British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.01070.x
www.brjpharmacol.org

RESEARCH PAPER

Scutellarin alleviates interstitial fibrosis and cardiac dysfunction of infarct rats by inhibiting TGFβ1 expression and activation of p38-MAPK and ERK1/2

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Keywords
cardiac infarction; ERK1/2; fibrosis; p38-MAPK; scutellarin; TGFβ1

Received
26 April 2010
Revised
24 August 2010
Accepted
24 September 2010

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BACKGROUND AND PURPOSE

Interstitial fibrosis plays a causal role in the development of heart failure after chronic myocardial infarction (MI), and anti-fibrotic therapy represents a promising strategy to mitigate this pathological process. The purpose of this study was to investigate the effect of long-term administration of scutellarin (Scu) on cardiac interstitial fibrosis of myocardial infarct rats and the underlying mechanisms.

EXPERIMENTAL APPROACH

Scu was administered to rats that were subjected to coronary artery ligation. Eight weeks later, its effects on cardiac fibrosis were assessed by examining cardiac function and histology. The number and collagen content of cultured cardiac fibroblasts exposed to angiotensin II (Ang II) were determined after the administration of Scu *in vitro*. Protein expression was detected by Western blot technique, and mRNA levels by quantitative reverse transcription-PCR.

KEY RESULTS

The echocardiographic and haemodynamic measurements showed that Scu improved the impaired cardiac function of infarct rats and decreased interstitial fibrosis. Scu inhibited the expression of FN1 and TGFβ1, but produced no effects on inflammatory cytokines (TNFα, IL-1β and IL-6) in the 8 week infarct hearts. Scu inhibited the proliferation and collagen production of cardiac fibroblasts (CFs) and the up-regulation of FN1 and TGFβ1 induced by Ang II. The enhanced phosphorylation of p38-MAPK and ERK1/2 in both infarct cardiac tissue and cultured CFs challenged by Ang II were suppressed by Scu.

CONCLUSIONS AND IMPLICATIONS

Long-term administration of Scu improved the cardiac function of MI rats by inhibiting interstitial fibrosis, and the mechanisms may involve the suppression of pro-fibrotic cytokine TGFβ1 expression and inhibition of p38 MAPK and ERK1/2 phosphorylation.

Abbreviations

Ang II, angiotensin II; CFs, cardiac fibroblasts; CVF, collagen volume fraction; ERK1/2, extracellular signal-regulated kinase; FN1, fibronectin 1; FS, fractional shortening; IL-1β, interleukin 1β; IL-6, interleukin 6; LVDd, left ventricular diastolic diameter; LVDP, left ventricular distolic pressure; LVEF, left ventricular ejection fraction; LVSd, left ventricular

systolic diameter; LVSP, left ventricular systolic pressure; p38MAPK, p38 mitogen-activated protein kinase; pERK1/2, phosphorylated extracellular signal–regulated kinase; p-P38MAPK, phosphorylated p38 mitogen-activated protein kinase; Scu, scutellarin; TGFβ1, transforming growth factor β1; TNFα, tumour necrosis factor α

Introduction

Currently, chronic heart failure (CHF) remains one of the leading causes of morbidity and mortality worldwide, with cardiac remodelling after myocardial infarction (MI) as the most common underlying factor (Pfeffer, 1995; Sutton and Sharpe, 2000; Yousef *et al.*, 2000). Significant fibrosis, occurring in both the infarcted and non-infarcted myocardium, represents a characteristic pathological alteration of post-infarct remodelling and is recognized to be a major determinant of the progressive deterioration of ventricular function after MI (Pfeffer, 1995; Sutton and Sharpe, 2000; Yousef *et al.*, 2000). Generally, fibrosis is modulated by a cascade of biochemical intracellular signalling processes that is triggered by necrotic myocardium in the context of MI in combination with increased preload and afterload (Pfeffer, 1995; Sutton and Sharpe, 2000; Yousef *et al.*, 2000).

Transforming growth factor-β1 is a key signalling molecule that induces cardiac fibrosis by activating the proliferation and collagen production of cardiac fibroblasts (Bujak and Frangogiannis, 2007). Intracellular mitogen-activated protein kinase (MAPK) signalling cascades were also proven to play an important role in the pathogenesis of cardiac fibrosis (Muslin, 2008). dministration of RWJ-67657, a p38α and p38β inhibitor, to long-term MI rats resulted in suppressed interstitial fibrosis and improved cardiac function (See *et al.*, 2004). Cardiac fibroblasts (CFs), which rapidly proliferate in MI, are responsible for the main deposition of extracellular matrix (ECM). The augmentation of extracellular signal-regulated kinase (ERK1/2) activity caused by the up-regulation of miR-21 enhances CF proliferation and thereby the interstitial fibrosis and cardiac dysfunction (Thum *et al.*, 2008). The activation of the ERK1/2 cascade underlies the angiotensin II (Ang II)-induced proliferation of CFs (Schorb *et al.*, 1995; Stockand and Meszaros, 2003). Although major therapeutic advances have been made in the management of MI, post-infarction CHF is still a common cause of morbidity, hospitalization and premature death. Efficient strategies that could regress fibrosis after infarction would open up a new route for preventing the exacerbation of cardiac function and improving the post-infarct prognosis (Dorn, 2009). Agents that can affect the molecular pathways that are elicited during post-infarct remodelling are promising therapeutic candidates.

Scutellarin (Scu) (Figure 1) is a flavonoid extracted from a traditional Chinese herb, *Erigeron breviscapus (vant.)* Hand-Mazz. *E. breviscapus* Hand Mazz is the main component of the Chinese herbal drug that has been used to treat cardiovascular diseases in China for a long time. Nowadays, various preparations of Scu are still widely used clinically to treat cardiovascular diseases such as hypertension, angina pectoris, coronary heart disease and stroke. Recent experimental studies have indicated that Scu exhibits a variety of pharmacological properties, including decreasing the infarct size of rats with myocardial ischaemia (Li *et al.*, 2004; Lin *et al.*, 2007; Jia *et al.*, 2008), suppressing cardiac hypertrophy (Pan *et al.*, 2010), relaxing rat isolated aortic rings (Pan *et al.*, 2008) and protecting PC12 cells from cobalt chloride-induced apoptosis (Wang *et al.*, 2007). However, there remains a lack of evidence for the role of Scu in post-infarct remodelling. The present study was performed to explore the effects of Scu on myocardial fibrosis after long-term MI and the underlying mechanisms in rats.

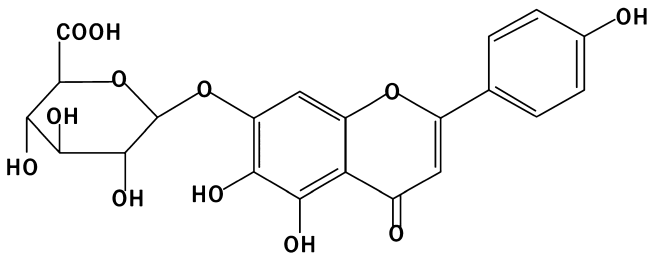

Figure 1
Chemical structure of scutellarin.

Methods

Animals

Healthy male Wistar rats (200–250 g) used in the current study were kept under standard animal room conditions (temperature 21 ± 1°C; humidity 55–60%) with food and water available *ad libitum* for 1 week before the experiment. All experimental procedures were in accordance with the Institutional Animal Care and Use Committee of Harbin Medical University, China.

MI and Scu administration

Male Wistar rats were subjected to left anterior decending (LAD) coronary artery ligation to induce MI as described previously (Yang *et al.*, 2007). Briefly, rats were anaesthetized with 1% sodium phenobarbital (40–60 mg kg^{-1}, i.p.) and intubated orally with a polyethylene tube for artificial respiration (UGO Basile S.R.L. Biological Research Apparatus, Comerio VA, Lombardy, Italy). A thoracotomy was performed at the fourth intercostal space, and the LAD branch was ligated approximately 2 mm from its origin. Sham-operated animals underwent the same procedure but the coronary ligature was left untied (Sham group, $n = 8$). The rats with MI that survived were randomly assigned into four groups: MI, MI + Scu (3, 10 and 30 mg kg^{-1} day^{-1}, i.p.), with nine rats in each group. Drug administration was started 3 days after surgery, and continued for 8 weeks. Sham and MI control rats were injected with the same volume of saline containing DMSO (final concentration = 0.1%). Two additional groups of normal rats ($n = 8$

each) were injected with Scu (30 mg kg^{-1}) (Scu group) or the same volume of saline containing DMSO (final concentration = 0.1%) (normal group).

Echocardiographic and haemodynamic measurements

Four and eight weeks after drug administration, the changes in left ventricular function were evaluated by transthoracic echocardiography with an ultrasound machine (Vivid 7, GE Medical, USA) equipped with a 10-MHz phased-array transducer. Left ventricular systolic diameter (LVSd) and left ventricular diastolic diameter (LVDd) were measured at the same time, and left ventricular ejection fraction and fractional shortening (FS) were calculated from M-mode recording. Haemodynamic studies were carried out 8 weeks after drug administration by use of a pressure volume control unit (Scisense Inc., London, Ontario, Canada) with the degree of anaesthesia adjusted down to the level where rats just started responding to toe pinch. A pressure-sensing catheter (1.9F, Scisense Inc.) was inserted into the left ventricle of the rats via the right common carotid artery. Parameters including heart rate, left ventricular systolic pressure, left ventricular distolic pressure, +dP/dtmax and –dP/dtmax were collected. After the haemodynamic measurements had been obtained, the rats were killed and the heart was removed rapidly and washed in ice-cold 0.9% saline. Parts of the heart were frozen in liquid nitrogen or fixed in 4% paraformalin for later use.

Histological analysis of collagen deposition and infarct size

Eight weeks after drug administration, rats were anaesthetized with 1% sodium phenobarbital (40–60 mg kg^{-1}, i.p.) and the hearts were collected. The right ventricle and atrium were cut off, and the left ventricle were fixed in 4% paraformalin, subjected to paraffin, then cross-sectionally cut into 5 μm thick sections along the centre of the fibrotic scar. Haematoxylin and eosin staining was used to distinguish the infarct and normal area. Masson's trichrome staining was used to evaluate collagen deposition. Sections were imaged at 200× magnification by bright-field microscopy (IX71, Olympus, Tokyo, Japan). The extent of cardiac fibrosis in the peri-infarct region was assessed by calculating collagen volume fraction. Infarct size was assessed by examining images obtained at low magnification and calculated as the ratio of scar average circumferences to LV average inner circumferences. All quantitative evaluations were carried out by ImagePro Plus software (version 6.0, Media Cybernetics, Bethesda, MD, USA).

Quantitative reverse transcription-PCR

Total RNA was extracted using TRIZOL reagent from cultured neonatal cardiac myocytes and cardiac tissues. After reverse transcription, the cDNA obtained was used in quantitative reverse transcription-PCR analysis to determine the expression of TGFβ1, FN1, IL-1β, IL-6, TNFα. The mRNA levels were quantified by SYBR Green incorporation on ABI 7500 fast Real Time PCR system (Applied Biosystems, Carlsbad, CA, USA), and GAPDH was used as an internal control. The sequences of primers were: TGFβ1, forward, 5′-GCGCCTGCAGAGATTCAAGTCAAC-3′, reverse, 5′-GTATCAGTGGGGGTCAGCAGCC-3′; FN1, forward, 5′-ACTTCTGGTCCTCTCCCGTGTCC -3′, reverse, 5′- CGCCC TCTCCAGGAGGCTAGT -3′; IL-1β, forward, 5′- GCT AGGGAGCCCCCTTGTCGAG-3′, reverse, 5′- AGGCAGG GAGGGAAACACACGTT-3′; IL-6, forward, 5′- TCCGCAAGA GACTTCCAGCCAG-3′, reverse, 5′- TGTGAAGTAGGGAA GGCAGTGGC-3′; TNFα, forward, 5′- GCCTCTTCTC ATTCCTGC-3′, reverse, 5′-CTTCTCCTCCTTGTTGGG-3′; GAPDH, forward, 5′- TCTACATGTTCCAGTATGACTC-3′, reverse, 5′-ACTCCACGACATACTCAGCACC-3′.

Isolation and culture of cardiac fibroblasts

The cultured CFs used in these experiments were obtained from neonatal Wistar rats as previously described (Yang *et al.*, 2007), with slight modifications. Briefly, after being treated with 0.25% trypsin solution, fibroblasts were isolated by the removal of myocytes through selective adhesion of non-myocytes at a 1.5 h pre-plating interval. CFs were maintained in DMEM supplemented with penicillin and streptomycin (1%v v^{-1}) and foetal bovine serum (10% v/v). CFs at the 3rd or 4th passage were used in the experiments.

Fibroblast proliferation assay

CF proliferation was measured by counting cell number. CFs were seeded in 24-well tissue culture plates (2×10^4 cells per well) in DMEM supplemented with 10% fetal bovine serum (FBS). After the CFs had been deprived of serum for 24 h by being placed in serum-free DMEM, angiotensin II (Ang II, 1×10^{-7} mol mL^{-1}) alone or in combination with scutellairn (0, 10, 30, 100 μM) was then added to the medium and the CFs were incubated for another 48 h. The CFs were then trypsinized and counted by using a haemocytometer.

Measurement of collagen production

To test the total collagen production, primary cardiac myofibroblasts were seeded in equal numbers in 100-mm cell culture dishes and grown until confluent in DMEM with 10% FBS. The cells were deprived of serum for 48 h before the addition of Ang II and Scu. Then after being incubated for 48 h, the hydroxyproline content in CFs was measured using a hydroxyproline assay kit according to the manufacturer's instruction. The results are expressed as degree of alteration compared with values from control CFs.

Protein isolation and western blot

The total amount of protein was extracted from the cultured CFs and the left ventricular peri-infarct region of rats for immunoblotting analysis, by use of procedures essentially the same as described in detail elsewhere (Yang *et al.*, 2007). Briefly, the protein content was determined by use of a bicinchoninic acid protein assay kit using bovine serum albumin as the standard. Protein samples (100 μg) were subjected to 10% SDS-PAGE and blotted to nitrocellulose. The blots were blocked by 5% non-fat milk and dissolved in phosphate-buffered saline (PBS) for 1 h, then probed with ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-ERK (Santa Cruz Biotechnology), p38-MAPK (Santa Cruz Biotechnology), phospho-p38-MAPK (Cell Signaling Technology, Danvers, MA, USA) antibodies and GAPDH (Kangcheng Inc., Shanghai, China) in PBS and incubated overnight at 4°C. Membranes were washed three times, 15 min each time, with

PBS containing 0.5% Tween 20 (PBS-T) and incubated with secondary antibody (Alexa Fluor, Molecular Probes, Eugene, OR, USA) for 1 h. Western blot bands were collected by using Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and quantified with Odyssey v1.2 software (LI-COR Biosciences, Lincoln, NE, USA) by measuring the band intensity (area × OD) in each group and normalizing this to that obtained with GAPDH as an internal control.

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's *t*-test. Differences were considered to be statistically significant when $P < 0.05$.

Materials

Scu (purity >95%) was purchased from Yunnan Yuxi Wangzilong Pharmaceutical Co., Ltd. (Yuxi City, Yunnan, China). Scu was dissolved in DMSO and diluted with saline before use. The final concentration of DMSO was less than 0.1%. The hydroxyproline assay kit was obtained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Angiotensin II was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Results

Mortality, infarct size and heart weight

One rat in the MI group died after the commencement of treatment 3 days post-MI induction; there was no mortality in the Scu-treated or sham groups. Infarct size was similar in all the groups (Figure 2), indicating that the degree of pro-fibrotic stimulation was the same for all the drug-treated groups. Body weight and left ventricle/body weight ratio were not significantly altered among the groups. The increase in heart/body weight ratio was significantly attenuated with Scu treatment (Table 1). Scu produced no effects on these parameters in normal rats.

Effect of Scu on cardiac function evaluated by echocardiography

Echocardiography was performed at 4 and 8 weeks post-Scu administration (Table 2). At both time points, the MI hearts were significantly dilated as evidenced by an increase in LVDd and LVSd ($P < 0.01$), while eject fraction (EF) and FS were significantly decreased, indicating impaired cardiac function. Scu treatment significantly attenuated the increase in left ventricle dimension and the deterioration of left ventricular performance as indicated by increased EF and FS (Table 2). Scu (30 mg kg^{-1}) produced no effects on the echocardiographic parameters of normal rats.

Effect of Scu on haemodynamic parameters

Eight weeks after Scu treatment, the haemodynamic parameters of rats were measured before scarification. Compared with control rats, MI rats demonstrated a significant increase

Table 1
Tissue weights of rats before and after MI and treatment with scutellarin

Group	Normal ($n = 8$)	Scutellarin (30 mg kg^{-1} day^{-1} $n = 8$)	Sham ($n = 8$)	MI ($n = 8$)	MI + Scu (3 mg kg^{-1} day^{-1} $n = 9$)	MI + Scu (10 mg kg^{-1} day^{-1} $n = 9$)	MI + Scu (30 mg kg^{-1} day^{-1} $n = 9$)
Body weight (g)	366 ± 13	361 ± 11	367 ± 12	348 ± 13	362 ± 12	358 ± 11	368 ± 9
HW/BW ratio (mg g^{-1})	3.13 ± 0.14	3.15 ± 0.07	3.16 ± 0.09	3.74 ± 0.11$^{\#}$	3.61 ± 0.16	3.51 ± 0.14	3.22 ± 0.08*
LVW/BW ratio (mg g^{-1})	2.34 ± 0.07	2.37 ± 0.1	2.36 ± 0.08	2.71 ± 0.09	2.6 ± 0.09	2.47 ± 0.07	2.43 ± 0.1

$^{\#}P < 0.05$ versus sham; *$P < 0.05$ versus MI.
Data are expressed as mean ± SEM.
MI, myocardial infarction; Scu, scutellarin; HW, heart weight; BW, body weight; LVW, left ventricle weight.

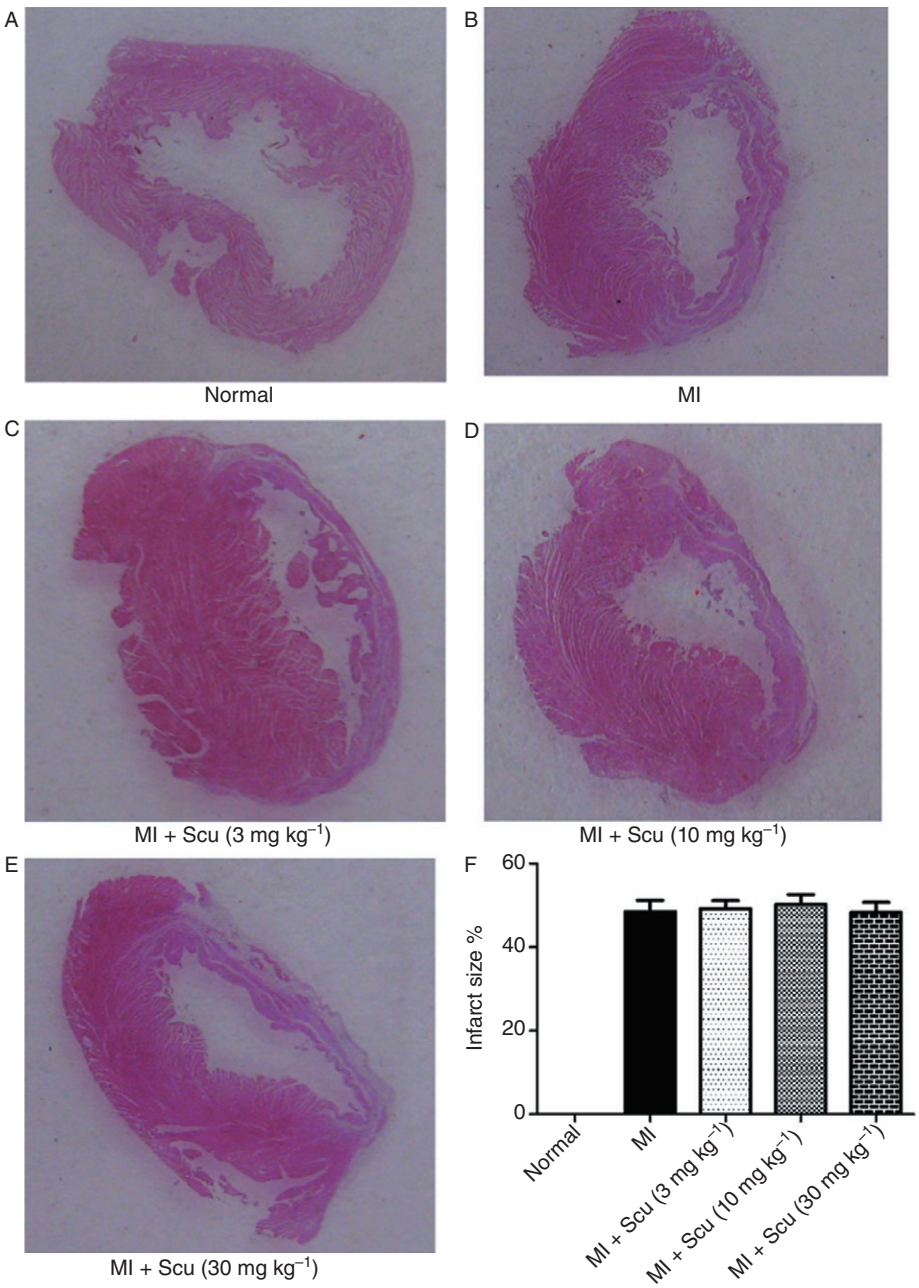

Figure 2

Effects of scutellarin (Scu) on infarct size. (A–E) Representative pictures of infarct heart. (F) Histogram depicting the effects of scutellarin on infarct size. Data are expressed as mean ± SEM, n = 8–9. MI, myocardial infarction.

in left ventricular end-diastolic pressure (LVEDP) (4.1 ± 0.68 vs. 14.6 ± 1.72 mm Hg for MI, $P < 0.01$) and decreases in dP/dtmax (7483 ± 135 vs. 5716 ± 199 mm Hg s^{-1} for MI, $P < 0.01$) and –dP/dtmax (6456 ± 210 vs. 4816 ± 177 mmHg s^{-1} for MI, $P < 0.01$) (Figure 3). These changes were alleviated by Scu in a dose-dependent manner. At the dose of 30 mg kg^{-1}, Scu markedly improved cardiac function by decreasing LVEDP (7.1 ± 0.8 mm Hg) and increasing dP/dtmax (7029 ± 212 mm Hg s^{-1}) and –dP/dtmax (6130 ± 275 mm Hg s^{-1}) ($P < 0.01$ vs. MI) (Figure 3). Scu (30 mg kg^{-1}) did not affect the haemodynamic parameters of normal rats (Figure 3). The mean arterial pressure among the groups did not change (Figure 3E).

Effect of Scu on cardiac interstitial fibrosis post-myocardial infarction

ECM deposition in the peri-infarct region was assessed using Masson's trichrome staining of the histological sections of the hearts. The interstitial fibrotic area of MI rats increased significantly compared with control animals (0.8 ± 0.1% for

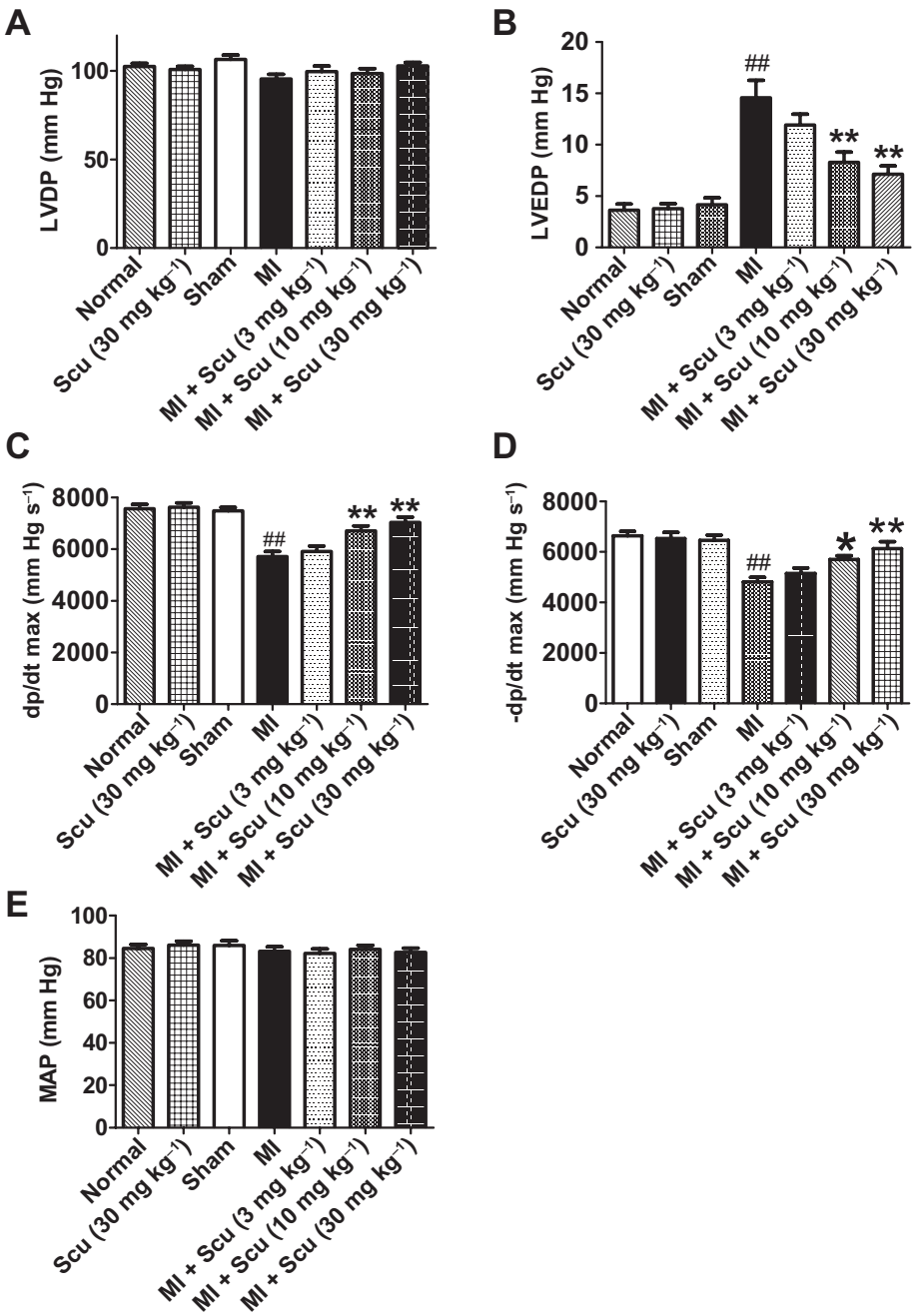

Figure 3

Effects of scutellarin (Scu) treatment on the haemodynamic effects of myocardial infarction (MI) in rats. (A) Left ventricular developed pressure, LVDP; (B) left ventricular end-diastolic pressure, LVEDP; (C) +dP/dt max; (D) –dP/dt max; (E) MAP, mean arterial pressure. Data are expressed as mean ± SEM, n = 8–9. ##P < 0.01 versus sham; *P < 0.05, **P < 0.01 versus MI.

control vs. 16 ± 1.5% for MI, P < 0.01) (Figure 4). Treatment with Scu at doses of 3, 10 and 30 mg kg^{-1} resulted in a dose-dependent reduction in ECM deposition, with a fibrotic area of 2.4 ± 0.3% in the 30 mg kg^{-1} Scu group (Figure 4). Scu (30 mg kg^{-1}) did not affect the cardiac collagen deposits of normal rats (Figure 4).

The mRNA expression of fibronectin 1 (FN1), a glycoprotein component of the ECM, was examined. In the peri-infarct region of 8-week MI rats, FN1 increased by 5.8-fold compared with the sham group (P < 0.01). Treatment with Scu significantly attenuated this increase in FN1. At the doses of 10 and 30 mg kg^{-1}, the FN1 levels decreased by 38 and 71%, respectively, compared with untreated MI rats (Figure 5A).

In addition, the mRNA levels of the pro-fibrotic cytokine TGFβ1 and the pro-inflammatory cytokines IL-1β, IL-6, TNFα were determined in the peri-infarct cardiac tissues. In 8-week MI hearts, the expression of TGFβ1 was significantly

Table 2
Echocardiography of MI rats after treatment with scutellarin for 4 and 8 weeks

Group	Normal (n = 8)	Scutellarin (30 mg kg^{-1} day^{-1} n = 8)	Sham (n = 8)	MI (n = 8)	MI + Scu (3 mg kg^{-1} day^{-1} n = 9)	MI + Scu (10 mg kg^{-1} day^{-1} n = 9)	MI + Scu (30 mg kg^{-1} day^{-1} n = 9)
4 weeks							
LVDd (mm)	6.09 ± 0.21	6.17 ± 0.13	6.15 ± 0.17	9.35 ± 0.29$^{\#\#}$	8.94 ± 0.66	8.57 ± 0.45	8.31 ± 0.23
LVSd (mm)	3.56 ± 0.17	3.67 ± 0.18	3.73 ± 0.14	8.01 ± 0.21$^{\#\#}$	7.5 ± 0.59	6.87 ± 0.37	6.57 ± 0.33
FS (%)	40.2 ± 2.16	38.9 ± 1.87	39.5 ± 1.59	14.6 ± 1.13$^{\#\#}$	16.3 ± 1.14	19.8 ± 1.54	22 ± 2.04*
EF (%)	75.8 ± 2.21	74.3 ± 1.96	76.6 ± 1.65	27.7 ± 1.5$^{\#\#}$	36.9 ± 1.36	43.5 ± 2.67**	50.1 ± 3.66**
8 weeks							
LVDd (mm)	6.49 ± 0.27	6.71 ± 0.18	6.58 ± 0.2	9.84 ± 0.32$^{\#\#}$	9.67 ± 0.44	9.48 ± 0.45	8.9 ± 0.55
LVSd (mm)	4.04 ± 0.16	3.93 ± 0.23	3.9 ± 0.21	8.36 ± 0.15$^{\#\#}$	8.17 ± 0.48	7.43 ± 0.27*	6.92 ± 0.28**
FS (%)	42.4 ± 2.01	43.5 ± 1.69	41.3 ± 1.07	15.1 ± 1.08$^{\#\#}$	16.1 ± 1.48	20.5 ± 1.37	22.9 ± 1.73**
EF (%)	77.5 ± 2.81	78.3 ± 1.49	79.4 ± 1.38	25.1 ± 1.06$^{\#\#}$	32.3 ± 1.36	42 ± 2.91**	48.9 ± 2.49**

$^{\#\#}P < 0.01$ versus sham; *$P < 0.05$ versus MI; **$P < 0.01$ versus MI.
Data are expressed as mean ± SEM.
MI, myocardial infarction; Scu, scutellarin; EF, eject fraction; FS, fractional shortening; LVDd, left ventricle diastolic diameter; LVSd, left ventricle systolic diameter.

increased compared with that in sham hearts ($P < 0.01$), and this increase was attenuated by the administration of Scu (Figure 5B). The expressions of IL-1β, IL-6, TNFα did not differ between the groups (Figure 5C).

Effect of Scu on the proliferation of cultured CFs and the expression of FN1 and TGFβ1

The effect of Scu on the proliferation of CFs was assessed by counting the number of cells. Treatment of CFs with 100 nM Ang II led to a 40% increase in the number of CFs, and the concomitant administration of Scu dose dependently inhibited this proliferation of CFs (Figure 6A). To determine whether the protein level of the main component of ECM (i.e. collagen) is altered by Scu, total collagen content was measured in cultured CFs using a hydroxyproline assay. The exposure of CFs to Ang II (100 nM) caused a significant up-regulation of collagen production ($P < 0.01$), which was attenuated by the co-application of Scu at doses of 30 and 100 μM respectively (Figure 6B). Scu also significantly suppressed the expression of FN1 (Figure 6C) and TGFβ1 (Figure 6D) at doses of 30 and 100 μM.

Effect of Scu on ERK and p-ERK expression

To further explore the underlying molecular mechanism of Scu's effect on collagen production and deposition, the expression of ERK and p-ERK was examined in the cultured CFs and in the peri-infarct region of cardiac infarct rats. Treatment of CFs with Ang II (100 nM) significantly activated the phosphorylation of ERK, with p-ERK increased by about 68% ($P < 0.01$) compared with control cells, while the expression of total ERK was not affected (Figure 7A,B). Co-application of Scu significantly inhibited the activation of ERK at doses of 10, 30 and 100 μM. The same qualitative results were obtained from the infarct hearts of rats (Figure 7C,D), with pERK increased in the peri-infarct region and suppressed by the administration of Scu, but with no change in total ERK levels.

Effect of Scu on p38MAPK and p-p38MAPK expression

The enhanced activation of p38MAPK has been reported to participate in cardiac fibrosis (See *et al.*, 2004). We found that in cultured CFs, AngII (100 nM) caused a significant increase in the phosphorylation of p38MAPK, with p-p38MAPK increased by 72% in the Ang II group compared with the control group. Co-application of Scu significantly inhibited the activation of p38MAPK (Figure 7E). The expression of total p38MAPK did not change (Figure 7F). In infarct hearts of rats, the expression of p-p38MAPK was also increased by about 120%; this was inhibited by the 8 week application of Scu (Figure 7G). Similarly, the expression of total p38MAPK was not altered (Figure 7H).

Discussion and conclusions

At the early stage, fibrosis after MI is an integral component of the reparative process, maintaining structural integrity of the necrotic area by the formation of a connective tissue scar. However, in the long run, the continuous remodelling of the

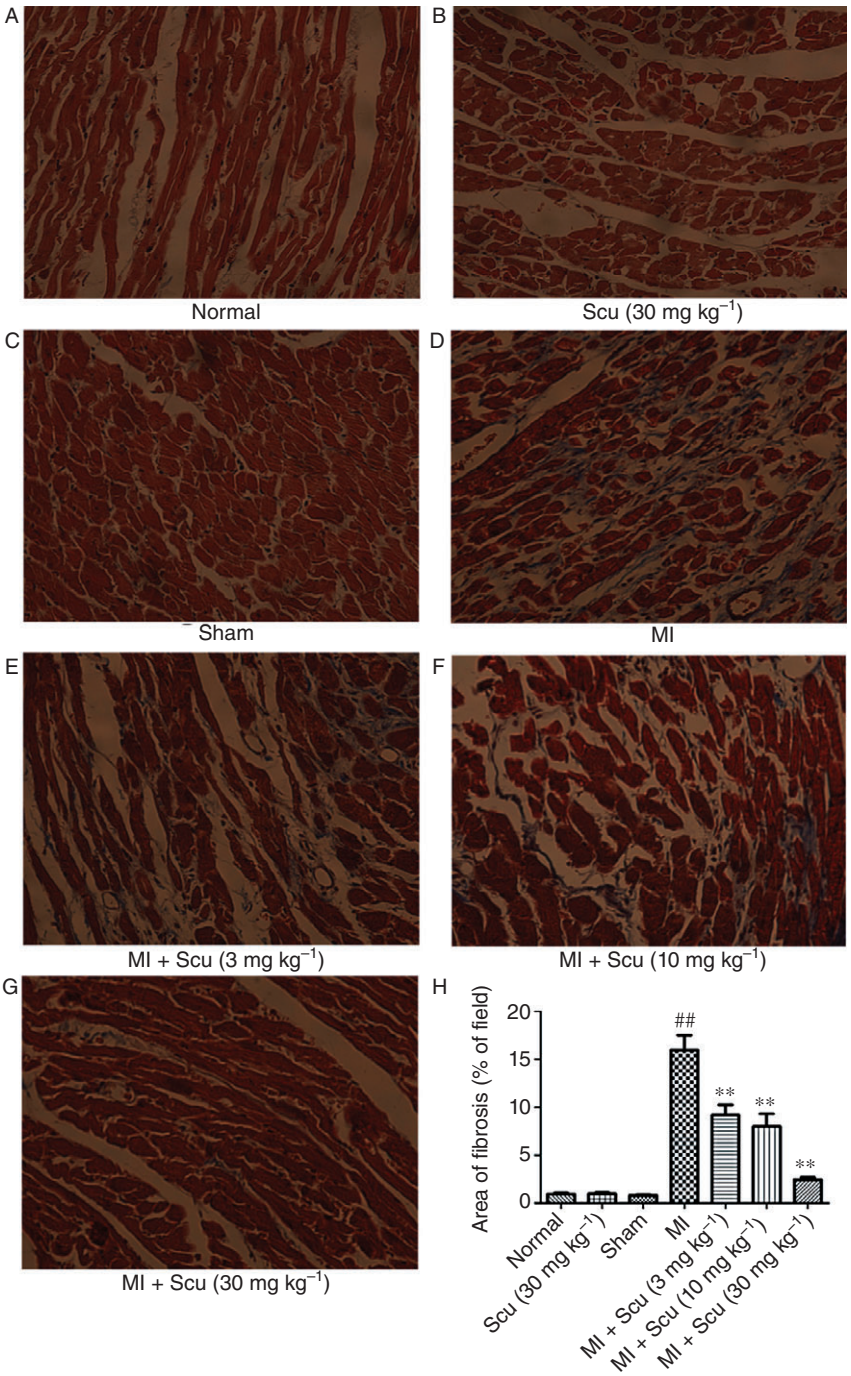

Figure 4

Effects of scutellarin (Scu) on the deposition of collagen in the peri-infarct region during myocardial infarction (MI) in rats. (A–G) Representative sections of heart stained with Masson's trichrome viewed at a magnification of 200×. The fibrotic area is stained blue and the viable area red. (H) Collagen deposition was quantified by automated image analysis and expressed as percentage of tissue area. Data are expressed as mean ± SEM, $n = 5$. ##$P < 0.01$ versus sham; **$P < 0.01$ versus MI.

infarct left ventricle will lead to progressive ventricular dilatation, decreased cardiac performance and eventually chronic heart failure (Weber, 1997; Kurrelmeyer *et al.*, 1998). In this process, the accumulation of collagenous material remote from the site of infarction is deeply involved; this contributes greatly to the reduced myocardial elasticity and impaired contractility (Boluyt *et al.*, 1994; Hein *et al.*, 2003). The extent of the detrimental remodelling predicts morbidity and mortality (Pfeffer and Braunwald, 1990; White and Braunwald, 1998). Despite the use of agents such as angiotensin converting enzyme-inhibitors, angiotensin II type 1 receptor blockers and β-adrenoceptor blocking agents, heart failure can still

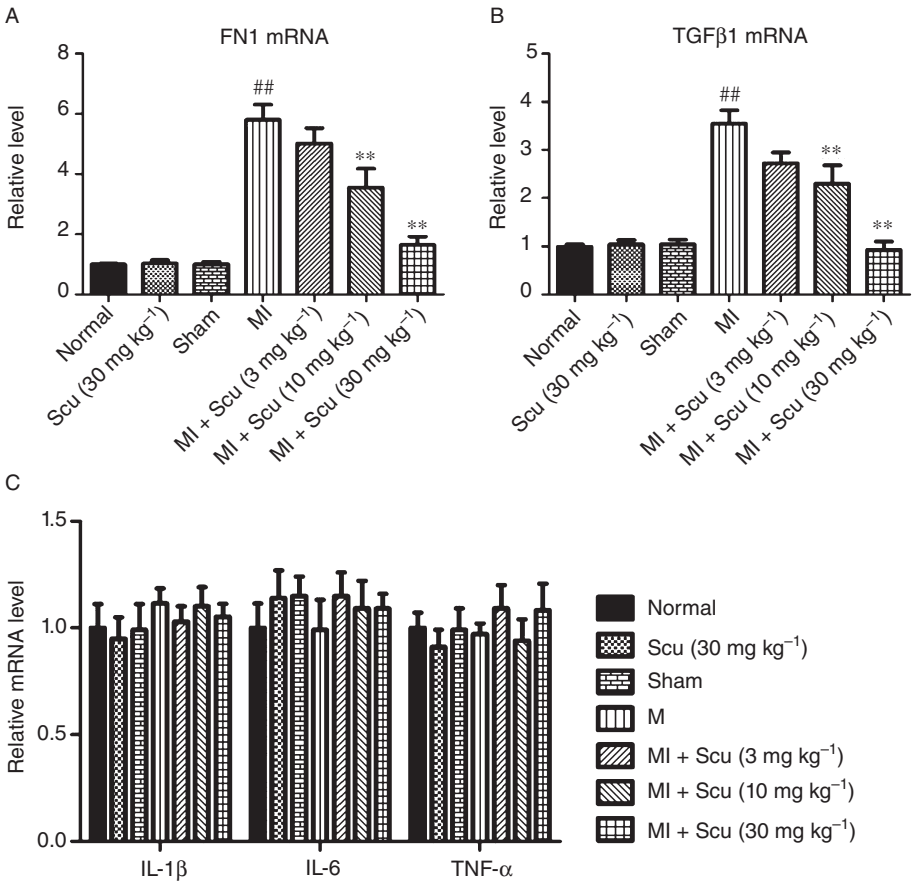

Figure 5

Effects of scutellarin (Scu) on the mRNA levels of fibronectin 1 (FN1) (A), TGFβ1 (B) and pro-inflammatory cytokines (IL-1β, IL-6, TNFα) (C) in peri-infarct cardiac tissues. All values are expressed as mean ± SEM (n = 4). ##P < 0.01 versus sham; **P < 0.01 compared with MI group.

occur. Strategies that inhibit cardiac remodelling such as fibrosis following MI represent one of the major therapeutic goals of modern cardiology (Vanhoutte *et al.*, 2006). Strikingly, herbal compounds such as sodium tanshinone IIA (Yang *et al.*, 2009) and resveratrol (Lin *et al.*, 2008) were demonstrated to produce beneficial effects on the infarct heart when applied for a long period. In this study, we found that Scu, a compound extracted from the traditional Chinese medicine *E. breviscapus* Hand Mazz, can improve the impaired cardiac function of chronic MI rats.

Other studies (Li *et al.*, 2004; Lin *et al.*, 2007; Jia *et al.*, 2008) have demonstrated that Scu protects rats from cardiac ischaemic injury, and the mechanism may involve the ability of Scu to attenuate the increment of intracellular free calcium in cultured neonatal ventricular myocytes (Li *et al.*, 2004). However, although these studies clarified the protective effects of Scu against acute cardiac injury, it was still unclear whether post-infarct administration of Scu could hinder the progressive deterioration of cardiac function. Our study confirmed that the treatment of MI rats with Scu for either 4 or 8 weeks significantly improved left ventricular function, as manifested by changes in the haemodynamic parameters, increased dP/dtmax and decreased LVEDP, and echocardiographic parameters, increased FS and EF, in comparision with sham rats.

Reactive fibrosis in the non-infarcted region, which adversely affects myocardial stiffness, is one of the major pathological alterations that participate in the development of heart failure after MI. In this study, we found that treatment with Scu apparently attenuated interstitial fibrosis, indicating a possible mechanism for its ability to improve cardiac function. The rapid proliferation of cardiac fibroblasts is responsible for the deposition of ECM and fibrosis, thus we examined the effects of Scu on the proliferation of cardiac fibroblasts and collagen production. We found that Scu significantly inhibited the increase in the number of cardiac fibroblasts induced by Ang II and the production of collagen, which explains the inhibitory effects of Scu on interstitial fibrosis after MI.

The molecular mechanism of interstitial fibrosis is very complex, involving the participation of a cascade of biochemical intracellular signalling processes. TGFβ1 is a key pro-fibrotic cytokine that is markedly elevated in experimental MI, and anti-TGF gene therapy mitigates cardiac remodelling by affecting cardiac fibrosis and infarct tissue dynamics (Okada *et al.*, 2005). The finding that Scu inhibited the expression of TGFβ1 in this study indicates that the suppression of the TGFβ1 pathway underlies the anti-fibrotic action of Scu on post-infarct hearts. The p38 mitogen activated protein kinase (p38 MAPK) cascade has also been shown to

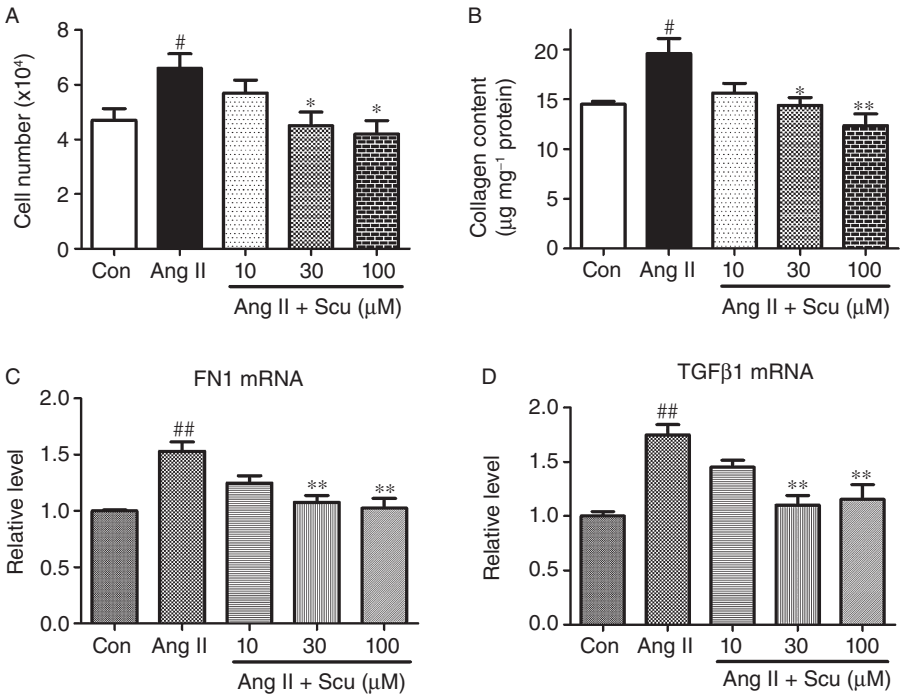

Figure 6

Inhibitory effects of scutellarin on angiotensin II (Ang II, 100 nM)-induced cardiac fibroblast proliferation. (A) Cell number; (B) collagen production; (C) FN1 expression; (D) TGFβ1 expression. Data are expressed as mean ± SEM, $n = 4$ to 5. #$P < 0.05$, ##$P < 0.01$ versus control (Con); *$P < 0.05$, **$P < 0.01$ versus Ang II.

play a critical role in the pathogenesis of cardiac fibrosis (Kompa *et al.*, 2008). In a recent study, Ren *et al.* (2005) demonstrated that long-term (12 weeks) daily treatment of rats with the p38 MAPK inhibitor RWJ67657 and the ACE inhibitor ramipril resulted in a reduction in cardiac fibrosis and hypertrophy accompanied by a beneficial outcome on cardiac function after MI, while brief (1 week) inhibition of p38 MAPK activity after MI seemed to have no ameliorating effect on the progressive ventricular remodelling process (Kompa *et al.*, 2008). Moreover, CFs, which rapidly proliferate after MI, are responsible for the main deposition of ECM, and activation of p38 MAPK underlies the proliferation and collagen production of CFs induced by Ang II (Chen and Mehta, 2006; Wu *et al.*, 2009). Therefore, we hypothesize that the suppression of p38 MAPK cascade may be one underlying mechanism of Scu's beneficial effects on MI hearts. Our data indicate that long-term (8 weeks) treatment of MI rats with Scu significantly inhibited the up-regulation of the active form of p38 MAPK, phospho-p38 MAPK, while having no effect on the total p38 MAPK, the expression of which did not alter after MI. Scu also repressed Ang II-induced activation of p38 MAPK in CFs. These results strongly indicate that the inhibition of p38 MAPK accounts for the anti-fibrotic action of Scu.

Another MAPK cascade, extracellular signal regulated kinase (ERK1/2), was also implicated in the proliferation of CFs and interstitial fibrosis (Thum *et al.*, 2008; Yeh *et al.*, 2010). The activation of ERK1/2 by the up-regulation of miR-21 enhances cardiac fibroblast proliferation and thereby the interstitial fibrosis and cardiac dysfunction (Thum *et al.*, 2008). The ERK1/2 signalling pathway accounts for Ang II-induced collagen I synthesis in the CFs (Olson *et al.*, 2008; Gao *et al.*, 2009). Treatment with resveratrol has been shown to inhibit the activation of ERK1/2 and the proliferation of CFs induced by Ang II (Olson *et al.*, 2005). Our results showed that the activation of ERK1/2 induced by Ang II in CFs was significantly inhibited by Scu, as indicated by a decreased phospho-ERK1/2 expression. Moreover, we found that the expression of phospho-ERK1/2 in the heart of MI rats was significantly up-regulated and this was attenuated by long-term treatment with Scu. These data suggest that the suppression of ERK1/2 activation by Scu represents one of the possible mechanisms accounting for its beneficial effects on impaired cardiac function after MI. However, in terms of the role of ERK1/2 in interstitial fibrosis after MI *in vivo*, the findings so far are controversial. Some researchers have shown that the activity of ERK1/2 is increased after MI in mice (van Deel *et al.*, 2008; Yeh *et al.*, 2010) and rats (Shimizu *et al.*, 1998), while others reported no change after MI in rabbits (Kobayashi *et al.*, 2008) and mice (Takenaka *et al.*, 2009). These disparities may be due to the different animal species used, or MI duration employed. However, the exact mechanism of Scu is currently unclear. Other studies have indicated that the action of Scu may be pleiotropic, as different pathways such as NFKB (Tan *et al.*, 2010), caspase-6 (Chan *et al.*, 2009), PKC (Yan *et al.*, 2010), Ca^{2+} (Pan *et al.*, 2008; Pan *et al.*, 2010) have been shown to be involved in its action. In this study, long-term suppression of p38MAPK and ERK1/2 by Scu was demonstrated to be beneficial for the post-infarction heart. However, as the

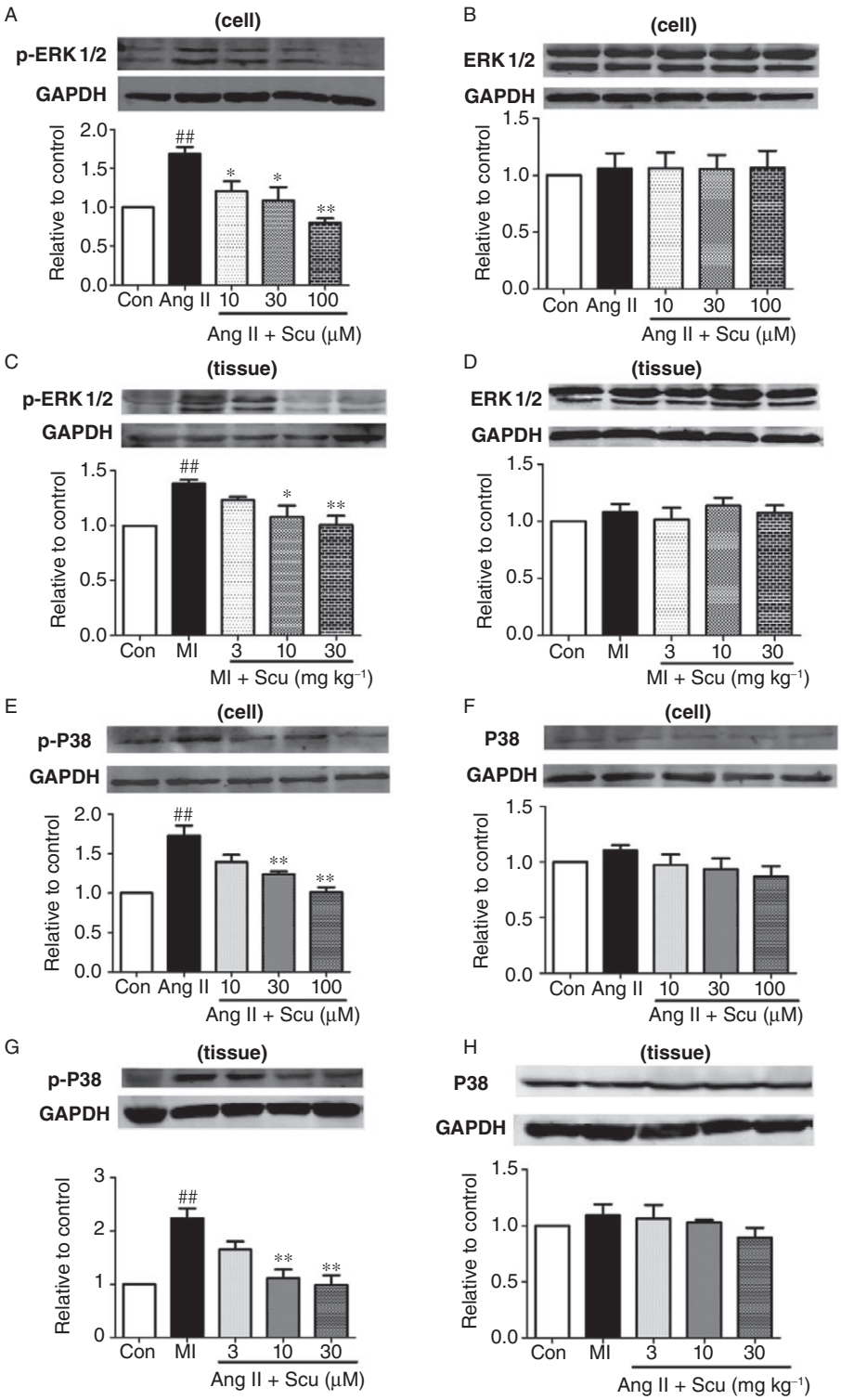

Figure 7

Effects of scutellarin (Scu) on the protein expression of ERK1/2, p-ERK1/2 and P38-MAPK, p-P38-MAPK in cultured cardiac fibroblasts and peri-infarct cardiac tissues. (A and B) p-ERK1/2 and ERK1/2 in cardiac fibroblasts; (C and D) p-ERK1/2 and ERK1/2 in cardiac tissues; (E and F) p-P38-MAPK and P38-MAPK in cardiac fibroblasts; (G and H) p-P38-MAPK and P38-MAPK in cardiac tissues. Ang II, angiotensin II (100 nM); MI, myocardial infarction. All values are expressed as mean ± SEM ($n = 4$). ##$P < 0.01$ versus sham; *$P < 0.05$, **$P < 0.01$ compared with Ang II or MI group.

activation of p38MAPK and ERK1/2 is critical for cardioprotection (Fan *et al.*, 2010), care should be taken when interpreting our results. Long-term suppression of the kinases by Scu may compromise the capacity of the heart to adapt to detrimental stimuli.

Taken together, the results of this study indicate that long-term application of Scu improves the cardiac function of MI rats by inhibiting interstitial fibrosis, irrespective of its other cardiovascular actions. The mechanisms may involve the suppression of TGFβ1 expression and inhibition of p38 MAPK and ERK1/2 phosphorylation. These data highlight the potential of Scu as an anti-fibrotic agent in the future.

Acknowledgements

This work was supported by the Ph.D. Programs Foundation of the Ministry of Education of China: Young Teacher, Opening Projects of Bio-Pharmaceutical Key Laboratory of Heilongjiang Province-Incubator of State Key Laboratory (to ZP), and by the National Basic Research Program of China (973 Program; 2007CB512000/2007CB512006; to BY).

Conflict of interest

None

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