

Original Article

PDGF-CC induced rat myocardial fibrosis is mediated by ERK1/2 signaling pathway and potential mechanism

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Abstract: *Objective:* This study aimed to investigate the role of ERK1/2 signaling pathway in the PDGF-CC induced rat myocardial fibrosis and its potential mechanism. *Methods:* Heart was collected from neonatal SD rats for the separation and purification of cardiac fibroblasts (CFs) which were then divided into 3 groups: control group (CON), PDGF-CC group (P) and PDGF-CC+U0126 (ERK1/2 inhibitor) group (PU). CFs proliferation was evaluated. The mRNA expression of PDGF- α receptor (PDGFR- α), PDGFR- β , ERK1, ERK2, type I collagen (Col I) and type III collagen (Col III) was detected. The protein expression of phosphorylated PDGFR- α (p-PDGFR- α), ERK1/2, p-ERK1/2, Col I and Col III was tested. *Results:* CFs proliferation was significantly promoted in P group as compared to CON group ($P < 0.01$), but addition of ERK inhibitor (PU group) markedly inhibited cells proliferation ($P < 0.01$) when compared with P group; In P group, the PDGFR- α mRNA expression increased significantly as compared to CON group ($P < 0.001$). The mRNA expression of ERK1, ERK2, Col I and Col III in P group was significantly higher than in CON group ($P < 0.001$), but that in PU group was markedly reduced as compared to P group ($P < 0.01$); The protein expression of p-PDGFR- α , ERK1/2, p-ERK1/2, Col I and Col III in P group was significantly higher than in CON group ($P < 0.001$), but addition of ERK inhibitor inhibited the protein expression dramatically as compared to P group ($P < 0.001$). *Conclusions:* PDGF-CC may bind to PDGFR- α to activate ERK1/2 signaling pathway to promote CFs proliferation and induce collagen synthesis, resulting in myocardial fibrosis.

Keywords: Myocardial fibrosis, cardiac fibroblast, platelet-derived growth factor, extracellular signal regulated kinase

Introduction

Myocardial fibrosis is a pathological change in the end stage of cardiovascular diseases and characterized by cardiac fibroblast hyperplasia and excess synthesis of collagen in the cardiac interstitium [1]. Heart tissue is composed of myocytes, myocardial interstitial cells and extracellular matrix (ECM). Under physiological condition, cardiac fibroblasts are regularly arranged around the myocytes. CFs are a major cell type in myocardial interstitium, and ECM is mainly composed of Col I and Col III. Cardiac fibroblasts are the main cell type responsible for the synthesis of both Col I and Col III. Generally, there is a few amount of collagen in the myocardial interstitium, which is essential for the maintenance of mechanical and electronic activities. Under pathological conditions, cardiac fibroblasts show hyperplasia, transform into myofibroblasts and secrete a large amount

collagen to ECM, which is one of causes of myocardial fibrosis [2].

Platelet-derived growth factors (PDGFs) belong to a polypeptide family with mitogenic activity and can bind to corresponding receptors to induce the proliferation and chemotaxis of fibroblasts. Currently, the signaling pathways related to PDGFs are still studied. Our previous studies showed PDGF-BB could bind to its receptor PDGFR- β to activate PI3K/AKT signaling pathway, which induced the proliferation of cardiac fibroblasts and the synthesis of collagen [3]. There is evidence showing that bleomycin induced lung fibrosis in mice is positively related to PDGF-CC expression, but negatively associated with PDGF-DD expression [4]. In addition, study also reveals that PDGF-CC may induce the proliferation and transformation of kidney fibroblasts, which is involved in the pathogenesis of renal failure [5]. Extracellular

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Table 1. Primers used for RT-PCR

Genes		Primer sequence (5'→3')
PDGFR- α	Sense	CCTGGCATGATGGTTGATTCTACTT
	Anti-sense	GGTCTCTTTTCGGGTTCACCTGTTT
PDGFR- β	Sense	GCACCGAAACAACACACCTT
	Anti-sense	ATGTAACCACCGTCGCTCTC
ERK1	Sense	TCCATCGACATCTGGTCTGT
	Anti-sense	AGCTGGTCCAGGTAGTGCTT
ERK2	Sense	TGAAGTTGAACAGGCTCTGG
	Anti-sense	AGTCGTCCAACCTCATGTCA
Col I	Sense	ACGCATGAGCCGAAGCTAAC
	Anti-sense	AGGGACCCTTAGGCCATTGT
Col II	Sense	ATAGACCTCAAGGCCCAAG
	Anti-sense	CCACCCATTCTCCGACT
β -actin	Sense	AGTGTGACGTTGACATCCGT
	Anti-sense	TGCTAGGAGCCAGAGCAGTA

Signal-Regulated Protein Kinases (ERK) include ERK1 and ERK2. The phosphorylated ERK1/2 acts on the cytoplasmic substrate proteins to regulate cell growth and differentiation. Moreover, ERK signaling pathway is involved in the pathophysiology after several factors induced myocardial injury such as myocardial hypertrophy, high glucose, and myocardial infarction [6-8]. The ERK1/2 expression and activation may be associated with the pathogenesis of kidney fibrosis in mice with diabetic nephropathy [9]. However, the role of ERK1/2 signaling pathway in the myocardial fibrosis has been rarely reported. In this study, rat cardiac fibroblasts were separated and treated with exogenous PDGF-CC, then the proliferation of cardiac fibroblasts was determined, and the expression of molecules in ERK1/2 signaling pathway and its downstream molecules (Col I and Col III) was measured to explore the mechanism underlying the PDGF-CC induced myocardial fibrosis.

Materials and methods

Animals

This study has been approved by the Ethics Committee of Anhui Provincial Hospital. SD rat pups (n=30) aged 1-3 days were purchased from the Experimental Animal Center of Anhui Medical University as described previously [3, 10].

Main reagent

Fetal bovine serum (FBS, Sijiqing, China), TRIZOL (Ambion, USA), Methyl thiazolyl tetrazole (MTT) (Sigma, USA), PDGF-CC (Pepro Tech, USA), U0126 (Beyotime, China), rabbit anti-rat IgG (Beijing Zhongshan Biotech), Primers for PCR (Sangon, China), p-PDGFR- α , ERK1/2, p-ERK1/2, Col I, Col III, β -actin (rabbit anti-rat, Bioworld, USA), Reverse transcription kit (Thermo, USA) and Real time quantitative PCR kit (Qiagen, USA) were used in this study.

Cell culture

Rat pups were sterilized with 75% ethanol, and the heart was collected under an aseptic condition. The apex was harvested and washed in PBS containing 1% antibiotics (penicillin and streptomycin) thrice. The tissues were cut into blocks, and mixed with 5 volumes of 0.08% trypsin, followed by incubation at 37°C for 3 min. The tissue blocks were pipetted for 1 min, and the residual tissues were digested again with above procedures. The supernatant was harvested, and centrifuged at 1200 rpm for 4 min. The supernatant was removed, and high glucose DMEM (HG/DMEM) was used to re-suspend cells. Cells were transferred into a 25 cm² dish, followed by incubation at 37°C for 90 min in an environment with 5% CO₂. The supernatant was removed, and equal volume of medium was added. Thereafter, the medium was refreshed once every 2 days. When the cell confluence reached 70-80%, cells were passaged at a ratio of 1:2. Cells of generation 2-4 were used in following experiments. The medium was HG/DMEM containing 10% FBS. Cardiac fibroblasts of generation 2 were subjected to immunofluorescence staining of vimentin and nuclear DAPI for identification.

Grouping and treatments

CFs in logarithmic growth phase would be divided into 3 groups: (1) control group (CON): cells were maintained in medium alone; (2) PDGF-CC group (P): cells were maintained in medium containing 20 ng/ml PDGF-CC; and (3) PDGF-CC+U0126 group (PU): cells were main-

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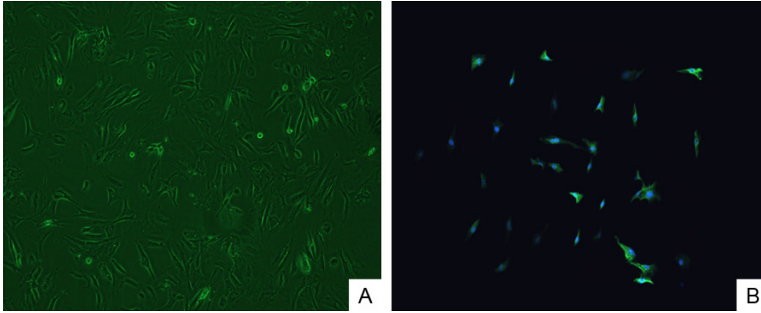


Figure 1. A: Primary cardiac fibroblasts under a light microscope ($\times 100$): cardiac fibroblasts were culture for 3-4 days, and cells became long spindle-shaped or irregular. B: Purified cardiac fibroblasts of generation 2 under a fluorescence microscope ($\times 200$): green fluorescence (Vimentin expression) and blue fluorescence (nucleus; DAPI) were observed in these cells.

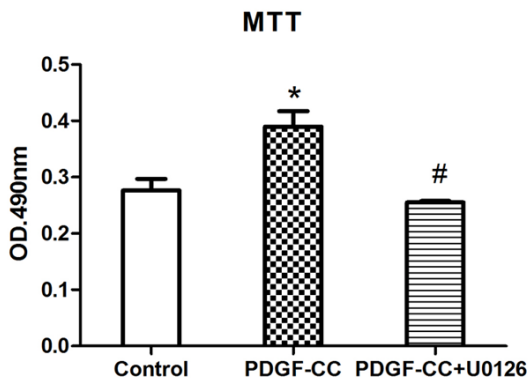


Figure 2. Proliferation of cardiac fibroblasts: * $P < 0.01$ vs. CON group ($n=3$); # $P < 0.01$ vs. P group ($n=3$).

tained in medium containing 20 ng/ml PDGF-CC+10 $\mu\text{mol/l}$ U0126 (ERK1/2 inhibitor).

Cell proliferation by MTT assay

Cells were seeded into 96-well plates with 6 wells in each group. Drugs were added independently, followed by incubation for additional 48 h. Then, 5 mg/ml MTT (20 $\mu\text{l/well}$) was added, followed by incubation at 37°C for 4 h in an environment with 5% CO_2 . The medium was removed, and DMSO (150 $\mu\text{l/well}$) was added to each well, followed by incubation for 10 min. The optical density (OD) was measured at 490 nm. The OD reflects the number of cardiac fibroblasts: the higher the OD is, the more rapid the cell proliferation is.

Detection of mRNA expression by quantitative RT-PCR

Cells were seeded into 6-well plates (10⁶/well), followed by incubation at 37°C for 24 h in an environment with 5% CO_2 . After treatment with

different drugs for 48 h, total RNA was extracted with TRIZOL reagent, and RNA quality and quantity were determined by measuring the absorbance (A) at 260 nm and 280 nm. The A260/A280 ranging 1.8-2.0 suggests a favorable RNA purity, and total RNA was then used in following experiment. Then, 5 μg of total RNA was used for reverse transcription into cDNA, and quantitative RT-PCR was performed with as follows: pre-denaturation at

95°C for 2 min, 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 10 s. The Ct value of each well was determined, and $2^{-\Delta\Delta\text{Ct}}$ method was employed for the calculation of mRNA expression of PDGFR- α , PDGFR- β , ERK1, ERK2, Col I and Col III. $\Delta\text{Ct} = \text{Ct}(\text{target gene}) - \text{Ct}(\text{reference gene})$. Primers are shown in **Table 1**, and β -actin was an internal reference.

Western blotting

Cells in logarithmic growth phase were seeded into 6-well plates (10⁶/well) and incubation at 37°C in an environment with 5% CO_2 for 24 h. Cells were treated with different drugs for 48 h, and then digested. After centrifugation, cells were harvested and lysed in RIPA lysis buffer (1 ml) containing 1 mM PMSF, followed by centrifugation at 12000 rpm for 10 min. The supernatant was collected, and protein concentration was determined with BCA method. Total protein was subjected to SDS-PAGE at 100 V, and then transferred onto PVDF membrane, followed by blocking at room temperature for 2 h. The membrane was incubated with primary antibody (1:500) at 4°C over night. After washing in TBST thrice, the membrane was incubated with HRP conjugated secondary antibody (1:1000) at room temperature for 2 h, followed by washing in TBST thrice. Visualization was done with ECL. ImageJ densitometry software was used to determine the OD of protein bands, and β -actin served as an internal reference.

Statistical analysis

Data are expressed as mean \pm standard deviation. Statistical analysis was performed with SPSS 14.0. Paired comparisons were done with

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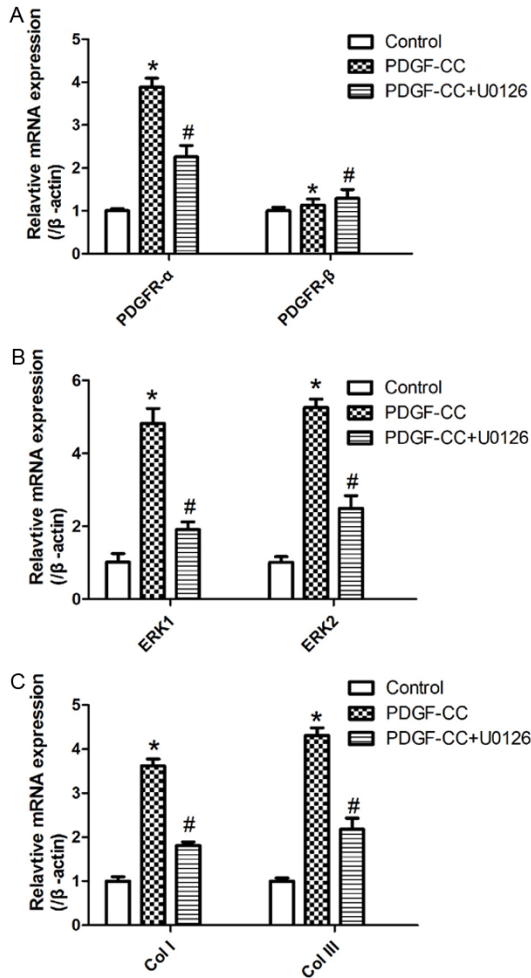


Figure 3. A: PDGFR- α mRNA expression: * $P < 0.001$ vs. CON group ($n=3$); * $P < 0.01$ vs. P group ($n=3$); PDGFR- β mRNA expression: * $P > 0.05$ vs. CON group ($n=3$). B: ERK1 and ERK2 mRNA expression. * $P < 0.001$ vs. CON group ($n=3$); * $P < 0.001$ vs. P group ($n=3$). C: Col I and Col III mRNA expression. * $P < 0.001$ vs. CON group ($n=3$); * $P < 0.001$ vs. P group ($n=3$).

Dunnett-t test, and a value of $P < 0.05$ was considered statistically significant.

Results

Physiological characteristics and identification of cardiac fibroblasts

After purification of primary cells, the purity of cardiac fibroblasts was as high as 99%. Cells were long spindle-shaped or polygonal. After culture for 3-4 days, cell confluence was observed under a light microscope, there was overlapping growth, cells were closely arranged, and spontaneous beating was not

noted. When cell confluence reached 70-80%, passaging was performed. Immunofluorescence staining showed green fluorescence (Vimentin expression) in these cells, and the nucleus was blue (DAPI) (Figure 1A and 1B).

PDGF-CC on cell proliferation

MTT assay was employed to detect the cell proliferation in different groups. After PDGF-CC treatment in P group, the proliferation of cardiac fibroblasts increased significantly as compared to CON group ($P < 0.01$). However, in the presence of ERK1/2 inhibitor (PU group), the cell proliferation was markedly inhibited as compared to P group ($P < 0.01$, Figure 2).

mRNA expression of PDGFR- α , PDGFR- β , ERK1, ERK2, Col I and Col III

In P group, the PDGFR- α mRNA expression was significantly higher than in CON group ($P < 0.001$); after ERK1/2 inhibitor treatment, the PDGFR- α mRNA expression in PU group was significantly lower than in P group ($P < 0.01$). There was no marked difference in PDGFR- β mRNA expression between P group and CON group (1.13 ± 0.145 vs. 1.001 ± 0.053 , $P > 0.05$). In P group, the mRNA expression of ERK1 and ERK2 was markedly higher than in CON group ($P < 0.001$), but it in PU group was significantly lower than in P group ($P < 0.01$). The mRNA expression of Col I and Col III in P group increased significantly as compared to CON group ($P < 0.001$), but addition of ERK1/2 inhibitor significantly reduced it in PU group ($P < 0.001$ vs. P group, Figure 3A-C).

Protein expression of PDGFR- α , p-PDGFR- α , ERK1, ERK2, and Col III

Western blotting showed p-PDGFR- α protein expression in P group was significantly higher than in CON group ($P < 0.001$). In addition, the ERK1/2 and p-ERK1/2 protein expression in P group increased significantly as compared to CON group ($P < 0.001$). The protein expression of downstream Col I and Col III in P group was also markedly higher than in CON group ($P < 0.001$). After addition of U0126 in PU group, the protein expression of p-PDGFR- α , ERK1/2, p-ERK1/2, Col I and Col III

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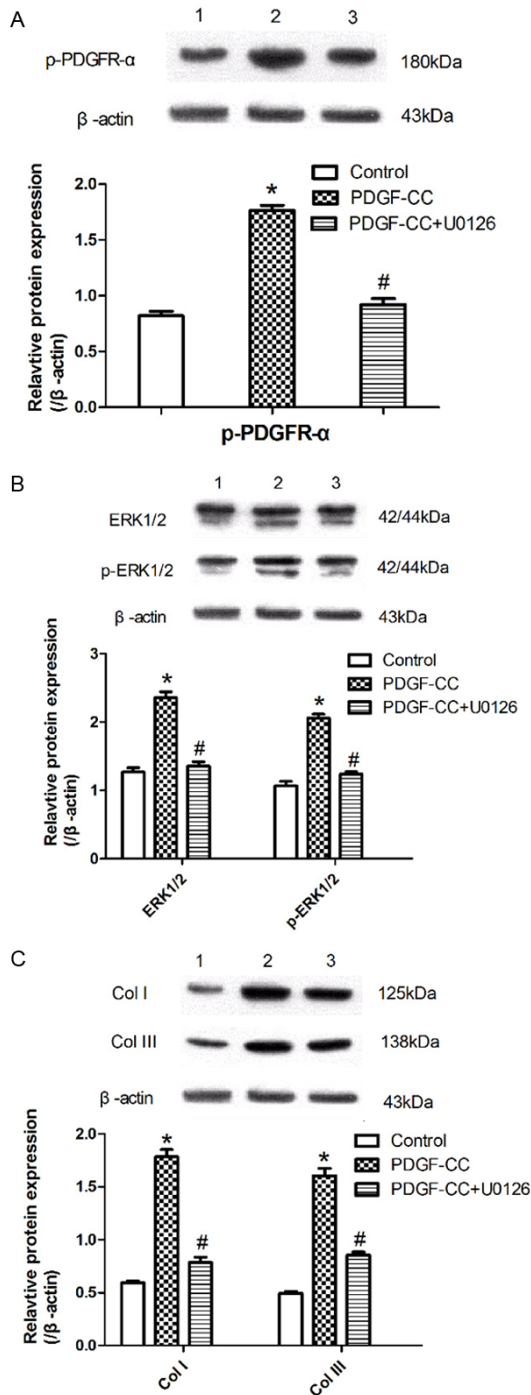


Figure 4. A: p-PDGFR- α protein expression: * $P < 0.001$ vs. CON group ($n=3$); # $P < 0.001$ vs. P group ($n=3$). B: ERK1/2 and p-ERK1/2 protein expression: * $P < 0.001$ vs. CON group ($n=3$); # $P < 0.001$ vs. P group ($n=3$). C: Col I and Col III protein expression: * $P < 0.001$ vs. CON group ($n=3$); # $P < 0.001$ vs. P group ($n=3$). 1: CON group; 2: P group; 3: PU group.

reduced dramatically as compared to P group ($P < 0.001$, **Figure 4A-C**).

Discussion

Myocardial fibrosis is a chronic compensatory response of the heart to the endogenous and/or exogenous stimuli and characterized by the imbalance between production and clearance of ECM. The over-stimulation of growth factor may induce the compensatory hyperplasia of cardiac interstitial cells (mainly cardiac fibroblasts), which is accompanied by the excess deposition of collagen in the heart [11]. The myocardial fibrosis may significantly compromise the ventricular compliance, affect the diastolic and systolic functions of the heart, and cause the abnormal electrical conduction of the heart, leading to severe heart failure and other malignant arrhythmias [12]. In the cardiac interstitium, the cardiac fibroblasts with hyperplasia may transform into myofibroblasts and the production of collagen also increases significantly, which is a major pathophysiology in myocardial fibrosis [3, 11, 13].

PDGFs are a group of mitogenic factors synthesized in fibroblasts and can be divided into 5 subtypes: PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and PDGF-AB. The PDGF receptors induce PDGFR- α and PDGFR- β [14]. Under physiological condition, only a small amount of PDGF is produced to maintain the normal cell proliferation. When the production of cytokines is accelerated after stimulation, the fibroblasts may become hyperplastic [15]. Our previous study showed that PDGFs/PDGFR is involved in the pathology of myocardial fibrosis [16, 17]. We also demonstrated that PDGF-BB mainly binds to PDGFR- β to activate PI3K/AKT signaling pathway, leading to the hyperplasia of cardiac fibrosis and transformation into myoblasts as well as the production of collagen [3]. In this study, purified cardiac fibroblasts were treated with PDGF-CC, and results showed their proliferation was significantly accelerated, suggesting that PDGF-CC is able to promote the growth of cardiac fibroblasts. There is evidence showing that PDGF-CC can bind to PDGFR- α on cardiac fibroblasts [18]. However, another study revealed that PDGF-CC on human vascular smooth muscle cells and microvascular endothelial cells may activate PDGFR- $\alpha\beta$ and ERK dependent signaling pathway to induce the expression of cytokines [19]. PDGF-CC mediated neovascularization in mouse cornea may be related to the PDGF- $\alpha\alpha$ and $\beta\beta$

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receptors [20]. Inactivated PDGFR- α and PDGFR- β exist in the form of monomers. Once PDGFR- α and PDGFR- β bind to PDGFs, they may form dimers (such as PDGFR- $\alpha\alpha$, PDGFR- $\beta\beta$ and PDGFR- $\alpha\beta$). In the present study, after treatment with PDGF-CC in cardiac fibroblasts, PDGFR- β mRNA expression remained unchanged, but PDGFR- α mRNA expression increased significantly. Thus, PDGFR- α on cardiac fibroblasts in the form of dimers bind to PDGF-CC, which is different from the dimers formed after binding of PDGF-CC to PDGFR.

Mitogen-activated protein kinase (MAPK) is one of important signal pathways in eukaryotes. In recent years, MAPK signaling pathways are divided into ERK1/2, JNK, p38 MAPK and ERK5. JNK and p38 MAPK signaling pathways play important roles in cell apoptosis and inflammation, and ERK1/2 signaling pathway is responsible for the regulation of cell growth and differentiation. Huang *et al.* [21] found that ERK1/2 signaling pathway is involved in renal interstitial fibrosis induced by high glucose concentration on renal tubular epithelial cell. Lin *et al.* [22] revealed that suppression of ERK/MAPK pathway can relieve hepatic stellate cell proliferation and reduce collagen deposition. ERK1/2 pathway is also expressed in the myocardium. There is evidence showing that STAT1/3 signaling pathway mediates the high glucose induced proliferation and collagen secretion of cardiac fibroblasts [23], in which ERK1/2 signaling pathway cooperated to regulate the fibrosis. In addition, study also reveals that ERK1/2 signaling pathway activation is involved in the PDGF-CC induced hyperplasia of renal fibroblast [5]. The activation of ERK1/2 signaling pathway may accelerate the proliferation of fibroblasts. In the present study, results showed the ERK1/2 mRNA and protein expression in cardiac fibroblasts increased significantly after PDGF-CC treatment, but addition of ERK1/2 inhibitor markedly reduced the ERK1/2 mRNA and protein expression. This indicates that PDGF-CC may induce the mRNA expression of ERK1/2 and its protein expression of Col I and Col III also increased significantly, indicating that PDGF-CC induced activation of ERK1/2 in cardiac fibroblasts may cause the synthesis of

downstream collagen, leading to myocardial fibrosis.

Ras/Raf/MEK/ERK is a main pathway of ERK signaling pathway. Extracellular stimulation may activate surface receptors, which induce the release guanosine diphosphate by Ras. The guanosine diphosphate binds to guanosine triphosphate, leading to Ras activation. Activated Ras binds to the N terminal of Raf, and then Raf translocates from cytoplasm into cell membrane. The C terminal of activated Raf may bind to MEK1/2, leading to MEK1/2 activation. The activated MEK activates ERK1/2, leading to the nuclear translocation of ERK1/2 to regulate the expression of target genes and then mediate the regulation of cell growth, differentiation and apoptosis. U0126 is an efficient noncompetitive inhibitor of MEK1/2 and can inhibit the activated and inactivated MEK1/2, leading to the activation of ERK1/2. There is evidence showing that U0126 may block ERK pathway to inhibit the FasL induced progression of dilated cardiomyopathy and chronic heart failure in mice, suggesting the anti-fibrotic effect of U0126 [24]. In the present study, results showed U0126 could attenuate the PDGF-CC induced proliferation of cardiac proliferation, which was related to the inhibition of ERK1/2 signaling pathway and the reduction of collagen production in cardiac fibroblasts. These findings indicate that U0126 may inhibit the ERK1/2 activation in cardiac fibroblasts after PDGF-CC treatment, exerting anti-fibrotic effect.

Taken together, PDGF-CC may bind to PDGFR- α to activate ERK1/2 signaling pathway, which up-regulates the expression of Col I and Col III, leading to the proliferation of cardiac fibroblasts. This may be involved in the pathogenesis of myocardial fibrosis. The mechanism underlying the PDGFs/PDGFRs signaling pathway mediated myocardial fibrosis is very complex, and both PI3K/AKT and ERK1/2 signaling pathways are involved in the progression of myocardial fibrosis. Thus, it is necessary to further elucidate the role of different subtypes of PDGFs in the pathogenesis of myocardial fibrosis, which may explain the potential mechanism of myocardial fibrosis.

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Disclosure of conflict of interest

None.

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