Phosphocreatine attenuates angiotensin II-induced cardiac fibrosis in rat cardiomyocytes through modulation of MAPK and NF-κB pathway

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Abstract. – OBJECTIVE: Transforming growth factor β (TGF-β) plays crucial roles in Ang II-induced cardiac fibrosis (CF). Phosphocreatine (PCr), one of the important players involved in cellular energy metabolism, is widely used in the treatment of clinical heart failure. However, whether it participates in CF is still unclear. This study aimed to identify the mechanisms involved in PCr and CF.

MATERIALS AND METHODS: Rat cardiomyocytes (H9C2) were induced by Ang II followed by treatment of PCr. ERK1 siRNA, ERK2 siRNA and NF-κB siRNA were applied to identify the molecular mechanism. Then CF-related proteins were analyzed by western blot and real-time PCR to confirm the influence of the mechanisms involved in PCr.

RESULTS: PCr did protect cardiomyocytes from Ang II-induced fibrosis. Meanwhile, PCr suppressed Ang II-induced up-regulation of TGF-β. By detecting TGF-β-mediated or MAPK pathway associated proteins, PCr inhibited MAPK and NF-κB pathway, thus suppressed Ang II-induced cardiac fibrosis, which was further confirmed by siRNA transfection.

CONCLUSIONS: Our study determined that PCr protected cardiomyocytes from Ang II-induced CF through inhibition of MAPK and NF-κB pathway.

Key Words: Cardiac fibrosis, Ang II, Phosphocreatine, TGFβ, MAPK, NF-κB.

Introduction

Cardiac fibrosis (CF), one of the leading reasons of death in cardiac disease, results in cardiac dysfunction and arrhythmias1. Renin-angiotensin-aldosterone system (RAAS) plays critical roles in development and progression of myocardial fibrosis (MF). Angiotensin-converting enzyme (ACE) and Ang II play important roles in the cardiovascular system, for example, cardiomyocyte hypertrophy, vascular smooth muscle cell hypertrophy and increase of extracellular matrix2. Ang II could up-regulate NF-κB level, increase the expression of myofibrillogenesis regulator-1 (MR-1), and induce the secretion of the cytokines (for example, TGF-β or TNF-α); consequently, it promotes fibrosis3,4.

TGF-β, contributing to cardiac fibrosis, is considered to stimulate cell growth, apoptosis and differentiation, increase collagen and matrix protein production, maintain fibroblast viability, and inhibit production of metalloproteinase which facilitates collagen degradation5,6. TGF-β signaling activation in cardiomyocyte serves as a bridge in cardiac fibrosis7, which is influenced by pathogenic factors like RAAS activation or inflammation. Therefore, blocking RAAS activation, especially to inhibit TGF-β activity, is the key point to reverse cardiac fibrosis.

Phosphocreatine (PCr), an energy-rich phosphate compound, not only provides energy when cardiomyocytes suffer from ischemia and hypoxia8, but also protects cardiomyocytes from being attacked by harmful substances like free radicals9. Additionally, Wei et al10 in 2015 suggested that PCr might play important roles in cardiac fibrosis. However, exact mechanism involved in PCr and CF is still unclear.

Our study used the Ang II-induced fibrosis model in rat cardiomyocytes, to identify the mechanisms involved in PCr and TGF-β pathway in cardiomyocytes, and further investigate on its associated downstream signaling related with TGF-β.
Materials and Methods

Cells and Reagents

Rat cardiomyocyte H9C2 was obtained from the Chinese Academy of Sciences (Institute of Shanghai Cell Biology and Chinese Type Culture Collection, China), which was cultured in DMEM containing 10% fetal bovine serum (FBS). Ang II was obtained from Sigma. Phosphocreatine sodium was bought from Sangon Biotech, Shanghai, China. Small molecular inhibitors U0126, SP600125 and PD98059 were bought from Sigma-Aldrich (St. Louis, Co, USA).

siRNA Transfection

ERK siRNA and NF-κB siRNA were generated by Ribobio in Guangzhou, China. H9C2 was cultured till 70% confluence and treated with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and siRNA mixture. Followings are the sequences of siRNAs used in this study:

- **ERK1 siRNA (S):** 5'-GGACCAGCUCAACCA-CAUU dTdT-3'
- **ERK1 siRNA (AS):** 5'-AAUGUGGUUGAGCUUGCC dTdT-3'
- **ERK2 siRNA (S):** 5'-GCUCUUGAAGACAC-GC dTdT-3'
- **ERK2 siRNA (AS):** 5'-GUGCUGUGUCUUCAAGCC dTdT-3'
- **NF-κB siRNA (S):** 5'-AAGCUGCUGAAACUCUGAG dTdT-3'
- **NF-κB siRNA (AS):** 5'-CUCAGAGUUUCAGCAGCUU dTdT-3'

Western Blot Assay

After cell culture, cells were collected for total protein extraction. The samples were loaded in 12% polyacrylamide gel and kept running for 2 h under a pressure of 80-120 V in Tris-Glycine buffer (25 mM Tris, 250 mM Glycine, 0.1% SDS). Then the proteins were transferred from the gel to cellulose nitrate membranes by semi-dry transferring method. The membranes were soaked for 1h at room temperature in 5% BSA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) on a shaker and incubated with specific primary antibodies, including anti-collagen I (Abcam, Cambridge, UK), anti-CTGF (Abcam), anti-Fibronectin (Abcam), anti-TGF-β (Cell Signaling), anti-JNK (Abcam), anti-phospho-JNK (Cell Signaling, Danvers, MA, USA), anti-ERK (Cell Signaling), anti-phospho-ERK (Cell Signaling), anti-p38 (Cell Signaling), anti-phospho-p38 (Cell Signaling), anti-Smad2/3 (Cell Signaling), anti-phospho-Smad2/3 (Abcam), anti-p65 (Abcam), anti-phospho-p65 (Abcam), anti-phospho-p38 (Cell Signaling), anti-p38 (Cell Signaling), and anti-Histone H3 (Abcam) in 4°C overnight. The next day, the membranes were incubated in HRP-labeled secondary antibodies (Beyotime, Nanjing, China) at room temperature for 1 h, colored with Fluorescent substrates and developed on films (Kodak, Rochester, NY, USA).

Real-Time PCR

Total RNA was extracted with RNA simple total RNA kit (Qiagen, Shanghai, China) following the instruction. Extracted total RNA was dissolved in 0.1% DEPC-treated deionized water. After dissolution, determine the purity and content of RNA with an ultraviolet spectrophotometer. CTGF copy numbers were determined with SYBR-Green and ∆∆CT relative quantitative measurement.

- **CTGF PCR primers are listed as follow:**
  - **CTGF (S):** 5'-TAGCCTCAAACTCCAAACACC -3'
  - **CTGF (As):** 5'-CCTCGTGGAATCTGACCAGT -3'
- **GAPDH PCR primers are listed as follow:**
  - **GAPDH (S):** 5'-GGT ATC GTG GAA GGA CTC ATG AC-3'
  - **GAPDH (As):** 5'-ATG CCA GTG AGC TTC CCG TTC AGC-3'

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted with Nucleo-protein Extraction Kit (Sangon, Shanghai, China) following the instruction. 2 ul nuclear extracts combined with biotin-labeled NF-κB probe (5'-TCAACTCCCCTGAAAGGGTCCG-3'). Conjugates were loaded in 4% polyacrylamide gel in 0.5 X TBE running buffer under the pressure of 10 V/cm. Dry the polyacrylamide gel in the drier and determined by X-ray film.

Statistical Analysis

One-way analysis of variance (ANOVA) was carried out by SPSS 13.0 (SPSS Inc, Chicago, IL, USA). $p < 0.05$ was considered as statistical differences.

Results

Ang II-Induced Cardiac Fibrosis

Cultured rat cardiomyocytes were treated with Ang II (1 nM, 10 nM, 100 nM) for 48 h. As shown in Figure 1A, expressions of collagen I, CTGF and Fn protein increased significantly when the cells were treated with 10 nM Ang II, which was
supported by CTGF mRNA expression tested with Real-time PCR (Figure 1B).

**Specific Small Molecular Inhibitors Blocked Ang II-Induced Cardiac Fibrosis and TGF-β Pathway Activation**

Rat cardiomyocytes were pre-treated with MAPK pathway specific inhibitors (ERK inhibitor U0126, JNK inhibitor SP600125, P38 inhibitor PD98059) for 1h, followed by cell culture in the presence or absence of Ang II (10 nM) for 48 h. As results shown in Figure 2A, U0126, SP600125 and PD98059 could significantly down-regulate expressions of collagen, CTGF, Fn and TGF-β, which suggested that Ang II-induced CF was mediated by MAPK pathway. MAPK associated

![Figure 1. Ang II induced CF. (A) Expression of CF associated proteins were identified in H9C2 treated with Ang II (1nM, 10nM, 100nM) for 48h. (B) real-time PCR was performed to determine CTGF mRNA levels. "p < 0.05, "p < 0.01 (compared with control group) was presented as significant difference.](image1)

![Figure 2. U0126, Sp600125, PD98059 treatment regulated Ang II-induced CF. (A) Expression of collagen I, CTGF, Fn, TGF-β, JNK, ERK, p38, Smad2/3 tested by Western blot. (B) NF-κB in nuclear extraction was determined. (C) CTGF mRNA levels was analyzed by real-time PCR. "p < 0.05 and "p < 0.01 (compared with control group) were presented as significant difference; "p < 0.05 and "p < 0.01 (compared with Ang II group) were presented as significant difference.](image2)
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protein levels also showed that Ang II could activate MAPK pathway, which could be inhibited by U0126, SP600125 and PD98059. Nuclear extraction determination showed that Ang II activated NF-κB. However, this could be blocked by U0126, SP600125 and PD98059.

**PCr Could Block Ang II-Induced NF-κB Activation**

Cardiomyocytes were pre-treated with phosphocreatine sodium (10 nM, 20 mM), followed by cell culture in the presence or absence of Ang II for 48 h. In Figure 3A, results showed that PCr could inhibit Ang II-induced expressions of collagen I, CTGF and Fn protein, thus inhibited rat CF, which was further confirmed by CTGF mRNA levels (Figure 3B). Interestingly, Ang II-induced TGFβ decreased significantly when treated with PCr (10 mM and 20 mM). CF is closely associated with TGF-β pathway activation. To identify how PCr regulates TGF-β pathway and then inhibits CF, we tested the PCr’s effects on MAPK pathway. Results showed that PCr could inhibit Ang II-induced activation of ERK and p38. Meanwhile, PCr could also block NF-κB activation induced by Ang II by checking with nuclear extraction.

**MAPK Pathway Was Involved in the Inhibition of Ang II-Induced CF by PCr**

Rat cardiomyocytes were transfected with ERK-siRNA and pre-treated with PCr for 1 h, followed by cell culture in the presence or absence of Ang II (10 nM) for 48 h. In Figure 4A, results showed that ERK knockdown could inhibit collagen I, CTGF and Fn protein expressions induced by Ang II, and then prevent rat CF. However, when ERK-siRNA was transfected, Ang II-induced expressions of collagen I, CTGF and

Figure 3. PCr regulated Ang II-induced CF, which was confirmed by (A) the expression of collagen I, CTGF, Fn, TGF-β, ERK, p38 and NF-κB was analyzed by Western blot. (B) NF-κB in nuclear extraction was analyzed. (C) CTGF mRNA levels were determined by real-time PCR. *p < 0.05 and **p < 0.01 (compared with control group) were presented as significant difference; ^p < 0.05 and ^^p < 0.01 (compared with Ang II group) were presented as significant difference.
Fn protein could not be significantly reverted by phosphocreatine, which suggested that ERK served as important regulators during reversal of CF by PCr. CTGF mRNA levels tested by real-time PCR further confirmed this observation (Figure 4B). Meanwhile, after ERK-siRNA was transfected, Ang II-induced TGF-β was significantly decreased, activities of p38 and Smad2/3 were also significantly down-regulated. NF-κB activity became lower as well when investigating nuclear extraction. After ERK knockdown, the activity of PCr to reverse Ang II-induced TGF-β expression and the activations of p38, Smad2/3, NF-κB also decreased. DNA binding activity of nuclear transcription factor NF-κB was checked by electrophoretic mobility shift assay (EMSA), in Figure 4C. When ERK-siRNA was transfected, DNA binding activity of NF-κB was weaker. The capacity of PCr to reverse increased DNA binding activity of NF-κB induced by Ang II also became weaker.

**NF-κB Knockdown by siRNA Confirmed its Roles in the Mechanism of Anti-Cardiac Fibrosis by PCr**

Rat cardiomyocytes were transfected with NF-κB siRNA, pre-treated with phosphocreatine sodium (20 mM) for 1 h and, then, followed by cell culture in the presence or absence of Ang II (10 nM) for 48 h. In Figure 5A, results showed that NF-κB knockdown could inhibit the expressions of collagen I, CTGF and Fn protein induced by Ang II. However, PCr could not reverse Ang II-induced CTGF expression significantly. CTGF

![Figure 4](image)

**Figure 4.** MAPK was involved in the mechanism of PCr and Ang II-induced CF. (A) the expressions of collagen I, CTGF, Fn, TGF-β, ERK, p38, Smad2/3 and NF-κB were detected by western blot upon siRNA transfection with PCr or Ang II treatment. (B) NF-κB in nuclear extraction was detected. (C) CTGF mRNA levels were determined by real-time PCR. *p < 0.05 and **p < 0.01 (compared with control group) were presented as significant difference; *p < 0.05 and ***p < 0.01 (compared with Ang II group) were presented as significant difference; *p < 0.05 and ***p < 0.01 (compared with ERK-siRNA group) were presented as significant difference. (D) DNA binding activity of NF-κB was determined by EMSA.
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mRNA levels checked with real-time PCR also confirmed it (Figure 5B). Meanwhile, after NF-κB was transfected, TGF-β level induced by Ang II was down-regulated, activities of ERK, p38 and Smad2/3 became significantly lower. The capacity of phosphocreatine to reverse TGF-β pathway activated by Ang II reduced upon ERK knockdown.

Discussion

CF is the key indicator in cardiac remodeling. This progression developed including that (i) CF overgrew, (ii) collagen synthesis increased, (iii) ratio imbalanced and (iv) disorders occurred under pathological conditions. Finally, MF is developed. MF is the risk factor in development and progression of cardiovascular events. It is related to cardiac insufficiency, arrhythmias and sudden cardiac death. It is reported that RASS and several cytokines (e.g. TGF-β1) play roles in MF development through activation of intracellular signal transduction.

Ang II upregulation is the main cause of CF. It is considered that Ang II promotes cardiac fibrosis through receptor-mediated activation of extracellular signal-regulated kinases (ERK) in a tyrosine kinase-dependent way, which promotes CF proliferation, stimulates expressions of collagens, fibronectins and integrins. Recently, connections between TGF-β1 and Ang II were determined. PCr is the native active substance that contributes to cellular energy metabolism. PCr, a myocardial protective agent, is clinically used in the treatment of CF, myocardial infarction and some cardiac surgeries. It serves protective roles in myocardial ischemia and reperfusion injury. PCr is widely used in the treatment of clinical heart failure. However, whether it is involved in CF is still not investigated well.

Figure 5. NF-κB was involved in the mechanism of PCr and Ang II-induced CF. (A) the expressions of collagen I, CTGF, Fn, TGF-β, ERK, p38, Smad2/3 and NF-κB were detected by western blot upon siRNA transfection with PCr or Ang II treatment. (B) NF-κB signaling in nuclear extraction was detected. (C) CTGF mRNA levels were determined by real-time PCR. *p < 0.05 and **p < 0.01 (compared with control group) were presented as significant difference; #p < 0.05 and ##p < 0.01 (compared with Ang II group) were presented as significant difference; ^p < 0.05 and ^^p < 0.01 (compared with ERK-siRNA group) were presented as significant difference.
In this study, we found that Ang II-induced indicators of CF (collagen I, CTGF, Fn) were significantly reduced after the treatment of PCr in Ang II-induced rat cardiomyocytes. At the same time, TGF-β protein level significantly decreased. TGF-β is considered to promote CTGF synthesis in fibroblasts, vascular smooth cells and endothelial cells, activate NF-κB, increase secretions of adherence factors and inflammatory factors, upregulate extracellular matrix proteinosis as well as expressions of collagen I gene and fibronectins, and finally result in CF.

Overexpression of TGF-β1 gene was induced by Ang II during MF development. Therefore, suppression of TGF-β1 might delay cardiac fibrosis. We make conclusions that PCr contributes to activation and regulation of TGF-β1 in myocardial tissue, thereby inhibiting cardiac fibrosis and protecting heart.

In the following study, we further investigate how PCr regulates TGF-β1 signaling pathway and prevent CF. MAPK pathway is an important signal transduction pathway, which participates in TGF-β-induced fibrosis. It could regulate cellular growth, transformation, differentiation, proliferation, as well as cell survival, cell death and so on. MAPK pathway consists of three main enzymes: extracellular signal regulated kinase (ERK), p38 and stress activated protein kinase (JNK). We found that PCr could inhibit activation of ERK and p38 induced by Ang II, suggesting that PCr reversed Ang II-induced CF through inhibition of MAPK pathway.

NF-κB is the key player in fibroblast growth and collagen expression mediated by Ang II and TNF-α. In the previous study, PCr suppressed Ang II-induced NF-κB activation. The capacity of PCr to reverse cardiac fibrosis and Ang II-induced activation of TGF-β-β associated pathway became weaker upon NF-κB-siRNA transfection, suggesting that NF-κB played critical roles in anti-cardiac fibrosis activity of PCr.

Conclusions

This study found out that myocardial protective agent PCr suppressed CF through regulation of TGF-β activity in the myocardial tissue, which involved in inhibition of MAPK pathway and NF-κB pathway. This study identified the potential clinical application of PCr in the anti-cardiac fibrosis and prevention of cardiomyocyte remodeling.

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Conflicts of interest

The authors declare no conflicts of interest.

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