miR-185 inhibits endoplasmic reticulum stress-induced apoptosis by targeting Na⁺/H⁺ exchanger-1 in the heart

Jin Ock Kim, Eun Jeong Kwon, Dong Woo Song, Jong Sub Lee & Do Han Kim*
School of Life Sciences and Systems Biology Research Center, Gwangju Institute of Science and Technology (GIST), Gwangju 61005, Korea

Prolonged ER stress (ERS) can be associated with the induction of apoptotic cell death in various heart diseases. In this study, we searched for microRNAs affecting ERS in the heart using in silico and in vitro methods. We found that miR-185 directly targets the 3'-untranslated region of Na⁺/H⁺ exchanger-1 (NHE-1), a protein involved in ERS. Cardiomyocyte ERS-triggered apoptosis induced by 100 ng/ml tunicamycin (TM) or 1 μM thapsigargin (TG), ERS inducers, was significantly reduced by miR-185 overexpression. Protein expression of pro-apoptotic markers such as CCAAT/enhancer-binding protein homologous protein (CHOP) and cleaved-caspase-3 was also markedly reduced by miR-185 in a dose-dependent manner. Cariporide (20 μM), a pharmacological inhibitor of NHE-1, also attenuated ERS-induced apoptosis in cardiomyocytes and CHOP protein expression, suggesting that NHE-1 plays an important role in ERS-associated apoptosis in cardiomyocytes. Collectively, the present results demonstrate that miR-185 is involved in cardio-protection against ERS-mediated apoptotic cell death. [BMB Reports 2016; 49(4): 208-213]

INTRODUCTION
The endoplasmic reticulum (ER) is a cellular organelle that plays an essential role in multiple processes such as protein folding, calcium homeostasis, and lipid biosynthesis (1). Various stimuli such as cardiac pressure-overload, ischemia, oxidative stress, gene mutation, and elevated protein synthesis can potentially lead to the accumulation of unfolded proteins, a condition referred to as ER stress (ERS) (2). When ERS occurs, the ER chaperone Bip/GRP78 dissociates from the three ER trans-membrane sensors such as protein kinase RNA-like ER chaperone Bip/GRP78 dissociates from the three ER stresses referred to as ER stress (ERS) (2). When ERS occurs, the potentially lead to the accumulation of unfolded proteins, a condition referred to as ER stress (ERS) (2). When ERS occurs, the censes/by-nc/4.0) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
miR-185 inhibits cardiomyocyte apoptosis via NHE-1
Jin Ock Kim, et al.

RESULTS

**miR-185 directly targets the 3′-UTR of NHE-1 in two distinct sites**

A bioinformatic target prediction using TargetScan showed that two putative binding sites for miR-185 are present in the 3′-UTR of NHE-1 and well-conserved between human, mouse, rat, and chimpanzee (Fig. 1A). To determine whether NHE-1 is a direct target of miR-185, WT or mutated sequences of each predicted miR-185 sites in the 3′-UTR of NHE-1 were inserted in the pmirGLO Dual-Luciferase miRNA target expression vector (Fig. 1B). miR-185 decreased the luciferase activity of the 3′-UTR of WT NHE-1 at both sites significantly, but it did not affect that of the mutant NHE-1 (Fig. 1C).

We next examined whether miR-185 overexpression could suppress NHE-1 mRNA and protein expression by qRT-PCR and western blotting, respectively. Both NHE-1 mRNA and protein expression were markedly reduced by miR-185 overexpression in neonatal rat ventricular myocytes (NRVMs) (Fig. 2A, B). Taken together, these data suggest that miR-185 negatively regulates NHE-1 expression both at the mRNA and protein levels and that the negative effect is mediated by direct binding of miR-185 to the 3′-UTR of NHE-1 mRNA.

**miR-185 prevents ERS-induced cardiomyocyte apoptosis**

A recent study using NHE-1 transgenic mice presented a significant increase in ERS responsive proteins such as GRP78, GRP94, p-eIF2α, and CCAAT/enhancer-binding protein homologous protein (CHOP) in the heart and spontaneously developed heart failure (16), suggesting that NHE-1 is a critical protein involved in ERS-mediated myocardial apoptosis. To investigate whether miR-185 has an inhibitory effect on myocardial apoptosis through targeting NHE-1, NRVMs were treated with miR-185 mimic or negative control (NC) mimic in conjunction with 100 ng/ml TM, a well-known ERS inducer for 48 h treatment. The results showed that TM induced apoptosis in NRVMs as evidenced by the TUNEL assay results, but this response was significantly attenuated by miR-185 overexpression (Fig. 3A, B).

The most significant ERS-induced apoptotic pathway is mediated through CHOP, which is regulated by the ATF4 and...
miR-185 inhibits cardiomyocyte apoptosis via NHE-1
Jin Ock Kim, et al.

ATF6 pathways. A recent study suggested that CHOP plays a key role in the transition from cardiac hypertrophy to heart failure, where occurrence of myocardial apoptosis prevails (17). Therefore, to determine whether miR-185 overexpression could inhibit CHOP production, CHOP expression level was measured in TM-treated NRVMs in the presence or absence of miR-185. As shown in Fig. 3C, the transfection with increasing concentration of miR-185 caused a progressive reduction of CHOP expression. A dose-dependent reduction of the proteolytic cleavage of caspase-3 was also observed when miR-185 was overexpressed in NRVMs as shown in Fig. 3D. Similar results were obtained when NRVMs were treated with 1 μM TG, an ERS inducer by irreversible inhibition of sarco/endoplasmic reticulum calcium ATPase (SERCA), in miR-185 overexpressing cells (Fig. S1A-D).

Taken together, these data demonstrate that miR-185 overexpression protects NRVMs from ERS-induced apoptosis.

Pharmacological inhibition of NHE-1 inhibits ERS-induced apoptosis
To confirm whether NHE-1, a target of miR-185, is functionally associated with ERS-induced myocardial apoptosis, we treated
NRVMs with 20 μM cariporide, a specific blocker of NHE-1 isomor, followed by 100 ng/ml TM treatment for 48 h. Subsequently, the extent of apoptosis was measured by using the TUNEL assay. NRVMs exposed to TM showed approximately 50% of TUNEL positivity and this was significantly reduced in 20 μM cariporide pretreated samples (Fig. 4A, B). The elevated level of CHOP expression by TM was also significantly reduced in cariporide pretreated cells (Fig. 4C). Similarly, cardiomyocyte apoptosis by 1 μM TG treatment was significantly inhibited by 20 μM cariporide pretreatment (Fig. S2A-D), suggesting that NHE-1 inhibition protects cardiomyocytes from apoptotic cell death through suppression of ERS.

Collectively, our data indicate that miR-185 has a cardio-protective effect against ERS-induced apoptosis mediated through direct repression of NHE-1.

**DISCUSSION**

The ER is the primary site for biosynthesis and maturation of secretory proteins and Ca^{2+} storage. For proper protein folding in the ER, a balance between the levels of Ca^{2+}, molecular chaperones, and redox status in the ER lumen must be maintained. The importance of the ER homeostasis is reflected by its critical roles in the pathogenesis of many diseases such as tumor growth, inflammation, and neurodegenerative disorders, including Parkinson and Alzheimer diseases (18, 19). Especially, ERS has been implicated in a number of cardiac symptoms and diseases such as cardiac hypertrophy, heart failure, cardiomyopathy, and I/R injury (5).

Recently, miRNAs have emerged as central regulators of ER homeostasis and key modulators of UPR signaling. For example, overexpression of the miR-23a-27a-24-2 cluster up-regulates ERS-related proteins such as CHOP, PERK, and IRE1α, inducing apoptosis (20). miR-122 overexpression represses UPR signaling via the CDK4-PSMD10 pathway, thereby altering tumorigenic properties of hepatocellular carcinoma cells (21).

In the present study, we attempted to identify microRNAs regulating UPR- and ERS-induced apoptosis in the heart. The major findings of this study are as follows: 1) miR-185 over-
expression prevents ERS-induced apoptosis as shown by TUNEL assay. 2) miR-185 overexpression significantly inhibits CHOP expression and the cleavage of caspase-3, indicative of apoptosis. 3) miR-185 directly targets two distinct sites in the 3′-UTR of NHE-1, thereby inhibiting NHE-1 expression. 4) Pharmacological inhibition of NHE-1 by cariporide significantly inhibited ERS-induced apoptosis. Taken together, the present data suggest that inhibition of NHE-1 activity by either overexpression of miR-185 or treatment with cariporide can protect cardiomyocytes from apoptotic cell death.

The involvement of NHE-1 in the pathogenesis of cardiovascular diseases such as cardiac hypertrophy (22), IR injury (23), cardiac fibrosis, and heart failure (24) has been reported. Evidence also suggests a cardio-protective effect of NHE-1 inhibition against those cardiac diseases. The possible cardio-protective mechanisms through NHE-1 inhibition involve 1) the blockade of NHE-1-dependent Na⁺ influx and subsequent attenuation of Ca²⁺ overload, preventing mitochondrial Ca²⁺ accumulation and mitochondrial permeability transition (MPT) pore opening (25, 26) and 2) the inhibition of reactive oxygen species (ROS) production, a crucial factor involved in cardiomyocyte apoptosis (27), through direct mitochondrial action (28). However, the effect of NHE-1 inhibition in the ERS-induced cardiomyocyte apoptosis has been largely unknown. In the present study, we identified a novel molecular mechanism linking the inhibition of NHE-1 to the attenuation of ERS and subsequent prevention of cardiomyocyte apoptosis.

Several studies indicated the correlation between ERS and cardiac hypertrophy. For example, hypertrophic hearts present elevated protein synthesis and morphological expansion of the ER (29). ER chaperones were substantially induced in mice with hypertrophic and failing hearts induced by transverse aortic constriction (17). The oral administration of the chemical chaperone PBA (4-phenylbutyric acid) could alleviate ERS and prevented cardiac hypertrophy and ERS-induced apoptosis (30), suggesting that ERS is a part of the hypertrophic response and may contribute, at least in part, to cardiac apoptosis observed during the transition from cardiac hypertrophy to failure.

Since miR-185 was previously shown to simultaneously target several pro-hypertrophic genes such as CaMKIIδ, NCX1, and Nuclear Factor of Activated T-cell (NFAT) C3 (15), we further investigated their roles in ERS-induced cardiomyocyte apoptosis. However, pretreatment of NRVMs with specific inhibitors such as KN-62 (CaMKIIδ inhibitor (31)), cyclosporin A (calcineurin-NFATc signaling inhibitor (32)), and SEA0400 (NCX1 inhibitor (33)) did not significantly affect ERS-induced cardiomyocyte apoptosis, as assessed by CHOP protein expression (Fig. S3), suggesting that the primary effect of miR-185 on the inhibition of ERS-mediated apoptotic cell death was associated with NHE-1 inhibition.

In conclusion, the present study suggests that NHE-1 is an important therapeutic target for the prevention of ERS-mediated apoptotic cell death and miR-185 represents a potential therapeutic strategy for heart diseases associated with apoptotic cell death.

**MATERIALS AND METHODS**

Materials and methods are available in the Supplemental information.

**ACKNOWLEDGEMENTS**

This study was supported by the 2015 GIST Systems Biology Infrastructure Establishment Grant and the National Research Foundation of Korea (NRF) grants funded by the Korea Government, the Ministry of Science, ICT & Future Planning (NRF-2013M3A9A7046297).

**REFERENCES**

1. Ron D and Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 8, 519-529
2. Groenendyk J, Agellon LB and Michelak M (2013) Coping with endoplasmic reticulum stress in the cardiovascular system. Annu Rev Physiol 75, 49-67
3. Ryoo HD (2015) Drosophila as a model for unfolded protein response research. BMB Rep 48, 445-453
4. Arduino DM, Raquel Esteves A, Filipa Domingues A et al (2009) ER-mediated stress induces mitochondrial-dependent caspases activation in NT2 neuron-like cells. BMB Rep 42, 719-724
5. Groenendyk J, Sreenivasaiah PK, Kim DH, Agellon LB and Michelak M (2010) Biology of endoplasmic reticulum stress in the heart. Circ Res 107, 1185-1197
6. Valencia-Sanchez MA, Liu J, Hannon GJ and Parker R (2006) Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 20, 515-524
7. Leite-Moreira AM, Lourenço AP, Falcão-Pires I and Leite-Moreira AF (2013) Pivotal role of microRNAs in cardiac physiology and heart failure. Drug Discov Today 18, 1243-1249
8. Varga Z V, Zvara A, Faragó N et al (2014) MicroRNAs associated with ischemia-reperfusion injury and cardioprotection by ischemic pre- and postconditioning: pro- and anti-miRNAs. Am J Physiol Heart Circ Physiol 307, H216-H227
9. He S, Liu P, Jian Z et al (2013) MiR-138 protects cardiomyocytes from hypoxia-induced apoptosis via MLK3/NK/c-jun pathway. Biochem Biophys Res Commun 441, 763-769
10. Nakamura TY, Iwata Y, Arai Y, Komamura K and Waku-bayashi S (2008) Activation of Na⁺/H⁺ exchanger 1 is sufficient to generate Ca²⁺ signals that induce cardiac hypertrophy and heart failure. Circ Res 103, 891-899
11. Chakrabarti S, Hoque AN and Karmazyn M (1997) A rapid ischemia-induced apoptosis in isolated rat hearts and its attenuation by the sodium-hydrogen exchange inhibitor HOE 642 (cariporide). J Mol Cell Cardiol 29, 3169-3174
12. Gumina RJ, Mizumura T, Beier N, Schelling P, Schultz JJ

**miR-185 inhibits cardiomyocyte apoptosis via NHE-1**

Jin Ock Kim, et al.
miR-185 inhibits cardiomyocyte apoptosis via NHE-1
Jin Ock Kim, et al.

and Gross GJ (1998) A new sodium/hydrogen exchange inhibitor, EMD 85131, limits infarct size in dogs when administered before or after coronary artery occlusion. J Pharmacol Exp Ther 286, 175-183

13. Knight DR, Smith AH, Flynn DM et al (2001) A novel sodium-hydrogen exchanger isomorph-1 inhibitor, zoniporide, reduces ischemic myocardial injury in vitro and in vivo. J Pharmacol Exp Ther 297, 254-259

14. Alvarez B V and Villa-Abril MC (2013) Mitochondrial NHE1: a newly identified target to prevent heart disease. Front Physiol 4, 152

15. Kim JO, Song DW, Kwon EJ et al (2015) miR-185 plays an Anti-Hypertrophic Role in the Heart via Multiple Targets in the Calcium-Signaling Pathways. PLoS One 10, e0122509

16. Cook AR, Bardswell SC, Pretheshan S et al (2009) Paradoxical resistance to myocardial ischemia and age-related cardiomyopathy in NHE1 transgenic mice: a role for ER stress?. J Mol Cell Cardiol 46, 225-233

17. Okada K, Minamino T, Tsukamoto Y et al (2004) Prolonged endoplasmic reticulum stress in hypertrophic and failing heart after aortic constriction: possible contribution of endoplasmic reticulum stress to cardiac myocyte apoptosis. Circulation 110, 705-712

18. Ma Y and Hendershot LM (2004) The role of the unfolded protein response in tumour development: friend or foe?. Nat Rev Cancer 4, 966-977

19. Lindholm D, Wootz H and Korhonen L (2006) ER stress and neurodegenerative diseases. Cell Death Differ 13, 385-392

20. Chhabra R, Dubey R and Saini N (2011) Gene expression profiling indicate role of ER stress in miR-23a~27a~24-2 cluster induced apoptosis in HEK293T cells. RNA Biol 8, 648-664

21. Yang F, Zhang L, Wang F et al (2011) Modulation of the unfolded protein response is the core of microRNA-122-involved sensitivity to chemotherapy in hepatocellular carcinoma. Neoplasia 13, 590-600

22. Cingolani HE and Ennis IL (2007) Cariporide (HOE642), a selective Na+-H+ exchange inhibitor, inhibits the mitochondrial death pathway. Circulation 108, 2275-2281

23. Von Harsdorf R, Li PF and Dietz R (1999) Signaling pathways in reactive oxygen species induced cardiomyocyte apoptosis. Circulation 99, 2934-2941

24. Garciaarena CD, Caltz C, Correa MV et al (2008) Na^-H^+ exchanger-1 inhibitors decrease myocardial superoxide production via direct mitochondrial action. J Appl Physiol 105, 1706-1713

25. Maron BJ, Ferrans VJ and Roberts WC (1975) Myocardial ultrastructure in patients with chronic aortic valve disease. Am J Cardiol 35, 725-739

26. Eckstein LA, Van Quill KR, Bui SK, Uusitalo MS and O'Brien JM (2005) Cyclosporin A inhibits calcineurin/nuclear factor of activated T-cells signaling and induces apoptosis in retinoblastoma cells. Invest Ophthalmol Vis Sci 46, 782-790

27. Matsuda T, Arakawa N, Takuma K et al (2001) SEA0400, a novel and selective inhibitor of the Na^-Ca^2+ exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. J Pharmacol Exp Ther 298, 249-256