Int J Clin Exp Med 2018;11(12):13208-13216
www.ijcem.com /ISSN:1940-5901/IJCEM0078421

Original Article

QKI-5 regulates FOXO1 to inhibit apoptosis during myocardial ischemia and reperfusion injury in H9C2 myocardiocytes

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Received September 1, 2017; Accepted September 10, 2018; Epub December 15, 2018; Published December 30, 2018

Abstract: *Aims:* In this study, the roles of QKI and possible mechanisms underlying the functions of pro-apoptotic transcriptional factor FOXO1 in I/R (I/R)-induced apoptosis were investigated. *Methods:* Neonatal rat cardiomyoblasts H9C2 were used to study the roles of QKI-5 in cardiomyocyte I/R injury, which was induced by 2 h of hypoxia plus glucose and serum deprivation, followed by 6 h of recovery. After QKI-5 and FOXO1 were silenced or overexpressed, respectively, the protein expression of QKI-5, FOXO1 and caspase-3 was detected by Western blot. The apoptosis of H9C2 cells was detected by flow cytometry after the expression of QKI-5 in the cells was altered in different ways. *Results:* In H9C2 cells, the deprivation of culture medium alone could slightly inhibit QKI-5 expression, but such intervention showed no effects on QKI-6 expression. In contrary, the QKI-5 expression was decreased sharply after 6 h of complete ischemia. After 2 h of ischemia plus 6 h of reperfusion, the apoptosis rate of the QKI siRNA group was significantly higher than that in the negative control group. In cells infected with adeno-QKI-5I/R, the apoptosis rate dropped to 4.6 ± 0.8%, which was notably lower than that in cells infected with ad-CMV-Null (14.3 ± 1.7%, $P<0.01$). PARP and caspase-3 experiments also confirmed that the apoptosis rate in the Ad-QKI-5 group was significantly lower than that in the Ad-CMV-Null group. In the QKI-5-siRNA group, I/R-induced expression of FOXO1 was more significantly increased, while the overexpression of QKI-5 suppressed FOXO1 expression. In the group with overexpressed FOXO1, I/R-induced expression of caspase-3 was much higher, but FOXO1-siRNA inhibited the expression of caspase-3. *Conclusion:* This study demonstrated that QKI5 proteins exerted a strong effect to prevent I/R-induced apoptosis in cardiomyocytes, and the effect of QKI5 may be exerted through the inhibition of FOXO1, a pro-apoptotic transcription factor.

Keywords: Coronary heart disease, ischemia/reperfusion, H9C2 cells, apoptosis, QKI, FOXO1

Introduction

Ischemic heart diseases (IHD) threaten human health worldwide. With the development of medical technology, ischemic myocardium can be reperfused by vascular reconstruction as early as possible after an ischemic heart event [1, 2]. Ischemia/reperfusion (I/R) can prevent the significant death of ischemic myocardium. Paradoxically, I/R is associated with an inherent risk. For instance, the process of I/R can induce I/R injury, which is featured by fatal myocardial damages and the increase in the size of infarction area [3]. Given the frequent applications of I/R therapy today, it is crucial to reduce I/R induced myocardial damages in the treatment of severe cardiovascular diseases.

The mechanisms of cardiac I/R injury are complex, among which the apoptosis and necrosis of cardiomyocytes have become one of the key pathologic processes. It was shown that the pharmacological inhibition of I/R-induced apoptotic signaling attenuated cardiomyocyte apoptosis [4, 5], which is regulated by multiple genes in the apoptotic signaling cascades, such as anti-apoptosis and pro-survival kinase signaling cascades [6, 7]. However, the current methods used in I/R treatments are unable to fully prevent I/R-induced cardiac damages, which are caused by many factors. Therefore, new strategies of I/R are needed to reduce the incidence of I/R-induced cardiac damages.

RNA binding protein QKI belongs to the evolutionarily conserved STAR family and plays a

critical role in the myelination of CNS development [8]. However, the expression of QKI in numerous other tissues has also been implicated in many fundamental biological processes, such as vascular development, cell apoptosis, cell adhesion, cell growth and organogenesis [9, 10]. Based on the 1400 target mRNAs of QKI predicted in a Nat Stuct Mol Bio article, many of these putative downstream targets, such as Bid, FOXO1 and Sirt1, are related to apoptosis and implicate QKI in the regulation of apoptosis.

Therefore, the objective of the present study was to investigate whether QKI protects against cardiomyocyte apoptosis in a myocardiocyte model of I/R. Furthermore, the mechanisms underlying the cardioprotective effects of QKI were explored by targeting FOXO1 expression.

Materials and methods

In vitro cardiomyocyte I/R models

Neonatal rat cardiomyoblasts H9C2 were seeded in a 6-well plate at the concentration of 3×10^5/well. For the induction of I/R, the cells were washed once with PBS, and cultured in a glucose free and serum free DMEM medium in a 37°C, 5% CO_2, and 1% O_2 incubator for 2 h, followed by culturing in a high glucose (4500 mg/L) DMEM medium containing 10% FBS in a normal cell incubator for 6 h. Mock control cells were incubated in a normal cell incubator with a high glucose DMEM medium containing 10% FBS.

SiRNA synthesis and transfection

SiRNA sequences specific for the silencing of QKI-5 and FOXO1 were designed and synthesized by Invitrogen as follows: 5'-GGUACACAUUCAAUUCAGCAAUCAU-3' 5'-AUGAUUGCUGAAUUGAAUGUGUACC-3'; and 5'-GCCGTGCTACTCGTTTGC-3' 5'-CTTGGGTCAGGCGGTTC-3'. The control sequences were 5'-GGUACACUUUAAACUACGUAACCAU-3' and 5'-AUGGUUACGUAGUUUAAAGUGUACC-3'. Transfection was performed using Lipofectamine 2000 following the procedure provided by Invitrogen. Forty-eight hours after transfection, the treatments of IR or RNA interference was performed.

Construction and infection of adenovirus vectors

H9C2 cells were infected with QKI-5 siRNA- or control siRNA-expressing adenoviruses at a MOI of 30. In brief, the cells were seeded (1×10^6 cells per well) into six-well plates. After 24 h of incubation, the culture medium was replaced with an Opti-MEM medium containing an appropriate amount of the virus vectors. The cells were then incubated with the virus for another 48 h before they were harvested.

Transfection of FOXO1 overexpressed plasmids

H9C2 cells were transfected with a pcDNA3-GFP-FOXO1 overexpressed plasmid. In brief, H9C2 cells were cultured in Petri dishes at 24 h before transfection. Lipo2000 and siRNA were diluted with a MEM and incubated at room temperature for 20 min to form siRNA-Lipo2000 complexes, which were then added onto H9C2 cells. After 48 h, the cells were collected for PCR to measure the mRNA expression of FOXO1.

Flow cytometry analysis

Conditioned H9C2 cells were stained with PI and Annexin V-FITC dyes, and then fixed with paraformaldehyde. The proportion of apoptotic cells was measured by flow cytometry.

Western blot analysis

The protein expression of QKI and FOXO1 was measured by a standard Western blot technique as previously reported. In brief, QKI and FOXO1 proteins were prepared using a QKI and FOXO1 Extraction Kit (Thermo Scientific, Grand Island, NY, USA). Western blotting was carried out by probing the transferred PVDF membranes with corresponding primary antibodies, followed by incubating with HRP-conjugated secondary antibodies. The HRP enzyme activity was visualized with enzyme-linked chemiluminescence (Millipore, Billerica, MA, USA) to reflect the content of target proteins on the membrane. The antibodies against PARP and GAPDH were purchased from Cell Signaling Technology (Cell Signaling, Danvers, MA, USA). All experiments for Western blot analyses were repeated at least 3 times independently.

Statistical analysis

All data in this manuscript were expressed as mean ± standard error (SE). All in vitro experiments were conducted with at least 3 independent replications. $P<0.05$ indicated significant difference. Graphpad Prism 5 was used for statistical analysis. Intergroup differences were

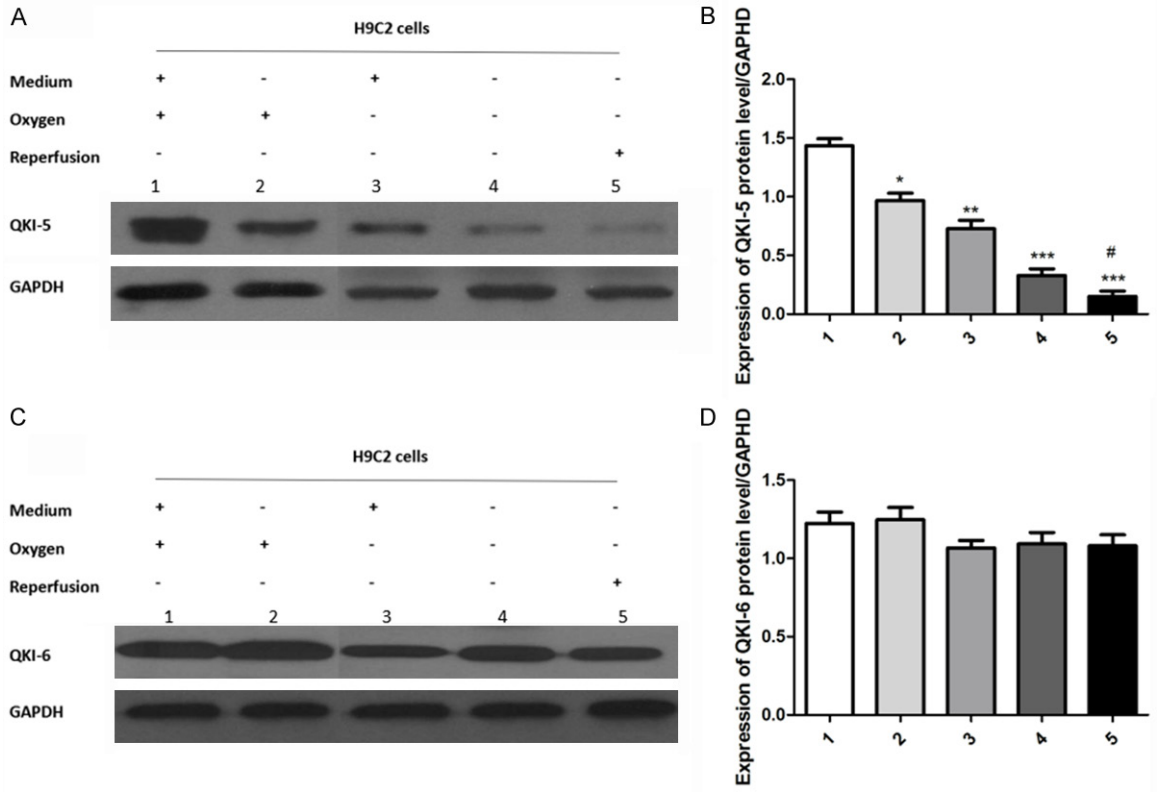

Figure 1. Ischemia/reperfusion suppressed QKI-5 expression. A. Western blotting showed levels of QKI-5 in H9C2 cells. B. Analysis of QKI-5 protein expression. Error bars represent standard deviations. Statistical analyses were done with one-way ANOVA. *P<0.05, **P<0.01 and ***P<0.001, respectively, vs. 1 group. #P<0.05 vs. 4 group. C. Western blotting showed levels of QKI-6 in H9C2 cells. D. Analysis of QKI-6 protein expression.

compared using one-way ANOVA where appropriate.

Results

I/R suppressed QKI-5 expression

As depicted in **Figure 1**, a QKI monoclonal antibody was used in this study to determine QKI expression in H9C2 cells. The results suggested that the major QKI isoforms were QKI-5 and -6 in cardiomyocytes. Simultaneously, QKI expression under I/R conditions was also measured by Western blotting. In H9C2 cells, ischemia was induced by replacing the culture medium with a Tyrode's buffer and the deprivation of oxygen, while reperfusion was simulated by the restoration of culture medium and oxygen. Deprivation of culture medium alone could slightly inhibit QKI-5 expression, but showed no effects on QKI-6 expression. In contrary, the QKI-5 expression was decreased sharply after 2 h of complete ischemia.

Silencing of QKI-5 expression elevated I/R-induced apoptosis in H9C2 cells

To evaluate the role of reduced QKI-5 expression in cardiomyocytes under I/R, RNA interference was performed to silence QKI-5 expression in H9C2 cells. H9C2 cells were harvested at 2 h, 24 h and 48 h after the transfection with QKI-5 siRNA, and Western blotting was performed to observe the effect of RNA interference. The results showed that the expression of QKI-5 was effectively silenced at 24 h after the transfection, and the silencing effect was most obvious at 48 h after the transfection (**Figure 2A**, **2B**). After 2 h of ischemia plus 6 h of reperfusion, the cells transfected with QKI-5 siRNA showed an apoptosis rate of 27.6 ± 2.4%, significantly higher than 14.2 ± 1.7% in the negative control cells (P<0.01) (**Figure 2C**, **2D**). These results suggested that the presence of QKI-5 was critical for the protection against apoptosis in cardiomyocytes.

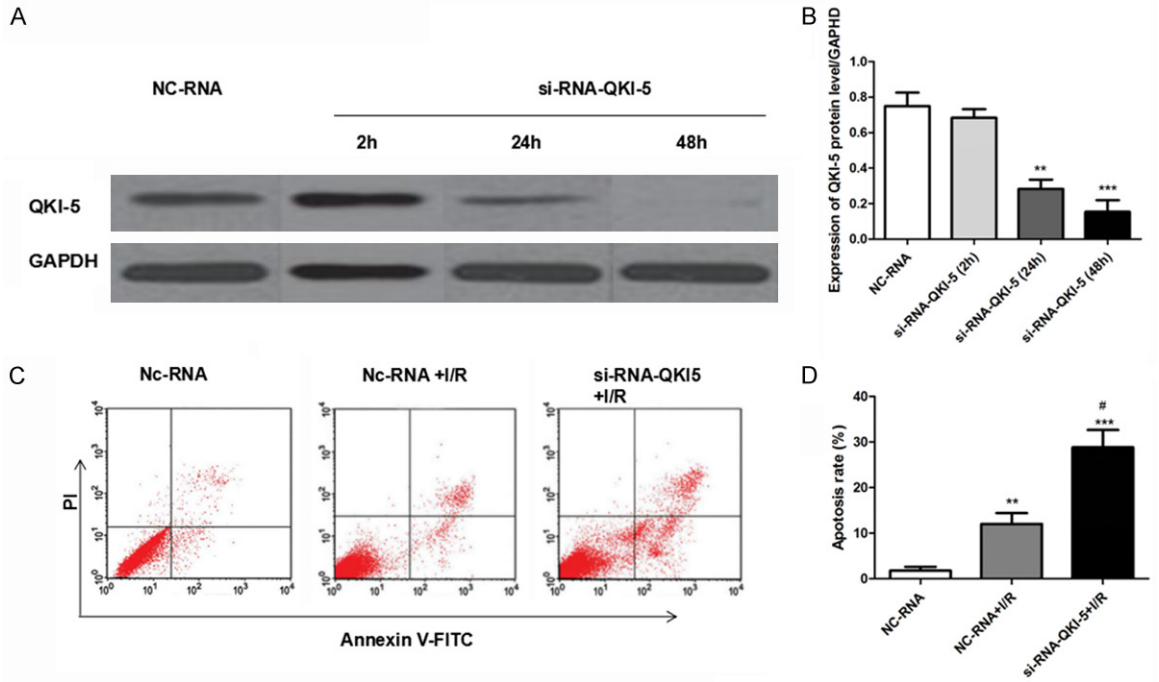

Figure 2. Silencing QKI-5 expression elevated the sensitivity to mimicked ischemia/reperfusion-induced apoptosis in H9C2 cells. A. The expression of QKI-5 protein was detected by Western blotting after transfection of QKI siRNA for 2 hr, 24 hr and 48 hr. B. Analysis of QKI-5 protein expression. Error bars represent standard deviations. Statistical analyses were done with one-way ANOVA. **P<0.01 and ***P<0.001, respectively, vs. NC-RNA group. C. si-RNA-QKI-5 significantly increased the early apoptotic cell percentage of H9c2 cells. D. Analysis of apoptotic cell percentage. Statistical analyses were done with one-way ANOVA. **P<0.01 and ***P<0.001, respectively, vs. NC-RNA group; #P<0.05 vs. NC-RNA+I/R group.

Over-expression of QKI-5 inhibited I/R-induced apoptosis in cardiomyocytes

QKI-5-expressing adenoviruses were constructed to investigate the direct effects of QKI-5 on I/R-induced apoptosis. After infecting the cells with adenoviruses carrying QKI5 downstream of a CMV promoter, the Western blotting results showed that the overexpression of QKI-5 was evident (**Figure 3A**, **3B**). Apoptosis was determined by flow cytometry after the infected cells were subjected to I/R stress. I/R induced an apoptosis rate of 4.6 ± 0.8% in the cells infected with ad-QKI-5, notably lower than that in the cells infected with ad-CMV-Null (14.3 ± 1.7%, P<0.01) (**Figure 3C**, **3D**). Furthermore, the expression of PARP and caspase-3, two key apoptotic genes, was also measured. The results showed that the overexpression of QKI-5 down-regulated the expression of PARP (**Figure 3E**, **3F**) and caspase-3 (**Figure 3G**, **3H**).

The effect of QKI-5 on the expression of FOXO1 proteins in I/R

The silencing of QKI-5 did not affect FOXO1 expression under normal conditions (**Figure 4A**). However, I/R could dramatically elevate FOXO1 expression, while the magnitude of FOXO1 elevation was more prominent in cells treated with QKI-5 siRNA (**Figure 4B**). To confirm QKI-5-mediated negative regulation of FOXO1 expression, the effects of aden-QKI-5 on FOXO1 expression were also determined. As mentioned above, under normal conditions, FOXO1 was expressed at a relative low level and was not influenced by over-expressed QKI-5 (**Figure 4C**). However, I/R greatly increased the expression of FOXO1 in cells infected with the control virus (**Figure 4D**). These results suggested that QKI-5 acted as a critical and negative regulator of FOXO1.

Silencing or overexpression of FOXO1 in apoptosis

In order to verify the involvement of FOXO1 in the regulation of apoptosis, the expression of caspase-3 was measured after FOXO1 was silenced or overexpressed. The results showed that overexpression of FOXO1 in H9C2 cells significantly upregulated the expression of caspase-3 proteins (**Figure 5A**, **5B**). In the I/R cell

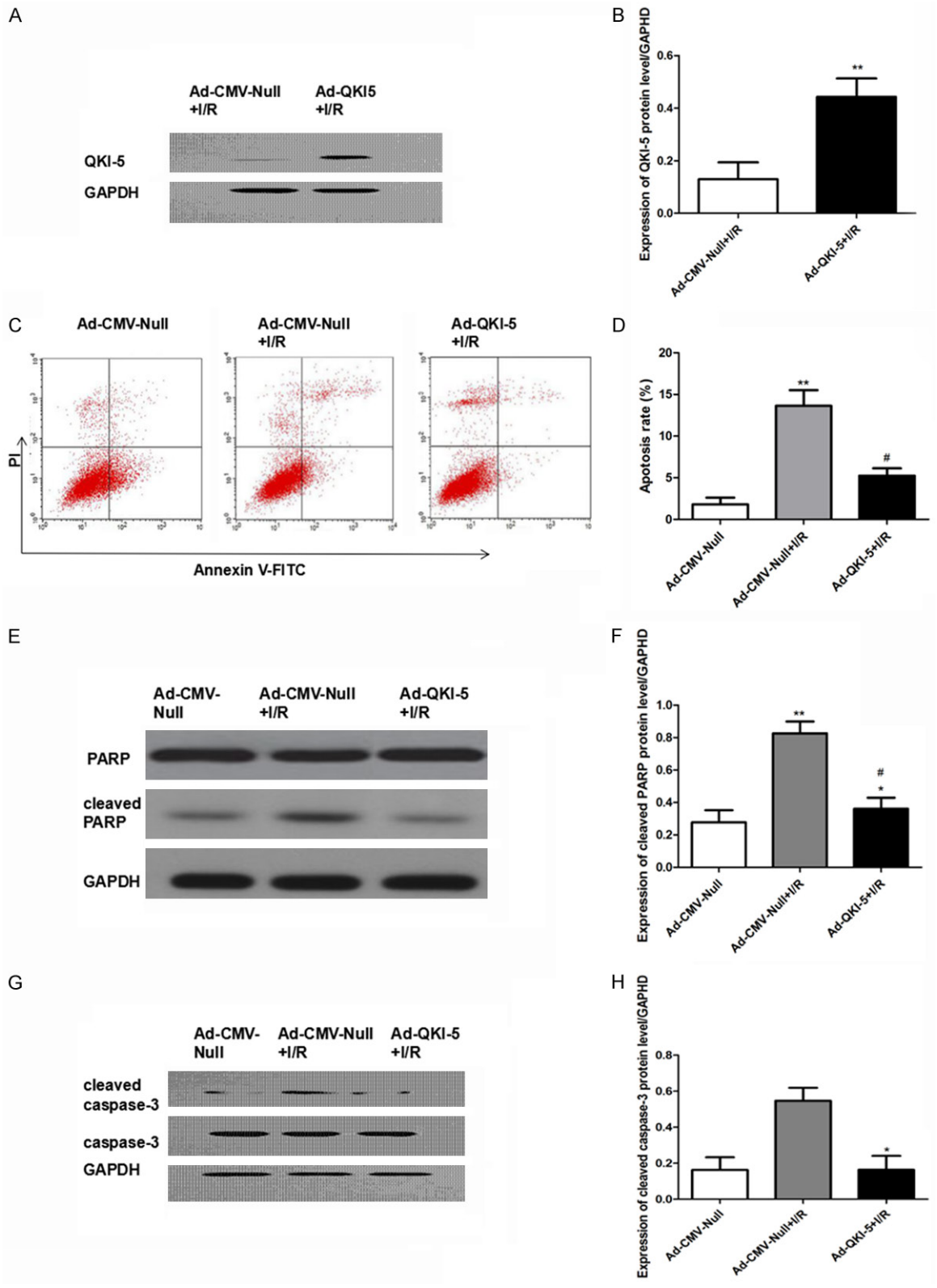

Figure 3. Over-expression of QKI-5 inhibited mimicked ischemia/reperfusion-induced apoptosis in cardiomyocytes. A. In I/R cell model, overexpression of QKI-5 significantly up-regulated QKI-5 protein expression. B. Analysis of QKI-5 protein expression. Statistical analyses were done with one-way ANOVA. **P<0.01 vs. Ad-CMV-Null+I/R group. C. Overexpression of QKI-5 significantly decreased the early apoptotic cell percentage of H9c2 cells compared with

Ad-CMV-Null+I/R group. D. Analysis of apoptotic cell percentage. Statistical analyses were done with one-way ANOVA. **P<0.01 vs. Ad-CMV-Null group. #P<0.05 vs. Ad-CMV-Null+I/R group. E and G. Western blotting showed levels of PARP and caspase-3 in H9C2 cells. F and H. Analysis of PARP and caspase-3 protein expression. Statistical analyses were done with one-way ANOVA. *P<0.05 and **P<0.01, respectively, vs. Ad-CMV-Null group. #P<0.05 vs. Ad-CMV-Null+I/R group.

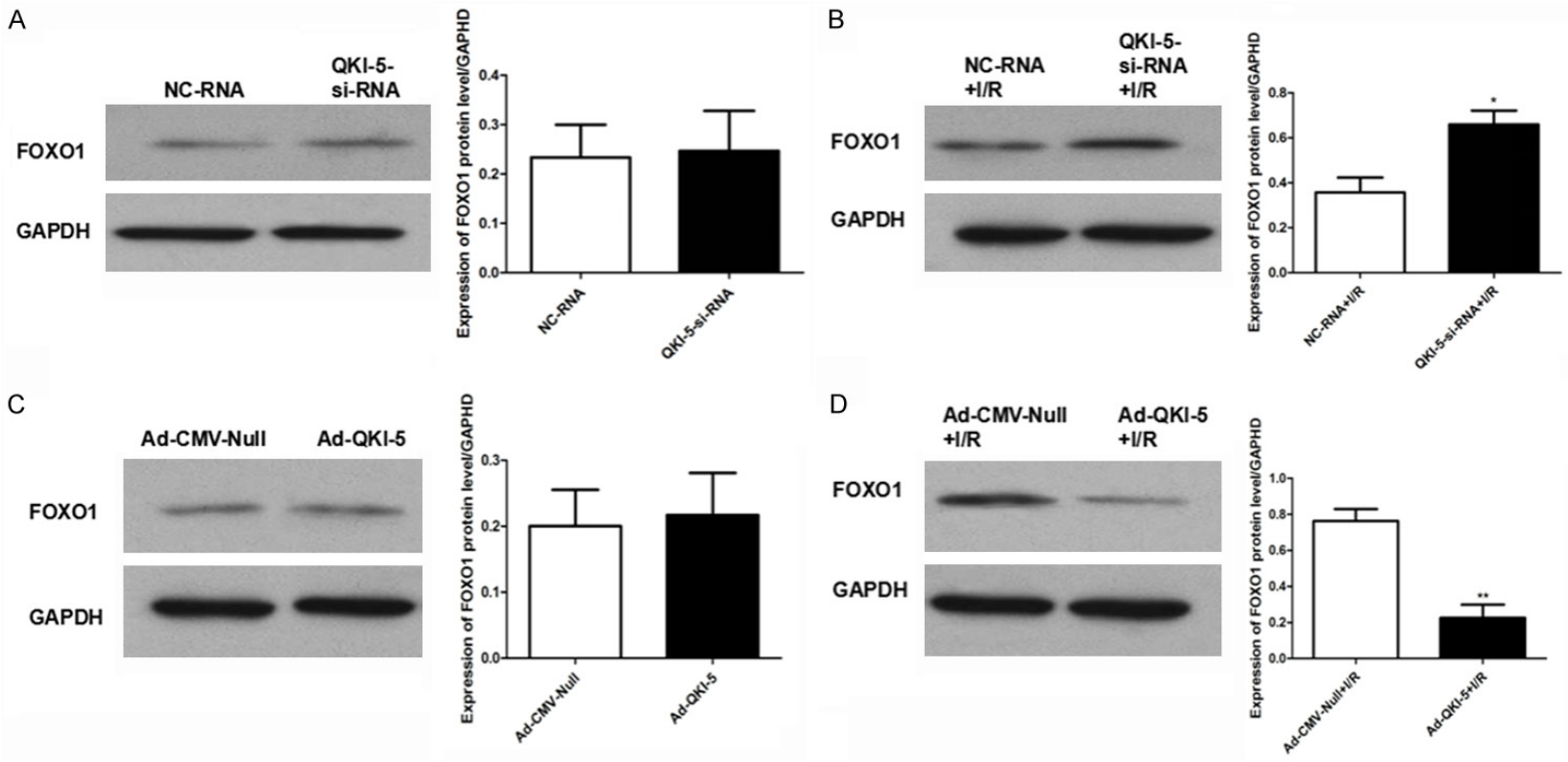

Figure 4. Effect of silencing or overexpression of QKI-5 on FOXO1 protein expression by ischemia/reperfusion. A. Western blotting analysis showed silencing QKI-5 did not affect FOXO1 expression under normal conditions. B. Silence of QKI-5 significantly increased the levels of FOXO1 compared with NC-RNA+I/R group. And analysis of FOXO1 protein expression. Statistical analyses were done with one-way ANOVA. *P<0.05 vs. NC-RNA+I/R group. C. Western blotting analysis showed overexpression of QKI-5 did not affect FOXO1 expression under normal conditions. D. Overexpression of QKI-5 significantly decreased the levels of FOXO1 compared with Ad-CMV-Null+I/R group. And analysis of FOXO1 protein expression. Statistical analyses were done with one-way ANOVA. **P<0.01 vs. Ad-CMV-Null+I/R group.

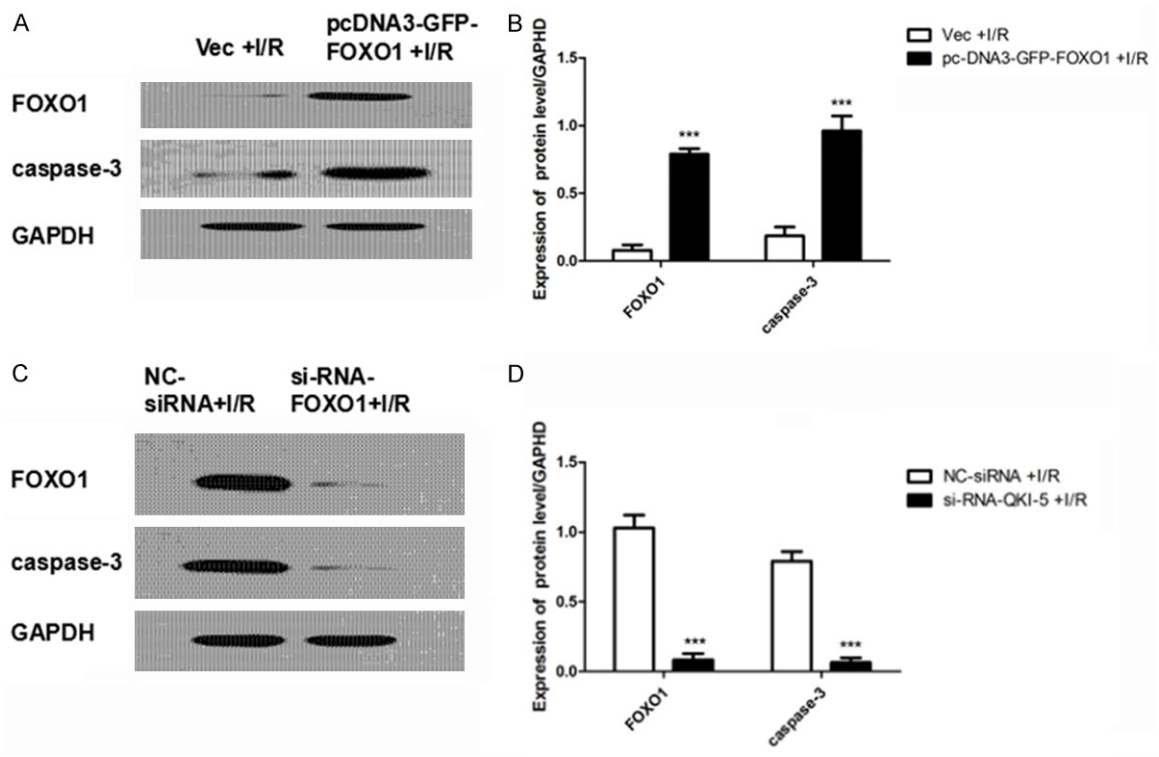

Figure 5. Silencing or overexpression of FOXO1 on apoptosis. A. Overexpression of FOXO1 can up-regulate caspase-3 protein expression. B. Analysis of caspase-3 protein expression. Statistical analyses were done with one-way ANOVA. ***P<0.001 vs. Vec+I/R group. C. In I/R cell models, silencing FOXO1 significantly down-regulated caspase-3 protein expression. D. Analysis of caspase-3 protein expression. Statistical analyses were done with one-way ANOVA. ***P<0.001 vs. Nc-RNA+I/R group.

model, the silencing of FOXO1 inhibited the expression of caspase-3 proteins (**Figure 5C**, **5D**).

Discussion

This study focused on the mechanisms responsible for the protective effect of QKI-5 in isolated cardiomyoctes exposed to simulated I/R. In the present study, it was revealed that QKI-5 attenuated I/R-induced apoptosis of H9C2 cardiomyocytes by negatively regulating the expression of FOXO1. These results suggested the therapeutic potential of QKI-5 in the treatment of I/R-induced myocardial injury.

This experiment confirmed that myocardial I/R significantly decreased QKI-5 expression. Previous research showed that QKIs were mainly expressed in the brain, heart, lungs, and testes [11-13]. It was also confirmed that QKI homozygous mutant mice showed enlarged heart, pericardial effusion and other cardiac defects during the embryonic period, suggesting that QKI-5 may play an important role in the heart [14, 15]. In this study, the expression of QKI-5 in cardiomyocytes-derived myoblast H9C2 cells was measured, and the results showed that cardiomyocytes mainly expressed QKI-5. Furthermore, it was also found that the removal of culture medium resulted in decreased QKI-5 expression, while complete ischemia (removal of both culture medium and oxygen supply) further reduced QKI5 expression. In contrary, reperfusion almost completely eliminated the expression of QKI-5. Interestingly, QKI-6 expression did not change significantly during these treatments.

To determine the possible role of QKI-5 in cardiomyocyte apoptosis, siRNAs specifically targeting QKI-5 expression were designed to test the effect of QKI-5 expression on the susceptibility to apoptosis in cardiomyocytes. H9C2 cells were chosen for the QKI-5 silencing experiment because H9C2 cells share many common properties with the cardiomyocytes widely used in molecular cardiology. The results of this

study showed that the silencing of QKI-5 did not induce apoptosis. Therefore, the decrease of QKI-5 during I/R was not the direct cause of cardiomyocyte apoptosis. However, when these cells were given the I/R treatment, their susceptibility to apoptosis became different. The silencing of QKI-5 expression increased the susceptibility of the cells to apoptosis [16]. This indicated that the early reduction of QKI-5 expression exacerbated cardiomyocyte damages caused by I/R. For instance, QKI-5 proteins may act as a protective factor in cells. As a result, the reduction of QKI-5 protein expression by I/R would inevitably aggravate cell damages.

In order to find out the possible mechanisms underlying the role of QKI-5 in apoptosis, the target molecules of QKI-5 involved in I/R induced cardiomyocyte apoptosis were investigated. FOXO family members were originally found in nematodes and were named as the transcription factor DAF16. The three homologues of DAF16, FKHR (FOXO1), FKHRL1 (FOXO3a) and AFX (FOXO4), were found in human tumor chromosome breakpoints in mammals, suggesting that the FOXO family is closely linked to cell growth and survival [17, 18]. In differentiated cells, FOXO protein dephosphorylation directly activates the transcription of pro-apoptotic proteins such as TNF, TRAIL and FasL [19]. In addition, the phosphorylation of FOXO in some cells leads to the ubiquitylation of FOXO. The results of this study showed that the reduced expression of QKI-5 did not lead to the upregulation of FOXO1. Therefore, the decline of QKI-5 expression was not the direct cause of FOXO1 upregulation and apoptosis. It is possible that FOXO1 is activated by other mechanisms in I/R, thus resulting in the loss of proper control of FOXO1 expression and apoptosis. After the cells were given the I/R treatment in this study, the results showed that the silencing of QKI-5 expression increased FOXO1 expression. Finally, the effect of FOXO1 silencing or overexpression on the expression of apoptotic factor caspase-3 was investigated in I/R treated cells. It was found that the overexpression of FOXO1 could significantly upregulate the expression of caspase-3 proteins, while the silencing of FOXO1 showed an opposite effect. These results further confirmed that FOXO1 indeed regulated apoptosis during I/R. Therefore, it can be concluded that the pretreatment of H9C2 cells with QKI-5 can alter their susceptibility to apoptosis, probably due to the regulation of FOXO1 expression by QKI-5.

In summary, this study identified a previously unrecognized mechanism, by which QKI-5 protects I/R-induced cardiomyocyte injury. This study suggested that the QKI-5 treatment down-regulated FOXO1 expression and protected cardiomyocytes against I/R injury-induced apoptosis. These findings provide further insights into the protective role of QKI-5 in ischemic heart diseases. However, in this study, the protective effect of QKI-5 was only determined in an in vitro model of cardiomyocyte I/R injury. Therefore, an in vivo investigation of the above effect is required in the future to further validate this effect. In addition, this study did not investigate the signaling pathways responsible for the effect of QKI-5. Therefore, further investigations are required to address this issue.

Disclosure of conflict of interest

None.

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