Quercetin inhibition of myocardial fibrosis through regulating MAPK signaling pathway via ROS

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Abstract: Mitogen-activated protein kinase (MAPK) cascades are important players in the cellular signal pathways, and the deregulation of MAPKs is involved in a variety of diseases, especially cardiovascular disorders. This study was designed to investigate the effects of quercetin on proliferation of cardiac fibroblasts, measured the secretion of Col I & Col III by ELISA and the expression of extra cellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) p38 by eastern blotting in cardiac fibroblasts challenged with angiotensin (Ang-II). Results showed that Ang-II significantly increased the DNA synthesis and collagen secretion. In contrast, quercetin reversed such effects and inhibited cardiac fibroblasts proliferation. Furthermore, reactive oxygen species (ROS) stimulated the phosphorylation of ERK, p38 and JNK, while pre-administration of quercetin significantly ($P<0.05$) reduced the phosphorylation. All these evidences revealed that quercetin inhibited the MAPK pathway activation via ROS.

Keywords: Quercetin, MAPK signaling, cell proliferation, cardiac fibrosis.

INTRODUCTION

The pathogenesis of myocardial fibrosis is associated with various factors, including the renin angiotensin aldosterone system, endothelin-derived factors, catecholamine, growth factors, chemokines and ROS (Matsumoto-Ida *et al*., 2005). Angiotensin II (Ang-II), a peptide derived from Ang-I cleaved by ACE (angiotensin I converting enzyme), can promote the hypertrophy of cardiac muscle cells, stimulate the proliferation of cardiac fibroblasts and induce myocardial fibrosis (Ren *et al*., 2005; Muslin, 2008). Previous study found that rats with over-expression of Ang-II showed focal necrosis accompanied with infiltration of macrophages, lymphocytes and neutrophils. Overt perivascular fibrosis was even observed in left ventricle and right ventricle (Li *et al*., 2011).

Mitogen-Activated Protein Kinases (MAPKs) are members of a kinase family, which mediate transfer of signals from cell membrane to nucleus or to other intracellular targets. They are involved in regulating multiple cellular processes, including cell growth, differentiation, cycling, apoptosis and also the progression of cardiovascular diseases. ERK, JNK/SAPK and p38MAPK are the three main MAPK cascades in cardiac muscle cells (Ahn *et al*., 2008). Takeishi and coworkers found that ERK1/2 signaling pathways were activated in myocardium of patients with heart failure caused by dilated cardiomyopathy, whereas JNK/SAPK had no obvious changes and p38 signaling was decreased significantly (Min *et al*., 2007). In addition, previous study demonstrated that MAPK pathway regulated cell fibrosis, which was closely related to TGF–β/Smad signaling (Weng *et al*., 2011).

Quercetin has been proven to be highly effective on anti-tumor, cardio-protection and numerous other pharmacological aspects (Chen *et al*., 2012; Nam *et al*., 2008). Previous studies also reported that quercetin could regulate viability of various cells through suppressing the intracellular ROS formation (Li *et al*., 2013). However, its role in myocardial fibrosis is still unknown.

Recent studies confirmed that low doses of quercetin (2μM) were capable of decreasing the activity of about 16 cell cycle-related kinases (Yan *et al*., 2013). Besides, the effect of quercetin on cardio-protection is closely related with its anti-inflammatory and antioxidant properties. What's more, quercetin has been revealed to inhibit the inflammation induced by fat cells and macrophage cells in *in vitro* studies by regulating MAPK signaling (Iles *et al*., 2008; Serra *et al*., 2012). In consideration of the potential of quercetin in protecting cardiovascular system, as well as the important functions of MAPK pathway in cardiovascular diseases, recent work probed into the role of quercetin in cardiac fibroblast proliferation and myocardial fibrosis, which will provide a theoretical basis to develop new drugs for myocardial fibrosis.

MATERIALS AND METHODS

Isolation and primary culture of rat cardiac fibroblasts

A 1d Wistar-rat from specific-pathogen-free apparatus was disinfected with 75% alcohol and slaughtered. The heart was taken out quickly and rinsed in precooled sterile PBS buffer three times. Subsequently, the heart was cut into $1mm^3$ tissue blocks and put in a clean tube suspended

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with 25% trypsin for 10min. After digestion, the tissue was centrifuged at 1000 rpm for 10min. The supernatant was poured off and gently re-suspended the remaining debris in 3mL DMEM medium containing 10% fetal bovine serum (FBS). Then cells were plated in a 10cm cell dish and maintained at 37°C in 5% CO_2, 5% O_2 and 90% N_2 with maximum humidity.

Detection of cardiac fibroblasts proliferation

2×10^4 cells were seeded into 96-well plates. When cells reached a confluence of approximately 70%, cell proliferation was assessed following the instructions of a commercial kit (Biovision, USA). Briefly, BrdU (5-bromo-2-deoxyuridine) was incorporated into the newly synthesized DNA of proliferating cells during the 4h incubation. Incorporated BrdU was then detected using a mouse anti-BrdU antibody. Subsequently, a secondary antibody conjugated with a HRP (horseradish peroxidase) enzyme was utilized to recognize the BrdU-primary antibody complex, which was followed by addition of TMB (a HRP substrate). Cell proliferation was indicated as the extent of color development.

Expression of coli and col Iii in cardiac fibroblasts

Supernatant of cultured cardiac fibroblasts was collected and ELISA was performed to quantify the content of Col I, Col III, SOD and MDA by using commercial kits (Abnova, USA). Accordingly, samples and standards were diluted in 96-well plates and 50µL of conjugate solution was added into each well. After an incubation of 2h at room temperature, the wells were washed three times. Then, 200µL of substrate solution was added. The plate was incubated for 10-15 min and the color development was stopped. Absorbance of each well was determined at 450nm in a microplate reader. A standard curve was constructed by plotting the absorbance of standards against the known concentration and the sample content was deduced from the standard curve.

Detection of ROS levels in cardiac fibroblasts

Cardiac fibroblasts were seeded in 24-well plates. The cells were cultured in serum-free medium for two days. When the cells reached a confluence of 70%, they were washed with PBS thrice. The cells were then incubated with 50mmol/L of DCFH-DA for 30min. To completely remove the DCFH-DA, 3 times of PBS was performed to wash the cells. Finally, the cardiac fibroblasts were observed and photographed under the fluorescence microscope, the fluorescence intensity was detected and the level of ROS in the cells was analyzed.

Expression of P38, JNK and MAPK ERK protein

Total protein was extracted by using RIPA lysis (Beyotime, China) and protein concentration was measured according to the instructions of a BCA kit (Beyotime, China). 40ug of protein was denatured by heating at 100°C for 10 min and was then loaded in a 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel. After 90min of electrophoresis, proteins were transferred on to a PVDF membrane with a transfer module. The membrane was blocked in 5% non-fat milk solution for 2h and was incubated in 1:500 diluted primary antibodies (Sigma, USA) & consecutively in secondary antibodies (Santa Cruz, Germany). Ultimately, proteins were visualized on an X-ray film by using a chemiluminiscence kit (Thermo Scientific, USA). Signals were analyzed with Image J software.

STATISTICS ANALYSIS

Data was represented as mean ±standard deviation (SD). SPSS 21 software was used and two-tailed T-test was performed to evaluate the statistical significance between two means of equal variance. $P<0.05$ was considered as statistically significant.

Ethical approval

The ethical approval of this study was taken from the institutional ethical committee of Sichuan Medical College, Sichuan, PR China. The reference No. is 78/ERB/2017.

RESULTS

Effect of NAC and quercetin on cardiac fibroblasts

Cardiac fibroblasts were successfully isolated from the rat and DNA synthesis rate of cardiac fibroblasts was detected by BrdU-Elisa assay. Results showed that cells treated with Ang-II propagated faster than the control (cells without Ang-II stimulation), whereas addition of NAC (ROS inhibitor) and QUE (quercetin) aborted the high proliferation rate as shown in fig. 1A. In addition, Ang-II dramatically up-regulated the production of Col I and Col III in supernatant of the medium. Accordingly, NAC and QUE suppressed the secretion of Col I and Col III as given in fig. 1B. Evidences indicated that ROS inhibitor and quercetin elicited opposed activities against Ang-II in cardiac fibroblasts proliferation & collagen secretion, and quercetin exhibited even more robust potency.

Quercetin inhibited the production of ROS in cells induced by Ang II

ROS was measured in cardiac fibroblasts treated with Ang-II or quercetin. Results showed that the content of ROS in the Ang-II group was more abundant compared with the control counterpart. However, quercetin significantly ($P<0.05$) inhibited the intracellular ROS production induced by Ang-II. This result suggested that quercetin suppressed the production of ROS in cardiac fibroblasts, which were able to resist the oxidative stress induced by Ang-II as shown in fig. 2.

Ang-II induced activation of MAPK signaling pathway in cardiac fibroblasts

As MAPK signaling played crucial roles in cell fibrosis a fore mentioned, study interested in whether this pathway was involved in cardiac fibrosis. Hence, the protein expression of ERK, p(phosphorylated)-ERK, p38 MAPK, p38, JNK, p-JNK was detected by using western blot, after the cardiac fibroblasts were challenging with 100μM of Ang-II for 5, 10, 15, 30, 60 min respectively. As depicted in fig. 3, ERK phosphorylation level continued to increase after Ang-II stimulation, and reached a peak at 30min. p38 phosphorylation began to appear at 10min and increased in a time-dependent manner. Ang-II also induced JNK phosphorylation. These results proved that Ang-II significantly ($P<0.05$) activated MAPK signaling, and 30min as the optimal challenging time for further study was chosen.

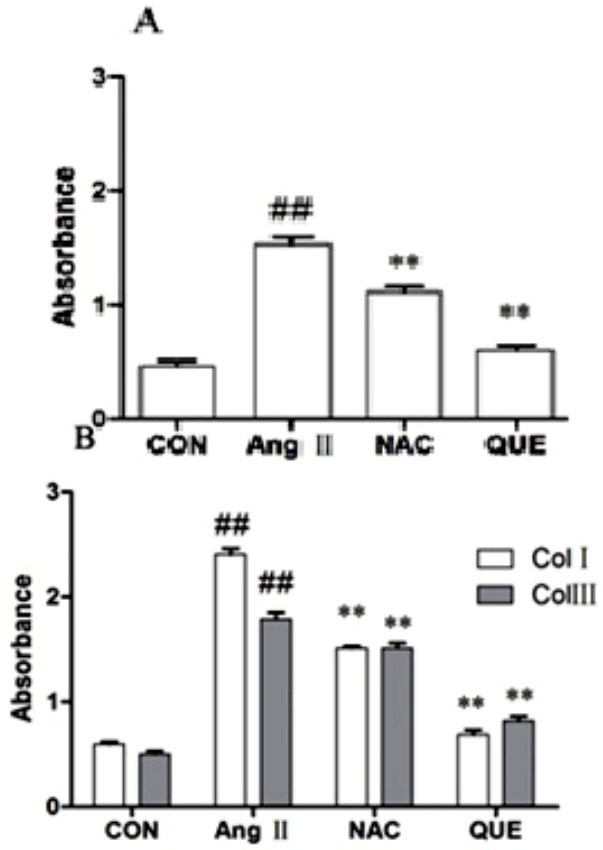

Fig. 1: Effect of ROS inhibitor on the proliferation and collagen secretion of fibroblasts. (A)The proliferation of cardiac fibroblasts induced by Ang II, NAC or QUE; (B) Secretion of ColI and ColIII in cardiac fibroblasts induced by Ang II, NAC or QUE.

Quercetin inhibited the activation of MAPK

To explore the function of quercetin on MAPK pathway, the phosphorylation levels of ERK, p38 and JNK were measured. Results here showed that the phosphorylation levels of ERK, p38 and JNK were significantly increased by Ang-II, which was in agreement with findings in fig. 3. By contrast, when the cells were treated with quercetin or ROS inhibitor, the phosphorylation level of these molecules was significantly down-regulated ($P<0.01$) as shown in fig. 4, which indicated that quercetin and ROS inhibitor decreased Ang-II-induced activation of MAPK signaling pathway in cardiac fibroblasts.

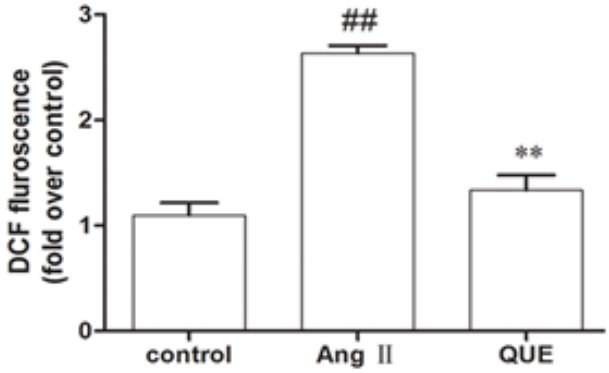

Fig. 2: ROS content of cardiac fibroblasts threated with Ang-II or quercetin

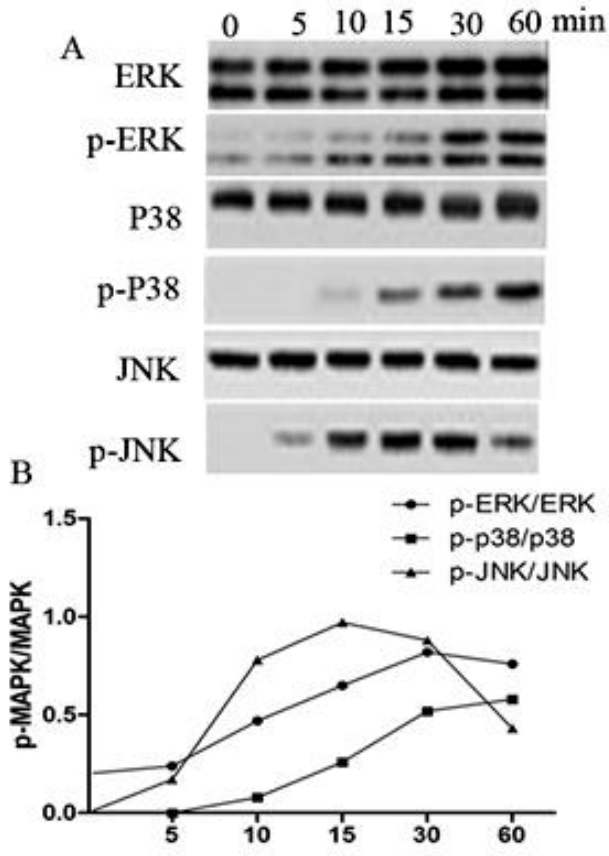

Fig. 3: Activation of MAPK signal pathway in cardiac fibroblasts treated with AngII; (A) Phosphorylation levels of ERK, p38 and JNK at different challenging time by western blot; (B) Relative expression of the degree of phosphorylation;

DISCUSSION

Myocardial fibrosis causes a variety of cardiovascular disorders such as myocardial infarction and hypertension, eventually leads to myocardial remodeling and even fundamental dysfunction of the heart. Therefore, prevention and improvement of myocardial fibrosis is essential to cardiac functions (Li *et al*., 2013). In this study, Ang-II was used to induce cardiac fibroblasts proliferation and the effect of quercetin on Ang-II function was studied. The study found that Ang-II significantly enhanced the DNA synthesis of rat cardiac fibroblasts, the secretion of collagen I and collagen III, and the expression of TGF and CTGF. By contrast, quercetin inhibited the induction caused by Ang-II, suggesting that quercetin was a robust antagonist against Ang-II in cardiac fibroblast activities.

Quercetin is a flavonoid widely distributed in plants, exists mainly in the form of glycoside (Panchal *et al*., 2012). The present work investigated the effect of ROS scavenger NAC on the proliferation of cardiac fibroblasts induced by Ang-II and found that NAC significantly inhibited the DNA synthesis, decreased the secretion of collagen I and collagen III and reduced ROS production. To determine whether quercetin inhibits the proliferation of cardiac fibroblasts induced by Ang-II through the ROS pathway, the present study examined the effect of quercetin on the ROS content of cardiac fibroblasts in condition of Ang-II challenging. Results showed that ROS in the Ang-II group was significantly increased compared to the control group, whereas quercetin pretreatment overtly suppressed the production of ROS in the cells. Data here implied that quercetin could inhibit myocardial fibrosis through ROS pathway.

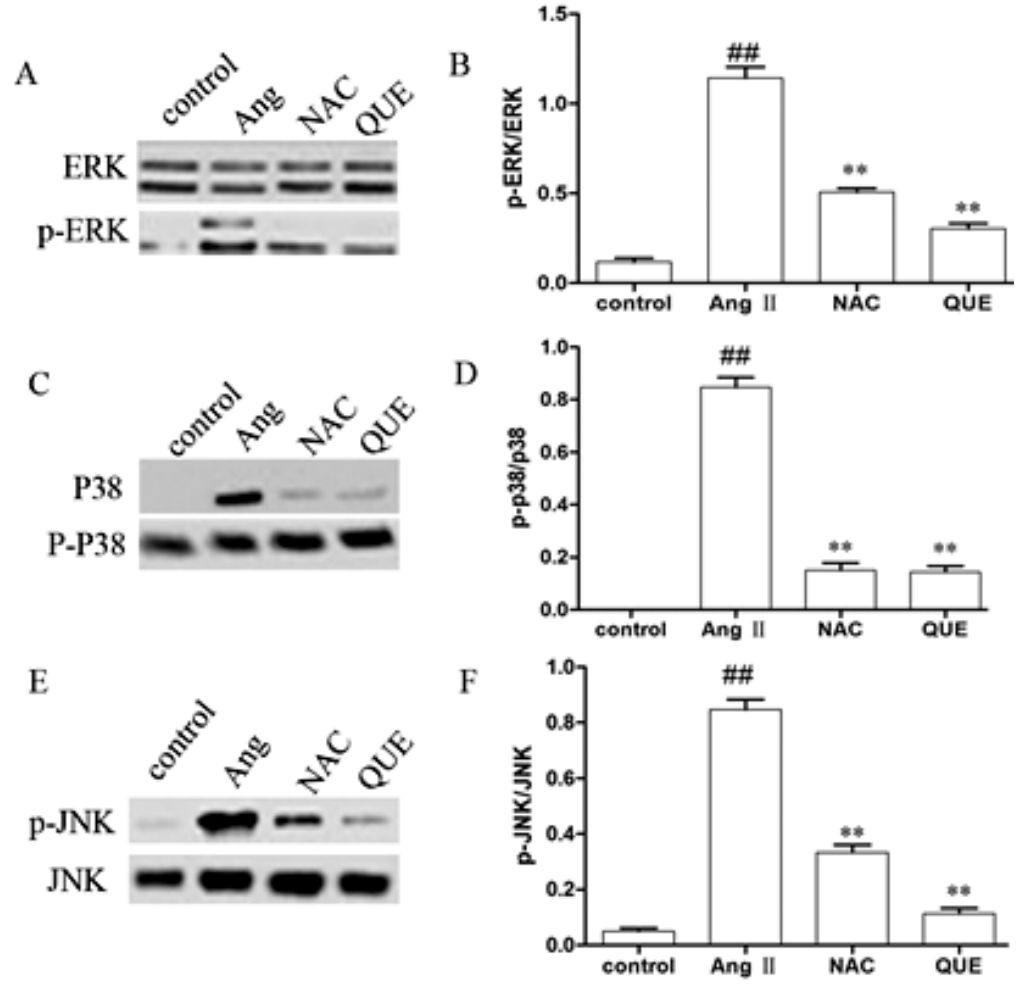

Fig. 4: Inhibitory effect of quercetin on MAPK signaling pathwaya

Oxidative stress damage occurs when the ROS production increases or the cellular antioxidant defense ability is impaired. This process plays an important role in myocardial ischemia, myocardial reperfusion and heart failure (Ren *et al*., 2005). Studies have pointed out that the excessive accumulation of ROS or antioxidant enzymes resulted in myocardial injury and myocardial fibrosis in rats. Present research work investigated the effect of quercetin on proliferation, collagen expression and the activation of MAPK signaling in cardiac

fibroblasts. NAC and QUE were observed to inhibit the cell DNA synthesis and collagen secretion and the phosphorylation of ERK, p38 and JNK induced by Ang-II, which showed that quercetin and ROS mediated Ang-II induced activation of MAPK signaling pathway. Therefore, quercetin inactivated the MAPK signaling by suppressing ROS formation, thereby decelerated the proliferation of cardiac fibroblasts induced by Ang-II and ultimately caused beneficial effects against myocardial fibrosis.

CONCLUSION

The results of this study revealed that quercetin inhibited the MAPK pathway activation via ROS and reversed such effects and inhibited cardiac fibroblasts proliferation.

REFERENCES

Ahn J, Lee H, Kim S, Park J and Ha T (2008). The anti-obesity effect of quercetin is mediated by the AMPK and MAPK signaling pathways. *Biochem. Biophy. Res. Com*., **373**(4): 545-549:

Chen SF, Nieh S, Jao SW, Liu CL, Wu CH and Chang YC (2012). Quercetin suppresses drug-resistant spheres via the p38 MAPK-Hsp27 apoptotic pathway in oral cancer cells. *PLoS. One*, **7**(11): e49275.

Iles L, Pfluger H, Phrommintikul A, Cherayath J, Aksit P and Gupta SN (2008). Evaluation of diffuse myocardial fibrosis in heart failure with cardiac magnetic resonance contrast-enhanced T1 mapping. *J. Am. Col. Cardiol*., **52**(6): 1574-1580.

Li L, Fan D, Wang C, Wang JY, Cui XB and Wu D (2011). Angiotensin II increases periostin expression via Ras/p38 MAPK/CREB and ERK1/2/TGF-beta1 pathways in cardiac fibroblasts. *Cardiol. Res*., **91**(1): 80-89:

Li M, Jiang Y, Jing W, Sun B, Miao C and Ren L (2013). Quercetin provides greater cardioprotective effect than its glycoside derivative rutin on isoproterenol-induced cardiac fibrosis in the rat. *Can. J. Phyiol. Pharmacol*., **91**(10): 951-959.

Matsumoto-Ida M, Takimoto Y, Aoyama T, Akao M, Takeda T and Kita T (2005). Activation ofTGF-beta1-TAK1-p38 MAPK pathway in spared cardiomyocytes is involved in left ventricular remodeling after myocardial infarction in rats. *Am. J. Physiol. Heart. Circ. Physiol*., **290**(2): 709-715

Min YD, Choi CH, Bark H, Son HY, Park HH and Lee S (2007). Quercetin inhibits expression of inflammatory cytokines through attenuation of NF-kappaB and p38 MAPK in HMC-1 human mast cell line. *Inf. Res*., **56**(5): 210-215.

Muslin AJ (2008). MAPK signalling in cardiovascular health and disease: Molecular mechanisms and therapeutic targets. *Clin. Sci*., **115**(7): 203-218.

Nam TW, Yoo CI, Kim HT, Kwon CH, Park JY and Kim YK (2008). The flavonoid quercetin induces apoptosis and inhibits migration through a MAPK-dependent mechanism in osteoblasts. *J. Bone. Miner. Metab*., **26**(6): 551-560.

Panchal SK, Poudyal H and Brown L (2012). Quercetin ameliorates cardiovascular, hepatic and metabolic changes in diet-induced metabolic syndrome in rats. *J. Nutrition*, **142**(7): 1026-1032.

Ren J, Zhang S, Kovacs A, Wang Y and Muslin AJ (2005). Role of p38alpha MAPK in cardiac apoptosis andremodeling after myocardial infarction. *J. Mol. Cell. Cardiol*., **38**: 617-623:

Serra A, Bryant N, Motilva MJ, Blumberg JB and Chen CY (2012). Fetal programming of dietary fructose and saturated fat on hepatic quercetin glucuronidation in rats. *Nutrition*. **28**(2): 1165-1171:

Weng CJ, Chen MJ, Yeh CT and Yen GC (2011). Hepatoprotection of quercetin against oxidative stress by induction of metallothionein expression through activating MAPK and PI3K pathways and enhancing Nrf2 DNA-binding activity. *Nat. Bio. Technol*., **28**(3): 767-777.

Yan L, Zhang JD, Wang B, Lv YJ, Jiang H and Liu GL (2013). Quercetin inhibits left ventricular hypertrophy in spontaneously hypertensive rats and inhibits angiotensin II-induced H9C2 cells hypertrophy by enhancing PPAR-gamma expression and suppressing AP-1 activity. *PLoS. One*., **8**(9): e72548