

Original Research Article

Role of miR-134 in angiotensin II-induced vascular cell pathological changes in atherosclerosis

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Abstract

Purpose: To investigate the role of miR-134 in vascular smooth muscle cell dysfunction-related cardiovascular disease.

Methods: The effect of miR-134 was evaluated after human aortic smooth muscle cells (HASMCs) were transfected with miR-134 mimics. The expression levels of p-Akt, mechanistic target of rapamycin (mTOR), cleaved caspase-3, p53, and β -actin were evaluated by immunoblotting. Terminal deoxynucleotidyl transferase dUTP nick-end labeling was used to measure cell apoptosis. Reactive oxygen species levels were assayed by fluorescence microscopy after staining with 2',7'-dichlorofluorescein diacetate.

Results: Angiotensin II treatment induced miR-134 expression and Akt/mTOR activation, and inhibited cell viability in HASMCs ($p < 0.01$). Co-treatment with miRNA-134 reversed Ang II-induced HASMC dysfunction ($p < 0.01$). Overexpression of miR-134 is protective in Ang II-induced oxidative stress and apoptosis via the Akt/mTOR pathway ($p < 0.05$).

Conclusion: MicroRNA-134 in HASMCs is a potential therapeutic target for preventing Ang II-induced cardiac dysfunction via modulating Akt/mTOR pathway.

Keywords: MiR-134, Akt/mTOR pathway, Oxidative stress, HASMCs, Atherosclerosis

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INTRODUCTION

Atherosclerosis is a common cardiovascular disease which often leads to vascular lesions, arterial intimal thickening, increased infiltration of inflammatory cells, and the formation of a lipid-rich fibrous plaques [1,2]. The presence of atherosclerosis influence coronary arteries due to the build-up of plaque. Vascular smooth muscle cell (VSMC) dysfunction plays an important role on the pathogenesis of cardiovascular disease[3].

Previous studies showed that multiple mechanisms are involved in cardiac remodeling among these, the renin-angiotensin system has been associated with the progression of cardiovascular diseases through angiotensin II (Ang II), which acts as a growth factor regulating cardiovascular homeostasis and blood volume [4]. Angiotensin II is involved in regulating migration, proliferation and hypertrophy in VSMCs [5]. Angiotensin II can also induce the activation of calcineurin and the mitochondria-

dependent apoptotic pathway [5]. Importantly, Ang II causes rapid activation of Akt, mitogen-activated protein kinase (MAPK), MAPK/ERK kinase (MEK), and c-Jun N-terminal kinase (JNK), accompanied by cellular survival, hypertrophy, or apoptosis [6,7].

Previous studies have shown that both angiotensin II and reactive oxygen species (ROS) stimulation leads to the initiation of downstream signaling events, such as activation of the Akt/mTOR pathway [8,9]. Reactive Oxygen Species generation and its associated inflammatory responses including enhanced lipid metabolism and cell death are involved in the causation of different cardiovascular diseases, including atherosclerosis. Mammalian target of rapamycin (mTOR) and Akt both improve cardiac protection in vascular disorder, hypertension, and cardiovascular abnormalities. Interestingly, recent research reported that inhibition of the Akt/mTOR pathway inhibit atherosclerosis progression and raise the stability of atherosclerotic plaques [10].

MicroRNAs are endogenous, non-coding small RNA molecules which play key roles in cell differentiation, apoptosis, and cardiovascular diseases. Some microRNAs, such as miR-134, miR-9, and miR-219 are significantly differentially expressed in coronary artery disease. For example, miR-134 serves a critical regulator in various cardio-biological processes including lipid accumulation and proinflammatory cytokine secretion. Previous research reported that miR-134 is a factor related to heart failure, and serves as a biomarker for myocardial infarction [11]. However, the pathogenic mechanisms of miR-134 in VSMC dysfunction-related cardiovascular disease remain unknown. Therefore, this study aims to investigate the role miR-134 in the function of vascular smooth muscle cells.

EXPERIMENTAL

Cell culture and transfection

Human aortic smooth muscle cells (HASMCs) were purchased from the American Type Culture Collection (ATCC), and were maintained in Dulbecco's Modified Eagle's Medium high-glucose medium supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere of 5 % CO₂. Human aortic smooth muscle cells were plated at a density of 6 × 10⁴ cells per well into six-well plates. miR-134 mimics were synthesized for miR-134 overexpression and transfected into HASMCs using Lipofectamine™ 2000 (Thermo Fisher Scientific)

48 h before Ang II treatment. The efficiency of transfection was evaluated using qRT-PCR and immunoblotting.

Quantitative real-time PCR

Human aortic smooth muscle cells were harvested and total RNA was isolated using the TRIzol reagent (Invitrogen), and the miR-134 level was measured by qRT-PCR using the Universal cDNA synthesis and SYBR Green Master Mix kits. The expression of miR-134 was normalized to rRNA U6. Gene expression data were analyzed using the cycle threshold method.

The sequence of the miR-134 mimic primer used in the present study was: 5'-UGUGACUGGUUGACCAGAGGGG-3'.

Western blotting

Human aortic smooth muscle cells were lysed and extracted in lysis buffer. The concentration of the protein was determined using Lowry's method and the proteins were separated using 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and incubated in blocking solution (5 % non-fat milk). The membranes were then incubated with 1:1000 dilution primary antibody (anti-AKT, anti-pAKT, anti-mTOR, anti-pmTOR, anti-cleaved caspase-3, anti-p53, anti-β-actin) overnight at 4 °C. Subsequently, the membranes were incubated with secondary antibody for 1 h and visualized using a chemiluminescence system.

Cell viability assay

Human aortic smooth muscle cells viability was detected using a Viability Assay kit (Abcam, ab112120). HASMCs were washed with phosphate-buffered saline, then incubated in fresh medium containing dye-loading solution for 1 h at 37°C. After the removal of dye-loading solution, the absorption values were measured at 405/460 nm using ELISA reader.

TUNEL assay

Terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay was conducted following a previously described protocol [12].

Monitoring ROS generation

Dichlorofluorescein dye (non-fluorescent CM-H2DCFDA), which can diffuse through the cell

membrane, was used to evaluate ROS levels. The levels of ROS in HASMCs were determined using a fluorescence microscope with CM-H2DCFDA dye, after incubation with the miR-134 mimic, Ang II, or Akt activator. The fluorescence of DCFDA was measured with an excitation wavelength of 488 nm, and its emission was detected using a 520 nm band-pass filter.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance followed by t-test for multiple comparisons, using GraphPad Prism. Data are expressed as mean \pm SEM. $p < 0.05$ was considered to indicate statistically significant difference.

RESULTS

Effect of Ang II on miR-134 expression, AKT/mTOR activation, and cell viability of HASMCs

To determine whether miR-134 expression was changed with Ang II treatment in HASMCs, the expression level of miR-134 was measured using qRT-PCR. As shown in Figure 1 A, Ang II drove the time-dependent decrease of miR-134 expression in HASMCs. Based on these analyses, the 48-h treatment duration with Ang II was used for subsequent experiments.

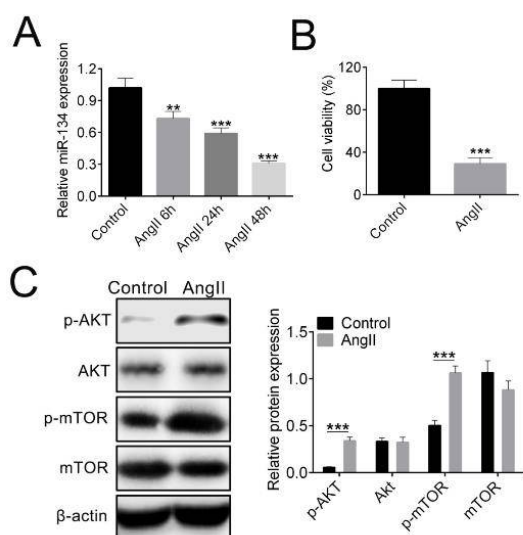


Figure 1: Effect of Ang II treatment in HASMCs. (A) Time-course of miRNA-134 expression following Ang II treatment, determined by qRT-PCR. (B) HASMC viability was assessed using Viability Assay kit. (C) The expression of indicated proteins in HASMCs treated with or without Ang II was detected by Western blot. (Data are mean \pm SEM, Student's t-test, ** $p < 0.01$, *** $p < 0.005$)

To assess the effect of Ang II on the activation of Akt signaling and cell apoptosis, HASMCs were treated with 100 nM of Ang II for 48 h. As seen in Figures 1 B and C, Ang II significantly decreased cell viability ($p < 0.01$; Figure 1 B) and enhanced the activation of Akt and mTOR ($p < 0.01$; Figure 1 C). These data indicate that Ang II treatment can induce AKT/mTOR activation and inhibit cell viability and miR-134 expression in HASMCs.

Overexpression of miRNA-134 protect against Ang II-induced HASMC dysfunction

To demonstrate the protective effect of miR-134 on Ang II-induced ROS generation and apoptosis, HASMCs were transfected with miR-134 mimic and co-treated with Ang II. The expression of miR-134 in all groups was evaluated by quantitative RT-PCR ($p < 0.01$; Figure 2 A). Intracellular ROS production was assessed following Ang II treatment using H2DCFDA ($p < 0.01$; Figure 2 C). The results are presented in Figure 2 C, and demonstrate that treatment with Ang II significantly increased the cellular oxidative stress in HASMCs. This trigger effect of Ang II was completely reversed by miR-134. In addition, treatment with miR-134 mimic alone did not significantly alter ROS production. Previous studies demonstrated that Ang II induces hypertrophy, proliferation, or apoptosis in VSMCs [13-15].

Thus, to investigate whether the miR-134 is involved in the mechanism of apoptosis, HASMC apoptosis was assayed using the TUNEL assay and western blot. Angiotensin II treatment alone caused a marked increase the number of cells that were positive for TUNEL staining ($p < 0.01$; Figure 2 C). Conversely, overexpression of miR-134 markedly attenuated this effect ($p < 0.01$; Figure 2 B). Consistent with the results of the TUNEL assay, the expression of cleaved caspase-3 and p53 were significantly increased in the Ang II group ($p < 0.01$; Figure 2 D). However, compared with the Ang II group, the group treated with miR-134 + Ang II showed significantly decreased expressions of cellular apoptosis biomarkers ($p < 0.01$; Figure 2 D). These results suggest that miR-134 overexpression may protect HASMCs from Ang II-induced oxidative stress and apoptosis.

Upregulation of miR-134 attenuates the Ang II-induced viability decrease in HASMCs

To explore the effects of miR-134 in Ang II-induced HASMC viability, a cell viability assay was performed. Treatment of HASMCs with Ang II significantly suppressed cell viability ($p < 0.01$; Figure 3), whereas overexpression of miR-134

with Ang II treatment significantly reversed the Ang II-induced effect ($p < 0.01$; Figure 3). These results showed that Ang II-inhibited cellular viability is apparently reversed upon overexpression of miR-134.

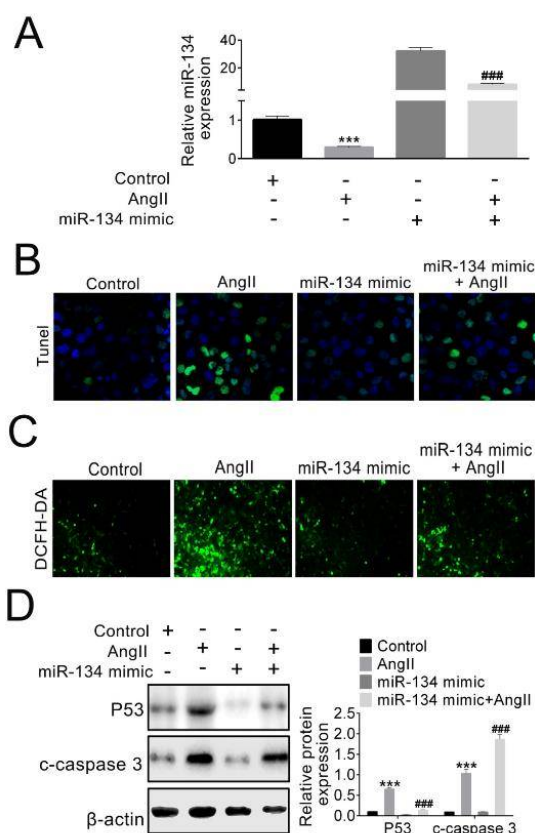


Figure 2: Effect of miR-134 in Ang II-induced cellular apoptosis and oxidative stress. (A) The expression level miR-134 after transfection with miR-134 mimic or Ang II treatment, determined by qRT-PCR. (B) Cell apoptosis was determined by TUNEL assay. (C) Cells were stained with oxidized dye to measure intracellular ROS, and then detected by DCF-derived fluorescence. (D) The expression of indicated proteins was detected by Western blot. (Data are mean \pm SEM, Student's t-test, ### $p < 0.01$ vs Ang II, *** $p < 0.005$ vs control).

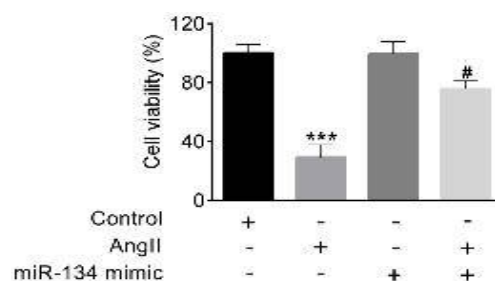


Figure 3: Effect of miR-134 on Ang II-induced viability inhibition. (A) miR-134 overexpression against Ang II-induced injury was determined by viability assay. (Data are mean \pm SEM, Student's t-test, # $p < 0.05$ vs Ang II, *** $p < 0.005$)

MiR-134 suppresses Ang II-induced cardiac dysfunction via Akt/mTOR pathway

To investigate the molecular mechanism of miR-134 in protecting Ang II-mediated cardiac dysfunction, this study determined whether the protective effect of miR-134 in Ang II treatment is mediated through the Akt/mTOR pathway. Cells exhibiting Ang II-induced cardiac dysfunction were pretreated with the Akt activator SC79 and pre-transfected with miR-134. Since miR-134 has anti-apoptotic and antioxidant effects, this study then assessed whether miR-134 affects the Akt activation induced by Ang II.

Western blot showed that miR-134 significantly inhibited the Ang II-induced activation of Akt and mTOR ($p < 0.05$; Figure 4C). Thus, overexpression of miR-134 attenuate Ang II-induced Akt/mTOR pathway activation. To ascertain whether Ang II-induced Akt activation and cardiomyocyte dysfunction is associated with cellular ROS generation, this study first analyzed the effect of Akt activation on Ang II-induced increases in intracellular ROS production in HASMCs, using DCFDA staining. As shown in Figure 4A, Ang II treatment significantly increased ROS production when compared with the control and miR-134 only groups, whereas miR-134 + Ang II treatment markedly attenuated these effects. Conversely, combined treatment with miR-134 + SC79 suppressed the protective effects of miR-134 ($p < 0.05$; Figure 4A). On the other hand, compared with the miR-134 + Ang II group, the cell apoptosis, as measured by TUNEL staining and the expression levels of cleaved caspase-3 and p53, were significantly increased upon miR-134 + SC79 treatment ($p < 0.05$; Figure 4B & C). These results indicate that miR-134 provides a protective mechanism in Ang II-induced cardiac dysfunction via the Akt/mTOR pathway.

DISCUSSION

There are multiple physiological and pathological processes involved in atherosclerosis, such as foam cell formation, activation of the inflammatory response, and ROS generation [16]. A large body of clinical and laboratory data are now available confirming that the renin-angiotensin system is mechanistically relevant in the pathogenesis of atherosclerosis [4,17]. Renin-angiotensin system has been well established as a major physiological system that regulates blood pressure, sodium balance, and cardiovascular status, and is involved in atherosclerosis development and vascular NAD(P)H oxidase activation [17].

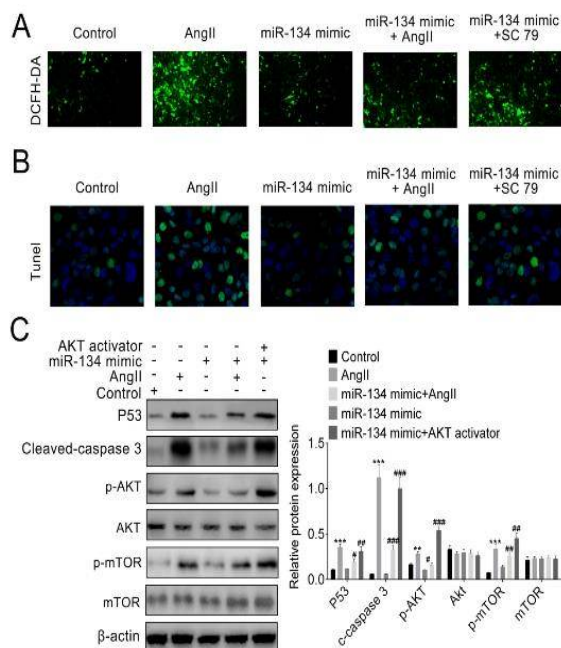


Figure 4: miR-134 modulate Ang II-induced cell dysfunction in HASMCs via the Akt/mTOR signaling pathway. (A) Cells were stained with oxidized dyes to measure intracellular ROS, which was then detected by DCF-derived fluorescence. (B) Apoptosis rate was determined by the TUNEL assay. (C) The expression of indicated proteins was detected by Western blot. (data are mean \pm SEM, Student's t-test, ## $p < 0.01$ vs Ang II, *** $p < 0.005$)

Activation of the renin–angiotensin system accelerates HASMC hypertrophy and inhibits cell viability, thereby contributing to cardiovascular disease. [18,19]. Recent research has reported that Ang II augments proinflammatory activity in the vascular wall by inducing ROS generation and inflammatory cytokines. However, the signaling pathway involved in regulating the cell viability and apoptosis of HASMCs in atherosclerosis remains unclear.

In the present study, this study first determined that the overexpression of miR-134 could attenuate Ang II-induced oxidative stress and apoptosis in HASMCs. These results revealed that the expression level of miR-134 was significantly decreased after Ang II treatment, compared with the untreated group. This finding was supported by the results of quantitative RT-PCR. Furthermore, Ang II significantly decreased cell viability and enhanced the activation of Akt and mTOR. These data are consistent with recent findings that Ang II activates the Akt/mTOR pathway through the small GTPase Rac1 and a NAD(P)H oxidase [20,21]. Activation of the Akt/mTOR pathway is important for the regulation of metabolism and proliferation in HASMCs.

Recent studies showed that the Akt/mTOR pathway is a target of ROS, and ROS play a role as second messengers mediating the trigger effect of Ang II [22]. MicroRNA-134, located on 14q32.31, has been reported to regulate the pathological processes of various vascular diseases [23,24]. Considering that Akt/mTOR pathway activation and oxidative stress and miRNA-134 are important factors in the process of cardiovascular disease, this study analyzed the functional relevance of the Akt/mTOR pathway, oxidative stress, and miR-134 in Ang II-induced HASMC dysfunction.

In this study, the Ang II treatment model in HASMCs was used to mimic the characteristic vascular pathologies of atherosclerosis *in vitro*. In the course of atherosclerotic disease, the balance between cell apoptosis and survival of VSMCs is associated with atherosclerotic plaque instability or stability [25]. Although Ang II could stimulate HASMC proliferation, a previous study showed that long-term treatment with Ang II may induce cell apoptosis. The effects of miR-134 on Ang II-induced cell apoptosis was found.

A previous research has shown that the protective effect of AKT/mTOR pathway could modulate intracellular ROS generation and regulate vascular smooth muscle dysfunction [26]. This study found that the overexpression of miR-134 significantly increased the expressions of cellular antioxidant factors, thereby protecting HASMCs from Ang II-induced cardiac dysfunction via the Akt/mTOR pathway. These findings identified miR-134 as a potential therapeutic target in preventing Ang II-induced cardiac dysfunction.

CONCLUSION

These results demonstrate that Ang II induces cellular apoptosis and increased oxidative stress. The expression of miR-134 in HASMCs will be critical for cardiac therapeutic applications. microRNA-134 provides a protective mechanism in Ang II-induced cardiac dysfunction via the Akt/mTOR pathway. This study reveals that miR-134 exerts a protective effect in cardiac dysfunction and also provides an alternative therapeutic approach for atherosclerosis.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. JC and QH designed all the experiments and revised the paper. BZ and XL formed the experiments, SY and HJ wrote the paper.

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