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Original article

QKI deficiency promotes FoxO1 mediated nitrosative stress and endoplasmic reticulum stress contributing to increased vulnerability to ischemic injury in diabetic heart

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ABSTRACT

Hearts of diabetic individuals are susceptible to ischemia/reperfusion (I/R) injury. The RNA-binding protein Quaking (QKI) is known to link intracellular signaling to cellular survival and QKI dysregulation may contribute to human diseases. However, the function of QKI in diabetic hearts remains unknown. The current study attempted to identify new molecular mechanisms that potentially contribute to the susceptibility to ischemic injury in diabetic myocardium. Diabetic *ob*/*ob* mice or wild-type C57BL/6J mice were subjected to *in vivo* myocardial I/R. Myocardial infarct size and apoptosis, QKI5 and FoxO1 expression, nitrosative stress (NS) and ER stress were compared. Knockdown of FoxO1 was obtained by intramyocardial injection of FoxO1 specific small interfering RNA (siRNA, 20 μg), and upregulation of QKI5 was acquired by injecting adenovirus encoding-QKI5. Obvious NS stress was observed in the myocardium of *ob*/*ob* mice represented by elevated iNOS expression, total NO content and nitrotyrosine content. Administration of 1400W or M40401 partly reduced the caspase-3 activity in *ob*/*ob* myocardium encountering I/R ($P < 0.05$). Higher ER stress was also observed represented by increased p-PERK, p-eIF2α and expression of CHOP in *ob*/*ob* myocardium. ER stress inhibitor did not affect the excessive NS stress, but partially reduced I/R-induced caspase-3 activity in *ob*/*ob* hearts ($P < 0.05$). FoxO1 was overactivated in *ob*/*ob* myocardium, and knockdown of FoxO1 attenuated both levels of NS stress and ER stress ($P < 0.05$). Furthermore, QKI5 expression was deficient in *ob*/*ob* myocardium. Upregulation of QKI5 diminished FoxO1 expression together with NS and ER stress in *ob*/*ob* myocardium, further reducing MI/R injury. Finally, QKI5 overexpression destabilized FoxO1 mRNA in cardiomyocytes. These results suggested that QKI5 deficiency contributed to the overactivation of FoxO1 in *ob*/*ob* animals and subsequently magnified nitrosative stress and ER stress, which enhances the ischemic intolerance of diabetic hearts.

1. Introduction

Epidemiologic studies have demonstrated that diabetes mellitus (DM) contributes to more than 3 million cardiovascular deaths worldwide each year. Type 2 diabetes mellitus (T2D) is the predominant prevalent DM. Experimental and clinical data demonstrated that the cardiovascular dysfunction caused by T2D directly and indirectly aggravates ischemic heart injury, which results in larger infarct size and more severe heart failure after myocardial ischemia [1,2]. These available evidences strongly support the notion that patients with T2D have higher mortality after acute myocardial infarction (AMI) than non-diabetic patients [3,4]. However, the core molecular mechanism of the susceptibility to ischemia/reperfusion (I/R) injury in diabetic hearts is still to be determined.

Nitrosative stress is namely intracellular protein nitration induced by peroxynitrite ($ONOO^-$), a reaction product of nitric oxide (NO^-) and superoxide (O_2^-). Tao et al. found that iNOS-derived reactive nitrogen species and resultant nitrosative stress increase the sensitivity of the heart to I/R injury [5]. Furthermore, interventions that inhibited iNOS activity or scavenged peroxynitrite reduced nitrosative stress and attenuated tissue injury [6,7]. Besides, perturbations of ER function cause endoplasmic reticulum (ER) stress leading to the unfolded protein response (UPR), which includes the inhibition of protein synthesis, protein refolding and clearance of misfolded proteins [8]. The UPR favors cellular homeostasis; however, prolonged ER stress can trigger apoptosis [9]. Large amounts of studies have shown that severe ER stress results in cardiomyocyte apoptosis *in vivo* and *in vitro* [10–12].

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Inhibiting ER stress can protect the heart from pathological changes including apoptosis [11,13–15]. Identifying the cellular and molecular basis that links nitrosative or ER stress with the vulnerability to MI/R injury may reveal new therapeutic approaches for cardiovascular diseases related to diabetes.

Forkhead box O1 (FoxO1) is a critical transcription factor that plays an important role in regulating cellular metabolism, proliferation, oxidative stress response, immune homeostasis and cell death [16]. Recent studies have found that FoxO1 might play important roles in cardiovascular diseases including diabetes [17,18]. Pathological conditions such as obesity, insulin resistance, and diabetes lead to unregulated activation of FoxO1, which can result in metabolic disturbances and cell death pathway activation. It was also reported that FoxO1 could activate the expression of iNOS and mediate cardiomyocyte apoptosis [17,18]. However, the molecular regulation mechanisms responsible for FoxO1 in diabetes mellitus have not been fully identified. Yu et al. found that RNA binding protein QKI has posttranscriptional regulation on FoxO1 in breast cancer cells [19]. Our previous study suggested that QKI5 was a cardiac endogenous protective protein that inhibited apoptosis. More importantly, we also found that FoxO1 was one of target genes inhibited by QKI5 in cultured cardiomyocytes [20]. Here, we hypothesize that QKI5 dysfunction promotes FoxO1 overactivation mediated myocardial cell death and increases the vulnerability to ischemic injury in diabetic heart.

2. Materials and methods

2.1. Animals

Male leptin-deficient (*ob*/*ob*) mice and their wild-type (WT) C57BL/6 mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China, AAALAC accredited) at ages of 8–10 weeks and utilized at ages of 12 weeks in this study. The *ob*/*ob* mice were fed normal chow diet, and sex matched wild-type mice served as controls. The experiments were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care.

2.2. Experimental protocols

Mice were anesthetized with 2% isoflurane, and myocardial infarction (MI) was induced as described in our previous studies [21]. After 30 min of MI, the slipknot was released, and the myocardium was reperfused (R) for 4 h or 24 h (cardiac function only). Blood samples (1 ml) were drawn after 4-hour reperfusion. Lactate dehydrogenase (LDH) was spectrophotometrically assayed using a kit from Sigma Chemical Co. To investigate the effects of nitrosative inhibitors on I/R injury of *ob*/*ob* myocardium, 10 min before reperfusion, *ob*/*ob* mice were randomized to receive either vehicle (PBS, pH 7.5), 1400W (2 mg/kg), or M40401 (0.25 mg/kg) by i.p. injection [6]. To investigate the effect of salubrinal (SAL) on the I/R injury of *ob*/*ob* myocardium, *ob*/*ob* mice were randomized to receive either vehicle (PBS, pH 7.5) or SAL (1 mg/kg) [22] by i.p. injection 10 min before reperfusion. To investigate the effects of FoxO1 downregulation, FoxO1 specific siRNA (against murine) contains the targeting sequence 5′-GAGCGTGCCCTACTTC AAG-3′, which targets to a coding region of mouse FoxO1 gene (GenePharma, Shanghai, China). 20 μg of FoxO1 siRNA or scrambled siRNA was diluted in 30 μl of vivo-jetPEITM (Invitrogen, USA) and 10% glucose mixture. 30 μl of vehicle, scrambled siRNA, or FoxO1 siRNA solution was delivered *via* six separate intramyocardial injections (5 μl per injection) into the left ventricle apex and anterolateral wall using a 30-gauge needle [21,23]. After 48 h of siRNA or vehicle injection in hearts, mice were subjected to cardiac surgery (MI/R). To study the role of QKI5 overexpression, *ob*/*ob* mice were randomized to receive myocardium injection of either vehicle (PBS, pH 7.5), 2×10^8 plaque forming units of Ad-QKI5 or control adenovirus (Ad-EGFP) 48 h before I/R treatment [20]. Normal C57BL/6J (WT) mice were used as control animals. Full-length QKI5 cDNA was inserted to pcDNA3.1 (+), producing QKI5 expression plasmid. Recombinant adenoviruses encoding these genes were generated *via* an AdEasy adenovirus construction kit following the manufacturer's instruction. Viral supernatants were clarified by centrifugation and titered by plaque assays. Aliquots of virus were stored frozen at −80 °C.

2.3. Intraperitoneal glucose tolerance test (IPGTT)

After a 16 h fast, alert mice were challenged with a glucose load of 1.5 g/kg administered *via* IP injection. Tail blood was taken 0, 15, 60, and 120 min after administering glucose, and blood glucose levels were determined using a OneTouch II glucose meter (Lifescan, USA).

2.4. Determination of myocardial apoptosis and myocardial infarct size

Myocardial apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and caspase-3 activity as described in our previous study [24,25]. Myocardial infarct size was determined by Evans blue-2,3,5-triphenyl tetrazolium chloride (TTC) double staining methods [21].

2.5. Determination of cardiac function

Cardiac function was determined by echocardiography as well as left ventricular catheterization methods 24 h after reperfusion before the chest was reopened, as described in previous studies [4].

2.6. Nitric oxide and nitrotyrosine content

Concentrations of nitrites (NO_2^-) and nitrates (NO_3^-), the stable end products of nitric oxide (NO), were determined at the end of reperfusion in the ventricular tissue by a NO colorimetric assay kit (BioVision, Inc., California). The values of cardiac NO production were expressed as the total nitrate and nitrite levels. Cardiac nitrotyrosine, a footprint of peroxynitrite was measured with chemiluminescence detection (Millipore, Billerica, MA, USA) according to the manufacturer's instruction [5].

2.7. Western blotting

The level of protein expression was analyzed using a Western blotting assay. In brief, heart tissues were homogenized in ice cold lysis buffer (Tris 50 mM, NaCl 150 mM, NP40 1%, SOD 0.25%, EDTA 1 mM, NaF 1 mM, Na_3VO_3 1 mM, PMSF 1 M, Rocheproteinase inhibitor cocktail); the total protein was measured using a Bradford protein assay. The lysates were loaded on SDS-polyacrylamide gel and transferred onto nitrocellulose membranes, which were then immunoblotted with respective antibodies. Quantification of Western blots was obtained by multiplying the area and intensity of each band using Image J software. The nuclear–cytoplasmic fraction of heart tissue was conducted using the NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermo, Fisher Scientific, Rockford, IL, USA).

2.8. RNA immunoprecipitation

RNA immunoprecipitation was done as described before [19,26]. The RNA in the immunoprecipitated complex and the RNA in the previously saved input fraction were extracted. Specific primers used to detect the presence of FoxO1 mRNAs were synthesized as follows: sense 5′-ttgttacatagtcagcttg-3′ and antisense 5′-tcactttcctgcccaaccag-3′.

2.9. Statistical analysis

Differences between 2 groups were compared with Student *t*-test. For groups more than 2, differences were compared by analysis of variance (ANOVA) followed by the Scheffe's correction for post-hoc *t*-test comparison. Probabilities of 0.05 or less were considered statistically significant.

3. Results

3.1. Diabetes increased susceptibility to myocardial I/R injury

12-Week-old *ob*/*ob* mice presented diabetes mellitus. *ob*/*ob* mice displayed significantly greater body and heart weights compared with age-matched WT mice. The heart size (when normalized to tibial length) and body fat composition were also significantly increased in *ob*/*ob* mice in comparison with WT mice, as expected (Table 1). Glucose tolerance and insulin sensitivity were significantly impaired in *ob*/*ob* mice when compared with WT controls ($P < 0.05$, Figs. 1A,B). By echocardiographic measurement, the average of the end systolic diameter (LVESD), the end diastolic diameter (LVEDD) and the ejection fraction (EF%) were comparable to that seen in two groups under basal condition (Table 1), excluding the dilated cardiomyopathy. Based on this, when subjected to *in vivo* myocardial I/R, diabetic *ob*/*ob* mice manifested profoundly greater cardiac injury than WT controls. MI/R-induced LDH release and infarct size were enlarged in *ob*/*ob* mice ($P < 0.05$, Figs. 1C,D).

3.2. FoxO1 was over-activated in ob/ob myocardium

The activation of FoxO1 is dependent on its translocation to the nucleus [27]. The content of FoxO1 in the nucleus would represent the FoxO1 activity. To determine whether diabetes causes alterations in FoxO1 levels and activity, the cardiac FoxO1 expression levels in the nuclei, cytoplasm, and whole-heart lysates from WT and *ob*/*ob* mice hearts were examined (Fig. 2). Total cardiac FoxO1 expression was significantly higher in *ob*/*ob* mice than WT animals ($P < 0.05$). We extracted the nucleus protein and determined FoxO1 levels both in the nucleus and cytoplasm. Nuclear FoxO1 in *ob*/*ob* mice hearts was significantly increased *vs.* WT animals, while cytoplasmic FoxO1 in *ob*/*ob* mice hearts was significantly decreased as compared to WT animals (both $P < 0.05$; Figs. 2B,C). These results demonstrated that cardiac FoxO1 is over-activated under conditions of diabetes.

3.3. Excess nitrosative stress and ER stress in ob/ob hearts

FoxO1 is a major inducer of iNOS in the heart [16]. iNOS induced nitrosative stress contributes to myocardial apoptosis during I/R [5]. We next hypothesized that FoxO1 over-activation would promote iNOS expression and result in nitrosative stress in the diabetic heart. Indeed, iNOS expression was significantly increased in the *ob*/*ob* mice heart ($P < 0.05$, Fig. 3A), There was no change in eNOS expression in cardiac tissues obtained from *ob*/*ob* mice, whereas eNOS phosphorylation was reduced ($P < 0.05$). Total NO production was also markedly increased in diabetic hearts ($P < 0.05$, Fig. 3B), resulting in a markedly enhanced nitrotyrosine formation as evidenced by a >1.8-fold increase in nitrotyrosine content in the *ob*/*ob* cardiac tissue (Fig. 3C sham group). These data showed that there was significant nitrosative stress in *ob*/*ob* myocardium. To determine whether such nitrosative stress in *ob*/*ob* mice would increase the vulnerability of diabetic hearts to I/R injury, we further treated *ob*/*ob* mice with 1400W (selective iNOS inhibitor, 2 mg/kg) or M40401 (a peroxynitrite scavenger, 0.25 mg/kg) 10 min before reperfusion. As summarized in Fig. 3, treatment with 1400W or M40401 effectively blocked excess nitrotyrosine content from iNOS-induced protein nitration in MI/R *ob*/*ob* mice cardiac tissue. Similarly, cardiomyocyte apoptosis, represented by caspase-3 activity, was more severe in *ob*/*ob* comparing to WT myocardium when subjecting to MI/R treatment. Administration of 1400W or M40401 reduced the caspase-3 activity in *ob*/*ob* myocardium encountering I/R ($P < 0.05$, Fig. 3D). It is noteworthy that despite the blockage of excess nitrosative stress from iNOS, treatment with 1400W or M40401 effectively, rather than completely, reduced additional cardiomyocyte apoptosis in *ob*/*ob* animals (Fig. 3D). These results demonstrate that blocking iNOS activity or scavenging peroxynitrite partially improved hearts against MI/R-induced cardiomyocyte apoptosis in *ob*/*ob* mice, and further suggest that there are other potential mechanisms involved in accelerating MI/R injury observed in diabetic heart.

Previously research suggests that the excessive ER stress transmitted pro-apoptotic signals and resulted in cellular apoptosis [9]. Furthermore, there is emerging evidence that over-activation of FoxO1 is intertwined with excessive ER stress [16]. We next compared the ER stress level in WT and *ob*/*ob* mice hearts. Three key molecular sensors are involved in the ER stress pathway, protein kinase RNA (PKR)-like ER kinase (PERK), eIF2α, and C/EBP homologous protein (CHOP). Phosphorylation of PERK induces CHOP-mediated apoptotic signaling, and mediates phosphorylation of eIF2α and subsequent cell death pathway. The ER stress could be represented by the level of CHOP and the phosphorylation of PERK and eIF2α [8]. We found that the expression of CHOP, and the phosphorylation of PERK and eIF2α were all up-regulated in *ob*/*ob* myocardium comparing to the WT one ($P < 0.05$). But, treatment with the aforementioned 1400W or M40401 did not change the ER stress level in *ob*/*ob* hearts (Fig. 4A). Moreover, treating with the ER stress inhibitor, SAL, did not affect the enhanced nitrosative stress in *ob*/*ob* hearts ($P < 0.05$, Fig. 4B). It was showed that the ER stress related cleaved-caspase-12 was exacerbated in diabetic MI/R hearts, and inhibited by SAL treatment ($P < 0.05$, Figs. 4C,D). But, treatment of SAL partially, rather than completely, reduced MI/R-induced caspase-3 activity in diabetic MI/R hearts ($P < 0.05$, Fig. 4E). Combined with the above-mentioned data in nitrosative stress, these results showed that both the nitrosative stress and ER stress mediated the ischemic intolerance of diabetic hearts.

Table 1
General features WT or *ob*/*ob* mice (12-week-old).

	WT	*ob*/*ob*
Body weigh (g)	26.1 ± 0.2	44.8 ± 1.03*
Heart weigh (mg)	119.7 ± 2.02	144.8 ± 1.79*
Tibial length (mm)	17.83 ± 0.28	17.88 ± 0.32
HW/TL (mg/mm)	6.71 ± 0.17	8.1 ± 0.14*
Body fat composition (%)	11.88 ± 0.21	71.36 ± 4.3*
Heart rate (bpm)	501 ± 25	495 ± 34
End diastolic dimension (mm)	3.98 ± 0.07	4.12 ± 0.09
End systolic dimension (mm)	2.50 ± 0.06	2.54 ± 0.08
EF (%)	61.52 ± 1.3	59.4 ± 1.4

Mean ± SEM.
* $P < 0.05$ *vs.* WT, n = 5 per group.

3.4. Down-regulation of FoxO1 improved the tolerance of diabetic heart to I/R injury

We confirmed that nitrosative and ER stress mediated the increased vulnerability of diabetic *ob*/*ob* myocardium to I/R injury. FoxO1 is the upstream regulated factor of both stresses [16]. FoxO1 over-activation in diabetic hearts may engage both harmful stresses to induce cardiomyocyte apoptosis. To obtain more evidence to support our hypothesis, we knocked down FoxO1 level in *ob*/*ob* mice hearts. After intramyocardial injection of FoxO1 specific siRNA into the LV wall of *ob*/*ob* hearts, the level of cardiac FoxO1 expression was decreased (Figs. 5A,B). Accordingly, the knockdown of FoxO1 effectively inhibited the iNOS expression ($P < 0.05$, Fig. 5C) and nitrotyrosine content in *ob*/*ob* myocardium ($P < 0.05$, Fig. 5F). Also, knocking down FoxO1 inhibited the ER stress level represented by decreased ER stress

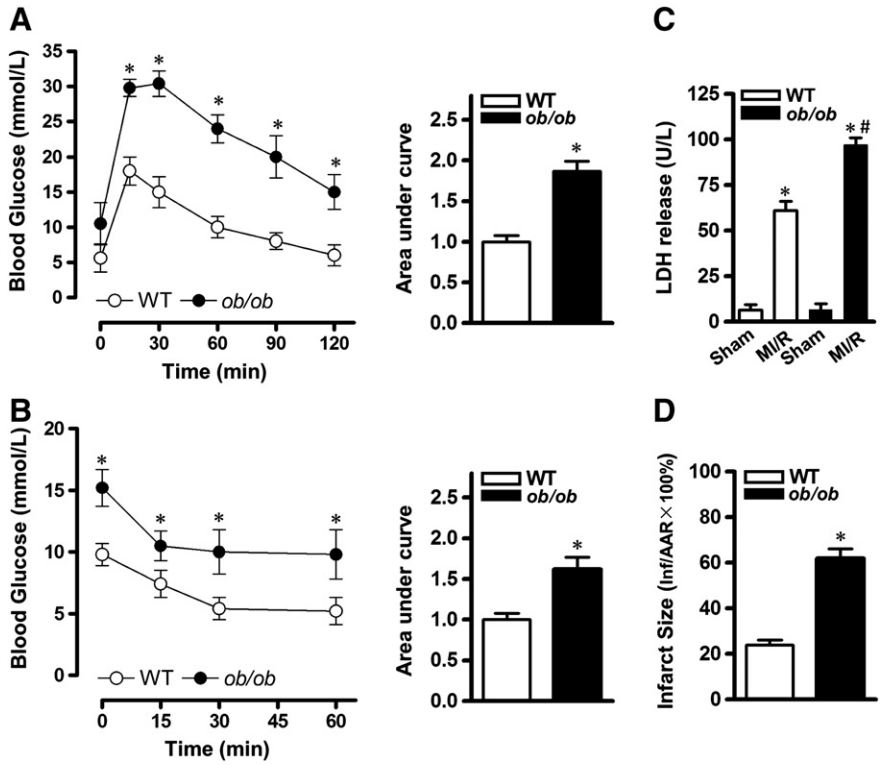

Fig. 1. Diabetes increased susceptibility to myocardial I/R injury. Glucose tolerance and insulin sensitivity were assessed in wild type (WT) and *ob*/*ob* mice. (A) Intraperitoneal glucose tolerance test (IPGTT) and area under the curve in IPGTT; (B) insulin tolerance test (ITT) and area under the curve in ITT (n = 6 per group. $^*P < 0.05$ *vs.* WT). WT and *ob*/*ob* mice were subjected to a 30-minute coronary artery ligation followed by a 4-hour reperfusion *in vivo*; plasma lactate dehydrogenase (LDH) levels (C) and myocardial infarct size (D) were detected (n = 6 per group. $^*P < 0.05$ *vs.* WT; $\#P < 0.05$ *vs.* WT + MI/R).

markers (phosphorylation of IRE1α and expression of GRP78) in *ob*/*ob* myocardium (both $P < 0.05$, Figs. 5D,E).

We further examine the cardiac protective effects of FoxO1 knockdown on I/R injury. Knockdown of FoxO-1 didn't impact cardiac function under basal conditions. 30 min of ischemia followed by 24 h of reperfusion resulted in impaired cardiac function. *ob*/*ob* manifested significantly enhanced functional impairment post-MI/R ($P < 0.05$, Fig. 6A). Knockdown of cardiac FoxO1 obviously ameliorated left ventricular ejection fraction post-MI/R ($P < 0.05$, Fig. 6A), while control siRNA injection was ineffective. Along with cardiac function, the infarct size of *ob*/*ob* mice was 2.5-fold larger than that of WT mice (Fig. 6B). However, knocking down FoxO1 obviously reduced infarct size by 44% in *ob*/*ob* mice ($P < 0.05$, Fig. 6B), while control siRNA injection had no apparent discrepancy in myocardial infarction compared to *ob*/*ob* hearts (Fig. 6B). These results suggested that over-activation of FoxO1 in *ob*/*ob* myocardium mediated the 'excess' injury in *ob*/*ob* myocardium after subjecting to I/R.

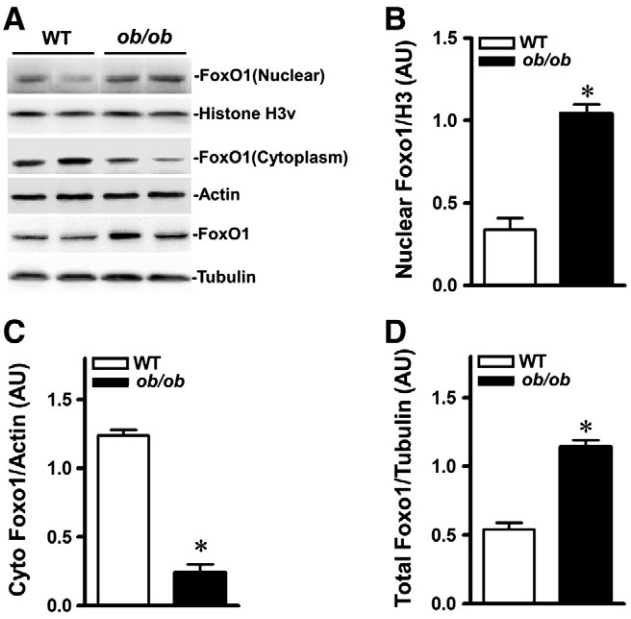

Fig. 2. FoxO1 overactivation in the myocardium of *ob*/*ob* mice. The hearts of wild type (WT) and *ob*/*ob* mice were isolated, and nuclear protein was extracted. Nuclear, cytoplasm and total lysates were applied to Western blotting to determine the relative levels of FoxO1 in nuclear, cytoplasm and total protein. (A) Representative gel blots depicting relative levels of (B) nuclear FoxO1, (C) cytoplasmic FoxO1 and (D) total FoxO1 in WT and *ob*/*ob* hearts (n = 6 per group. $^*P < 0.05$ *vs.* WT).

3.5. QKI5 up-regulation reduced FoxO1 and prevented diabetic hearts against I/R injury

The data above demonstrated that FoxO1 mediated the increased nitrosative and ER stresses, and consequently I/R injury in diabetic *ob*/*ob* myocardium, but the upstream mechanism of FoxO1 over-activation was not observed in *ob*/*ob* hearts. Our previous studies have demonstrated that the RNA binding protein QKI5 inhibits the expression and activation of FoxO1 in cardiomyocytes [20]. We then tested a hypothesis that the lack of QKI5 may increase FoxO1 activation in diabetic hearts *in vivo*, and thus render cardiomyocytes more susceptible to I/R injury. First, the expression level was determined by a significant down-regulation of QKI5 in *ob*/*ob* myocardium (Fig. 7A). Second, the upregulation of QKI5 by intramyocardial injection of Ad-QKI5 in an *ob*/*ob* heart availably inhibited the FoxO1 overexpression, resulting in a decrease of iNOS, GRP78 expression and p-IRE1α level ($P < 0.05$, Fig. 7B). Third, I/R-induced cardiomyocyte excessive apoptosis in *ob*/*ob* myocardium was observed to be inhibited by the overexpression of QKI5

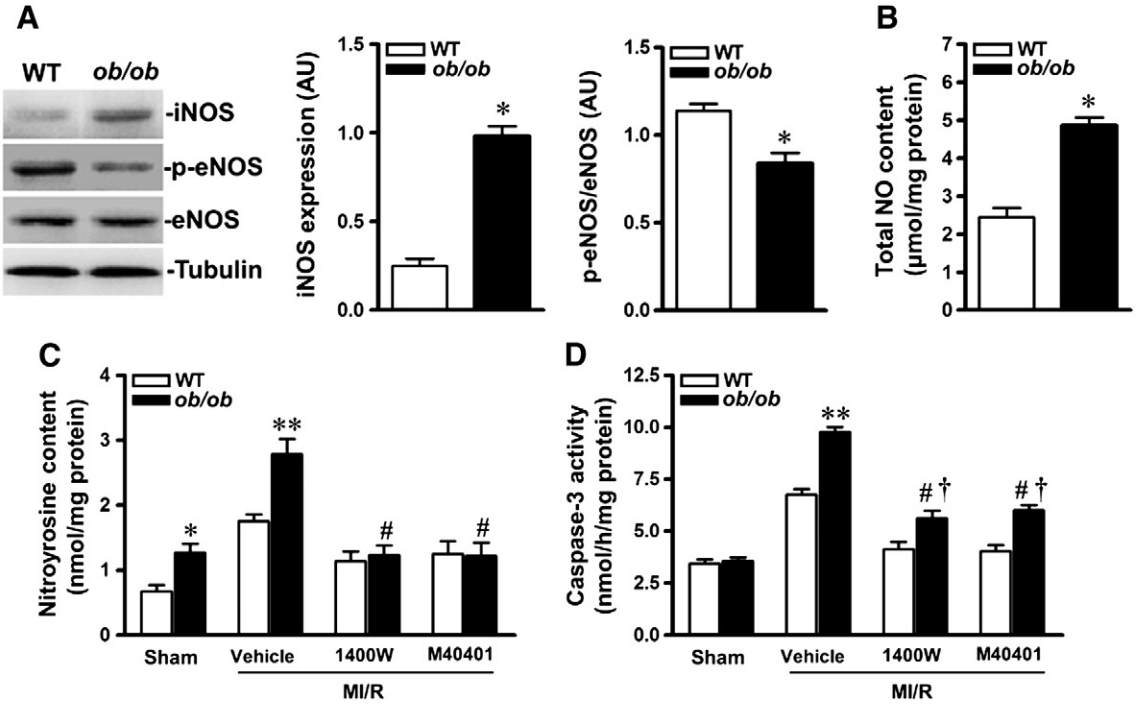

Fig. 3. Nitrosative stress in the myocardium of *ob*/*ob* mice. The hearts of wild type (WT) and *ob*/*ob* mice were isolated, and homogenized. (A) iNOS, eNOS expression and phosphorylation of eNOS and (B) total content of nitric oxide were assessed (n = 6 per group. $^{*}P < 0.05$ *vs.* WT). WT and *ob*/*ob* mice were subjected to a 30-minute coronary artery ligation followed by a 4-hour reperfusion *in vivo*; 1400W (a selective iNOS inhibitor) and M40401 (a peroxynitrite scavenger) or vehicle was administered by *i.p.* injection 10 min before reperfusion, respectively. Myocardial nitrosative stress (measured by nitrotyrosine content) and cardiomyocyte apoptosis (measured by caspase-3 activity) were detected (n = 8 per group. $^{*}P < 0.05$ *vs.* WT sham; $^{**}P < 0.01$ *vs.* MI/R + WT vehicle; #$P < 0.05$ vs MI/R + *ob*/*ob* vehicle; †$P < 0.05$ *vs.* MI/R + WT 1400W or WT M40401, respectively).

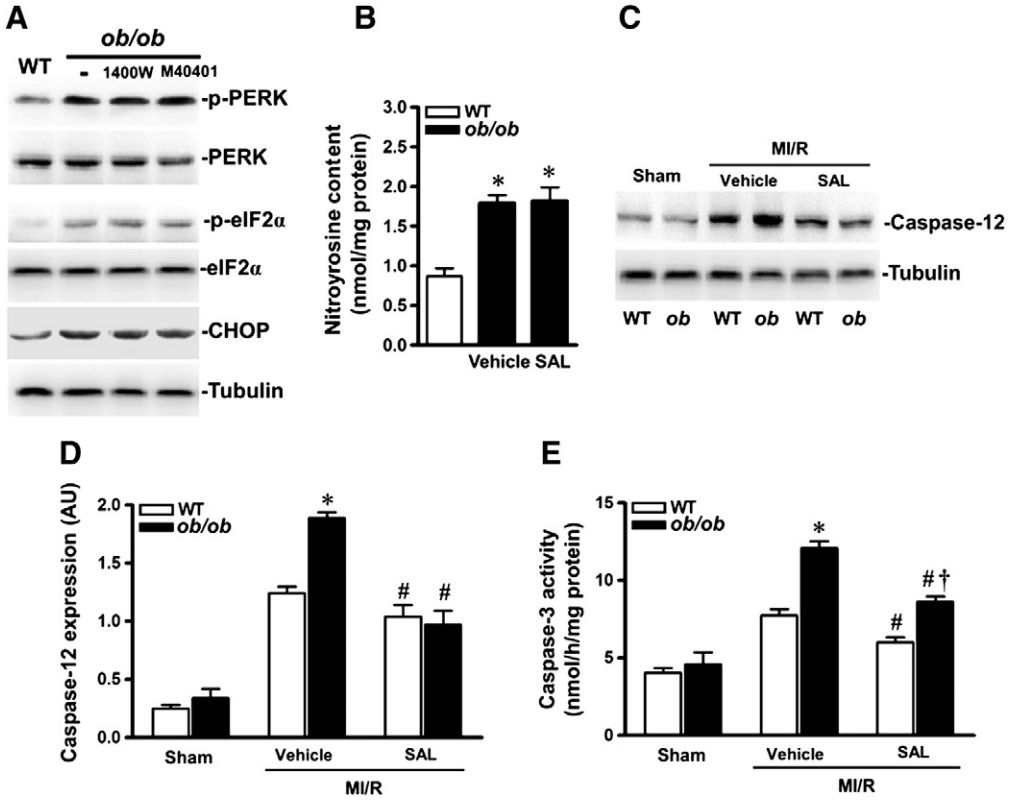

Fig. 4. Endoplasmic reticulum (ER) stress in the myocardium of *ob*/*ob* mice. (A) Representative gel blots depicting the levels of p-PERK, p-eIF2α and CHOP in wild type (WT) and *ob*/*ob* mice hearts with or without 1400W or M40401 treatment. (B) The *ob*/*ob* animals were treated by SAL (ER stress inhibitor) or vehicle; normal WT mice were used as control. Myocardial nitrotyrosine content was measured (n = 6 per group. $^{*}P < 0.05$ *vs.* WT). (C) WT and *ob*/*ob* mice were subjected to a 30-minute coronary artery ligation followed by a 4-hour reperfusion *in vivo*, SAL or vehicle was administered by *i.p.* injection 10 min before reperfusion, respectively. Myocardial caspase-12 expression (D) and caspase-3 activity (E) were compared (n = 8 per group. $^{*}P < 0.05$ *vs.* MI/R + WT vehicle; #$P < 0.05$ vs MI/R + *ob*/*ob* vehicle; †$P < 0.05$ *vs.* MI/R + WT SAL).

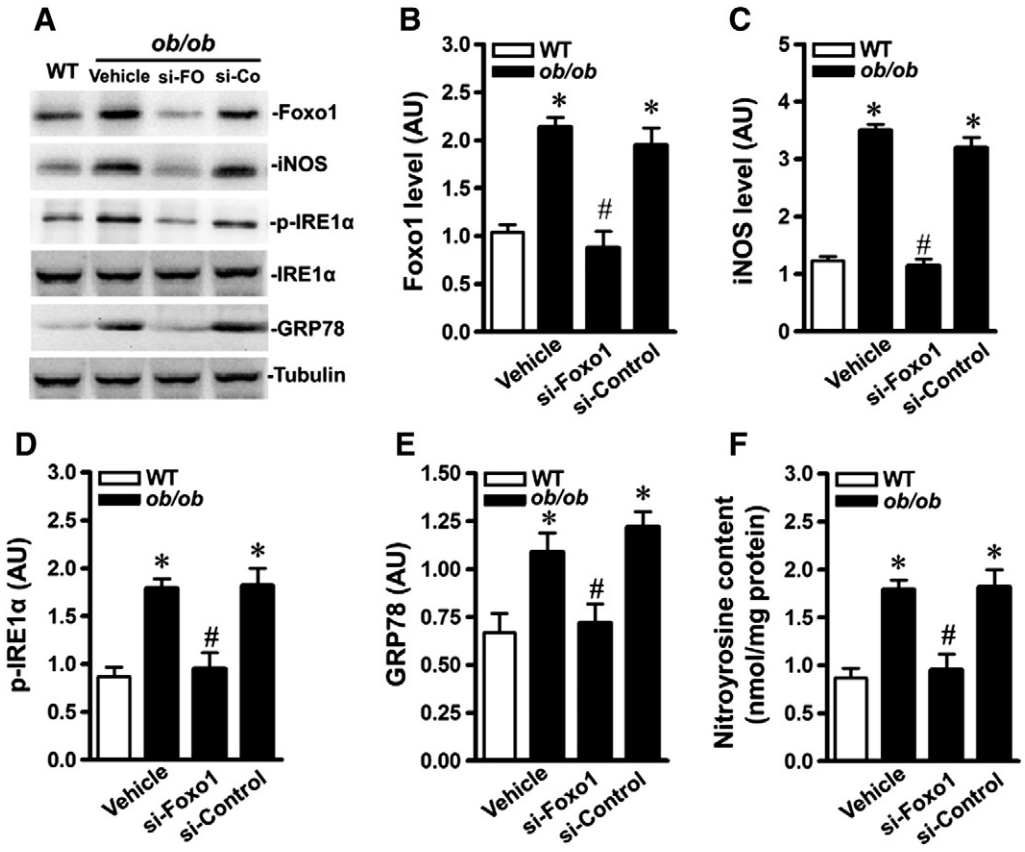

Fig. 5. The regulation of nitrosative and endoplasmic reticulum (ER) stresses by FoxO1 in the myocardium of *ob*/*ob* mice. (A) The *ob*/*ob* animals were subjected to intramyocardial injection of FoxO1 siRNA (20 μg), control siRNA or vehicle; normal WT mice were used as control. Quantification showing the relative expression of (B) FoxO1, (C) iNOS, (D) p-IRE1α and (E) GRP78. (F) Myocardial nitrotyrosine content was measured (n = 6 per group. **P* < 0.05 *vs.* WT; #*P* < 0.05 *vs.* *ob*/*ob* vehicle).

(*P* < 0.05, Fig. 7C). Furthermore, QKI5 overexpression also obviously improved cardiac functional recovery in an *ob*/*ob* heart (*P* < 0.05, Fig. 7D), while control adenovirus (Ad-EGFP) injection failed to increase cardiac ejection fraction. In *ex vivo* perfused mouse hearts, we found that the myocardial infarct was significantly larger in *ob*/*ob* hearts than in WT hearts. QKI5 overexpression exerted directly cardioprotection in *ob*/*ob* myocardium, as evidenced by the reduced myocardial infarct size (*P* < 0.05, Fig. 7E). Consistent with our previous findings, QKI5 reduced myocardial FoxO1 expression and exerts cardioprotective effects. Collectively, these *in vivo* experimental results provide evidence that the loss of QKI5 is an important cause of FoxO1 over-activation in *ob*/*ob* hearts, and subsequently magnified nitrosative stress and ER stress. Most importantly, recovery with QKI5 in diabetic hearts markedly attenuated MI/R injury.

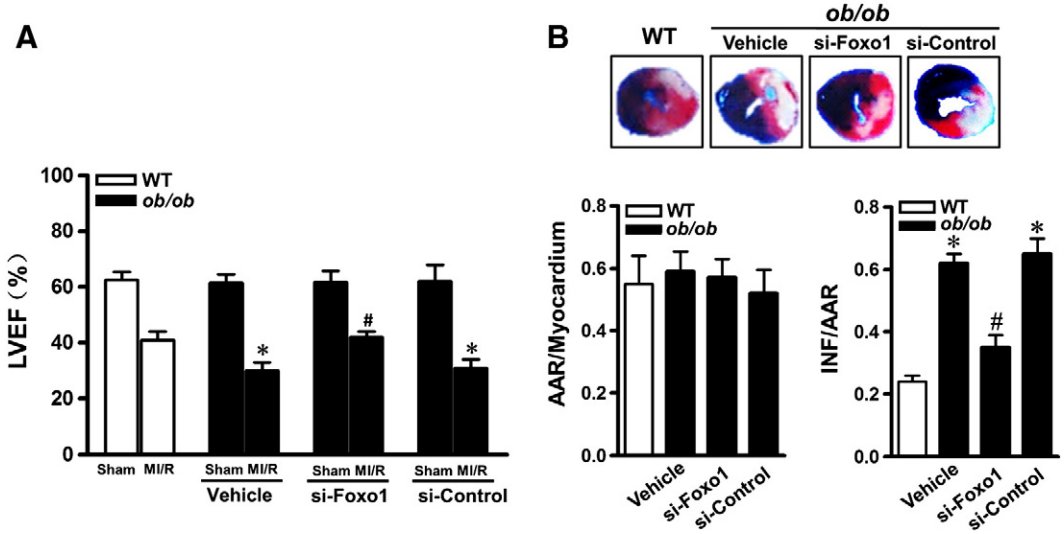

Fig. 6. FoxO1 knockdown improved the tolerance of diabetic heart to I/R injury. The *ob*/*ob* animals were treated by intramyocardial injection of FoxO1 siRNA (20 μg), control siRNA or vehicle, 48 h before ischemia (30 min)/reperfusion (4-hour) treatment. WT MI/R mice were used as control. (A) Cardiac function was determined by echocardiography. (B) Summary of myocardial infarct size expressed as a percent of total ischemic–reperfused area (area-at-risk, AAR) (n = 6 per group. **P* < 0.05 *vs.* MI/R + WT; #*P* < 0.05 *vs.* MI/R + *ob*/*ob* vehicle).

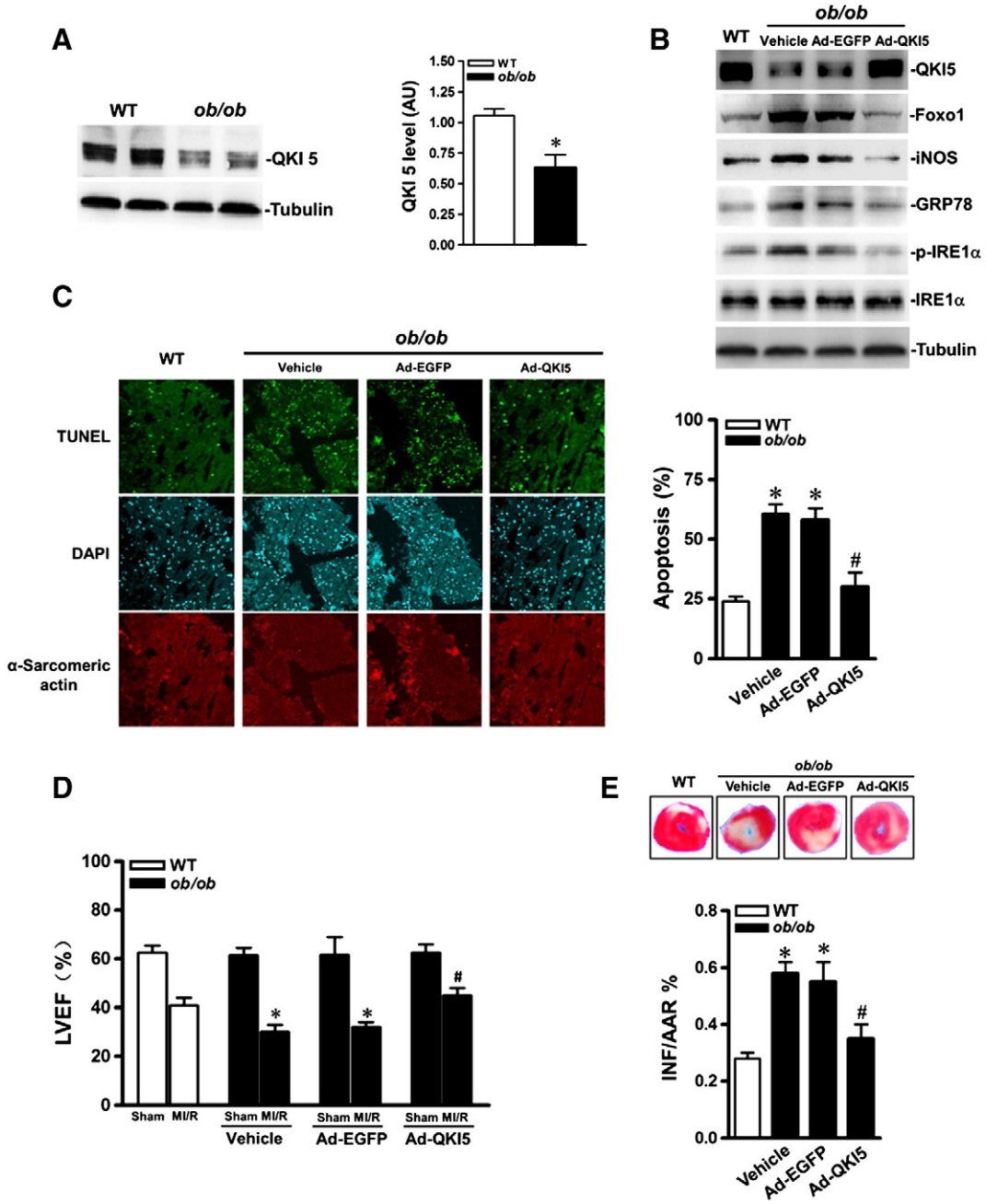

Fig. 7. QKI5 upregulation reduced FoxO1 expression and cardiomyocyte apoptosis after ischemia/reperfusion in diabetic hearts. (A) Representative gel blots depicting QKI5 protein expression in wild type (WT) and *ob/ob* mice hearts (n = 6 per group; $^{*}P < 0.05$ *vs.* WT). (B) The *ob/ob* animals were subjected to intramyocardial injection of adenovirus encoding-QKI5 (Ad-QKI5), Ad-EGFP or vehicle; normal WT mice were used as control. After 48 h, the myocardial QKI5, FoxO1, iNOS, GRP78 and p-IRE1α expression were measured. (C) WT and treated *ob/ob* mice were subjected to a 30-minute ischemia followed by a 4-hour reperfusion *in vivo*. TUNEL assay was performed to evaluate the effects of QKI5 upregulation on MI/R induced cardiomyocyte apoptosis. (D) Cardiac function was determined by echocardiography. (E) The percent of infarct size of isolated WT and *ob/ob* hearts with or without QKI5 upregulation (n = 6 per group. $^{*}P < 0.05$ *vs.* MI/R + WT; $\#P < 0.05$ *vs.* MI/R + *ob/ob* vehicle).

3.6. QKI destabilized FoxO1 mRNA

To verify the negative regulation of FoxO1 by QKI5, we next determined the possible mechanism of QKI5-mediated repression of FoxO1. Associated with the decreased FoxO1 protein levels, the mRNA levels of FoxO1 were markedly reduced by QKI5 *in vivo* overexpression in an *ob/ob* heart ($P < 0.05$, Fig. 8A). In order to detect the interaction between QKI5 and FoxO1, an RNA co-immunoprecipitation experiment was performed. Primary culture of neonatal cardiomyocytes was transfected with pcDNA3.1(+)-EGFP-QKI5 for 24 h, and total cellular extracts were then incubated with either an anti-EGFP or a nonspecific antibody (IgG) (Fig. 8B). By RT-PCR analysis, the anti-EGFP immunoprecipitate contained FoxO1 mRNA whereas the one with non-specific IgG did not (Fig. 8 C). We found that overexpression of QKI5 inhibited the mRNA and protein levels of FoxO1 (Fig. 8 D). To verify whether QKI post-transcriptionally regulates the mRNA stability of FoxO1, a transcription inhibitor (actinomycin D) was used to block the transcription initiation in cardiomyocytes infected with Ad-QKI5. Ad-EGFP was used as control virus. Cardiomyocytes were harvested at different time points, and real-time PCR was employed to determine the decaying rate of FoxO1 mRNA. As shown in Fig. 8E, the half-life of FoxO1 mRNA was shortened in QKI5 overexpression.

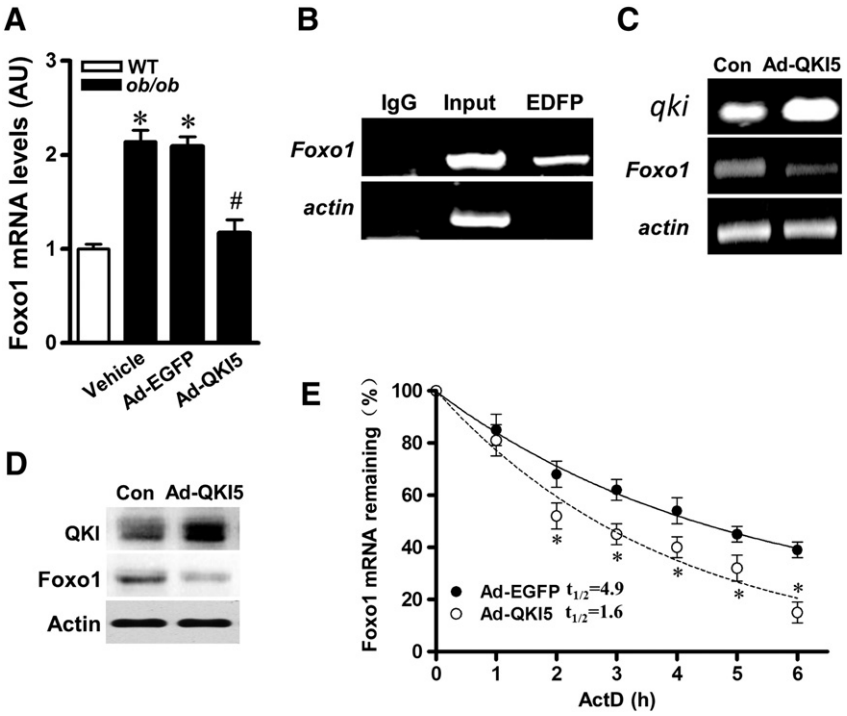

Fig. 8. QKI5 repressed FoxO1 expression. (A) FoxO1 mRNA levels in the WT and *ob*/*ob* hearts with or without QKI5 overexpression (n = 5 per group; $^{*}P < 0.05$ *vs.* MI/R + WT; $\#P < 0.05$ *vs.* MI/R + *ob*/*ob* vehicle). (B) The association of QKI5 and FoxO1 was detected by RNA immunoprecipitation. Culture of cardiomyocytes were seeded in 100-mm dishes and transfected with recombinant adenoviruses expressing EGFP-tagged QKI5 for 24 h and treated with formaldehyde to cross-link protein to RNA. Cells were lysed and incubated with either anti-EGFP or nonspecific antibodies (IgG) and protein A Sepharose. Cytoplasmic extract without antibody incubation was saved as an 'input' sample. The immunoprecipitated RNAs were isolated and FoxO1 mRNAs were amplified by PCR. A nonspecific antibody was used as a negative control (IgG). (C) RT-PCR and (D) Western blotting were performed to detect FoxO1 expression in cardiomyocytes with QKI5 overexpressing. (E) Half-life of FoxO1 in cardiomyocytes with or without QKI overexpressing. 5 mg/ml actinomycin D was added to the cardiomyocytes. At the indicated time points, RNAs were isolated, and the mRNA concentrations of FoxO1 and actin were determined by real-time PCR. The relative amount of FoxO1 mRNA without actinomycin D treatment was set to 100%, and the percentage of FoxO1 mRNA treated with actinomycin D was calculated accordingly (n = 3 per group. $^{*}P < 0.05$ *vs.* Ad-EGFP).

These data suggest that QKI5, as an RNA binding protein, destabilizes FoxO1 mRNA.

4. Discussion

The potential molecular mechanism responsible for the increased vulnerability of diabetic hearts to ischemic injury and the resultant higher mortality has not been established. In this study, we provide direct evidence linking QKI5 deficiency to FoxO1 overactivation in the hearts of *ob*/*ob* mice. We have proved that QKI5 repressed FoxO1 expression *via* decreasing its mRNA stability in cardiomyocytes. We have provided additional evidence that over-activation of FoxO1 possessed detrimental properties on diabetic myocardium. FoxO1 aggravated I/R injury *via* induction of nitrosative stress and ER stress, highlighting the role of FoxO1 in I/R injury of diabetic myocardium. Last but not the least, QKI5 up-regulation could inhibit FoxO1-mediated nitrosative and ER stresses in *ob*/*ob* myocardium. Targeting QKI-mediated repression of FoxO1 may serve as a novel strategy to reduce diabetic patient mortality in those with ischemic heart disease.

As one of the signal transduction and activation of RNA (STAR) family members, QKI exerts its function by mediating the upstream kinase signals to directly modulate various RNA metabolism processes, including pre-mRNA splicing, mRNA export, mRNA stability and protein translation [28]. QKI is abundantly expressed in the brain and heart. The role of QKI in the nervous system has been well characterized, but its function in the cardiovascular system is still poorly understood. The deficiency of QKI was correlated with the reduction of transcriptional genes which were critical for blood vessel development, highlighting the link between abnormal QKI expression and cardiovascular system dysfunction [29]. Although recent research has implied that QKI is a critical regulator of VSMC physiological function and vascular repair [30], the additional roles of QKI in diabetic hearts remain to be discovered. Whether aberrant QKI expression occurs in diabetic myocardium and what are the possible cellular and physiological consequences remain unexplored. A bipartite consensus sequence NACUAAY-N1-20-UAAY was designated as a QKI response element (QRE) and as many as 1430 new mRNA targets were predicted to be QKI direct targets [31]. FoxO1 mRNA was among these predicted targets [19,20]. The *qk* gene produces several alternative splicing variants and encodes 3 major QKI isoforms, namely QKI5, -6 and -7, respectively. Connecting to our previous study, we first demonstrated the anti-apoptotic effects of QKI5 in cultured cardiomyocytes and the underlying mechanism was involved in the repression of FoxO1 [20]. The present study reports that QKI5 is deficient in diabetic myocardium and FoxO1 is the downstream target of cardiac QKI5 during diabetes. We thereupon hypothesized that an abnormal QKI5 level might contribute to the dysregulation of FoxO1 activation and subsequent myocardial injury in diabetic hearts. Our results showed that *ob*/*ob* hearts expressed a very low level of QKI5. Furthermore, the up-regulation of QKI5 reduced diabetes-induced FoxO1 expression, along with subsequent myocardial NS stress, ER stress, and cardiomyocyte apoptosis in *ob*/*ob* animals. Our present data again provided evidence that QKI5 inhibited the expression and activation of cardiac FoxO1 *in vivo*. We demonstrate for the first time that QKI5 is a natural molecular factor that inhibits cardiac FoxO1 overexpression in diabetic cardiac tissue, and thus attenuates the vulnerability of diabetic hearts to ischemic injury.

FoxO1 has important roles in systemic homeostasis. FoxO1 is over-activated under conditions of nutrient excess, insulin resistance and ischemia, which can result in metabolic disturbances and cell death pathway activation in respective tissues [16]. FoxO1 participates in the regulation of all types of cell death seen during insulin resistance and diabetes. Although FoxO1 has a well defined role in the induction of apoptosis, whether this mode of programmed cell death contributes to increased vulnerability in diabetic heart is debated. Previous studies

have demonstrated that both nitric oxide (NO) and superoxide (O_2^-) production are increased in diabetic or high-glucose-treated cardiomyocytes. Peroxynitrite ($ONOO^-$), the reaction product of superoxide and nitric oxide, is highly cytotoxic. Nitrosative stress, initiated by excess NO production and mediated by peroxynitrite, is suggested to be another crucial response that leads to cell death in diabetic cardiomyocytes besides oxidative stress. Previous studies suggested that the inhibition of peroxynitrite formation, either by detoxifying peroxynitrite or iNOS inhibitors, is able to reduce I/R injury [32]. Our study presented similar results that inhibition of peroxynitrite formation attenuated caspase-3 activity in diabetic *ob*/*ob* animals [4,5]. ER stress is also involved in cardiac apoptosis in the diabetic rat model [33]. Investigations have also demonstrated that ER stress induces cell apoptosis independently from mitochondria- and death receptor-dependent pathways. ER stress reduces SERCA2a protein leading to LV diastolic dysfunction in T2D [34]. These experimental data suggested that ER stress was initiated in the diabetic heart. The present study suggested that ER stress was involved in the hypersensitivity of cardiomyocytes to I/R in the diabetic mouse strain, and that inhibition of ER stress significantly attenuated the injury of diabetic cardiomyocytes when subjected to I/R, which is similar with what was found in diabetic cardiomyopathy. Although concurrent nitrosative stress also contributes to the liability to I/R injury of cardiomyocytes in the diabetic heart, nitrosative stress pathway inhibitors (1400W or M40401) did not affect the ER stress represented by the activation of PERK, p-eIF2α, IRE1α, GRP78 and CHOP in an *ob*/*ob* heart, suggesting that there is no cross talk between nitrosative stress and ER stress. However, the ER stress was demonstrated to be initiated by reactive oxygen species (ROS) and calcium handling [35]. The PERK/ATF4/CHOP branch of the UPR transmits pro-apoptotic signals. The precise mechanism of ER stress-induced cardiomyocyte apoptosis remains elusive; however, recent data suggest that suppression of Puma, a member of Bcl-2 family, prevented both ER stress and I/R-induced cardiomyocyte loss [36]. Another study suggested that Puma activation was also mediated by FoxO1 activation in diabetic cardiomyocytes [37]. Combined with our data, FoxO1-mediated ER stress might be transduced by Puma. But the present study did not detail this aspect of the mechanism. Previous studies suggested that FoxO1 brought about cell death through its ability to increase the expression of caspases and cell death receptors, both of which are reported to be augmented during diabetes. FoxO1 has been shown to directly increase the expression of some pro-apoptotic proteins like Bim and Puma. A role was also suggested for FoxO1 in regulating pro-apoptotic BAD, a mechanism mediated through PP2A [27]. Here, we found that FoxO1 activated two crucial pathways that lead to cardiomyocyte death, nitrosative stress and ER stress, establishing new pathways that contribute to the hypersensitivity to I/R injury of diabetic cardiomyocytes, which highlights the crucial roles of FoxO1 in post I/R injury of diabetic hearts.

As an RNA-binding protein, QKI post-transcriptionally regulates the mRNA stability, translation efficiency or RNA transportation of target genes *via* specifically binding to the 3′-untranslated region (3′UTR). Our study observed that QKI5 and FoxO1 were inversely expressed in cardiomyocytes, and the level of FoxO1 mRNA could be inhibited by QKI5 overexpression. Our current data demonstrated that QKI5 restrained the expression of FoxO1 *via* decreasing the mRNA stability of FoxO1 in cardiomyocytes. Although we demonstrated that QKI5 downregulated FoxO1 by posttranscriptional regulation, the molecular mechanism for the deficient expression of QKI5 in diabetic hearts remains to be elucidated. Previous studies demonstrated that QKI expression is absent or very lowly expressed in colon cancer cells because of the high methylation of the promoter region [26]. Some studies reported that global DNA hypermethylation is associated with aging or diabetes mellitus and increased mortality in cardiovascular disease [38]. Such intriguing possibilities all warrant direct investigation. The precise mechanisms responsible for QKI5 deficiency are likely complex and cannot be answered in a single experiment.

In conclusion, QKI5 deficiency promotes FoxO1 mediated nitrosative stress and ER stress which constitute a novel mechanism for rendering diabetic hearts more vulnerable to enhanced MI injury. The present study demonstrates that QKI5 is an endogenous therapy target that relieves the vulnerability of the diabetic heart to I/R injury. These results suggest that therapeutic interventions preserving QKI5 expression in the diabetic patient may further improve patient outcomes after myocardial infarction.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2014.07.010.

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