Role of the specific miRNAs on the reactive oxygen species stimulated cardiomyocytes

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Role of the specific miRNAs on the reactive oxygen species stimulated cardiomyocytes

Directed by Professor Byung-Chul Chang

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Hwan-Kyu Roh

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This certifies that the Doctoral Dissertation of Hwan-Kyu Roh is approved.

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"You might take Graduate program, right?"
Twenty years ago, my lifetime teacher Professor Meyun Shick Kang asked me the question. Even though I never had interest in obtaining Master or Doctor degree but I could not against my teacher’s word. I said, "Of course sir, I will". My long graduate course was started just like that.

My graduate course ended up without difficulties. After leaving the university, however, I had to live as a businessman, a practicing doctor, or civic activist. It became difficult to finish my doctor degree. Since that time, the burden on my shoulder of my life was too much for me to handle ; I wanted to give up many times. But Professor Byung-Chul Chang who my advisor and another teacher of my lifetime taught me that ‘the way of medical doctor itself is not giving up of passion to learn’. That coaching made it possible for me to finish Ph.D. thesis. I have countless deep respects and thanks to Professor Meyun Shick Kang for giving advices about the importance of the study and to Professor Byung-Chul Chang for encouraging every time whenever when I met the difficulties.

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My doctor degree which was from 26 years later since graduating the Medical School, I wish to make a conclusion of this letter to start to thanks to my dear parents who waited longtime for the release of the doctoral dissertation. At last time, I thanks to my wife who cheer me up all the time whatever I do, I wish to show my love to my son who always obedient and loving.

Hwan-Kyu Roh

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ABSTRACT

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Oxidative damage has been suggested to play a critical role in pathophysiology of various cardiovascular diseases. A staggering amount of evidences implicates that reactive oxygen species (ROS) is a common denominator in the development of most cardiovascular diseases. It has been reported that miRNAs play important roles in physiological regulation of cardiac biology as well as in pathological events such as hypertrophy, apoptosis, and heart failure. However, the study on microRNAs in ROS-induced cardiac apoptosis and hypertrophy has been rarely carried out. ROS regulates two face of cardiac myocyte phenotype: hypertrophy occurred at low levels (30 μM) and apoptosis occurred at higher levels (100–150 μM) of hydrogen peroxide (H_2O_2) in primary

cultured neonatal rat cardiomyocytes. Hypertrophy was determined by significant (≥30%) increase of cell size and hypertrophic markers, including α-MHC, MLC, NFATC1, ANP and BNP, and apoptosis was measured by cell viability, Annexin V / PI staining, and caspase-3 activity. In this study, miRNAs that are involved in ROS-mediated regulation of cardiomyocyte phenotype were elucidated. A set of miRNAs (miR-1, 26a, 1331, and 145) were identified to abound in cardiomyocytes. These miRNAs were abundantly expressed in neonatal and adult heart tissue. Moreover, H_2O_2-mediated up-regulation and down-regulation of specific miRNAs was confirmed and their regulation by H_2O_2 were determined. At a concentration of H_2O_2 that caused hypertrophy (30 μM) the expression of miR-1 was decreased and overexpression of miR-1 inhibited H_2O_2-induced hypertrophy. At a concentration of H_2O_2 that caused apoptosis (100 μM) miR-26a expression was increased while miR-26a mimic enhanced H_2O_2 -induced apoptosis. The results indicate that different spectrums of miRNAs affect H_2O_2-mediated cardiomyocyte hypertrophy and apoptosis.

Key words: cardiomyocytes, hypertrophy, apoptosis, miRNA, hydrogen peroxide

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I. INTRODUCTION

Ischemic heart disease - distinguished by reduced blood supply to the heart muscle - is the leading cause of death worldwide. Atherosclerosis, aortic stenosis, or valvular diseases can cause transient or prolonged cardiac ischemia. Depending on the cell type and the duration of the ischemic period, ischemia induces a broad range of cell responses including loss of adhesion and cell death.[1,2] During ischemic period, reactive oxygen species (ROS) including superoxide, H_2O_2 and hydroxyl radicals are found in the myocardium. ROS can act as signaling molecules to stimulate either hypertrophy or apoptosis in cardiomyocytes.[3-5] Many evidence suggested that ROS have been implicated in the pathophysiology of myocardial failure.[6-8] Various cell types within the heart,

including cardiomyocytes, endothelial cells, vascular smooth muscle cells, fibroblasts, and infiltrating inflammatory cells generate ROS.[9,10] Traditionally ROS have been viewed as destructive molecules because they induced cell death through damaging key cellular molecules such as DNA and lipids. But recently, ROS have been shown to contribute to cell proliferation, migration, and survival as well.

ROS has been shown to cause cell death in many experimental systems. Fatal arrhythmia, myocardial infarction, cardiomyopathy, and heart failure have been reported to involve cell death.[4,5,11] Oxidative stress is well known to induce cardiomyocyte apoptosis either indirectly, through damage of DNA, lipids, and proteins, or more directly via the activation of pro-apoptotic signaling molecules such as apoptosis signal-regulating kinase 1 (ASK-1), JNK, extracellular signal-regulated kinase (ERK) 1/2, and p38 MAPK.[6,7,10,12] In cardiomyocytes, a small increase in ROS due to inhibition of superoxide dismutase (SOD) causes hypertrophy, whereas a larger increase due to more complete inhibition of SOD causes apoptosis.[12-14] Similarly, direct addition of ROS leads to apoptosis in NRVM (neonatal rat cardiac myocytes) and H9C2 cells, while hypertrophy has been reported in cells that survived ROS exposure.[2,5,15] Other studies using antioxidants suggest that ROS are involved in mediating the apoptotic effects of pathophysiologically-relevant stimuli such as

mechanical strain, neurohormonal stimulation and inflammatory cytokines.[16-19] While the source(s) of ROS activated by these pathophysiological stimuli is not yet known, several studies have investigated the mechanism by which ROS activate apoptotic phenotypes in myocytes.[20,21] ROS are known to activate ERK1/2, p38 kinase, JNK, and Akt in many cell types. For example, activation of ERK1/2 in the absence of JNK, p38 kinase, or Akt activation occurs in ARVM (adult rat cardiac myocytes) in an ROS-dependent manner after a-adrenergic receptor stimulation, leading to apoptosis. In contrast, in ARVM with high concentrations of H_2O_2 cause apoptosis and JNK activation and overexpression of a dominant-negative SEK1 construct inhibits both JNK activation and apoptosis. H_2O_2 activates ERK1/2 and JNK in a concentration-dependent manner.[3,22-24] Taken together, these studies led to our hypothesis that differential, concentration-dependent activation of kinase cascades determines whether oxidative stress leads to myocyte apoptotic cell death.

A number of studies have investigated the role of NADPH oxidase-derived ROS in cardiac hypertrophy.[6,25-29] An abnormal thickening of the heart muscle occurs in response to increased blood pressure that preserves myocardial wall stress, chamber size, and contractile function. Cardiac hypertrophy, which is a risk factor for cardiovascular disease, can be induced by a number of stimuli,

like long-term exposure to ROS, and if untreated it can lead to cardiomyopathy and heart failure. Cardiac hypertrophy is an end point of many heart diseases. Under pathological conditions, cardiac hypertrophy is often asymmetric and does not result in an increase in cardiac output. Sustained hypertrophy can lead to cardiomyopathy and is a transition to heart failure.[5,6,12-14] The causes of pathological cardiac hypertrophy beyond hypertension or hemodynamic overload remain unknown. The fact that many heart diseases involve both oxidative damage and cardiac hypertrophy suggests a possible cause and effect relationship. Under many circumstances, cell death by apoptosis in the heart is observed concurrently with cardiomyocyte hypertrophy.[3,5] For example, both hypertrophy and cell loss is observed in the hearts from aged individuals and from spontaneously hypertensive rats. Ischemia–reperfusion, hypoxia –reoxygenation, and work overload can cause both hypertrophy and apoptosis. These observations suggest the possibility that oxidants can trigger both apoptosis and hypertrophy at the cellular level.

MicroRNAs are small (21-25 nucleotide) and highly conserved non-coding RNA molecules. There are estimated to be as many as 1000 miRNAs encoded by the human genome. MicroRNAs regulate gene expression by binding to the 3’-untranslated regions (3’-UTRs) of specific mRNAs. There are also repressed mRNA targets through binding sites in 5’-UTRs or coding regions. Recently,

the study on microRNAs renovated our understanding about the gene regulation in cardiac physiology and pathology.[30] The cardiac-specific knockout of Dicer, an endonuclease critical for processing of pre-miRNAs into mature miRNAs, leads to rapidly progressive dilated cardiomyopathy, heart failure, and postnatal lethality.[31,32] Targeted deletion of the muscle specific miRNA reveals numerous functions in the heart including regulation of cardiac morphogenesis, electrical conduction, and cell-cycle control.[33-36] These results suggest that miRNAs play critical roles in maintaining normal cardiac structure and function.

Cardiomyocyte apoptosis is related to cardiac disorders such as myocardial infarction, cardiomyopathy, cardiac hypertrophy, and anthrax cycline-induced cardiotoxicity. The growing evidence demonstrates that miRNAs can regulate apoptosis.[11,13,17,20] Because, the adult heart has only limited regenerative capacities, an excessive loss of cardiomyocytes after myocardial ischemia or infarction can significantly decrease cardiac performance. Apoptosis can be initiated by a variety of miRNAs. In response to ischemia/reperfusion, miR-320 expression is decreased in the heart. miR-206 prevents apoptosis by targeting notch3.[37]. miR-17-92 cluster is a novel target for p53-mediated transcriptional repression under hypoxia, and overexpression of miR-17-92 cluster markedly inhibits hypoxia-induced apoptosis.[38] In addition, p53 is also regulated by miR-125b.[39] Although the functions of the above miRNAs in apoptosis have

been tested in other cell types, it can be speculated that they may have an impact in cardiomyocyte apoptosis.

In addition, many studies on specific miRNAs in animal models and human heart have identified essential roles of miRNAs in the development of cardiac hypertrophy. Cardiac overexpression of miR-195 was able to increase the left ventricular wall size, upregulate the expression of ANP, BNP and β-MHC and reduce cardiac output.[40] miR-1 regulates cardiomyocyte growth responses by negatively regulating the expression of Mef2a and Gata4, the key components of calcium signaling pathways that are necessary for agonist-induced cardiomyocyte hypertrophy.[41] While bioinformatics analysis and several lines of evidence indicate that Rhoa, Cdc42, and NELFA/Whsc2 are regulated by miR-133 in cardiac hypertrophy.[42] Another upregulated miRNA in mice and human hypertrophic hearts is miR-208. miR-208 regulates thyroid hormone-associated protein 1 and myostatin 2, negative regulators of muscle growth, and hypertrophy to induce hypertrophic growth.[43]

In this study, we assessed estimating miRNAs in H_2O_2-induced cardiomyocyte response. Our studies that several specific miRNAs detected in cardiomyocytes, and these miRNAs expressions were modulated by H_2O_2. We aimed to inhibit hypertrophy by miR-1, and induce apoptosis via miR-26a in H_2O_2 stimulated cardiomyocyte.

II. MATERIALS AND METHODS

1. Isolation of rat ventricular cardiomyocytes

Neonatal rat cardiomyocytes were isolated and purified by a previously described method. Briefly, hearts of 1−2 day old Sprague Dawley rat pups are dissected. Then the ventricles were washed with Dulbecco's phosphate-buffered saline solution (pH 7.4, Gibco BRL) lacking Ca^{2+} and Mg^{2+}. Using micro-dissecting scissors, hearts are minced to pieces of approximately 1 mm_3 and treated with 10 ml of collagenase II (0.12 mg/ml, 280 units/mg, Gibco BRL) for 5 min at 37°C. The supernatant is then removed and the tissue is treated with fresh collagenase II solution for an additional 5 min. The cells in the supernatant were transferred to a tube containing cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL). The tubes were centrifuged at 2000 rpm for 3 min at room temperature, and the cell pellets were resuspended in 5 ml of cell culture medium. The above procedures were repeated 7−9 times until little tissue was left. The cell suspensions were then collected and incubated in 100 mm tissue culture dishes for 2 hr to reduce fibroblast contamination. The non-adherent cells were collected and seeded to achieve a final concentration of 5×10^5 cells/ml. After incubation for 4−6 hr, the cells were rinsed twice with cell

culture medium and 0.1 μM BrdU was added. The cells were then cultured at 37°C in a CO_2 incubator with 10% (v/v) FBS.

2. Treatment of Cells with Hydrogen Peroxide

1 day after isolation, cardiomyocytes were further incubated with α-MEM containing 1% FBS. The cells were then rinsed twice with PBS. H_2O_2, at various concentrations, was added to the medium and incubated with the cells for an indicated time. For negative controls, cells were incubated with the medium alone for equivalent amounts of time.

3. Cell Survival Assay

Cell survival was measured using the CCK assay kit (Dojindo, Japan). Cardiomyocytes were seeded at the density of 5×10^3 cells per well of 96-well plates. These cells were incubated with varying concentrations of H_2O_2 for 6 hr in 0.1% a-MEM. CCK assay kit was diluted with 0.1% a-MEM then added 100ul of it were added to each well and incubated for 2 hr at 37°C. The absorbance of the samples was measured at 450 nm using a microplate reader against a background control.

4. Measurement of Caspase-3 Activity

Relative caspase-3 activity was determined using the Homogeneous Caspases Assay, fluorimetric according to the manufacturer's instructions (Roche). Cells were cultured in microplates and apoptosis was induced to activate caspases. Caspase substrate, prediluted in incubation buffer, was added and incubated for 2 hr at 37°C. During this incubation period, the incubation buffer lysed the cells. Free R110 is determined fluorimetrically at max = 521 nm. The developed fluorochrome was proportional to the concentration of activated caspases and could be quantified by a calibration curve.

5. Annexin V/PI Staining

For immunocytochemistry staining, cardiomyocytes were cultured in a 4-well culture dish (1×10^4 cells/well) and pretreated with a control medium or media containing varying concentrations of H_2O_2 for 6 hr. The dishes were washed with ice-cold PBS for 5 min and fixed with 4% formalin for 10 min. After being blocked with the Annexin-binding buffer at 500 μl, the cells were stained with Annexin V-FITC at room temperature in the dark for 15 min. The dishes were washed with ice-cold PBS and stained with PI and diluted with Annexin-binding buffer at room temperature in the dark for 5 min. The number of Annexin V / PI-positive

cells was counted under a fluorescence microscope.

6. Western blot

Cardiomyocytes were washed once in PBS and lysed in lysis buffer (cell signaling, Beverly, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM PMSF. Protein concentrations were determined using the BCA protein assay kit (Pierce). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to the PVDF membrane (Millipore Co, Bedford, MA, USA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% non-fat dried milk for 1 hr at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibodies for 1 hr at room temperature or for overnight at 4°C. The membrane was washed three times with TBS-T for 10 min and then incubated for 1 hr at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using the

Photo-Image System (Molecular Dynamics, Uppsala, Sweden).

7. RT-PCR analysis

The expression levels of various proteins were analyzed by the reverse transcription polymerase chain reaction (RT-PCR) technique. Total RNA was prepared by Ultraspect^{TM}-II RNA system (Biotecx Laboratories, Inc., USA), and single-stranded cDNA was then synthesized from isolated total RNA by Avian Myeloblastosis virus (AMV) reverse transcriptase. A 20 μl reverse transcription reaction mixture containing 1 μg of total RNA, 1X reverse transcription buffer (10 mM Tris–HCl, pH 9.0, 50mM KCl, 0.1% TritonX-100), 1mM deoxynucleoside triphosphates (dNTPs) 0.5 U of RNase inhibitor, 0.5 μg of oligo(dT)15, and 15 U of AMV reverse transcriptase was incubated at 42 °C for 15 min, heated to 99 °C for 5 min, and then incubated at 0–5 °C for 5 min. PCRs were performed for 35 cycles with 3′ and 5′ primers based on the sequences of various genes. GAPDH was used as the internal standard. The signal intensity of the amplification product was normalized to its respective GAPDH signal intensity.

8. MicroRNA transfection

Transfections of miRNA were performed using silentfact reagent. Briefly, neonatal rat cardiomyocytes were seeded at the density of 2×10^5 cells per 35mm culture plate. Silentfact reagent was diluted with Opti-MEM and combined with indicated miRNA mimic for each plate. The miRNA and silentfact reagent were added to each plate containing fresh medium and cells. After 4 hr incubation in a CO_2 incubator at 37°C, the medium was changed to conditioned α-MEM.

9. Real Time-PCR

Total RNA was prepared by Ultraspect™-II RNA system (Biotecx Laboratories, Inc., USA), and single-stranded cDNA was then synthesized from isolated total RNA by Avian Myeloblastosis virus (AMV) reverse transcriptase. In brief, 10 ng purified total RNA was used for reverse transcriptase (Taqman® MicroRNA Reverse Transcriptase Kit, Applied Biosystems) in combination with Taqman® MicroRNA Assays for quantification of specific miRNAs and U6 control transcripts, according to the manufacturers' conditions. Amplification and detection of specific products were performed in a MyIQ single-color real-time PCR system (Bio-Rad) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The threshold cycle (Ct)

of each target gene was automatically defined, located in the linear amplification phase of the PCR, and normalized to control U6 (ΔCt value). The relative difference in expression levels of each miRNA in proliferating and apoptosis Cardiomyocytes (ΔΔCt) was calculated and presented as fold induction (2-ΔΔCt).

10. Statistical analysis

Results are expressed as means ± SEM. Statistical differences were determined by Student's t-test. P-value < 0.05 was considered statistically significant.

III. RESULTS

1. Effect of exogenous H_2O_2-stimulated rat neonatal ventricular cardiomyocytes in cell survival

To investigate the influence of exogenous H_2O_2 on the survival and proliferation of rat neonatal ventricular cardiomyocytes, the cells were cultured with various concentrations of H_2O_2. Cardiomyocytes were exposed to increasing concentrations of H_2O_2 ranging from 10 to 200 μM for 6 hr and viability was assessed by CCK assay (Fig. 1). There was no change in cell viability at H_2O_2 concentrations of 10 - 30 μM. On the other hand, at H_2O_2 concentrations of 100 -150 μM, cell viability decreased slightly.

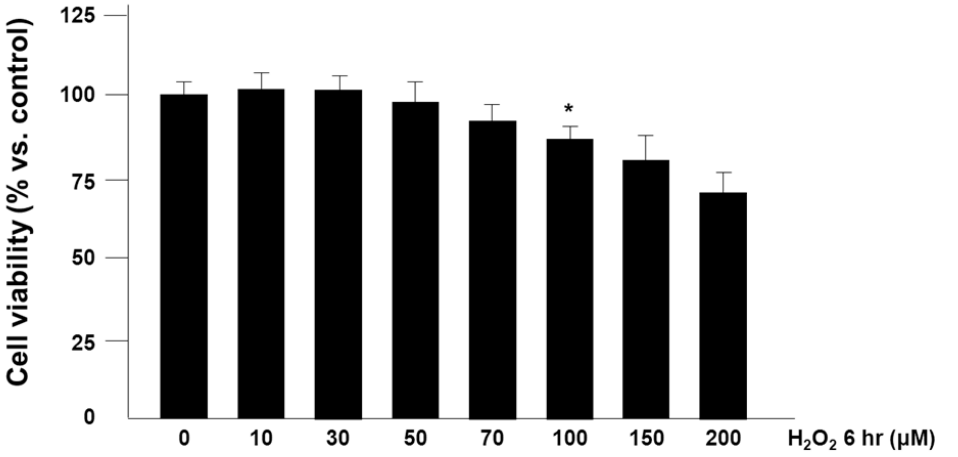


Figure 1. Concentration-dependent effect of H_2O_2 on viability in cardiomyocytes. Cardiomyocytes were exposed to various concentration of H_2O_2 for 6 hr and viability was assessed by CCK assay. *$p<0.001$ compared with control.

2. Effect of H_2O_2 on cardiac hypertrophy

Cardiomyocyte hypertrophy is typically characterized by cell enlargement and increase in total sarcomeric myosin heavy chain. Here, we sought to determine the impact of H_2O_2-induced ROS expression on cell hypertrophy response by two complementary approaches, measurement of a monoclonal antibody staining sarcomeric myosin heavy chain immuno-florescent, expression of hypertrophic genes. Exposure of cardiomyocytes to H_2O_2 led to an increase in cell size as assessed by myosin staining intensity (Fig. 2A). H_2O_2 at concentrations of 30 μM increased expression of hypertrophic genes, including α-MHC, MLC, NFATC1, ANP, and BNP (Fig. 2B).

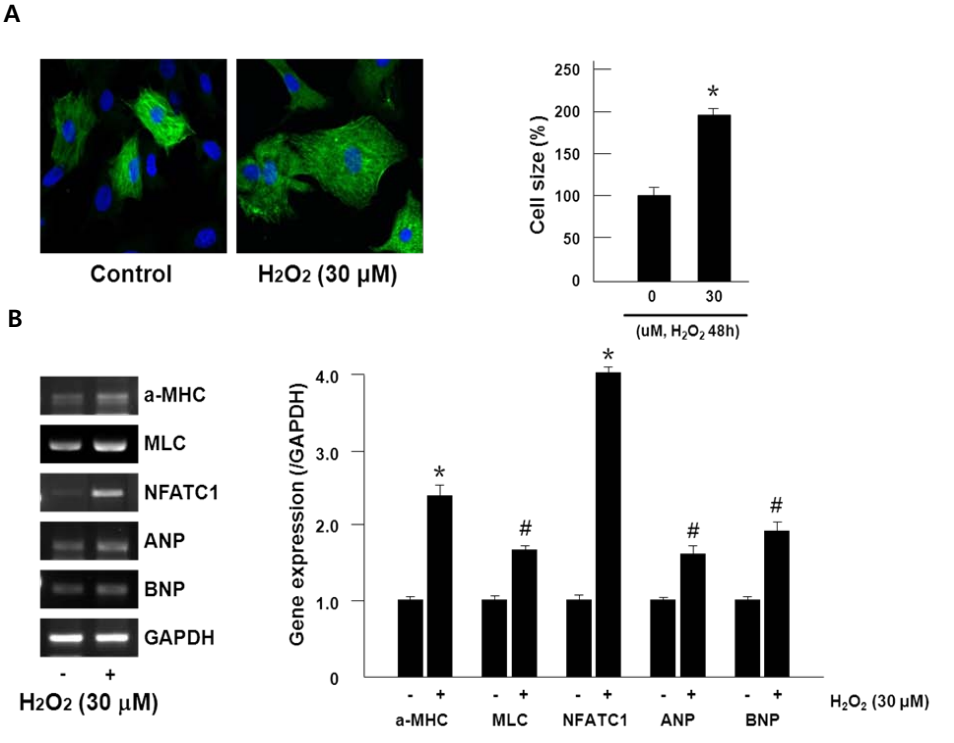


Figure 2. Treatment of H_2O_2 induces hypertrophy in cardiomyocytes. A. Cardiomyocytes stained for cardiac markers cardiac myosin heavy chain on H_2O_2 30 μM in 48 hr. Nuclei were stained blue by DAPI. B. Expression of cardiac markers in cardiomyocytes for H_2O_2 30 μM. *p<0.001, #p<0.05 compared with normal.

3. Higher concentrations of H_2O_2 stimulate apoptosis

H_2O_2 concentrations of 10 - 30 μ did not affect the frequency of apoptosis of cardiomyocytes as assessed by Annexin V (An V) and PI co-staining in unfixed cells. At 100 μM (high concentration), however, AnV staining was increased in the absence of nuclear PI staining, consistent with apoptosis (Fig 3A). In addition, we found that high concentration of H_2O_2 significantly increased caspase-3 activity by 75% compared with that of negative controls (Fig 3B). Apoptotic cell death is known to be triggered by extrinsic, receptor-mediated, and intrinsic (Bax and Bcl-2) mitochondria-mediated signaling pathways that induce death-associated proteolytic and/or nucleolytic activities. We observed that H_2O_2 increased the expression of Bax and active caspase-3, but significantly decreased the expression of anti-apoptotic Bcl-2 compared with that of the controls (Fig. 3C,D). These results suggest that H_2O_2 induces or enhances cardiac apoptosis through intracellular ROS overload.

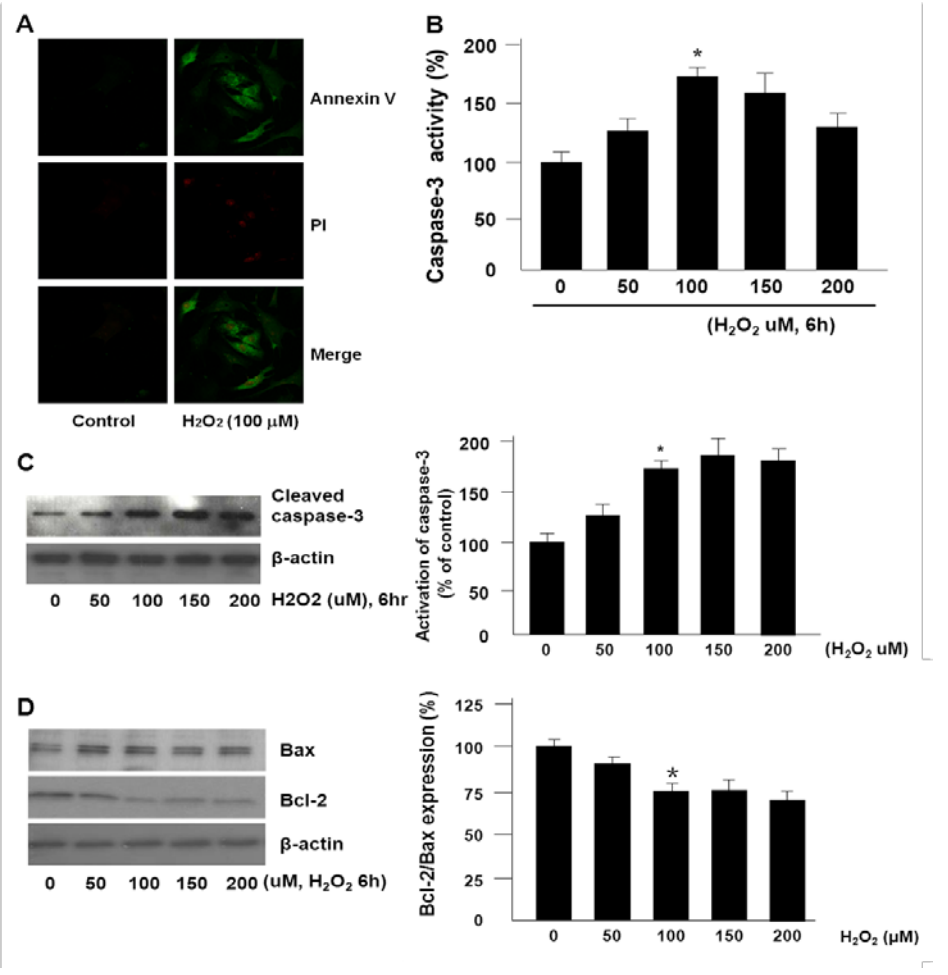


Figure 3. Concentration-dependent effect of H_2O_2 on apoptosis in cardiomyocytes. A. Cardiomyocytes were exposed to various concentrations of H_2O_2 for 6 hr, and viability was assessed by AnV and PI staining. B, C. Cardiomyocytes were treaeted with H_2O_2 for 6 hr. Caspase-3 activity and cleaved caspase-3 level were determined in cardiomyocytes through caspase-3 activity kit and western blotting. D. Expressions of Bax and Bcl-2 in H_2O_2-induced cardiomyocytes. The cells were treated with various concentrations of H_2O_2 for 6 hr. β-actin is loading control. *p<0.001, compared with normal.

4. Expression profile of miRNAs in cardiomyocytes

Real-time PCR showed high expression of miR-1, 26a, 133a and 145 in normal cardiomyocytes (Fig. 4A). We also evaluated miRNA expression profiles of neonatal and adult heart samples (Fig. 4B,C). Overall, miR-1, miR-26a, miR-133a, and miR-145 have often been found to be upregulated in heart tissue when compared with neonatal cardiomyocytes.

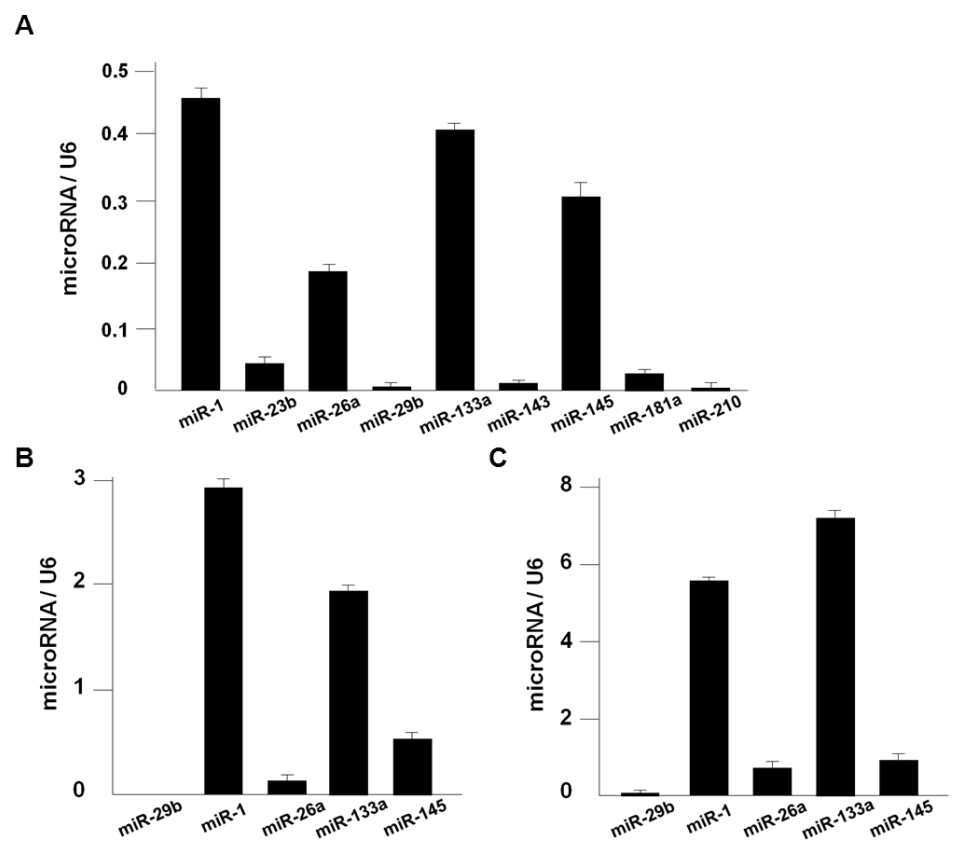


Figure 4. Expression of microRNAs in cardiomyocytes. A. Expression of selected miRNAs in rat adult heart. B, C. miRNAs in neonatal cariomyocytes and adult cardiomyocytes parallels miRNA expression. miRNA levels were determined by qRT-PCR. U6 was used for normalization.

5. The effect of H_2O_2 on specific miRNAs expression in cardiomyocytes

To investigate the miRNAs expression in cardiomyocytes in response to ROS, cardiomyocytes were incubated with H_2O_2. Cultured neonatal rat cardiomyocytes were treated with H_2O_2 30 μM for 48 hr. miRNA levels were determined by real-time PCR (Fig. 5A). miR-1 was significantly decreased in 30 μM H_2O_2 compared with control cardiomyocytes in time-dependent manner (Fig. 5B). To assess the expression of miRNAs by apoptotic H_2O_2 concentration, short-time exposure (6 hr) of cardiomyocytes to H_2O_2 (100 μM) treated, expression of miRNAs were determined by real-time PCR (Fig. 5C). miR-26a was significantly increased in H_2O_2 compared with control cardiomyocytes in dose-dependent manner (Fig. 5D). These results indicated that expression of specific miRNAs was modulated in H_2O_2-induced cardiomyocyte hypertrophic or apoptotic models.

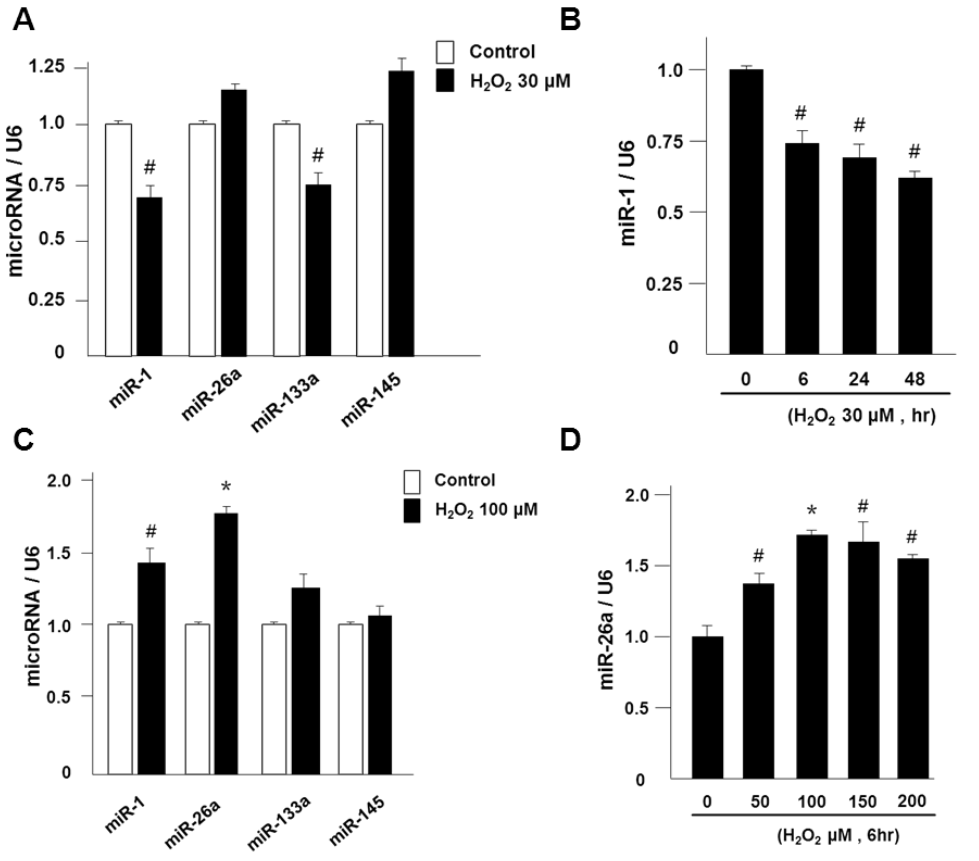


Figure 5. The effect of H_2O_2 on miRNA expression in cultured cardiomyocytes. A. Real-time RT-PCR analysis showing up/down regulated miRNAs expression in low concentration H_2O_2-induced cardiomyocytes compared to controls. B. Time-dependent treatment of H_2O_2 30 μM in cardiomyocytes showed that decreased miR-1 expression. C. Real-time RT-PCR analysis showing increased miRNAs expression in high concentration H_2O_2-induced cardiomyocytes compared to controls. D. Expression of miR-26a increased cardiomyocytes with dose-dependent treatment of H_2O_2 for 6 hr. *$p<0.001$, #$p<0.05$ compared with normal.

6. The effect of miR-1 on H_2O_2-induced cardiac hypertrophy

To gain further insight into the possible functional roles of miRNA-1 in hypertrophy, miR-1 levels in cardiomyocytes were modulated by transfecting the cells with miR-1 mimic. A negative control miRNAs mimic was used as a control. Cardiomyocytes transfected with miR-1 mimic significantly increased the expression of miR-1 (Fig. 6A). Increased cell size as well as elevated mRNA levels of α-MHC, MLC, NFATC1, ANP and BNP were observed in cultured cardiomyocytes in H_2O_2 for 48 hr. Using cardiomyocyte cell surface area analysis, our studies showed that up-regulation of miR-1 in H_2O_2-induced cardiomyocyte and significantly decreased cell size of cardiomyocytes (Fig. 6B). More importantly, we also found that overexpression of miR-1 cardiomyocytes markedly decreased α-MHC, MLC, NFATC1, ANP, and BNP mRNA expression level (Fig. 6C). These results suggested that up-regulation of miR-1 was sufficient to inhibit hypertrophy of cardiomyocytes.

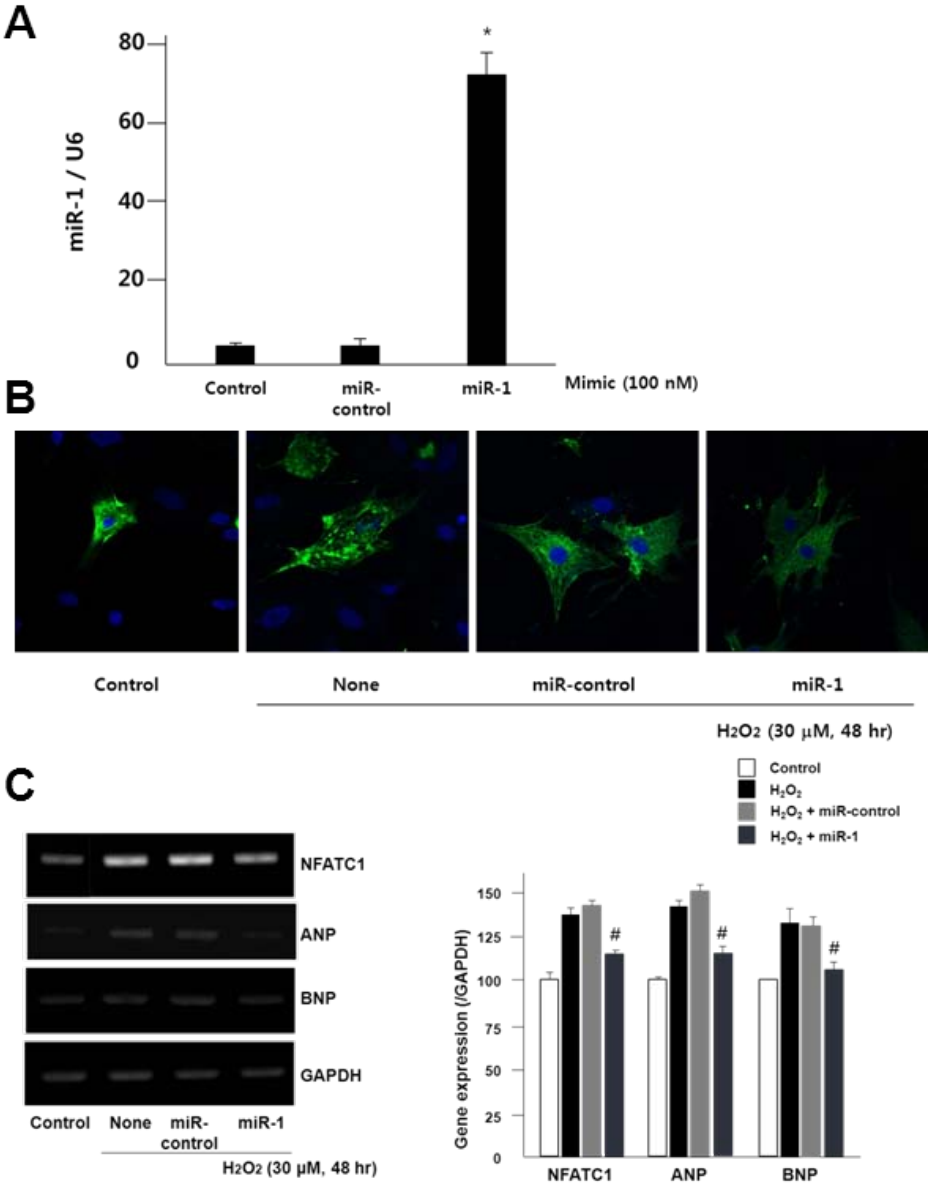
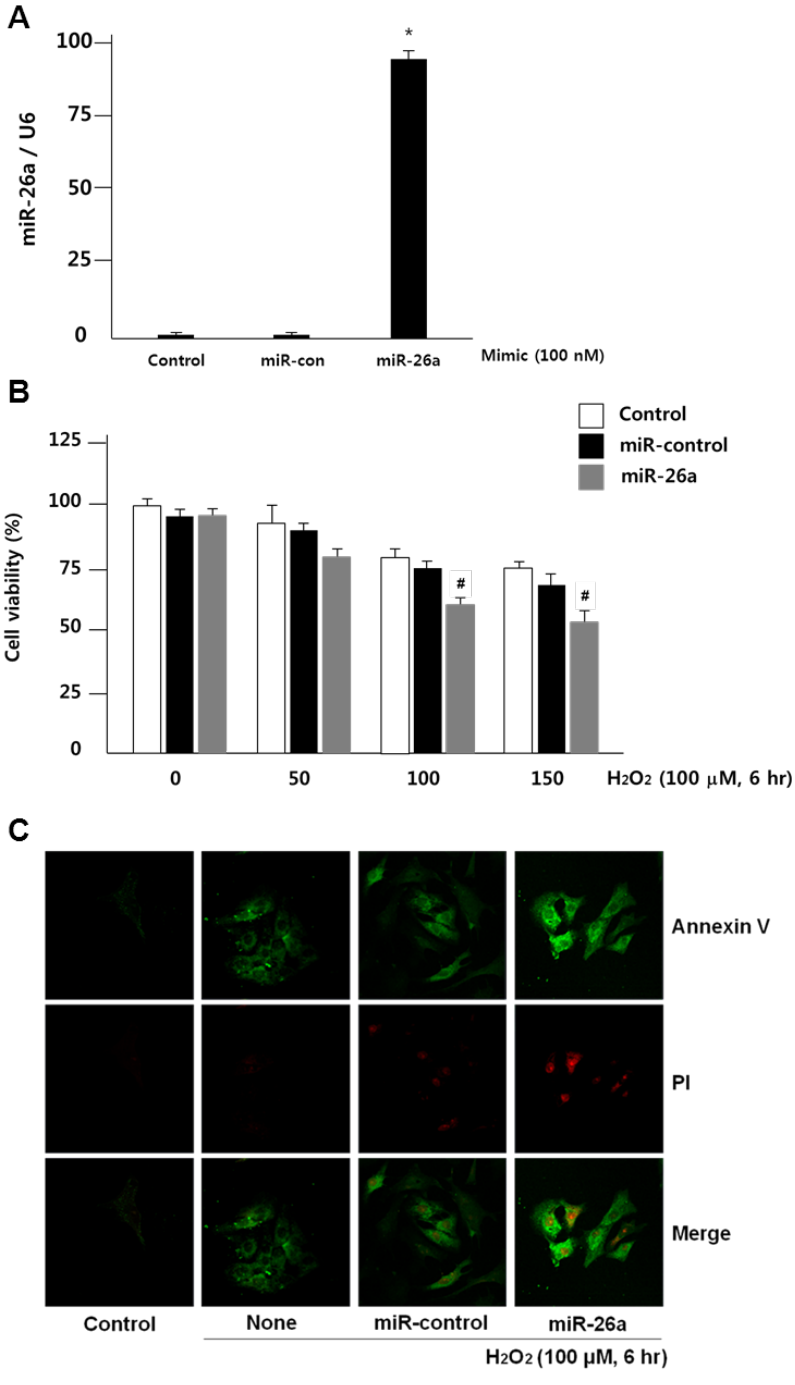

A
80
60
40
20
0
miR-1 / U6
*
Control
miR-control
miR-1
Mimic (100 nM)
B
Control
None
miR-control
miR-1
H2O2 (30 μM, 48 hr)
C
NFATC1
ANP
BNP
GAPDH
Control
None
miR-control
miR-1
H2O2 (30 μM, 48 hr)
Control
H2O2
H2O2 + miR-control
H2O2 + miR-1
Gene expression (/GAPDH)
150
125
100
75
50
25
0
#
NFATC1
ANP
BNP

Figure 6. The roles of miRNA-1 in hypertrophy. A. Cultured rat cardiomyocytes transfected with miR-control, miR-1 mimic. The data are averaged from normal cardiomyocytes. B. Cell size decreased by miR-1 transfection in H_2O_2 stimolation. Representative images of cardiomyocytes stained with anti- sarcomeric myosin heavy chain (green) and DAPI (blue) are shown. C. Representative mRNA expression of NFAT-1, ANP and BNP is analyzed RT-PCR. *$p<0.001$, compared with normal. #$p<0.05$, compared with H_2O_2 treated cardiomyocytes.

7. The effect of miR-26a on H_2O_2-induced cardiac apoptosis

To determine the role of miR-26a in apoptosis, cardiomyocytes were transfected with miR-26a mimic. The level of miR-26a was analyzed 4 hr after transfection (Fig. 7A). All measurements were performed at this time in order to exclude any influences from transfection time. Cardiomyocytes were incubated with H_2O_2 (50-200 μM) for 6 hr, cell survival was determined by CCK assay. Consistent with previous studies, high concentration of H_2O_2 treatment decreased survival in cardiomyocytes compared with the control. However, these effects of H_2O_2 on survival were significantly more decreased by miR-26a mimic (Fig. 7B). Apoptosis was then determined by Annexin V / PI staining, caspase-3 activity, and detection of pro-apoptotic molecules. To demonstrate the role of miR-26a further, we introduced miR-26a mimic into cardiomyocytes and then incubated these cells in H_2O_2 for 6 hr. These effects of H_2O_2 on apoptosis were significantly increased by miR-26a mimic (Fig. 7C-F). Furthermore, overexpression of miR-26a decreased survival in normal cardiomyocytes (Fig. 8). Thus, these results indicate that miR-26a promotes apoptosis in H_2O_2-stimulated cardiomyocytes.


A
100
75
50
25
0
miR-26a / U6
*
Control
miR-con
miR-26a
Mimic (100 nM)
B
Control
miR-control
miR-26a
125
100
75
50
25
0
Cell viability (%)
#
#
0
50
100
150
H2O2 (100 μM, 6 hr)
C
Annexin V
PI
Merge
Control
None
miR-control
miR-26a
H2O2 (100 μM, 6 hr)

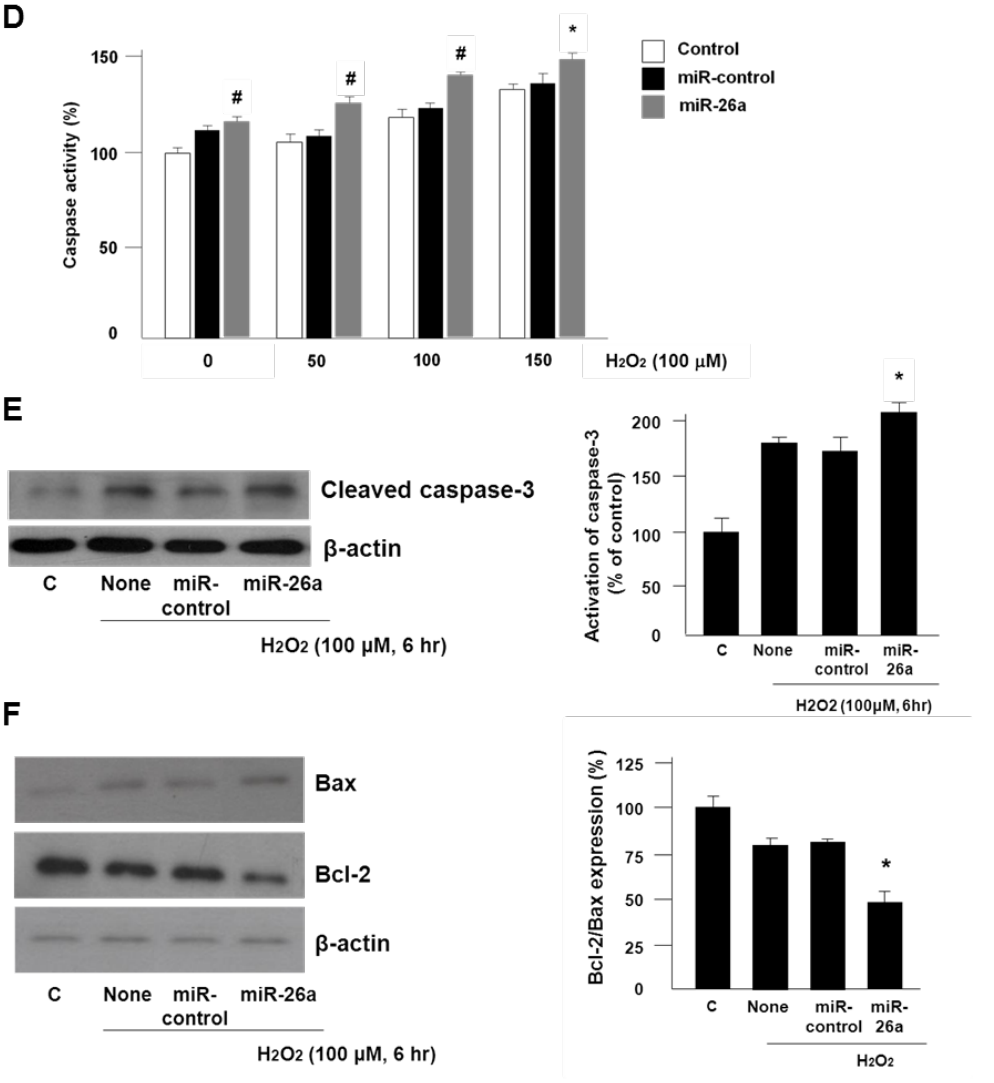

D
Caspase activity (%)
150
100
50
0
#
#
#
*
Control
miR-control
miR-26a
0
50
100
150
H2O2 (100 μM)
E
Cleaved caspase-3
β-actin
C
None
miR-control
miR-26a
H2O2 (100 μM, 6 hr)
Activation of caspase-3 (% of control)
200
150
100
50
0
*
C
None
miR-control
miR-26a
H2O2 (100μM, 6hr)
F
Bax
Bcl-2
β-actin
C
None
miR-control
miR-26a
H2O2 (100 μM, 6 hr)
Bcl-2/Bax expression (%)
125
100
75
50
25
0
*
C
None
miR-control
miR-26a
H2O2

Figure 7. The effect of miRNA-26a on H_2O_2-induced cardiomyocytes apoptosis. Cultured rat cardiomyocytes pre-treated with miR-control or miR-26a mimic. Then, Cells were treated with H_2O_2 for 6 hr. A. miR-26a levels without and with transfection of miRNA mimic. The data are averaged from normal cardiomyocytes. B. The effect of miR-26a on H_2O_2-induced apoptosis. C. Representative AnV and PI stained cell photographs, and their merged photographa from cells treated with miR-control, miR-26a mimic. D, E. Cardiomyocytes were transfected with miR-26a mimic or miR-control as a control, and then incubated with H_2O_2 for 6 hr. Caspase-3 activity and cleaved caspase-3 were determined in cardiomyocytes. F. Western blot was performed to determine Bax and Bcl-2 proteins in cardiomyocytes. *$p<0.001$, #$p<0.05$; compared with normal.

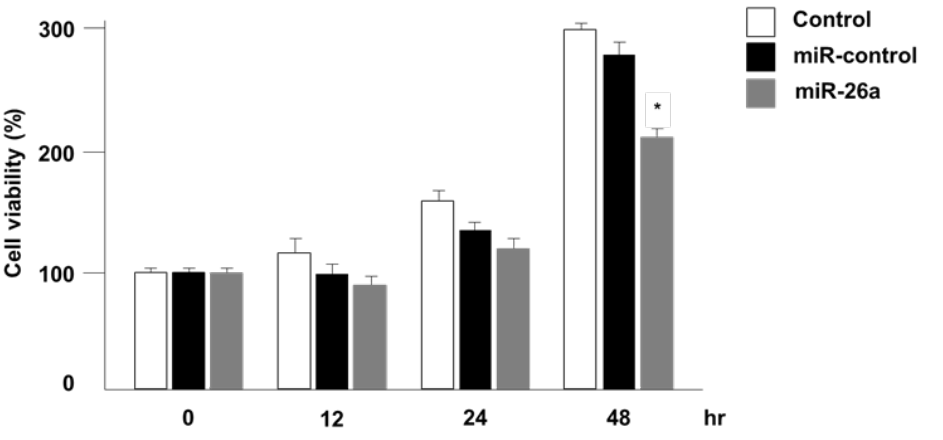


Figure 8. Time-dependent effect of miR-26a on viability in cardiomyocytes. Cultured rat cardiomyocytes treated with negative miRNA, miR-26a mimic (100 nM). The effect of miR-26a on normal cardiomyocyte viability. *p<0.001, compared with normal.

IV. DISCUSSION

The results of the present studies demonstrate the following (1) H_2O_2 caused concentration-dependent effects on cardiomyocyte phenotype, resulting in hypertrophy at low concentrations and apoptosis at higher concentrations; (2) specific microRNAs (miR-1, miR-26a, miR-133a and miR-145) were expressed in cardiomyocytes and neonatal and adult hearts, and these miRNAs were modulated by H_2O_2 in cardiomyocytes; (3) miR-1 has an anti-hypertrophic effect in H_2O_2-mediated cardiomyocyte hypertrophy; (4) induction of miR-26a expression promotes apoptosis in H_2O_2-stimulated cardiomyocytes.

Reactive oxygen species (ROS) play an important role in many heart diseases. ROS function as signal transduction intermediates to induce transcription factor activation, gene expression, cell growth, and apoptosis.[3,5] The concentration-dependent hypertrophic and apoptotic phenotype induced by H_2O_2 in NRNM is similar to what previous reports showed in ARVM and H9C2.[7,21] Hypertrophy is a complex process involving cell size increase as well as changes in gene expression. These results show that mild doses of H_2O_2 promotes hypertrophy determined by cell size increase and changes in hypertrophic markers (α-MHC, MLC, NFATC1, ANP and BNP).[26,28] Stimulation with Ang II increased protein synthesis, sarcomere organization, and the re-expression of embryonic genes, such as ANP and MLC-2v, in rat cardiac cardiomyocytes. The hypertrophic

effect of Ang II is mediated by ROS. Isoproterenol induced expression of α-MHC, MLC-2v, and BNP mediated hypertrophic growth.[26] Many studies have demonstrated that ischemia/reperfusion generates ROS and that ROS induce various cardiomyocyte abnormalities, including cell death.[30]. It has previously reported that ROS strongly induces apoptosis in cultured cardiac myocytes. Also, this result supports that ROS promotes cardiomyocyte apoptosis, through H_2O_2 induced Annexin V positive cell population, caspase-3 activity, cleaved caspase-3, and Bax/Bcl2 ratio.[18]

In the cardiovascular field, miRNAs are now acknowledged as fundamental factor in regulating the expression of genes that governs physiological and pathological myocardial adaptation to stress. Given that mature miRNAs are abundant, easily measurable, and relatively stable in the plasma/serum.[44,45] Peripheral blood may be a particularly attractive source for the routine clinical measurement of these molecules in diseased settings. Recently, several reports have revealed important roles of miRNAs in cardiac hypertrophic growth and heart failure. During the adaptive response of the heart to stress stimuli, microarray analyses have shown upregulated, downregulated, or unchanged miR expression when compared to normal heart.[46] Moreover, differential regulation of subsets of miRNAs has been shown to characterize various etiologies. We further identified that miR-1, miR-26a, miR-133a, and miR-145

were the most abundant families in normal neonatal cardiomyocytes. These results were similar to that of the neonatal heart and the adult heart.[35,47,48] MicroRNAs expression profiles analyzed by northern blot analysis, microarray, or real time PCR. Although RNA is not amplified to a similar manner, real-time PCR represents yet another technological leap forward that has opened up new and powerful applications for researchers throughout the world. This is in part because the enormous sensitivity of PCR has been coupled to the precision afforded by "real-time" monitoring of PCR products as they are generated. [49]

One of the earliest changes observed after applying pressure overload on a mouse heart was the down regulation of miR-1, the increase in cardiac mass, or the contractile dysfunction. Consistently, miR-1 is down-regulated in genetic models of cardiac hypertrophy, including the calcineurin and Akt transgenic hearts. In humans, miR-1 is down-regulated in the hypertrophied left ventricle of acromegalic patients and in those with aortic stenosis, in which the ejection fraction is preserved (50–60%). Both in-vitro and in-vivo data suggest that the reduction in miR-1 is required for an increase in cell mass. IGF-1 is a member of a family of proteins involved in mediating growth and development. IGF-1 stimulation and transfection of Akt controls miR-1 expression. As expected, overexpression of Akt in neonatal cardiomyocytes reduces miR-1 expression.[50] In this study, expression of miR-1 was down-regulated by hypertrophic

concentration of H_2O_2. Overexpression of miR-1 decreased cell size and hypertrophy-related molecules expression.

The functional study reveals that overexpression of miR-320 enhances apoptosis in cardiomyocytes, whereas knockdown of miR-320 can attenuate cell death upon ischemia/reperfusion.[37] Programmed cell death 4 also is a direct target of miR-21.[32] miR-199a inhibits hypoxia-inducible factor (Hif)-1α and its stabilization of p53, thereby reducing apoptosis.[51] miR-1 participates in the activation of apoptosis by reducing the expression levels of heat shock protein-60 and heat shock protein-70, whereas miR-133 antagonizes apoptosis by repressing caspase-9 expression.[52] Also, miR-1 regulated cardiomyocyte apoptosis, which is involved in post-transcriptional repression of Bcl-2.[53] MiR-195 promotes palmitate-induced apoptosis in cardiomyocytes by targeting Sirt1, a known anti-apoptotic protein.[54] But, the role of miR-26a in cardiac apoptosis is not cleared. miR-26a is abundantly expressed in rat hearts and decreased in ischemic preconditioning (IP).[55] miR-26a initiated cell apoptosis through both extrinsic and intrinsic pathways with caspase-8 and caspase-9 activation, respectively, in breast cancer cell. In nasopharyngeal carcinoma (NPC) cell line C666-1 cells, ionizing radiation (IR)-induced apoptosis was dependent on ROS and exogenous expression of miR-26a led to significant toxicity in cells.[56] In this study, miR-26a is abundantly expressed in

cardiomyocytes and increased in apoptotic concentration of H_2O_2. Also, specific miR-26a promotes H_2O_2-induced cardiac myocytes apoptosis.

This study has shown that specific miRNAs expressions are cell type or tissues specific. Hypertrophic or apoptotic concentration of H_2O_2 in modulation of miRNA has different aspects. Also, miR-1 decreased cardiac hypertrophy and miR-26a promotes apoptosis in cardiomyocytes. Mainly, this study focuses on the protective effect of miRNAs on ROS-induced cardiomyocyte response.

V. CONCLUSION

In summary, these results assessed miRNAs in H_2O_2-induced cardiomyocyte response. In this study we confirmed that several specific miRNAs were detected in cardiomyocytes, and these miRNAs expressions were modulated by H_2O_2. And these results show that some miRNA promotes H_2O_2-induced apoptosis. Our study suggests that specific miRNA regulates hypertrophy and apoptosis.

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Abstract (in Korean)

활성산소종에 의해 자극된 심근세포에서의 특정 miRNA의 역할

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노 환 규

산화에 의한 손상은 많은 심장혈관계 질병에서 중요한 역할을 할 것이라고 제안되어왔다. 많은 연구자들에 의해 대부분의 심장혈관계 질병의 발전이 활성산소종(ROS)에 의한 것임을 보여주었다.

MicroRNA들은 작은 non-cording RNA로 post-transcriptional 유전자 silencing을 중재한다. miRNA들은 심근의 비대, 심근세포의 사멸과 심부전과 같은 심장의 형태학적 병리학적 조절에 중요한 작용을 한다. 그러나 활성산소종에 의해 유도된 심근세포 사멸과 심근비대에서의 microRNA의 연구는 충분하지 않다. ROS는 심근세포의 표현형의 두 가지 면을 조절한다; 심근비대는 과산화수소의 낮은 수준(30uM)에서 발생하며 세포사멸은 높은 수준(100-150uM) 에서 발생한다. 심근세포 비대는 세포의 크기나 α-MHC, MLC, NFATC1, ANP와 BNP등 비대에서 특이적으로 발현하는 표지인자들에 의하여 확인되며, 세포사멸은 세포의 생존능력,

Annexin V/PI 염색, 그리고 caspase-3 활성에 의하여 측정된다.

본 연구에서는 실시간 유전자 증폭기법을 이용하여 심근세포에서 발현하는 miR-1, 26a, 133a 그리고 145를 확인했다. 또한 이 miRNA들은 신생아, 성인의 심장조직에서 발현하였다. 과산화수소에 의해 중재된 특정 miRNA의 발현의 증감을 확인하였다. 과산화수소의 심근비대성 농도는 miR-1의 발현을 저해시켰고, miR-1의 과발현은 과산화수소에 의한 심근비대를 감소시켰다. 과산화수소의 세포사멸 농도는 miR-26a의 발현을 증가 시켰고, miR-26a mimic 은 과산화수소에 의한 세포사멸을 향상시켰다.

이러한 결과는 miRNA의 발현시점과 양, 특정 miRNA 조절에 의해 과산화수소에 의한 심근세포의 비대와 세포사멸에 영향을 미친다는 것을 시사한다.

핵심되는 말: 심근세포, 비대, 세포사멸, miRNA, 과산화수소