Research Paper

MicroRNA-135a Regulates Apoptosis Induced by Hydrogen Peroxide in Rat Cardiomyoblast Cells

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Abstract

Oxidative stress and apoptosis are the most important pathologic features of ischemic heart disease. Recent research has indicated that microRNAs (miRs) play an essential role in apoptosis. However, whether miRs might regulate B cell lymphoma-2 (Bcl-2) protein in apoptosis during ischemic heart disease is still unclear. The aim of this study, therefore, was to confirm the regulation of microRNA-135a (miR-135a) in oxidative stress injuries induced by hydrogen peroxide (H_2O_2) in rat cardiomyoblast cells H9c2. To this end, we analyzed the effects of H_2O_2 treatment on miR-135a expression in rat cardiomyocytes. Furthermore, we upregulated and inhibited miR-135a using mimics and inhibitors, respectively, and examined the effects on cell viability and apoptosis-related proteins. We observed that miR-135a was markedly up-regulated under H_2O_2 treatment in rat cardiomyoblast cells. Overexpression of miR-135a blocked the Bcl-2 protein and enhanced the apoptosis induced by H_2O_2, and miR-135a inhibition restored Bcl-2 protein expression. Interestingly, miR-135a inhibition did not attenuate H_2O_2-induced apoptosis with Bcl-2 knockdown. The results of the present study indicate that miR-135a regulates H_2O_2-induced apoptosis in H9c2 cells via targeting Bcl-2, and that miR-135a may be a novel therapeutic target for ischemic heart disease.

Key words: MicroRNA-135a, Rat cardiomyoblast cells, Apoptosis, Bcl-2, Hydrogen peroxide.

Introduction

Ischemic heart disease is a common health problem and one of the leading causes of cardiac dysfunction in the world [1]. One potential therapeutic approach for ischemic heart disease is to reduce oxygen consumption, inhibit cardiomyocyte apoptosis, increase coronary flow, and induce revascularization [2, 3]. Ischemia and reperfusion activate cardiac myocyte apoptosis and oxidative stress, which is an important feature in the progression of ischemic heart disease [4, 5]. Hydrogen peroxide (H_2O_2)-induced cardiac cell apoptosis plays a key role in the pathogenesis of cardiac oxidative stress injury [6-8].

Apoptosis is a type of programmed cell death, and there are two major apoptotic signaling pathways in the cardiovascular system [9]. Both the intrinsic pathways and extrinsic pathways are associated with ischemic heart diseases [10, 11]. The intrinsic pathway is mainly regulated by the B cell lymphoma-2 (Bcl-2) superfamily, such as Bax, Bak, Bcl-2 and Bcl-XL [12]. Bcl-2 has been shown to regulate the apoptotic cascade, and it can block apoptosis by preventing cytochrome *c* release and mitochondrial permeabilization in ischemic heart disease [13, 14]. Recent studies have demonstrated that overexpression of Bcl-2 in cardiac myocytes significantly reduced cardiac myocyte apoptosis and cardiac dysfunction in ischemic heart disease [15-17]. Furthermore, the ratio of antiapoptotic to proapoptotic proteins plays an important role in the regulation of apoptosis in cardiac cell injury [17, 18]. However, the detailed mechanism by which Bcl-2 is regulated during disease progression requires further investigation.

MicroRNAs (miRs) are small noncoding RNAs that play important roles in gene expression by inhibiting translation or inducing target molecules degradation [19-21]. MiRs also have roles in the functions of the healthy and diseased heart, such as cardiac hypertrophy, myocardial infarction, myocardial fibrosis, cardiac arrhythmia and heart failure [22-26]. An increasing number of reports have confirmed that miRs function as a critical apoptotic regulator in heart cells [27-29]. MiRs can function as either pro-apoptotic or anti-apoptotic factors [29-31]. Our previous studies have found that the expression of miRNA is different in rat myocardial injury models induced by psychological stress and microRNAs-135a (miR-135a) is significantly downregulated in chronic psychological stress [32]. Several studies have shown that miR-135a acts as a pro-apoptotic factor in many cancer cell lines [33-35].

However, whether miR-135a might regulates Bcl-2 during the development of cardiac cell injury, thereby affecting ischemic heart disease progression is not known. Therefore, our aim was to unravel the effects of miR-135a on apoptosis, and to investigate the underlying mechanisms in H9c2 cells. In the present study, we exposed the cells to H_2O_2 and detected the expression of miR-135a.

Materials and methods

Cell culture

The embryonic rat cardiac myoblast H9c2 cell line was obtained from Department of Cardiology of the Second Hospital of Jilin University. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Carlsbad, USA) supplemented with 10% fetal bovine serum (Hyclone, Australia). The cells were propagated at 37°C under 5% CO_2.

Cell viability assays

H9c2 cells were plated in 96-multiwell plates at 1×10^4 cells/well at 24 h before treatment. The cells were then treated with increased concentrations of H_2O_2 (0, 0.1, 0.25, 0.5, and 1 mM) for 24 h. Each treatment was repeated in five wells. H9c2 were then incubated with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (10 μl; 5 mg/ml in PBS; Sigma, St. Louis, MO, USA) for 4 h at 37°C under 5% CO_2. Subsequently, 150 μl of dimethylsulfoxide was added to dissolve the formazan crystals of each group. After shaking for 10 min, the absorbance values were measured at a wavelength of 570 nm by a Microplate Reader (Molecular Devices, Sunnyvale, CA).

Transfection

H9c2 cells were transfected using Lipofecta-mine® 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were plated on six-well plates and transfected at 80% confluence with the negative control (NC), miR-135a mimics or miR-135a inhibitor (GenePharma, Shanghai, CHINA).

Small interfering RNA-mediated gene silencing

H9c2 cells were seeded in 6-well plates. Small interfering RNAs targeting Bcl-2 or a scramble sequence (GenePharma, Shanghai, CHINA) were transfected into H9c2 cells using Lipofectamine® 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA from H9c2 cells was extracted using a miRcute miRNA Isolation Kit (Tiangen Biotech, Beijing, China). To quantify the RNA expression levels, cDNA was synthesized using the miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen Biotech, Beijing, China) and qPCR was carried out using the miRcute miRNA qPCR Detection Kit (SYBR Green; Tiangen Biotech, Beijing, China). All reactions were conducted in triplicate in a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland). Fold changes in miR-135a expression between treatments and controls were determined using $2^{-\Delta\Delta Ct}$ method [36] and normalized to U6 RNA expression as an internal reference. The PCR forward primer for mature miR-135a was designed as 5'-GCGCCGTATGGCTTTTTATTCCTA-3', and the PCR reverse primer for mature miR-135a was obtained from the commercial miRcute miRNA qPCR Detection Kit. Primer sequences are as follows: U6, 5'-AACGCTTCACGAATTTGCGT-3' (sense) and 5'CTCGCTTCGGCAGCACA-3' (antisense), Bcl-2, 5'-TGCAGAGATGTCCAGTCAGC-3' (sense) and 5'-CATCCACAGAGCGATGTTGT-3' (antisense), and GAPDH, 5'-AACGACCCCTTCATTGAC-3' (sense) and 5'-TCCACGACATACTCAGCAC-3' (antisense).

Flow cytometry analysis

After exposure to different experimental conditions, cells were trypsinized and incubated with propidium iodide (PI, 1μg/ml) and Annexin V-FITC (1 μg/ml; Invitrogen, Carlsbad, CA) for 15 min at 37°C. Samples were then analyzed for apoptosis by a FACScan flow cytometer (FCM; Bechman Coulter, CA).

Western blot analysis

H9c2 cells grown in 100-mm Petri dishes were treated with 1mM H_2O_2 for 24h. Total proteins were

extracted from H9c2 cells using RIPA buffer, and then the cell lysates were centrifuged at 3,000×g for 30 min. The concentrations of protein were determined using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA). For western blot analysis, equivalent amounts of proteins were resolved on 12% gels by SDS-polyacrylamide gel electrophoresis and then transferred onto PVDF membranes (Millipore, Bedford, MA). After blocking in 10% nonfat dry milk at room temperature for 1 h, the membranes were incubated with the relevant primary antibody overnight at 4°C. The β-actin, Bcl-2, Bax and caspase-3 antibodies (all used at 1:1000 dilution) were obtained from Cell Signaling Technology (CST, BSN, USA). On the following day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Thermo, Waltham, MA, USA) for 1 h at room temperature. The immunoreactive bands were visualized by the ECL detection system (Amersham/GE Healthcare, Pittsburgh, PA). The reactive bands were measured by a Tanon GIS gel imager system and the protein levels were quantified by densitometry using the Quantity One software.

Statistical analysis

All of the data are representative of three independent experiments performed as triplicate determinations. Statistical analyses of the data were evaluated using one-way ANOVA. Values of $P < 0.05$ were considered statistically differences.

Results

Expression of miR-135a is decreased in H9c2 cells treated with H_2O_2

We treated H9c2 cells with increasing doses of H_2O_2 for 24 h or 1 mM H_2O_2 for 2, 4, 8, 12 and 24 h, and examined the relative expression of miR-135a by qPCR. We found that H_2O_2 treatment upregulated the expression of miR-135a in H9c2 cells (Fig. 1A and B). MTT assays indicated that H_2O_2 inhibited the cell viability in a dose-dependent manner (Fig. 1C). H9c2 cells were subjected to PI and Annexin V-FITC staining. Flow cytometry analysis was used to quantify apoptotic cell populations. H_2O_2 treatment resulted in an increase in the apoptotic cell population (Fig. 1D and E). These findings demonstrate that H_2O_2 induced apoptosis of embryonic rat cardiac myoblasts H9c2 cells and increased the expression of miR-135a.

MiR-135a is functionally involved in apoptosis induced by H_2O_2 in H9c2 cells

To determine whether miR-135a regulates apoptosis, we transfected miR-135a mimics, inhibitor or NC into H9c2 cells. The expression of miR-135a was markedly changed after transfection (Fig. 2A and

B). We increased the expression of miR-135a in H9c2 cells exposed to H_2O_2 for 24 h, and then examined the growth inhibition and apoptosis ratio. Transfection of the miR-135a mimics decreased the cell viability and increased apoptosis after H_2O_2 treatment compared to the NC + H_2O_2 group (Fig. 2C, D and E). As shown in Fig. 2F, G and H, inhibition of miR-135a expression increased the cell viability and decreased apoptosis after H_2O_2 treatment compared to the NC + H_2O_2 group. Together, these findings demonstrate that miR-135a promotes H_2O_2-induced apoptosis in H9c2 cells, and that inhibition of miR-135a attenuates apoptosis.

The effect of miR-135a mimics on apoptotic proteins in H9c2 cells

To examine the mechanism underlying the induction of apoptosis in more detail, we examined the expression of Bcl-2, Bax and caspase-3 proteins. Western blot analysis showed that H9c2 cells transfected with miR-135a mimics showed a decrease in Bcl-2 protein expression compared to the NC group. Importantly, the level of the Bcl-2 protein was markedly decreased in the group of miR-135a mimics + H_2O_2 group compared to the NC + H_2O_2 group (Fig. 3A). Furthermore, the level of apoptotic proteins Bax and cleaved caspase-3 were increased after miR-135a mimics transfection following H_2O_2 treatment compared to the NC + H_2O_2 group (Fig. 3B and C). However, there was no change in the expression of Bcl-2 mRNA in either group (Fig. 3D). These results suggest that miR-135a overexpression can affect apoptosis-associated proteins under H_2O_2 exposure.

Inhibition of miR-135a attenuates the apoptotic proteins induced by H_2O_2

We inhibited miR-135a expression by using an inhibitor in H9c2 cells exposed to H_2O_2. As shown in Fig. 4A, the inhibition of miR-135a upregulated the level of Bcl-2 protein compared to the NC group, and the expression of the Bcl-2 protein was increased in the miR-135a inhibitor + H_2O_2 group compared to the NC + H_2O_2 group. The levels of Bax and cleaved caspase-3 protein were decreased in the miR-135a inhibitor + H_2O_2 group compared to the NC + H_2O_2 group (Fig. 4B and C). However, there is no change of the expression of Bcl-2 mRNA in either group (Fig. 4D). This data indicate that inhibition of miR-135a directly increases the expression of Bcl-2 protein and decreases the pro-apoptosis proteins caused by H_2O_2.

MiR-135a regulates apoptosis induced by H_2O_2 via targeting Bcl-2 protein

To further confirm whether Bcl-2 protein is involved in this apoptosis induction, we specifically

knocked down Bcl-2 in H9c2 cells by using siRNA. We found that the level of Bcl-2 protein was lower in the si-Bcl-2 group than in the si-NC group (Fig. 5A). To determine whether Bcl-2 is directly involved in the apoptosis pathway of miR-135a in H9c2 cells exposed to H_2O_2, we used Bcl-2 siRNA after the downregulation of miR-135a by inhibitor in H9c2 cells. As shown in Fig. 5B, C and D, the transfection of si-Bcl-2 decreased the cell viability and increased cell apoptosis after H_2O_2 treatment compared to the NC +

H_2O_2 group. Moreover, co-transfection with miR-135a and si-Bcl-2 decreased the cell viability and increased apoptosis after H_2O_2 treatment in comparison with the miR-135a inhibitor + H_2O_2 group. In the NC + H_2O_2 group (with normal Bcl-2 expression), miR-135a inhibition protected cells from apoptosis under H_2O_2 exposure. Interestingly, in the group treated with Bcl-2 siRNA, miR-135a inhibition did not protect cells from apoptosis induced by H_2O_2.
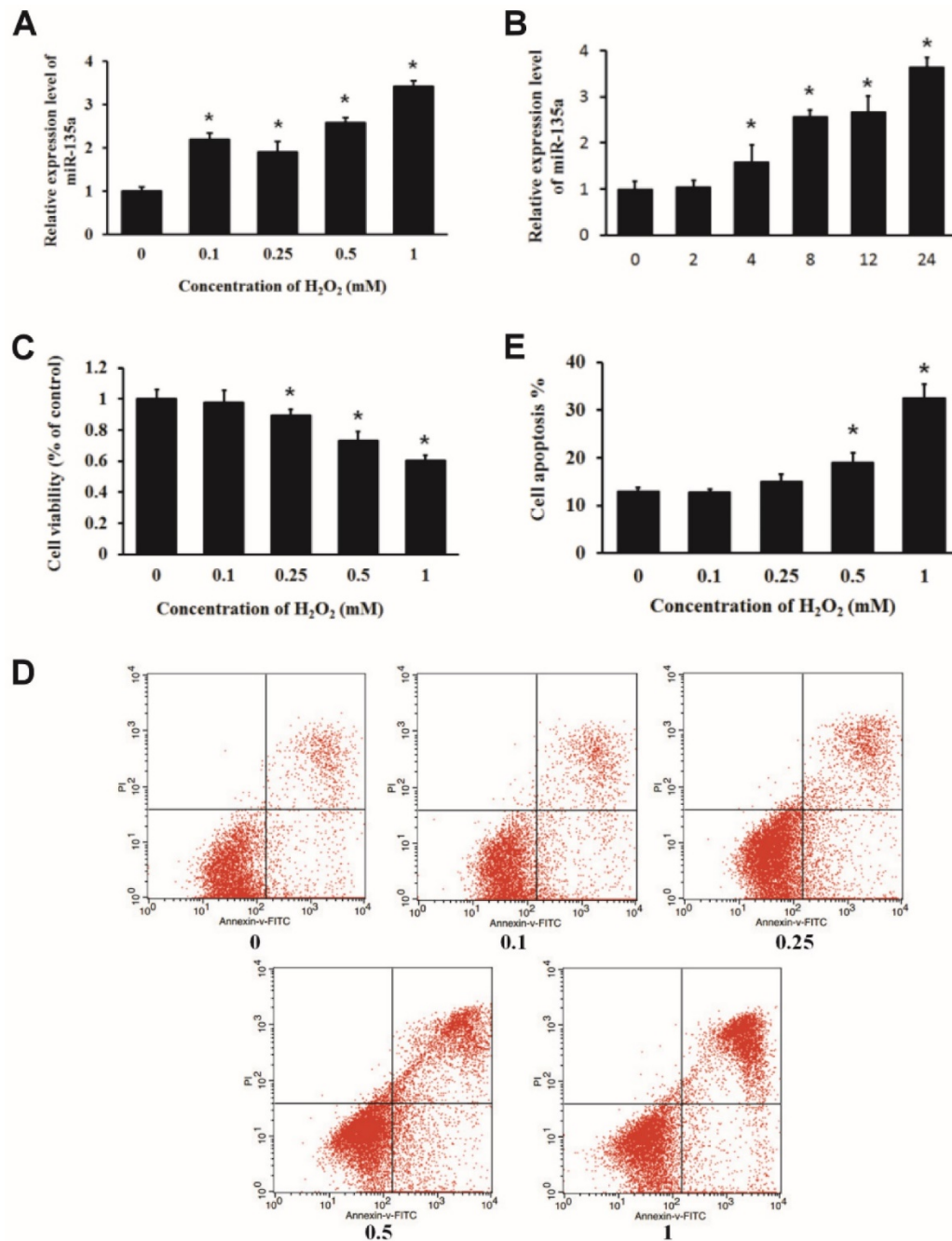

Fig. 1 Expression of miR-135a is increased in H9c2 cells treated with H_2O_2. The relative expression of miR-135a was measured by quantitative real-time PCR (qPCR). (A) H9c2 cells were treated with 0, 0.1, 0.25, 0.5 or 1 mM of H_2O_2 for 24 h, or with (B) 1 mM of H_2O_2 for 0, 2, 4, 8, 12, or 24 h. (C) Cells were treated with varying doses of H_2O_2 for 24 h. Cell viability was determined by the MTT assay. (D) H9c2 cells were stained with propidium iodide (PI) and Annexin V-FITC. The apoptosis positive stained cells were counted by flow cytometry. (E) Quantitation of the apoptosis rate. All data are presented as means ± SD ($n = 3$). *$P < 0.05$ vs. control.
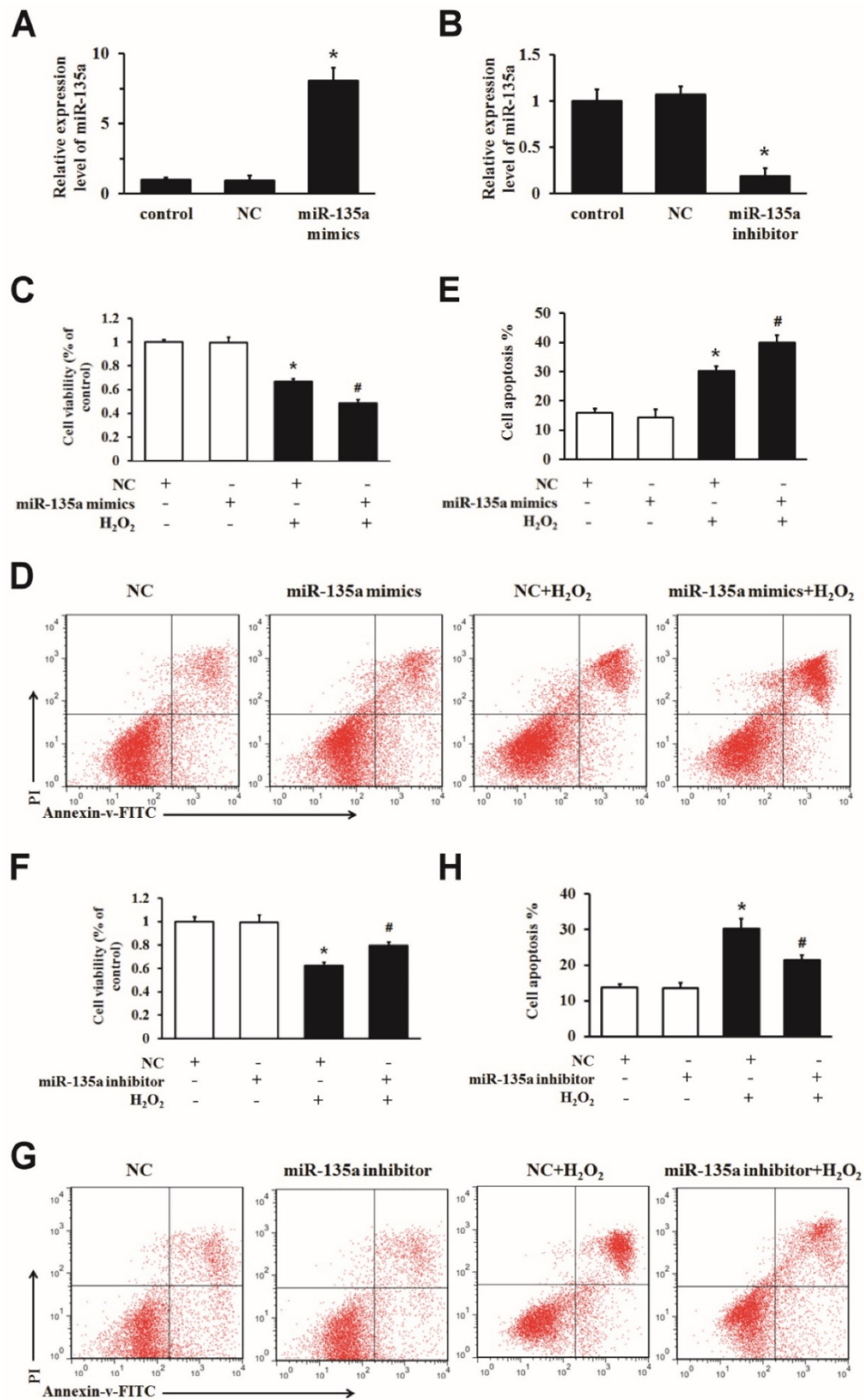
Fig. 2 miR-135a is functionally involved in apoptosis induced by H_2O_2 in H9c2 cells. qPCR was used to determine the expression of miR-135a in H9c2 cells. Cells were transfected with negative control (NC) or miR-135a mimics (A), or with NC or miR-135a inhibitor (B). Cells were subsequently treated with 1 mM H_2O_2 for 24 h and cell viability was assessed using the MTT assay (C and F). The cells were stained with PI and Annexin V-FITC. The positive stained cells were counted by flow cytometry (D and G). Quantitation of the apoptosis rate (E and H). All data are presented as means ± SD (*n* = 3). *P < 0.05 vs. NC. #P < 0.05 vs. NC + H_2O_2.
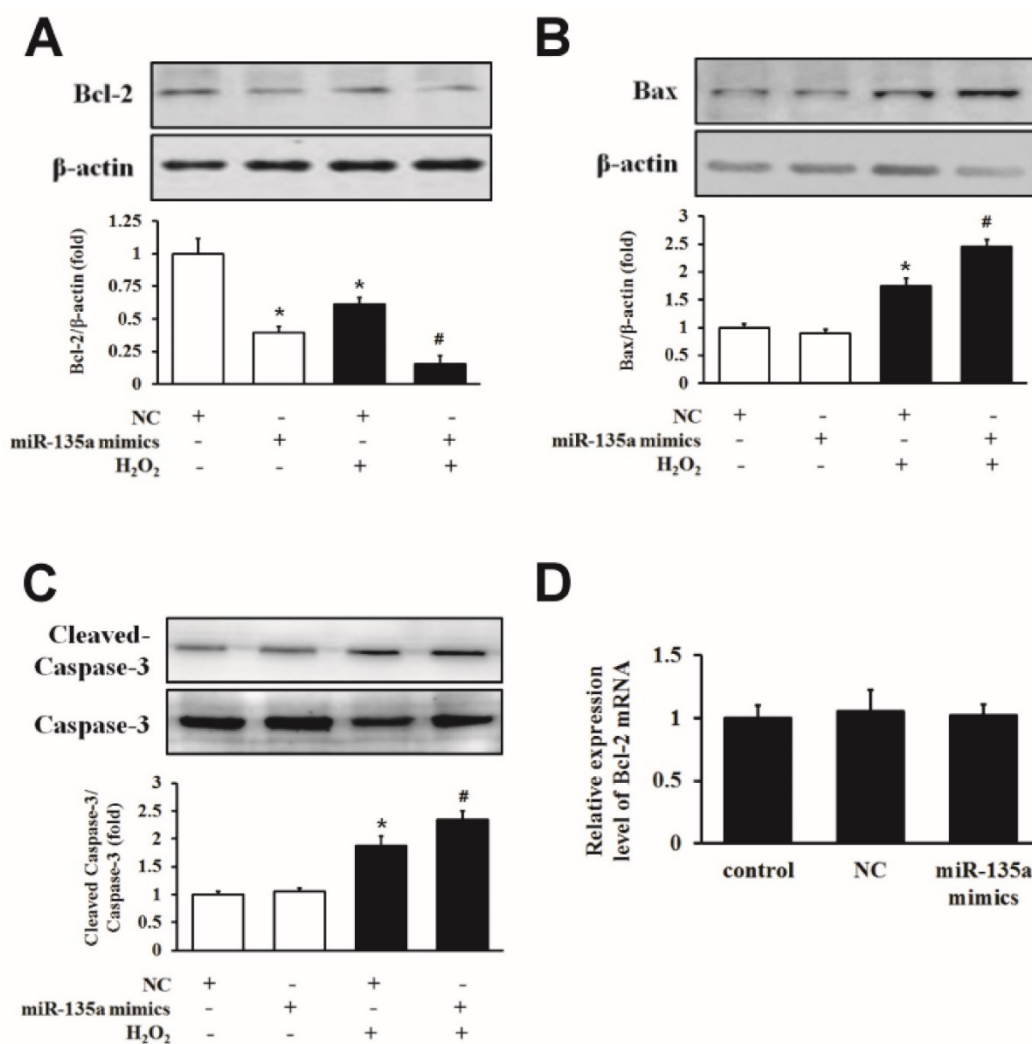
Fig. 3 The effect of miR-135a mimics on apoptotic proteins in H9c2 cells. Cells were treated with 1 mM H_2O_2 for 24 h after transfection with NC or miR-135a mimics. Western blot analysis for the expressions of Bcl-2 (A), Bax (B) and cleaved caspase-3 (C). Data are presented as means ± SD ($n = 3$). $*P < 0.05$ vs. NC. $\#P < 0.05$ vs. NC + H_2O_2. (D) qPCR was used to analyze the expression of Bcl-2 after transfection with miR-135a mimics. Data are presented as means ± SD ($n = 3$).

Discussion

Clinical observations have suggested that myocardial infarction is one of the leading causes of death in the world [37, 38], and apoptosis may be involved in the process of ischemic cardiomyoblast cells [30]. Apoptosis is a major feature of ischemic heart disease [39, 40], and Bcl-2 protects cells from apoptotic stimuli [41-43]. H_2O_2 has been widely used to simulate conditions of oxidative stress, and H_2O_2 is considered to play a key role in ischemic cardiomyoblast cells by stimulating apoptosis [44, 45]. We treated rat cardiomyoblast cells H9c2 with H_2O_2 and found significant apoptosis accompanied by caspase-3 activation. Previous studies have indicated that Bcl-2, an anti-apoptotic protein, plays an important role by controlling the permeabilization of the mitochondrial outer membrane [46, 47]. We evaluated the expression of Bcl-2 protein by western blot and found that the level of Bcl-2 was downregulated in cardiomyocytes treated with H_2O_2. These results suggested that Bcl-2 is involved in apoptosis under H_2O_2 treated conditions.

Recent data have suggested that miRs regulate apoptosis in ischemic heart disease [48]. In our study, we found that miR-135a plays a significant role in regulating apoptosis in rat cardiomyoblast cells. The expression of miR-135a was strongly upregulated in H9c2 cells after H_2O_2 treatment in a dose-dependent manner. To determine the potential role of miR-135a in regulating apoptosis induced by H_2O_2, the expression of miR-135a was modulated by miR-135a mimics and inhibitor. Upregulation of miR-135a by miR-135a mimics in H9c2 cells exacerbated apoptosis induced by H_2O_2. In contrast, inhibition of miR-135a expression attenuated cardiomyocyte apoptosis. These results suggested that miR-135a participates in the regulation of H_2O_2-mediated apoptosis and may be a new target for the treatment of cardiac cell injury.
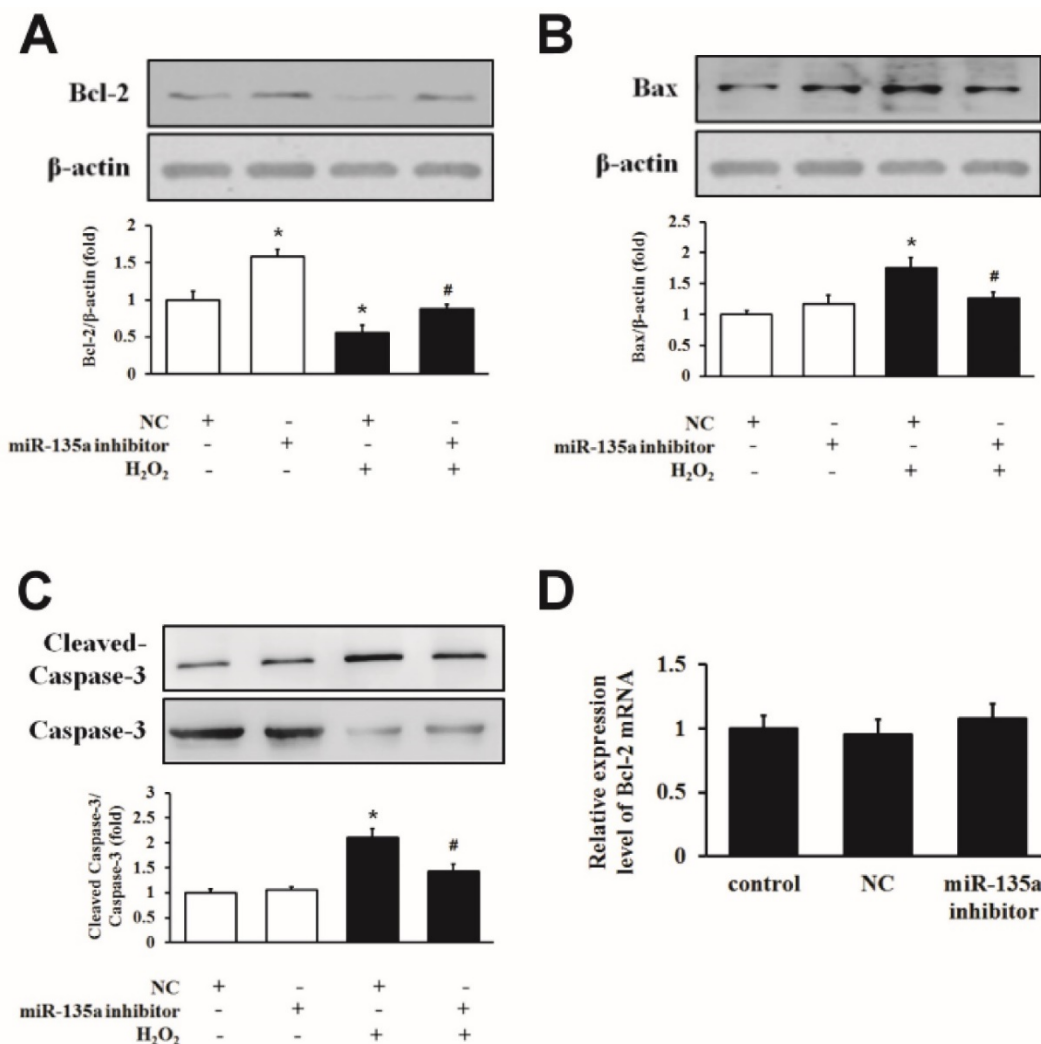
Fig. 4 Inhibition of miR-135a attenuates the induction of apoptotic proteins by H₂O₂. H9c2 cells were treated with 1 mM H_2O_2 for 24 h after transfection with NC or miR-135a inhibitor. Western blot was used to determine the expression of Bcl-2 (A), Bax (B) and cleaved caspase-3 (C). Data are presented as means ± SD (n = 3). *P < 0.05 vs. NC. #P < 0.05 vs. NC + H_2O_2. (D) Expression of Bcl-2 mRNA after treatment with miR-135a inhibitor. Data are presented as means ± SD (n = 3).

To determine whether Bcl-2 is involved in miR-135a-mediated H₂O₂-induced cardiomyocytes apoptosis, we evaluated the expression of Bcl-2 proteins after transfected with miR-135a mimics and inhibitor. We found that the level of Bcl-2 protein was downregulated in cardiomyocytes treated with miR-135a mimics, and the suppression of miR-135a directly enhanced the expression of Bcl-2 protein. Interestingly, we did not detect any changes in Bcl-2 mRNA expression after treatment with the miR-135a mimics and inhibitor. Furthermore, we knocked down the expression of Bcl-2 in H9c2 cells with si-Bcl-2. We found that inhibition of miR-135a did not increase the cell viability or attenuate apoptosis in cells with Bcl-2 knockdown. These results demonstrated that the Bcl-2 protein might be a target

of miR-135a in cardiomyocytes, and miR-135a regulates apoptosis partly through the regulation of Bcl-2 protein. In summary, our data investigate for the first time that miR-135a regulated H₂O₂-induced apoptosis via the modulation of Bcl-2.

In summary, our findings demonstrate that the expression of miR-135a in rat cardiomyoblast cells is upregulated under H₂O₂ treatment. MiR-135a participates in apoptosis induced by H₂O₂ in H9c2 cells by downregulating Bcl-2. Overexpression of miR-135a blocked the Bcl-2 protein and increased the apoptosis induced by H₂O₂. Together, our results suggest that miR-135a might play a significant role in apoptosis of rat myocardial cells, and miR-135a may lead to a novel therapeutic target for ischemic heart disease.
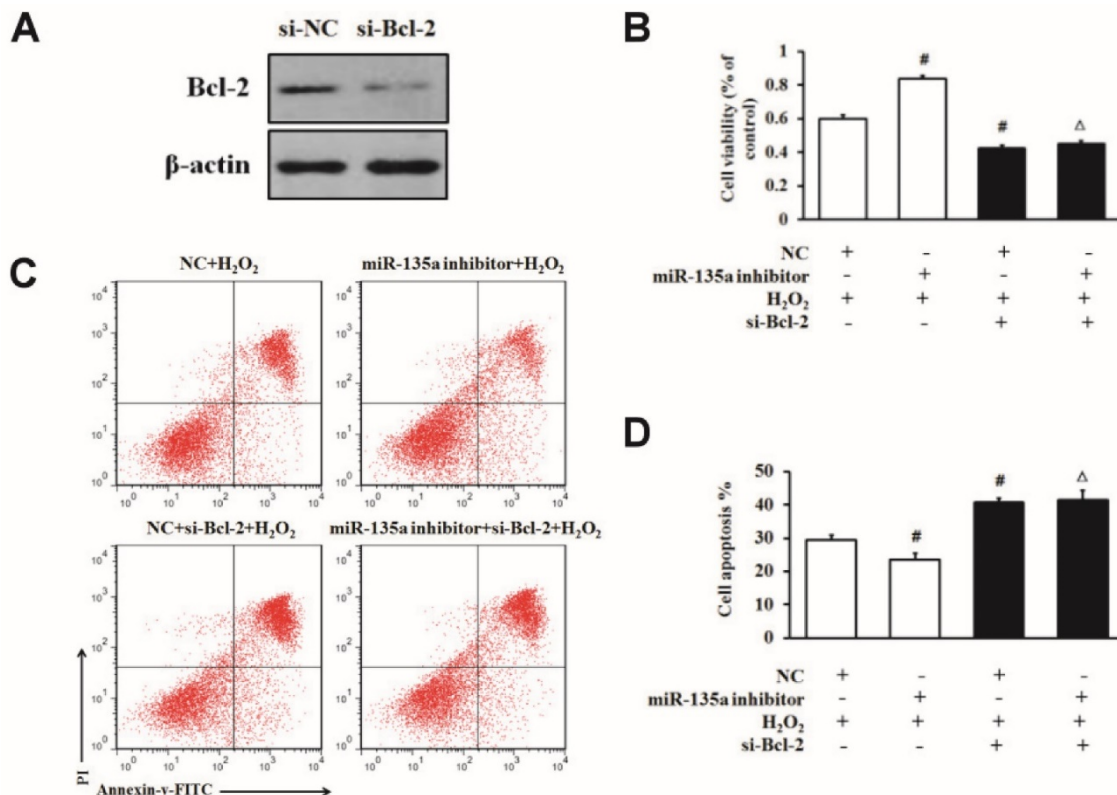
Fig. 5 miR-135a regulates H$_2$O$_2$-induced apoptosis via Bcl-2 modulation. (A) The expression of Bcl-2 protein was detected by western blot in cells transfected with si-Bcl-2. (B) H9c2 cells were transfected with the miR-135a inhibitor, si-Bcl-2 or both, followed by treatment with 1 mM H$_2$O$_2$ for 24 h. Cell viability was determined by the MTT assay. (C) Cells were stained with PI and Annexin V-FITC. Positively stained cells were counted by flow cytometry. (D) Quantitation of the apoptosis rate. All data are presented as means ± SD (n = 3). #P < 0.05 vs. NC + H$_2$O$_2$. △P < 0.05 vs. miR-135a inhibitor + H$_2$O$_2$.

Abbreviations

IHD: ischemic heart disease; H$_2$O$_2$: hydrogen peroxide; Bcl-2: B cell lymphoma-2; miR: microRNA; miR-135a: microRNA-135a; DMEM: Dulbecco's modified Eagle's medium; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; qPCR: quantitative real-time PCR; NC: negative control; siRNA: small interfering RNA; PI: propidium iodide; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF: polyvinylidene difluoride; ECL: enhanced chemiluminescence.

Acknowledgements

Competing Interest

The authors have declared that no competing interest exists.

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