Curcumin Suppress Cardiac Fibroblasts Activities by Regulating Proliferation, Migration, and the Extracellular Matrix

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Background: Cardiac fibrosis plays a critical role in the pathophysiology of cardiovascular disease. It has been observed that curcumin has several cardiovascular effects. The purpose of this study was to evaluate whether curcumin can attenuate cardiac fibroblasts activity.

Methods and Results: We evaluated the migration, proliferation, collagen production, and transcription signaling in rat cardiac fibroblasts isolated from Sprague-Dawley rats (males, weighing 300-350 g) that were or were not incubated with curcumin (25 μM) and the co-administration of transforming growth factor (TGF)-β1 (10 ng/ml) or angiotensin (Ang) II (100 nM) by a cell migration analysis, proliferation assay, and Western blot analysis. Compared to those without curcumin, curcumin-treated cardiac fibroblasts exhibited lower migratory, proliferative abilities and collagen production at the baseline and after the co-administration of TGF-β1 or Ang II. Curcumin-treated cardiac fibroblasts had increased matrix metalloproteinase (MMP)-2 activity in the presence of Ang II treatment. Curcumin-treated cardiac fibroblasts down-regulated phosphorylated protein kinase B (Akt) and phosphorylated Smad2/3 expression irrespective of TGF-β1 treatment. Curcumin also decreased phosphorylated extracellular signal-regulated kinase (ERK)1/2 levels in the presence of Ang II.

Conclusion: Curcumin attenuated Akt, Smad2/3, and ERK1/2 phosphorylation which were mediated by TGF-β1 and angiotensin II. This resulted in decreased cardiac fibroblast activation and supports the assertion that curcumin is an effective antifibrotic agent which can be used to treat heart failure.

Key Words: Angiotensin • Curcumin • Fibroblasts • Heart failure • Transforming growth factor

INTRODUCTION

Cardiac fibroblasts are the main contributors to the non-myocyte portion of myocardial tissues,1 and cardiac fibrosis plays a critical role in the pathophysiology of cardiovascular disease.2 Activated cardiac fibroblasts can transform into myofibroblasts3 and demonstrate augmented proliferation, migration, and collagen-secretion abilities.4,5 In response to myocardial injury, cardiac fibroblasts can also support the integrity of myocardial tissues by maintaining a balance between the synthesis and degradation of the extracellular matrix (ECM).6 During the initial phase of myocardial injury, degradation of the ECM due to increased matrix metalloproteinase (MMP) expression is dominant.3 During the later phase, net ECM deposition by enhanced collagen and tissue inhibitor of MMPs (TIMPs) is dominant.3,7 The net accumulation of ECM in the myocardium and activation of cardiac fibroblasts are major features of cardiac fibro-
sis. However, a more comprehensive understanding as to the regulation of cardiac fibroblasts remains incomplete.

Curcumin (diferuloylmethane), a polyphenol accounting for the yellow color of turmeric (a curry spice), is widely used in Asian countries and has a diverse range of molecular targets; it was proposed to possess therapeutic potential due its anti-inflammatory, antioxidant, and antifibrotic effects. Curcumin ameliorated the left ventricle (LV) function in pressure-overloaded rabbits through inhibiting myocardial collagen remodeling. It attenuated type I collagen production in amiodarone-induced pulmonary fibrosis in rats. Curcumin suppressed hepatic fibrosis in a rodent model by inhibiting hepatic stellate cell activation. It also improved renal fibrosis in obstructive nephropathy in rats. Nevertheless, the effects and molecular mechanisms of curcumin on cardiac fibroblasts have not yet been evaluated. Transforming-growth factor (TGF)-β and angiotensin (Ang) II are two main partners in fibroblast activation. Therefore, the purpose of this study was to test the hypothesis that curcumin can regulate activation of cardiac fibroblasts through modulating TGF-β and Ang II signaling.

METHODS

Isolation of cardiac fibroblasts

Cardiac fibroblasts were isolated from male Sprague-Dawley rats (weighing 300-350 g) using a modified version of a previously described method. After anesthetization with an isoflurane overdose, the rat hearts were rapidly removed and mounted on a Langendorff apparatus to perfuse them with phosphate-buffered saline (PBS) containing 0.02% collagenase (Sigma, St. Louis, MO, USA) at 37 °C for 35 minutes. The LV was excised and gently shaken in PBS until single fibroblasts were obtained. Cells were filtered through a 40-μm cell strainer and then centrifuged at 300 g for 10 minutes.

Isolated cardiac fibroblasts were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Before each assay, cardiac fibroblasts were cultured with serum-free medium for 24 hours. Cardiac fibroblasts from passage 1 were used in the experiments with curcumin (25 μM), TGF-β1 (10 ng/ml), or Ang II (100 nM).

Cell migration analysis

Cardiac fibroblast cell migration was assessed by a wound-healing assay through scraping a cell monolayer with a P200 pipette tip in 6-well culture plates treated with curcumin (25 μM), TGF-β1 (10 ng/ml), or Ang II (100 nM) for 24 hours. Each gap length was analyzed by SPOT software (Diagnostic Instruments, Sterling Heights, MI, USA) and calculated from 12 averaged regions. The net migration distance after 24 h was subtracted from that at the baseline.

Proliferation assay

Cardiac fibroblasts proliferation was measured using a commercial MTS kit (Promega, Madison, WI, USA). Cells were plated in a 96-well culture dish at a density of 3000 cells/well. After growing to 50% confluence, cells were cultured with serum-free medium for 24 hours. Cell growth was analyzed using the MTS reagent, which was added 4 hours before performing the spectrophotometric analysis.

Picro-Sirius red staining

Staining was carried out as previously described. After growing to confluence, cells were cultured in serum-free medium with and without (control) administration of curcumin, TGF-β1, or Ang II for 24 hours. Cardiac fibroblasts were fixed in methanol, and incubated in a Sirius red staining solution (Biocolor, Belfast, Northern Ireland) as per the manufacturer’s instructions. A bright-field image was obtained with a 10× objective lens.

Zymographic analysis of MMP activity

MMP-2 activity was quantified by a zymographic analysis. Supernatants were collected and concentrated in a freeze-dryer from cardiac fibroblasts with curcumin (25 μM), TGF-β1 (10 ng/ml), or Ang II (100 nM) for 24 hours. Concentrated supernatants were mixed with Tris-glycine sodium dodecyl sulfate (SDS) sample buffer (1 M Tris-HCl at pH 6.8, glycerol, SDS, and bromophenol blue) and loaded into 7.5% SDS gels containing 1 mg/ml gelatin. After electrophoresis, the gels were incubated with renaturing buffer (2.5% Triton X-100) with gentle agitation for 30 minutes. Then, the renaturing buffer was replaced with fresh developing buffer (50 mM Trizma hydrochloride and 5 mM calcium chloride).
chloride dihydrate), and the mixture was incubated at 37 °C for at least 4 hours. The gels were stained with 0.5% Coomassie Brilliant Blue R-250 in acetic acid/methanol/distilled water for at least 30 min. Then, the gels were destained in a 10% acetic acid and 50% methanol solution until the bands were clear. Finally, the gels were photographed, and the active form of MMP-2 was quantified with an enhanced chemiluminescence (ECL) detection system (Millipore, Billerica, MA, USA) and analyzed with ALPHAEASEFC software (Alpha Innotech, San Leandro, CA, USA). Targeted bands were normalized to the protein concentration of the cell lysate.

**Western blot analysis**

The procedure of Western blotting was as described previously. Cardiac fibroblasts were homogenized and lysed in RIPA buffer containing 50 mM Tris at pH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktails (Sigma). The protein concentration was determined with a Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA, USA). Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions and electrophoretically transferred onto an equilibrated polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). Blots were probed with primary antibodies against TGF-β receptor type I (TGFβR1, Millipore), Ang II type I receptor (AT1R, Abcam, Cambridge, UK), Smad2/3 (Cell Signaling Technology, Beverly, MA, USA), protein kinase B (Akt) (Cell Signaling Technology), extracellular signal-regulated kinase (ERK)1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and secondary antibodies conjugated with horseradish peroxidase. Bound antibodies were detected with the ECL detection system (Millipore) and analyzed with ALPHAEASEFC software (Alpha Innotech). Targeted bands were normalized to cardiac sarcomeric actin (Sigma) to confirm equal protein loading.

**Statistical analysis**

All quantitative data are expressed as the mean ± SEM. An unpaired t-test and one-way analysis of variance (ANOVA) with a post-hoc Tukey's test were used to compare cardiac fibroblasts under different conditions. A p value of < 0.05 was considered statistically significant.

**RESULTS**

**Effects of curcumin on the proliferation, migration and collagen production in cardiac fibroblasts**

As shown in Figure 1, curcumin-treated cardiac fibroblasts had a lower migratory ability than control fibroblasts. In addition, curcumin-treated cardiac fibroblasts had less collagen production than control fibroblasts. The MTS assay showed that curcumin-treated cardiac fibroblasts also had lower proliferative capabilities than control fibroblasts (Figure 1C).
Effects of curcumin on TGF-β1’s actions in cardiac fibroblasts

In the presence of TGF-β1, as shown in Figure 2, curcumin-treated cardiac fibroblasts had a lower migratory ability than control fibroblasts. However, curcumin decreased the migratory ability of fibroblasts under stimulation with TGF-β1 (n = 5) to a lesser extent (37% ± 12% vs. 64% ± 2%, p < 0.05) than control fibroblasts without TGF-β1 (n = 5). Similarly, curcumin-treated cardiac fibroblasts had less collagen production than control fibroblasts in the presence of TGF-β1 (Figure 2). Curcumin attenuated collagen production to a similar extent (16% ± 4% vs. 15% ± 5%, p > 0.05) in cardiac fibroblasts in the presence (n = 4) and absence (n = 4) of TGF-β1. In addition, in the presence of TGF-β1, curcumin-treated cardiac fibroblasts also exhibited decreased proliferative capabilities than control fibroblasts. Curcumin attenuated the proliferation to similar extents (46% ± 12% vs. 33% ± 5%, p > 0.05) in cardiac fibroblasts in the presence (n = 6) and absence (n = 6) of TGF-β1.

Effects of curcumin on the actions of Ang II in cardiac fibroblasts

In the presence of Ang II, as shown in Figure 3, curcumin-treated cardiac fibroblasts had a lower migratory ability compared to control fibroblasts. Similarly, curcumin-treated cardiac fibroblasts exhibited less collagen production and proliferation than control fibroblasts in the presence of Ang II (Figure 3). Curcumin attenuated the migration (60% ± 5%, n = 5), collagen production (39% ± 18%, n = 4), and proliferation (45% ± 4%, n = 6) in the presence of Ang II to similar extents compared to those without Ang II. Moreover, as shown in Figure 4, curcumin significantly decreased the suppressive effects of Ang II on MMP2 activity in cardiac fibroblasts.

Curcumin’s effects on TGF-β and Ang II signaling

There were similar levels of TGFβR1 protein expressions in cardiac fibroblasts in the presence and absence of curcumin; nevertheless, curcumin significantly downregulated expressions of phosphorylated Smad2/3 and phosphorylated Akt (Figure 6). Curcumin significantly suppressed expressions of AT1R, phosphorylated ERK1/2, and phosphorylated Akt (Figure 7).

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DISCUSSION

Curcumin was used in various studies of cardiovascular diseases in animals and humans. Curcumin prevents diabetic oxidative heart damage by modulating NF-κB signaling pathway. In rats with myocardial infarction, curcumin improved systolic function and reduced myocardial hypertrophy in non-infarcted myocardium. In this study, we found that curcumin changed the baseline cardiac fibroblast’s proliferative, migratory, and collagen-producing abilities. These findings suggest that curcumin directly modulates cardiac fibroblast activity, which may contribute to the beneficial cardiovascular effects of curcumin.

TGF-β can augment the proliferative, migratory, and collagen-producing abilities of cardiac fibroblasts by inducing myofibroblast differentiation. Ang II possesses profibrotic action and induces cardiac fibroblasts proliferation, increases ECM protein synthesis, decreases MMP activity, and increases TIMP activity. In this study, curcumin attenuated the suppressive effect of Ang II on MMP2 activity, which may explain why collagen production significantly decreased in cardiac fibroblasts co-administered with curcumin and Ang II. Our findings highly support curcumin being able to regulate cardiac fibrosis by modulating the effects of TGF-β1 and Ang II. A previous report proved that curcumin can attenuate fibrotic signaling of TGF-β in hepatic stellate cells by suppressing gene expressions of TGF-β receptors. It diminishes the proliferation and differentiation of lung fibroblasts by suppressing the phosphorylation of Smad2 and Smad3 and the downstream signaling of TGF-β. We found that in cardiac fibroblasts, curcumin did not influence TGF-β receptor expression but down-regulated phosphorylated Smad2/3 protein expression.
We also found that curcumin suppressed phosphorylated Akt signaling in cardiac fibroblasts. Phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) signaling, a downstream signal pathway of TGF-β and Ang II, plays an important role in the cellular proliferation pathway of cardiac fibroblasts. Curcumin was proven to inhibit leptin-induced hepatic stellate cell activation by interrupting Akt signaling. Therefore, it is possible that curcumin can interfere with TGF-β1-induced cardiac proliferation.

**Figure 5.** Effects of curcumin on signal transduction in cardiac fibroblasts under baseline conditions. There were similar transforming growth factor (TGF)-β receptor type 1 (TGFβR1) (n = 6 independent experiments) protein expressions in cardiac fibroblasts in the presence (25 μM) and absence of curcumin. Curcumin (25 μM) significantly decreased phosphorylated Smad 2/3 (pSmad2/3) (n = 6 independent experiments), angiotensin (Ang) II type 1 receptor (AT1R) (n = 7 independent experiments), phosphorylated ERK 1/2 (pERK1/2) (n = 5 independent experiments), and phosphorylated Akt (pAkt) (n = 7 independent experiments) protein expressions as determined by Western blotting. Expressions of the TGFβR1, pSmad2/3, AT1R, pERK1/2, and pAkt proteins were normalized to β-actin as the internal control, and then normalized to the value of control cells. *p < 0.05, †p < 0.005.

**Figure 6.** Effects of curcumin on signal transduction in cardiac fibroblasts in the presence of transforming growth factor (TGF)-β1. When co-administered with TGF-β1 (10 ng/ml), there were similar TGF-β receptor type 1 (TGFβR1) (n = 6 independent experiments) protein expressions in cardiac fibroblasts with and without curcumin (25 μM). However, in the presence of TGF-β1, curcumin significantly decreased phosphorylated Smad 2/3 (pSmad2/3) (n = 3 independent experiments), and phosphorylated Akt (pAkt) (n = 4 independent experiments) protein expressions as determined by Western blotting. Expressions of the TGFβR1, pSmad2/3, and pAkt proteins were normalized to β-actin as the internal control, and then normalized to the value of control cells. *p < 0.05.
fibroblast activation by inhibiting multiple downstream signal pathways of TGF-β. Wang et al. found that curcumin can reduce interstitial fibrosis and increase mass of viable myocardium in rats with experimental myocardial infarction.\textsuperscript{38} In this study, we also found the direct inhibitory effects of curcumin in isolated cardiac fibroblasts. However, it is not clear whether the curcumin concentration (25 μM) used in this study is relevant to that in the whole animal experiments.

Curcumin can attenuate the effect of Ang II on the collagen I gene in aortas of transgenic mice.\textsuperscript{39} Curcumin can also reduce Ang II-mediated cardiomyocyte growth by suppressing AT1R receptor expression.\textsuperscript{40} In our study, curcumin downregulated AT1R receptor expression, and also inhibited phosphorylated ERK1/2 expression. These findings may explain curcumin’s effects on Ang II-induced cardiac fibroblasts activation.

We evaluated the different responses of curcumin to TGF-β1 and Ang II and found that when co-stimulated with Ang II, curcumin decreased migration to a similar extent compared to fibroblasts without Ang II. However, in the presence of TGF-β1, curcumin decreased migration of fibroblasts to a lesser extent than control fibroblasts without TGF-β1. Curcumin had no significant effect on the TGF-β receptor, which may explain the different responses of curcumin suppressing cell migratory abilities triggered by TGF-β and Ang II.

**CONCLUSIONS**

In conclusion, curcumin blocks TGF-β and Ang II profibrotic effects on cardiac fibroblasts through opposing multiple downstream targets of both TGF-β and Ang II signal pathways. The in vitro data provided here support the possibility that curcumin could be an effective antifibrotic agent for treating cardiac fibrosis.

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DISCLOSURES

None.

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