Salvianolic Acid B-Alleviated Angiotensin II Induces Cardiac Fibrosis by Suppressing NF-κB Pathway *In Vitro*

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Salvianolic acid B (SalB) is the representative component of phenolic acids derived from the roots and rhizomes of *Salvia miltiorrhiza* Bge (Labiatae), which has been used widely in Asian countries for clinical therapy of various cardiovascular dysfunction-related diseases. However, cardiac protection effects and the underlying mechanism for clinical application are still poorly understood. Here, we investigated the potential anti-myocardial fibrosis effect and mechanism of SalB on Angiotensin II (Ang II)-induced cardiac fibrosis *in vitro*.

Material/Methods: The proliferation and migration capacity of cardiac fibroblasts (CFBs) were measured by MTT assay and scratch analysis, respectively. The colorimetric assay determined the hydroxyproline content in medium. Western blotting detected the protein expressions of nuclear transcription factor-kappa B (NF-κB) pathway-associated proteins, fibronectin (FN), collagen type I (Coll I), α-smooth muscle actin (α-SMA), and connective tissue growth factor (CTGF). The expression of α-SMA protein was observed by immunofluorescence staining. qRT-PCR detected the mRNA expression of NF-κB.

Results: SalB attenuated Ang II-induced the proliferation and the migration ability of CFBs. Ang II-induced the extracellular matrix protein Coll I, FN, and α-SMA, the pro-fibrotic cytokine CTGF protein expression was inhibited, and the nuclear translocation of NF-κB p65 subunit was reduced by SalB. Western blotting and qRT-PCR confirmed that SalB blocked the activation of NF-κB induced by Ang II. PDTC (the NF-κB inhibitor) also inhibited proliferation of CFBs and reduced α-SMA and Coll I expression induced by Ang II.

Conclusions: SalB can alleviate Ang II-induced cardiac fibrosis via suppressing the NF-κB pathway *in vitro*.

MeSH Keywords: **Angiotensin II • Fibroblasts • NF-kappa B**

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Background

Cardiovascular diseases are a serious health threat and are the leading cause of human death worldwide. Understanding the mechanisms leading to cardiovascular diseases and developing novel drugs have long been important research areas [1]. Cardiac fibrosis plays a critical pathophysiological role in cardiovascular disease [2], which is characterized by interstitial fibroblast proliferation and excessive production and deposition of myocardial extracellular matrix (ECM), including collagens and fibronectin (FN) and increased expression of pro-fibrotic cytokines such as connective tissue growth factor (CTGF) [3,4]. Cardiac fibroblasts (CFBs) have been identified as the most important participants in the development of cardiac fibrosis [5]. A previous study suggested that excessive activation of cardiac fibroblasts (including proliferation, migration, collagen synthesis, and transformation) contribute to occurrence and development of myocardial fibrosis [6]. Therefore, searching for novel agents that can inhibit the proliferation, migration, and deposition of collagen, as well as increased expression of fibrotic cytokines in cardiac fibroblasts, is important for prevention and cure of myocardial fibrosis.

Angiotensin II (Ang II) has been reported to be aberrantly activated in various cardiovascular diseases, playing a major role in cardiac remodeling by promoting myofibroblasts differentiation and deviant ECM production and degradation [7]. It is well known that nuclear transcription factor-kappa B (NF-κB) activation plays a key role in the progression of cardiac fibrosis, and translocation of p65 into nuclear fraction from the cytoplasm regulates target genes that are mediated by NF-κB activation. Suppressing NF-κB activation and nuclear translocation can alleviate cardiac fibrosis [8]. Recent studies suggest that Ang II promotes NF-κB activation and can increase collagen expression in CFBs [9]. Subsequently, in this study, the NF-κB inhibitor PDTC was used to inhibit NF-κB activation in order to investigate the role of NF-κB activation in Ang II-induced fibroblast-to-myofibroblast differentiation of CFBs.

Salvianolic acid B (SalB), a polyphenolic acid, is a main water-soluble bioactive ingredient of *Radix salvia*, which is a condensate of 3 molecules of danshennol and 1 molecule of caffeic acid. It was proposed to possess therapeutic potential role due to its antioxidant, anti-inflammatory, anti-apoptosis, and anti-arrhythmic effects [10]. There is growing evidence that it has antifibrotic effects on a variety of organs and tissues, including heart [11], liver [12], kidney [13], and lung [14]. In addition, SalB can prevent arsenic trioxide-induced cardiotoxicity *in vivo* and enhance its anticancer activity *in vitro* [15]. Previous studies have shown that SalB can inhibit myofibroblast transdifferentiation and decrease oxidative stress via activating Nrf2 in experimental pulmonary fibrotic lung fibroblasts [16]. SalB inhibited Ang II-stimulated HSC activation including cell proliferation

and expression of collagen I (Coll I) and α-smooth muscle actin (α-SMA) production *in vitro*, reduced the gene expression of transforming growth factor beta (TGF-β), and downregulated AT1R expression and ERK and c-Jun phosphorylation [17]. SalB could attenuate renal ischemic reperfusion injury via suppressing oxidative stress and inflammation through the PI3K/Akt signaling pathway [18]. Our previous study showed that the anti-myocardial fibrosis effect of SalB may be associated with downregulating protein expressions of type III collagen, MMP-9, and Smad2/3 and upregulating protein expressions of Smad7 [19].

However, the inhibitory effect of SalB on myocardial fibrosis and the underlying mechanism remain unclear. Therefore, the purpose of the present study was to investigate the antifibrotic effect of SalB on the CFBs induced by Ang II and to identify the possible underlying mechanism.

Material and Methods

Ethics statement

All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by Guizhou Medical University and were approved by the Committee for Experimental Animal Ethics of Guizhou Medical University.

Materials

SalB (purity 98%, lot no. D1516060) was purchased from Jingchun biochemical Technology Co., Ltd., Shanghai, China; Ang II (purity 98%, lot no. SLBL5396V) was from Sigma, USA; Trypsin was from Solarbio (Beijing, China); Dulbecco's modified Eagle's medium (DMEM) was from GIBCO (Gaithersburg, USA); penicillin and streptomycin were from Sigma (St. Louis, MO, USA); Hydroxyproline assay kits were obtained from Jiancheng Bioengineering (Nanjing, China); the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) and pyrrolidinedithiocarbamate (PDTC, NF-κB inhibitor) were obtained from Sigma-Aldrich (St. Louis, MO, USA); fibronectin (FN) rabbit polyclonal antibody and GAPDH polyclonal antibody were obtained from Immunoway; collagen type I (Coll I) rabbit polyclonal antibody, α-SMA rabbit polyclonal antibody, and IκBα polyclonal antibody were purchased from Proteintech; nuclear transcription factor-kappa B (NF-κB) p65 rabbit polyclonal antibody, phospho-NF-κB p65 (Ser536) antibody, and phospho-IκBα (Ser32) rabbit polyclonal antibody were all obtained from the Cell Signaling Technology.

Isolation and purified culture of primary neonatal rat CFBs

CFBs were isolated and purified from 1- to 3-day-old Sprague-Dawley rats. Briefly, the hearts of 1–3-day-old Sprague Dawley rats were isolated and digested in 6 mL of phosphate-buffered

saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2–7.4) containing 0.08% trypsin for 5 min at 37°C, which was repeated 4–6 times. After each digestion step, the medium containing suspended cells was removed and an equal volume of DMEM containing 10% fetal bovine serum solution was added. Primary cultures of rat cardiac stromal cells were grown in DMEM supplemented with 15% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) at 37°C in a humidified atmosphere of 5% CO_2. The differential adhesion method was used to separate out other cells after 4 h, and primary neonatal rat CFBs were used for subculture. CFBs at the second or third passage were used for the following experiments.

Immunohistochemical staining

CFBs were cultured in 24-well plates at 37°C in a humidified atmosphere of 5% CO_2 for 24 h and then washed 3 times with PBS, followed by fixing with ice-cold 4% paraformaldehyde for 15 min and then permeabilization with 0.5% Triton X-100 at room temperature for 20 min. Immunocytochemical staining was performed by using the streptavidin peroxidase (SP) combined with Immunohistochemical 3'-3'-diaminobenzidine (DAB; ZSGB-BIO; OriGene Technologies, Inc., Beijing, China; cat. no. ZLI-9018) staining method. The primary antibody, mouse monoclonal anti-rat vimentin (Boster Biological Technology, Pleasanton, CA, USA; cat. no. BM0135) was added at a dilution of 1: 200 and incubated with CFBs overnight at 4˚C. Following incubation, CFBs were washed 3 times with PBS and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (ZSGB-BIO; OriGene Technologies, Inc.; cat. no. PV-6002; used at the working dilution recommended by the manufacturer) for 20 min at 37°C. This was followed by DAB and hematoxylin staining for 10 s at room temperature, then observation under a light microscope (Leica DMi 1; Leica Microsystems GmbH, Wetzlar, Germany). Cells were visualized at ×200 magnification and 5 fields of view were analyzed.

CFBs proliferation assay

Cultured CFBs were exposed to Ang II (1 μM) alone for 24 h or pretreated with different concentrations of SalB (12.5 μM, 25 μM, and 50 μM) for 1 h before exposure to Ang II for 24 h in 96-well plates. Then, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at 20 μl/well (final concentration 5 mg/mL) in sterile conditions, and the plates were incubated for 4 h at 37°C in a 5% CO_2 incubator, then the medium was discarded and washed 3 times with PBS. Formazan salt crystals were dissolved by addition of 150 μL dimethylsulfoxide per well and the absorbance values were determined at 490 nm by using a microplate reader (ELX800; General Electric, Fairfield, CT, USA).

Scratching assay

CFBs migration capacity was assessed by wound-healing assay through scratching a cell monolayer with a 10-μl pipette tip in 24-well culture plates. After 3 washes with PBS, FBS-free medium was added to each well and cells were incubated for 6 h. Then, cells were pretreated with SalB (12.5 μM, 25 μM, and 50 μM) for 1 h before stimulation with Ang II (1 μM) for 24 h. The wounded area was imaged by microscopy at 0 h and 24 h. The width of the wound was measured using Image J software and calculated from 5 averaged regions. The net migration distance after 24 h was subtracted from that at the baseline. Results are reported as the percentage of migration using the equation: % migration=[(wound distance at T0 h–wound distance at T24 h)/wound distance at T0 h)]×100%.

Hydroxyproline colorimetric assay

The hydroxyproline (Hyp) content of cell supernatants was quantified by using a commercial Hyp detection kit. The OD values of the samples were measured at 550 nm using an ELX800 microplate reader.

Immunofluorescence staining

CFBs cultured on 24-well plates were fixed in 4% paraformaldehyde for 30 min, washed with PBS 3 times, and permeabilized with 0.5% Triton X-100 in PBS for 20 min. After rinsing with PBS 3 times, the cells were blocked with 5% BSA at room temperature for 1 h. Next, the cells were incubated with a rabbit polyclonal anti-a-SMA primary antibody (1: 50 dilution) overnight at 4°C. Primary antibody was removed, then cells were washed with PBS 3 times and then incubated in FITC-secondary antibody (1: 80 dilution) for 1 h at room temperature. Following the washing with PBS, cells were labeled with 4',6-diamidino-2-phenylindole (DAPI) to show the nucleus. Fluorescence microscopy (Nikon Corporation, Tokyo, Japan) was used to take images.

Western blotting

CFBs were harvested and protein was extracted using a lysis buffer containing PMSF and RIPA (PMSF: RIPA=1: 99). Cell lysates were prepared in lysis buffer and spun at 12 000 rpm at 4°C for 20 min. A commercial BCA protein assay kit (Beyotime Institute of Biotechnology) was used to quantify the protein concentration of the cell lysates. The nuclear and cytoplasmic protein extract was prepared by using the Nuclear and Cytoplasmic Protein Extraction Kit (Bestbio Institute of Biotechnology) according to the manufacturer's instructions. Equal amounts of protein (45 μg/lane) were fractionated to 10% stacking sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked
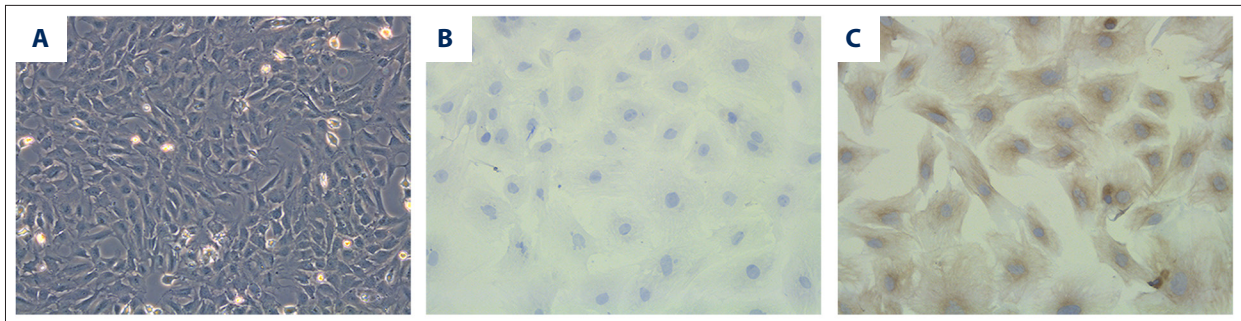
Figure 1. Identification of cardiac fibroblasts (CFBs). (**A**) Morphology image of the second-passage CFBs (magnification, ×100). (**B**) Representative image of negative control-stained cells (PBS was used instead of primary antibody, magnification, ×200). (**C**) Representative image of cells stained with the anti-vimentin antibody (magnification, ×200). CFBs – cardiac fibroblasts.

with 5% albumin bovine serum (BSA) at room temperature for 1 h, followed by washing with TBST, and the membranes were incubated with primary antibody at 4°C overnight. Primary anti-bodies were used as follows: anti-phosphorylated P65 (#3033, Ser536, Cell Signaling, 1: 1000), anti-phosphorylated IκBα (#2859, Ser32, Cell Signaling, 1: 1000), anti-P65 (10745-1-AP, Proteintech, 1: 500), anti-IκBα (10268-1-AP, Proteintech, 1: 1000), anti-FN (YT1733, Immunoway, 1: 1000), anti-Coll I (I4695-I-AP, Proteintech, 1: 1000), anti-CTGF (ab6992, abcam, 1: 1000), anti-α-SMA (YT5053, Immunoway, 1: 1000), anti-GAP-DH (60004-1-1g, Immunoway, 1: 10 000), anti-Tubulin (10094-1-AP, Proteintech, 1: 10,000) and anti-Lamin B1 (12987-1-AP, Proteintech, 1: 10 000). After washing the blots to remove excessive primary antibody binding, the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1: 10 000) at room temperature after washing 3 times, followed by enhanced chemiluminescence (ECL) detection (7Sea Biotechnology, Shanghai, China). Densitometric measurements of the Western blot band analysis were performed by using the Syngene Gel Imaging System (Bio-Rad). The results were normalized to those of GAPDH and the data from 3 independent experiments were analyzed.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted using the E.Z.N.A.® Total RNA Kit I (R6834-01, OMEGA) from CFBs according to the standard protocol. RNA was reverse-transcribed into complementary DNA (cDNA) using PrimeScript™ RT reagent kit (AK6003, Takara Bio, Inc.) and a SimpliAmp Thermal Cycler (Applied Biosystems, Life Technologies). Quantitative real-time PCR was performed on CFX Manager 3.0 Real-Time PCR System (Bio-Rad) and SYBR® Premix Ex Taq™ II (Takara Bio, Inc.). GAPDH was used as the internal control for mRNA expression. The primers that we designed were as follows: rat NF-κB forward, 5'-ACATCCCTCAGCACCATCAA-3', reverse, 5'-TTGGTACCATGGCTGAGGAG-3'; rat GAPDH, forward, 5'-GACATGCCGCCTGGAGAAAC-3, reverse, 5'-AGCCCA GGATGCCCTTTAGT-3'.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using GraphPad prism® 6.0 software (La Jolla, CA, USA). Statistical analysis was undertaken using ANOVA and *t* test for 2 groups, and one-way ANOVA for multiple comparisons. Data are expressed as the mean ±SEM. A value of $P<0.05$ was considered to be statistically significant. Data from *in vitro* studies were derived from at least 3 independent experiments.

Results

Identification of primary cardiac fibroblasts (CFBs)

Vimentin, an intermediate filament protein, is a key identifying biological marker of fibroblasts, which can be stained via immunocytochemistry to display its filamentous morphology. As shown in Figure 1A, we can observe that CFBs are flat and spindle-shaped with multiple projecting processes by the microscope. As shown in Figure 1B, control-stained cells exhibited a blue nucleus and no yellow cytoplasmic staining (replacing the anti-vimentin antibody with PBS). In Figure 1C, isolated primary cells stained with the anti-vimentin antibody exhibited a blue nucleus and a strong yellow cytoplasmic staining for vimentin. As the majority of cells were positive for vimentin, we successfully isolated primary CFBs. Primary CFBs were isolated from neonatal rats and used at passages 2–3 for subsequent experiments.

Salvianolic acid B (SalB) inhibits CFBs proliferation induced by Angiotensin II (Ang II)

The CFBs proliferation is the key pathophysiological process in myocardial fibrosis, and Ang II has been shown to promote the proliferation of cultured CFBs as an independent factor. The MTT assay was used to assess the ability of SalB to inhibit Ang II-induced CFBs proliferation. CFBs were pretreated with or without different concentrations of Sal B (12.5 µmol/L, 25 µmol/L, and 50 µmol/L) for 1 h and then stimulated with
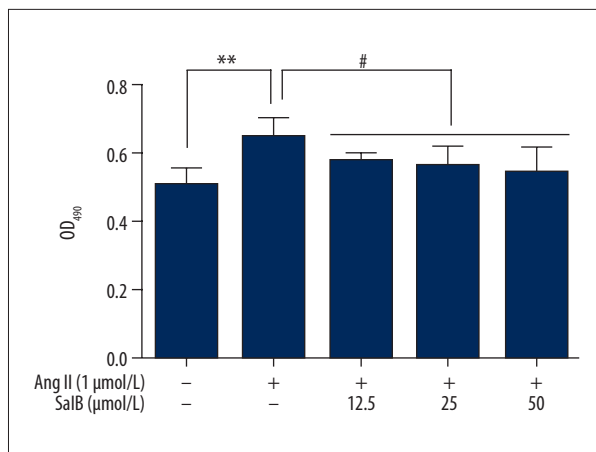
Figure 2. Effect of Salvianolic acid B (SalB) on Ang II-induced cardiac fibroblasts (CFBs) proliferation. CFBs were pretreated with SalB (12.5, 25, and 50 μmol/L) or without SalB for 1 h, and then co-incubated with Ang II (1 μmol/L) for 24 h. MTT assay. (i) Control; (ii) Ang II; (iii) SalB (12.5 μmol/L); (iv) SalB (25 μmol/L). (v) SalB (50 μmol/L). Results are presented as the mean ±SEM (* $P<0.05$ vs. control, ** $P<0.01$ vs. control; # $P<0.05$ vs. Ang II group, ## $P<0.01$ vs. Ang II group, n=6). Ang II – Angiotensin II; Sal B – salvianolic acid B.

Ang II (1 μmol/L) for 24 h. The MTT assay showed that Ang II significantly increased CFBs proliferation compared with control cells ($P<0.01$). However, pretreatment with SalB inhibited the Ang II-induced increase in CFBs proliferation ($P<0.05$) (Figure 2).

Inhibition effects of SalB on the migration ability of CFBs induced by Ang II

After exposure of CFBs to Ang II, which significantly enhanced cell migration ability compared to control (63.3±6.2% versus 32.1±6.3%), SalB significantly reversed the increase induced by Ang II (40.1±1.6% vs. 63.3±6.2% for SalB (12.5 μmol/L); 26.7±6.3% vs. 63.3±6.2% for SalB (25 μmol/L); and 22.9±1.8% vs. 63.3±6.2% for SalB (50 μmol/L)) detected by scratch wound-healing assay. The results are shown in Figure 3.

## Effects of SalB on Coll I, FN, and CTGF induced by Ang II in CFBs

Upregulation of CTGF in fibrotic tissue appears to be closely and positively correlated with the severity of fibrosis and is considered to be a critical marker of cardiac fibrosis. Coll I and FN are 2 key ECM proteins, which are indispensable for directing cell attachment and migration. As shown in Figure 4A–4C, the protein levels of Coll I, FN, and CTGF were significantly increased after Ang II (1 μmol/L) stimulation for 24 h ($P<0.01$), as shown by Western blotting analysis. However, pretreatment with SalB inhibited the Ang II-induced upregulation of Coll I, FN, and CTGF expression.

SalB blocked Ang II-induced activation of nuclear transcription factor-kappa B (NF-κB) in CFBs

Accumulating evidence confirms that NF-κB activation leads to myocardial hypertrophy, myocardial fibrosis, and heart failure. As shown in Figures 5A, 5C, in Ang II-treated CFBs, the expression of phospho-IκBα (p-IκBα) and phospho-p65 (p-p65) were significantly enhanced compared with control cells ($P<0.01$). Pretreatment with SalB attenuated the Ang II-induced increases in p-IκBα and p-p65 expressions in CFBs but had no influence on the total expressions of IκBα and P65 (Figure 5B, 5D). Subsequently, the relative nuclear level of NF-κB p65 subunit and the cytoplasmic level of NF-κB p65 were examined by Western blotting analysis. As shown in Figure 5E, 5F, the nuclear p65 level was significantly elevated in CFBs. Treatment with 50 μmol/L SalB for 1 h markedly blocked the increase in p65 expression induced by Ang II in nuclei. In addition, the mRNA expression of NF-κB as detected by qRT-PCR showed that exposure of CFBs to Ang II significantly increased the mRNA expression of NF-κB compared to control cells. However, pretreatment with SalB attenuated the Ang II-induced increases in the mRNA expression of NF-κB (Figure 5G). Therefore, pre-incubation of CFBs with SalB can block NF-κB activation induced by Ang II.

SalB inhibited cardiac fibrosis induced by Ang II via NF-κB signaling pathway in CFBs

In order to further investigate whether SalB inhibits AngII-induced myocardial fibrosis through the NF-κB signaling pathway, the cultured rat CFBs were treated with pyrrolidine dithiocarbamate (PDTC), an inhibitor for NF-κB, or SalB followed by AngII stimulation for 24 h. MTT results shown that Ang II significantly increased CFBs proliferation compared with control cells, while pretreatment with PDTC or Sal B (50 μmol/L) markedly inhibited Ang II-induced increase in CFBs proliferation (Figure 6A). Immunofluorescence staining was used to observe fluorescence staining signals for α-SMA expression (green), which is considered a biomarker of the differentiation of fibroblasts into myofibroblasts, and Western blot analysis was used to detect expression of Coll I and α-SMA in CFBs. After stimulation with Ang II (1 μmol/L) for 24 h, there was a significantly increased expression of Coll I (Figure 6B) and secretion of collagen in supernatant (Figure 6C) in CFBs compared with the control group, while the upregulation was markedly inhibited by co-incubation with PDTC or Sal B (50 μmol/L). As shown in Figure 6D, after exposure of CFBs to Ang II (1 μmol/L) for 24 h, CFBs exhibited bright fluorescence staining signals for α-SMA expression (green) compared with the control group, while pretreatment with PDTC or Sal B (50 μmol/L) markedly inhibited the expression of α-SMA. These results were also validated by Western blot analysis (Figure 6E). However, there was no significant difference of SalB and PDTC compared to
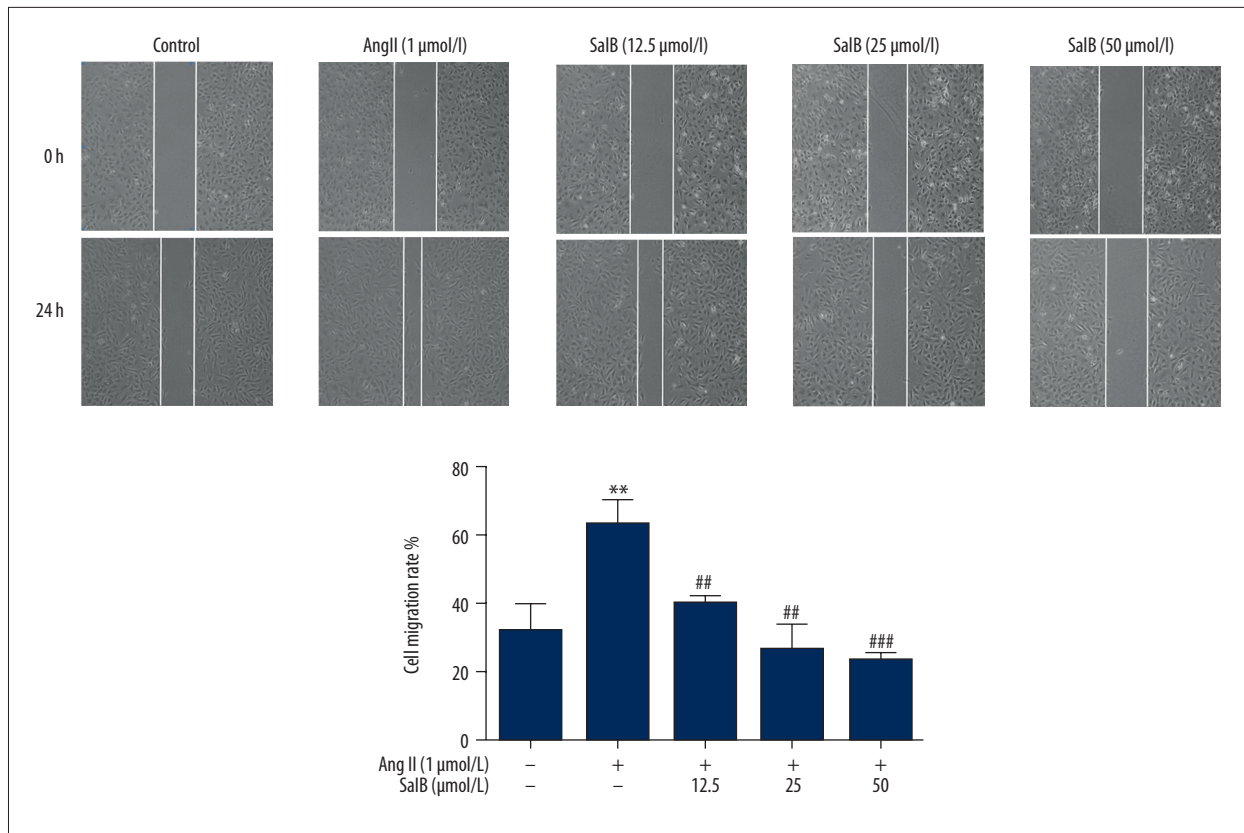
Figure 3. Effects of treatments with Salvianolic acid B (SalB) on the migration ability of cardiac fibroblasts (CFBs) induced by Ang II (magnification, ×50). Cells were treated with SalB (12.5, 25, and 50 μmol/L) or without SalB for 1 h, and then co-incubated with Ang II (1 μmol/L) for 24 h. Data are expressed as the mean ±SEM (n=3). * $P<0.05$ *vs.* control, ** $P<0.01$ *vs.* control; ## $P<0.01$ *vs.* Ang II group, ### $P<0.001$ *vs.* Ang II group. Ang II – Angiotensin II; Sal B – salvianolic acid B.

PDTC alone in CFBs proliferation and in levels of expression of Coll I and α-SMA induced by Ang II. Our results suggest SalB inhibits Ang II-induced cardiac fibrosis via NF-κB signaling.

Discussion

Cardiovascular diseases (CVDs) are a class of disorders that involve the heart and blood vessels, including coronary heart disease, rheumatic heart disease, peripheral vascular disease, heart failure, congenital heart disease, and cardiomyopathies [20,21]. It is becoming a global health burden and is still the leading cause of morbidity and mortality worldwide [22,23]. Cardiac fibrosis develops in pathological changes to various CVDs, including persisting hypertension, hypertropic cardiomyopathy, ischemic insults, and congenital heart defects [24,25]. Cardiac fibrosis is characterized by accumulation of cardiac fibroblasts (CFBs) and excessive deposition of extracellular matrix (ECM) [26]. Myofibroblasts differentiated from CFBs are mainly responsible for cardiac fibrosis [27]. Therefore, control of abnormal CFBs proliferation and differentiation into myofibroblasts is critical to attenuate cardiac fibrosis.

It has been confirmed that the renin-angiotensin-system (RAAS) is a key endocrine signal factor involved in organic fibrosis. Ang II, the major mediator of the RAAS, contributes to a range of cardiovascular pathologies. It has been confirmed that Ang II increases collagen expression, proliferation, and migration in CFBs by activating a variety of cell signaling pathways that promote the differentiation, proliferation, and migration of CFBs [28]. In this study, to contribute to the development of novel therapeutic strategies for cardiovascular disease, we investigated the ability of Salvianolic Acid B (SalB) to inhibit Ang II-induced CFBs proliferation and differentiation *in vitro* and explored the associated mechanisms.

Proliferation, migration, and differentiation of CFBs play a central role in cardiac fibrosis [29]. In addition, Coll I, FN, and CTGF, mainly produced by fibroblasts, are the important ECM proteins, and abnormal synthesis of these proteins indicate cardiac fibroblast activation [30]. In this study we found that Ang II stimulation significantly increased the proliferation and migration ability of CFBs, as well as the expression of Coll I, CTGF, and FN, which were significantly depressed by the pretreatment with SalB. Therefore, our findings reveal that SalB
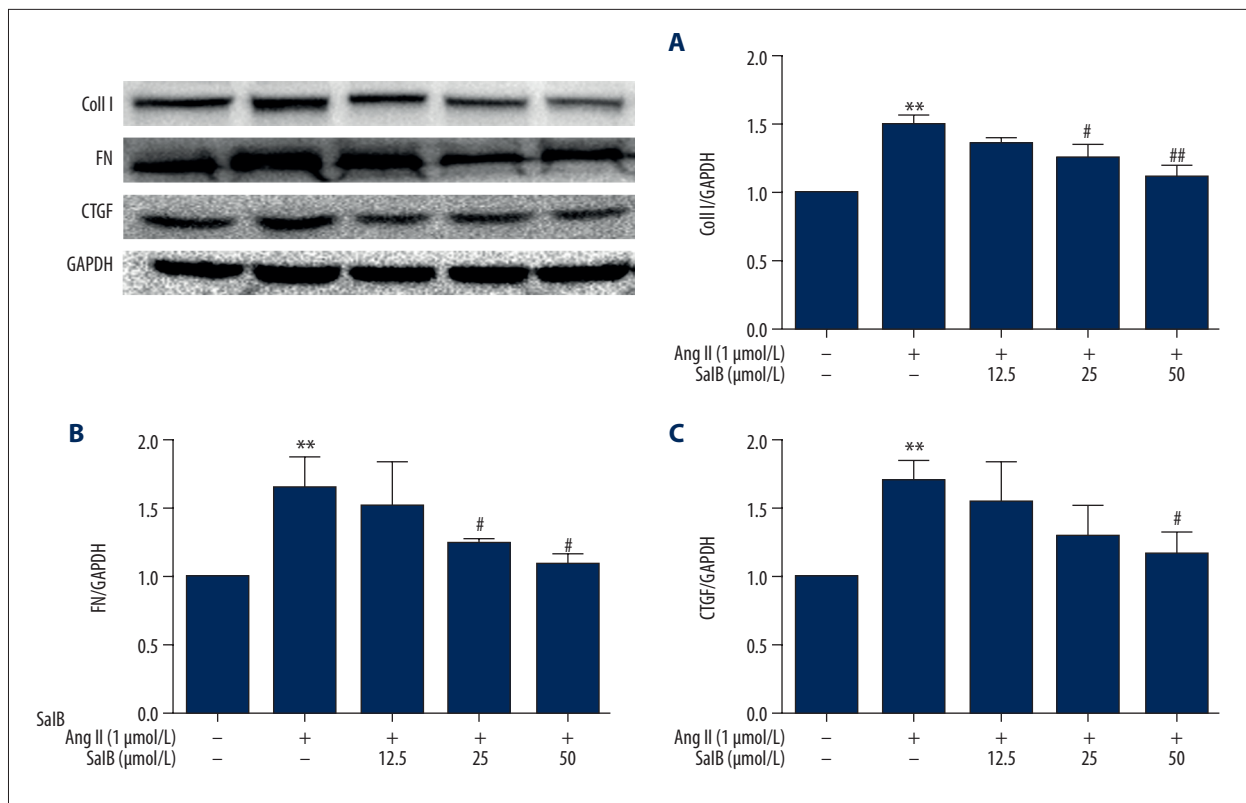
Figure 4. Effects of Ang II and Salvianolic acid B (SalB) on Ang II-induced upregulation of Coll I, FN, and CTGF in cardiac fibroblasts (CFBs). Cells were treated with Ang II (1 μmol/L) and/or SalB (12.5, 25, and 50 μmol/L, 1 h prior to Ang II stimulation) for 24 h. The expression of Coll I (**A**), FN (**B**), and CTGF (**C**) were identified by Western blot analysis. Data are expressed as mean ±SEM (n=3). * *P*<0.05 *vs.* control, ** *P*<0.01 *vs.* control; # *P*<0.05 *vs.* AngII group, ## *P*<0.05 *vs.* AngII group.

can ameliorate Ang II-induced cardiac fibrosis by suppressing the proliferation and migration ability of activated CFBs, inhibiting the differentiation of CFBs as evaluated by expression of α-SMA and reducing pro-fibrotic cytokine and ECM accumulation as assessed by CTGF, Coll I, and FN expression.

However, its effect and associated mechanisms need further study. NF-κB is a key regulator of inflammatory reaction. Activation of NF-κB signaling results in the synthesis and release of numbers of cytokine and chemotactic factors that can induce the expression of α-SMA (α-SMA is a protein marker of myofibroblasts) and other fibrosis-related genes, eventually contributing to myofibroblast differentiation and cardiac fibrosis [31]. Accumulating evidence suggests that NF-κB is involved in the development of pathologic cardiac remodeling. NF-κB activation leads to myocardial hypertrophy, myocardial fibrosis, and subsequent heart failure [32–35]. NF-κB is held inactive in a cytoplasmic complex with its inhibitor, IκB. NF-κB can be activated for IκBα phosphorylation, inducing NF-κB translocation to the nucleus [36–38]. NF-κB is the key player in fibroblast growth and collagen expression mediated by Ang II and TNF-α [39]. In this study, we revealed that Ang II significantly increased the expression of p-IκBα and p-p65 and translocation

of NF-κB p65 from the cytoplasm to the nucleus, which indicates that NF-κB is activated upon stimulation with Ang II. Our results show that Sal B can significantly inhibit Ang II-induced expression of p-IκBα and p-p65 and translocation of NF-κB p65 from the cytoplasm to the nucleus as compared with cells treated with AngII alone. qRT-PCR results show that pretreatment with SalB attenuated the Ang II-induced increases the mRNA expression of NF-κB. Nevertheless, the NF-κB inhibitor PDTC was applied to inhibit NF-κB activation in order to further investigate the role of NF-κB activation of SalB in inhibiting Ang II-induced cardiac fibrosis. Results from the Western blot analysis and MTT assay verified that pretreatment with PDTC or SalB also inhibited CFBs proliferation and reduced α-SMA and Coll I expression induced by Ang II. In addition, Hyp, a degradation production of collagen, is an index of collagen secretion, which, to an extent, can reflect myofibroblast secretion activity. Hydroxyproline (Hyp) was measured using a commercial colorimetric assay. Ang II increased the Hyp content of the cell supernatant of cultured CFBs. Compared to cells treated with Ang II, pretreatment with PDTC or SalB significantly reduced the Ang II -induced increase in the Hyp content of the cell supernatant.
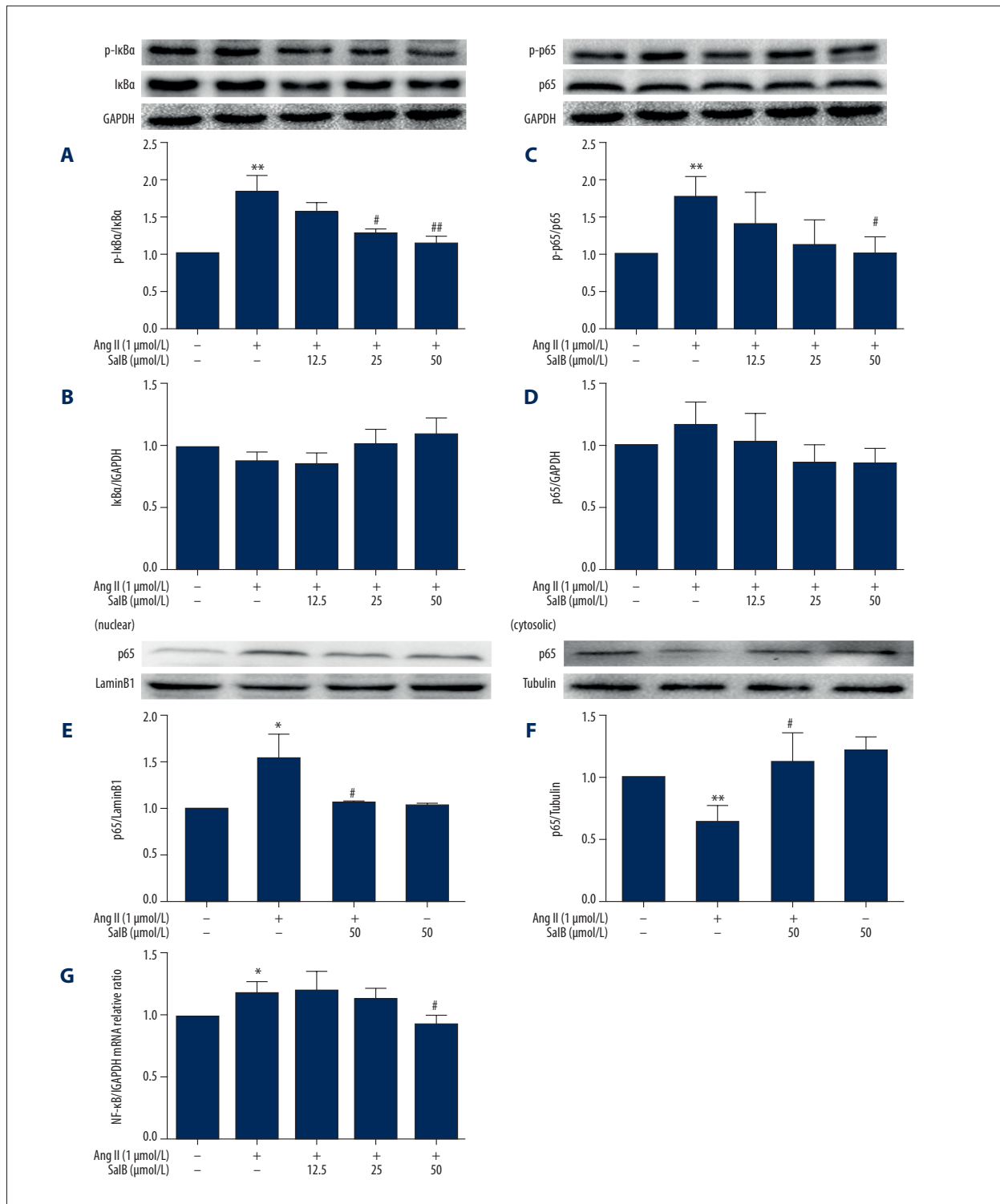
Figure 5. Salvianolic acid B (SalB) blocks Ang II-induced nuclear transcription factor-kappa B (NF-κB) activation in cardiac fibroblasts (CFBs). Cells were treated with SalB (12.5, 25, and 50 μmol/L) or with SalB (50 μmol/L) for 1 h, and then co-incubated with Ang II (1 μmol/L) for 24 h. Western blotting analysis of (**A**) p-IκBα, (**B**) IκBα, (**C**) p-p65, (**D**) p65, (**E**) nuclear factor-κB p65, and (**F**) cytosolic NF-κB p65. (**G**) qRT-PCR analysis the mRNA relative expression levels of NF-κB. Data are presented as the mean ± SEM (n=3). ** $P<0.01$ *vs.* control; # $P<0.05$ *vs.* Ang II group; ## $P<0.01$ *vs.* Ang II group. Ang II – Angiotensin II; Sal B – salvianolic acid B; p – phosphorylated; IκBα – inhibitor kappa-Bα; QRT-PCR – quantitative real-time PCR.
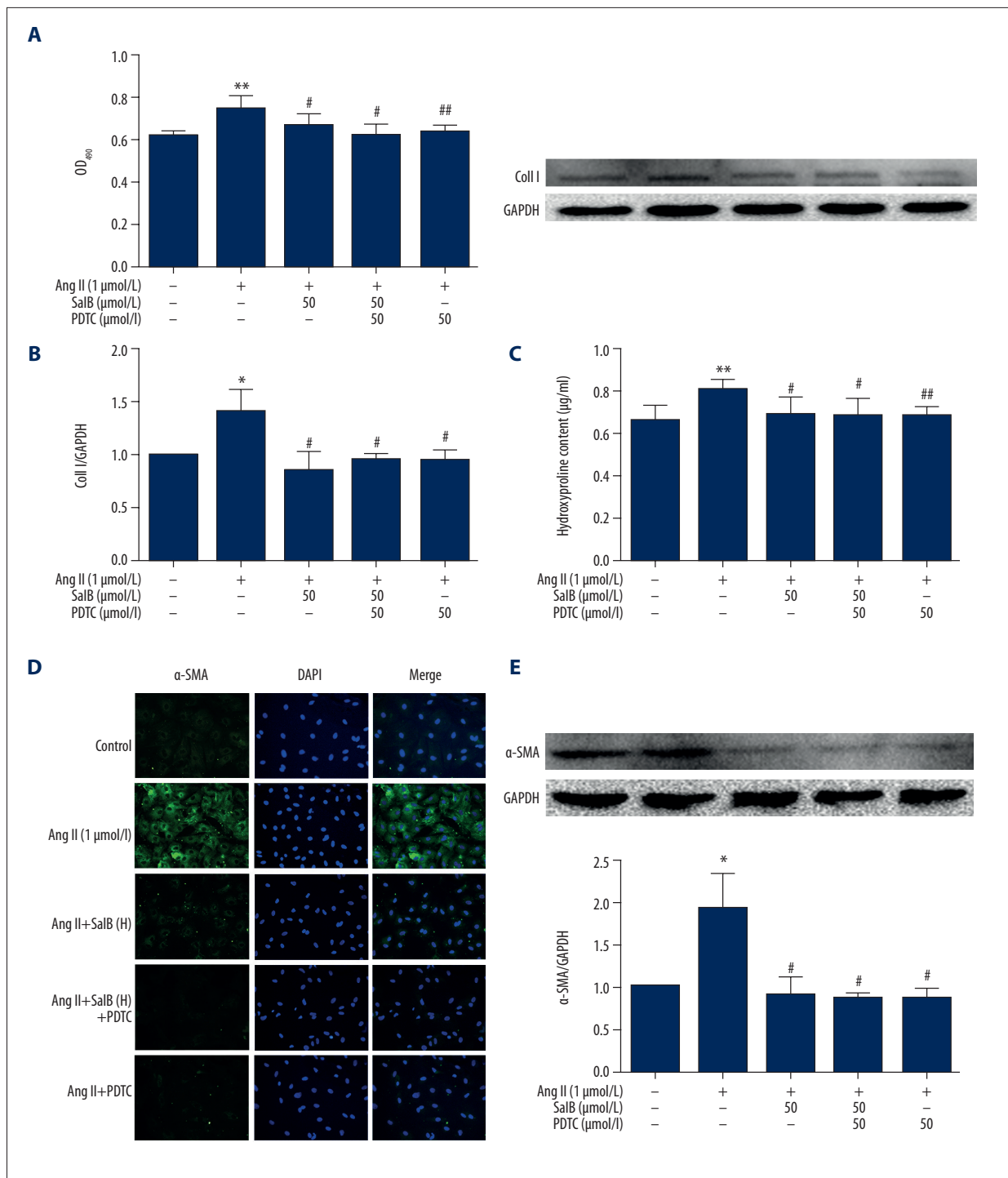
Figure 6. Salvianolic acid B (SalB) inhibits cardiac fibrosis induced by Ang II via NF-κB signaling pathway in cardiac fibroblasts (CFBs). Cells were pretreated with the inhibitor PDTC (50 µmol/L) for 30 min and SalB (50 µmol/L) for 1 h, and then co-incubated with Ang II (1 µmol/L) for 24 h. (**A**) MTT assay; (**B**) Western blot analysis of Coll I expression; (**C**) Analysis of hydroxyproline (Hyp) content in CFBs supernatant; (**D**) Immunofluorescence staining of α-SMA in CFBs. α-SMA and nuclei were stained with FITC (green) and DAPI (blue), respectively (magnification, ×200); (**E**) Western blot analysis of α-SMA expression. Representative Western blot images and quantification are shown. Data are presented as the mean ±SEM (n=3). * $P<0.05$ vs. control; ** $P<0.01$ vs. control; # $P<0.05$ vs. Ang II group; ## $P<0.01$ vs. Ang II group. Coll I – collagen type I; Ang II – Angiotensin II; SalB – salvianolic acid B; PDTC – pyrrolidine dithiocarbamate; CFBs – cardiac fibroblasts.

Our results demonstrate that SalB inhibited proliferation and migration ability, as well as α-SMA, Coll I, CTGF, and FN expression and the Hyp content of the cell supernatant induced by Ang II stimulation in CFBs. On a molecular level, SalB pretreatment inhibited the expression of p-IκBα and p-p65 and translocation of NF-κB p65 from the cytoplasm to the nucleus induced by Ang II in CFBs. In addition, SalB pretreatment reduced the Ang II-induced mRNA expression of NF-κB in CFBs. Therefore, the present study shows that SalB may prevent cardiac fibrosis by decreasing Ang II-induced NF-κB activation, thereby reversing the process of myocardial fibrosis.

Conclusions

Salvianolic acid B can alleviate Ang II-induced cardiac fibrosis via suppressing the NF-κB pathway *in vitro*.

Conflicts of interests

None.

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