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Original Article

Mir-23a inhibition attenuates ischemic/reperfusion-induced myocardial apoptosis by targeting XIAP

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Abstract: MicroRNAs are a group of single-strand, non-coding RNAs that inhibit the translation of protein-coding genes. Recent studies indicated that miRNAs are broadly involved in the development of cardiovascular diseases, including arrhythmia, hypertrophy, heart failure and cardiac injury. In this study, we report that miR-23a, a tumor suppressor, acts as an apoptotic promoter in rats undergoing ischemic/reperfusion. In rats subjected to ischemic/reperfusion injury, the expression of miR-23a in heart tissue was upregulated significantly. The infarct area and the apoptosis rate also increased. In contrast, knockdown of miR-23a by tail injection of antagomir-23a attenuated the ischemic/reperfusion injury. Moreover, we used Western blots to determine that miR-23a targeted XIAP to influence the expression of caspase and the NFkB pathway. In summary, miR-23a was shown to be part of a novel regulatory pathway that contributed to ischemic/reperfusion injury.

Keywords: miR-23a, ischemic/reperfusion, XIAP

Introduction

MicroRNAs (miRNAs) are small non-coding single-strand RNAs approximately 21 to 25 nucleotides (nt) long that inhibit the translation of protein-coding genes by targeting mRNAs with RNA-induced silencing complexes (RISC), which cause translational repression or degradation of the mRNA [1, 2]. MicroRNAs play important roles in various biological processes, such as development, differentiation, proliferation, and apoptosis.

There are approximately 500 known mammalian miRNAs, and each miRNA may regulate hundreds of protein-coding genes, which indicates miRNA probably affects entire signal pathways [3]. Myocardial infarction is a leading cause of death worldwide and leads to apoptosis, cardiac fibrosis, inflammation and pathological remodeling [4]. The current typical treatment for myocardial ischemia is rapid reperfusion, which can attenuate myocardial infarction, reduce cardiomyocyte apoptosis and restore contractile dysfunction. However, the subsequent ischemia-reperfusion injury may still cause extensive cardiomyocyte death and acute heart dysfunction [5]. Numerous studies have suggested miRNAs are involved in the regulation of myocardial ischemia reperfusion (I/R) injury [6]. Inhibition of miR-29a or miR-29c by antisense inhibitors reduced cell death induced by SIR injury [7]. Knockdown of endogenous miR-320 provides protection against I/R induced cardiomyocyte death and apoptosis by targeting Hsp20 [8]. Mir-1 plays an important role in the regulation of cardiomyocyte apoptosis, which is involved in posttranscriptional repression of Bcl-2 [9]. MiR-494 leads to cardioprotective effects against I/R-induced injury by targeting both pro-apoptotic and anti-apoptotic proteins that activate the Akt pathway [10]. MiR-204 may inhibit autophagy induced by ischemia-reperfusion during hypoxia-reoxygenation by regulating LC3 [11]. Na_2S induces cardioprotective effects through miR-2-dependent attenuation of ischemic and inflammatory injury in cardiomyocytes [12]. Complete loss of miR-

92a in mice attenuated infarct-related myocardial dysfunction to a larger extent than cardiomyocyte-specific miR-92a deletion [13]. MiR-7a/b is sensitive to I/R injury and protects myocardial cells against I/R-induced apoptosis by negatively regulating PARP expression in vivo and in vitro [14]. MiR-21 plays a protective role in myocardial apoptosis through the PTEN/Akt signaling pathway [15]. Mir-23a, as a tumor suppressor, reduces proliferation and increases apoptosis of tumor cells such as prostate cancer, neuroblastoma, hepatocellular cancer, and gastric cancer [16-19]. However, the role of miR-23a in cardiac I/R injury has yet to be examined in detail. A microarray assay revealed that miR-7 was upregulated in murine hearts after I/R [20]. In this study, we detected expression of miR-23a with real-time PCR and investigated its role in myocardial I/R injury in vivo and examined possible targets for miR-23a.

Materials and methods

I/R injury model in rats

The animal experiments conformed to the Animal Management Rules of the Chinese Ministry of Health (document No. 55, 2001). Male Wister rats (260-280 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). After anesthesia, the animals were placed in the supine position and the limbs were placed subcutaneously to record an electrocardiogram (ECG). An incision was made between the third and fourth ribs to expose the left anterior descending coronary artery (LAD) followed by a ligation with 4-0 silk suture for 30 minutes. Ischemia was achieved when cyanotic left anterior ventricular wall and local wall distension were observed as well as elevated ST segments and peak T waves on the electrocardiogram.

After occlusion, the knot was relaxed and the heart was reperfused for 2 hours. Following reperfusion for 120 min, the rat hearts were harvested. Sham surgery animals underwent the entire surgical procedure without occlusion of the LAD.

TUNEL assay

Heart samples were sliced horizontally, immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin, and cut at a 5 mm thickness. Apoptosis was detected using a TUNEL assay according to manufacturer's instructions (TUNEL fluorescence FITC kit, Roche, Indianapolis, IN, USA). After TUNEL staining, the heart sections were immerged into DAPI (Sigma-Aldrich) solution to stain cell nuclei. The number of apoptotic cardiac myocytes was calculated as the percentage of the total cells counted.

In vivo administration of antagomiR-23a

Chemically modified antisense oligonucleotides (antagomiR) have been used to inhibit microRNA expression in vivo and have successfully knocked out target miRNAs [10, 21]. We treated rats with antagomiR-23a in three consecutive daily tail vein injections of cholesterol-modified antagomiR-23a (100 mg/kg).

Quantitative PCR assay

Total RNA was extracted from the rat myocardium with Trizol (Invitrogen, USA) according to the manufacturer's instructions. Then, real-time quantitative PCR was performed to quantify the expression level of miR-23a and mRNA of XIAP with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The qRT-PCR was performed on an IQ50 (Bio-Rad) for 38 cycles. The comparative Ct (threshold cycle) value was used for measurement with U6 and β-actin serving as internal controls.

Measurement of myocardial infarct size

At the end of reperfusion, the LAD coronary artery was reoccluded and Evans blue dye solution (3 ml, 2% wt/vol) was injected into the left ventricle to identify ischemic (area at risk) and non-ischemic (area not at risk) regions. The hearts were refrigerated at -4°C overnight and sliced horizontally to yield five slices that were approximately 2 mm thick followed by incubation in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) for 15 min at 37°C. The infarct area was identified as the unstained region, whereas the living tissues were stained red. The area at risk (AAR; ischemic area) and the infarct area were measured using Image ProPlus 5.0 software (Media Cybernetics, USA).

Western blot

The total protein was extracted from the tissue samples with a total protein extraction kit.

Then, we measured the density of the samples according to the instructions included with the BCA protein fluorometric kit. Following extraction, 40 μg total protein was separated by 10% (w/v) SDS-PAGE, transferred onto a nitrocellulose membrane and blocked with 5% (w/v) nonfat dried milk. The membranes were probed with the primary antibody followed by a HRP-labeled secondary antibody. Next, the signal was detected by an ECL Western blotting system and quantified with Image-Pro Plus 6.0. The signal was normalized to the internal standard GAPDH.

Immunohistochemistry

The myocardial tissues were fixed in 10% formalin, embedded in paraffin, cut into 5 μm sections, and mounted on slides. After baking, dewaxing and a wash with PBS, paraffin sections were put into EDTA solution and received microwave repair heating for 14 min at 96°C. Next, the samples were washed in blocking buffer containing 5% goat serum for 30 minutes at room temperature. The primary antibody was diluted 1:200 in blocking buffer and incubated overnight at 4°C. A secondary goat anti-rabbit antibody was added and incubated for 1 hour at room temperature. The sections were incubated in 3,3 N-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. Digital images of the stained sections were acquired using a Nikon ECLIPSE E100 microscope with a digital camera system.

Results

Overexpression of miR-23a in myocardial I/R rats

It has been reported that a group of miRNAs, including miR-15b, miR-21, miR-23a and miR-499, are abnormally expressed in response to myocardial I/R injury. Among these miRNAs, the expression of miR-23a was downregulated in ischemic post-conditioning models, which indicates miR-23a may be an important regulator of myocardial I/R injury. To investigate the potential role of miR-23a involved in I/R injury, quantitative real-time PCR was used to detect the expression levels of miR-23a in myocardial I/R rats. The results showed miR-23a was significantly upregulated in the myocardium of I/R rats compared to animals that received the sham surgery (**Figure 1A**).

Effects of miR-23a on infarct size of I/R hearts and the apoptosis of cardiomyocytes

In this study, myocardial I/R rats were treated with antagomir-23a to explore the effects of miR-23a during I/R injury. The infarct size in the hearts was measured with TTC and Trypan blue staining. Histomorphometric analysis revealed that the myocardial infarct size was larger in the I/R group than the antagomir-23a group (**Figure 1D**). The IA/AAR (ischemic area/area at risk) ratio was also significantly reduced in the antagomir-23a group relative to the I/R group. However, the AAR was nearly the same in both groups (**Figure 1D**). To further determine the mechanism of I/R injury that miR-23a may be involved in, a TUNEL assay was performed to detect apoptosis of cardiomyocytes. Few TUNEL positive cells were observed in the sham group, whereas the number of apoptotic cells in the I/R rats increased remarkably compared to the sham group (46.98±3.92%). However, apoptosis of cardiomyocytes in the I/R rats treated with antagomir-23a decreased to 32.65±3.94% (**Figure 1B**, **1C**). These results indicate that myocardial I/R injury may be mediated by upregulation of miR-23a, whereas silencing miR-23a attenuated myocardial infarct and cardiomyocyte apoptosis.

Expression of XIAP mediated by miRNA-23a during myocardial I/R injury

TargetScan was used to analyze target genes of miRNA-23a (**Figure 2A**). X-linked inhibitor of apoptosis protein (XIAP) is an inhibitor of apoptosis proteins. To identify XIAP as the predicted miRNA target, quantitative real-time RT-PCR and Western blotting were used to assess the expression of XIAP in the myocardium of rats. Interestingly, the amounts of XIAP mRNA were similar in the sham I/R and anagomir-23a groups (**Figure 2C**), but the protein expression of XIAP was lower in the I/R and anagomir-23a groups compared to the sham group (**Figure 2B**, **2D**). Specifically, XIAP was significantly upregulated in the anagomir-23a group relative to the I/R group (**Figure 2B**, **2D**). To confirm this observation, immunohistochemical staining with XIAP was performed. The I/R group displayed the least XIAP expression, whereas the anagomir-23a group showed more expression than the I/R group (**Figure 2E**). This observation confirmed that miR-23a participates in regulat-

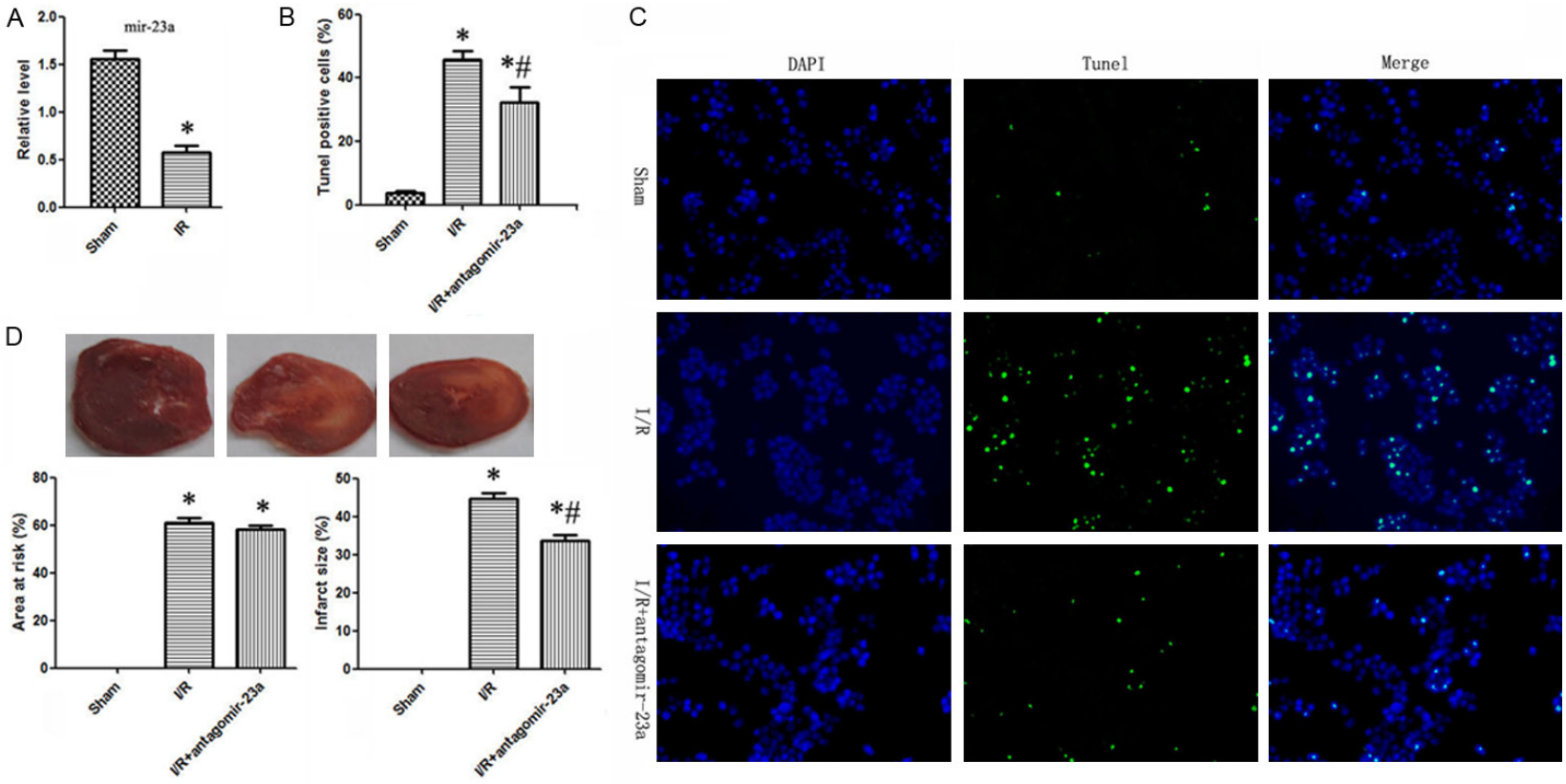

Figure 1. A. qPCR was used to detect expression of miR-23a in myocardial tissue of rats subjected to ischemic/reperfusion. The expression of miR-23a was significantly elevated in the I/R group. B, C. A TUNEL assay was used to evaluate the effect of miR-23a on myocardial apoptosis. The number of apoptotic cells in I/R rats increased remarkably compared to the sham group (46.98±3.92%). Nevertheless, the apoptosis of cardiomyocytes in I/R rats treated with anagomir-23a decreased to 32.65±3.94%. D. The myocardial infarct size was larger in the I/R group compared to the sham group. However, the myocardial infarct size decreased in the antagomir-23a group.

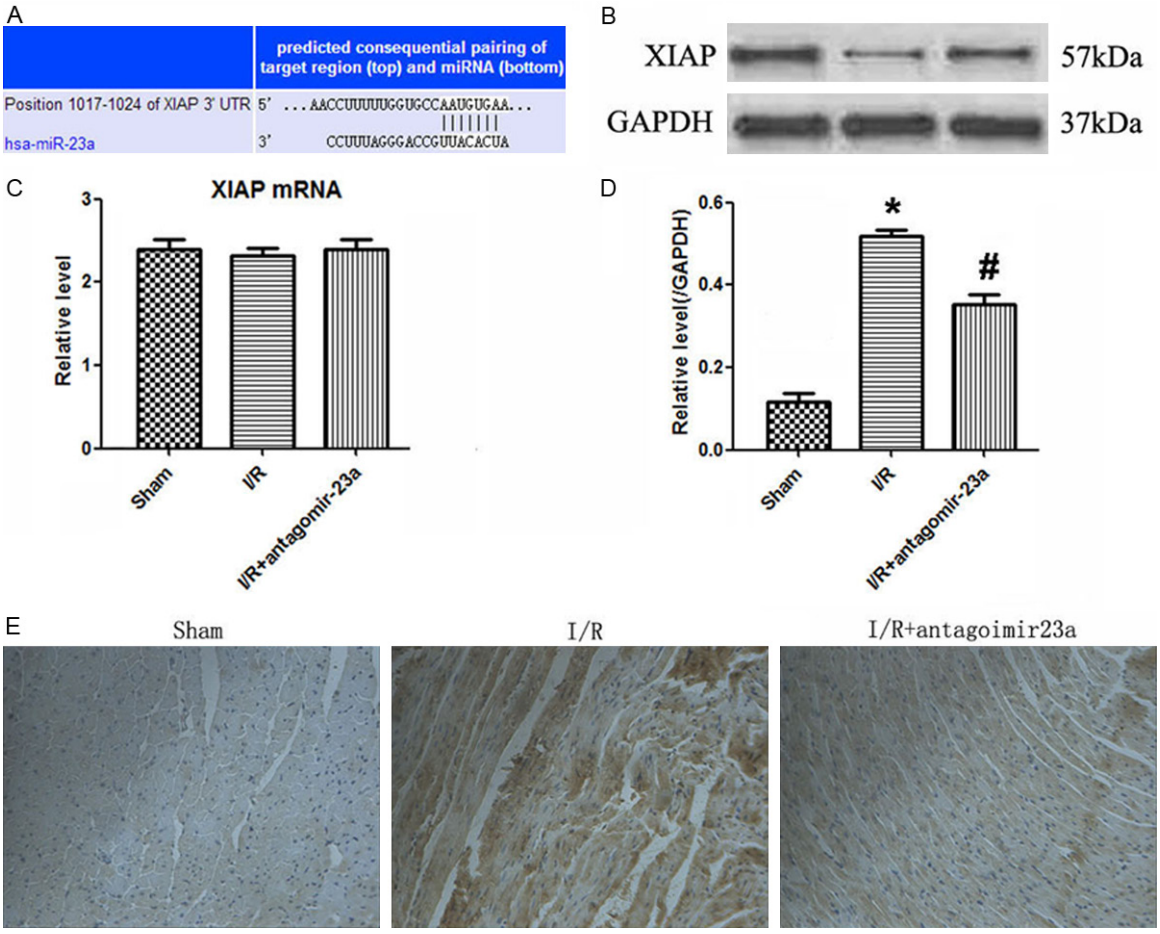

Figure 2. A. XIAP was shown to be the target of miR-23a using TargetScan. B, D. Western blot assays demonstrated that the expression of XIAP in the I/R group was significantly downregulated compared to the sham group. Additionally, antagomir-23a treatment reduced the upregulation. C. qPCR was used to evaluate the mRNA expression of XIAP and no significant differences were detected among the three groups. E. Immunohistochemistry was applied to detect XIAP expression. Similar to the Western blot results, expression of XIAP in the I/R group was significantly downregulated compared to the sham group. Additionally, antagomir-23a treatment reduced the upregulation of XIAP.

ing the protein expression of XIAP at a level other than the mRNA level.

Regulation of NF-κB mediated apoptosis in I/R injured myocardium

Furthermore, we aimed to explore possible pathways for mediating the cardiomyocyte apoptosis during I/R injury. Myocardial apoptosis is a severe consequence of I/R injury. An important function of XIAP is its role in signaling NF-κB activation during apoptosis [22]. To assess whether miRNA-23a induced myocardial apoptosis through XIAP-mediated NF-κB alteration, Western blots were performed to detect activation of cytoplasmic p65 and nuclear p65 of NF-κB as well as degradation of IκB-α. The results showed that expression of cytoplasmic NF-κB was almost the same in the sham, I/R and anagomir-23a groups, but expression of nuclear NF-κB was significantly enhanced in the I/R and anagomir-23a groups compared to the sham groups. Nevertheless, it is worth mentioning that expression of nuclear p65 was remarkably inhibited in the anagomir-23a group compared to the I/R group. Consistent with nuclear p65 results, the degradation of IκB-α significantly increased in the I/R group and decreased in the anagomir-23a group (**Figure 3A**, **3B**). One mechanism by which NF-κB inhibits apoptosis is the regulation of mitochondrial pathway. We investigated some proteins included in this pathway. The results revealed that expression of caspase 3, caspase 9, Bax and cyto-C increased after I/R treatment, whereas these proteins were reduced to lower levels in

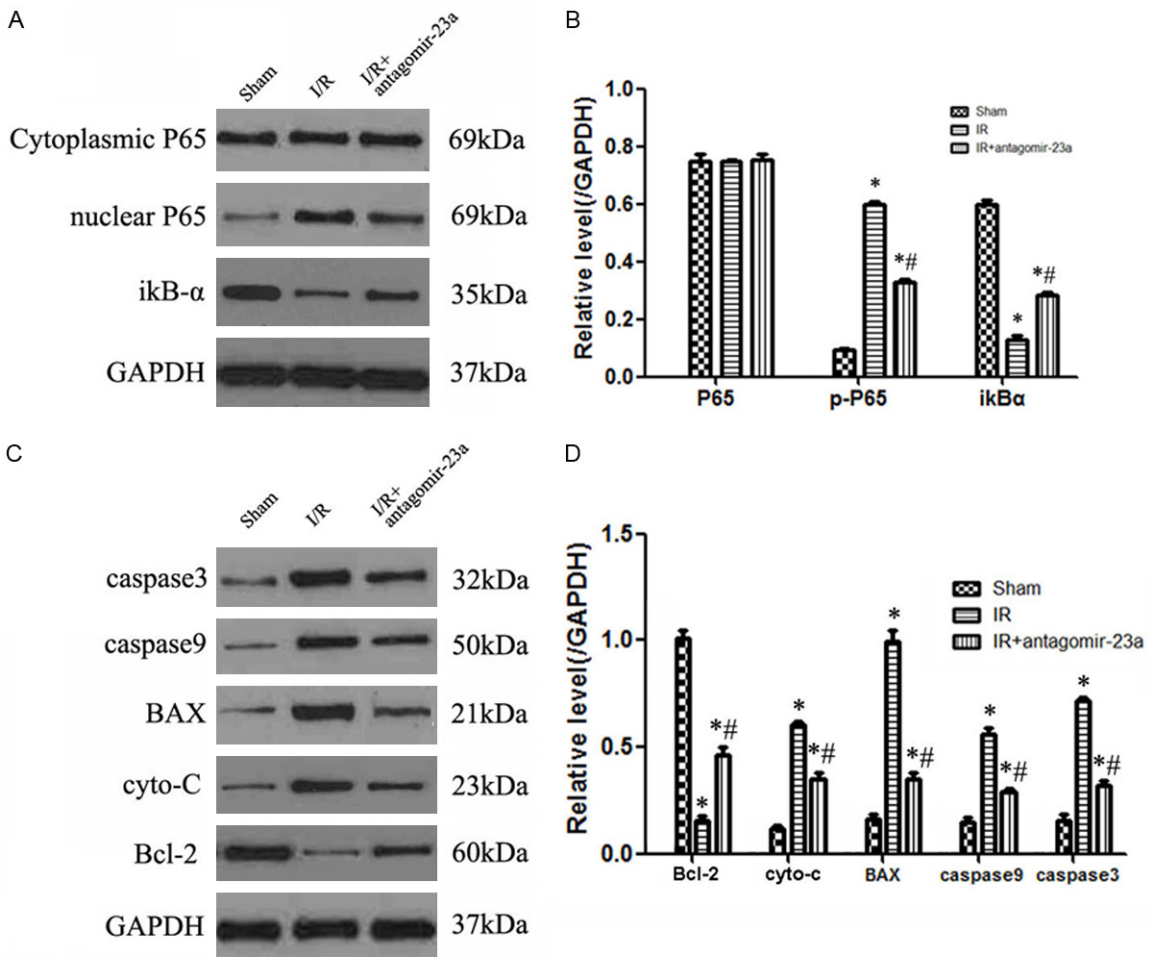

Figure 3. Western blots were used to evaluate different proteins related to apoptosis in the NF-κB pathway. A, B. Cytoplasmic p65, nuclear p65 and IκB-αwere assayed with Western blots. The expression of cytoplasmic NF-κB was similar in the sham, I/R and anagomir-23a groups, but the expression of nuclear NF-κB was significantly enhanced in the I/R and anagomir-23a groups compared to the sham groups. The degradation of IκB-α was significantly increased in the I/R group, whereas degradation of IκB-α decreased in the anagomir-23a group. C, D. Apoptosis-related proteins caspase 3, caspase 9, Bax and cyto-C expression increased after I/R treatment, whereas these proteins were reduced to lower levels in the antagomir-23a rats. In contrast, Bcl-2 was downregulated in the I/R group. However, antagomir-23a could inhibit Bcl-2 downregulation.

the antagomir-23a rats. Additionally, Bcl-2 was downregulated in the I/R group and upregulated in the antagomir-23a treatment group (**Figure 3C**, **3D**).

Discussion

In the last decades, increased attention has been paid to certain microRNAs (miRNAs), including miR-23a. As a tumor suppressor, mir-23a has anticancer functions through different mechanisms. For example, miR-23a can enhance migration and invasion in osteosarcoma cells by downregulating PTEN and SATB1 [23, 24]. Additionally, miR-23a can reduce ER stress in primary hepatocytes and reduce IL-6R expression, which has a critical role in inflammation in prostate cancer [25]. In addition, miR-23a overexpression enhanced the chemoresistance of colorectal cancer cells by directly targeting ABCF1 [26]. Not only a tumor suppressor, miR-23a can also affect NF-κB activity and the expression of downstream NF-κB-target genes that encode proinflammatory mediators, such as IL-6 and TNF-α [27]. Overexpression of miR-23a reduced telomere-bound TRF2 and increased telomere dysfunction-induced foci (TIFs) by targeting the 3' untranslated region (3'UTR) of TRF2 in fibroblast cells [28]. Atrophy-inducing conditions downregulate miR-23a in muscle through mechanisms involving attenuated Cn/NFAT signaling and selective packag-

ing into exosomes [29]. MiR-23a promotes myelination in the central nervous system as well [30].

Previous studies have indicated that a variety of miRNAs are involved in myocardial ischemia. More recently, the roles of miR-23a in cardiovascular disease have been investigated. It has been demonstrated that mir-23a can mediate hypertrophic signals by regulating Foxo3a, NFATc3 and LPA1 [31-33]. In previous studies, a collection of miRNAs, including miR-23a, were abnormally expressed in ischemic mouse hearts in response to I/R injury after ischemic post-conditioning, which indicated that miR-23a may have a protective effect during I/R injury [15]. In this study, we detected mir-23a in rats subjected to ischemia/reperfusion and a sham group using quantitative real-time RT-PCR analysis. As expected, mir-23a was upregulated significantly in the I/R group compared to the sham group. Cardiomyocyte apoptosis critically contributes to cardiac I/R [34]. Accumulating evidence indicates that miRNAs play a critical role in regulating apoptosis not only in tumor cells but also in heart cells. In addition to functioning as a physiological regulator of cell density, excessive apoptotic cell death may be the primary cause of degenerative diseases such as myocardial infarction [35]. With a TUNEL assay, we observed that knockdown of mir-23a using antagomir-23a significantly attenuated I/R-induced myocardial apoptosis. To further explore the mechanism by which miR-23a protected ischemic cardiomyocytes, we searched for potential targets of miR-24 using bioinformatic algorithms, and we predicted miR-23a would bind at the 3' UTR of XIAP. A Western blot assay determined that expression of XIAP as well as caspase 3 and caspase 9 were upregulated in the myocardial tissue of I/R rats. Meanwhile, knockout of mir-23a restrained this upregulation. The IHC results were consistent with the Western blots, which further confirmed that XIAP played a crucial role in myocardial injury in the I/R rats.

XIAP, an inhibitor of apoptosis proteins, is considered to be a potent endogenous inhibitor of apoptosis. MiR-23a played an important role in ischemic sexual dimorphism by directly binding the 3'-UTR of XIAP [36]. XIAP is a family of endogenous caspase inhibitors that share a common baculoviral IAP repeat (BIR) domain. XIAP carrying three functional domains, including the BIR, linker and Ring-finger domains, may be the best-characterized IAP protein with respect to its structure and biochemical mechanisms. XIAP inhibits apoptosis by inhibiting caspases 3, 7 and 9, but not caspases 1, 6, 8 and 10 [37]. A recent study demonstrated that XIAP appears to stimulate the NF-κB signaling pathway upstream of the point at which signals from TNFa and IL-1 receptors converge by regulating IKBα [22]. We hypothesized that miR-23A targets XIAP by regulating stimulation of the NF-κB signaling pathway. As expected, the Western blot results validated this prediction. We next explored the effect of NF-κB on cardiomyocyte apoptosis. One mechanism by which NF-κB inhibits apoptosis is upregulation of the anti-apoptotic Bcl-xL protein. Bcl-xL, in turn, blocks mitochondrial permeabilization, which results in inhibition of cytochrome C release as well as preventing mitochondrial depolarization [38-40]. Consequently, we evaluated the expression of proteins in the mitochondrial pathway. The expression of Bax, cytoC, caspase 9 and caspase 3 were all significantly upregulated in the I/R group and suppressed by antagomir-23a. In contrast, Bcl-2 was downregulated in the I/R group.

Disclosure of conflict of interest

None.

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