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Original Research

Role of microRNA-124 in cardiomyocyte hypertrophy inducedby angiotensin II

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Abstract: Cardiac hypertrophy is a crucial predictor of heart failure and is regulated by microRNAs. MicroRNA-124 (miR-124) is regarded as a prognostic indicator for outcomes after cardiac arrest. However, whether miR-124 participates in cardiac hypertrophy remains unclear. Therefore, our study aimed to determine the role of miR-124 in angiotensin II(AngII)-induced myocardial hypertrophy and the possible mechanism. Primary cultured rat neonatal cardiomyocytes(NCMs) were transfected with miR-124 mimics or inhibitor, followed by AngII stimulation. Quantitative RT-PCR, western blot analysis and determination of cell surface area of NCMs were used to detect the hypertrophic phenotypes. We observed that miR-124 was elevated in AngII-induced hypertrophic cardiomyocytes. Cell surface area of NCMs and mRNA expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC), indicators of myocardial hypertrophy, were higher in NCMs transfected with miR-124 mimics in the presence of AngII. On the contrary, knockdown of miR-124 by its specific inhibitor could restore these courses. Furthermore, downregulation of miR-124 alleviated the increased protein level of endoplasmic reticulum (ER) stress markers 78-kDa glucose-regulated protein (Grp78) and calreticulin(CRT) in AngII-induced NCMs. In conclusion, our study shows that inhibition of miR-124 effectively suppresses AngII-induced myocardial hypertrophy, which is associated with attenuation of ER stress.

Key words: miR-124; Cardiac hypertrophy; Angiotensin II; ER stress.

Introduction

Cardiac hypertrophy is a crucial progression manifesting itself as larger myocardial cells, which leads to deterioration of cardiac pump function and increases the risk of cardiovascular diseases, such as heart failure, coronary atherosclerotic heart disease, and cardiac arrest (1, 2). Multiple mechanisms concerning cardiac hypertrophy are explored so far. Renin–angiotensin–aldosterone system (RAAS) is an important compensatory mechanism to maintain cardiac output through increasing blood pressure, fluid volume, peripheral arterial vasoconstriction and some inflammatory factors (3). Angiotensin II (AngII), the key element in RAAS, can bind with Angiotensin II type I receptor (AT1R) to promote vasoconstriction. Plenty studies have revealed that AngII can disturb the balance of endoplasmic reticulum (ER) (4, 5). It will trigger ER stress showing as accumulation of unfolded/misfolded proteins in ER and motivate unfolded protein response (UPR) to restore the homeostasis of ER, which is associated with cardiac hypertrophy (6-8).

MicroRNAs (miRNAs) are a class of short, non-coding RNAs, which can inhibit mRNA translation via binding with 3'UTR of target genes (9). Studies have reported that many miRNAs such as miR-455, miR-19a/b, and miR-30 can promote or attenuate cardiac hypertrophy via regulating ER stress (10-12). For example, activation of transcription factor 6 (ATF6), an ER stress branch, contributes to protective effects in the heart via down-regulation of miR-455 to augment calreticulin (CRT) expression (10). It is worth noting that miR-124 is associated with dilated cardiomyopathy (DCM) and may make for early diagnosis of heart failure (13). Furthermore, miR-124 regulates bone marrow-derived mesenchymal stem cells to differentiate into cardiomyocytes via targeting signal transducer and activator of transcription (STAT3) signaling (14), suggesting that miR-124 may be involved in some pathological processes of cardiovascular events. However, the role of miR-124 in myocardial hypertrophy remains unknown.

Therefore, this study aimed to explore whether miR-124 participates in myocardial hypertrophy, and to investigate the underlying mechanism.

Materials and Methods

Cell culture

Primary cultured rat neonatal cardiomyocytes (NCMs) were performed as previously described (15). Briefly, the hearts were removed from newborn 0-to-3-day-old Sprague–Dawley rats, and quickly placed in icy phosphate buffer solution (PBS). After cutting off the atria, the ventricles were rinsed several times and cut into approximately 1mm^3 pieces. NCMs were isolated by digesting with 0.05% collagenase type II and 0.05% pancreatin (Gibco, USA). Cell suspensions were centrifuged at 1,000 rpm for 10 minutes. Then the harvest cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) (Hyclone Laboratories, USA) supplemented with 10% serum fetal bovine serum (FBS, biological industries, Israel), penicillin (100 U/ml)/streptomycin (100 U/ml) and cultured in a flask for 90 minutes to eliminate fibroblast. Finally, NCMs were

Table 1. List of product codes (GeneCopoeia).

Name	Product codes
Rno-miR-124 specific mimics	RmiR-SN0074
Rno-mimics scramble	CmiR-SN0001-SN
Rno-miR-124 specific inhibitor	RmiR-AN0074-SN-10
Rno-inhibitor scramble	CmiR-AN0001-SN
Rno-miR-124 primer	RmiRQP0074
Rno-U6	RmiRQP9003

seeded on six-well or twelve-well plates. After 2 days, NCMs were exposed to angiotensinII (AngII) (10^{-5}M, Sigma-Aldrich, USA) for 24h after starvation in serum-free DMEM for 4-6h.

Transfections

Transfection was performed according to manufacturer's instruction. NCMs were transfected with miR-124 mimics (50nM/100nM), inhibitor (100nM) or scramble (100nM) (GeneCopoeia, USA) mixed with cationic lipid Lipofectamine® 3000(Invitrogen, USA), and 48h later AngII was added into six-well or twelve-well plates. The product code numbers about miR-124 specific mimics or inhibitor were shown in Table 1.

Immunofluorescence Staining

Upon different treatments, cultured NCMs were fixed in 4% paraformaldehyde for 15 minutes. The cells were penetrated by 0.5% Triton X-100 for 10 minutes, followed by primary antibody (anti α-actinin, 1:200, Sigma-Aldrich) incubation at 4℃ overnight. Then cells were incubated with an anti-mouse Alexa Fluo 488-conjugated secondary antibody (1:200, Invitrogen). 1h later, NCMs were stained with DAPI (1:1000, Sigma-Aldrich) for 5 minutes after a brief wash. Eclipse TE2000-U fluorescent microscope system (Nikon, Japan) was applied to visualize the cells, followed by determination of cell surface area with Image-J software (National Institutes of Health).Four microscopic fields were observed, and about 100 NCMs were randomly selected to determine the surface area of NCMs through semi-quantifying the fluorescence intensity in every group.

Quantitative real-time PCR

Total RNAs from cardiomyocytes were extracted with Trizol reagent (Invitrogen, USA) according to our previous study (16). cDNA was generated from 1μg of mRNA using reverse transcription kits (Toyobo, Japan for mRNA and GeneCopoeia, USA for miRNA) and quantitative real-time PCR assay was performed on cDNAs with CFX96™Real-Time PCR Detection System (Bio-Rad, USA) using SYBR® Green Supermix kit(Bio-Rad, USA). β-actin was used as the control of mRNAs, and U6 was the control of miRNAs. Primers for miRNAand mRNAwere shown in Table 1 and Table 2 respectively.

Table 2. List of primers used in qRT-PCR.

Gene	Sequences
ANP	F: ACCAAGGGCTTCTTCCTCT
	R: TTCTACCGGCATCTTCTCC
BNP	F:AGAACAATCCACGATGCAGAAG
	R: AAACAACCTCAGCCCGTCACA
β-MHC	F:GCCCGGCATGATTGCG
	R:TGGCGTCCGTCTCATACT
AT1R	F:GGAAACAGCTTGGTGGTGAT
	R: ATAAGTCAGCCAAGGCGAGA
β-actin	F: ACTATCGGCAATGAGCGGTTC
	R: ATGCCACAGGATTCCATACCC

Western Blot Analysis

Western blot analysis was completed to determine the protein expression. Total protein samples were extracted from NCMs using radio-immunoprecipitation assay (RIPA) buffer as described previously (17). Samples(25μg) were subjected to SDS-PAGE and then transferred onto PVDF membrane (Millipore, USA), followed by incubation with primary antibodies, GRP78 (Cell Signaling, #3183), Calreticulin (Cell Signaling, #12238) and β-actin (Santa Cruz), overnight at 4℃. After a simple wash, the membrane was incubated with the secondary antibody for 1h. Finally, enhanced chemiluminescence detection kit (Pierce, USA) was used to visualize the brands and protein expression was analyzed by Quantity One software. β-actin was regarded as the equal-loading control.

Statistics

Data of our study are shown as mean ± SEM. One-way *ANOVA* or *t*-test was used for statistical evaluation among multiple groups or between two groups correspondingly with the help of SPSS 17.0 software. A *P* value that was less than 0.05 was taken to consider statistically significant differences.

Results

MiR-124 is up-regulated in hypertrophic cardiomyocytes.

To detect whether miR-124 is involved in cardiomyocyte hypertrophy, we established an *in vitro* hypertrophic phenotype model. Cell surface area of primary cultured NCMs as well as mRNA level of gene markers of cardiac hypertrophy, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC), was determined by immunofluorescence staining and quantitative RT-PCR separately. Upon AngII stimulation, we observed obvious cardiomyocyte hypertrophy based on larger cell surface area expanded nearly 2-fold and higher level of ANP, BNP and β-MHC that correspondingly increased 2.3-fold, 2.1-fold and 3.2-fold (Figure 1A and Figure 1B), indicating that our *in vitro* cell model was successfully established. Furthermore, the expression of miR-124 was elevated almost 5-fold in hypertrophic NCMs compared with the untreated control (Figure 1C), suggesting that miR-124 may play a crucial role in cardiac hypertrophy.

Knockdown of miR-124 alleviates AngII-induced myocardial hypertrophy.

To evaluate the possible action of miR-124 in hypertrophic NCMs, overexpression or specific inhibition of miR-124 was performed to investigate whether miR-124 participated in the regulation of myocardial hypertrophy. As shown in Figure 2A, forced transient expression of miR-124 by its mimics increased its expression

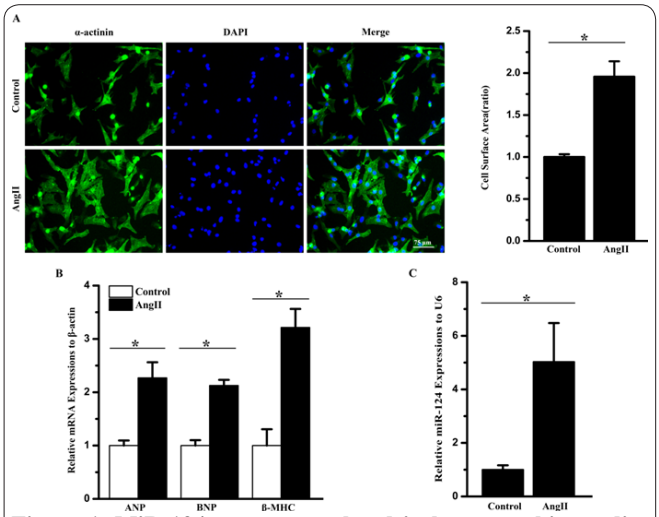

Figure 1. MiR-124 was up-regulated in hypertrophic cardiomyocytes. (A)determination of cell surface area of NCMs with AngII (10^{-5}M for 24h) treatment; Cell surface area (ratio) stands for the ratio of cell surface area to cell numbers in every field. (B) mRNA level of hypertrophic markers(ANP, BNP and β-MHC) in AngII (10^{-5}M for 24h)-induced myocardial hypertrophy; (C) miR-124 expression in AngII (10^{-5}M for 24h)-induced myocardial hypertrophy. (Data were represented as Mean ± SEM, n=4, **P* < 0.05 *vs.* Control).

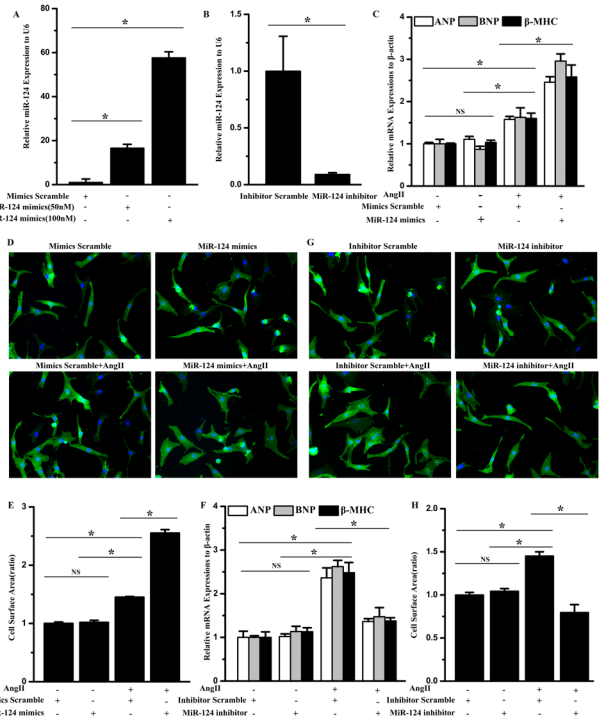

Figure 2. Knockdown of miR-124 alleviated AngII-induced myocardial hypertrophy. (A) miR-124 expression in NCMs transfected with miR-124 mimics;(B) miR-124 expression in NCMs transfected with miR-124 inhibitor;(C) mRNA level of hypertrophic markers(ANP,BNP and β-MHC) in NCMs transfected with miR-124 mimics in the presence or absence of AngII(10^{-5}M for 24h); (D,E)determination of cell surface area of NCMs transfected with miR-124 mimics in the presence or absence of AngII(10^{-5}M for 24h); Cell surface area (ratio) stands for the ratio of cell surface area to cell numbers in every field.(F) mRNA level of hypertrophic markers(ANP,BNP and β-MHC) in NCMs transfected with miR-124 inhibitor in the presence or absence of AngII(10^{-5}M for 24h);(G,H) determination of cell surface area of NCMs transfected with miR-124 inhibitor in the presence or absence of AngII(10^{-5}M for 24h). Cell surface area (ratio) stands for the ratio of cell surface area to cell numbers in every field.(Data were represented as Mean ± SEM, n=4, A:**P* < 0.05 *vs.* mimics scramble, B: **P* < 0.05 *vs.* inhibitor scramble, C,E: **P* < 0.05 *vs.* mimics scramble or mimics scramble+AngII, F,H: **P* < 0.05 *vs.* inhibitor scramble or inhibitor scramble+AngII) (*"+"* means something including mimics scramble/miR-124 mimics/AngII/inhibitor scramble/miR-124 inhibitor exists while*"-"*means it absents. "NS" means no significant difference).

about 58-fold at the final concentration of 100nM. In contrast, miR-124 was significantly reduced up to 9% after transfection of miR-124 inhibitor (100nM) in NCMs (Figure 2B).

Moreover, gain-of-function study showed that miR-124 promoted myocardial hypertrophy in the presence of AngII, manifesting that miR-124 further elevated the increased mRNA level of ANP, BNP and β-MHC almost 1.6-fold,1.8-fold,1.6-fold (Figure 2C) and the larger cell surface area of NCMs about 2-fold (Figure 2D and Figure 2E). On the contrary, the increase of ANP, BNP and β-MHC levels (Figure 2F) and cell surface area of NCMs (Figure2G and Figure 2H) were remarkably suppressed by 42.4%,44.9%,44.4% and 44.8% separately after knockdown of miR-124 by its inhibitor. The results indicated miR-124 as an important factor to promote myocardial hypertrophy induced by AngII.

Inhibition of miR-124 attenuates AngII-induced activation of Grp78 and CRT.

AngII fulfills its pro-hypertrophic role via binding with AT1R and activating the downstream pathways. Suggested by the effect of miR-124 on AngII-induced myocardial hypertrophy, we hypothesized that miR-124 could influence AT1R expression to play its role in myocardial hypertrophy. QRT-PCR was performed to test the hypothesis. However, our results showed that AT1R mRNA expression was not changed after transfection with miR-124 mimics or inhibitor in AngII-treated NCMs (Figure 3A).

Moreover, previous studies have demonstrated that ER stress is a critical pathway that regulates myocardial hypertrophy, which would be triggered by AngII (18). Therefore, we examined whether miR-124 affected the expression of 78-kDa glucose-regulated protein (Grp78) and CRT, two known ER stress parameters, in hypertrophic cardiomyocytes induced by AngII treatment. As shown in Figure 3B, AngII could significantly increase the protein level of Grp78 and CRT about 1.4-fold and 2.6-fold. Overexpression of miR-124 by its mimics further augmented the increased expressions of Grp78 and CRT approximately 4-fold and 4.6-fold (Figure 3C). In contrast, downregulation of miR-124 by its inhibitor apparently inhibited the increasement of Grp78 and CRT up to 54% and 53% separately (Figure 3D).

Discussion

In the current study, we used AngII to induce myocardial hypertrophy and gained several findings: (1) miR-124 was up-regulated in NCMs with AngII treatment; (2) forced transient expression of miR-124 by its mimics promoted AngII-induced myocardial hypertrophy, whereas inhibition of miR-124 attenuated this pro-

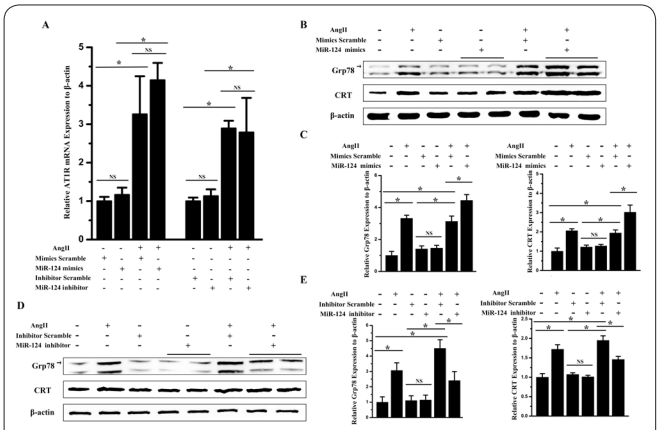

Figure 3. Inhibition of miR-124 attenuated AngII-induced activation of Grp78 and CRT. (A) mRNA expression of AT1R in NCMs after transfection of miR-124 mimics or inhibitor with or without AngII(10^{-5}M for 24h) stimulation; (B,C) protein expression of ER stress markers(Grp78 and CRT) in NCMs after transfection of miR-124 mimics with or without AngII(10^{-5}M for 24h) stimulation; Arrow indicates the main band. (D,E) protein expression of ER stress markers(Grp78 and CRT) in NCMs after transfection of miR-124 inhibitor with or without AngII(10^{-5}M for 24h) stimulation; Arrow indicates the main band. (Data were represented as Mean ± SEM, n=4, $*P < 0.05$ *vs.* control or mimics scramble or mimics scramble+AngII or inhibitor scramble or inhibitor scramble+AngII) (*"+"*means something including mimics scramble/miR-124 mimics/AngII/inhibitor scramble/miR-124 inhibitor exists while *"-"*means it absents. "NS" means no significant difference).

cess; (3) AT1R expression was unchanged upon miR-124 overexpression or inhibition; and (4) knockdown of miR-124 might restrain the activation of ER stress.

A substudy of a randomized clinical trial has investigated the relationship between circulating miR-124 level and the outcome after out-of-hospital cardiac arrest (19). The plasma level of miR-124 could serve as a prognostic marker for outcomes after cardiac arrest, meaning that miR-124 may fulfill a crucial role in cardiovascular events. It is speculated that miR-124 participates in myocardial hypertrophy induced by AngII. In our study, we observed, for the first time, that miR-124 was markedly elevated in AngII-induced myocardial hypertrophy. Additionally, overexpression of miR-124 was able to efficiently increase the mRNA level of ANP, BNP and β-MHC and the cell surface area of NCMs in the presence of AngII, whereas knockdown of miR-124 notably inhibited the hypertrophic response induced by AngII. Taken together, miR-124 may exacerbate AngII-induced cardiac hypertrophy.

AngII plays critical roles during the process from myocardial hypertrophy to heart failure, and takes part in myocardial hypertrophy via binding with its type I receptor, AT1R (20, 21). The AT1R mediates the principal biological effects of Ang II, and activation of AT1R signaling is reported to play an important role in cardiac hypertrophy. Previous studies have reported that AngII can regulate several miRNAs expressions, such as miR-132, miR-212, miR-29b, and miR-129-3p (22-24). Some microRNAs could also modulate AT1R, for example, miR-155 regulates AT1R expression (25). The expression of miR-133 is reduced in thyroid hormone-mediated cardiac hypertrophy partially via AT1R (26). Therefore, we investigated whether miR-124 could regulate AT1R expression in hypertrophic cardiomyocytes. The results showed that overexpression or knockdown of miR-124 could not change the mRNA expression of AT1R, indicating that the effect of miR-124 on cardiomyocyte hypertrophy might be independent of activation of AT1R.

In addition, AngII could also induce ER stress which is involved in cardiac hypertrophy. Takayanagi *et al* found that AngII might induce vascular remodeling via activation of epidermal growth factor receptor (EGFR) and ER stress involving cardiac hypertrophy (27). Furthermore, some miRNAs also participate in cardiac hypertrophy via ER stress. For example, miR-19a/b is sufficient to induce hypertrophy in rat neonatal cardiomyocytes via elevating the pro-hypertrophic calcineurin/ nuclear factor of activated T-cells (NFAT) signaling under ER stress conditions (11). Chen *et al* have informed that downregulation of miR-30 contributed to ER stress and the associated upregulation of GRP78 in the cardiovascular system, which may be a therapeutic strategy for moderating cardiac disorders associated with ER stress (28). Therefore, there is a possibility that the role of miR-124 in myocardial hypertrophy is linked with AngII-induced ER stress. Here, we observed that forced expression or inhibition of miR-124 could influence the expression of Grp78 and CRT, two ER stress markers, in hypertrophic cardiomyocytes. These data suggests that miR-124 promotes myocardial hypertrophy partly via activation of ER stress in NCMs exposed to AngII stimulation. However, further studies are necessary to determine the target gene of miR-124 in hypertrophic cardiomyocytes.

In conclusion, our findings demonstrate that miR-124 inhibition effectively suppresses AngII-induced myocardial hypertrophy, and this action may be associated with attenuation of ER stress.

Acknowledgements

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