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Full paper

# Scoparone attenuates angiotensin II-induced extracellular matrix remodeling in cardiac fibroblasts

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ABSTRACT

Scoparone is a biologically active constituent isolated from *Artemisia capillaris* and possesses a variety of pharmacological activities, such as anti-inflammatory, anti-tumor, anti-allergic and anti-cardiovascular activities. However, there are no studies focusing on the effects of scoparone against cardiac fibrosis. Therefore, the aim of this study was to investigate the effects of scoparone on Angiotensin II (Ang II)-induced extracellular matrix (ECM) remodeling and its possible mechanism in cardiac fibroblasts (CFs). Our results demonstrated that scoparone effectively attenuated CFs proliferation in Ang II-stimulated CFs. Scoparone also prevented the differentiation of CFs to myofibroblasts and ECM proteins (type I collagen and fibronectin) expression in Ang II-stimulated CFs. Furthermore, scoparone prevented Ang II-induced the activation of TGF-β1/Smad signalling in CFs. Taken together, these studies indicated that scoparone attenuated Ang II-induced ECM remodeling in CFs, at least in part, by inhibiting TGF-β1/Smad signalling. These findings suggest that scoparone may be used a novel therapeutic agent against cardiac fibrosis.

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## Introduction

Acute myocardial infarction is a major cause of death and disability in the world. Cardiac fibrosis is an important pathological feature of acute myocardial infarction.[1] It is characterized by proliferation of cardiac fibroblasts (CFs) and aberrant deposition of extracellular matrix (ECM) proteins.[2] CFs are enmeshed in the endomysial interstitial matrix that surrounds cardiomyocytes and play an important role in the maintenance of myocardial structure and function.[1,3]

Angiotensin II (Ang II) is a major component of rennin-angiotensin system that plays an important role in the development of cardiac fibrosis.[4] In response to Ang II, cardiac fibroblasts migrate to the injured myocardial site where undergo transdifferentiation to myofibroblasts.[5] Thus, inhibiting Ang II-induced CFs transdifferentiation might be a promising strategy against cardiac fibrosis.

Scoparone is a biologically active constituent isolated from *Artemisia capillaries*.[6] Studies have demonstrated that scoparone possesses important pharmacological activities, such as immunosuppressive, anti-inflammatory, anti-tumor and anti-allergic activities.[7–10] In addition, it was reported that scoparone could suppress the proliferation of vascular smooth muscle cells through inhibiting the STAT3 signaling pathway.[11] However, there are no studies focusing on the effects of scoparone against cardiac fibrosis. Therefore, the aim of this study was to investigate the effects of scoparone on Ang II-induced ECM remodeling and its possible mechanism in CFs. Our results demonstrated that scoparone attenuates Ang II-induced ECM remodeling in CFs, at least in part, by inhibiting TGF-β1/Smad signalling.

## Materials and method

### *Cell culture and treatment*

Animal experiments were conducted in accordance with the guidelines of Ethical Committee for Animal Research of The Second

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People's Hospital of Jiaozuo, The First Affiliated Hospital of Henan Polytechnic University (China). Primary cultures of neonatal rat CFs were prepared as previously described.[12] In brief, ventricles from rats were minced and digested in collagenase (450 U/ml; Sigma, St. Louis, MO, USA). Cells were pelleted and cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and a combination of penicillin-streptomycin at 37 °C in a humidified 5% $CO_2$ atmosphere. The medium was replaced every 3 days, and cells were sub-cultured or subjected to experimental procedures at 90% confluence. Cells were then pre-treated with various concentrations of scoparone (1, 5 and 10 μM; Sigma) for 24 h, and exposed to Ang II (100 nM; Sigma) for different times. Scoparone was purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO; Sigma).

### *Cytotoxicity assay*

Cell viability was measured using the release of lactate dehydrogenase (LDH). Following treatment with different concentrations of scoparone (0, 1, 5 and 10 μM) for the specified time periods (24, 48 and 72 h), the culture medium was collected and evaluated using the LDH Cytotoxicity Detection Kit (Invitrogen) according to the manufacturer's instructions. The absorbance at 530 nm was examined and the cell survival rates were expressed as percentages of the value of normal cells.

### *Cell proliferation assay*

Cell proliferation was measured by the MTT assay. Briefly, CFs were seeded into 96-well plates at $1 \times 10^4$ cells/well. When cells reached confluence, cells were serum starvation for 24 h. Then cells were pretreated with or without scoparone (1, 5 and 10 μM) for 4 h, and then stimulated by Ang II (100 nM) for 24 h. Then, 20 μl of MTT (5 mg/ml; Sigma) was added to each well and incubation at 37 °C for another 4 h, followed by removal of the culture medium and addition of 100 μl of dimethyl sulfoxide (DMSO; Sigma). The optical density was measured at a wavelength of 490 nm using an ELISA microplate reader (Invitrogen, Carlsbad, CA, USA). BrdU incorporation assay was also used to detected cell proliferation. Briefly, after treatment, CFs were trypsinized, and then plated into a 96-well plate and grown in complete culture medium with 10 μM BrdU for 24 h. BrdU incorporation into cellular DNA was measured using an ELISA microplate reader at 450 nm (Invitrogen).

### *Quantitative real-time polymerase chain reaction (qRT-PCR) analysis*

Total RNA was isolated from the CFs was extracted by use of TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. About 5 μg of total RNA for each sample was reverse-transcribed into first strand cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). The mRNA levels were analyzed by real-time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA, USA). PCR amplification was performed using the following primers: α-smooth muscle actin (α-SMA), forward: 5′-GCTATT CAGGCTGTGCTGTC-3′, and reverse: 5′-GGTAGTCGGTGAGATCTCGG-3'; TGF-β1, forward: 5′-GCGCCTGCAGAGATTCAAGTCAAC-3′, and reverse: 5′- GTATCAGTGGGGGTCAGCAGCC-3'; and β-actin, forward: 5′-TTAGTTGCGTTACACCCTTTC-3′ and reverse: 5′-ACCTTCACCGTTCCAGTTT-3'. The β-actin was used as a quantitative and qualitative control to normalize the gene expression. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

### *Western blot analysis*

Proteins were extracted from CFs using RIPA lysis buffer (Beyotime, Nantong, China), and the protein concentrations were determined by using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). Aliquots (about 30 μg per lane) were subjected to 12% SDS PAGE electrophoresis and transfer to polyvinylidene fluoride membranes, which were then blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat milk for 1 h. After blocking, the membranes were incubated with primary antibodies over night at 4 °C. The primary antibodies were anti-α-SMA, anti-type I collagen, anti-fibronectin, anti-TGF-β1, anti-Smad2/3, anti-p-Smad2/3 and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The protein bands were visualized by enhanced chemiluminescence (ECL) reagents according to the manufacturer's instruction. To show western blot results quantitatively, the densitometry analysis of the target proteins was performed through Gel-Pro Analyzer version 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

### *Statistical analysis*

All results are presented as mean ± standard deviation (SD). The statistical significance was assessed using one way analysis of variance followed by Tukey's post hoc test for multiple group comparisons. Differences between experimental groups were considered to be significant at $p < 0.05$.

## Results

### *Scoparone inhibits Ang II-induced proliferation of CFs*

First, we detected the effect of scoparone on cellular toxicity using the LDH release assay. As shown in Fig. 1A, at the tested concentrations and time periods, scoparone had no significant cytotoxicity effect on cultured CFs. Then, we examined the effect of scoparone on cell proliferation in CFs exposed to Ang II. The results of MTT assay demonstrated that Ang II significantly induced the proliferation of CFs. In addition, we observed that pretreatment with scoparone remarkably reversed Ang II-induced cell proliferation, exhibiting a dose-dependent manner (Fig. 1B). BrdU incorporation assay also showed that scoparone inhibited Ang II-induced proliferation of CFs (Fig. 1C).

### *Scoparone prevents the differentiation of CFs to myofibroblasts*

The expression of α-SMA is a hallmark of myofibroblast differentiation.[13] Therefore, we investigated the effects of scoparone on the expression of α-SMA in CFs stimulated by Ang II. As indicated in Fig. 2, Ang II alone treatment efficiently induced α-SMA expression at both the mRNA and protein levels in CFs, when compared with the control group. However, pretreatment with scoparone prevented Ang II-induced α-SMA expression in CFs.

### *Scoparone inhibits Ang II-induced ECM proteins of CFs*

It has been reported that ECM accumulation plays a critical role in the development of cardiac fibrosis.[14] Therefore, we next investigated the effects of scoparone on ECM proteins expression in CFs exposed to Ang II. As shown in Fig. 3, Ang II alone treatment significantly induced the expression levels of type I collagen and

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