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MiR-124 promotes ischemia-reperfusion induced cardiomyocyte apoptosis by targeting sphingosine kinase 1

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Abstract. – OBJECTIVE: Ischemia-reperfusion (IR) injury of cardiomyocyte contributes to the cardiac dysfunction following myocardial infarction (MI). MiRNAs have been found to play a vital role in the pathogenesis of myocardial IR injury. In this study, the role of miR-124 in the myocardial IR injury was examined.

MATERIALS AND METHODS: Myocardial ischemia rats' model was established to examine the expression level of miR-124. The primary rat cardiomyocytes were isolated to determine in vitro oxygen-glucose deprivation and reoxygenation model. The expression of miR-124 was analyzed by quantitative Real Time-PCR (qRT-PCR). The cell viability was assessed by Cell Counting Kit-8 (CCK-8) and LDH release assay. Cell apoptosis was evaluated by flow cytometry. The expression of cleaved caspase-3, Bcl-2, and Bax was assessed by Western blot. The expression of miR-124 was manipulated by transfection with miR-124 mimics and inhibitors. The Luciferase activity assay was performed to verify whether SphK1 was a direct target of miR-124. The mRNA and protein expression of SphK1 was assessed by qRT-PCR and Western blot. The ectopic expression of SphK1 was achieved by transfecting with overexpressing plasmid.

RESULTS: Our results showed that miR-124 expression was elevated in the infarct zone. The expression level of miR-124 following OGD/R was also significantly increased. Our results showed that miR-124 mimics could enhance OGD/R-induced miR-124 increase while miR-124 inhibitors performed the opposite effect. Our findings also revealed that miR-124 mimics could augment OGD/R-induced cell death and apoptosis, while miR-124 inhibitors expressed the opposite effect. SphK1 was proposed to be a direct target of miR-124. SphK1 overexpression could abrogate the augmenting activities of miR-124 on OGD/R-induced cell injury.

CONCLUSIONS: In the pathogenesis of MI, miR-124 promotes myocardial IR-induced cell death and apoptosis in cardiomyocyte by targeting SphK1.

Key Words:
Myocardial infarction, Cardiomyocyte, Apoptosis, MiR-124, SphK1.

Introduction

Ischemic heart disease (IHD) ranks among the leading causes of human morbidity and mortality in both developing and developed countries[1]. Notably, the acute myocardial infarction (AMI) is a significant contributor to death[2]. At the current stage, the mainstream therapeutic regimen for AMI is to restore the blood flow by using thrombolysis agents or performing surgeries, including percutaneous transluminal coronary angioplasty and coronary artery bypass surgery[3]. Although timely restoration of blood flow significantly improves the survival of patients, reperfusion in previous ischemic myocardium itself can cause additional damage to the myocardium, including cardiomyocyte dysfunction and death[3]. It has been found that myocardial ischemia/reperfusion (I/R) injury involves a series of complicated factors. Among these factors, apoptosis of cardiomyocyte is a significant contributor to cardiac dysfunction following IR[4]. Therefore, a better understanding of the underlying mechanisms of cardiomyocyte apoptosis during myocardial IR injury will help to develop a new therapeutic regimen.

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs with 22 nucleotides (nt), which can regulate the gene expression after transcription. Some important biological processes, including proliferation, differentiation, apoptosis, and autophagy, are regulated by miRNAs[5]. It has been well established that miRNAs function by inhibiting the translation following the formation of the RNA-interference-induced silencing complex (RISC) or promoting the cleavage of mRNA

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of target genes[6]. The role of miRNAs in the development of normal heart has also been evidenced[7]. Moreover, miRNAs have been found to play a role in the pathological processes of a variety of cardiovascular disorders, including heart failure, cardiac fibrosis, arrhythmia, and coronary heart disease[7]. In the context of myocardial IR injury, a number of miRNAs are upregulated or downregulated following myocardial IR[7]. MiRNAs can function as biomarkers for MI and predict the prognosis of patients with AMI[7]. For instance, the levels of miR-1, miR-133a, miR-133b, and miR-208b are significantly elevated in the plasma of patients within 12 hours following the onset of STEMI and are correlated with the prognosis of patients[7]. This evidence showed that miRNAs dysregulation contributes to the myocardial IR injury.

MiR-124, firstly discovered in neuronal cells, is involved in a variety of human conditions[8]. In human malignancies, including ovarian cancer[9], breast cancer[10], and gastric adenocarcinoma[11], miR-124 is aberrantly lower expressed and functions as a tumor suppressor. The role of miR-124 in the IR injury was first recognized in the experimental stroke[12]. The data collected from *in vitro* and *in vivo* experiments provide further evidence that miR-124 plays a regulatory role in the apoptotic neuronal death upon cerebral IR injury[4]. The involvement of miR-124 in hepatic IR injury has also been documented[13]. For what it concerns AMI, a significantly elevated level of miR-124 in peripheral blood has been recorded in patients with AMI[12]. In the animal model of MI, miR-124 has also been found to be overexpressed in ischemic cardiac tissue[4]. However, the regulatory role of miR-124 in IR was not fully understood. In this study, our results showed that miR-124 overexpression promoted cardiomyocyte apoptosis by targeting SphK1.

Materials and Methods

Animals and Myocardial IR Procedure

All experimental protocols were operated following the approval of the Institutional Animal Care and Use Committee of the Zhangjiagang First People's Hospital and the Ethical Committee of our institution. The Sprague-Dawley (SD) rats of either sex (250-300 g; Guilin Medical Laboratory Animal Center, Guangxi, China) were fasted overnight, then the corresponding EGCG (10 mg/kg or 20 mg/kg) and Z-VAD-FMK (1 mg/kg) were injected into sublingual veins 30 min before ischemia. The rats were anesthetized with 10% Chloral hydrate at a dose of 3 ml/kg (i.p.). The respiration was maintained with a fraction of inspired oxygen of 0.80. Then, the left anterior-descending (LAD) was ligated with a 4-0 silk suture (except for the IR control group). After 30 min of ischemia, the ligation was loosened for 12 h. Blood samples were collected and centrifuged at 3600 g for 20 min to harvest the sera. The left ventricle of animals was immediately excised and stored at -80°C for the analyses described below.

Neonatal Rat Ventricular Cardiomyocyte Culture

Primary neonatal rat cardiomyocytes were isolated from newborn Sprague-Dawley rat heart ventricles by trypsin solution. Briefly, the hearts of newborn SD rats were aseptically removed after the rats were decapitated. Moreover, the hearts were minced in the serum-free Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, UT, USA) and digested in 0.25% trypsin solution. The cell suspension was centrifuged at 2500 rpm for 3 min and re-suspended in DMEM medium containing 10% fetal bovine serum. Then, the non-adherent cardiomyocytes were plated into culture flasks (non-coated). Cardiomyocytes were cultured under a condition of 5% CO_2 at 37°C.

Transfection of MiR-124 Mimics and MiR-124 Inhibitors

The cells were incubated in 2 mL DMEM at 1×10^5 cells/ well in a 6-well plate (Greiner, Bahlingen, Germany) containing 10% FBS (Gibco, Grand Island, NY, USA) and antibiotics (Sigma-Aldrich, St Louis, MO, USA). The miR-124 mimics and miR-124 inhibitors were purchased from Applied Biosystems (Foster City, CA, USA). These cells were transfected into cells with FuGENE HD6 (Roche China, Shanghai, China) after they reached 80% confluence following the manufacturer's protocols.

Quantitative Real Time-PCR (qRT-PCR)

Quantitative Real Time-PCR (qRT-PCR) was conducted for detecting miR-124 and SphK1 mRNA expression. cDNA was synthesized from the total RNA using the TaqMan miRNA Reverse Transcriptase Kit (ABI, Foster City, CA, USA) to detect the miRNA levels. Applied Biosystems ViiA 7 Sequence Detection System (ABI ViiA 7 SDS; Foster City, CA, USA) was used to perform qRT-PCR based on the manufacturer's protocols. MiR-124 expression was normalized to snRNA

U6. cDNA was synthesized from the total RNA to monitor SphK1 mRNA level by a High-Capacity cDNA Reverse Transcriptase Kit (ABI, Foster City, CA, USA), using primers as previously described[14]. The sequence for the forward primer was 5'-CTGTCACCCATGAACCTGCT-3'. The sequence for the reverse primer was 5'-TACAGGGAGGTAGGCCAGTC-3'. All reactions were performed in triplicate. At the same time, the relative expression was analyzed with the comparative cycle threshold method ($2^{-\Delta\Delta CT}$) according to the manufacturer's guidelines.

Cell Viability Measurement

Cell viability was measured using the Cell Counting Kit-8 (CCK-8). The cells were seed in 96-well plates at 5×10^3 cells /well. The cells were cultured with 10 μL CCK-8 solution for 2-3 h at 37°C. We detected 450 nm absorbance with a microplate reader (Tecan Infinite f200 Pro, Tecan Group Ltd, Männedorf, Switzerland).

Apoptosis Assessment Using Flow Cytometry

The apoptosis ratio of cells was determined according to the procedure used in previous studies. Briefly, the cells were stained with Annexin V-FITC/PI for the indicated time. Then, the cell apoptosis was examined and quantified by flow cytometer (Beckman Coulter Inc., Miami, FL, USA).

Luciferase Targeting Assay

The TargetScan predicted miR-124 targets. The 3' untranslated region (UTR) fragment of SphK1 mRNA containing the putative SphK1 binding sequence was cloned into a Firefly Luciferase reporter construct pGL3 (Promega, Madison, WI, USA) and transfected into the cells using PureFection transfection reagent. There was a target fragment of the 3' UTR mutation of SphK1 without the structure of the putative miR-124 binding sequence and was used for mutation control. After 48 h, the transfected cells were obtained and analyzed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The results were obtained from the three independent experiments and were exhibited in triplicate.

Western Blot

The Western blot analysis was performed according to the standard procedure. The total proteins were isolated and subjected to SDS-PAGE. The blots were then transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% skim milk powder. The membranes were incubated with primary antibodies at 4°C for overnight. Also, goat anti-rabbit IgG-HRP was used as secondary antibodies (Beyotime, Shanghai, China). β-actin was used as an internal reference. The proteins were evaluated using a chemiluminescent substrate (KPL, Guildford, UK) and calculated using BandScan software (Glyko, Novato, CA, USA).

SphK1 Overexpressing

SphK1 expression plasmid (and the empty vector) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used to transfect the cardiomyocytes by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The culture medium was changed 48 hours after transfection, and the harvested cells were used for the appropriate assay.

Statistical Analysis

The Statistical Product and Service Solution (SPSS 20; IBM Corp., Armonk, NY, USA) was used as the analysis software for the experiment, and the data were expressed as mean ± standard deviation (SD). The Student's *t*-test or One-Way ANOVA followed by Dunnett's *t*-test analyzed the differences between two or more groups. $p<0.05$ was statistically significant.

Results

MiR-124 Expression is Elevated in Animal and Cellular Model of MI

Before we examined the role of miR-124 in MI, the level of miR-124 in the infarct zone and remote zone in animal MI model was compared. As shown in Figure 1A, miR-124 in the infarct zone was significantly higher relative to the remote zone, implying the role of miR-124 in myocardial injury. Then, the OGD/R cellular model was established to verify the role of miR-124 in the myocardial IR injury. As shown in Figure 1B, the expression level of miR-124 was significantly elevated in cardiomyocyte following OGD/R. Then, the expression of miR-124 was manipulated by transfecting miR-124 mimics and inhibitors, respectively. As shown in Figures 1C and 1D, the expression level of miR-124 was effectively elevated by miR-124 mimics while miR-124 inhibitors effectively repressed the expression of miR-124. Then, the effect of OGD/R procedure on

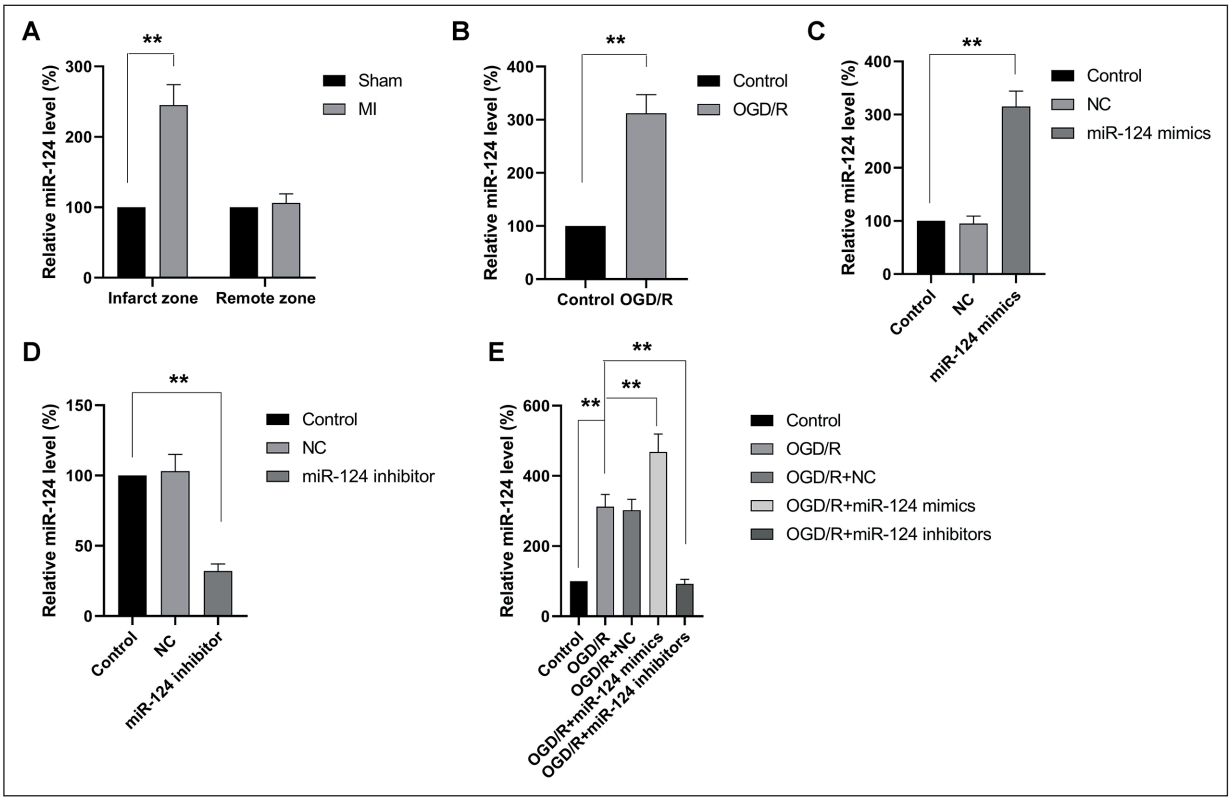

Figure 1. MiR-124 expression is elevated in *in vivo* and *in vitro* IR models. ***A,*** The level of miR-124 was higher in the infarct zone compared with the remote zone. ***B,*** The level of miR-124 was higher following OGD/R procedure. **C,** The expression of miR-124 was elevated following transfection with miR-124 mimics. ***D,*** The expression of miR-124 was repressed following transfection with miR-124 inhibitor. ***E,*** The expression of miR-124 following OGD/R procedure was further elevated by miR-124 mimics while attenuated by miR-124 inhibitor. $^{**}p<0.01$.

the expression level of miR-124 was examined. As shown in Figure 1E, the expression of miR-124 was significantly elevated in cardiomyocyte following OGD/R procedure. Besides, our results showed that miR-124 mimics further increased the expression level of miR-124 in cells while miR-124 inhibitors abolished the OGD/R-induced increase in miR-124 levels (Figure 1E).

MiR-124 Modulates OGD/R-Induced Cell Death and Promotes Cell Apoptosis

Next, the change in the proportion of viable cells was examined using the CCK-8 assay. As shown in Figure 2A, OGD/R caused a significant loss in the number of viable cells and transfection with miR-124 mimics augmented the loss in some viable cells. The cell injury was also examined by LDH release assay. As shown in Figure 2B, OGD/R significantly increased the LDH release from cells. Also, miR-124 mimics enhanced LDH release from cardiomyocyte. Meanwhile, our results showed that OGD/R significantly elevated the proportion of cell apoptosis (Figures 2C and 2D). To further examine the role of miR-124, the cardiomyocytes were transfected with miR-124 inhibitors before subjected to OGD/R. As shown in Figure 3A, miR-124 inhibitors effectively rescued OGD/R-induced cell death in cardiomyocytes. The protective activities of miR-124 inhibitor against OGD/R-induced cell death were also confirmed by the fact that miR-124 inhibitors reduced LDH release (Figure 3B). Moreover, the cardiomyocyte apoptosis induced by OGD/R was also reduced by miR-124 inhibitors (Figures 3C and 3D).

SphK1 is a Direct Target of MiR-124

The TargetScan performed the prediction of miR-124 targets. We chose to focus on SphK1 as the mediator of the regulatory activities of miR-124. To verify that SphK1 was a direct target of miR-124, the binding region on SphK1 was mutated as presented in Figure 4A. Then, the Luciferase activity assay was used to examine whether

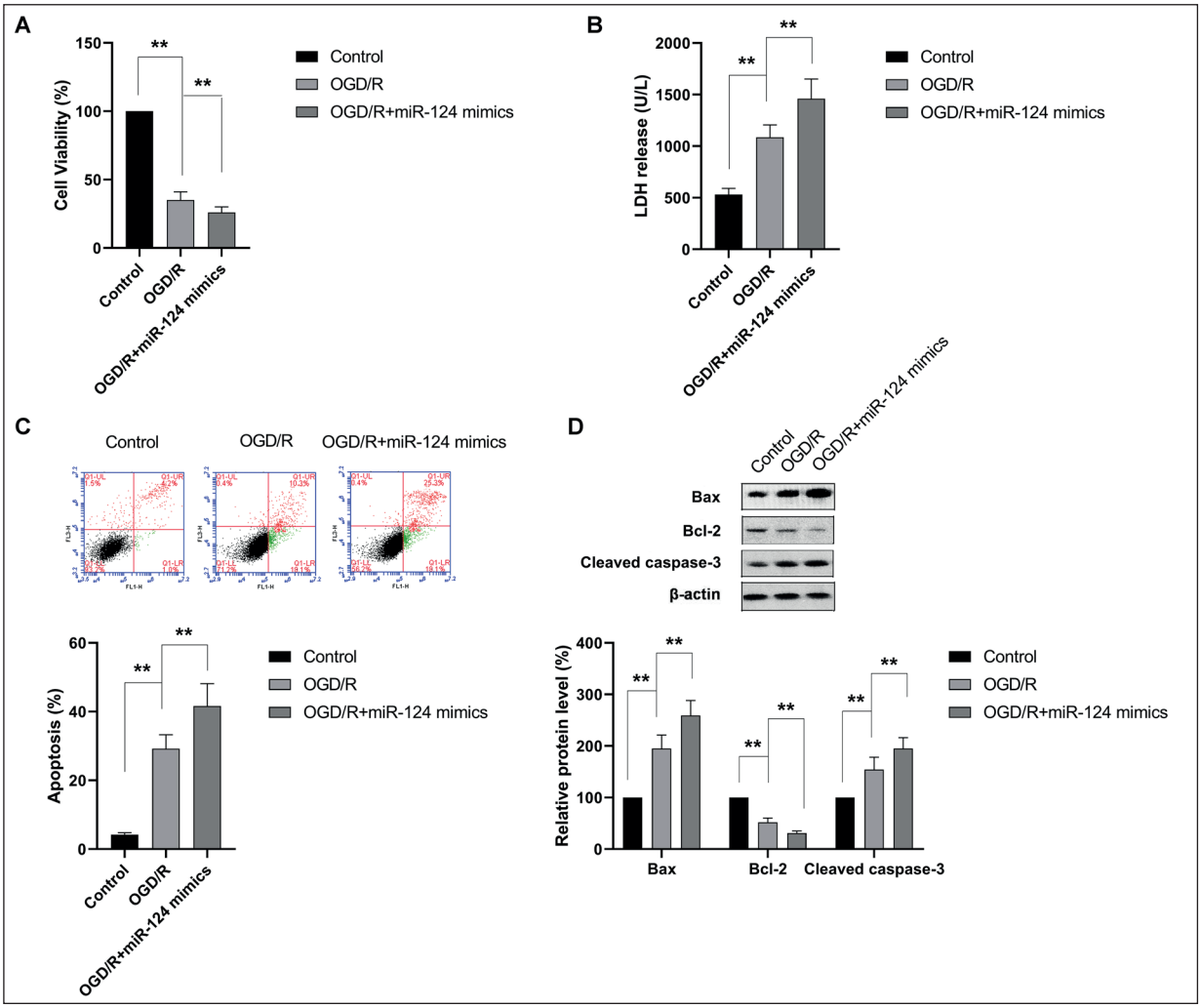

Figure 2. MiR-124 augments OGD/R-induced cell death and apoptosis. ***A,*** OGD/R effectively reduced the cell viability and miR-124 enhanced its effect. ***B,*** OGD/R effectively promoted LDH release and miR-124 enhanced its effect. ***C,*** OGD/R effectively promoted cell apoptosis. ***D,*** OGD/R effectively promoted cleavage of caspase-3, Bcl-2, and Bax, and miR-124 enhanced its effect. $^{**}p<0.01$.

miR-124 was directly able to bind to the 3'UTR of SphK1. As shown in Figure 4B, compared with the mutant sequence, the Luciferase activities were markedly reduced following transfection with miR-124 mimic, indicating that SphK1 was a direct target of miR-124. Then, the cardiomyocytes were transfected with miR-124 mimics or miR-124 inhibitors, and the SphK1 mRNA expression was assessed using qRT-PCR. The results from qRT-PCR exhibited that the transfection with miR-124 mimic could reduce the SphK1 mRNA expression by more than half (Figure 4C). Following transfection with miR-124 inhibitors, the SphK1 mRNA expression was increased by about 1.5 folds (Figure 4C). Correspondingly, miR-124 mimics markedly suppressed the protein expression of SphK1 while miR-124 inhibitors were able to significantly enhance the protein expression of SphK1 (Figure 4D). These results showed that miR-124 was able to regulate the expression of SphK1.

Ectopic Expression of SphK1 Reverses the Augmenting Effect of miR-124 on OGD/R-Induced Cell Injury

The next part of our study was to examine whether miR-124 regulated OGD/R-induced cell injury by targeting SphK1. Firstly, the mRNA and protein expression of SphK1 were examined following transfection with miR-124 mimics or inhibitors and underwent OGD/R procedure. As shown in Figures 5A and 5B, miR-124 mimics

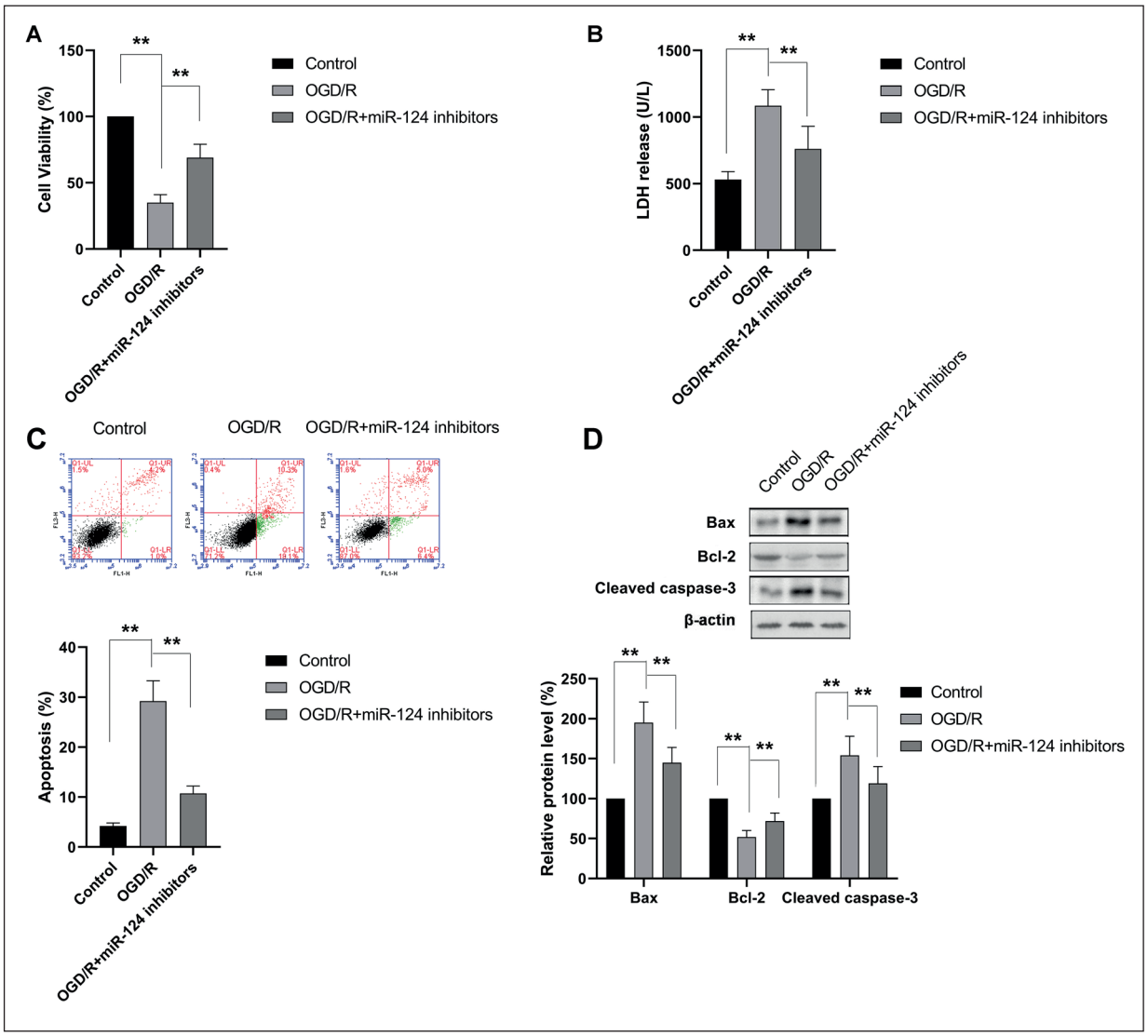

Figure 3. MiR-124 inhibitor attenuates OGR/R-induced cell death and apoptosis. ***A,*** MiR-124 inhibitor rescued OGD/R-induced cell death. ***B,*** MiR-124 inhibitor attenuated OGD/R-induced LDH release. ***C,*** MiR-124 inhibitor protected OGD/R-induced apoptosis. ***D,*** MiR-124 inhibitor compromised OGD/R-induced caspase-3 cleavage, Bcl-2 downregulation, and Bax upregulation. $^{**}p<0.01$.

was able to decrease both mRNA and protein expression of SphK1 following OGD/R. By contrast, miR-124 inhibitors were able to reverse the repressing effect of OGD/R on SphK1 expression (Figures 5A and 5B). Then, the cardiomyocytes were transfected with SphK1 overexpressing vector. As shown in Figures 5C and 5D, both mRNA and the protein expression of SphK1 were all effectively elevated in cardiomyocytes. The effect of SphK1 overexpressing the vector on OGD/R was examined. As shown in Figure 5E, the overexpression of SphK1 significantly abolished the augmenting activities of miR-124 mimics on OGD/R-induced cell death. Correspondingly, the enhancing effect of miR-124 mimics on LDH release was also compromised by the SphK1 overexpression (Figure 5F). Meanwhile, our results showed that OGD/R promoted cell apoptosis, which was significantly enhanced by miR-124 mimics and attenuated by the SphK1 overexpression (Figures 5G and 5H).

Discussions

MiRNAs have been identified in more than 160 organisms. Intriguingly, miRNAs have been found to play a crucial regulatory role in a variety

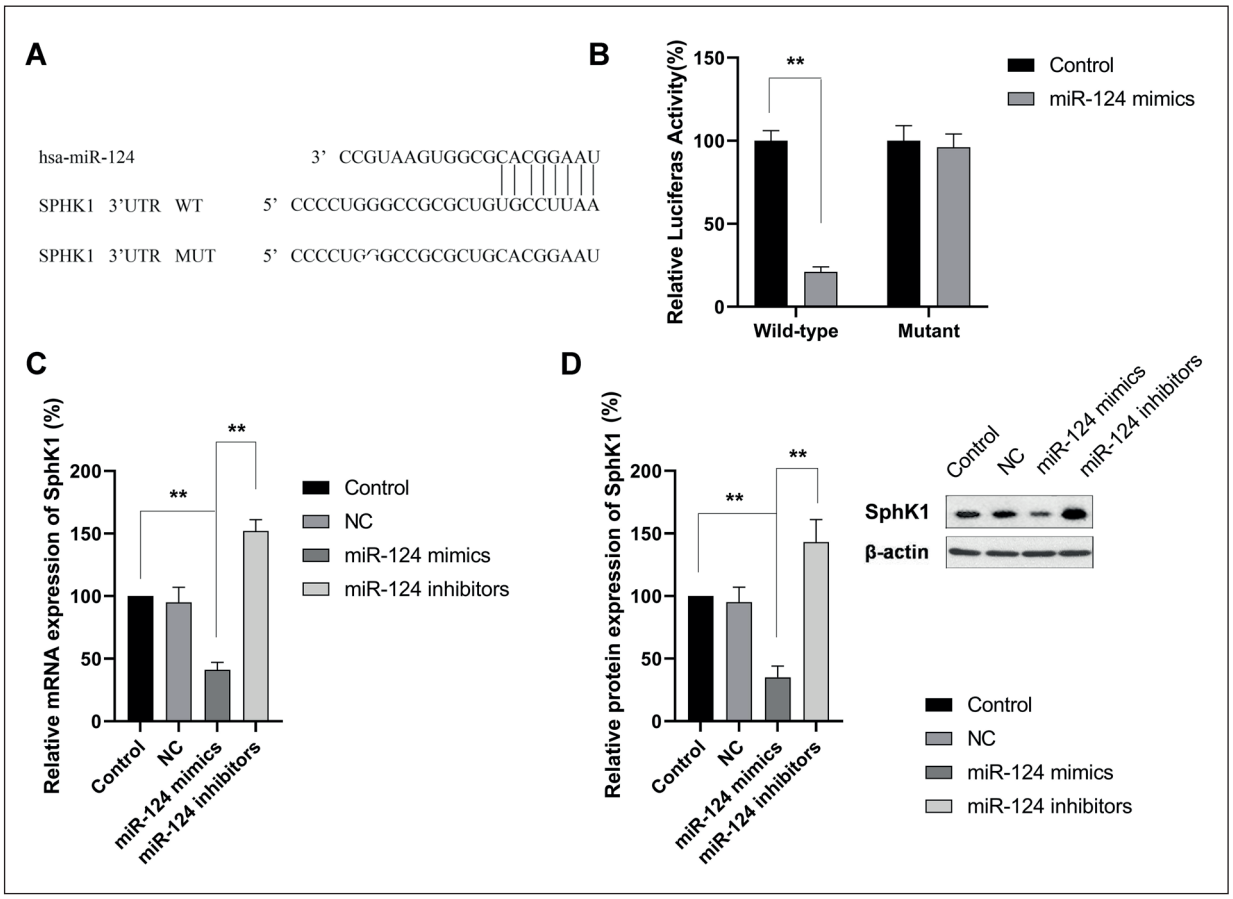

Figure 4. SphK1 is identified as a direct target of miR-124. ***A,*** The sequence of miR-124, the binding region of SphK1 with miR-124, and the mutated sequence. ***B,*** The Luciferase activity assay showed that SphK1 was a direct target of miR-124. ***C,*** Whether miR-124 was able to affect the mRNA expression of SphK1 was assessed by qRT-PCR. ***D,*** Whether miR-124 was able to affect the protein expression of SphK1 was assessed by the Western blot. $^{**}p<0.01$.

of cellular biological function and human disorders[15]. As highly conserved component in either exonic or intronic regions of the genome, miRNAs were converted into mature miRNAs following transcription[15]. After insertion in RNA-induced silencing complex (RISC), miRNAs bind to a specific target sequence often located in the 3'UTR of messenger RNAs (mRNAs) in a partly complementary way. The binding between miRNA and 3'UTR of mRNA results in either translational repression or degradation of the mRNA and eventually, inhibition of protein expression[16-18]. In the context of myocardial IR injury, several miRNAs have also been found to function as positive and negative regulators. As early as in 2011, miR-1 was found to be downregulated in rats model following the coronary artery occlusion and reperfusion[19]. The anti-apoptotic role of miR-1 against IR-induced cardiomyocyte injury was attributed to its direct regulation of Bcl-2[19]. The role of miR-21 in myocardial IR injury has also been well established. It has been documented that miR-21 was upregulated in the area of myocardial infarction and played a regulatory role by targeting PTEN and related downstream signaling[20]. In this study, our experiments were aimed to explore the role of miR-124 in cardiac IR injury. Li et al[21] by using the bioinformatics network analysis, identified miR-124 as a critical modulator of the cardiac IR injury. Moreover, the negative regulation of miR-124 alleviates hypoxia injury in H9c2 cells[22]. In agreement with these previous studies, our results showed that miR-124 was upregulated in the infarct zone. Also, miR-124 mimics were found to augment OGD/R-induced cell death and apoptosis in cardiomyocytes. MiR-124 inhibitors were able to protect the cardiomyocytes from OGD/R-induced

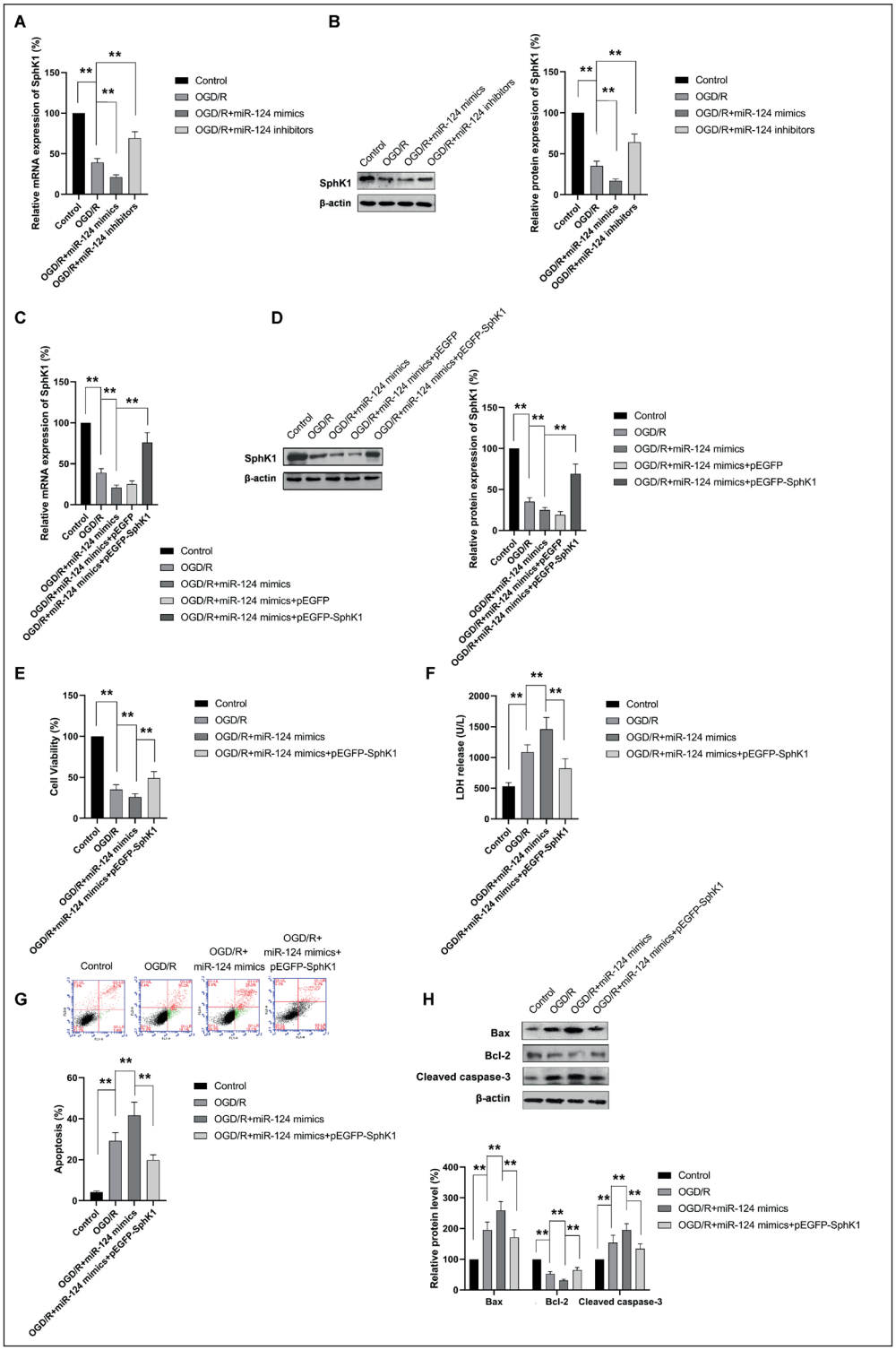

Figure 5. Ectopic SphK1 expression abolishes the augmenting activities of miR-124 on OGD/R-induced cell death and apoptosis. ***A,*** MiR-124 mimics or inhibitors were able to regulate the mRNA expression of SphK1 following OGD/R. ***B,*** MiR-124 mimics or inhibitors were able to regulate the protein expression of SphK1 following OGD/R. ***C,*** The transfection of SphK1 overexpressing the vector elevated the mRNA expression of SphK1. ***D,*** The transfection of SphK1 overexpressing the vector elevated the protein expression of SphK1. ***E,*** Ectopic expression of SphK1 reversed the augmenting effect of miR-124 mimic on OGD/R-induced cell death. ***F,*** Ectopic expression of SphK1 reversed the augmenting effect of miR-124 mimic on OGD/R-induced cell death. ***G,*** Ectopic expression of SphK1 reversed the augmenting effect of miR-124 mimic on OGD/R-induced apoptosis. ***H,*** Ectopic expression of SphK1 reversed the augmenting effect of miR-124 mimic on OGD/R-induced caspase-3 cleavage, Bcl-2 downregulation, and Bax upregulation. $^{**}p<0.01$.

cell death and apoptosis. Moreover, our results showed that miR-124 functioned in myocardial IR injury by direct targeting SphK1. Sphingosine 1-phosphate (S1P), which is a member of sphingolipids, has been found involved in a variety of cellular functions[23]. SphK1 is a critical enzyme that plays the catalyzing role in the conversion from sphingosine to S1P by phosphorylation[24]. Given the crucial role of S1P in cellular activities, the activation of SphK1 has been found to correlate with various human disorders, including cardiovascular conditions[24]. It has been found that the cardiac SphK1 was significantly upregulated following MI and that PF543 (an SphK1 inhibitor) could ameliorate the cardiac remodeling and dysfunction in rats following MI procedure[25]. Zak et al[26] also confirmed the role of SphK1 in cardiac remodeling. In the context of myocardial IR injury, it has been found that the specific activator of SphK1 was able to attenuate the OGD/R-induced cell death[27]. In our study, we found that the overexpression of SphK1 attenuated OGD/R-promoted cell death and apoptosis, further supporting the role of SphK1 as a prosurvival factor against OGD/R cell injury.

Conclusions

Our results showed that miR-124 augmented ischemia/reperfusion injury in cardiomyocyte by directly targeting SphK1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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