig. 5D), indicating that the AB-activated complex consisted of p50 and p65. The addition of excess unlabeled NF-κB oligonucleotide caused complete disappearance of the band, whereas mutated oligonucleotide had no effect on the DNA binding. Consistent with the AB model, NF-κB activity was also completely blocked by allicin in cardiomyocyte subjected to Ang II stimulation (Fig. 5E).

3.6. Allicin repressed collagen synthesis and fibrosis in vitro and in vivo

To explore whether allicin can regulate fibrosis in cardiac hypertrophy, we determined the collagen synthesis by [^{3}H]-proline incorporation. In our study, allicin dose-dependently reduced [^{3}H]-proline incorporation induced by 1 μM Ang II stimulation (Fig. 6A). In order to confirm the effect of allicin on [^{3}H]-proline incorporation was specific to the synthesis of collagen or not, we performed luciferase assay to assess the promoter activity of Col1a1. As shown in Fig. 6A, elevated Col1a1 promoter activity was also inhibited by allicin in a dose dependent manner. Our in vivo analysis further demonstrated that allicin markedly blocked fibrosis induced by 8 weeks AB (Fig. 6B). Moreover, increased protein and mRNA expressions of known markers of fibrosis, CTGF, Collagen I and III resulting from 8 weeks AB were also clearly decreased by treatment of allicin (Fig. 6C and D). To further elucidate the mechanisms underlying these effects of allicin, we assessed the Smad cascade in the hearts after AB. The increased phosphorylation of reactive Smad, Smad 2 and Smad 3, were completed suppressed by allicin after 4 weeks pressure overload, but not Smad 4 (Fig. 6E and F). Therefore, our results suggested that the anti-fibrosis effect

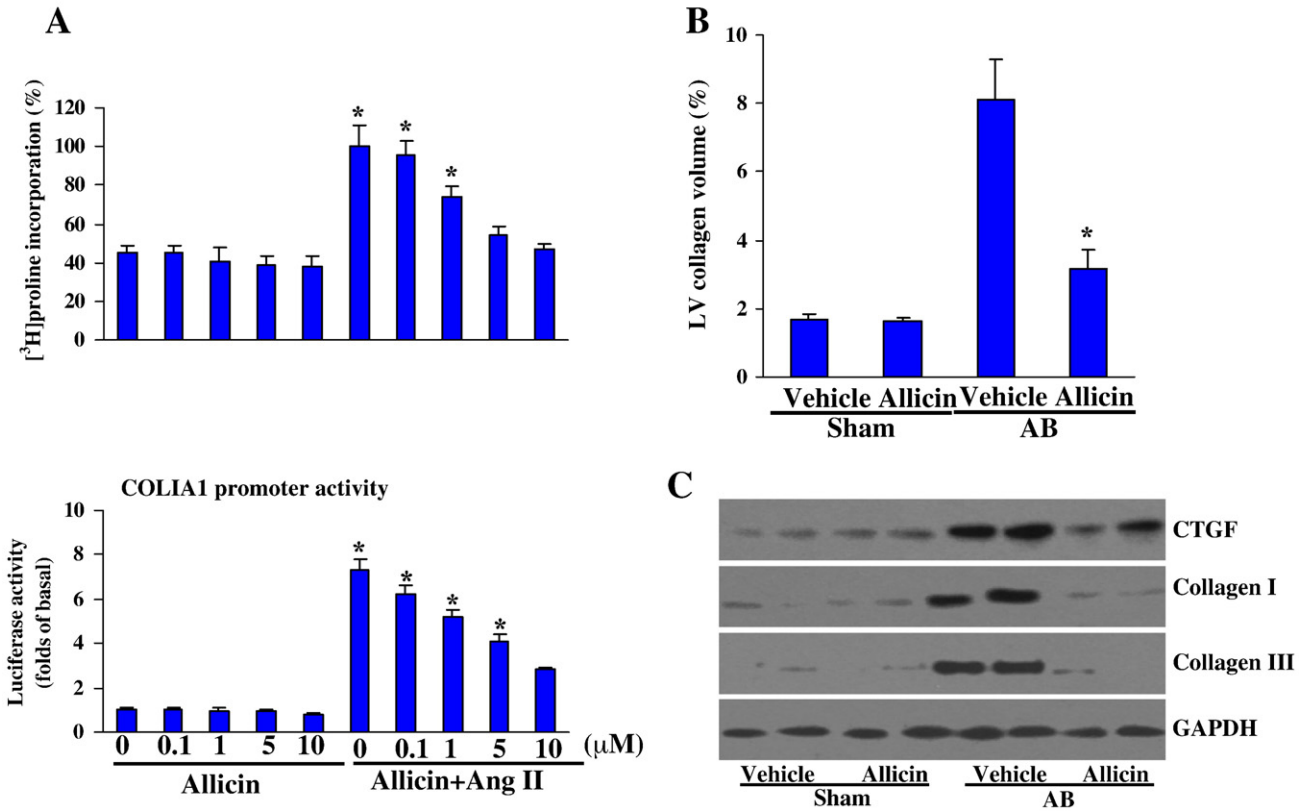

Fig. 6. Allicin reduced collagen synthesis in vitro and fibrosis induced by pressure overload. (A) Allicin inhibited Ang II-induced [^{3}H]-proline incorporation and COL1A1 promoter activity. Cardiac fibroblasts were pretreated with different dose of allicin for 60 min and then incubated with 1μM Ang II for up to 48 h. Collagen synthesis and promoter activity of Col1a1 were determined by [^{3}H]-proline incorporation and luciferase assay, respectively (n=6). (B) Quantitative analysis of left ventricle interstitial collagen volume fraction in indicated groups (n=6). (C) Protein expression of CTGF, collagen I and III were tested by Western blot analysis. GAPDH was used as internal control (n=4). (D) Real-time PCR analysis of TGF-β1, Col1a1, Col1a3 and CTGF mRNA level (n=4). (E and F) Representative Western blot and quantification of phosphorylated and total protein expression of Smad 2, 3 and 4 in the indicated groups (n=4). *P<.05 vs. vehicle treatment after AB.

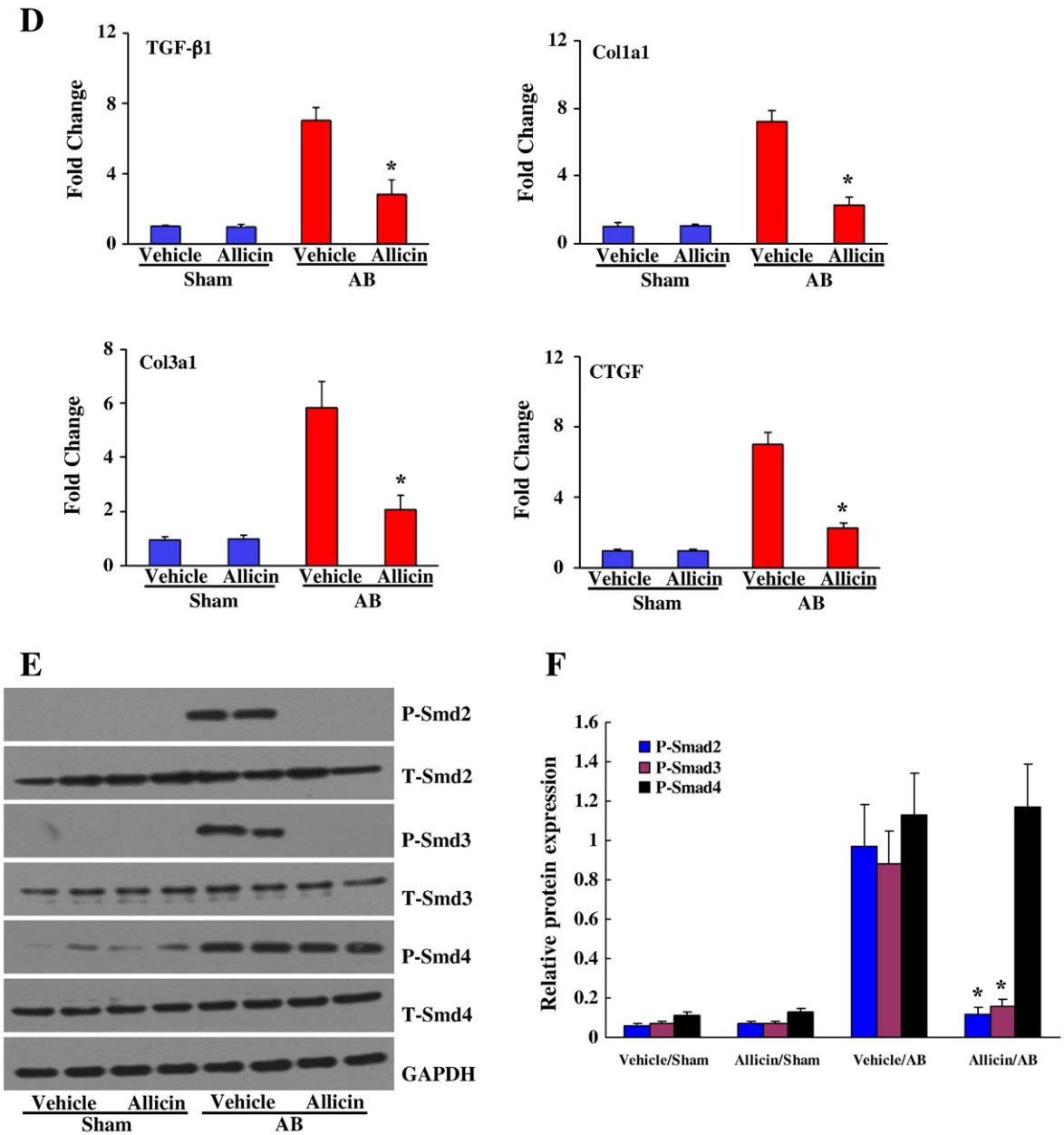

Fig. 6 (*continued*).

of allicin resulted from the inhibition of collagen synthesis and the blockage of Smad cascade.

3.7. Role of ROS in Ang II-induced cardiac hypertrophy and collagen synthesis, as well as ERK1/2, JNK1/2, AKT and Smad 2/3 signaling

In order to confirm the role of ROS in Ang II-induced hypertrophy and collagen synthesis, *N*-acetylcysteine (NAC, 100 mM), a typical antioxidant and diphenyleneiodonium (DPI;10 μM, an inhibitor of NADPH oxidase) were used. Both NAC and DPI strongly inhibited [^{3}H]-leucine and [^{3}H]-proline incorporation induced by Ang II, indicating Ang II-induced cardiac hypertrophy and collagen synthesis were ROS dependent. Moreover, phosphorylated ERK1/2, JNK1/2 and AKT as well as Smad 2/3 were also obviously attenuated by NAC and DPI in response to Ang II treatment (Fig. 7A and 7B). These data indicated that Ang II-induced cardiac hypertrophy and fibrosis mainly depends on oxidative stress.

4. Discussion

In the present study, we provided the first evidence that allicin not only improved cardiac performance but inhibited the development of cardiac hypertrophy in vitro and in vivo. The cardioprotective effects of allicin were medicated by reducing the production of ROS and blocking ROS-dependent ERK1/2, JNK1/2, AKT, NF-κB and Smad signaling, which led to the inhibition of hypertrophy, inflammation and fibrosis, ultimately preventing cardiac dysfunction. These findings supported the notion that allicin could be a promising candidate for therapies against cardiac hypertrophy and progression of heart failure.

Mounting reports demonstrated that ROS played important roles in injury of cardiomyocyte and exerted a significant effect on the development of cardiac hypertrophy [29–32]. Tsujimoto et al. reported that pressure overload induced cardiac hypertrophy accompanied by increased ROS level, while treatment with free

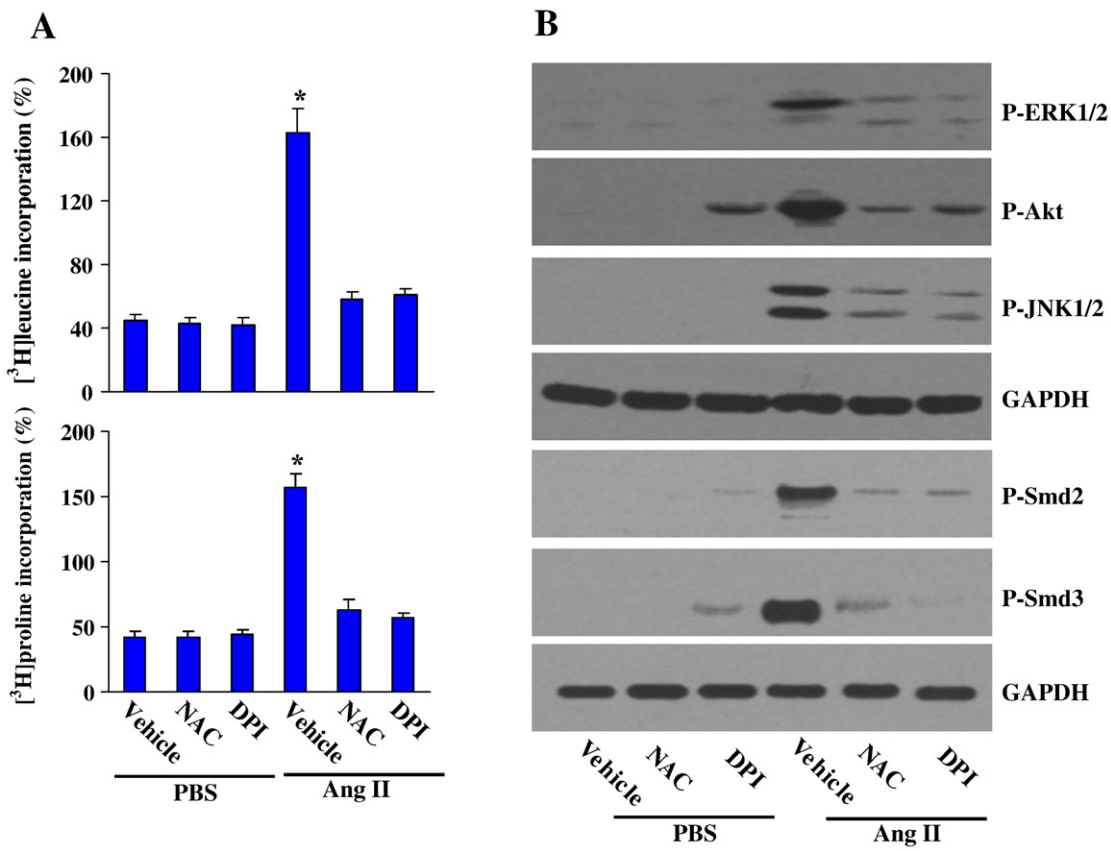

Fig. 7. Role of ROS in Ang II-induced cardiac hypertrophy and collagen synthesis, as well as ERK1/2, JNK1/2, AKT and Smad 2/3 signaling. Summary data of the effects of NAC and DPI on [^{3}H]-leucine incorporation and [^{3}H]-proline incorporation in the heart with or without 48 h Ang II stimulation (n=6). (B) Western blot analysis of the effects of NAC and DPI on phosphorylation of ERK1/2, JNK1/2, Akt, Smad 2 and 3. GAPDH was used as internal control (n=4). Cardiac myocytes or fibroblasts were pretreated with DPI or NAC for 60 min and then incubated with 1μM Ang II for up to 60 min. Western blot was performed for phosphorylation of ERK1/2, JNK1/2, and Akt in cardiac myocytes and Smad 2/3 in cardiac fibroblasts. *P<.05 vs. corresponding control.

radical scavenger significantly attenuated hypertrophic response [33]. An antioxidant *N*-2-mercaptopropionyl glycine was shown to reduce cardiac hypertrophy induced by pressure overload [11]. Additionally, in vitro studies reported Ang II could induce cardiac hypertrophy through the increased ROS, and pretreatment with antioxidants remarkably eliminated this effect [34,35]. Garlic has long been considered as a natural remedy against a range of human illnesses. Up to now, many biologically active sulfur species were found in garlic; among them, allicin has been mostly studied in garlic research. Due to the prominent antioxidative property of allicin, the role of allicin in cardiac hypertrophy was tested in our study. We demonstrated allicin ameliorated cardiac function, blocked cardiac dilation and attenuated cardiac hypertrophy in vivo and in vitro. Moreover, allicin could eliminate the ROS generation in hearts and cultured cardiomyocytes subjected to pressure overload and Ang II stimulation, respectively. Therefore, allicin might inhibit the development of cardiac hypertrophy mainly depended on its antioxidative function.

It is known that the effects of ROS on cardiac intracellular signaling cascades are essential to elucidate the molecular mechanism underlying cardiac hypertrophy. Tsujimoto et al. reported that increased ROS could activate ASK1 and ASK1 downstream kinases of JNK1/2 and p38, so as to initiate hypertrophic responses in response to cardiac stimuli [33]. Tanaka et al. showed that ROS-dependent activation of ERK1/2 but not those of JNK1/2 and p38 mainly related to cardiac hypertrophy induced by ET-1 and PE in cultured cardiomyocytes [36]. As for Akt, the effect of ROS on Akt is controversial. One study has showed that Akt was involved in the pro-hypertrophic effect of ROS [28]. Conversely, Kwon et al. reported Akt was not affected in ROS-induced hypertrophic cardiomyocytes [9]. These discrepancies may be due to different stimuli of cardiac hypertrophy. Therefore, it is necessary to figure out the specific signaling related to the anti-hypertrophic effects of allicin. We found that the phosphorylation of ERK1/2, JNK1/2 and Akt induced by Ang II were disrupted by treatment with potent antioxidants NAC and DPI, indicating the activation of ERK1/2, JNK1/2 and Akt was dependent on ROS. Moreover, our data clearly demonstrated allicin almost completely ablated ERK1/2 and JNK1/2 activation in vivo and in vitro without regulating the phosphorylation of p38. In addition, allicin significantly reduced the phosphorylation of p85, the regulatory subunit of PI3K, which is essential to the activation of downstream molecules, Akt and GSK3β, which are important in the development of cardiac hypertrophy. Collectively, it suggested that allicin exerted the inhibitory effects on cardiac hypertrophy through the blockage of ROS-dependent signaling including ERK1/2, JNK1/2 and PI3K/Akt/GSK3β pathway. Importantly, it is worthwhile to point out the inhibition of Akt by allicin is more potent than by NAC and DPI, suggesting the negative regulation of Akt by allicin is both ROS dependent and independent.

Inflammation plays an important role in the development of cardiac hypertrophy. Several studies demonstrated that cardiac specific overexpressed cytokines stimulated the progression of cardiac hypertrophy and heart failure [37–39]. Allicin has been shown to have anti-inflammation effect [16,40,41]. In line with these findings, we observed cytokines including TNF-α, IL-6 and MCP-1 were markedly induced by pressure overload and such increases were

blocked by administration of allicin. To investigate the mechanism attributed to the anti-inflammation property of allicin, we further tested the transcription factor, NF-κB, which is pivotally responsible for transcription of proinflammatory cytokines. Allicin blunted NF-κB activity as well as disrupting its DNA binding activity. Therefore, our data indicated allicin inhibited inflammation by blocking NF-κB in the heart. In addition, accumulated studies showed NF-κB signaling is critical for the development of cardiac hypertrophy in response to multiple stimuli not simply based on the proinflammatory property, and inhibition of NF-κB greatly prevented hypertrophic response and heart failure. Accordingly, NF-κB maybe serves as a common factor combining hypertrophic and inflammatory pathway which ultimately results in heart failure [42]. The inhibition of NF-κB by allicin could impair both hypertrophy and inflammation and, in turn, obtained reduced cardiac hypertrophy and conserved cardiac function.

Myocardial fibrosis is one of the pathological features of chronic pressure overload [43]. Increased fibrosis decreases myocardial compliance, impairs diastolic relaxation and causes cardiac dysfunction. We observed allicin observably reduced the cardiac fibrosis in vivo and inhibited collagen synthesis in vitro. To further investigate the molecular mechanism, we evaluated the role of allicin in Smad signaling, which is responsible for transcription of profibrotic genes. It was demonstrated that allicin abrogated Smad 2/3 phosphorylation. Intriguingly, NAC and DPI also disrupted Smad 2/3 activation in response to hypertrophic stimuli, indicating Smad 2/3 activation is ROS dependent. Therefore, the restrained cardiac fibrosis by allicin may be due to blocking ROS-dependent Smad activation. In addition, it is documented that cytokines play a positive role in the development of cardiac fibrosis. Hereby, limited fibrosis by allicin may partly result from decreased cytokines in cardiac hypertrophy.

Taken together, our present work showed the first evidence that allicin was effective in inhibiting cardiac hypertrophy and fibrosis and preserving cardiac function. Allicin prevented hypertrophy by blocking ROS-dependent ERK1/2, JNK1/2 and PI3K/Akt/GSK3β signaling pathway in vivo and in vitro. In addition, allicin also inhibited inflammation and fibrosis through abrogating NF-κB- and ROS-dependent activation of Smad cascades, respectively. Overall, our study suggested the potential clinical efficacy of allicin for prevention of cardiac hypertrophy. It is tempting for the application of allicin to be a useful therapeutic method in patients with cardiac hypertrophy and fibrosis.

Acknowledgments

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