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Silibinin Attenuates Cardiac Hypertrophy and Fibrosis Through Blocking EGFR-Dependent Signaling

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ABSTRACT

Cardiac hypertrophy is a major determinant of heart failure. The epidermal growth factor receptor (EGFR) plays an important role in cardiac hypertrophy. Since silibinin suppresses EGFR in vitro and in vivo, we hypothesized that silibinin would attenuate cardiac hypertrophy through disrupting EGFR signaling. In this study, we examined this hypothesis using neonatal cardiac myocytes and fibroblasts induced by angiotensin II (Ang II) and animal model by aortic banding (AB) mice. Our data revealed that silibinin obviously blocked cardiac hypertrophic responses induced by pressure overload. Meanwhile, silibinin markedly reduced the increased generation of EGFR. Moreover, these beneficial effects were associated with attenuation of the EGFR-dependent ERK1/2, PI3K/Akt signaling cascade. We further demonstrated silibinin decreased inflammation and fibrosis by blocking the activation of NF-κB and TGF-β1/Smad signaling pathways in vitro and in vivo. Our results indicate that silibinin has the potential to protect against cardiac hypertrophy, inflammation, and fibrosis through blocking EGFR activity and EGFR-dependent different intracellular signaling pathways. J. Cell. Biochem. 110: 1111–1122, 2010.

KEY WORDS: SILIBININ; CARDIAC HYPERTROPHY; EGFR; FIBROSIS; SIGNALING PATHWAY

Cardiac hypertrophy is an adaptive process in response to increased hemodynamic overload, characterized by an increase in the size of individual cardiac myocytes and whole-organ enlargement. Although it may be compensatory initially, sustained pathologic hypertrophy is deleterious and frequently decompensate into congestive heart failure [Haq et al., 2001; Wettschureck et al., 2001; Chen et al., 2002]. It is well known that the effects of hypertrophic stimuli such as mechanical stretch and angiotensin II (Ang II) on cardiac intracellular signaling cascades are crucial to elucidate the molecular mechanism underlying cardiac hypertrophy [Haq et al., 2001; Lips et al., 2003; Frey et al., 2004]. These signaling pathways include the epidermal growth factor receptor (EGFR) pathway, the mitogen-activated protein kinases (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, the NF-κB pathway, and TGF-β1/Smad pathway [Homcy, 1998; Selvetella and Lembo, 2005; Zhai et al., 2006], and Insulin/IGF-1 signaling pathway [Zi et al., 2000; Singh and Agarwal, 2006]. Therefore, pharmacological interventions of these signaling pathways may provide promising approaches in treating cardiac hypertrophy and heart failure [Li et al., 2005].

Silibinin is a natural polyphenolic flavanoid extracted from fruits and seeds of milk thistle (Silybum marianum), which has been extensively used clinically as an anti-hepatotoxic agent for treatment of liver diseases [Di Sario et al., 2005]. In addition, anti-tumor efficacy of silibinin is shown in prostate, skin, renal, colon, and bladder cancer models [Ramasamy and Agarwal, 2008]. It is also reported that silibinin possesses anti-oxidant, anti-apoptotic, anti-inflammatory and anti-fibrotic properties [Li et al., 2008; Ramasamy and Agarwal, 2008]. However, the effect of silibinin on cardiac hypertrophy and the related signaling mechanisms still remain unclear. Although silibinin has been shown to inhibit EGFR activation, very little is known about whether this inhibitory effect is

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related to the protective role in cardiac hypertrophy [Singh et al., 2006; Tyagi et al., 2008]. Therefore, we aimed to determine whether silibinin attenuates cardiac hypertrophy in vitro and in vivo by interfering EGFR-dependent pathways.

MATERIALS AND METHODS

MATERIALS

The antibodies used to recognize total and phosphorylation of EGFR, ERK1/2, P38, JNK1/2, P85, AKT, GSK3β, IκBα, Smad2, Smad, Smad 4, as well as phospho-IKKβ, Cox-2, Cyclin D1, MMP-9, CTGF, Collagen I, and Collagen III were purchased from Cell Signaling Technology. All other antibodies were purchased from Santa Cruz Biotechnology. The BCA protein assay kit was purchased from Pierce (Rockford, IL) and the IKK activity kit was obtained from B&D Bioscience. [^{3}H]-Leucine and [^{3}H]-proline were purchased from Amersham. NF-κB-luc, CTGF, and COL1A1-luc report constructs was described previously [Bian et al., 2009]. Fetal calf serum (FCS) was obtained from Hyclone. Silibinin and all other reagents were obtained from Sigma. Silibinin was derived from plant, its purity is high-performance liquid chromatographic (HPLC) $\geq$ 98% and dissolved in DMSO medium for all in vitro studies.

CULTURED NEONATAL RAT CARDIOMYOCYTES AND FIBROBLASTS

Primary cultures of cardiac cardiomyocytes were prepared as described previously [Bian et al., 2009]. Cells from the hearts of 1- to 2-day-old Sprague–Dawley rats were seeded at a density of 1×10^6/well onto six-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, silibinin alone, or silibinin followed by Ang II (1 μM) were added to the medium and the cultures were incubated for the indicated time. Viability was determined by MTT assay.

Cultures of neonatal rat cardiac fibroblasts were described previously [Bian et al., 2009]. Briefly, hearts obtained from neonatal rats were enzyme-digested as described above for cardiomyocytes. The adherent non-myocyte fractions obtained during preplating were grown in 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 >95% cardiac fibroblasts as determined by positive staining for vimentin and negative staining for smooth muscle actin and von Willebrand factor. For cell infection, 1×10^6/well cardiac cardiomyocytes or cardiac fibroblasts were cultured in six-well plates and exposed to 2×10^8 pfu of each virus in 1 ml of serum-free medium for 24 h. The cells were then washed and incubated in serum-containing media for 24 h.

[^{3}H]-LEUCINE INCORPORATION, SURFACE AREA, AND COLLAGEN SYNTHESIS ASSAY

[^{3}H]-leucine incorporation was measured as described previously [Bian et al., 2009]. Briefly, cardiac cardiomyocytes were pretreated with silibinin for 60 min and subsequently stimulated with Ang II (1 μM) and coincubated with [^{3}H]-leucine (1 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 (TCA) 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 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 [Bian et al., 2009]. In brief, cardiac fibroblasts were made quiescent by culturing in 0.1% FCS DMEM for 24 h, pretreating with silibinin for 60 min and subsequently incubating with Ang II and 2μCi/ml [^{3}H]-proline for the indicated time. Cells were washed with PBS twice, treated with ice-cold 5% TCA for 1 h and washed with distilled water twice. Cells were then lysed with NaOH (1 N) solutions and counted in a liquid scintillation counter. The count representing the amount of newly synthesized collagen was normalized to the cell number.

REPORTER ASSAYS, WESTERN BLOTTING, AND QUANTITATIVE REAL-TIME RT-PCR

Cardiac cardiomyocytes 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 silibinin for 60 min and then treated with Ang II. Cells were harvested using passive lysis buffer (Promega, Madison, WI) 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 blotting, cardiac tissue and cultured cardiomyocytes or fibroblasts were lysed in RIPA lysis buffer. Fifty micrograms of cell lysate was used for SDS–PAGE, and proteins were then transferred to an Immobilon-P membrane (Millipore). Specific protein expression levels were normalized to either the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) protein for total cell lysate and cytosolic protein, or the Lamin-B1 protein for nuclear protein signal on the same nitrocellulose membrane. For real-time PCR, total RNA was extracted from frozen, pulverized mouse tissues using TRIzol (Invitrogen) and synthesized cDNA using oligo (dT) 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.

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). Nuclear proteins were isolated as described previously. Protein concentrations were measured by BCA Protein Assay Reagents (Pierce) using bovine serum albumin (BSA) as a standard.

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 C57BL/6 mice (8–10 weeks old) were purchased from the Jackson Laboratory and acclimatized for 1 week prior to experimental use. Mice were randomly assigned into four groups. Silibinin 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 comprising solely of the vehicle solution (0.5% carboxy methylcellulose). Aortic banding (AB) was performed as described previously [Bian et al., 2009]. Treatment with 50 mg/kg/day of silibinin or vehicle for 8 weeks after AB surgery or sham operation allowed for critical evaluation. Doppler analysis was performed to ensure that physiologic constriction of the aorta was induced. The internal diameter and wall thickness of LV were assessed by echocardiography. 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 mice. Mice was anesthetized with isoflurane, echocardiography was performed by SONOS 5500 ultrasound (Philips Electronics, Amsterdam) with a 15-MHz linear array ultrasound transducer. The left ventricle (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. 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) inserted into the right carotid artery and advanced into the left ventricle for hemodynamic measurements.

MEASUREMENTS OF CONCENTRATIONS OF SILIBININ IN PLASMA AND HEARTS

The concentrations of silibinin in plasma and hearts were measured by HPLC assay as described in our previous study [Zhu et al., 2010].

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 thickness) were prepared and stained with hematoxylin and eosin (HE) for histopathology and picrosirius red (PSR), Masson Trichrome for collagen deposition and then visualized by light microscopy. For myocyte cross-sectional area, sections were stained with HE. A single myocyte was measured with an image quantitative digital analysis system (Image Pro-Plus 4.5). The outline of 100–200 cardiomyocytes was traced in each group.

STATISTICAL ANALYSIS

Data are expressed as mean ± SEM. Differences among groups were tested by one-way ANOVA. Comparisons between two groups were performed by unpaired Student's *t*-test. A value of $P < 0.05$ was considered to be significantly different.

RESULTS

SILIBININ INHIBITED CARDIAC HYPERTROPHY IN VITRO

To determine the possible cytotoxity of silibinin in cardiomyocytes, we evaluated cell viability by MTT assay with different concentrations of silibinin in cardiomyocytes. Our data showed that silibinin was non-cytotoxic for cardiomyocytes in all tested concentration and no significant differences in cell viability were found between normal cardiomyocytes and cardiomyocytes treated with 20 μM silibinin for 48 h (data not shown). To examine the effects of silibinin on cardiac hypertrophy, we used Ang II to induce hypertrophy in vitro. Cardiomyocytes were preincubated with indicated doses of silibinin for 60 min and then subsequently treated with Ang II (1 μM) for 48 h. The results showed that silibinin reduced the increase of [^{3}H]-leucine incorporation induced by Ang II in a dose-dependent manner, and the maximal inhibitory effect is 20 μM (Fig. 1A). Additionally, the inhibition of [^{3}H]-leucine incorporation by 20 μM silibinin began at 12 h after Ang II treatment. Similar to the changes of [^{3}H]-leucine incorporation, the increased size of cardiomyocyte was also prevented by silibinin (Fig. 1B). Further experiments showed that silibinin inhibited the activities of atrial natriuretic peptide (ANP) promoter (Fig. 1C), and Western blotting showed the increased β-MHC, ANP, and BNP protein expression were blocked by silibinin treatment (Fig. 1D). Therefore, these data suggested that silibinin could inhibit cardiac hypertrophy in vitro.

SILIBININ LEVELS IN PLASMA AND HEART

To investigate the pharmacokinetic properties of silibinin in our murine models in vivo, we used a highly sensitive and reliable analytical method (HPLC) for quantification of the concentration of silibinin in plasma and hearts. Our pharmacokinetic results demonstrated that the peak plasma and hearts concentrations were 14.84 ± 2.12 μM and 17.67 ± 2.18 μM ($n = 8$), respectively. These were achieved at 30 min after oral treatment of 50 mg/kg/day consumption of silibinin.

SILIBININ INHIBITED CARDIAC HYPERTROPHY IN VIVO

To further confirm the inhibitory effect of silibinin on cardiac hypertrophy, we used AB model to induce hypertrophy in vivo. In order to assess the effect of silibinin on cardiac hypertrophy, mice were randomly assigned into four groups. Pretreatment with vehicle or 50 mg/kg/day of silibinin for 1 week prior to AB surgery or sham operation allowed for critical evaluation. As shown in Table I, silibinin pretreatment significantly decreased heart HW/BW ratio, LW/BW ratio, and cardiomyocyte cross-sectional area in aortic-banded mice. Furthermore, treatment with silibinin prevented ventricular dysfunction, as evidenced by improvements in LVESD, LVEDD, and percent fractional shortening (FS). No significant changes were observed in the sham-operated mice treated with silibinin or vehicle. Gross heart, H&E staining further confirmed the inhibitory effect of silibinin on cardiac hypertrophy in AB hearts (Fig. 2A). To determine whether silibinin affected the expression of

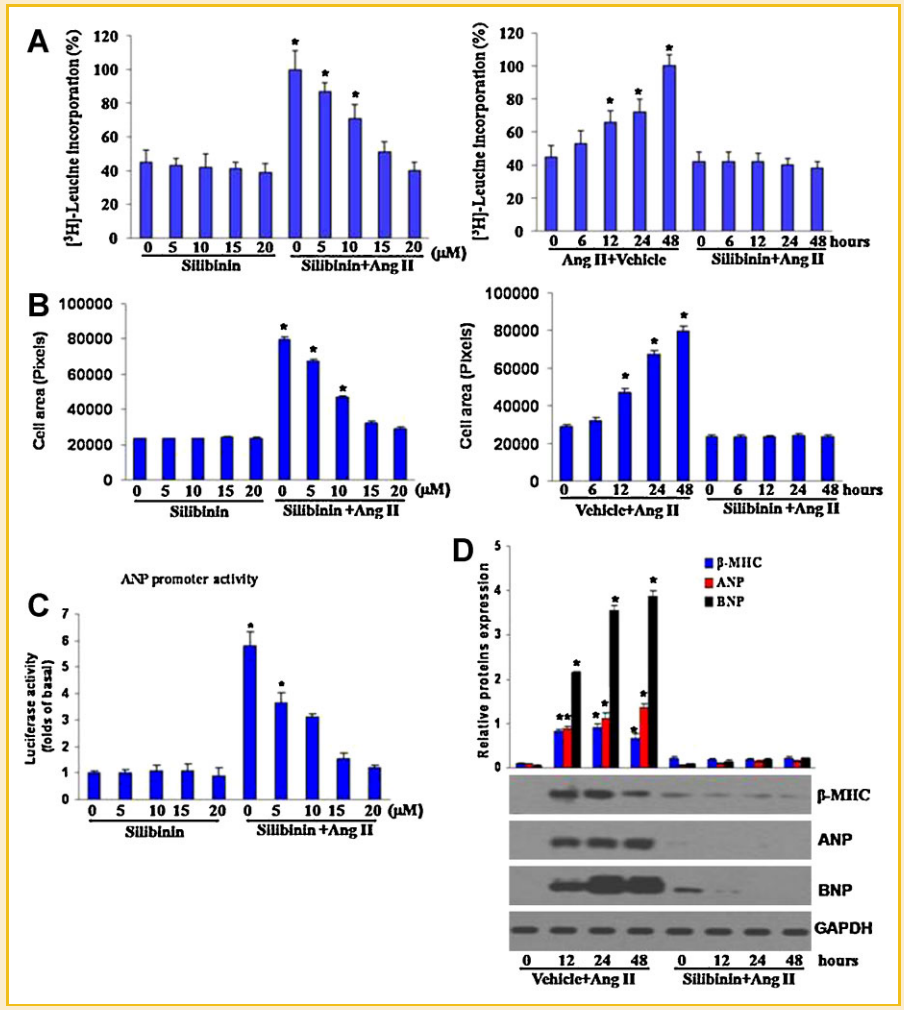

Fig. 1. Silibinin inhibits cardiac hypertrophy in vitro. A: Silibinin inhibited Ang II-induced [^{3}H]-leucine incorporation. Cardiomyocytes were treated with different dose of silibinin for 60 min and then incubated with 1 μM Ang II for 48 h. B: Quantification of cell cross-sectional area by measuring 50 random cells. C: Silibinin blunted Ang II-induced ANP promoter activity. D: Silibinin blocked Ang II-induced β-MHC, ANP, and BNP protein expression levels by Western blot. Cardiac myocytes were incubated with 1 μM Ang II or pretreated with 20 μM silibinin for 60 min and then incubated with 1 μM Ang II for indicated time. $^*P < 0.05$ versus control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Echocardiographic Data Showed the Effects of Silibinin on Cardiac Hypertrophy Induced by Aortic Banding

Parameter	Vehicle-Sham	Silibinin-Sham	Vehicle-AB	Silibinin-AB
Number	n = 13	n = 14	n = 12	n = 13
BW (g)	26.5 ± 1.7	27.7 ± 2.1	27.5 ± 1.6	26.9 ± 1.4
HW/BW (mg/g)	4.75 ± 0.14	4.68 ± 0.15	7.61 ± 0.24*	5.30 ± 0.12**
LW/BW (mg/g)	4.56 ± 0.14	4.63 ± 0.15	7.77 ± 0.31*	5.25 ± 0.15**
CSA (μm^2)	268 ± 25	259 ± 18	439 ± 22*	315 ± 25**
SBP (mmHg)	110.8 ± 2.7	113.5 ± 1.5	157.4 ± 4.1*	147.3 ± 4.5*
HR (beats/min)	469 ± 21	478 ± 18	481 ± 32	468 ± 27
PWT (mm)	1.24 ± 0.01	1.27 ± 0.04	2.44 ± 0.05*	1.79 ± 0.03**
LVEDD (mm)	3.65 ± 0.04	3.59 ± 0.03	5.36 ± 0.04*	4.12 ± 0.05**
LVESD (mm)	2.35 ± 0.03	2.35 ± 0.03	3.36 ± 0.05*	2.57 ± 0.06**
IVSD (mm)	0.61 ± 0.01	0.63 ± 0.02	1.52 ± 0.04*	0.84 ± 0.03**
LVPWD (mm)	0.62 ± 0.02	0.61 ± 0.03	1.31 ± 0.02*	0.82 ± 0.04**
FS (%)	56.2 ± 1.8	55.4 ± 2.9	33.1 ± 2.7*	46.1 ± 1.5***

BW, body weight; HW, heart weight; LW, lung weight; CSA, cardiomyocyte cross-sectional area; SBP, systolic blood pressure; HR, heart rate; PWT, posterior wall thickness; 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.
$^*P < 0.01$ was obtained for the Vehicle-sham values.
$^{**}P < 0.01$ was obtained for the Vehicle-AB values after AB.

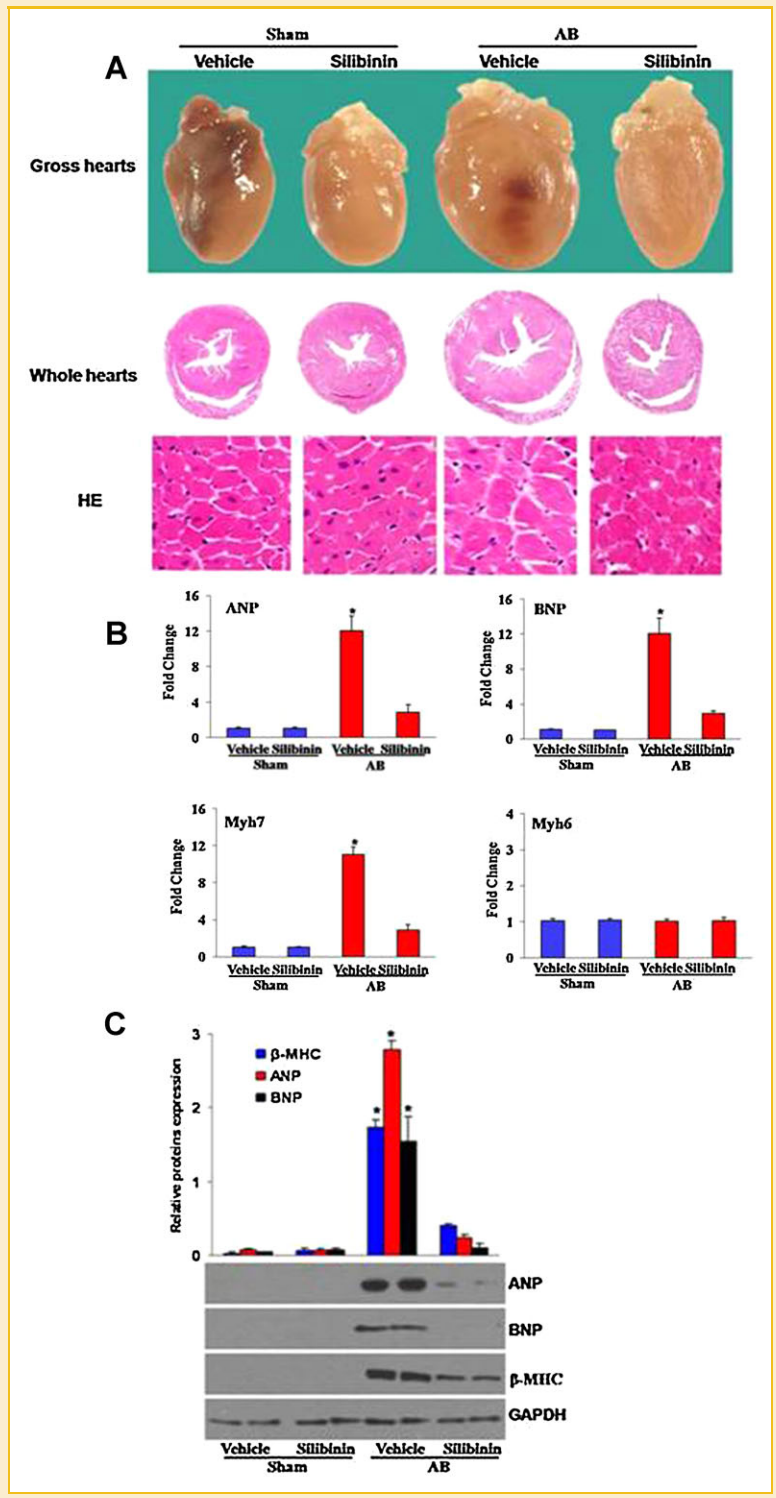

Fig. 2. Silibinin inhibits cardiac hypertrophy in vivo. A: Gross heart and HE staining of mice treated with vehicle or silibinin at 8 weeks after surgery ($n = 6$). B,C: Silibinin prevent the expression of hypertrophic markers induced by AB. Total RNA was isolated from hearts of mice ($n = 4$), and the expression of transcripts for ANP, BNP, Myh7, and Myh6 were determined by real-time PCR (B); β-MHC, ANP, and BNP protein expression were detected by Western blot analysis, GAPDH was used as loading control (C). $^{*}P < 0.05$ versus corresponding control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cardiac hypertrophy markers ANP, BNP, Myh7, and Myh6, we performed real-time PCR and Western blotting analysis. The results demonstrated that silibinin attenuated the observed increase in hypertrophic marker expression caused by AB (Fig. 2B,C). These findings indicate that silibinin prevents the development of cardiac hypertrophy in vivo.

EFFECTS OF SILIBININ ON EGFR TRANSACTIVATION IN VITRO AND IN VIVO

Since the EGFR pathway plays an important role in regulating the development of cardiac hypertrophy [Zhai et al., 2006], we first assessed the effect of silibinin on EGFR transactivation. As shown in Figure 3, significant phosphorylation of Tyr1068 and Tyr1173 in EGFR were observed both in Ang II-treated cardiomyocytes and in AB mice. Importantly, silibinin almost inhibited such EGFR transactivation completely, which was not due to the decrease of total EGFR protein levels.

EFFECTS OF SILIBININ ON MAPKs AND PI3K/Akt/GSK3β SIGNALING IN VITRO AND IN VIVO

Based on the inhibitory effect of silibinin on EGFR transactivation in response to hypertrophic stimuli [Hannay and Yu, 2003; Tyagi et al., 2008], we further assessed its effect on the downstream signaling targets such as MAPK and PI3K/Akt/GSK3β pathways. Our data showed that ERK1/2, JNK1/2, and p38 were significantly phosphorylated both in Ang II-treated cardiomyocytes and in AB mice. However, the phosphorylation of ERK1/2 was almost completely impaired by silibinin, whereas JNK1/2 and p38 were not significantly affected (Fig. 4A,B). The observed inhibitory effect of silibinin on ERK1/2 activation was not due to the decrease in total ERK1/2 protein levels.

To determine whether silibinin also blocks the PI3K/Akt/GSK3β pathway in response to hypertrophic stimuli, we examined the activation of p85 subunit of PI3K, the key PI3K targets Akt and its downstream GSK3β. 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 such changes were inhibited by treatment with silibinin (Fig. 4C,D). Taken together, our findings imply that both ERK1/2 pathway and PI3K/AKT/GSK3β signaling play important roles in silibinin-mediated inhibitory effects on cardiac hypertrophy.

SILIBININ BLUNTED INFLAMMATORY RESPONSE IN VIVO AND IN VITRO

A growing body of evidence has suggested that inflammation plays an important role in the development of cardiac hypertrophy and progression to heart failure [Li et al., 2007a,b; Hamid et al., 2009]. To determine whether silibinin can suppress the inflammatory responses in the heart, we first examined NF-κB activity. Our data revealed that silibinin significantly decreased NF-κB activation induced by Ang II or AB mice (Fig. 5A,B). To further determine the molecular mechanisms through which silibinin blocks NF-κB activation, we analyzed IκBα phosphorylation and IKKβ activation processing in vivo. Silibinin markedly impaired IκBα phosphorylation and degradation, as well as IKKβ activation mediated by AB (Fig. 5C). We next examined whether silibinin has the ability to inhibit the induction of NF-κB responsive proteins, such as COX-2, Cyclin D1, and MMP-9 (Fig. 5D). The treatment of silibinin resulted in marked inhibition of AB-induced expression of COX-2, CyclinD1, and MMP-9 proteins. We further investigated the effects of silibinin on the expression of inflammatory mediators IL-6, TNF-α, and MCP-1 in cardiac tissue. As shown in Figure 5E, silibinin significantly decreased the levels of IL-6, TNF-α, and MCP-1 mRNA expression compared with vehicle-treated AB mice. These results indicate that silibinin inhibits inflammation through blocking NF-κB signaling in response to chronic pressure overload.

SILIBININ INHIBITED CARDIAC FIBROSIS IN VIVO AND COLLAGEN SYNTHESIS IN VITRO

To explore whether silibinin can regulate fibrosis in cardiac hypertrophy, we determined cardiac fibrosis by PSR staining. Our data showed that the increase of LV collagen volume induced by AB

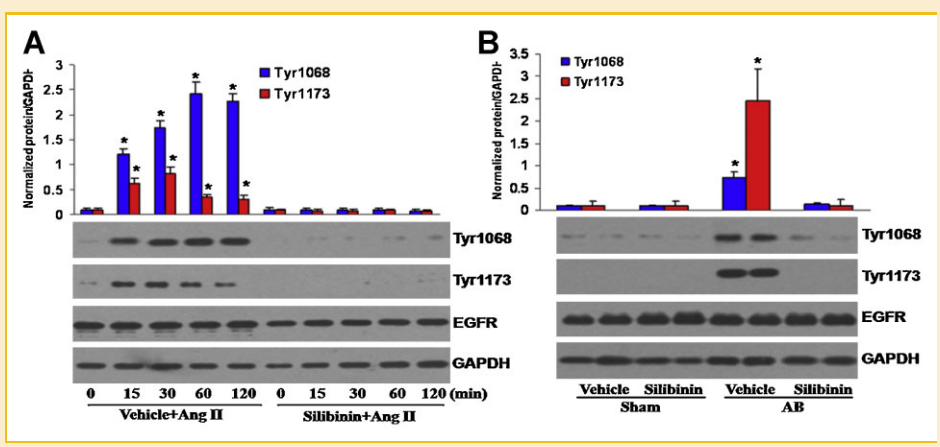

Fig. 3. Effects of silibinin on EGFR transactivation in vitro and in vivo. A: Silibinin inhibited phosphorylation of Tyr1068 and Tyr1173 in EGFR in Ang II-treated cardiomyocytes by Western blot analysis. Cardiac myocytes incubated with Ang II with/without silibinin for indicated times. $^{*}P < 0.05$ compared with silibinin treatment group. B: Silibinin inhibited phosphorylation of Tyr1068 and Tyr1173 in AB mice by Western blot analysis ($n = 4$). $^{*}P < 0.05$ versus corresponding control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

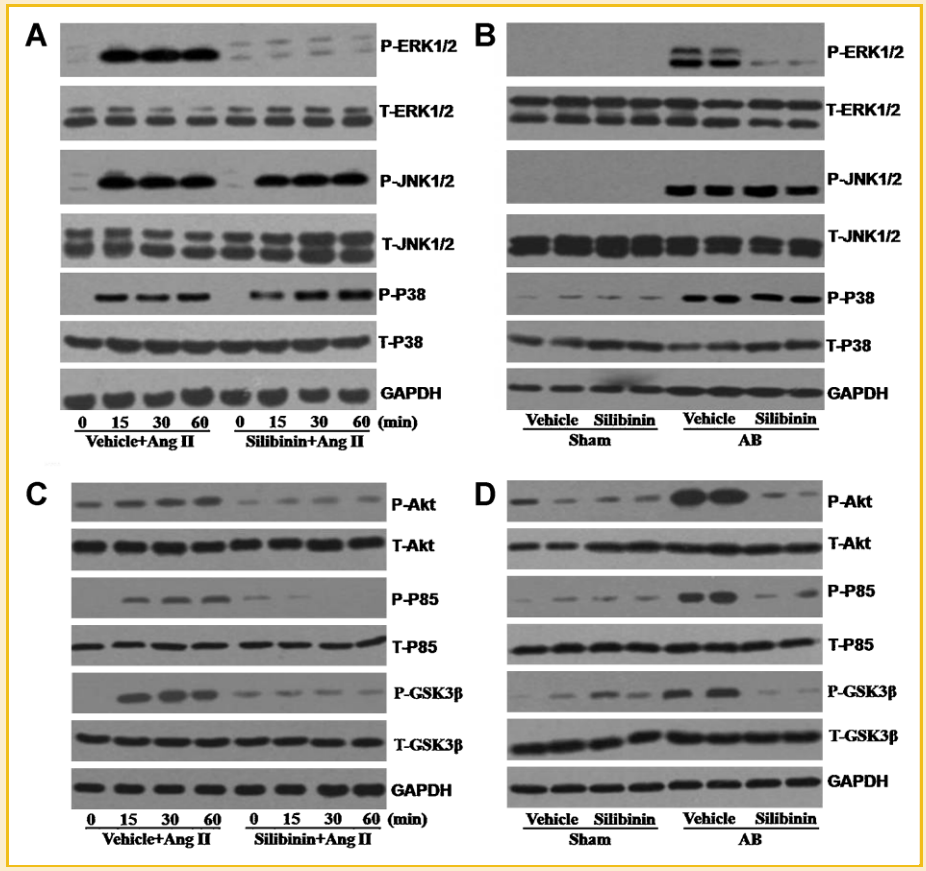

Fig. 4. Effects of silibinin on MAPKs and PI3K/Akt/GSK3β signaling in vitro and in vivo. A,B: The protein expression level of ERK1/2, JNK1/2, and p38 were significantly phosphorylated in Ang II–treated cardiomyocytes (A) and in AB mice (B) (n = 4). ERK1/2 was almost completely impaired by silibinin by Western blot analysis. C,D: Silibinin prevented the protein expression level of p85, Akt, and GSK3β phosphorylated by Western blot analysis in Ang II–treated cardiomyocytes (C) and in AB mice (D) (n = 4).

was remarkably attenuated after treatment with silibinin (Fig. 6A). We further examined the ability of silibinin to inhibit collagen synthesis induced by Ang II in cardiac fibroblasts. Cells were serum-starved for 24 h in 0.5% FCS and then treated with 1 μM Ang II for the indicated time. Ang II significantly increased [^{3}H]-proline incorporation and COL1A1 promoter activity, which was remarkably reduced by silibinin pretreatment in a dose-dependent manner (Fig. 6B). Subsequent analysis of protein expression levels of known mediators of fibrosis including CTGF, Collagen I, and Collagen III, demonstrated a blunted response following silibinin administration compared to the vehicle-treated group (Fig. 6C). To further elucidate the molecular mechanisms through which silibinin inhibits cardiac fibrosis, we examined the effects of silibinin on Smad cascade activation in hearts subjected to AB. Immunoblot analysis demonstrated that silibinin suppressed Smad 2/3/4 phosphorylation (Fig. 6D).

ROLE OF EGFR IN Ang II–INDUCED CARDIAC HYPERTROPHY AS WELL AS ERK1/2, PI3K/AKT, NF-κB, AND SMAD SIGNALING

To identify whether EGFR inhibition was responsible for the protective role of silibinin on cardiac hypertrophy, SU1428, a typical EGFR inhibitor was used. We found that SU1428 almost completely abrogated both [^{3}H]-leucine and [^{3}H]-proline incorporation in response to Ang II (Fig. 7A). To further examine the mechanisms involved, we investigated whether EGFR is involved in Ang II-induced activation of ERK1/2, PI3K/AKT, NF-κB, and Smad signaling. As shown in Figure 7B,C, blocking EGFR activity by SU1428 remarkably attenuated Ang II-induced activation of ERK1/2, AKT, P85, IκBα, IKKβ, Smad2, Smad3, and Smad4. These results clearly indicate that silibinin blocks cardiac hypertrophy by disrupting EGFR-dependent ERK1/2, PI3K/AKT, NF-κB, and Smad2/3/4 signaling pathways.

DISCUSSION

The present study demonstrates for the first time that silibinin protects against cardiac hypertrophy induced by Ang II in vitro and pressure-overload induced cardiac hypertrophy in vivo. The cardioprotection of silibinin is mediated by direct interruption of EGFR-dependent ERK1/2, PI3K/AKT, NF-κB, and Smad2/3/4 signaling, which leads to the inhibition of cardiomyocyte growth, inflammation, and fibrosis and ultimately preventing the progress of cardiac hypertrophy. These findings suggest that silibinin could be an effective therapeutic candidate against cardiac hypertrophy and progression to heart failure.

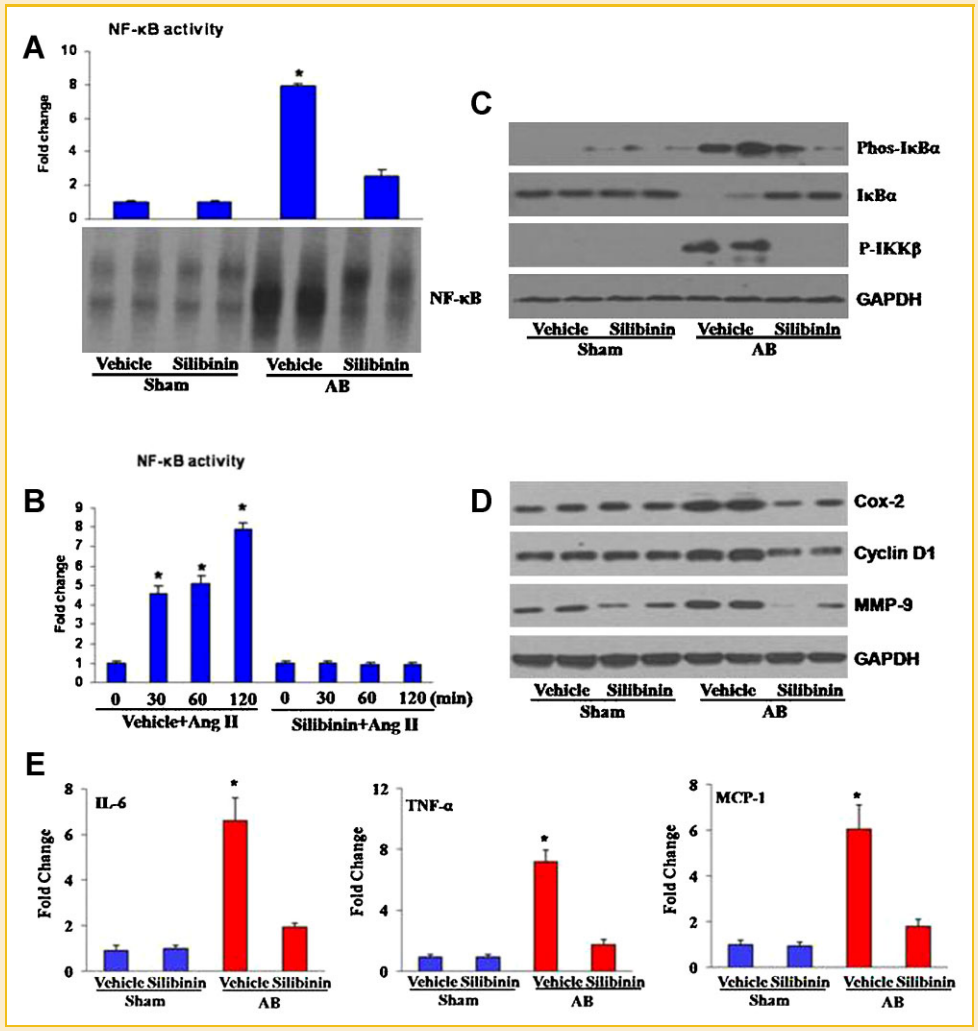

Fig. 5. Silibinin blunted inflammatory response in vivo and in vitro. A: Silibinin prevented NF-κB activation induced by AB, the representative electrophoretic mobility shift assay of NF-κB activity in myocardium decreased significantly in silibinin treatment group (n = 5). B: Silibinin prevented NF-κB activation induced by Ang II-treated cardiomyocytes. C: Western blot analysis of total IκBα and phosphorylation in IκBα and IKK-β of myocardium obtained from four groups of animals (n = 4). D: Silibinin inhibited expression of COX-2, CyclinD1, and MMP-9 proteins by AB-induced (n = 5). E: Silibinin decreased the levels of IL-6, TNF-α, and MCP-1 mRNA expression in AB mice compare to sham group (n = 4). $^{*}P < 0.05$ versus corresponding control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Recently, silibinin has been shown to protect against isoproterenol-induced rat cardiac myocyte injury [Zhou et al., 2006]. However, the effect of silibinin on cardiac hypertrophy has not yet been clarified. In this study, we found that silibinin not only attenuated cardiac hypertrophy in vitro and in vivo in response to hypertrophic stimuli, but also improved cardiac performance and reduced chamber dimensions. However, silibinin treatment did not affect blood pressure which increased significantly in AB mice. This indicates that the primary target of silibinin action is cardiac protection, rather than lowering blood pressure. An understanding of pharmacokinetics is critical for defining the pharmacological and toxicological profile of silibinin. There have some reports to show the pharmacokinetics of silibinin [Zhao and Agarwal, 1999; Singh et al., 2002]. Our pharmacokinetic results from current study demonstrated that the peak plasma and heart concentrations are 14.84 and 17.67 μM, respectively. They were achieved at 30 min after oral treatment of 50 mg/kg/day consumption of silibinin. These results were close to Agarwal group's results that the peak plasma concentration of silibinin was 25 μM and achieved at 30 min after oral consumption of 100 mg/kg silibinin in mice [Singh et al., 2002]. However, in our in vitro experiments, the effective dose of silibinin is from 5 μM and the maximum effect is 20 μM. Therefore, the physiological dose of silibinin in animal study can be effective against cardiac hypertrophy in vitro.

The mechanism by which silibinin exerts an anti-hypertrophic effect remains largely unclear. The EGFR pathway activation has been shown to contribute to the development of cardiac hypertrophy [Zhai et al., 2006; Zhou et al., 2009]. Importantly, previous studies showed silibinin could inhibit EGFR activation in various tumor cells [Ramasamy and Agarwal, 2008]. Therefore, the inhibitory mechanisms of silibinin on cardiac hypertrophy were examined for its effect on EGFR activation. We found that silibinin blocked EGFR

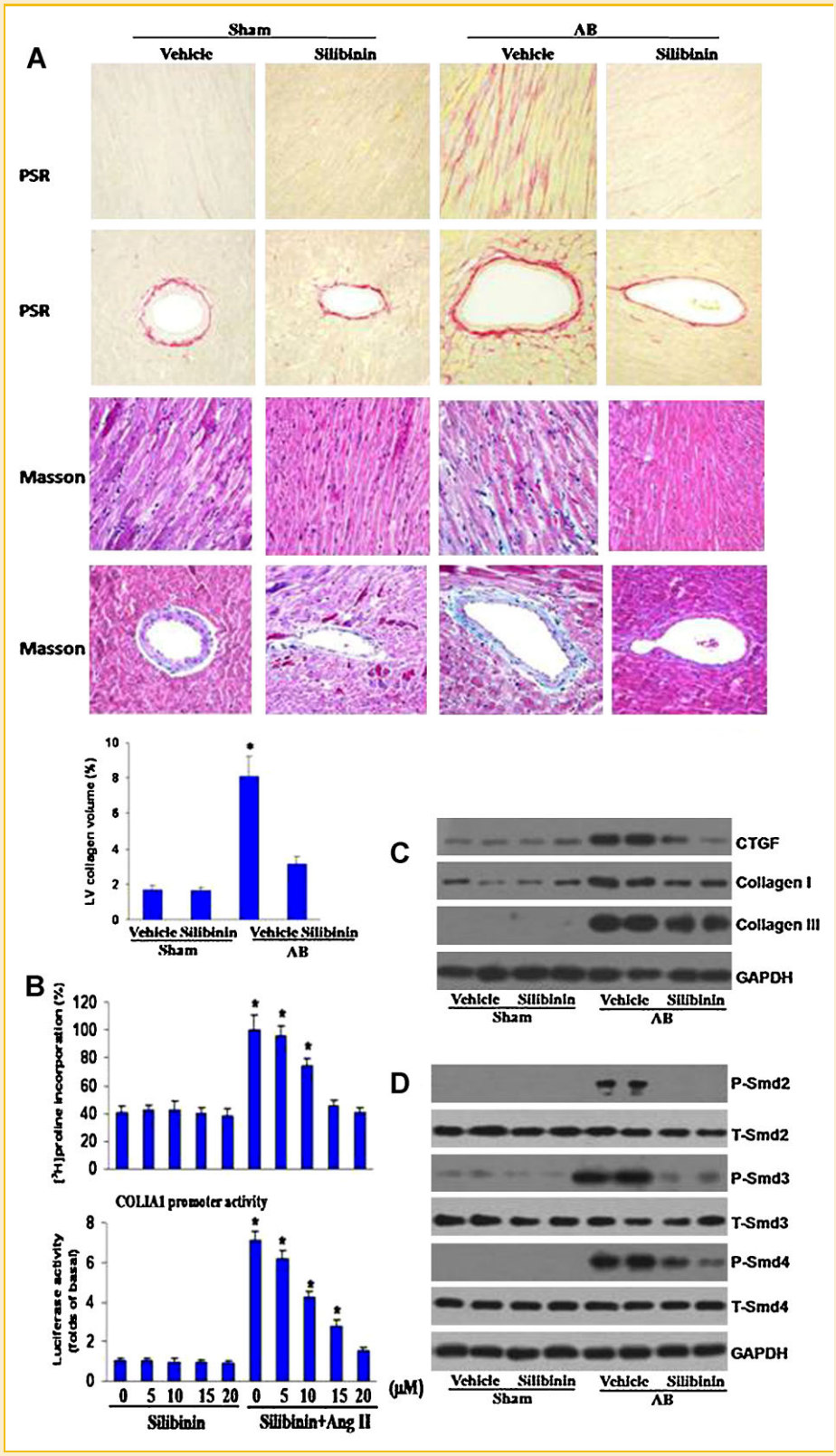

Fig. 6. Silibinin inhibited cardiac fibrosis in vivo and collagen synthesis in vitro. A: Silibinin attenuated the increase in LV collagen volume induced by AB, analyzed by PSR in four groups of animals ($n = 4$). B: [^{3}H]-proline incorporation and COL1A1 promoter activity induced by Ang II were suppressed by silibinin treatment in a dose-dependent manner in indicated time. C: Western blot analysis of CTGF, Collagen I, and Collagen III of myocardium obtained from four groups of animals ($n = 4$). D: Western blot analysis of total and phosphorylation Smad2, Smad3, and Smad4 in four groups of animals ($n = 4$). $^*P < 0.05$ versus corresponding control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

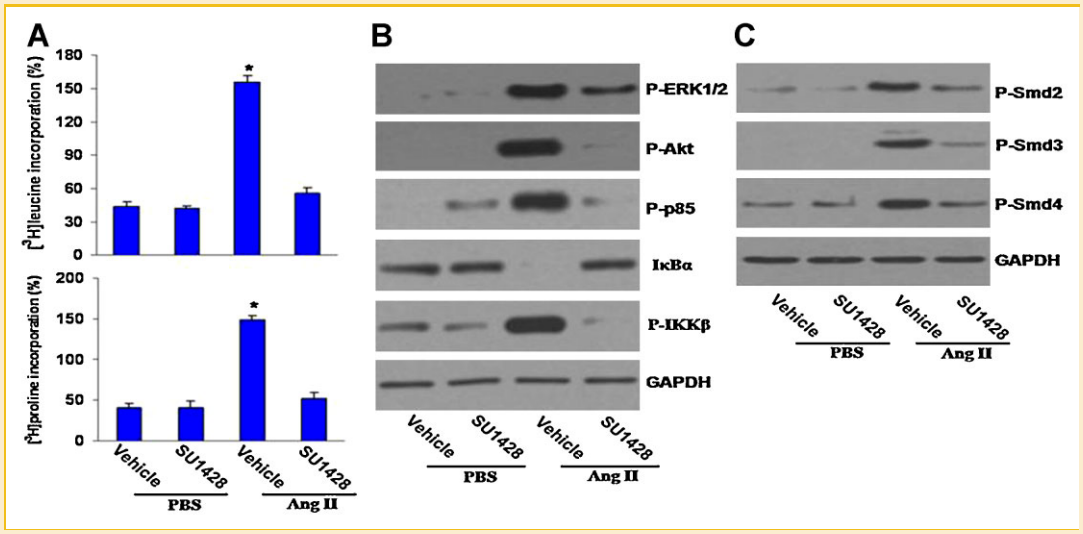

Fig. 7. Role of EGFR in Ang II-induced cardiac hypertrophy and collagen synthesis as well as ERK1/2, PI3K/AKT, NF-κB, and Smad signaling. A: SU1428, an inhibitor of EGFR, decreased both [^{3}H]-leucine and [^{3}H]-proline incorporation in response to Ang II-induced protein and collagen synthesis in vitro. Cardiomyocytes were treated with SU1428 or vehicle for 60 min and then incubated with 1 μM Ang II or PBS for another 48 h. [^{3}H]-leucine and [^{3}H]-proline incorporation were measured as described in the Methods Section. B,C: Western blot analysis of the effect of SU1428 on Ang II-induced activation of ERK1/2, AKT, P85, IκBα, IKKβ (B), Smad2, Smad3, and Smad4 (C). Cardiomyocytes were treated with SU1428 or vehicle for 60 min and then incubated with 1 μM Ang II or PBS for another 60 min. Western blot was used to examine the phosphorylation and total of ERK1/2, AKT, P85, IκBα, IKKβ as well as Smad2, Smad3, and Smad4. $^*P < 0.01$ was obtained for PBS/control value. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

transactivation induced by Ang II or pressure overload. These results suggest that such effect may be involved in the cardioprotective effect of silibinin on cardiac hypertrophy. Using SU1428, an EGFR inhibitor, we further demonstrated that EGFR inhibition attenuated Ang II-induced [^{3}H]-leucine incorporation in cardiac myocytes, confirming a necessary role for EGFR in cardiac hypertrophy. Taken together, these results indicate that the anti-hypertrophic effect of silibinin can be in part ascribed to blocking EGFR transactivation.

It is well known that EGFR transmits signals to prominent downstream pathways, such as MAPKs and PI3K/Akt pathways [Kagiyama et al., 2003; Liu et al., 2007]. Among the MAPKs, ERK1/2 has been considered as the essential regulator of a hypertrophic response, although JNK and p38 were recently examined in regulating cardiac hypertrophy [Bueno and Molkentin, 2002; Duquesnes et al., 2009]. Our data clearly demonstrated that ERK1/2, JNK1/2, and p38 were all significantly phosphorylated both in Ang II-treated cardiomyocytes and in AB mice. Importantly, silibinin markedly blocked only ERK1/2 activation in vivo and in vitro in response to hypertrophic stimuli, without affecting the activation of JNK1/2 and p38. We further found that ERK1/2 activation induced by Ang II in cardiomyocyts was EGFR dependent. These findings confirmed ERK1/2 as a pivotal regulator of cardiac hypertrophy and revealed that the anti-hypertrophic effect of silibinin is achieved through blocking EGFR-dependent ERK1/2 signaling. In accordance with our findings, Li et al. reported that silibinin effectively inhibits the renal cancer carcinoma Caki-1 cell proliferation through inhibiting the activation of EGFR-ERK1/2 pathways but not through inhibition of the activation of JNK1/2 and p38 pathways [Li et al., 2008]. Singh et al. also demonstrated that silibinin treatment strongly inhibited UVB-caused phosphorylation of ERK1/2, without any substantial response on JNK1/2 and p38 in mouse epidermal JB6 cells [Singh et al., 2006]. However, inconsistent with our results, they showed silibinin treatment under similar conditions strongly inhibited EGF-induced ERK1/2, JNK1/2, and p38 phosphorylation. In other study, silibinin treatment caused an inhibition of EGFR activation followed by that of ERK1/2 in both androgen-dependent (LNCaP) and -independent (DU145) advanced human prostate carcinoma cells. Meanwhile, silibinin caused strong inhibition of JNK1/2 activation in LNCaP cells and a strong induction in JNK1/2 activation was observed in DU145 cells [Tyagi et al., 2008]. The discrepancy on MAPKs activation may be explained by different stimuli and different cell types.

In addition, a similar profile was observed with respect to PI3K/Akt pathway. Silibinin blocked the activation of p85 and Akt in response to hypertrophic stimuli, which was dependent on EGFR activation. Our results are in agreement of the previous study that silibinin inhibit cancer cells growth partly through inhibition of Akt phosphorylation [Ramasamy and Agarwal, 2008; Garcia-Maceira and Mateo, 2009; Rajamanickam et al., 2009]. Our data imply that EGFR-dependent PI3K/Akt pathway is also implicated in silibinin-mediated inhibitory effects on cardiac hypertrophy.

Downstream targets of MAPKs and PI3K/Akt include transcription factors such as NF-κB and Smad, which have been shown to regulate inflammation and fibrosis, respectively [Liehn et al., 2006; Verheyen, 2007; Bian et al., 2009]. Mounting evidence has strongly suggested that inflammation and fibrosis play a key role in the development of cardiac hypertrophy and heart failure [Tokuda et al., 2004; Turner et al., 2007; Li et al., 2007a,b; Hamid et al., 2009]. Silibinin has been shown to suppress UVB- or EGF-induced NF-κB activation in mouse epidermal JB6 cells and significantly reduce liver fibrosis in patients with chronic hepatitis [Singh et al., 2006; Ferenci et al., 2008; Tyagi et al., 2008]. Therefore, we investigated

whether silibinin possesses anti-inflammatory and anti-fibrotic properties in the hypertrophic hearts. Our results showed that silibinin significantly abrogated NF-κB activation through blocking the phosphorylation and degradation of IκBα, IKKβ activation as well as inhibited the expression of NF-κB responsive proteins and inflammatory mediators (IL-6, TNF-α, and MCP-1). Similarly, silibinin attenuated cardiac fibrosis characterized by decreased LV collagen volume and expression levels of known mediators of fibrosis (CTGF, Collagen I, and Collagen III) in vivo as well as diminished [^{3}H]-proline incorporation and COL1A1 promoter activity in vitro. We further demonstrated that silibinin suppressed Smad2/3/4 phosphorylation in hypertrophied hearts, indicating a mechanism underlying the inhibitory effect of silibinin on fibrosis. More importantly, we found that blocking EGFR activation led to inhibition of phosphorylation of IκBα, IKKβ, Smad2, Smad3, and Smad4. Collectively, these results suggest that silibinin blocks EGFR-dependent NF-κB and Smad signaling and subsequently attenuates cardiac inflammation and fibrosis, which eventually preventing the progress of cardiac hypertrophy.

In conclusion, we demonstrate for the first time that silibinin inhibits cardiac hypertrophy in vitro and in vivo by blocking EGFR-dependent hypertrophy, inflammation, and fibrosis. We further confirm that EGFR-dependent ERK1/2, PI3K/Akt, NF-κB, and TGF-β1/Smad signaling pathways are targets of silibinin's inhibitory actions. This study serves to elucidate the inhibitory effect of silibinin on cardiac hypertrophy and related molecular mechanisms. More importantly, our results provide experimental evidence for the application of silibinin in the treatment of cardiac hypertrophy and heart failure. Indeed, future clinical trials are required to prove the new potential clinical use of silibinin.

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