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Breviscapine Protects Against Cardiac Hypertrophy Through Blocking PKC-α-Dependent Signaling

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ABSTRACT

Breviscapine is a mixture of flavonoid glycosides extracted from the Chinese herbs. Previous studies have shown that breviscapine possesses comprehensive pharmacological functions. However, very little is known about whether breviscapine have protective role on cardiac hypertrophy. The aim of the present study was to determine whether breviscapine attenuates cardiac hypertrophy induced by angiotensin II (Ang II) in cultured neonatal rat cardiac myocytes in vitro and pressure-overload-induced cardiac hypertrophy in mice in vivo. Our data demonstrated that breviscapine (2.5–15 μM) dose-dependently blocked cardiac hypertrophy induced by Ang II (1 μM) in vitro. The results further revealed that breviscapine (50 mg/kg/day) prevented cardiac hypertrophy induced by aortic banding as assessed by heart weight/body weight and lung weight/body weight ratios, echocardiographic parameters, and gene expression of hypertrophic markers. The inhibitory effect of breviscapine on cardiac hypertrophy is mediated by disrupting PKC-α-dependent ERK1/2 and PI3K/AKT signaling. Further studies showed that breviscapine inhibited inflammation by blocking NF-κB signaling, and attenuated fibrosis and collagen synthesis through abrogating Smad2/3 signaling. Therefore, these findings indicate that breviscapine, which is a potentially safe and inexpensive therapy for clinical use, has protective potential in targeting cardiac hypertrophy and fibrosis through suppression of PKC-α-dependent signaling. J. Cell. Biochem. 109: 1158–1171, 2010.

KEY WORDS: BREVISCAPINE; CARDIAC HYPERTROPHY; PKC-α; SMAD

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. It may initially be compensatory, however, sustained pathologic hypertrophy is deleterious and frequently decompensates into congestive heart failure [Heineke and Molkentin, 2006; Berk et al., 2007]. A growing body of evidence indicates that cardiac renin–angiotensin system is involved as mediator of cardiac hypertrophy in response to pressure-overload [Kagiyama et al., 2002; Reudelhuber et al., 2007; Kumar et al., 2008]. It is well known that angiotensin II (Ang II), the major bioactive peptide of the renin–angiotensin system, plays critical role on cardiac intracellular signaling cascades in Ang II-induced pathological cardiac hypertrophy [Dorn and Force, 2005]. These signaling pathways include the PKC pathway [Jalili et al., 1999], the mitogen-activated protein kinases (MAPK) pathway [Proud, 2004], and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [Shiojima and Walsh, 2006], which directly modify transcriptional regulatory factors promoting alterations in cardiac gene expression and result in cardiac hypertrophy [Rouet-Benzineb et al., 2000; Fleegal and Sumners, 2003]. Therefore, pharmacological interventions of these signaling pathways may provide promising approaches for preventing cardiac hypertrophy and progression to heart failure.

Breviscapine is a mixture of flavonoid glycosides extracted from the Chinese herb *Erigeron breviscapus* (Vant) Hand Mazz, the primary active ingredient of which is scutellarin. The use of preparations of breviscapine administered by injection has been extensively used clinically for treatment of ischemic cardiovascular

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and cerebrovascular diseases, such as angina pectoris, myocardial infarction, and focal cerebral infarction in China since the 1970s [Cao et al., 2008]. Previous studies have shown that breviscapine possesses comprehensive pharmacological functions. Breviscapine has been demonstrated to be a calcium channel blocker and a PKC inhibitor [Zhang et al., 2007; Zhu et al., 2008]. It can dilate vessels, inhibit platelet aggregation, reduce blood viscosity, and improve microcirculation [Zhou et al., 1992; Pan et al., 2008]. It is also a potent antioxidant and free radical scavenger [Li et al., 2004; Xiong et al., 2006]. Another important function of breviscapine is a strong anti-inflammatory activity [Luo et al., 2008]. In addition, Wu et al. [2007] demonstrated that treatment with breviscapine significantly reduced liver fibrosis in streptozotocin-induced diabetic rats. Importantly, the curative dosage of breviscapine (0.5 mg/kg, i.v.) in clinic is safe, without liver or kidney toxicity. Scutellarin monomer, as the active component in breviscapine compound, has been shown to have an anti-myocardial ischemia effect, and protect against cardiomyocyte apoptosis in MI rats [Lin et al., 2007]. Moreover, scutellarin has been demonstrated to reverse ventricular remodeling in spontaneously hypertensive rats [Zhou et al., 2002]. However, the effects of breviscapine on cardiac hypertrophy and the related signaling mechanisms still remain unclear. Although evidence demonstrates that breviscapine is a strong non-competitive PKC inhibitor, very little is known about whether this regulatory effect is related to a protective role in cardiac hypertrophy. Therefore, we aimed to determine whether breviscapine attenuates cardiac hypertrophy induced by Ang II in cultured neonatal rat cardiac myocytes in vitro and pressure-overload-induced cardiac hypertrophy in mice in vivo by interfering with PKC-dependent pathways.

MATERIALS AND METHODS

MATERIALS

The antibodies used to recognize total and phosphorylation of ERK1/2, P38, JNK1/2, P85, AKT, GSK3β, PKCα, PKCβI, PKCβII, and PKCε as well as phospho-Smad2/3, phospho-IKKβ, phospho-IκBα and IκBα were purchased from Cell Signaling Technology. [^{3}H]-Leucine and [^{3}H]-proline were purchased from Amersham. The BCA protein assay kit was purchased from Pierce and the IKK activity kit was obtained from B&D Bioscience. All other antibodies were purchased from Santa Cruz Biotechnology. Fetal calf serum (FCS) was obtained from Hyclone. COL1A1-luc report constructs was described previously [Bian et al., 2009]. Breviscapine was obtained from Sigma and all other reagents were from Sigma. Breviscapine was dissolved in dimethylsulfoxide (DMSO) medium for all in vitro studies.

CULTURED NEONATAL RAT CARDIAC MYOCYTES AND FIBROBLASTS

Primary cultures of cardiac myocytes were prepared as described previously [Li et al., 2007a]. 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, breviscapine alone, or breviscapine 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 [Li et al., 2006]. Briefly, hearts obtained from neonatal rats were enzyme-digested as described above for myocytes. 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 greater than 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 myocytes 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 [Li et al., 2007a]. Briefly, cardiac myocytes were pretreated with breviscapine 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 breviscapine 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% TCA 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.

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

Cardiac myocytes or fibroblasts were seeded in triplicate in six-well plates. Cells were transfected with 0.5 μg of luciferase reporter constructs using 10 μl of LipofectAMINE reagent (Invitrogen), according to the manufacturer's instructions. To normalize for transfection efficiency, we added 0.1 μg of pRL-TK Renilla luciferase reporter plasmid to each transfection. Firefly luciferase activities were normalized on the basis of Renilla luciferase activities. 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 breviscapine for 60 min and then treated with Ang II. Cells were harvested using passive lysis buffer (Promega) according to the manufacturer's protocol. 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 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, Madison, WI). Synthetic, double-stranded oligonucleotide for NF-κB is 5′-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCA-GGGAGGCGTGG-3′). Proteins were isolated as described previously [Li et al., 2006]. Protein concentrations were measured by BCA protein assay reagents (PIERCE, Rockford, IL) 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 C57/B6 mice (8- to 10-week old) were used in the current study. Aortic banding (AB) was performed as described previously [Tang et al., 2009]. Doppler analysis was performed to ensure that physiologic constriction of the aorta was induced. Breviscapine 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 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 breviscapine- 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) inserted into the right carotid artery and advanced into the left ventricle for hemodynamic measurements.

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 & eosin (H&E) for histopathology or picrosirius red (PSR) for collagen deposition and then visualized by light microscopy. For myocyte cross-sectional area, sections were stained with H&E. A single myocyte was measured with an image quantitative digital analysis system (Image Pro-Plus 4.5). The outline of 100–200 myocytes was traced in each section.

STATISTICAL ANALYSIS

Data are expressed as means ± 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

BREVISCAPINE INHIBITED CARDIAC HYPERTROPHY IN VITRO

In this study, breviscapine was dissolved in DMSO medium for the in vitro studies. To rule out the possibility of cytotoxicity, we determined the number of viable cells using MTT assay. When 2.5–15 μM breviscapine was applied to cultured neonatal cardiomyocytes alone or with Ang II, cells were observed to be healthy even in the presence of 15 μM breviscapine at the end of 48 h (Fig. 1A). There were also no observable adverse effects by the DMSO alone or with Ang II on cellular viability for any of the treatment conditions.

Cardiac hypertrophy is characterized by increased protein synthesis and myocardial cell size, as well as reactivation of fetal gene program. To evaluate the effects of breviscapine on cardiac hypertrophy, cardiac myocytes were pretreated with breviscapine at the indicated concentrations for 60 min and subsequently treated with Ang II for 48 h. We found that breviscapine decreased [^{3}H]-Leucine incorporation induced by Ang II in a dose-dependent manner, which showed maximal effects at 15 μM (Fig. 1B). We further investigated the inhibitory effect of breviscapine on cardiac myocyte size. The increase in cardiac myocyte size seen after 48 h of culture in the presence of Ang II was significantly attenuated after treatment with breviscapine (Fig. 1C). In addition, breviscapine markedly reduced atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) promoter activities (Fig. 1D) as well as β-MHC, ANP, and BNP protein expression levels induced by Ang II (Fig. 1E,F). The inhibition of cardiac hypertrophy in vitro by breviscapine was sustained for all tested times. However, breviscapine alone had no significant difference compared with control in [^{3}H]-Leucine incorporation, cardiac myocyte size or expression of β-MHC, ANP, and BNP. These data clearly demonstrate that breviscapine inhibits cardiac hypertrophy in vitro.

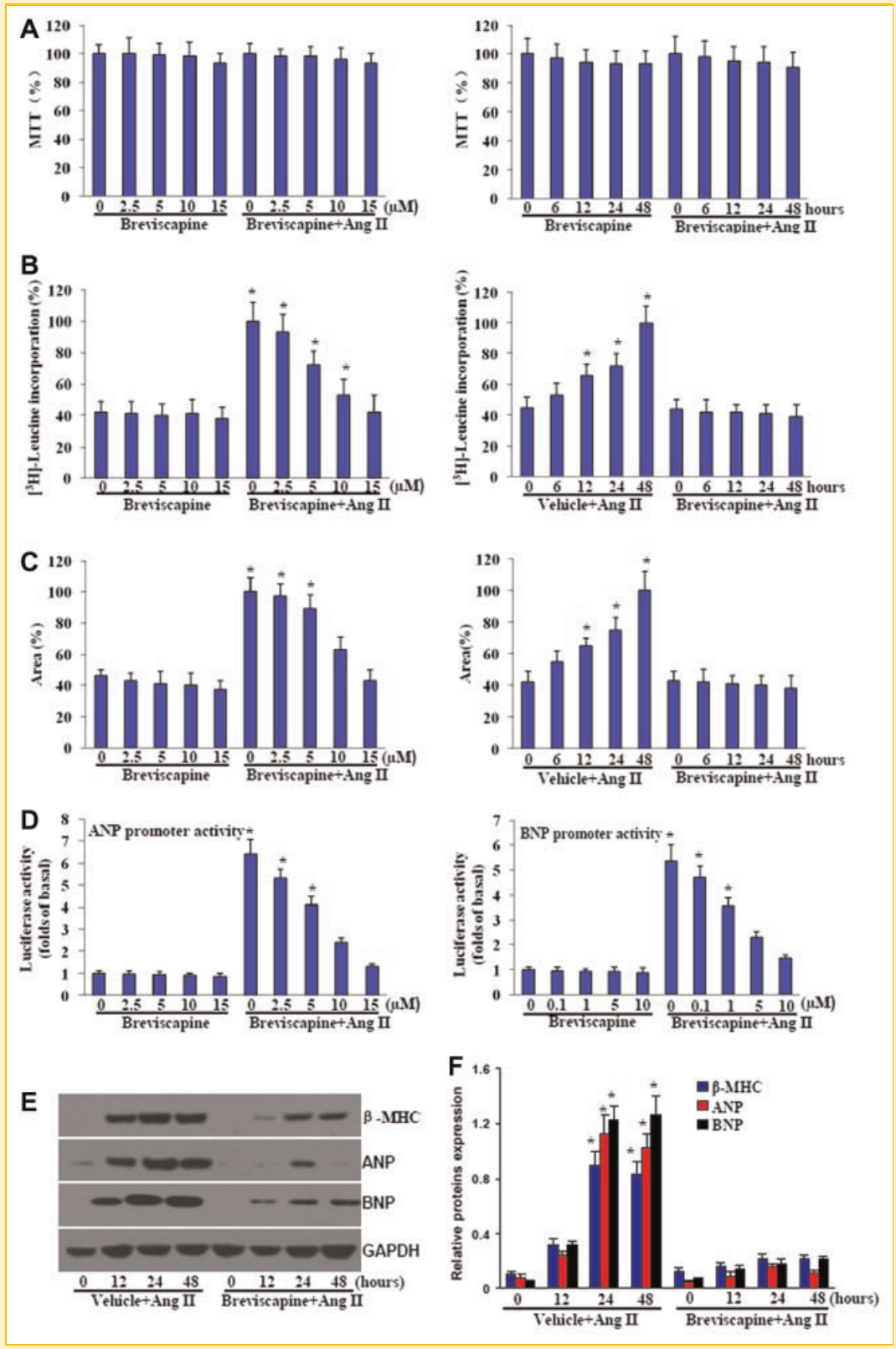

Fig. 1. Breviscapine inhibited cardiac hypertrophy in vitro. A: Effect of breviscapine and/or Ang II on cell viability in cardiomyocytes ($n = 6$). B: Breviscapine blocked Ang II-induced [^{3}H]-Leucine incorporation ($n = 6$). C: Quantification of cell cross-sectional area by measuring 100 random cells. D: Breviscapine inhibited Ang II-induced ANP and BNP promoter activities ($n = 3$). E,F: Breviscapine inhibited β-MHC, ANP, and BNP protein expression levels induced by Ang II ($n = 4$). Cardiomyocytes were incubated with different doses of breviscapine (2.5–15 μM) for 48 h or pretreated with 10 μM breviscapine for 60 min, and then incubated with 1 μM Ang II for indicated time. Cell viability, [^{3}H]-Leucine incorporation, Reporter assay and Western blot were measured as described in "Materials and Methods." The results were reproducible in three separate experiments. $^*P < 0.05$ versus exposed to control.

TABLE I. Echocardiographic Data Showed the Effects of Breviscapine on Cardiac Hypertrophy Induced By Aortic Banding

Parameter	Vehicle-Sham	Breviscapine-Sham	Vehicle-AB	Breviscapine-AB
Number (n)	13	12	11	10
BW (g)	25.3 ± 1.3	26.6 ± 1.5	26.1 ± 1.6	26.7 ± 1.3
HW/BW (mg/g)	4.49 ± 0.32	4.63 ± 0.15	7.83 ± 0.17*	5.67 ± 0.21**
LW/BW (mg/g)	4.57 ± 0.15	4.64 ± 0.17	5.89 ± 0.14*	4.95 ± 0.13**
CSA (μm^2)	267 ± 31	274 ± 38	442 ± 35*	336 ± 35**
SBP (mmHg)	112.3 ± 3.8	115.8 ± 3.6	155.5 ± 3.8*	142.0 ± 4.5*
HR (beats/min)	472 ± 35	479 ± 38	456 ± 30	478 ± 26
PWT (mm)	1.22 ± 0.03	1.18 ± 0.02	2.35 ± 0.05*	1.73 ± 0.05**
LVEDD (mm)	3.67 ± 0.03	3.61 ± 0.04	5.23 ± 0.05*	4.35 ± 0.04**
LVESD (mm)	2.38 ± 0.03	2.36 ± 0.04	3.61 ± 0.04*	2.67 ± 0.05**
LVSD (mm)	0.65 ± 0.03	0.61 ± 0.04	1.43 ± 0.03*	0.79 ± 0.05**
LVPWD (mm)	0.61 ± 0.05	0.60 ± 0.06	1.34 ± 0.05*	0.92 ± 0.01**
FS (%)	55.1 ± 4.6	54.9 ± 2.7	33.1 ± 1.7*	43.6 ± 1.2*,**

CSA, cross-sectional area; SBP, systolic blood pressure; HR, heart rate; BW, body weight; HW, heart weight; LW, lung weight; PWT, posterior wall thickness; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVSD, 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.

BREVISCAPINE INHIBITED CARDIAC HYPERTROPHY IN VIVO

To determine whether our in vitro findings have any physiological relevance, we investigated the effects of breviscapine in a murine pressure-overload model of cardiac hypertrophy in vivo. Mice were randomly assigned to four groups: pretreatment with vehicle or 50 mg/kg/day of breviscapine for 1 week prior to AB surgery or sham operation. As shown in Table I, breviscapine pretreatment significantly decreased HW/BW and LW/BW ratios, and cardiomyocyte cross-sectional area in aortic-banded mice. Furthermore, treatment with breviscapine prevented ventricular dysfunction, as evidenced by improvements in LVESD, LVEDD, and percent fractional shortening (FS; Fig. 2A). No significant changes were observed in the sham-operated mice treated with breviscapine or vehicle. Gross hearts, whole hearts, and H&E staining further confirmed the inhibitory effect of breviscapine on cardiac hypertrophy in AB mice (Fig. 2B). To determine whether breviscapine affected the expression of cardiac hypertrophy markers β-MHC, ANP and BNP, we performed real-time PCR and Western blotting analysis. The results demonstrated that breviscapine attenuated the observed increase in hypertrophic marker expression caused by AB (Fig. 2C–E). These findings indicate that breviscapine prevents the development of cardiac hypertrophy in vivo.

BREVISCAPINE DISRUPTED ERK1/2 AND PI3K/AKT/GSK3β SIGNALING PATHWAYS

MAPKs and PI3K/AKT signaling pathways are known to be involved in the regulation of cardiac hypertrophy induced by various types of hypertrophic stimuli [Epstein and Parmacek, 2005; Li et al., 2005]. To clarify the effects of breviscapine on MAPKs activation, the phosphorylation of individual MAPKs was detected. We found 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 breviscapine, whereas JNK1/2 and p38 were not significantly affected (Fig. 3A,B). The observed inhibitory effect of breviscapine on ERK1/2 activation was not due to the decrease in total ERK1/2 protein levels.

To determine whether breviscapine also blocks the PI3K pathway in response to hypertrophic stimuli, we examined the activation of p85 subunit of PI3K, the key PI3K targets Akt and its downstream GSK3β. As expected, Ang II induced a significant increase in the phosphorylated levels of P85, AKT, and GSK3β (Ser9) in cardiomyocytes. However, breviscapine pretreatment markedly blocked the activation of P85, AKT, and GSK3β and sustained for all tested time points (Fig. 3C). Consistent with these in vitro data, strongly hyperphosphorylated P85, AKT, and GSK3β were found in AB mice and such changes were inhibited by treatment with breviscapine (Fig. 3D). Taken together, our findings imply that breviscapine-mediated inhibitory effects on cardiac hypertrophy are mediated by disrupting both ERK1/2 pathway and PI3K/AKT/GSK3β signaling pathways.

BREVISCAPINE INHIBITED PKC-α SIGNALING IN RESPONSE TO HYPERTROPHIC STIMULI

PKC has been shown to activate MAPKs and PI3K cascades in Ang II-treated cardiac myocytes [Schonwasser et al., 1998; Li et al., 2005]. Thus, we investigated the possible involvement of PKC in the cardioprotective activity of breviscapine against cardiac hypertrophy induced by Ang II or pressure-overload. Activation of various PKC isoforms were examined by immunoblot analysis using antibodies specific for the phospho forms of PKC-α, PKC-βI, PKC-βII, and PKC-ε. As shown in Figure 4A, PKC-α, PKC-βI, PKC-βII, and PKC-ε were significantly phosphorylated in AB mice. However, the activation of PKC-α was almost completely impaired by breviscapine, but not other PKC isoforms. In order to further test this, we exposed cultured neonatal rat cardiac myocytes to 1 μM Ang II with or without breviscapine. Breviscapine treatment markedly abolished Ang II-induced phosphorylation of PKC-α and sustained for all tested points in time (Fig. 4B). These data suggest that the cardioprotective effect of breviscapine against cardiac hypertrophy may be associated with selectively inhibition of PKC-α isoform activation.

BREVISCAPINE 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 [Nian et al., 2004; Li et al., 2007a,b]. To

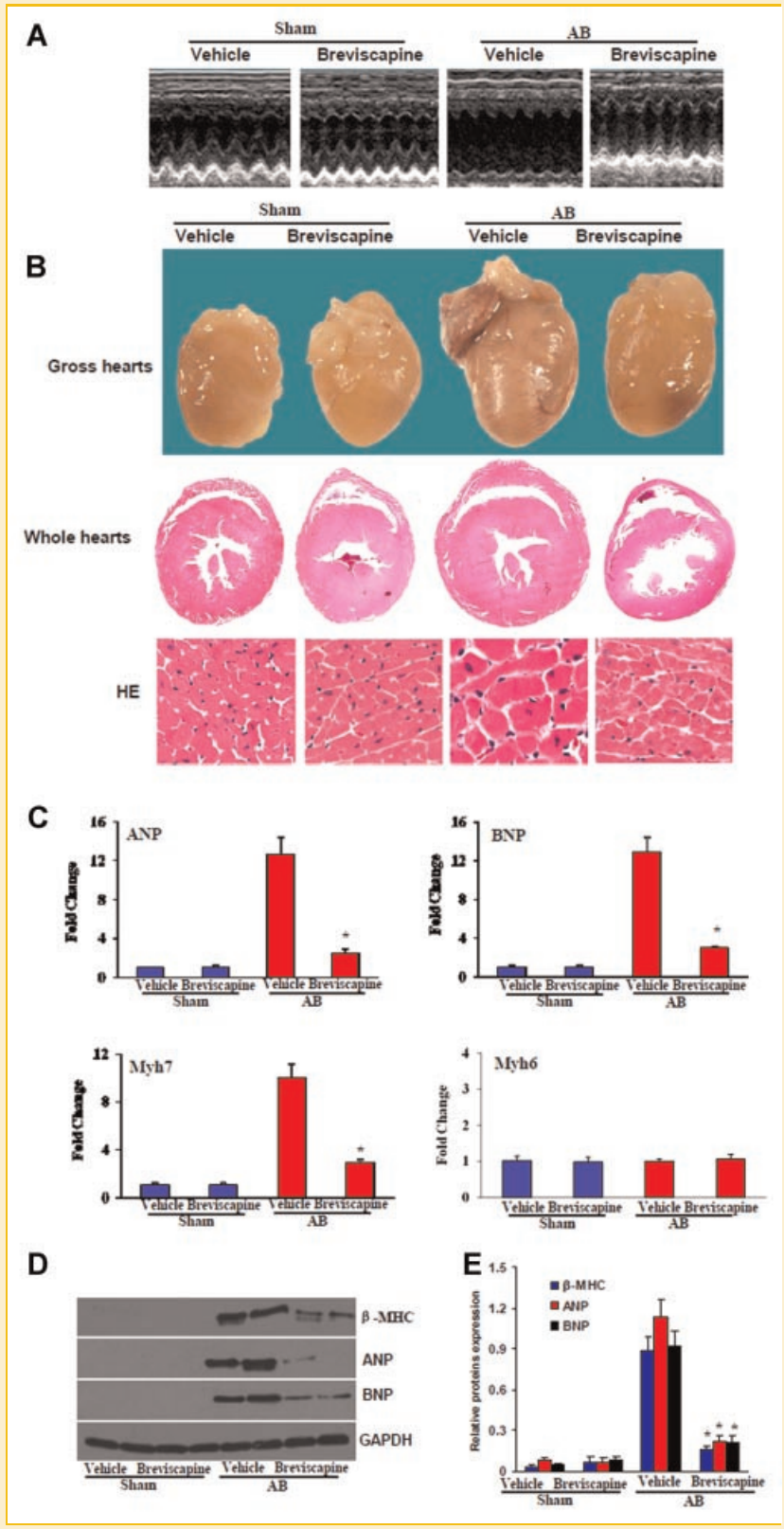

Fig. 2. Breviscapine inhibited cardiac hypertrophy in vivo. A: Examples of M-mode echocardiograms from AB- or sham-operated vehicle-treated and breviscapine-treated mice. B: Gross hearts (top), whole hearts (middle), and representative H&E staining (bottom) of Sham and AB mice at 8 weeks after surgery treated with vehicle or breviscapine. C: Real-time PCR analysis of hypertrophic markers, ANP, BNP, Myh6, and Myh7, from hearts of mice in the indicated groups. D,E: The protein expression levels of β-MHC, ANP, and BNP were determined by Western blot analysis. Data represent typical results of three to four different experiments as mean ± SEM (n = 4–6 mice/per group). $^{*}P < 0.05$ for difference from vehicle/AB values in the AB model.

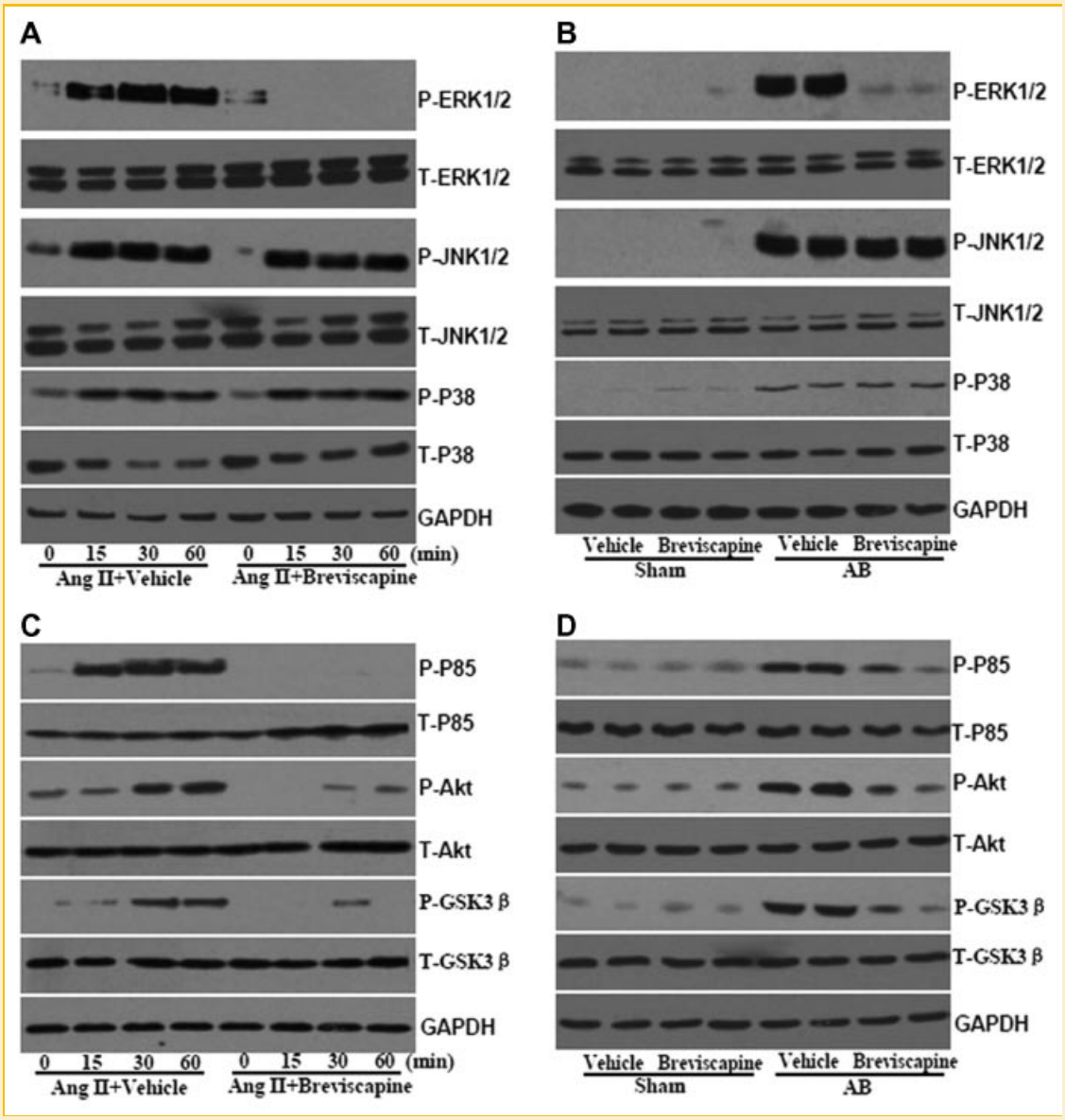

Fig. 3. Breviscapine disrupted ERK1/2 and PI3K/AKT/GSK3β signaling pathways. A: Time course of ERK1/2, JNK1/2, and p38 phosphorylation and their total protein expression after treated with Ang II for indicated time in vehicle or breviscapine pretreated-cardiomyocytes (n = 4). B: Representative Western blot of phosphorylated and total ERK1/2, JNK1/2, and p38 at 8 weeks after surgery in Sham and AB mice treated with vehicle or breviscapine (n = 4). C: Time course of P85, AKT, and GSK3β phosphorylation and their total protein expression after treated with Ang II for indicated time in vehicle or breviscapine pretreated-cardiomyocytes (n = 4). D: Representative Western blot of P85, AKT, and GSK3β phosphorylation and their total protein expression at 8 weeks after surgery in Sham and AB mice treated with vehicle or breviscapine (n = 4). The results were reproducible in three separate experiments.

determine whether breviscapine can suppress the inflammatory responses in the heart, we first examined NF-κB activity. Our data revealed that breviscapine abolished the increased activation of NF-κB observed in the myocardium of vehicle-treated mice 8 weeks post-AB (Fig. 5A). 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. 5B), 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. In line with this in vivo findings, breviscapine significantly decreased NF-κB activation induced by Ang II in cardiac myocytes (Fig. 5C). To further determine the molecular mechanisms through which breviscapine blocks NF-κB activation, we analyzed IκBα phosphorylation and IKKβ activation processing in vivo. Heart lysates from samples obtained from mice 8 weeks post-AB were prepared and Western blot analysis was performed. Breviscapine treatment markedly impaired IκBα phosphorylation and degradation, as well as IKKβ activation mediated by AB (Fig. 5D). We next examined whether breviscapine has the ability to inhibit the expression of

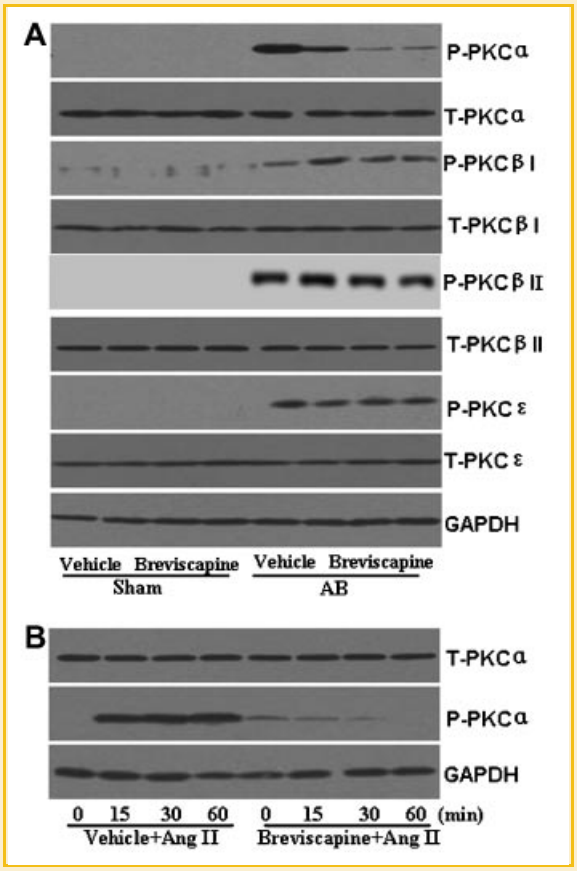

Fig. 4. Breviscapine inhibited PKC-α signaling in response to hypertrophic stimuli. A: Representative blots of PKC-α, PKC-βI, PKC-βII, and PKC-ε phosphorylation and their total protein expression at 8 weeks after surgery in Sham and AB mice treated with vehicle or breviscapine (n = 4). B: Time course of phosphorylated and total PKC-α protein expression after treated with Ang II for indicated time in vehicle or breviscapine pretreated-cardiomyocytes (n = 4). The results were reproducible in three separate experiments.

inflammatory mediators IL-6, TNF-α, and MCP-1 in cardiac tissue. As shown in Figure 5E, breviscapine significantly decreased the levels of IL-6, TNF-α, and MCP-1 mRNA expression compared with vehicle-treated AB mice. In order to examine the direct relationship between NF-κB and cytokine expression, we examined the effects of inhibition or activation of NF-κB by AdIκBα and AdIKKβ infection. As shown in Figure 5F, blocking NF-κB by AdIκBα infection inhibited Ang II-induced IL-6, TNF-α, and MCP-1 proteins expression, whereas activation of NF-κB by AdIKKβ infection augmented Ang II-induced IL-6, TNF-α, and MCP-1 proteins expression in cardiac myocytes. These results indicate that breviscapine inhibits inflammation through blocking NF-κB signaling in response to chronic pressure-overload.

BREVISCAPINE INHIBITED CARDIAC FIBROSIS IN VIVO AND COLLAGEN SYNTHESIS IN VITRO

Pathological cardiac hypertrophy is associated with increased fibrosis in the myocardium [Berk et al., 2007]. To further investigate the mechanism by which breviscapine inhibits cardiac hypertrophy, we examined the ability of breviscapine to inhibit fibrosis. The increase in LV collagen volume detected in the vehicle-treated AB mice by PSR staining was remarkably attenuated after treatment with breviscapine (Fig. 6A). TGF-β1 signaling plays an important role in cardiac fibrosis. Our results showed that breviscapine also attenuated the increased concentration of TGF-β1 induced by AB in plasma and heart tissue (Fig. 6B). Subsequent analysis of protein and mRNA expression levels of known mediators of fibrosis including TGF-β1, CTGF, Collagen I, and Collagen III, demonstrated a blunted response following breviscapine administration compared to the vehicle-treated group (Fig. 6C,D). To further elucidate the molecular mechanisms through which breviscapine inhibits cardiac fibrosis, we examined the effects of breviscapine on Smad cascade activation in hearts subjected to AB. Immunoblot analysis demonstrated that breviscapine suppressed phosphorylation of Smad-2, Smad-3, and Smad-4 as well as nuclear translocation of Smad-2 and Smad-3 (Fig. 6E).

In addition, we examined the ability of breviscapine 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 breviscapine pretreatment in a dose-dependent manner (Fig. 6F).

ROLE OF PKC-α IN Ang II-INDUCED CARDIAC HYPERTROPHY AS WELL AS ERK1/2, PI3K/AKT, NF-κB, AND Smad2/3 SIGNALING

To identify whether PKC-α inhibition was responsible for the protective role of breviscapine on cardiac hypertrophy, cells were infected with Ad-GFP, Ad-caPKC-α, or Ad-dnPKC-α. We found that overexpression of PKC-α significantly increased both [^{3}H]-leucine and [^{3}H]-proline incorporation in response to Ang II. On the other hand, blocking PKC-α activity almost completely abrogated Ang II-induced [^{3}H]-leucine and [^{3}H]-proline incorporation (Fig. 7A). We next performed an experiment with breviscapine in presence of exogenous PKC-α. As shown in Figure 7B, breviscapine eliminated Ang II-induced [^{3}H]-leucine and [^{3}H]-proline incorporation in cells that overexpressed PKC-α, which confirming that the cardioprotective effect of breviscapine against cardiac hypertrophy are mediated by selectively inhibition of PKC-α activation. To further examine the mechanisms involved, we investigated whether PKC-α is involved in Ang II-induced activation of ERK1/2, PI3K/AKT, NF-κB, and Smad2/3 signaling. As shown in Figure 7C, activation of PKC-α by infection with Ad-caPKC-α caused significant phosphorylation of ERK1/2, P85, AKT, IκBα, Smad-2, and Smad-3 induced by Ang II, but blocking PKC-α activity by Ad-dnPKC-α infection remarkably attenuated Ang II-induced activation of ERK1/2, P85, AKT, IκBα, Smad-2, and Smad-3. These results clearly indicate that breviscapine blocks cardiac hypertrophy by disrupting PKC-α-dependent ERK1/2, PI3K/AKT, NF-κB, and Smad2/3 signaling.

DISCUSSION

The present study demonstrates for the first time that breviscapine protects against cardiac hypertrophy induced by Ang II in vitro

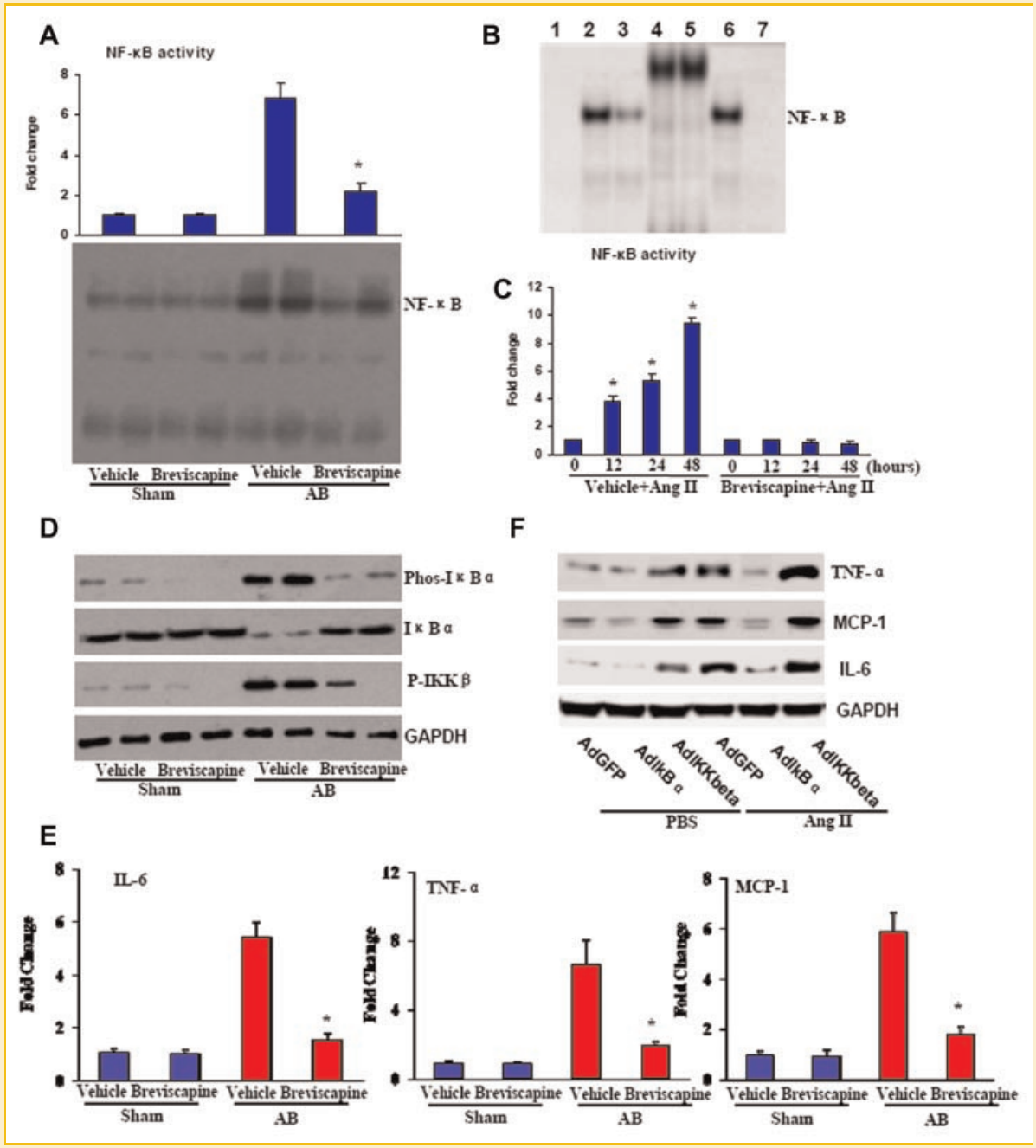

Fig. 5. Breviscapine blunted inflammatory response in vivo and in vitro. A: The DNA-binding activity of NF-κB was evaluated in the myocardium obtained from indicated animals at 8 weeks after AB (n = 6). $^{*}P < 0.05$ for difference from vehicle/AB values in the AB model. B: NF-κB induced by AB is composed 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). C: NF-κB activity was determined by Reporter assays in cardiomyocytes treated with Ang II for indicated time (n = 6). D: Representative blots of IκBα degradation, and IκBα and IKKβ phosphorylation in the myocardium obtained from indicated groups (n = 4). E: Real-time PCR analysis of IL-6, TNF-α, and MCP-1 mRNA expression in the myocardium obtained from indicated groups (n = 4). GAPDH was used as the sample loading control. $^{*}P < 0.05$ for difference from vehicle/AB values.

and pressure-overload induced cardiac hypertrophy in vivo. The cardioprotection of breviscapine is mediated by direct interruption of PKC-α-dependent ERK1/2, PI3K/AKT, NF-κB, and Smad2/3 signaling, which leads to the inhibition of cardiomyocyte growth, inflammation, and fibrosis and ultimately preventing the progress of cardiac hypertrophy. These findings suggest that breviscapine could be an effective therapeutic candidate against cardiac hypertrophy and progression to heart failure.

Breviscapine, a well-known traditional Chinese medicine with potent protective effects against the cardiovascular system, has been commonly prescribed by Chinese practitioners in treating heart diseases including hypertension, coronary heart disease, and heart

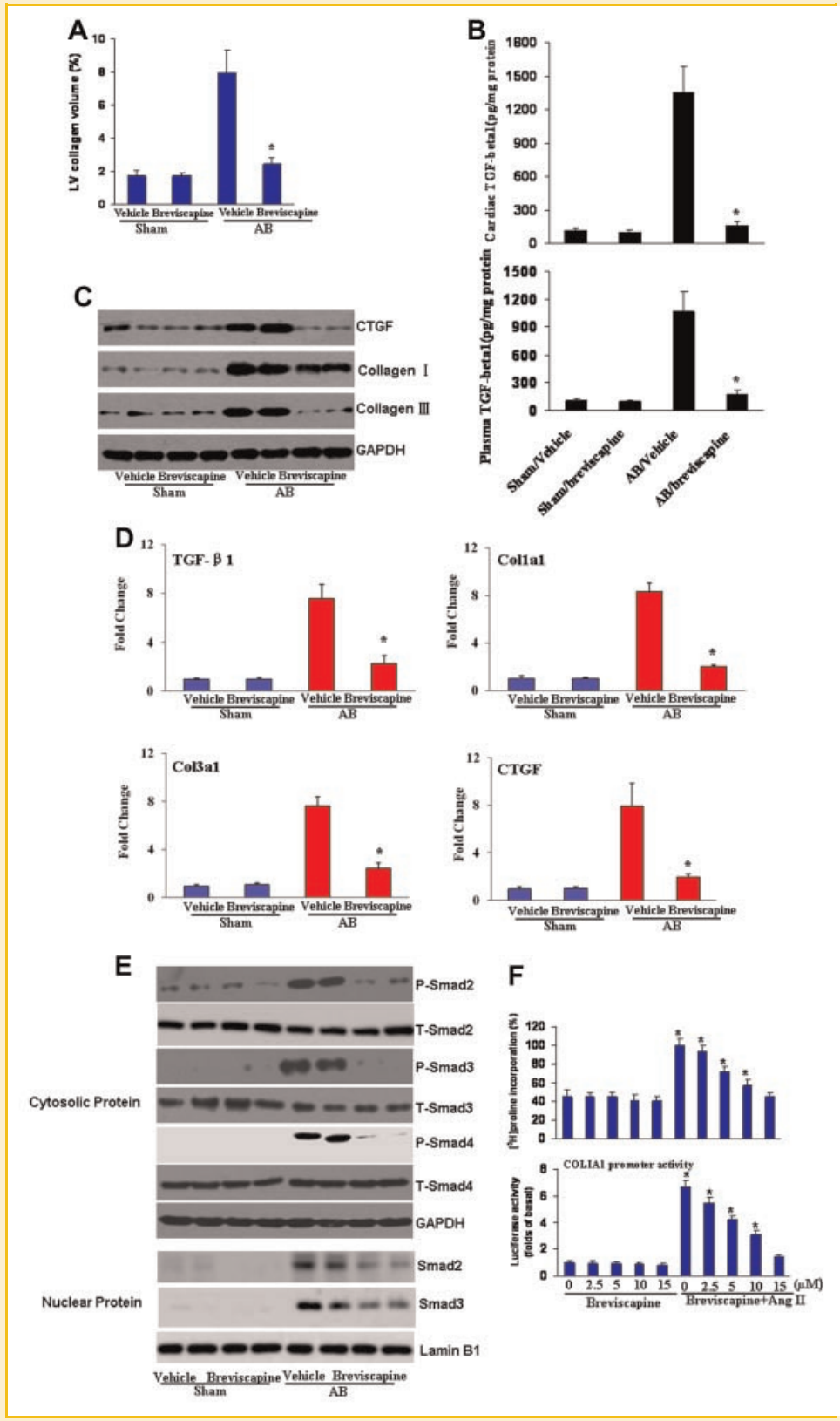

Fig. 6. Breviscapine inhibited cardiac fibrosis in vivo and collagen synthesis in vitro. A: Left ventricle interstitial collagen volume fraction was quantified using an image-analyzing system ($n = 6$). B: TGF-β1 content as determined by ELISA of plasma and homogenized protein obtained from the LV of the heart 8 weeks after AB ($n = 4$). $^{*}P < 0.05$ for difference from vehicle/AB values. C: Western blot analysis of CTGF, Collagen I, and Collagen III protein expression levels in the myocardium obtained from indicated groups ($n = 4$). D: Real-time PCR analysis of TGF-β1, Col1a1, Col1a3, and CTGF were performed to determine mRNA expression levels in indicated groups ($n = 4$). GAPDH was used as the sample loading control. $^{*}P < 0.05$ for difference from vehicle/AB values after AB. E: Representative blots of phosphorylated and total protein expression of Smad 2, 3, and 4 from indicated groups 8 weeks after AB ($n = 4$) as well as Smad-2/3 expression in the nucleus from each group 8 weeks after AB ($n = 3$). F: Breviscapine inhibited Ang II-induced [^{3}H] proline incorporation ($n = 6$) and COL1A1 promoter activity ($n = 3$). Cardiac fibroblasts were pretreated with either different doses of breviscapine for 60 min and then incubated with 1 μM Ang II for 48 h. [^{3}H]-proline incorporation and Luciferase assay were performed as described in "Materials and Methods." Each assay was performed in triplicate. $^{*}P < 0.05$ versus exposed to control.

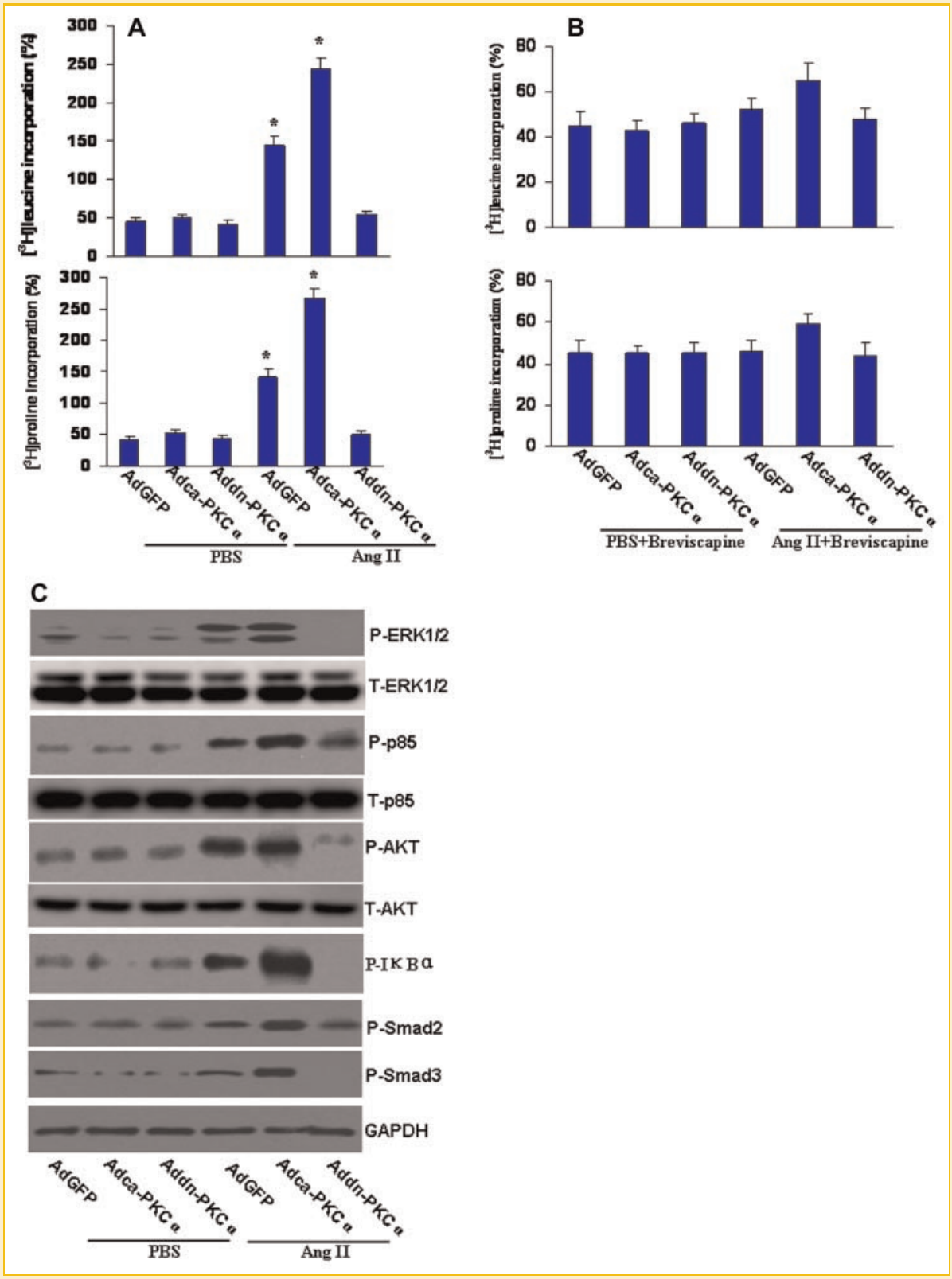

Fig. 7. Role of PKC-α in Ang II-induced cardiac hypertrophy as well as ERK1/2, PI3K/AKT, NF-κB, and Smad2/3 signaling. A: The effect of PKC-α on Ang II-induced [^{3}H]-Leucine and [^{3}H] proline incorporation ($n = 6$). B: The effect of breviscapine on Ang II-induced [^{3}H]-Leucine and [^{3}H] proline incorporation in presence of exogenous PKC-α ($n = 6$). C: Western blot analysis of the effect of PKC-α on Ang II-induced activation of ERK1/2, P85, AKT, IκBα, Smad-2, and Smad-3 ($n = 4$). Cells were infected with Ad-GFP, Ad-caPKC-α, or Ad-dnPKC-α for 24 h, and then incubated with 1 μM Ang II for up to 48 h. [^{3}H]-Leucine incorporation, [^{3}H] proline incorporation assay, and Western blot were performed as described in "Materials and Methods." The results were reproducible in three separate experiments. $^*P < 0.05$ versus exposed to control.

failure. However, the effect of breviscapine on cardiac hypertrophy has not yet been clarified. In this study, we found that breviscapine not only attenuated cardiac hypertrophy in vitro and in vivo in response to hypertrophic stimuli, but also improved cardiac performance and reduced chamber dimensions. Unexpectedly, we did not observe hypotensive effect of breviscapine in aortic-banded mice. In accordance with our finding, Zhou et al. [2002] reported that scutellarin, the primary active ingredient of breviscapine, reversed ventricular remodeling in spontaneously hypertensive rats. However, they demonstrated that scutellarin (10 mg/kg/day) can slightly reduce blood pressure in SHRs. The discrepancy on BP may be explained by different drug dosage and/or the difference in vasorelaxtion action between scutellarin monomer and breviscapine compound [Pan et al., 2008]. We also found that breviscapine showed no toxicity in the liver or kidney at dose of 50 mg/kg/day, which confirming the safety of breviscapine in clinic. These results allow a better understanding regarding the potential clinical therapeutic use of breviscapine in cardiac hypertrophy and heart failure.

The mechanism by which breviscapine exerts an anti-hypertrophic effect remains largely unclear. A number of reports have associated PKC activation with cardiac hypertrophy [Vijayan et al., 2004; Pan et al., 2005]. Importantly, increasing evidence has demonstrated that breviscapine possesses a strong inhibitory effect on PKC activation, which contributes to its protective role in cerebral ischemia and hepatic injury during brain-death [Xu et al., 2007; Zhang et al., 2007]. Therefore, the inhibitory mechanisms of breviscapine on cardiac hypertrophy were examined for its effect on PKC activation. Our data clearly revealed that breviscapine blocked PKC-α activation induced by Ang II or pressure-overload, whereas it failed to affect the activation of other PKC isoforms including PKC-βI, PKC-βII, and PKC-ε. These results implicate breviscapine as a selective inhibitor of PKC-α isoform and suggest that such effect may be related to the cardioprotective effect of breviscapine against cardiac hypertrophy. Using adenoviral-mediated gene transfer of wild-type and dominant negative mutant of PKC-α, we further demonstrated that overexpression of PKC-α increased whereas blocking PKC-α activity attenuated Ang II-induced [^{3}H]-leucine incorporation in cardiac myocytes, confirming a necessary role for PKC-α in cardiac hypertrophy. These results are consistent with the previous report that PKC-α is uniquely required for α-adrenergic agonist PE-induced cardiomyocyte hypertrophic growth [Braz et al., 2002]. Collectively, these findings indicate that anti-hypertrophic effect of breviscapine can be in part ascribed to specifically blocking PKC-α activation. The mechanism whereby breviscapine inhibit PKC-α is uncertain. Recent investigations have shown that scutellarin is a Ca^{2+} channel blocking agent with the ability to inhibit extracellular calcium influx [Pan et al., 2008], which is a possible mechanism in blocking PKC-α activation considering calcium as a robust activator of PKC-α. Indeed, future studies are required to investigate the specific mechanism.

It is noteworthy that PKC participates in the activation of MAPK cascades and PI3K/Akt pathway [Ueda et al., 1996; Schonwasser et al., 1998; Li et al., 2005], which are also important for developing a hypertrophic phenotype in cardiomyocytes. Therefore, we examined the effects of breviscapine on these signaling pathways, respectively. 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; Sadoshima et al., 2002; Petrich and Wang, 2004]. 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, breviscapine 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. Braz et al. [2002] also demonstrated that PKC-α activation increased the activity of ERK cascade in PE-treated cardiac myocytes and induced cell growth, but did not contribute significantly to the activation of the two other MAPK cascades. In line with their study, we found that ERK1/2 activation induced by Ang II in cardiomyocyts was PKC-α dependent. Taken together, these findings confirm ERK1/2 as a pivotal regulator of cardiac hypertrophy and reveal that the anti-hypertrophic effect of breviscapine is achieved through blocking PKC-α-dependent ERK1/2 signaling. In addition, a similar profile was observed with respect to PI3K/Akt pathway. Breviscapine blocked the activation of p85 subunit of PI3K, the key PI3K targets Akt and its downstream GSK3β in response to hypertrophic stimuli, which was dependent on PKC-α activation. Such data imply that PKC-α-dependent PI3K/AKT/GSK3β signaling is also implicated in breviscapine-mediated inhibitory effects on cardiac hypertrophy.

Downstream targets of the PKC, MAPKs, and PI3K/Akt include transcription factors such as NF-κB and Smad, which have been shown to regulate inflammation and fibrosis, respectively [Baeuerle, 1998; Li et al., 2006; Ruiz-Ortega et al., 2007]. Mounting evidence has strongly suggested that inflammation and fibrosis play a key role in the development of cardiac hypertrophy and heart failure. Recently, Zhang et al. [2007] reported that breviscapine decreased the release of inflammatory factors, and thus alleviates hepatic injury during brain-death. Wu et al. [2007] demonstrated that treatment with breviscapine significantly reduced liver fibrosis in diabetic rats. Therefore, we investigated whether breviscapine possesses anti-inflammatory and anti-fibrotic properties in the hypertrophic hearts. Our results showed that breviscapine significantly abrogated NF-κB activation by disrupting transcriptional activity through blocking the phosphorylation and degradation of IκBα, IKKβ activation as well as inhibited the expression of inflammatory mediators (IL-6, TNF-α, and MCP-1). Similarly, breviscapine attenuated cardiac fibrosis characterized by decreased LV collagen volume and expression levels of known mediators of fibrosis (TGF-β1, 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 breviscapine suppressed phosphorylation of Smad-2, Smad-3, and Smad-4 as well as nuclear translocation of Smad-2 and Smad-3 in hypertrophied hearts, indicating a mechanism underlying the inhibitory effect of breviscapine on fibrosis. More importantly, we found that activation of PKC-α resulted in significant upregulation, while blocking PKC-α activation led to inhibition of phosphorylation of IκBα, Smad-2, and Smad-3. Collectively, these results suggest that breviscapine blocks PKC-α-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 breviscapine inhibits cardiac hypertrophy in vitro and in vivo by blocking PKC-α-dependent hypertrophy, inflammation, and fibrosis. We further confirm that PKC-α-dependent ERK1/2, PI3K/AKT, NF-κB, and Smad2/3 signaling pathways are targets of breviscapine's inhibitory actions. This study serves to elucidate the inhibitory effect of breviscapine on cardiac hypertrophy and related molecular mechanisms. It also improves our knowledge about the dominant signaling pathways leading to cardiac hypertrophy, inflammation, and fibrosis in response to hypertrophic stimuli. More importantly, our results provide experimental evidence for the application of breviscapine in the treatment of cardiac hypertrophy and heart failure. Indeed, future clinical trials are required to prove the new potential clinical therapeutic use of breviscapine.

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