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MicroRNA-145 suppresses ROS-induced Ca^{2+} overload of cardiomyocytes by targeting CaMKIIδ

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ABSTRACT

A change in intracellular free calcium (Ca^{2+}) is a common signaling mechanism of reperfusion-induced cardiomyocyte death. Calcium/calmodulin dependent protein kinase II (CaMKII) is a critical regulator of Ca^{2+} signaling and mediates signaling pathways responsible for functions in the heart including hypertrophy, apoptosis, arrhythmia, and heart disease. MicroRNAs (miRNA) are involved in the regulation of cell response, including survival, proliferation, apoptosis, and development. However, the roles of miRNAs in Ca^{2+}-mediated apoptosis of cardiomyocytes are uncertain. Here, we determined the potential role of miRNA in the regulation of CaMKII dependent apoptosis and explored its underlying mechanism. To determine the potential roles of miRNAs in H_2O_2-mediated Ca^{2+} overload, we selected and tested 6 putative miRNAs that targeted CaMKIIδ, and showed that miR-145 represses CaMKIIδ protein expression and Ca^{2+} overload. We confirmed CaMKIIδ as a direct downstream target of miR-145. Furthermore, miR-145 regulates Ca^{2+}-related signals and ameliorates apoptosis. This study demonstrates that miR-145 regulates reactive oxygen species (ROS)-induced Ca^{2+} overload in cardiomyocytes. Thus, miR-145 affects ROS-mediated gene regulation and cellular injury responses.

1. Introduction

Myocardial infarction (MI) is associated with increased reactive oxygen species (ROS) production, heart failure, and increased mortality [1]. The calcium/calmodulin dependent protein kinase II (CaMKII) has emerged as a MI- and ROS-activated signaling molecule that regulates expression of apoptotic genes and affects adverse outcomes after MI [2–4]. Cardiac-specific transgenic overexpression of CaMKII results in cardiac hypertrophy, heart failure, and premature death [5]. CaMKII is constitutively activated by threonine-287 phosphorylation, and constitutively active CaMKII can lead to many of these MI-related adverse effects [6,7]. In recent *in vitro* experiments, we described an apoptotic pathway that involves increases in ROS produced by diesel exhaust particles, and activation of CaMKII [8].

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by targeting the 3′ untranslated region (3′UTR) of target mRNAs. As a result, translation is suppressed or target mRNA is rapidly degraded [9,10]. Recent studies showed that miRNAs are involved in cardiac physiology and pathology [11,12]. Many such studies have demonstrated that miRNAs can regulate cardiac apoptosis [13]. Myocardial-specific miR-1 and miR-133a may play an important role in cardiac apoptosis. miR-1 regulates cardiomyocyte apoptosis via post-transcriptional repression of IGF-1, Hsp60, and Bcl-2 [14–16]. A miR-133a mimic down-regulated caspase-9 protein expression and attenuated ischemia–reperfusion (I/R)-induced apoptosis [17]. miR-21, the miR-30 family, and miR-199a are anti-apoptotic miRNAs, and miR-195 and miR-320 are pro-apoptotic miRNAs [13,18].

A previous study demonstrated that miR-145 is abundantly expressed in smooth muscle [19]. One of the targets of miR-145 in vascular smooth muscle cells (VSMC) is CaMKII [20]. miR-145 in-

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duces smooth muscle cell (SMC) proliferation and differentiation [20,21]. Although miR-145 initiates apoptosis in cancer cells [22–24], the role of miR-145 in cardiomyocytes remains unclear. Therefore, our research focused on the effect of miR-145 expression in Ca^{2+} overload by activation of CaMKII in cardiomyocytes and determined whether miR-145 regulates ROS-induced cardiomyocyte apoptosis. Our data suggest that miR-145 may be a powerful therapeutic target for ischemic heart diseases.

2. Materials and methods

2.1. Isolation of rat ventricular cardiomyocytes

Animals were handled in compliance with the Guiding Principles in the Care and Use of Animals. All experimental procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine, and performed in accordance with the Committee's Guidelines and Regulations for Animal Care (NIH Publication No. 85-23, revised 1996). We isolated and purified neonatal rat cardiomyocytes. Briefly, 1- to 2-day-old Sprague Dawley rat pups were anesthetized with ether in batches of five at a time. Using microdissecting scissors, hearts were minced to pieces of approximately 1 mm^3 and the ventricles were washed with Dulbecco's phosphate-buffered saline solution (PBS, pH 7.4) free of Ca^{2+} and Mg^{2+}. The tissues were washed with PBS and enzymatically digested with 10 ml of collagenase II (0.8 mg/ml, 262 units/mg, Gibco BRL) for 5 min at 37 °C. The supernatant was then removed, and the tissue was 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 (FBS), Gibco BRL). The tubes were centrifuged at 1200 rpm for 4 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 remained. The cell suspensions were collected and incubated in 100 mm tissue culture dishes for 1–3 h 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 h, the cells were rinsed twice with cell culture medium, and 0.1 μM 5-bromo-2′-deoxyuridine (BrdU) was added in order to increase the purity of cardiomyocytes. The cells were then cultured with 10% (v/v) FBS in a CO_2 incubator at 37 °C.

2.2. Treatment of cells with hydrogen peroxide

One day following isolation, cardiomyocytes were rinsed twice with PBS. The cells were further incubated with α-MEM containing 1% FBS. Various concentrations of H_2O_2 were then added to the medium and cells were incubated for the indicated times. For negative controls, cells were incubated with medium lacking H_2O_2 for equivalent amounts of time.

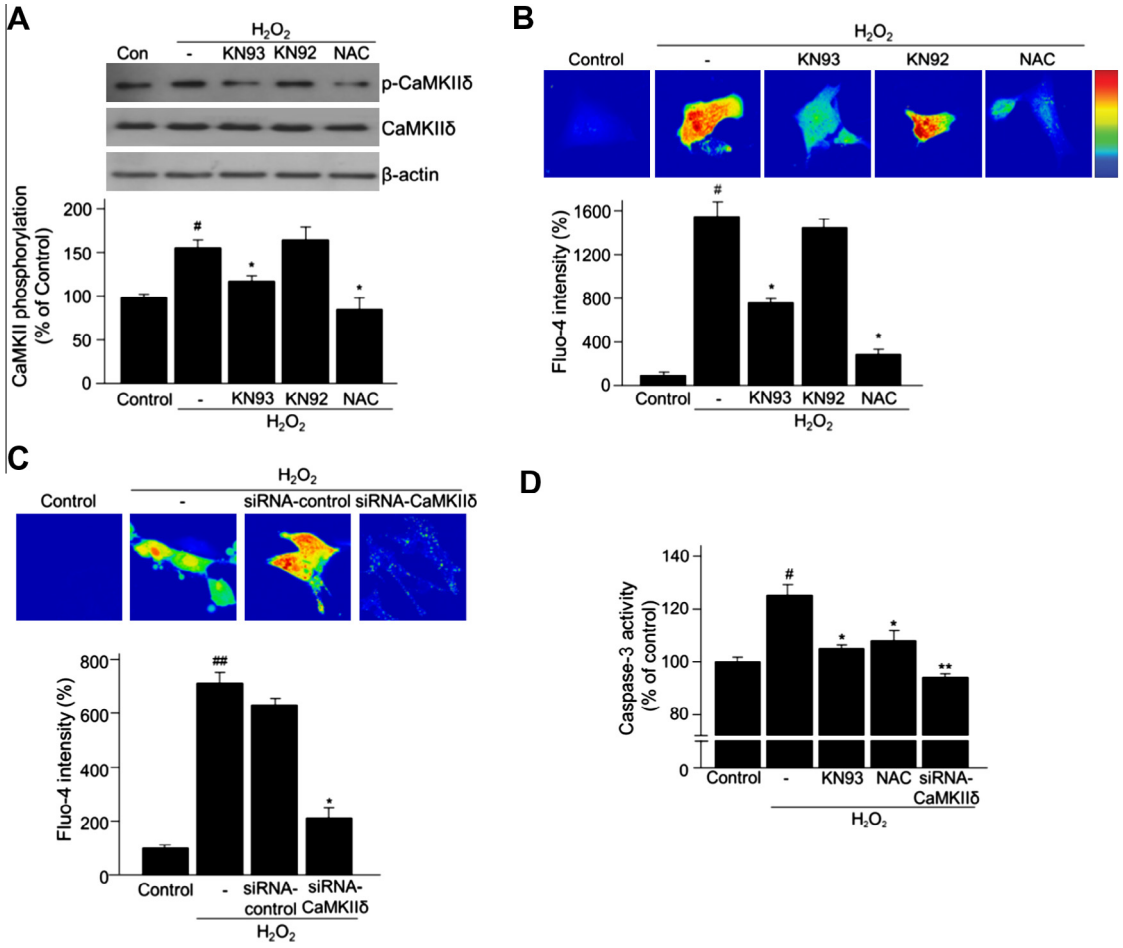


Fig. 1. H_2O_2 induces CaMKIIδ expression. (A) Western blot analysis of phosphorylated CaMKIIδ (p-CaMKIIδ), CaMKIIδ, and β-actin. (B) Cardiomyocytes treated with or without each inhibitor or analogue and H_2O_2. All inhibitors, including 5 μM KN93, 5 μM KN92, and 10 mM NAC were added for 30 min, then 100 nM H_2O_2 was added for 6 h. Cytosolic free Ca^{2+} concentration was determined by Fluo-4 intensity. (C) siRNA-CaMKIIδ was added for 6 h in a dose-dependent manner. (D) Apoptosis was measured by caspase-3 activity assay ($^{\#}p < 0.05$, $^{\#\#}p < 0.001$ vs control, $^{*}p < 0.05$, $^{**}p < 0.001$ vs H_2O_2).

2.3. RT-PCR analysis

The expression levels of various proteins were determined by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was prepared by TRIzol® Reagent (Life Technologies), and cDNA was then synthesized from isolated total RNA by AMV reverse transcriptase. A 20 μl reverse transcription reaction mixture containing 1 μg of total RNA, 1× reverse transcription buffer, 1 mM 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 4 °C for 5 min. PCRs were performed for 35 cycles with primers based on the sequences of the various genes. The primer sequences were as follows: CaMKIIδ, sense: 5′-TCAGAT GTTTTG

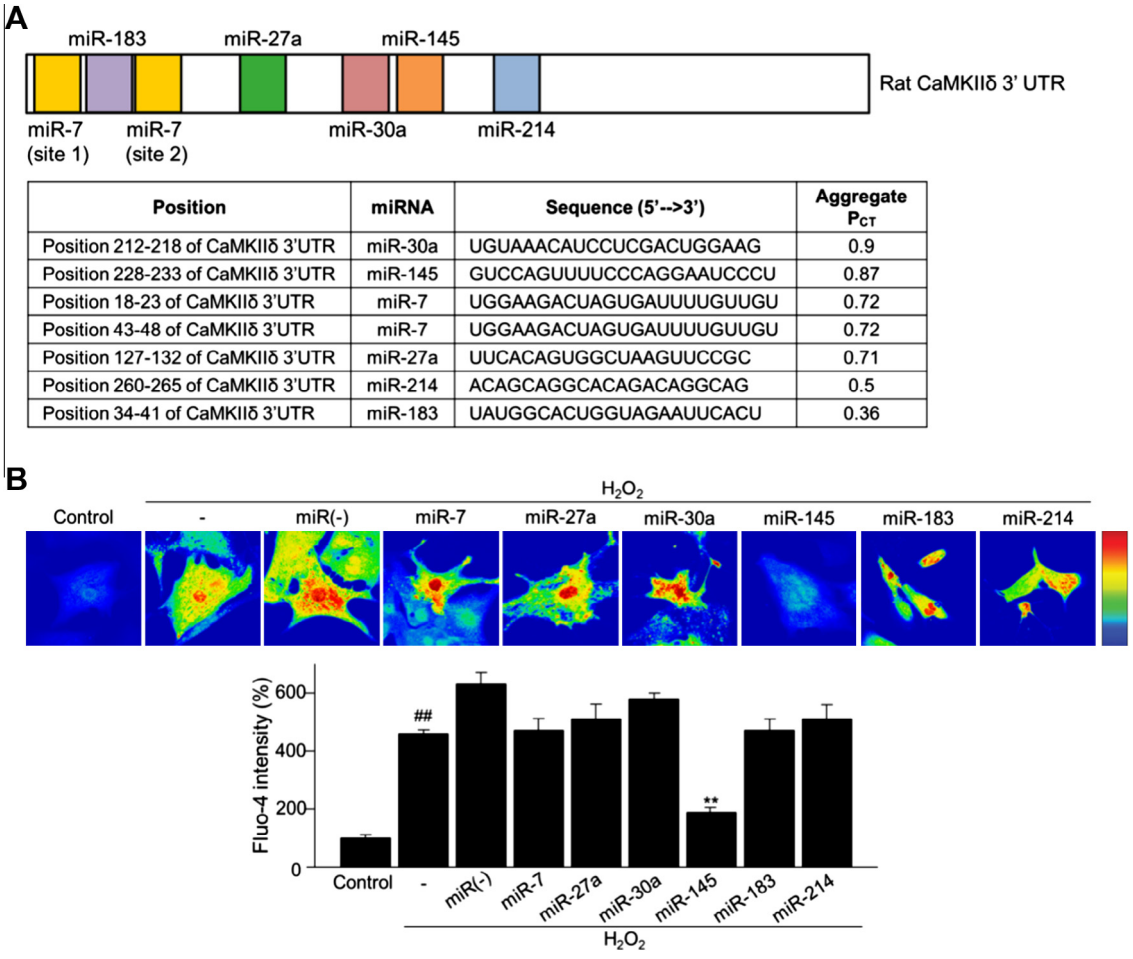
CCACAAAGAGGTGCCTCCT-3′ and anti-sense: 5′-CCGGATGGGG-TAAA GGAGTCAACTGAG AGCT-3′; RyR2, sense: 5′-CCAACATGC CAGACCCTACT-3′ and anti-sense: 5′-TTTCTCCATCCT CTCCCTCA-3′; NCX, sense: 5′-TGTCTGCGATTGCTTGTCTC-3′ and anti-sense: 5′-TCACTCAT CTCCACCAGACG-3′; SERCA2a, sense: 5′-TCCATCTGC-CTGTCCAT-3′ and anti-sense: 5′-GCGG TTACTCCAGTATTG-3′; PMCA, sense: 5′-TGCCTTGTTGGGA TTTCTCT-3′ and anti-sense: 5′-C ACTCTGGTTCTGGCTCTCC-3′; PLB, sense: 5′-GCTGAG CTCCCAG-ACTTCAC-3′ and anti-sense: 5′-GCGACAGCTTGTCACAGAAG-3′. GAPDH was used as the internal standard, and the signal intensity of each amplification product was normalized to the respective GADPH signal intensity.

2.4. Real-time PCR

Total RNA was isolated with TRIzol® Reagent (Life Technologies). In brief, 100 ng purified total RNA was used for reverse transcription (Taqman® MicroRNA Reverse Transcriptase Kit, Applied Biosystems, Bedford, MA) in combination with Taqman® MicroRNA Assays for quantification of specific miRNAs and U6 control transcripts, according to the manufacturer's conditions. Amplification and detection of specific products were performed in a Light Cycler 480 II (Roche) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. 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 cardiomyocytes (ΔΔCt) was calculated and presented as fold induction ($2^{-\Delta\Delta Ct}$).

2.5. Western blot

Cardiomyocytes were washed once in PBS and lysed in lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) with protease inhibitor cocktail. Proteins were separated by SDS–PAGE and transferred to PVDF membrane (Millipore Co, Bedford, MA). The membrane was incubated with primary antibodies (anti-phospho-CaMKII (Novus Biologicals, Littleton, CO) and anti-CaMKII (Santa Cruz Biotechnology, Inc., Dallas, TX)) overnight at 4 °C, and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive



Position	miRNA	Sequence (5'-->3')	Aggregate P_{CT}
Position 212-218 of CaMKIIδ 3'UTR	miR-30a	UGUAAACAUCCUCGACUGGAAG	0.9
Position 228-233 of CaMKIIδ 3'UTR	miR-145	GUCCAGUUUUCCCAGGAAUCCCU	0.87
Position 18-23 of CaMKIIδ 3'UTR	miR-7	UGGAAGACUAGUGAUUUUGUUGU	0.72
Position 43-48 of CaMKIIδ 3'UTR	miR-7	UGGAAGACUAGUGAUUUUGUUGU	0.72
Position 127-132 of CaMKIIδ 3'UTR	miR-27a	UUCACAGUGGCUAAGUUCCGC	0.71
Position 260-265 of CaMKIIδ 3'UTR	miR-214	ACAGCAGGCACAGACAGGCAG	0.5
Position 34-41 of CaMKIIδ 3'UTR	miR-183	UAUGGCACUGGUAGAAUUCACU	0.36

Fig. 2. miRNAs regulate Ca^{2+} overload. (A) Potential miRNAs targeting CaMKIIδ were found in Targetscan version 5.1. miRNAs were chosen on the basis of having higher than 0.2 aggregate P_{CT}. (B) Cytosolic-free Ca^{2+} concentration was measured for each miRNA. miRNAs were transfected at 100 nM for 6 h ($^{\#\#}p < 0.001$ vs control, $^{*}p < 0.05$, $^{**}p < 0.001$ vs H_2O_2).

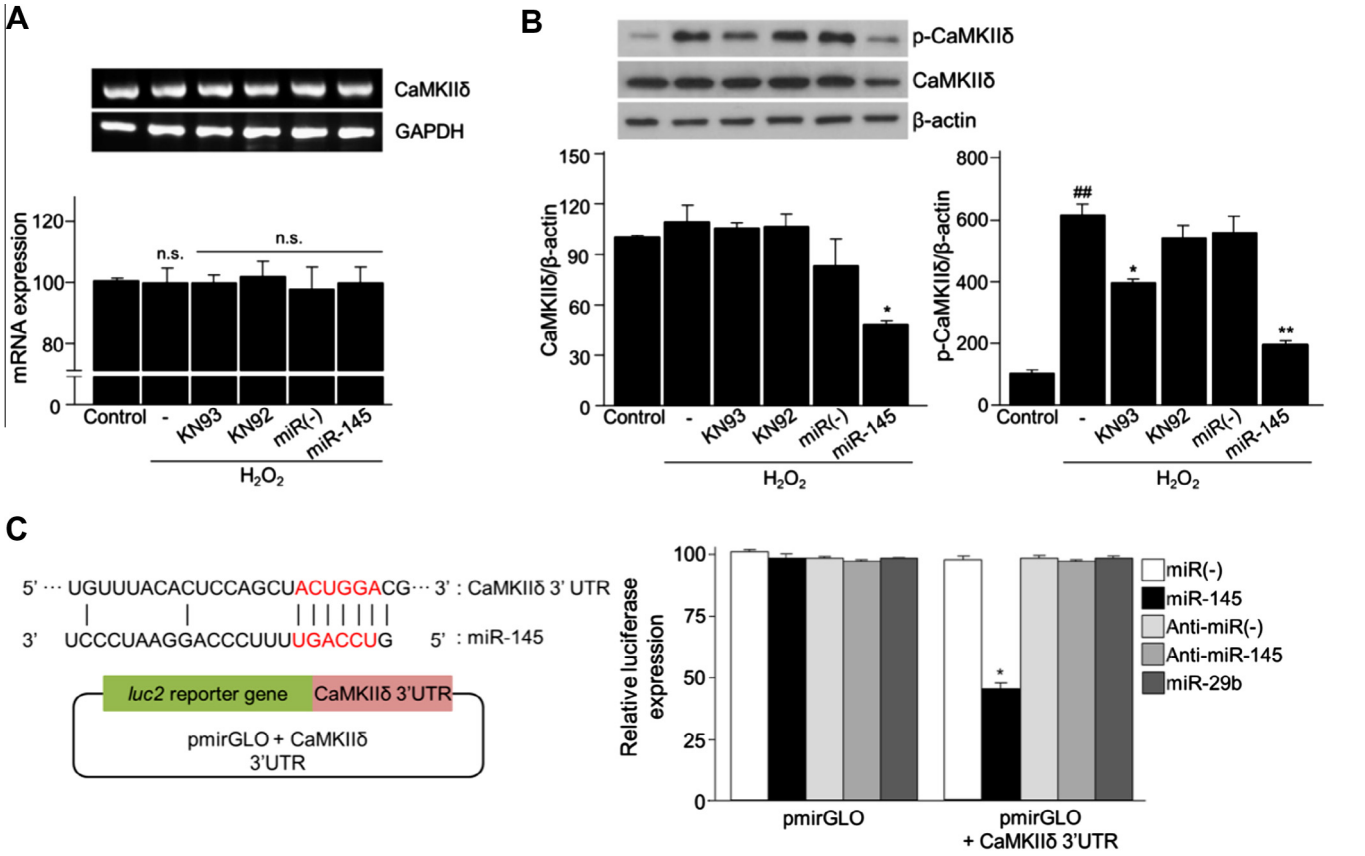


Fig. 3. miR-145 inhibits CaMKIIδ. CaMKIIδ was detected by (A) RT-PCR, and (B) western blot analysis. miRNAs were transfected at 100 nM for 6 h, and H_2O_2 at 100 nM was added for 6 h. (C) CaMKIIδ 3′UTR expression was measured by luciferase reporter assay. Control vector or CaMKIIδ 3′UTR vector was transfected with miR-control, miR-145, or miR-29. miR-29b lacks a binding site for CaMKIIδ's 3′UTR ($^{\#\#}p < 0.001$ vs control, $^{*}p < 0.05$, $^{**}p < 0.001$ vs H_2O_2, n.s., not significant).

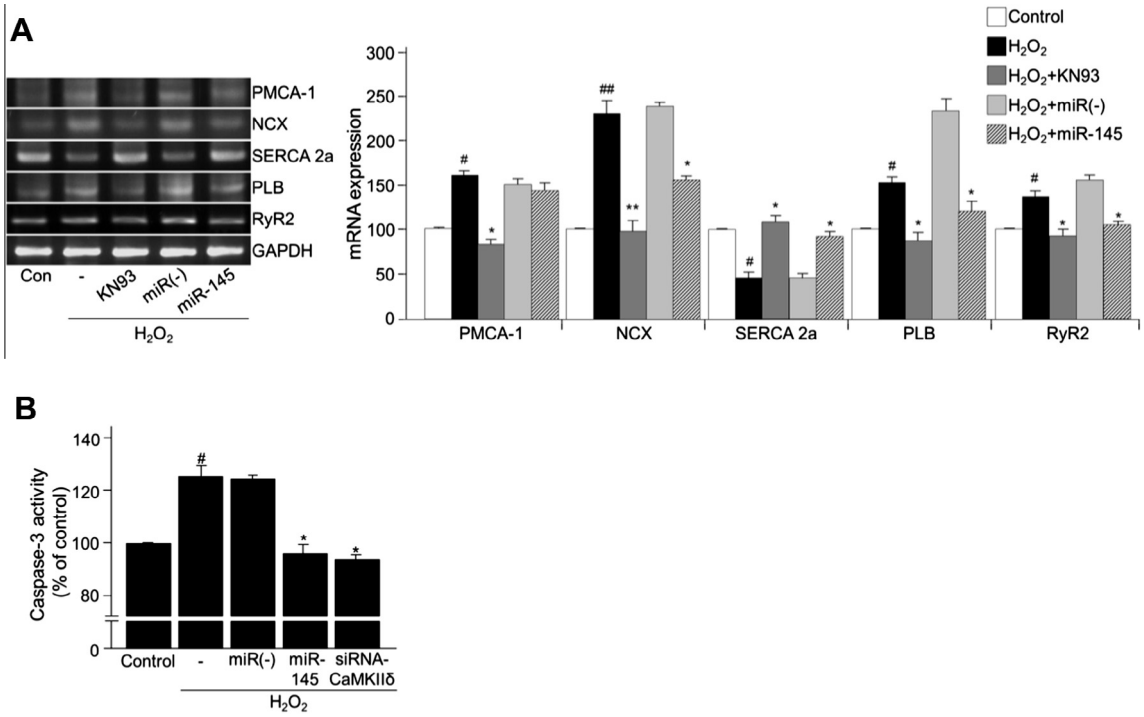


Fig. 4. miR-145 modulates CaMKIIδ-related signaling. (A) Ca^{2+}-regulated signaling was detected by RT-PCR. PMCA-1, sarcolemmal Ca^{2+} pump; NCX, Na^{+}/Ca^{2+} exchanger; SERCA2a, sarcoplasmic reticulum Ca^{2+}-ATPase; PLB, phospholamban; RyR2, ryanodine receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) Apoptosis was measured by caspase-3 activity assay ($^{\#}p < 0.05$, $^{\#\#}p < 0.001$ vs control, $^{*}p < 0.05$, $^{**}p < 0.001$ vs H_2O_2).

washing, proteins were detected by incubating the membranes in enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology). Signal intensities were quantified using the Photo-Image System (Molecular Dynamics, Sweden).

2.6. Intracellular Ca^{2+} measurement

To measure cytosolic free Ca^{2+}, cardiomyocytes were placed on glass coverslips coated with laminin (5 mg/cm^2) for 1 day in cell culture medium containing 10% FBS and 0.1 μM BrdU. Cells were washed with modified Tyrode's solution containing 0.265 g/L of $CaCl_2$, 0.214 g/L of $MgCl_2$, 0.2 g/L of KCl, 8.0 g/L of NaCl, 1 g/L of glucose, 0.05 g/L of NaH_2PO_4, and 1.0 g/L of $NaHCO_3$ and then loaded with 5 mM of Fluo-4 AM for 20 min in the dark at room temperature. Fluorescence images were obtained using an argon laser confocal microscope (Carl Zeiss MicroImaging Inc., Germany). The fluorochrome was excited by a 488 nm wavelength argon laser and emitted light was collected through a 510–560 nm band pass filter. Relative changes of free intracellular Ca^{2+} were determined by measuring fluorescent intensity.

2.7. miRNA transfection and RNA interference

Transfections of miRNA mimics were performed using siLentFect™ Lipid reagent (Life Science Research, Baltimore, MA). Mature miRNA mimics (Genolution Pharmaceuticals, Inc., Korea) were used at a final concentration of 100 nM. After 4–6 h incubation in a CO_2 incubator at 37 °C, the medium was changed to conditioned α-MEM. For function-blocking experiments, we used an siRNA molecule specific to CaMKIIδ mRNA. A 19-nt siRNA sequence was derived from rat tissue CaMKII mRNA sequences (GI: 1616021; sense, 5′-GACUUGCAUGGUAGUCUGU-3′; antisense, 5′-ACAG ACUACCAUGCAAGUC-3′).

2.8. Luciferase reporter assay

The predicted target gene of miR-145 was retrieved using a publicly available database (TargetScan, www.targetscan.org). We synthesized the 3′-UTR of CaMKIIδ containing the predicted binding sites for miR-145 and then cloned it into the pmirGLO vector. HeLa cells were plated at 1×10^5 in 12-well plates. After 24 h, the vector containing the CaMKIIδ binding site for miR-145 was co-transfected with miR-145 or miR-control using Lipofectamine 2000. Renilla luciferase was used to normalize the cell number and transfection efficiency. Luciferase activity was measured by the Dual Luciferase assay (Promega, Madison, WI) according to the manufacturer's instructions on the Luminometer (Promega).

2.9. Caspase-3 activity

Relative caspase-3 activity was determined using an ApopTarget™ Capase-3 Colorimetric Protease Assay used according to the manufacturer's instructions (Life Science Research). Briefly, 2×10^6 cardiomyocytes were harvested in lysis buffer with 1 M DTT after different treatments, and cell extracts were centrifuged to remove cellular debris. Aliquots (50 μl) of the cell extracts were incubated at 37 °C for 2 h in the presence of the chromophore-labeled substrate DEVD-pNA. Free DEVD-pNA was determined colorimetrically. The comparison of the absorbance of pNA from the apoptotic sample with an uninduced control allowed the determination of the amount of increase in capase-3 activity.

2.10. Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using Student's *t*-test. Relationships were considered statistically significant if *p* values were less than 0.05.

3. Results

3.1. H_2O_2 induces CaMKIIδ -mediated Ca^{2+} overload

To test H_2O_2-mediated induction of phosphorylated CaMKIIδ (p-CaMKIIδ), cardiomyocytes were treated with H_2O_2. H_2O_2 induced p-CaMKIIδ; however, the induction was markedly reduced by KN93 (a CaMKII selective inhibitor) and NAC (a ROS scavenger) (Fig. 1A). Cells treated with the analogue KN92 (which does not inhibit CaMKII) also exhibited induction of Ca^{2+}, but KN92 did not protect against H_2O_2-induced Ca^{2+} overload. However, KN93 and NAC-treated cells exhibited reduced Ca^{2+} overload (Fig. 1B). To determine the specific role of CAMKIIδ in Ca^{2+} overloaded cardiomyocytes, we knocked down CaMKIIδ expression with siRNA-CaMKIIδ. Significantly, siRNA-CaMKIIδ silenced CaMKIIδ expression at both the mRNA and protein level (Data not shown). Ca^{2+} overload and caspase-3 activity were reduced in siRNA-CaMKIIδ-treated cells (Fig. 1C and D). These results indicate that CaMKIIδ mediates Ca^{2+} overload in H_2O_2-treated cells and induces apoptosis of cardiomyocytes.

3.2. miRNAs regulate Ca^{2+} overload

We hypothesized that CaMKIIδ-targeting miRNAs might act to downregulate intracellular Ca^{2+} levels and that these miRNAs could affect Ca^{2+} overload-induced apoptosis in cardiomyocytes. To identify possible miRNAs targeting CaMKIIδ, we searched miRNAs in Targetscan version 5.1. Six miRNAs (miR-30a, -145, -7, -27a, -214, and -183) were selected on the basis of having higher than 0.2 aggregate P_{CT} (Fig. 2A). Next, we determined the inhibitory effect on Ca^{2+} overload in each miRNA-transfected cell. Only miR-145-overexpressing cells reduced Ca^{2+} overload (Fig. 2B).

3.3. miR-145 downregulates endogenous CaMKIIδ expression

We chose miRNA-145 to target CaMKIIδ. In order to measure the inhibitory effect of miR-145, the expression level of CaMKIIδ was determined by RT-PCR or immunoblot. In miR-145-treated cells, the expression of CaMKIIδ was decreased at the protein level but not at the mRNA level (Fig. 3A and B). Next, we confirmed the inhibitory effect of miR-145 using a luciferase activity assay. pmirGLO-CaMKIIδ vector was used to determine whether miR-145 interacts with its target sequence in the 3′UTR of CaMKIIδ in HeLa cells. Cells were transiently co-transfected with the pmirGLO-CaMKIIδ vector or pmirGLO control vector, in conjunction with a miR-145 mimic, microRNA negative control, anti-miRNA negative control, anti-miR-145, or miR-29b (a miRNA which does not bind the 3′UTR of CaMKIIδ). A marked decreased in luciferase activity was observed in miR-145-overexpressed cells (Fig. 3C). These results indicate that miR-145 binds to a seed sequence in the 3′UTR of CaMKIIδ mRNA.

3.4. miR-145 regulates CaMKIIδ-related signaling

The H_2O_2-mediated Ca^{2+} handling proteins, PMCA-1, NCX, SERCA2a, RyR2, and PLB were examined at the mRNA level. With the exception of SERCA2a, transcription of these Ca^{2+} handling proteins was induced by H_2O_2; however, when miR-145 was transfected, induction was partially suppressed (Fig. 4A). Furthermore, caspase-3 activity was decreased in miR-145 and siRNA-CaMKIIδ-overexpressing cells (Fig. 4B). Thus, these results show that miR-145 ameliorated apoptosis by regulating Ca^{2+} handling proteins.

4. Discussion

ROS are generated in MI, reperfusion injury, and mediated cell death [1,25]. As powerful regulators of gene expression, miRNAs are now acknowledged as novel multi-target agents for treating

cardiovascular pathology including MI. The present study demonstrates that overexpression of miR-145 protects against Ca^{2+} overload in cardiomyocytes under oxidative stress, in association with decreased CaMKIIδ expression.

Intracellular Ca^{2+} overload was initially considered an essential mechanism of reperfusion or other stresses, leading to cardiomyocyte death [26]. Increases in intracellular Ca^{2+} concentration can relay signals through various classes of Ca^{2+}-regulated enzymes, one of which is the calcium/calmodulin-dependent protein kinase (CaM kinase or CaMK) family [5]. CaMKII has been shown to be present in the heart, to induce cardiac hypertrophy and apoptosis, and to contribute to the development of heart failure [5–7]. Yang et al. showed that CaMKII inhibition reduces isoproterenol- (Iso) or MI-induced apoptosis, using mice with genetic myocardial CaMKII inhibition due to transgenic expression of a highly specific CaMKII inhibitory peptide [2]. As well, KN93 or AIP (CaMKII inhibitory peptide) prevented I/R-induced infarct size, necrosis, and apoptosis [3]. In the present study, we have shown that CaMKII inhibition (via KN93 or CaMKII-directed siRNA) decreases apoptosis in H_2O_2-treated cardiomyocytes. ROS-dependent alteration of CaMKII activity acts as a central signal in intracellular Ca^{2+} handling of myocytes [27]. Ca^{2+} handling proteins, including PMCA-1, NCX1, SERCA2a, PLB and RyR2 regulate intracellular Ca^{2+} concentration [28,29]. Our results show that CaMKII inhibition significantly lowered intracellular Ca^{2+} (Fig. 1B), downregulated PMCA-1, NCX, PLB, and RyR2 at the mRNA level, and upregulated SERCA2a at the mRNA level (Fig. 4A).

To identify novel regulators of Ca^{2+} overload-mediated cardiac apoptosis, we investigated whether miRNAs are involved in Ca^{2+} overload in oxidative stress-induced cardiomyocyte apoptosis. It has been reported previously that miR-214 represses Ca^{2+} overload and cell death during IR, and targets NCX1, BIM, CaMKIIδ, and Cyclophilin D (CypD) [29]. To determine which miRNA was closely related with CaMKIIδ in Ca^{2+} overload, we selected 6 candidate miRNAs that potentially target CaMKIIδ. These were the top 6 miRNAs according to aggregate P_{CT}. Among these miRNAs, miR-145 decreased H_2O_2-induced intracellular Ca^{2+} concentration and decreased the expression of CaMKIIδ protein, via a direct interaction between miR-145 and CaMKIIδ mRNA (Fig. 3B and C). A previous study validated CaMKIIδ experimentally as a direct target for miR-145 in SMCs [20].miR-145 is the most abundant miRNA in normal arteries and VSMCs [19]. In addition, miR-145 inhibits growth and induces cell death in various tumor cell types, including colon cancer, lung cancer, and breast cancer [22–24]. Even though the endogenous concentration of miR-145 may be sufficient to prevent calcium overload induced by ROS under normal condition, Li et al. [30] reported that miR-145 modulated the mitochondrial pathway by directly targeting Bnip3, an initiation factor of mitochondrial apoptotic pathway, resulting in protection against oxidative stress-associated cardiomyocyte apoptosis. However, previous studies of miR-145 on cardiomyocytes have been insufficient. In this study, we have shown that miR-145 represses Ca^{2+} overload and apoptosis in H_2O_2-treated cardiomyocytes. Consistent with inhibition of CaMKII, miR-145 overexpression inhibits H_2O_2-induction of PMCA-1, NCX, PLB, and RyR2, and eleveates mRNA levels of SERCA2a (Fig. 4A).

In summary, we have demonstrated that miR-145 is essential for Ca^{2+} overload regulation in cardiomyocytes by repressing CaMKIIδ. Furthermore, miR-145 protects against ROS-induced cardiomyocyte cell death. These findings have implications for our understanding of Ca^{2+} overload and Ca^{2+} related cell death in cardiomyocytes.

Acknowledgments

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