

Inhibition of miR-23 protects myocardial function from ischemia-reperfusion injury through restoration of glutamine metabolism

Y. KOU¹, W.-T. ZHENG², Y.-R. ZHANG³

¹Department of Cardiovascular Medicine, Binzhou People's Hospital, BinZhou, Shandong, China

²Incentive Care Unit, Binzhou People's Hospital, BinZhou, Shandong, China

³Medical Department, Binzhou People's Hospital, BinZhou, Shandong, China

Abstract. – OBJECTIVE: Myocardial disorders caused by ischemia/reperfusion (IR) continue to be among the most frequent causes of debilitating disease and death. The contribution of cellular metabolism through the production of metabolic intermediates during IR has been increasingly investigated.

MATERIALS AND METHODS: In this study, by using a rat IR injury model, we reported that the expression of microRNA miR-23 was induced by IR. In contrast, the glutamine metabolism was suppressed during IR. The glutamate, glutamine dehydrogenase activity, α -ketoglutarate, and glutaminase (GLS) mRNA expression were significantly decreased by IR. Moreover, the pre-treatment of glutamine could protect the myocardium from IR injury.

RESULTS: From microRNA target prediction analysis and results of luciferase assay, we found that miR-23 could directly target the 3'UTR of GLS. Finally, we demonstrated that inhibition of miR-23 protected myocardial function from IR through the restoration of glutamine metabolism.

CONCLUSIONS: This study reveals that inhibition of miR-23 renders protective effects on rat IR injury, highlighting the importance of miR-23 and glutamine metabolism during IR, and suggests a potentially clinical benefit.

Key Words:

miR-23, Ischemia-reperfusion injury, Glutamine.

ing to IR injury include microvascular dysfunction, local inflammation, incensement of reactive oxygen species, disruption of Ca^{2+} homeostasis, and activation of mitochondrial apoptosis pathway³. It has been known that reperfusion-induced oxidative stress is an essential pathological risk factor in this process⁴. Reactive oxygen accumulation aggravates serious acute and chronic heart damage⁵. Moreover, amino acids have been widely studied as essential cardioprotective substrates for maintaining the normal functions of the heart through their potential roles as anti-oxidative agents as well as cellular energy supplementation^{6,7}.

MicroRNAs (miRNAs) are a class of endogenous, small (~22 nucleotides) size, noncoding single-stranded RNAs⁸. MiRNAs repress target genes expression by complementary binding to sequences in the 3'-UTRs of target mRNAs⁹. Recently, miRNAs have been illustrated to play an important role in IRI. It was known that miR-208 was up-regulated, while miR-1 and miR-133a were down-regulated in IRI¹⁰. In addition, miRNA-126^{11,12} and miRNA-133^{13,14} have been reported as myocardial protective microRNAs in IRI. Therefore, analysis of the expression and function of myocardial I/R related miRNAs contributes to explore the mechanisms of myocardial I/R injury. In this study, the roles of miR-23a/b/c in the myocardial rat IRI has been studied. Also, the miR-23-regulated glutamine metabolism during IR has been investigated.

Introduction

Ischemia/reperfusion injury (IRI), as a consequence of vessel occlusion followed by multiple stresses during the restoration of blood flow to the tissue contributes significantly to postoperative mortality and morbidity^{1,2}. IRI is a complex pathophysiological event, leads to deterioration of heart function, and limits the benefits of reperfusion after acute myocardial infarction, causing a serious health problem². The factors contribut-

Materials and Methods

Animal Experiments

Twenty Sprague-Dawley rats were equally randomized to two groups: control group (n=10),

where the rats underwent thoracotomy only and IR group (n=10), where the rats were treated with ischemia for 15 or 30 min and reperfusion for 30 min. All animal procedures were approved by the Institutional Animal Care and Use Committee of Binzhou People's Hospital.

Infarct Size Measurement

Infarct size of the myocardium was measured as previously described¹⁴. Total left ventricular area (LV), infarct area (INF) and area at risk (AAR) were determined by computerized planimetry. The relative percentage of the INF/AAR was calculated.

Cell Culture

Primary cardiomyocyte cultures were isolated from 1-day-old neonatal rats (Harlan Sprague-Dawley) and cultured using Primary Cardiomyocyte Isolation Kit (Pierce, Waltham, MA, USA) according to the manufactory instructions.

Antibodies and Reagents

L-Glutamine was purchased from Sigma-Aldrich (Shanghai, China). Rabbit polyclonal anti-GLS antibody was purchased from Abcam (ab93434) (Cambridge, United Kingdom); Mouse monoclonal anti-GAPDH antibody was purchased from Abcam (ab8245) (Cambridge, United Kingdom).

Glutamine Metabolism Assays

The glutamate assay, glutamate dehydrogenase activity assay, and α -ketoglutarate assay were performed using glutamate assay kit (K629-100), glutamate dehydrogenase activity colorimetric Assay Kit (K729-100) and alpha-ketoglutarate colorimetric assay kit (K677-100) from Biovision (Milpitas, CA, USA) according to the manufacturer's instructions. The experiments were repeated three times and the results were normalized to the amount of total protein of the rat heart tissue in IR group compared with the control group.

microRNA Transfection In Vivo

Mimic and anti-miRNA oligonucleotides (AMO) of miRNA pretreatments *in vivo* were performed as previously described¹⁴. The chest of rats was opened with appropriate anesthesia. Then 100 nM synthesized miR-23 mimic or AMO-23 (50 mg/kg), or control microRNAs pretreated with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), was injected into the my-

ocardium of rats. The chests were closed after injection and the rats were allowed to recover for 48 hours, followed by IR treatments.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA of cells was isolated by using TRIzol reagent, and reverse transcribed according to the manufacturer's instructions (Fermentas, Waltham, MA, USA). For mRNA analysis, complementary DNA was randomly primed from 1 mg of total RNA using the Fermentas cDNA Synthesis kit. For evaluating the miR-23 expressing levels, quantification using the TaqMan microRNA assays was performed using two-step RT-PCR according to the manufacturer's instructions. PCR products were amplified from cDNA samples using the TaqMan MicroRNA Assays kit together with the TaqMan Universal PCR Master Mix (Applied Biosystems). The comparative Ct (threshold cycle) method with arithmetic formulae ($2^{-\Delta\Delta Ct}$) was used to determine relative quantitation of gene expression. All miRNA data are normalized to a RNU6B, which displays stable expression profile throughout all experiments and then expressed as relative changes. All experiments were repeated three times independently.

Western Blot

Tissue or cell protein extracts were obtained with radioimmunoprecipitation assay buffer (RIPA) following standard protocols. The protein extracts were denatured and 40-50 μ g of each sample were separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk (LabScientific, Inc.) in Tris-Buffered Saline-Tween (TBST) buffer, the membranes were probed with antibodies against GLS (1:1000) and GAPDH (1:3,000) at 4°C overnight. Membranes were washed with TBST for 3 $\times$ 5 mins followed by secondary antibody incubation at room temperature for 2 hours. Membranes were exposed using enhanced chemiluminescence (Roche, Basel, Switzerland) method following manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed by Prism 5.0 software. Quantitative data are presented as mean $\pm$ standard error. Statistical significance was determined using Student *t*-test. $p < 0.05$ was considered statistically significant.

Results

miR-23 is Induced by IR in Rat Heart

Since microRNAs have been reported to play important roles during myocardial ischemia-reperfusion injury¹⁰, we investigated the roles of miR-23 during the IR. By comparison of the expression of miR-23a/b/c between control and IR groups in the rat hearts, our results showed that miR-23a/b/c were significantly induced by IR injury at 15 minutes ischemia, 30 minutes reperfusion, and 30 minutes ischemia, 30 minutes reperfusion (Figures 1A, 1B and 1C), indicating miR-23 might involve in the myocardial IR injury.

Glutamine Metabolism is Suppressed by IR

It has been demonstrated that glutamine metabolism of heart involves in the protective effects against IR¹⁵. We started to assess the glutamine metabolism of rat heart by IR injury. As we expected, the glutamate amount, α -ketoglutarate, and glutamate dehydrogenase (GDH) activity were significantly decreased in IR injury group (Figures 2A, 2B and 2C). To further confirm the above results, we measured the expression of glutaminase (GLS) which converts the glutamine to glutamate¹⁶. Consistently, the GLS mRNA expressions were downregulated in IR injury group compared with control group (Figure 2D). Taken together, our results revealed that the rat heart with IR injury displayed impaired glutamine metabolism.

Glutamine Metabolism Contributes to the Recovery of Heart from IR

We next investigated whether the ameliorative glutamine metabolism could improve the myocardial functions against IR injury. We pre-treated rats with glutamine by intraperitoneal in-

jection 12 hours ahead of IR experiments. Rats that received glutamine and underwent 30 minutes of ischemia and reperfusion times of 30 minutes showed significantly less myocardial injury area compared with the control group (Figure 3), suggesting that improvement of glutamine metabolism might contribute to the reduction of myocardial infarction by IR.

miR-23 Targets GLS1 and Suppress Glutamine Metabolism

Our above results demonstrated a correlation between the IR injury-induced miR23 and suppressed glutamine metabolism; we, therefore, hypothesized that there is regulatory correlation between miR-23 and glutamine metabolism during IR. Potential targets of miR-23 were then investigated. We searched the public miRNA database (www.targetscan.org) for predicted targets of miR-23. The database predicted that 3'-UTR of glutaminase (GLS) contains a highly conserved binding site for miR-23a/b/c (Figure 4A). Importantly, the predicted binding site on 3'-UTR of GLS is conserved in multiple species including human and rat (Figure 4B). To determine whether miR-23 targets GLS, a mixture of miR-23a/b/c was transfected into rat cardiomyocytes. Overexpression of miR-23 significantly inhibited expression of the GLS protein (Figure 4C). Moreover, we investigated whether miR-23 directly targets the 3'-UTR of GLS mRNA. We co-transfected a vector containing pMIR reporter-luciferase fused with either the original 3'-UTR sequence or a sequence with a mutation in the predicted binding site of the 3'-UTR of GLS mRNA, and with either pre-miR-23 or control microRNAs. Our findings showed overexpression of miR-23 only decreased the luciferase activity of the reporter containing the wild-type 3'-UTR of GLS, compared with the

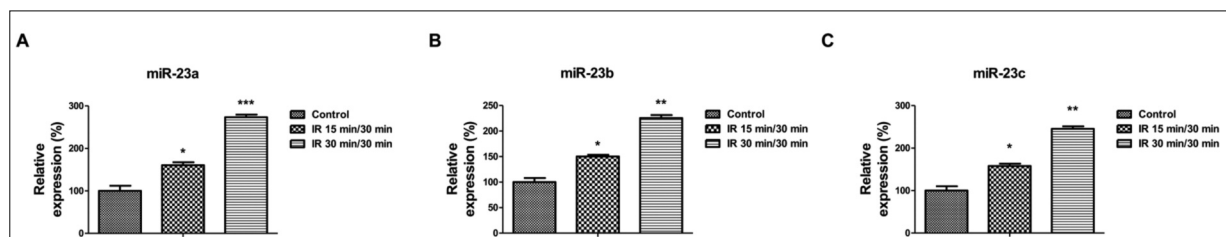


Figure 1. miR-23 is upregulated by ischemia-reperfusion. **A**, miR-23a or **(B)** miR-23b or **(C)** miR-23c was detected in rat hearts treated by ischemia 15 min, reperfusion 30 min and ischemia 30 min, reperfusion 30 min. The expressions of miR-23 were measured by qRT-PCR. U6 was used as an internal control. Columns, mean of three independent experiments; bars, SE. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

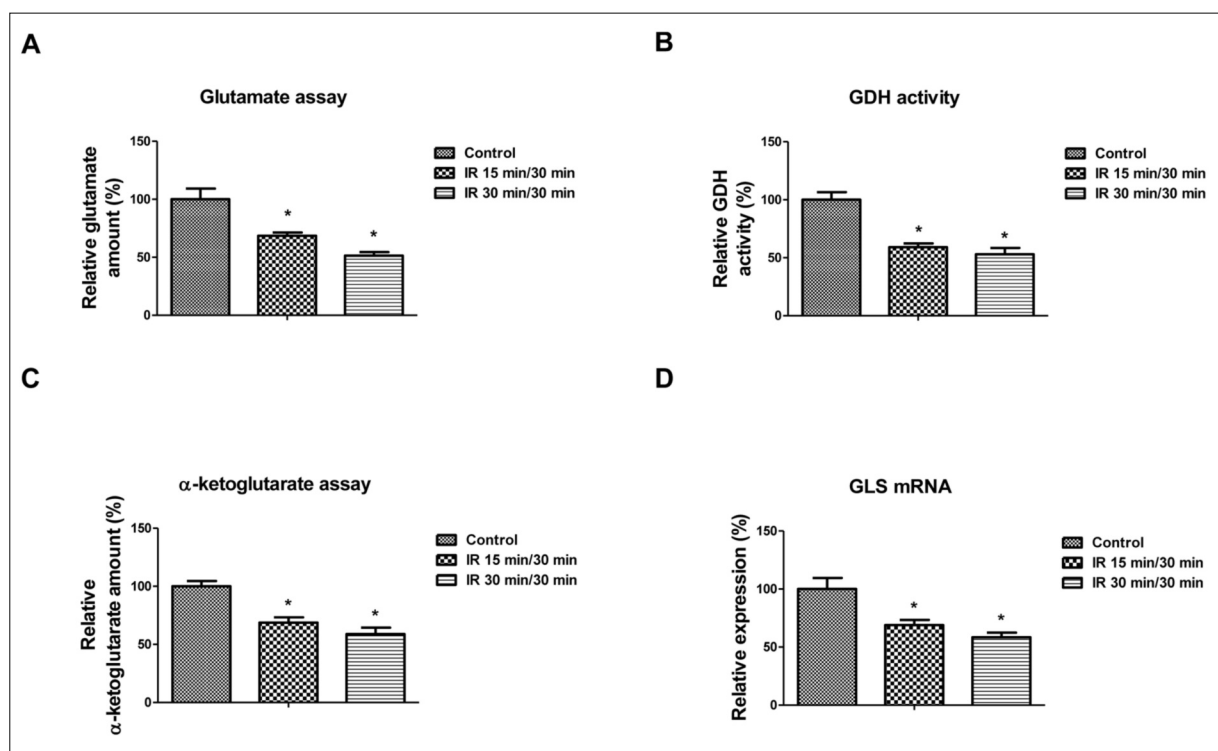


Figure 2. miR-23 inhibits glutamine metabolism. **A**, Glutamine amounts were measured in rat hearts from the control group, IR 15 min/30 min, or IR 30 min/30 min group. **B**, The activities of glutamine dehydrogenase were measured in rat hearts from the control group, IR 15 min/30 min, or IR 30 min/30 min group. **C**, The α -ketoglutarate amounts were measured in rat hearts from the control group, IR 15 min/30 min, or IR 30 min/30 min group. **D**, The mRNA expressions of GLS were measured in rat hearts from the control group, IR 15 min/30 min, or IR 30 min/30 min group. GAPDH is used as internal control. Columns, mean of three independent experiments; bars, SE. *, $p < 0.05$; **, $p < 0.01$.

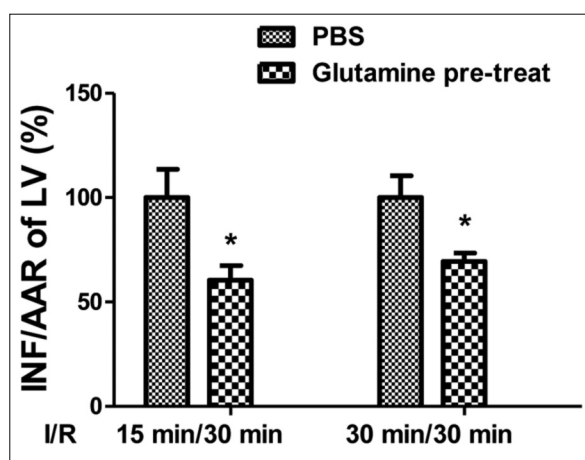


Figure 3. Glutamine treatment protects myocardial function from ischemia-reperfusion injury. Rats were intraperitoneally injected PBS or glutamine 12 hours before the IR 15 min/30 min or 30 min/30 min experiments. The relative INF/AAR ratio was measured and calculated. Columns, mean of three independent experiments; bars, SE. *, $p < 0.05$; **, $p < 0.01$.

mutation on 3'-UTR of GLS (Figure 4D). These results demonstrated that miR-23 directly targeted GLS in IR injury.

The above results demonstrated that miR-23 could target GLS, which is the commit step for the glutamine metabolism. We, therefore, suggested that miR-23 could suppress glutamine metabolism in rat heart. We transfected miR-23 mimic into rat myocardium (Figure 5A), then assessed the glutamine metabolism. As we expected, the glutamate amount, α -ketoglutarate, glutamate dehydrogenase (GDH) activity as well as the GLS mRNA and protein expression was significantly decreased by overexpression of miR-23 (Figures 5B-5F).

Inhibition of miR-23 Protects Myocardial from IR Through Restoration of Glutamine Metabolism

To investigate whether the miR-23-mediated suppression of glutamine metabolism correlates with IR *in vivo*, anti-miR-23 oligonucleotide

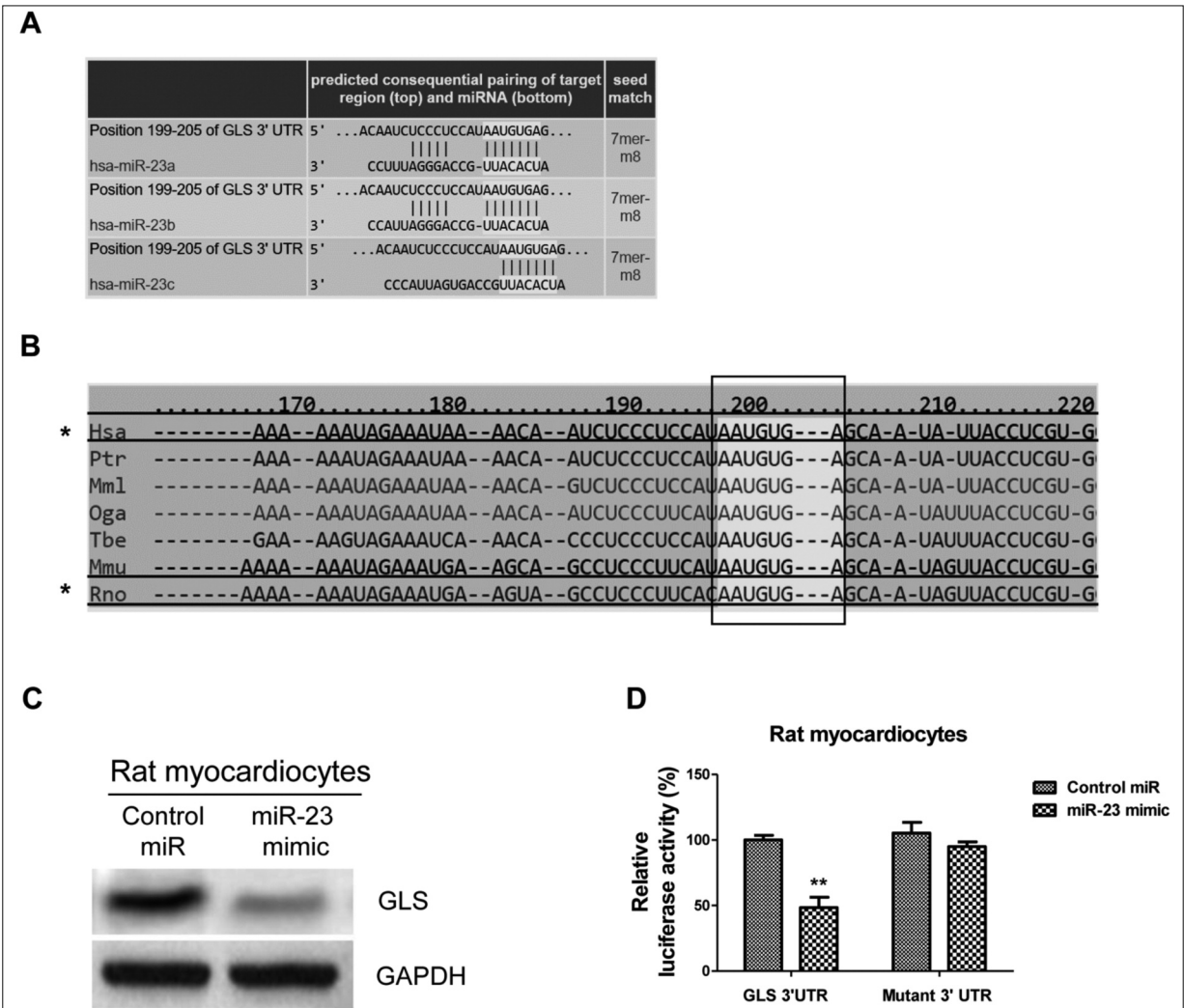


Figure 4. miR-23 targets glutaminase in rat heart. **A**, Target prediction from Targetscan.org shows the position 119-205 of GLS 3' UTR contains putative binding sites for miR-23a/b/c. **B**, Sequence analysis of GLS 3' UTR shows the putative binding sites for miR-23a/b/c were conserved in multiple species. **C**, Rat myocardiocytes were transfected with 100 nM control mimic or miR-23 mimic for 48 hrs. Cell lysates were prepared for Western blotting analysis. GAPDH was used as a loading control. **D**, Luciferase assay showed miR-23 mimic could target 3'UTR of wild-type GLS but not binding sites mutant GLS in rat myocardiocytes.

(AMO-miR-23) was exogenously transfected in the myocardium before IR. Consistently, inhibition of miR-23 in rat heart significantly restored GLS expression (Figure 6A). In addition, the glutamate amount, GDH activity and α -ketoglutarate were increased in the AMO-miR-23 treated rat hearts (Figure 6B, 6C and 6D). As we expected, the IR injury in rat heart with inhibition of miR-23 was significantly less than that of control microRNA transfection (Figure 6E), suggesting that anti-miR23 oligonucleotide might be considered a therapeutic agent for the protection of ischemia-reperfusion injury clinically.

Discussion

In this study, we investigated the roles of miR-23 in rat heart IRI. Our results consistently illustrated that miR-23 was induced in the isolated working rat heart model, suggesting that inhibition of miR-23 might be a protective approach against ischemia reperfusion injury.

The heart consumes 10% of the body's total oxygen uptake and produces upwards of 35 kg of ATP every day¹⁷. Therefore, the metabolic substrates, as well as oxygen supply through blood flow, are essential for maintaining the functions

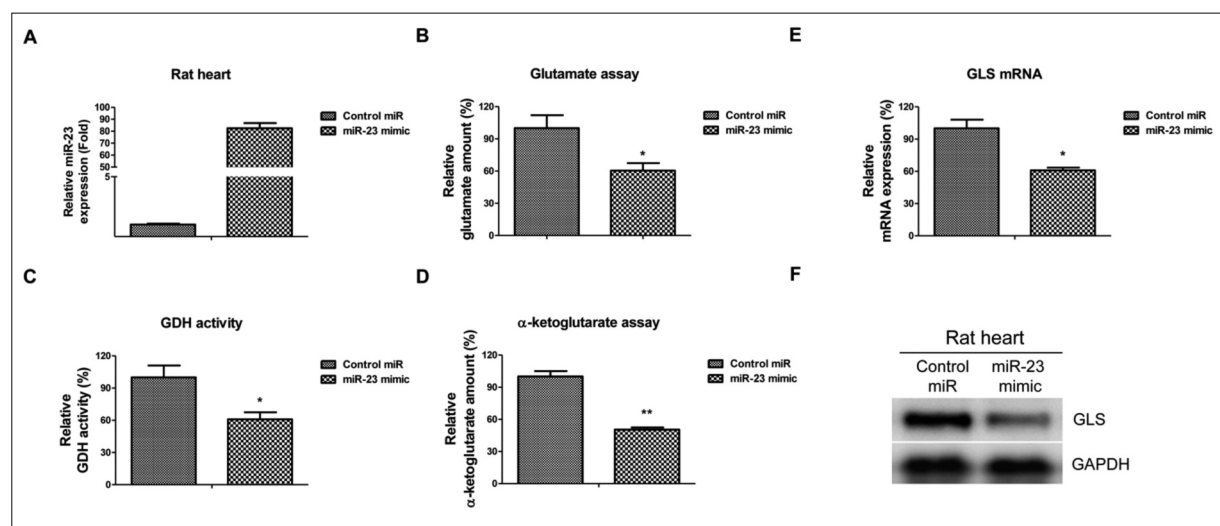


Figure 5. Overexpression of miR-23 inhibits glutamine metabolism in rat hearts. **A**, A mixture of miR-23a/b/c mimic or control mimic was transfected in rat hearts followed by the measurements of miR-23 expression by qRT-PCR. U6 was used as an internal control. **B**, Glutamate amounts were measured in control or miR-23 mimic transfected rat heart tissues. **C**, Glutamate dehydrogenase activities were measured in control or miR-23 mimic transfected rat heart tissues. **D**, α -ketoglutarate amounts were measured in control or miR-23 mimic transfected rat heart tissues. **E**, GLS mRNA expressions were measured in control or miR-23 mimic transfected rat heart tissues by qRT-PCR, GAPDH was used as an internal control. **F** GLS protein expressions were measured in control or miR-23 mimic transfected rat heart tissues by Western blot; GAPDH was used as an internal control. Columns, mean of three independent experiments; bars, SE. *, $p < 0.05$; **, $p < 0.01$.

of myocardium. Glucose, amino acids, fatty acids and other substrates metabolism are fundamental system that governs the heart's behavior evidenced by the recent increase in excellent review articles detailing the importance of cardiac amino acid metabolism^{6,7}. We found that the rats with pretreated glutamine before ischemia-reperfusion exhibited alleviated heart functions, consistently with previous reports that glutamine improved rat myocardial function following ischemia-reperfusion injury¹⁸. However, the detailed mechanisms for the glutamine-mediated anti-IRI are still unclear.

It has been reported that the Reactive Oxygen Species (ROS)-induced vascular endothelial dysfunction plays an important role in the development of IRI⁵. The application of low frequency pulse magnetic fields can effectively reduce ROS generation and subsequently attenuate myocardial IRI. Moreover, glutamine is significantly involved in the synthesis of reduced glutathione (GSH), which serves as a very important intracellular antioxidant and detoxication factor¹⁹. Our results supported that elevated glutamine metabolism contributes to the antioxidant effects on the myocardium, leading to the protection of heart in IRI.

Ischemia also depletes cellular ATP which inactivates ATPases (e.g., Na⁺/K⁺ ATPase), limit-

ing the reuptake of calcium by the endoplasmic reticulum (ER), thereby impair the myocardium functions²⁰. Importantly, the primary energy source of the normal myocardium is free fatty acids and metabolism of free fatty acids could increase toxic metabolite accumulation, resulting damage to the myocardium during ischemia²¹. We found that inhibition of miR-23 upregulated the glutamine metabolism, which might be an alternative nutrition supply for myocardiocytes to limit the toxic metabolite accumulation from a fatty acid. Moreover, glutamine metabolism generates a higher amount of GSH, which protects myocardium in IR through its anti-oxidative function.

Conclusions

This study demonstrated the roles of the axis miR-23-glutamine metabolism during IRI, providing a new aspect on the development of the microRNA-based therapeutic strategies against ischemia-reperfusion injury.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

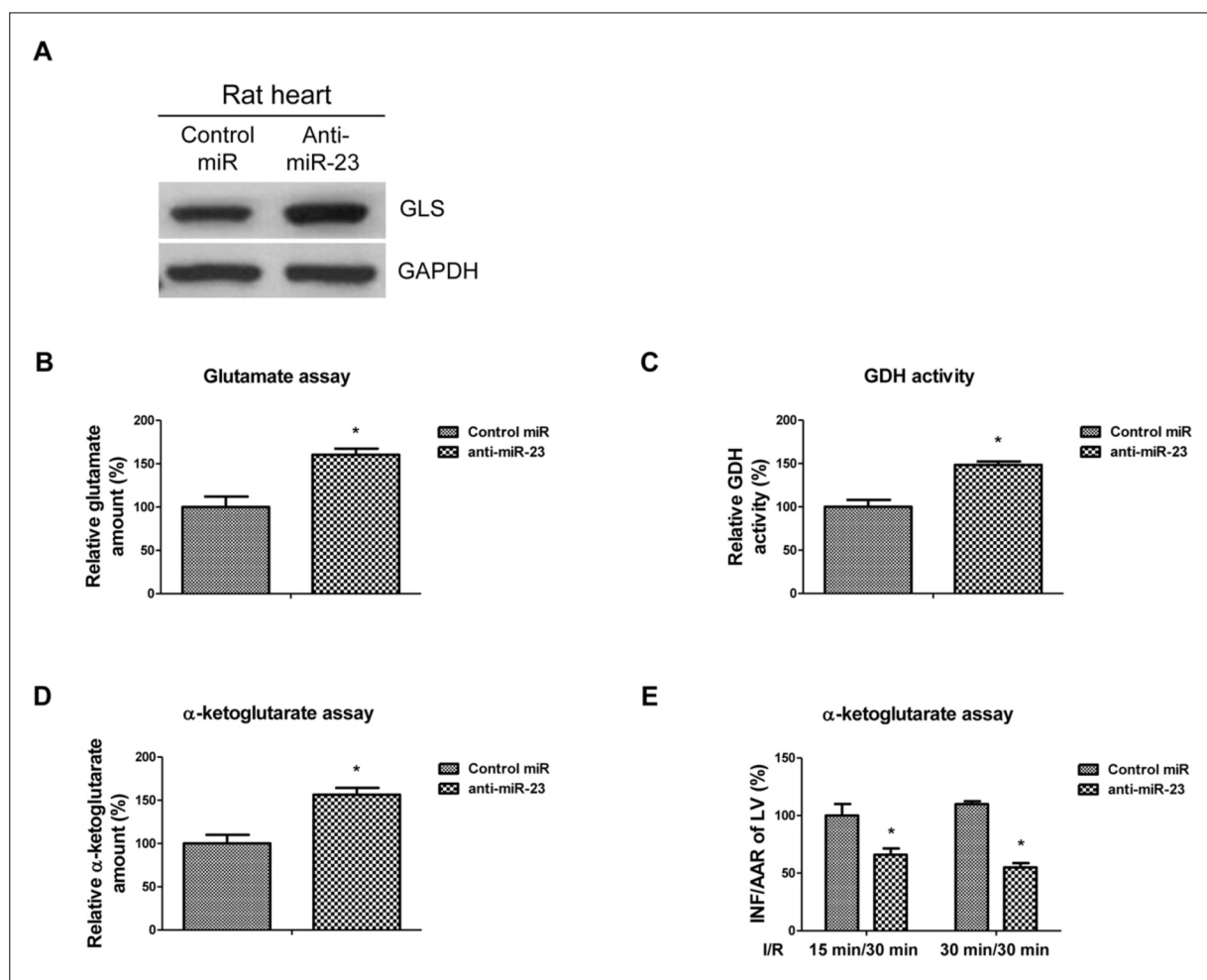


Figure 6. Inhibition of miR-23 protects myocardium from ischemia-reperfusion injury through upregulation of glutamine metabolism. **A**, Rat hearts were transfected with control miR or anti-miR-23 for 48 hours followed by tissue protein collection and Western blot analysis. GAPDH was a loading control. **B**, Rat hearts were transfected with control miR or anti-miR-23 followed by the analysis of glutamate amount, **C**, GDH activity and **D**, α -ketoglutarate amount. **E**, Rats hearts were transfected with control miR or anti-miR-23 48 hours before the IR 15 min/30 min or 30 min/30 min experiments. The relative INF/AAR ratio was measured and calculated. Columns, mean of three independent experiments; bars, SE. *, $p < 0.05$.

References

- HAUSENLOY DJ, YELLON DM. Ischaemic conditioning and reperfusion injury. *Nat Rev Cardiol* 2016; 13: 193-209.
- LI J, LI RJ, LV GY, LIU HQ. The mechanisms and strategies to protect from hepatic ischemia-reperfusion injury. *Eur Rev Med Pharmacol Sci* 2015; 19: 2036-2047.
- XIA Z, CHEN Y, FAN Q, XUE M. Oxidative stress-mediated reperfusion injury: mechanism and therapies. *Oxid Med Cell Longev* 2014; 2014: 373081.
- MADDIKA S, ELIMBAN V, CHAPMAN D, DHALLA NS. Role of oxidative stress in ischemia-reperfusion-induced alterations in myofibrillar ATPase activities and gene expression in the heart. *Can J Physiol Pharmacol* 2009; 87: 120-129.
- KALOGERIS T, BAO YI, KORTHUIS RJ. Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biol* 2014; 2: 702-714.
- PREM JT, EPPINGER M, LEMMON G, MILLER S, NOLAN D, PEOPLES J. The role of glutamine in skeletal muscle ischemia/reperfusion injury in the rat hind limb model. *Am J Surg* 1999; 178: 147-150.
- DRAKE KJ, SIDOROV VY, MCGUINNESS OP, WASSERMAN DH, WIKSWO JP. Amino acids as metabolic substrates during cardiac ischemia. *Exp Biol Med* (Maywood) 2012; 237: 1369-1378.
- CAO Y, LU L, LIU M, LI XC, SUN RR, ZHENG Y, ZHANG PY. Impact of epigenetics in the management of cardiovascular disease: a review. *Eur Rev Med Pharmacol Sci* 2014; 18: 3097-3104.

- 9) JONAS S, IZAURRALDE E. Towards a molecular understanding of microRNA-mediated gene silencing. *Nat Rev Genet* 2015; 16: 421-433.
- 10) FAN ZX, YANG J. The role of microRNAs in regulating myocardial ischemia reperfusion injury. *Saudi Med J* 2015; 36: 787-793.
- 11) BUKERK R, VAN SOLINGEN C, DE BOER HC, DE VRIES DK, MONGE M, VAN OEVEREN-RIETDIJK A, VAN DER VEER EP, SCHAAPHERDER AF, RABELINK TJ, VAN ZONNEVELD AJ. Silencing of miRNA-126 in kidney ischemia reperfusion is associated with elevated SDF-1 levels and mobilization of Sca-1+/Lin- progenitor cells. *Microna* 2014; 3: 144-149.
- 12) BAI Y, BAI X, WANG Z, ZHANG X, RUAN C, MIAO J. MicroRNA-126 inhibits ischemia-induced retinal neovascularization via regulating angiogenic growth factors. *Exp Mol Pathol* 2011; 91: 471-447.
- 13) LI S, XIAO FY, SHAN PR, SU L, CHEN DL, DING JY, WANG ZQ. Overexpression of microRNA-133a inhibits ischemia-reperfusion-induced cardiomyocyte apoptosis by targeting DAPK2. *J Hum Genet* 2015; 60: 709-716.
- 14) HE B, XIAO J, REN AJ, ZHANG YF, ZHANG H, CHEN M, XIE B, GAO XG, WANG YW. Role of miR-1 and miR-133a in myocardial ischemic postconditioning. *J Biomed Sci* 2011; 18: 22.
- 15) ZHANG WX, ZHOU LF, ZHANG L, BAO L, WANG CC, MENG HY, YIN W. Protective effects of glutamine preconditioning on ischemia-reperfusion injury in rats. *Hepatobiliary Pancreat Dis Int* 2011; 10: 78-82.
- 16) HENSLEY CT, WASTI AT, DEBERARDINIS RJ. Glutamine and cancer: cell biology, physiology, and clinical opportunities. *J Clin Invest* 2013; 123: 3678-3684.
- 17) TAEGTMEYER H. Energy-Metabolism of the Heart - from basic concepts to clinical-applications. *Curr Prob Cardiology* 1994; 19: 61-113.
- 18) BOLOTIN G, RAMAN J, WILLIAMS U, BACHA E, KOCHER-GINSKY M, JEEVANANDAM V. Glutamine improves myocardial function following ischemia-reperfusion injury. *Asian Cardiovasc Thorac Ann* 2007; 15: 463-467.
- 19) MATÉS JM, PÉREZ-GÓMEZ C, NÚÑEZ DE CASTRO I, ASENJO M, MÁRQUEZ J. Glutamine and its relationship with intracellular redox status, oxidative stress and cell proliferation/death. *Int J Biochem Cell Biol* 2002; 34: 439-458.
- 20) FULLER W, PARMAR V, EATON P, BELL JR, SHATTOCK MJ. Cardiac ischemia causes inhibition of the Na/K ATPase by a labile cytosolic compound whose production is linked to oxidant stress. *Cardiovasc Res* 2003; 57: 1044-1051.
- 21) LOPASCHUK GD, USSHER JR, FOLMES CD, JASWAL JS, STANLEY WC. Myocardial fatty acid metabolism in health and disease. *Physiol Rev* 2010; 90: 207-258.