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Original Article

Orientin prevents myocardial remodeling after MI through the PI3K/Akt signaling pathway

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Abstract: In the current study, the protective role of orientin in myocardial infarction (MI) and its underlying mechanism was explored. Male C57BL/6J mice were treated with anterior wall standard MI surgery and administered orientin one week later in mice for 14 days. Improved cardiac dysfunction was observed after orientin treatment detected by echocardiographic and hemodynamic evaluation. Additionally, H&E staining demonstrated an infarct size ratio and the cardiomyocyte cross-sectional area that was lower in the MI-Ori group compared with the MI-Veh group. In addition, the fibrotic area ratio was significantly lower in the MI-Ori group detected by Masson's staining. qRT-PCR analysis showed that orientin treatment remarkably decreased the expression of fibrotic and hypertrophic biomarkers. Western blot analysis showed that orientin improved cardiac dysfunction through targeting the MAPK and PI3K/Akt signaling pathways. Taken together, the results demonstrate that orientin may improve mouse left ventricular function and attenuate cardiac remodeling by inhibition of the MAPK and PI3K/Akt signaling pathway.

Keywords: Myocardial infarction (MI), orientin, PI3K/Akt signaling, infarct size, fibrotic

Introduction

Myocardial infarction (MI) is a serious disease caused by coronary atherosclerotic heart disease that induces myocardial cell death and triggers a series of repair reactions [1, 4, 15] A series of wound healing reactions occur in the heart after MI [16, 23]. Cardiomyocyte necrosis and apoptosis lead to molecular and cell remodeling, including inflammation, cardiac hypertrophy, and fibrosis [6, 8, 20]. Through inflammation, the changes of the heart play a compensatory role in maintaining normal cardiac function. However, due to continuous stress, cardiac remodeling will lead to progressive and irreversible dysfunction, which results in chronic heart failure or death [10, 18, 22, 27]. In the process of cardiac remodeling and oxidative stress, several cell toxicities, such as lipid peroxidation, protein oxidation, and DNA damage induce changes of calcium transport protein and activation of signaling pathways triggered myocardial hypertrophy, dysfunction, cell apoptosis, and proliferation of fibroblasts [7, 11, 14, 17]. To date, pharmaceutical drugs have been the main treatment for heart disease. Many pharmaceutical drugs, such as β-blockers, angiotensin converting enzyme inhibitors (ACEI), and angiotensin receptor blockers (ARB), play an important role in the prevention of myocardial remodeling [9, 21]. Despite their usage, the incidence and mortality of heart failure are still relatively high. Many studies have confirmed that intake foods rich in flavonoids can reduce the mortality of cardiovascular disease [25]. Recent studies have found that naringenin, a kind of flavonoid that exists in citrus fruits, reduces myocardial hypertrophy and interstitial fibrosis in stress-overload mice [19]. Orientin is a natural flavonoid compound, which has the effect of anti-tumor, antiproliferation, anti-inflammation, and anti-oxidation [13]. Recent studies have discussed the effect of Orientin on telomere integrity in human cells. The results have shown that Orientin could significantly increase the response of telomere to DNA damage. In addition, Orientin has proven to be an atrial-selective drug and can prolong the absolute non-reaction period of atrium, block transient outward potassium channel (Ito), and effectively prevent atrial fibrillation [2]. A study on neonatal rat cardiomyo-

cytes confirmed that Orientin has protective effects on hypoxia/reoxygenation stress [12]. However, it is not clear whether Orientin can improve the cardiac function and inhibit the remodeling after MI. In this study, C57BL/6 mice were used for a standard anterior wall MI to determine whether Orientin has a protective effect on myocardial remodeling. Furthermore, in order to analyze the mechanism underlying protection, expression of the MAPK and PI3K/Akt signaling pathways was examined. This article extends the medicinal value of Orientin, and Orientin, as a natural compound, can be used as a potential target for the treatment of coronary disease.

Materials and methods

Experimental mice

All experimental procedures were carried out in accordance with the guidelines of the Animal Care and Use Committee of Linzi People's Hospital. This study was approved by the Ethics Committee of Linzi People's Hospital. Eight to ten weeks old male C57BL/6J mice with body weights of 23-26 g were purchased from the Institute of Laboratory Animal Science (Beijing, China). Four groups were included: Sham-Ori, Sham-Veh (n=15), MI-Ori and MI-Veh (n=15). One week after MI operation, mice were treated with orientin (10 mg/kg/day) or only distilled water by gavage for 2 weeks.

Left coronary artery ligation operation

Standard anterior wall MI surgery or sham operation was performed according to the method described in the literature [3]. After adequate anesthesia, left thoracotomy was carried out to open the pericardium. The proximal left anterior descending branch (LAD), located at the top of the left auricle, was ligated with 7-0 silk suture. For the sham operation group, the silk suture was placed around the left anterior descending branch without ligation. In order to reduce the effect of acute inflammation and early fibrosis after MI, Orientin was administered to mice in one week after operation, in which the mice died within seven days after operation could be excluded.

Echocardiography and hemodynamic evaluation

Echocardiography was performed on the mice after anesthesia at three weeks after the operation. The anesthetic method was isoflurane inhalation. The left ventricular structure and function were evaluated using the MyLab 30CV system (Biosound Esaote, Inc.), as described in the previous article. The parameters were obtained from at least 3 heartbeats and each parameter was averaged. Invasive hemodynamic evaluation was performed using the 1.4-French Millar catheter-tip of micromanometer gauge catheter. The catheter-tip was inserted into the left ventricle through the right carotid artery. The Aria pressure volume conductance system was used to record heart rate, pressure, and volume continuously. The maximum pressure development rate (dp/dtmax) and the minimum pressure decay rate (dp/dtmin) were processed by PVAN data analysis software (Millar, Inc., Texas, USA). All surgical and data analysis were performed by blind method.

Analysis of heart morphology and morphometry

After echocardiography and hemodynamic data were recorded, the anesthetized mice were soon executed by cervical dislocation. The hearts of mice were removed during diastolic phase and placed in 10% potassium chloride. The heart and lungs were weighed. The heart weight/body weight ratio (HW/BW, mg/g), heart weight/tibia length ratio (HW/TL, mg/mm), and lung weight/body weight ratio (LW/BW, mg/g) were calculated. A high-magnification image was obtained under the light microscope. Morphological quantitative analysis was performed by HE staining. The infarct area at the level of the middle papillary muscle was detected by a quantitative digital analysis system. The infarct area was expressed as a percentage of the total left ventricular area. The cross sectional area of cardiac myocytes was analyzed by quantitative digital image analysis system and HE staining section. Evidence of interstitial collagen deposition was found by Masson staining and analyzed with Image-ProPlus 6.0.

Cell apoptosis detection

Apoptotic cells were detected by In situ cell death detection kit (Sigma Aldrich, USA) and using the TUNEL method. Tissue sections were prepared and stained according to the instructions. The TUNEL positive cells in the peripheral area of the heart infarction were determined by the quantitative digital analysis system and counted with the Image-Pro Plus 6 software.

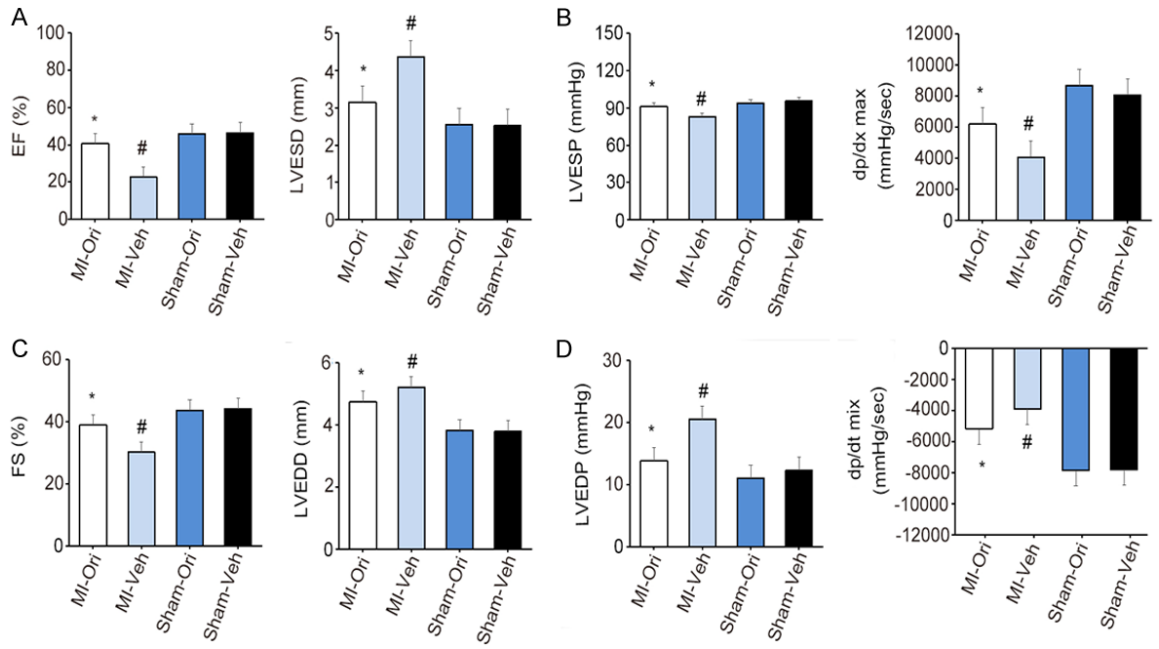

Figure 1. Effect of orientin on cardiac function. A: Orientin enhanced LV function and decreased LV diameter after MI; B: Hemodynamic data showing cardiac function. #P < 0.05 vs sham-Veh group, *P < 0.05 vs MI-Veh group; C: Left ventricular fractional shortening (FS) was reduced, and left ventricular end diastolic diameter (LVEDD) was increased after Orientin treatment; D: Left ventricular end diastolic pressure (LVEDP) and dp/dtmin change in different groups; #P < 0.05 vs sham-Veh group, *P < 0.05vs MI-Veh group.

qRT-PCR analysis

qRT-PCR analysis was carried out according to the procedures described in the literature [9]. Total RNA was extracted from the frozen heart of mice by Trizol. For each sample, the transcript of the first strand cDNA synthesis kit (Basel, Switzerland) and 2 μg RNA was used to synthesize cDNA. Then Light Cycler 480 SYBR Green 1 MasterMix (Roche) was used to perform QRT-PCR, GAPDH as a reference gene. Primers for Collagen Ia: Forward 5'-AGGCAGTCTTGGTTTGGATG-3', Reverse: 5'-CACCAGCAACACCATCGTTA-3'; Collagen IIIa, 5'-CCCAAATCCCCAGAGCCATT-3', 5'-GAAGGTGTCACACAGGGAGAGA-3'; GAPDH, 5'-ACTCCAGGCCTCACAAATTC-3', 5'-TCTCGGTGCATGGTAAGACA-3'.

Western blot analysis

The BCA protein quantitative assay kit (Thermo Scientific, MIT, USA) was used to detect the concentration of the total protein extracted from the frozen heart tissues of mice. The experiment was carried out according to the method described in the literature (Schroecksnadel et al., 2004), and GAPDH was used as a control. Primary antibodies were purchased from Cell Signaling Technology (MA, USA).

Statistical analysis

The data are expressed as the mean ± standard deviation (SD). The differences among groups were compared by a one-way analysis of variance (ANOVA) and Tukey's post hoc test. The average value of the two groups was compared by Unpaired Student's t test. P < 0.05 suggested a statistically significant difference.

Results

Orientin improves impaired myocardial function after MI

In order to analyze the effect of orientin on the progression of cardiac dysfunction, a ligation of the left anterior descending branch or a sham operation on the mice was performed. After 3 weeks, the thickness of the wall, the diameter of the heart cavity and the function of the left ventricle were measured by transthoracic echocardiography. No significant difference was found between the mice treated with distilled water and the mice treated with Orientin. However, compared with the MI-Veh group, the results of EF, FS, LVESD and LVEDD showed that cardiac dysfunction in the MI-Ori group was improved. The results of LVESP, LVEDP, dp/dtmax and dp/dtmin suggested that

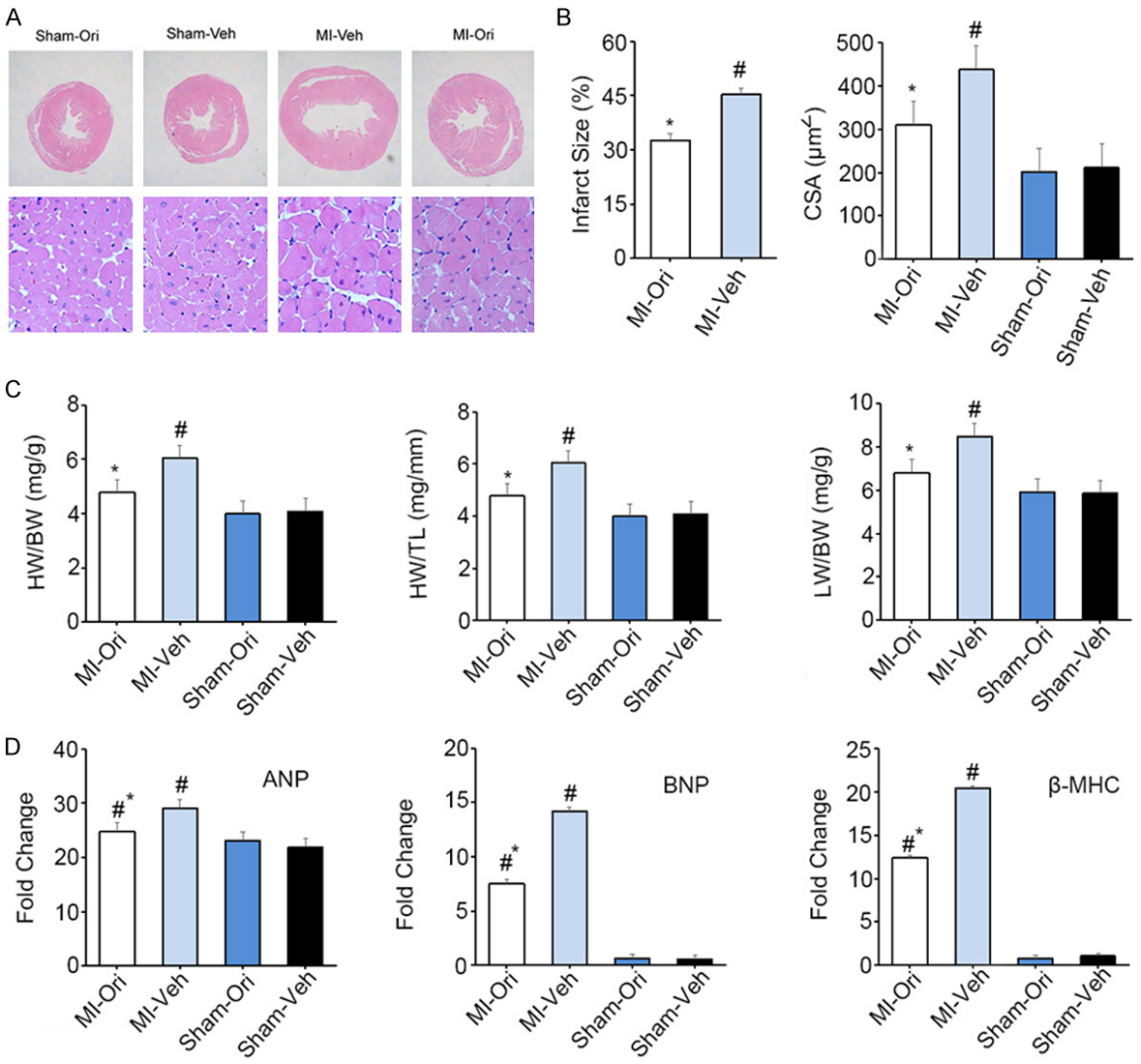

Figure 2. Orientin protects against cardiac hypertrophy after MI. A: HE staining of whole heart after MI; B: Infarct size ratio and cardiomyocyte CSA in different groups; C: Statistical results of HW/BW, HW/TL and LW/BWratios in different groups; D: Relative mRNA expression of hypertrophic markers were determined by qRT-PCR. #P < 0.05 vs sham-Veh group, *P < 0.05 vs MI-Veh group.

the hemodynamics in the MI-Ori group improved, indicating that Orientin treatment after MI has a positive effect (**Figure 1**).

Orientin prevents the occurrence of myocardial hypertrophy after MI

In order to analyze the effect of Orientin on myocardial hypertrophy after MI, we detected the myocardial hypertrophy by histology and qRT-PCR. The results of H&E staining showed that the proportion of infarct area in the MI-Ori group was significantly lower than that of the MI-Veh group. H&E staining also showed that Orientin could restrain the cross sectional area of myocardial cells after MI (**Figure 2A** and **2B**). After 3 weeks of treatment, the ratios of HW/BW, HW/TL and LW/BW in the MI-Veh group increased significantly compared with the sham-vector group. Compared with the MI-vector group, the ratios of HW/BW, HW/TL and LW/BW in the MI-Ori group decreased significantly ($P < 0.05$) (**Figure 2C**). In addition, according to the results of qRT-PCR, myocardial hypertrophy indexes including mRNA expression of ANP, BNP, and b-MHC increased after MI operation, while they were alleviated after Orientin treatment ($P < 0.05$) (**Figure 2D**). These results confirme the protective effect of Orientin on myocardial hypertrophy.

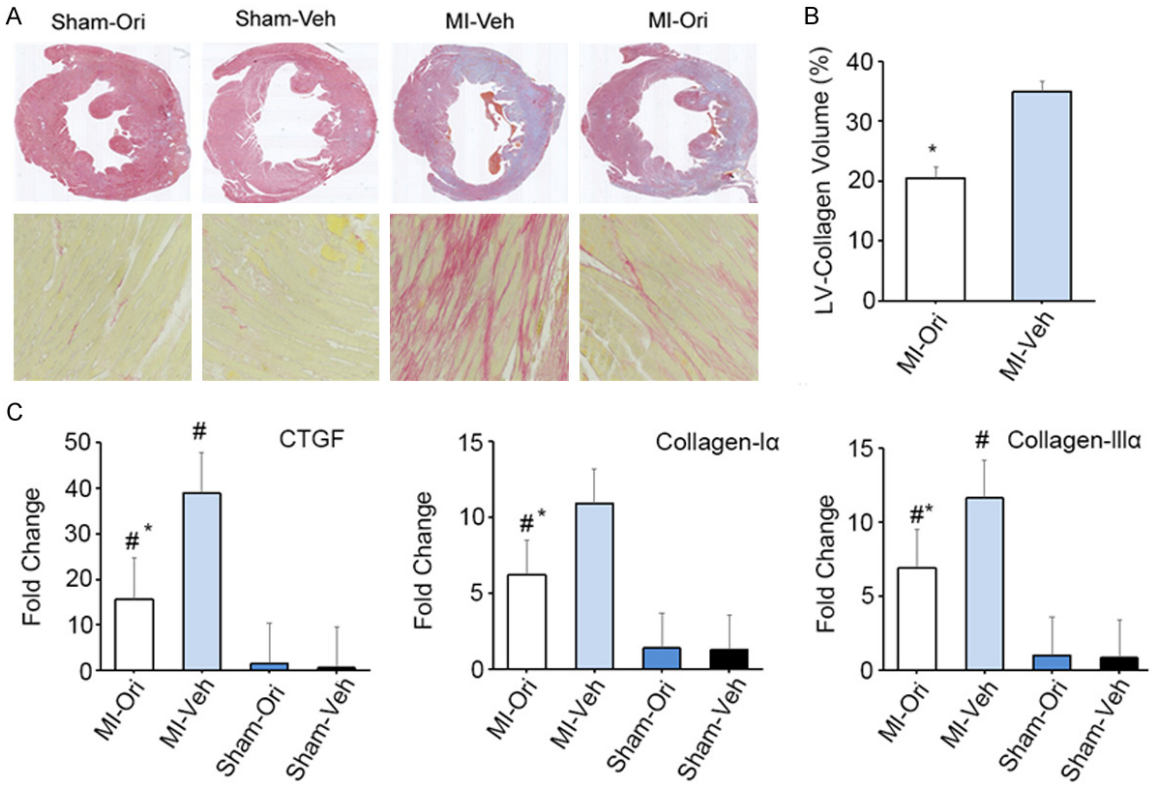

Figure 3. Orientin attenuates cardiac interstitial fibrosis subsequent to MI. A: Representative Masson's staining images of heart after MI; B: Quantitative fibrotic area ratio measurements; C: Relative mRNA expression of fibrotic markers in left ventricular samples from the indicated groups. #P < 0.05 vs sham-Veh group, *P < 0.05 vs MI-Veh group.

Orientin weakens the fibrosis reaction after MI

In order to analyze the effect of Orientin on interstitial and perivascular fibrosis, interstitial collagen deposition was detected in cardiac myocytes by Masson staining. There was no significant difference in the extent of myocardial fibrosis between the sham-operated mice and the Orientin-treated mice. However, the proportion of fibrotic area in the Orientin-treated group was significantly lower than that of the MI-Veh group after MI operation ($P < 0.05$) (**Figure 3A** and **3B**). To further analyze the effect of Orientin on collagen synthesis, we also examined mRNA expression of fibrosis mediators, such as collagen Ia, collagen IIIa, and CTGF. Compared with the MI-Veh group, expression of collagen Ia, collagen IIIa, and CTGF in the Orientin-treated group decreased after MI ($P < 0.05$) (**Figure 3C**).

Orientin alleviates myocardial remodeling by inhibiting the activation of MAPK and PI3K/Akt signaling pathways

In order to understand the molecular mechanism of Orientin affecting myocardial remodeling after MI, activation of proteins in MAPK and PI3K/Akt signaling was detected by Western Blot. The results showed that expression of phosphorylated ERK and phosphorylated JNK in the Orientin-treated group after MI were decreased ($P < 0.05$). However, there was no significant difference in the level of phosphorylated p38 between the MI-Veh group and the MI-Ori group ($P>0.05$). Moreover, the role of PI3K/Akt signaling pathway was also analyzed. The PI3K/Akt signaling pathway is the main mechanism to regulate the progression of pathological myocardial hypertrophy. It appeared that Orientin could reduce the expression level of phosphorylated PI3K and phosphorylated Akt ($P < 0.05$) (**Figure 4**).

Discussions

Orientin is a natural flavonoid from food that is also a traditional herbal medicine. A recent *in vivo* study showed that Orientin prodrug had a protective effect on acute ischemia/reperfusion injury [12]. We have found that Orientin has the effect of protecting against remodeling of the myocardium after MI. In addition, echo-

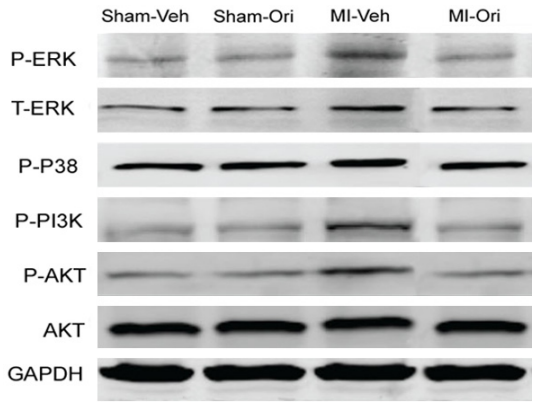

Figure 4. Effects of orientin on PI3K/AKT signaling after MI. Representative and quantitative expression of phosphorylated and total ERK1/2, JNK1/2, p38, PI3K and Akt in the heart tissues.

cardiography and hemodynamic evaluations showed that orientin could improve cardiac function and inhibit myocardial remodeling.

In this experiment, mice were treated with Orientin 1 week after the operation could avoid the effect of acute inflammation and early fibrosis after MI. This method excluded mice that died in the 7 days after the operation. The second factor was that the hearts in most of the survival mice were in a compensatory state at 3 weeks after ligation of the left anterior descending branch. The heart after MI undergoes a series of adaptations and this pathological process can be divided into the following stages: acute myocardial deaths caused by MI, expansion of infarction area due to inadequate perfusion, and compensatory myocardial hypertrophy, myocardial cell death, and fibrosis in the remote area of infarction. Despite adoption of new treatment strategies, all these changes will cause a rise in cardiac dysfunction and mortality. In the process of myocardial remodeling, extracellular stress can be transmitted from cell sensors to the cytoplasm and activate of the MAPK cascade. The MAPK cascade includes multiple kinases, such as ERK, JNK, and p38. For example, ERK1/2 can be activated by a G-protein coupled receptor that transfers extracellular signals, resulting in activation of kinase phosphorylation cascade (MAPKKK and MAPKK), and promoting cardiac hypertrophy. JNK1/2 cascade activation in the heart promotes pathological myocardial remodeling after MI, including cardiomyocyte apoptosis, inflammation, and fibrosis. Moreover, ischemia could stabilize activation of p38 MAPK in the isolated perfused heart of the rat. Our results show that orientin could relieve the activation of ERK and JNK. Akt is the downstream target of PI3K, which can be activated after the upstream signal factors remove the inhibitory isoforms. Expression of phosphorylated PI3K and phosphorylated Akt was also detected. Our results show that Orientin could decrease the expression of phosphorylated PI3K and phosphorylated Akt. When cardiac hypertrophy occurs, fibrous collagen deposition and abnormal expression of extracellular matrix can be detected. Collagen I and III are the main collagen types, and also the main factors that lead to interstitial fibrosis in the progression of heart failure [3]. Our results show that the mRNA expression of collagen Ia and collagen IIIa increased after MI, and Orientin could significantly alleviate this increase. In addition, the results of Masson's staining showed that Orientin could relieve the interstitial and perivascular fibrosis of myocardium after MI. A number of studies have confirmed that cell apoptosis occurs in many cardiovascular diseases, including myocardial hypertrophy, infarction, and heart failure [5, 26]. Bax is a proapoptotic protein, which can migrate to mitochondria when ischemia occurs, resulting in the release of cytochrome c and apoptosis of cardiac myocytes [24]. In this study, expression of p65, BAX, and cleaved Caspase3 protein was detected by TUNEL staining. Their expression in the MI-Ori group decreased significantly. These results suggest that Orientin may alleviate apoptosis of myocardial cells.

In conclusion, Orientin inhibits cardiac hypertrophy and fibrosis by suppressing the MAPK and PI3K/Akt signaling pathways, and by decreasing the up-regulation of phosphorylation of ERK and JNK, PI3K, and Akt. In addition, Orientin can significantly reduce levels of phosphorylated p65, BAX, and cleaved Caspase3 through the NF-κB signaling pathway, and then decrease cardiomyocyte apoptosis. These results partially illustrate pharmacological effects of Orientin on myocardial remodeling after MI and the signaling pathways associated with the protective effect after MI. This study provides a new strategy of pharmaceutical treatment for cardiac remodeling and prevention of heart failure.

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