Qiliqiangxin Protects Against Cardiac Ischemia-Reperfusion Injury via Activation of the mTOR Pathway

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Key Words
Qiliqiangxin • Cardiac ischemia-reperfusion injury • mTOR

Abstract
Background/Aims: Qiliqiangxin (QL) has been used for the treatment of chronic heart failure in China. Accumulating evidence suggests QL’s cardio-protective effects on continuous myocardial ischemia. However, it is unclear whether QL has beneficial effects on cardiac ischemia-reperfusion (I/R) injury. Methods: A mouse model of cardiac I/R was established by ligation of the left anterior descending coronary artery for 45 minutes followed by reperfusion. The mice were treated with QL for three days before surgery and continually after I/R. Triphenyltetrazolium chloride staining, echocardiography and Masson’s trichrome staining were used to determine infarct size, cardiac function, and fibrosis, respectively. Expression levels of phospho-mTOR (Ser2448), mTOR, phospho-4EBP (Ser65), 4EBP, phospho-Akt (Ser473) and Akt were detected by Western blotting. Results: At 1 day after I/R, QL treatment significantly reduced the infarct size of mice exposed to I/R. At 7 days after I/R, mortality was reduced in QL treated animals in comparison with the control group. In addition, QL treated mice showed increased left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) at 1 and 7 days after I/R. In agreement, Masson’s trichrome staining demonstrated that interstitial fibrosis was less pronounced in QL treated mice compared with controls, suggesting that adverse left ventricular remodeling is attenuated in QL treated mice. Moreover, western blotting analysis demonstrated that QL activated the mTOR pathway, while mTOR inhibition via Rapamycin abolished the protective effects of QL against I/R injury. Conclusion: This study suggests that QL attenuates the progression of cardiac remodeling after I/R likely via mTOR activation. This represents a new application for QL in the prevention of I/R injury.

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Introduction

Acute myocardial infarction (MI) caused by coronary artery occlusion is a common cause of cardiac dysfunction and heart failure (HF) [1]. Early restoration of myocardial perfusion is the primary goal of initial treatment for patients with acute MI [1]. Currently, advances in pharmacological therapy and percutaneous coronary intervention can restore coronary flow in a majority of patients within a short time after the onset of symptoms [2]. However, restoring blood flow itself may extend cardiac injury, a phenomenon referred to as reperfusion injury [3, 4]. Adverse left ventricular (LV) remodeling after acute MI, characterized by LV dilatation and fibrosis, is a critical determinant of the subsequent development of HF [5]. LV remodeling is also an important pathophysiological feature in ischemia-reperfusion (I/R) injury [6]. Therefore, identifying pathways that effectively protect against I/R injury and/or adverse remodeling could lead to novel therapeutic approaches to mitigating LV remodeling and HF after acute MI.

The mammalian target of rapamycin (mTOR) is an important mediator of the insulin-PI3K-Akt axis in multiple organs, including the heart [7]. mTOR forms two functional complexes: rapamycin-sensitive mTOR complex 1 (mTORC1) and rapamycin-insensitive mTOR complex 2 (mTORC2) [8]. mTORC1 activates p70S6 kinase that phosphorylates the ribosomal protein S6, and inhibits the binding of 4E-binding protein 1 (4EBP) to eukaryotic translation initiation factor 4E, resulting in the promotion of translation [9]. mTORC2 activates Akt, a key regulator of cardiomyocyte survival, by phosphorylation at Ser473 [10, 11]. Previous studies using pharmacological mTOR inhibitors, including rapamycin, in both in vivo and ex vivo models of MI have found discrepant effects. Some reports have demonstrated beneficial effects of mTOR activation in I/R models [12-16]; conversely, another report described cardio-protective effects of mTOR inhibition [10]. Thus, the role of mTOR in cardiac function and LV remodeling after I/R injury remains undefined.

Qiliqiangxin capsule (QL) is a Chinese patent drug that was shown to be effective and safe in our recent clinical trial for the treatment of patients with chronic heart failure [17]. QL includes 11 Chinese herbs, with Radix Astragali and Aconite Root containing the main active constituents [18]. Radix Astragali is known to attenuate adverse cardiac remodeling after MI and hypertrophy [19]. However, whether QL has a role in cardiac function and LV remodeling after I/R injury remains unknown. Therefore, the present study aimed to assess QL's beneficial effects on I/R injury in mice and explore potential mechanisms.

Materials and Methods

Animals

Eight to ten week old C57BL/6 male mice (20-25g) were obtained from Laboratory Animal Center of Nanjing Medical University and housed with 12-hour dark/light cycle and free access to food in accordance with the regulations on mouse welfare. This study complied with standards for the Care and Use of Laboratory Animals [Laboratory Animal Center of Nanjing Medical University], and all procedures were approved by the Ethics Review of Lab Animal Use Application of Nanjing Medical University.

In vivo I/R model

The I/R model was established according to previously reported studies [20-23]. Briefly, animals were anesthetized i.p. with ketamine and sevoflurane, intubated, and ventilated. A left thoracotomy was performed, and the left anterior descending coronary artery (LAD) was ligated using 7-0 silk sutures with a section of PE-10 tubing placed over the LAD, 1mm from the tip of the normally positioned left atrium. After 45-min ischemia, the LAD ligature was released and reperfusion was visually confirmed. During the procedure, body temperature was maintained with a 37°C warming plate. The operated mice were euthanized at 1 and 7 days after I/R for infarction size, fibrotic area, and signaling pathway assessment. Sham operations were carried in a similar fashion, but without sutureing the LAD.
In vivo QL and Rapamycin Treatment

QL was provided by Shijiazhuang Yiling Pharmaceutic (Hebei, China). The herbal drugs were authenticated and standardized with marker compounds according to the Chinese Pharmacopoeia 2005 (National Pharmacopoeia Committee, 2005). The QL powder was dissolved in normal saline (NS). Mice were randomized for intragastric treatment with QL (0.5 g/kg/day, n=25) or NS (n=25) [17, 24, 25]. Sham operated mice were also given QL (0.5 g/kg/day, n=10) or NS (n=10). All mice were treated for three days before surgery and afterward, until euthanasia. In order to investigate if mTOR activation is required for the protective effects of QL in I/R injury, Rapamycin (a specific mTOR inhibitor, 5mg/kg) was injected via the tail vein 10 min before cardiac I/R in the I/R+QL group.

Measurement of Myocardial Infarction Size

At 24 hours after I/R, mice were anesthetized i.p. with 0.4 to 0.75 mg/g tribromoethanol. Then, 1 ml Evans blue (0.01 g/ml; BioSharp, China) was slowly injected into inferior vena and the heart removed immediately [26, 27]. After storage for 10 min at -20°C, the heart was cut into 5 or 6 transverse slices (1 mm thickness) across the long axis. Then, the slices were stained with 1% triphenyltetrazolium chloride (TTC, Amresco, USA) in citrate buffer solution (ph=7.4) for 10 min at 37°C, to delineate the area at risk (AAR) [20-23]. Afterwards, all slices were fixed with 4% paraformaldehyde, photographed and analyzed. As previously reported [28, 29], the infarct area (IA) appeared white, while the non-infarct but at risk area was red. The final infarct size was expressed as the ratio of IA to AAR, calculated by computerized planimetry (Image J, version 1.44, NIH, Bethesda, MD).

Echocardiography Measurement

Cardiac function was evaluated on a high-frequency ultrasound system Vevo2100 (VisualSonics Inc, Toronto, ON, Canada) with a 30 MHz central frequency scan head at 1 and 7 days after I/R operation. Mice were anesthetized with 1–2% isoflurane vapor in a 1:1 oxygen mixture via a nose cone on a heating pad to maintain normothermia. The left ventricular ejection fraction (LVEF; %) and left ventricular fractional shortening (LVFS; %) were assessed according to the guidelines accompanying the Vevo2100 system.

Histological Analysis

To evaluate the morphological changes and the extent of cardiac fibrosis, the hearts were harvested at 7 days after I/R, washed in PBS, fixed in 4% paraformaldehyde overnight and embedded in paraffin. Each heart was cut into sections of 4 µm-thick and stained with hematoxylin and eosin (H&E) and Masson’s trichrome. Micrographs were acquired on a Nikon light microscope (Nikon, Japan).

Western Blotting Analysis

At 24 hours after reperfusion, total protein was obtained from left ventricular myocardial tissues after extraction with the RIPA lysis buffer (P0013B, Beyotime, China). Protein concentration was measured by the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). A total of 60 µg total protein were electrophoresed and separated on 4–12% SDS-PAGE, and transferred onto nitrocellulose membranes (Millipore, US). The membranes were blocked with 5% skim milk at room temperature for an hour, and incubated overnight at 4°C with primary antibodies raised in rabbits against mTOR (1:1000), phospho-mTOR (Ser2448, 1:1000), 4EBP (1:1000), phospho-4EBP (Ser65, 1:1000), Akt (1:1000), phospho-Akt (Ser473, 1:1000) purchased from Cell Signaling Technology (USA). Then, the membranes were incubated with HRP-conjugated secondary antibodies (1:2000; Cell Signaling Technology, USA) at room temperature for two hours. The signals were detected with a SuperSignal ECL kit (Thermo, USA) in a Western blotting detection system (Bio-Rad, CA, USA). HRP-conjugated monoclonal mouse anti-GAPDH (1:5000; KANGCHEN, China) was used to quantify GAPDH levels. Results were expressed as density values normalized to GAPDH.

Statistical Analyses

Data were presented as mean±standard error of mean (SEM). Group differences were analyzed by a two-tailed Student’s t-test. For multiple comparisons, one-way ANOVA with Bonferroni post hoc test was used. The overall mouse survival after I/R was evaluated using Kaplan-Meier curves and compared by log-rank test. All statistical analyses were conducted using SPSS 15.0 or GraphPad Prism 5. P<0.05 was considered statistically significant.
Results

QL Treatment Reduces Mortality and Preserves Cardiac Function in Mice after I/R in vivo

The Kaplan-Meier survival curves demonstrated lower mortality in QL treated mice compared with control untreated mice at 7 days after I/R. Indeed, the survival rate of QL treated animals was 80% and only 52% mice survived in the control group (log-rank test \( P = 0.0329 \); Fig. 1). Compared with untreated animals, echocardiography showed increased LVEF and LVFS at 1 and 7 days after I/R (Fig. 2A-C). One week after I/R, the heart weight/body weight (HW/BW) ratios were significantly lower in QL treated mice than in untreated animals (Fig. 2D).

QL Treatment Reduces Infarct Size

Evans blue and TTC staining methods were used to detect the size of infarct- and non-infarct but at risk areas [27]. The risk areas of the total LV were similar between QL and
untreated groups. However, treatment with QL resulted in significantly reduced infarct size compared with the untreated group (Fig. 3).

**Fig. 3.** QL reduces myocardial infarct size after I/R. (A) Hearts were cut transversely into 5-6 slices at 24 hours after I/R. Each slice was stained with Evans blue and TTC. The area at risk (AAR) of myocardium appears red, while the infarct area (IA) is white. (B) Infarct size in QL treated and untreated mice. Infarct size was expressed as the ratio of IA to AAR (n=10, QL treated mice; n=9, untreated mice). **P<0.01 I/R+QL vs I/R+NS.

**Fig. 4.** QL improves pathological changes in myocardial tissue after I/R. (A) Representative micrographs of I/R mouse hearts after H&E staining. These images demonstrated intense inflammatory infiltration and myocardial cells arranged irregularly after I/R. (B) Representative images of Masson’s trichrome-stained hearts from I/R mice. Blue indicates fibrosis. (C) Quantitative analysis of interstitial fibrosis examined by Masson’s trichrome staining. Percent fibrosis was determined using Image J software to quantify blue (fibrotic) versus non-blue (non-fibrotic) pixels (n=6). **P<0.01 I/R+QL vs I/R+NS.
QL Treatment Improves Pathological Changes in Myocardial Tissue

One week after I/R, surviving myocardial cells were found in border zones and arranged irregularly in untreated animals, as shown by H&E staining. However, most myocardial cells were arranged regularly in the QL treated group (Fig. 4A). In agreement, Masson trichrome staining demonstrated that QL treatment significantly decreased fibrosis and collagen deposition after I/R (Fig. 4B-C).

QL Treatment Activates mTOR Signaling

We assessed whether QL treatment affected mTOR signaling in the myocardium after I/R. As observed previously [12, 15, 16], cardiac mTOR signaling can be activated by I/R injury. The expression of phosphorylated mTOR (Ser2448) was significantly higher in the QL treatment group, in comparison with controls (Fig. 5A-B). Similarly, the expression of phosphorylated Akt was significantly higher in QL animals, while phosphorylated 4EBP-1 expression was significantly reduced, compared with values obtained for the untreated group (Fig. 5A-B).

Rapamycin Abolishes the Protective Effect of QL against I/R

To further investigate the role of mTOR activation in the therapeutic effect of QL in I/R, pharmacological intervention via rapamycin, an mTOR inhibitor, was used in QL treated I/R mice. The reduced infarct size and improved LVEF and LVFS in QL treated I/R was significantly
Fig. 6. Rapamycin abolishes the therapeutic effects of QL in mice after I/R. (A) TTC staining and quantitative analysis for infarct size in QL treated I/R mice with and without Rapamycin intervention at 24 hours after I/R. (n=10, I/R+QL mice; n=9, I/R+QL+Rapamycin mice). (B) Echocardiography for LVEF and LVFS at 24 hours after I/R. (n=10, sham+NA mice; n=10, I/R+NS mice; n=10, I/R+QL mice; n=9, I/R+QL+Rapamycin mice). (C) Representative immunoblots of mTOR signaling molecules in the left ventricular wall. Hearts were harvested at 24 hours after I/R. D: Densitometric quantification of immunoblots. n=6 mice/group. *P<0.05, **P<0.01, ***P<0.001.
reversed by Rapamycin (Fig. 6A-B). Meanwhile, the enhanced phosphorylation of mTOR, together with subsequent increased phosphorylated Akt and decreased phosphorylated 4EBP-1 levels in QL treated I/R, were also reversed by Rapamycin (Fig. 6C-D).

**Discussion**

Herein, we demonstrated that QL treatment after I/R in mice increased survival rate, decreased infarct size during the acute phase of I/R and prevented adverse LV remodeling in the chronic phase, resulting in the preservation of cardiac function. Moreover, mTOR activation is required for the protective effects of QL in preventing myocardial I/R injury.

A multicenter, randomized, double-blind, parallel-group, placebo-controlled study from our group have shown that in combination with standard treatment, QL further reduced NT-proBNP levels in patients with chronic HF [17]. In addition, QL efficacy against cardiac hypertrophy and remodeling has been demonstrated in several studies [24, 25, 29-36]. Herein, we demonstrated that QL treatment of mice after I/R increased the survival rate, decreased infarct size and decreased fibrosis after I/R, suggesting that QL may also confer cardiac protection against I/R injury.

Previously, it has been reported that mTOR inhibition by rapamycin increases infarct size at 24 h post-I/R in vivo [37]. However, previous reports have demonstrated that mTOR inhibition protected the heart in an **in vivo** MI model using an LAD permanent ligation without reperfusion [38, 39]. A recent report demonstrated that cardiac mTOR overexpression is sufficient to confer substantial cardio-protection against I/R injury and suppress the inflammatory response [16]. The two types of cardiac injury, MI (without reperfusion) and I/R injury, induce cell death with different pathophysiological features; thus, activation of mTOR signaling may have different cardio-protective effects on these two injury types [15]. In line with these studies, our data showed that administration of QL activated the mTOR pathway and reduced mortality as well as infarct size in mice after I/R. Indeed, QL treatment significantly increased p-mTOR levels, induced the expression of phosphorylated Akt and down-regulated phosphorylated 4EBP-1 in mice after I/R, as shown in this study. Moreover, Rapamycin, an mTOR inhibitor, abolished the therapeutic effects of QL in I/R injury. These data indicate that mTOR activation is essential to mediate the protective effect of QL against cardiac I/R injury.

To the best of our knowledge, this is the first study reporting that QL treatment can reduce mouse mortality and infarct size after I/R. Moreover, QL therapy may contribute to improvement of LV function after I/R by inhibiting myocardial fibrosis and enhancing cardiac repair. Indeed we simultaneously observed mTOR activation in QL treated animals, which may be involved in the prevention of myocardial I/R injury. However, several limitations should be highlighted in our study. First, whether a combination of QL with other medications could achieve better therapeutic effects needs further clarification. In addition, a quantitative analysis of leukocyte infiltration and myocardial cells arrangement after QL treatment in I/R is needed in the future.

In conclusion, we have shown that QL administration has notable benefits on cardiac function and attenuates the progression of heart remodeling after I/R, which is mediated by activation of the mTOR pathway. This study suggests a new application for QL in preventing I/R injury.

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