Overexpressed microRNA-506 and microRNA-124 alleviate H₂O₂-induced human cardiomyocyte dysfunction by targeting krüppel-like factor 4/5

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Abstract. Krüppel-like factors (KLFs) regulate a wide variety of cellular functions and modulate pathological processes. In the present study, a post-translational mechanism of microRNAs (miRs) was investigated in H₂O₂-induced human cardiomyocyte (HCM) injury. In H₂O₂-cultured HCM cells, reactive oxygen species and apoptotic cells were measured via flow cytometry. miR-506/-124 mimics and inhibitors were transfected to induce gain or loss of miR-506/-124 function. Cell proliferation was analyzed by an MTT assay. The targeted genes were predicted by a bioinformatics algorithm and confirmed by a dual luciferase reporter assay. The mRNA and protein expression levels were measured by reverse transcription-polymerase chain reaction analysis and western blotting, respectively. The results indicated that H₂O₂ induced significant apoptosis and increased the concentration of reactive oxygen species (ROS) in HCMs. H₂O₂ markedly upregulated the expression levels of KLF4 and KLF5, and downregulated the expression levels of miR-506 and miR-124 in the HCMs. In addition, bioinformatics analysis showed the potential miR-506 and miR-124 binding sites within the 3′-untranslated region of KLF4 and KLF5 in the HCMs. The overexpression of miR-506 and miR-124 inhibited the H₂O₂-induced upregulation of KLF4 and KLF5 in the HCMs. The overexpression of miR-506 and miR-214 reversed the H₂O₂-induced apoptosis and increase of ROS in the HCMs. In conclusion, the overexpression of miR-506 and miR-214 were confirmed to have a protective effect against H₂O₂-induced HCM injury by suppressing the expression of KLF4 and KLF5.

Introduction

Oxidative stress stimuli are critical in the pathogenesis of several cardiovascular diseases, including acute myocardial infarction (AMI), cardiomyocyte apoptosis and heart failure (1,2). Oxidative stress stimuli-induced reactive oxygen species (ROS) production can increase blood vessel permeability and accelerate cardiac cell injury (3). Although there has been progress in understanding the mechanisms of AMI to develop efficient treatment strategies, they remain to be fully elucidated. Previously, microRNAs (miRs) have been reported to be deregulated in several cardiovascular diseases and contribute to AMI (4). Accumulating evidence suggests the usefulness of circulating miRs as stable blood-based biomarkers for AMI, including miR-208, miR-499, miR-19a, miR-21 and miR-1 (5-7). In vitro, the expression of miR-874 is markedly increased in response to H₂O₂ treatment in cardiomyocytes, and miR-874 loss-of-function targets and upregulates caspase-8 to antagonize necrosis (8). In addition, miR-145 regulates ROS-induced Ca²⁺ overload and cellular injury responses in cardiomyocytes (9). However, the underlying functions of miR-506 and miR-124 in H₂O₂-induced human cardiomyocyte (HCM) injury remain to be elucidated.

Krüppel-like factors (KLFs) are distinguished by a highly conserved interfinger sequence space, and 17 KLFs have been identified in mammals (10,11). KLFs regulate a wide variety of cellular functions, including growth, apoptosis, angiogenesis and proliferation (12). For example, the overexpression of KLF15 in cardiomyocytes can inhibit cardiomyocyte hypertrophy through the suppression of cell size, protein synthesis and hypertrophic gene expression (13). KLF5 contributes to pulmonary artery smooth muscle proliferation and resistance to apoptosis in human pulmonary arterial hypertension (14), and KLF4 has been shown to repress smooth muscle cell proliferation (15). Of note, KLFs can be regulated by oxidative stress stimuli, including endothelin-1 (ET-1), H₂O₂ and inflammatory cytokines (16). Specifically, KLF4 and KLF5 are significantly upregulated in the presence of ET-1, and H₂O₂ increases the expression levels of KLF2, KLF4 and KLF6 (16). The post-translational mechanism of miRs indicates that miR-32 promotes gastric carcinoma tumorigenesis by targeting the 3′-untranslated region (3′-UTR) of KLF4 (17). In addition, KLF5 is regulated by miR-375 in oral
squamous cell carcinoma (18). However, the molecular mechanisms underlying the effects of miR-506 and miR-124 in HCMs by regulating KLF4/5 remain to be fully elucidated.

Although the suppression of epithelial-mesenchymal transition in hepatocellular carcinoma cells by miR-506 targets KLF4, the biological functions of miR-506 in response to H$_2$O$_2$-induced HCM dysfunction remain to be fully elucidated. The present study investigated whether the expression of KLF4/5 was regulated by miR-506 and miR-124, and whether the pro-apoptotic role of KLF4/5 was affected by miR-506 and miR-124 in HCMs.

Materials and methods

Cell culture. The Ethics Committees of the Binzhou People’s Hospital (Binzhou, China) approved the experimental protocol for the present study. The HCMs and AC16 cell lines were purchased from the American Type Culture Collection (Bethesda, MD, USA) and were cultured in the Medical Experimental Center of the Binzhou People's Hospital (Binzhou, China). The HCMs were cultured in Dulbecco’s modified Eagle’s medium (DMEM), which contained 10% fetal calf serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10% L-glutamine, 0.5% penicillin/streptomycin, 10% nonessential amino acids and 10% pyruvate, in a 5% CO$_2$ atmosphere at 37°C.

Cell viability detection using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The proliferation of the HCMs was monitored using an MTT Cell Proliferation/Viability Assay kit (R&D Systems, Inc., Minneapolis, MN, USA) in accordance with the manufacturer’s protocol.

Lactate dehydrogenase (LDH) activity. The HCMs were plated (1x10$^5$ cells/well) and treated in 96-well plates, which were incubated with H$_2$O$_2$ (0, 50, 100, 200 and 400 µM). After 24 h, centrifugation (12,000 x g for 15 min at 4°C) was performed to obtain the supernatant, and the level of LDH was measured. Measurements were recorded according to the manufacturer’s protocol (cat no. ab102526; Abcam, Cambridge, UK). Data were normalized with the protein concentration of podocyte lysates.

Measurement of ROS. The generation of ROS in the HCMs was evaluated using a fluorometric assay via the intracellular oxidation of dichlorodihydrofluorescein diacetate (DCFH-DA). The cells (4x10$^4$) were incubated in a 6-well plate for 24 h, and following DCFH-DA (50 µg/ml) incubation for 30 min at 37°C, the cells were harvested and washed twice with PBS, and finally added into 1 ml PBS. Cells were detected and analyzed using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The fluorescent product 2’, 7’-dichlorofluorescein (DCF) was detected at an emission wavelength of 530 nm and excitation wavelength of 485 nm, and the result was analyzed using the flow cytometry analysis software BD CellQuest (version 5.1; BD Biosciences).

Apoptosis assay. The quantitative assessment of apoptotic cells was performed using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method, which examines DNA-strand breaks during apoptosis, using the BD ApoAlert™ DNA Fragmentation Assay kit (BD Biosciences). The measurements were recorded according to manufacturer’s protocol. The cells were immediately analyzed using the FACSscan system and the CellQuest program (version 5.1; BD Biosciences).

miRs expression profiling. HCMs (1x10$^5$ cells/well) were incubated with H$_2$O$_2$ (0, 50, 100 and 200 µM). Total RNA was extracted with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). miRs were isolated from total RNA using the miRNA isolation kit (Invitrogen; Thermo Fisher Scientific, Inc.). Denaturing agarose gel electrophoresis was performed using 1% formaldehyde electrophoresis reagent. miRs were labeled with Hy3 or Hy5 fluorescence using the miRCURY™ Array Power Labeling kit (Exiqon, Inc., Woburn, MA, USA) to obtain the fluorescent probe that may be hybridized with the chip. The labeled probe was hybridized with the miRCURY™ chip under the standard condition using the MAUI hybridization system. The fluorescence intensity of the chip was scanned with the Agilent chip scanner and analyzed using Agilent feature extraction software (version 12). Differentially expressed miRs were screened based on the fold change ≥2, P<0.05 and FDR <0.05. Finally, the differentially expressed miRs were displayed by hierarchical clustering analysis, and the output was drawn using MeV software (version 4.2.6).

miRNA Target prediction. The target genes of miR-506 and miR-124 were predicted based on the use of computer-based bioinformatics software, including miRanda-mirSVR (http://www.microrna.org), DIANA TOOLS (http://diana.imis.athena-innovation.gr) and TargetScan (http://www.targetscan.org/).

Luciferase reporter gene activity assay. The 3’-UTR of the KLF4 and KLF5 genes containing the predicated target sites for miR-506 and miR-124 was obtained by polymerase chain reaction (PCR) amplification. The fragment was inserted into the multiple cloning sites in the pMIR-REPORT luciferase microRNA expression reporter vector (Ambion; Thermo Fisher Scientific, Inc.). The cells were co-transfected with 0.1 µg of luciferase reporters containing the KLF4 and KLF5 3’-UTR, and miR-506 and miR-124 mimics using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The cell lysates were harvested following transfection for 48 h, and luciferase activity was examined according to the manufacturer’s protocol.

Mimics and inhibitor of miRs. The FAM-modified 2’-Ome-oligonucleotides were chemically synthesized and purified using high-performance liquid chromatography (GenePharma, Shanghai, China). The 2’-Ome-mir-506 and miR-124 mimics were composed of RNA duplexes with the following sequences: 5’-UAAGGCACCUCUCUGAGUAG-3’ and 5’-UAAGGCACCGCGUGAUAUCC-3’, respectively. The sequences of 2’-Ome-mir-506 and miR-124 inhibitor were as follows: 5’-UCUACUCAGAGGGUGCCUA-3’ and 5’-GGCAUUCACCGCGUGCCUA-3’, respectively. The 2’-Ome-scramble oligonucleotides of miR-506 and miR-124 gene expression.
were as follows: 5'-UAGUGCUGGCCAUCCAGGGGAA-3' and 5'-GCCGAGUCGGAGGCCAUUGG-3', respectively. The cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 100 nM.

Reverse transcription (RT)-PCR analysis. RNA extraction was performed using TRIzol according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). The synthesis of cDNA was performed by RT reactions with 4 µg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) with oligo dt (15) primers (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The levels of miR-506 and miR-124 were quantified using the mirVana RT-qPCR miRNA detection kit (Ambion; Thermo Fisher Scientific, Inc.) in conjunction with real-time PCR with SYBR-Green. PCR reaction mixtures contained 12.5 µl SYBR-Green SuperMix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 1 µl cDNA, 300 nM of each primer, and DEPC H2O to a final volume of 25 µl. The cycling reactions were 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 58˚C for 30 sec, and 72˚C for 30 sec. Following the thermocycling reaction, the quantification cycle (Cq) was determined and the relative levels of miR-506 and miR-124 were calculated based on the Cq values (19) and normalized to the level of U6 in each sample. PCR analysis was performed with the following primers: KLF4 (175 bp forward, 5'-TCA AGAGCTCATGGCCAGG-3' and reverse, 5'-CTCTGCCTGT GTGAGTTCGCA-3'); KLF5 (212 bp forward, 5'-AGCTCAG CTGAGACTCATA-3' and reverse, 5'-GTGGCAGCTGCT CAGTTCT-3'; GAPDH (83 bp) forward, 5'-CTC GCC TGT AGA GCT CAT GCC ACC GG-3' and reverse, 5'-CTC GCC TGT AGA GCT CAT GCC ACC GG-3'.

Western blot analysis. The cells were extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation (12,000 x g for 15 min at 4˚C) to obtain the supernatant. Protein concentrations were determined using a bicinchoninic acid kit (catalog no. BCA1-IKT; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Samples containing 60 µg of protein were separated on a 10% SDS-PAGE gel, transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.) with appropriate horseradish peroxidase-conjugated antibody (catalog no. sc-516102; dilution: 1:10,000; Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); KLF4 with the appropriate horseradish peroxidase-conjugated anti-KLF4 (catalog no. ab191086; dilution: 1:1,000) from Abcam. Following saturation with 5% (w/v) non-fat dry milk in TBS-Tween-20 (TBST), the membranes were incubated with the following primary antibodies for 1.5 h at 37˚C: Caspase‑3 (catalog no. sc‑271028; dilution: 1:1,000) and caspase-9 (catalog no. sc‑17784; dilution: 1:500) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); KLF4 (catalog no. ab75486; dilution: 1:1,000) and KLF5 (catalog no. ab191086; dilution: 1:1,000) from Abcam. Following three washes with TBST, the membranes were incubated with the appropriate horseradish peroxidase-conjugated antibody for 1 h at 37˚C and visualized using chemiluminescence (Thermo Fisher Scientific, Inc.).

Statistical analysis. The data from the experiments are reported as the mean ± standard deviation for each group. All statistical analyses were performed using PRISM version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**H2O2-induced cytotoxicity, apoptosis and ROS in HCMs.** The present study first examined the effect of H2O2 on HCM viability. The HCMs were exposed to H2O2 at different concentrations for 24 h, and the results revealed that incubation with H2O2 resulted in a significant decrease in the survival of HCMs in a dose-dependent manner (Fig. 1A). The HCMs were cultured for 24 h, and a TUNEL assay was performed to examine apoptosis. H2O2 incubation led to significant increase in cell apoptosis of the HCMs in a dose-dependent manner (Fig. 1B). A concentration-dependent increase in extracellular LDH activity was also observed in the HCMs exposed to H2O2 (Fig. 1C). To determine the role of ROS in H2O2-induced HCM injury, ROS concentrations were measured using flow cytometry with DCFH-DA. It was found that H2O2 incubation at concentrations ≥100 µM increased the ROS concentration in the HCMs, compared with the control group (Fig. 1D).

H2O2 regulates the expression of KLF4 and KLF5 in HCMs. Using RT-qPCR analysis, the present study examined the effects of 200 µM H2O2 on the mRNA expression of KLF4 and KLF5. The mRNA expression of KLF4 was upregulated at all time-points, with maximal expression at 6 h (Fig. 2A). KLF5 was also upregulated at 12 and 24 h in response to H2O2 stimulation, although the response was delayed, compared with that of KLF4 (Fig. 2B).

miR-506 and miR-124 are downregulated in H2O2-incubated HCMs. To examine differentially expressed miRs in response to H2O2 in HCMs, a microarray assay was performed using small RNA libraries generated using total RNA extracted from HCMs exposed to different concentrations of H2O2 for 12 h. The results demonstrated that miR-506 and miR-124 were significantly downregulated in HCMs with different concentrations of H2O2, compared with the control group (Fig. 3A). Therefore, the functional roles of miR-506 and miR-124 were determined in HCMs in the presence of H2O2. To further examine the roles of miR-506 and miR-124 in H2O2-incubated HCMs, the mimics and inhibitors of miR-506 and miR-124 were transfected into HCMs. The miR-506 and miR-124 mimics were found to be sufficient to increase the expression levels of miR-506 and miR-124, respectively, whereas the miR-506 and miR-124 inhibitors had the opposite effect. These results suggested that the mimics and inhibitors of miR-506 and miR-124 had effects in the HCMs (Fig. 3B and C).

miR-506 and miR-124 targets KLF4/5 in HCMs. The Bioinformatics analysis revealed that KLF4 and KLF5 RNA contained one conserved target site of miR-506 and miR-124 (Fig. 4A and B). To confirm this possibility, the wild-type sequence of KLF4 or KLF5 (KLF4-wild or KLF5-wild) or their mutant sequence (KLF4-Mut or KLF5-Mut), as shown in Fig. 4A and B, were subcloned into the pMIR luciferase reporter and were then co-transfected with the mimics of miR-506 and miR-124, or their miR-NC,
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into HCMs, respectively. Luciferase assays were performed 24 h following transfection. As shown in Fig. 4C and D, reduced luciferase activities were found in the KLF4-wild and KLF5-wild HCMs transfected with miR-506 or miR-124, compared with those in the NC group. By contrast, following transfection of miR-506 or miR-124 into KLF4-Mut or KLF5-Mut HCMs, no significant difference in luciferase activity was observed, compared with the NC group.

Overexpression of miR-506 and miR-124 inhibits apoptosis and ROS in H\(_2\)O\(_2\)-incubated HCMs. RT-PCR analysis was further used to observe the effect of miR-506 and miR-124 on the mRNA levels of KLF4 and KLF5. As shown in Fig. 5A and B, transfection with the miR-506 and miR-124 mimics did not significantly alter the mRNA expression of KLF4 or KLF5 in the HCMs. The ability of miR-506 and miR-124 to regulate the protein expression of KLF4 and KLF5 in HCMs was verified using western blot analysis, the results of which indicated that the overexpression of miR-506 or miR-124 inhibited the H\(_2\)O\(_2\)-induced upregulation of KLF4 and KLF5 in the HCMs (Fig. 5C). The protein expression of apoptosis-associated markers were also measured using western blot analysis. The results showed that caspase-3 and caspase-9, which are key caspase pathway proteins closely associated with cell apoptosis, were significantly downregulated in the cells overexpressing miR-506 or miR-124 in combination with H\(_2\)O\(_2\), compared with those exposed to H\(_2\)O\(_2\) only (Fig. 5D). It was also found that the overexpression of miR-506 or miR-124 reversed the H\(_2\)O\(_2\)-induced increase in ROS concentrations in the HCMs, respectively (Fig. 5E and F).

Figure 1. H\(_2\)O\(_2\)-induced cytotoxicity, apoptosis and ROS in HCMs. (A) HCMs were incubated with H\(_2\)O\(_2\) in different concentration for 24 h, and the cell viability was examined using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. (B) HCMs were incubated with H\(_2\)O\(_2\) for 24 h, and TUNEL staining was measured by flow cytometry. (C) LDH levels were measured following HCM exposure to H\(_2\)O\(_2\) for 24 h. (D) Intracellular ROS production was measured according to changes in the fluorescence intensity of DCF, the oxidized derivative of DCF-DA, when treated for 12 h with H\(_2\)O\(_2\). Values are expressed as the mean ± standard deviation (n=3 in each group). *P<0.05, **P<0.01 and ***P<0.001, vs. control group. HCMs, human cardiomyocytes; TUNEL, triphosphate nick-end labeling; LDH, lactate dehydrogenase; ROS, reactive oxygen species; DCF-DA, dichlorodihydrofluorescein diacetate.

Figure 2. H\(_2\)O\(_2\) regulates the expression of KLF4 and KLF5 in HCMs. mRNA expression levels of KLF4 and KLF5 are regulated by H\(_2\)O\(_2\) (200 µM) in human cardiomyocytes. mRNA expression of (A) KLF4 and (B) KLF5 were measured using reverse transcription-quantitative polymerase chain reaction analysis at different time points. Values are expressed as the mean ± standard deviation (n=3 in each group). *P<0.05, **P<0.01 and ***P<0.001, vs. control group. KLF, krüppel-like factor.
Discussion

The results of the present study demonstrated that H_2O_2 incubation resulted in a significant increase in the mRNA levels of KLF4 and KLF5, and decreases in the levels of miR-506 and miR-214 in HCMs. The post-translational mechanism of miR-506 and miR-214 regulated the protein expression of KLF4 and KLF5 in H_2O_2-induced HCM injury. In addition, the...
overexpression of miR-506 and miR-124 reversed H$_2$O$_2$-induced apoptosis and ROS levels in the HCMs. Therefore, it was concluded that the overexpression of miR-506 and miR-124 had a protective effect against H$_2$O$_2$-induced HCM injury by suppressing the expression of KLF4 and KLF5.

A previous study showed that H$_2$O$_2$, as a potent generator of ROS, induces apoptosis in neonatal rat cardiomyocytes (20). Consistent with this report, the present study found that H$_2$O$_2$ incubation resulted in a significant decrease in cell survival, and an increase in the apoptosis and production of ROS in HCMs. It was found that a post-translational mechanism of miRs was involved in H$_2$O$_2$-induced HCM dysfunction. Increasing evidence has shown that miRs are critical in myocardial ischemia, for example miR-19b is downregulated in the infarct area of myocardial ischemia-reperfusion in mice (1), the down-regulation of miR-320 suppresses cardiomyocyte apoptosis, and protects against myocardial ischemia and reperfusion injury (21), and miR-93 inhibits ischemia-reperfusion-induced cardiomyocyte apoptosis (22). In the cardiomyocytes of newborn mice, H$_2$O$_2$ upregulates the expression of miR-137 and induces cell apoptosis, whereas miR-137 loss-of-function protects against cell apoptosis (23). In the present study, microRNA microarray analysis demonstrated that H$_2$O$_2$ significantly downregulated the expression of miR-506 and miR-124, whereas the overexpression of miR-506 or miR-124 reversed H$_2$O$_2$-induced apoptosis and increased ROS levels in HCMs.

The present study also investigated the underlying molecular mechanisms of miR-506 and miR-124 in HCMs. As a by-product of AMI, H$_2$O$_2$ can induce oxidative stress-associated apoptosis in cardiomyocytes (3,24). In cardiomyocytes, at least 11 KLFs are expressed, which have differing affinities for precise sequences in various gene promoters (16). Cyclin D1 is identified as a direct transcriptional target for KLF13, which may account for the proliferation defects observed in embryos with downregulated levels of KLF13 (25). The overexpression of KLF4 in neonatal rat ventricular myocytes inhibits three cardinal features of cardiomyocyte hypertrophy (26). KLF5 contributes to pulmonary artery smooth muscle proliferation and resistance to apoptosis in human pulmonary arterial...
hypertension (14). These findings suggest that KLF families have a developmental role in cardiomyocytes. However, whether miR-506 and miR-124 regulate the expression of KLF4 and KLF5 in H$_2$O$_2$-induced cardiomyocyte injury has not been reported. In hepatocellular carcinoma (HCC) cells, KLF4 induces the expression of miR-506, which inhibits EMT-enhanced HCC growth and invasion, whereas the overexpression of antisense of miR-506 eliminates the inhibitory effect of KLF4 on HCC cell growth and invasiveness (27). The present study found that the KLF4 and KLF5 3'-UTR contains one conserved target site of miR-506 and miR-124, and the overexpression of miR-506 and miR-124 inhibited the H$_2$O$_2$-induced upregulation of KLF4 and KLF5 in HCMs.

In conclusion, the present study demonstrated the beneficial role of the miR-506/-124 target to KLF4 and KLF5 signaling for inhibiting H$_2$O$_2$-induced apoptosis and ROS levels in HCMs. Although the present study was limited to clinical experiments, the data obtained indicated that miR-506 and miR-124 may be considered as potential biomarker for diagnosing and evaluating the onset and development of AMI.

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