Original Article

miRNA-134 suppresses angiotensin II-induced vascular smooth muscle cell dysfunction by targeting angiotensin II type 1 receptor (AT1R)

Yuan Zhang1,2, Xin Lu3, Zhi-Liang Li1, Feng Ding2, Hao Cheng2

1Department of Cardiology, Zhujiang Hospital of Southern Medical University, Guangzhou 510280, China; 2Department of Cardiology, Inner Mongolia People’s Hospital, Hohhot 010017, China; 3Department of Electrocardiogram, The Affiliated Hospital of Inner Mongolia Medical University, Hohhot 010050, China

Received January 15, 2016; Accepted April 6, 2016; Epub May 15, 2017; Published May 30, 2017

Abstract: MicroRNA-134 is a multifunctional post-translational modulator that participates in a variety of diseases. However, the relationship between Ang II, miRNA-134 and AT1R has yet to be fully explored in cardiovascular diseases. In this study, we hypothesized that a post-translational mechanism of microRNAs regulated the expression of AT1R in VSMCs in the present of Ang II. To identify differentially expressed miRNAs in response to Ang II in cultured VSMCs, we performed microarray assay and found that miRNA-134 expression was significantly suppressed in Ang II-treated VSMCs. Next, bioinformatic analysis showed the potential miRNA-134 binding sites within the 3’-UTR of AT1R in human sapiens. Luciferase assays verified significantly reduced luciferase activity in miRNA-134-transfected wild-type VSMCs compared with NC cells. In addition, miRNA-134 could inhibit Ang II-induced cell proliferation, secretory and mitochondrial dysfunction in VSMCs. Furthermore, overexpressed miRNA-134 could reverse Ang II-induced increase of ACE and AT1R and decrease of AT2R in VSMCs. In conclusion, these results demonstrated that miRNA-134 played a protective effect on Ang II-induced VSMCs dysfunction in vitro, and the underlying mechanism was mediated, at least partially, through the inhibition of AT1R signaling.

Keywords: MiRNA-134, vascular smooth muscle cell, angiotensin II, AT1R

Introduction

Vascular smooth muscle cells (VSMCs) dysfunction are a common pathological basis for hypertension, atherosclerosis and vascular restenosis. Moreover, various cardiovascular diseases risk factors lead to damage to the blood vessel wall and cellular infiltration [1, 2]. The renin-angiotensin system (RAS) has been implicated in the pathogenesis of cardiovascular diseases through its primary effector molecule angiotensin II (Ang II), which has a major impact on the cardiac and vascular function through regulation of systemic hemodynamics and blood volume [3]. Recent reports have demonstrated that Ang II can induce VSMCs proliferation, migration and hypertrophy [4-6]. Importantly, Ang II induces the expression of cytokines such as interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and osteopontin in VSMCs, which have a crucial role in monocyte recruitment, neointimal formation, and atherosclerosis [5, 7]. Ang II plays essential roles in the signal transduction via binding to two distinct high affinity plasma membrane receptors designated angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R), which are seven transmembrane-spanning G protein-coupled receptors. The current view is that the AT2R counteracts the “classical” Ang II responses in the cardiovascular and renal systems mediated via the AT1R [8, 9]. However, the underlying molecular mechanisms involved in Ang II-induced VSMC dysfunction remain incompletely understood.

MicroRNAs (miRNAs) are endogenous non-coding RNAs and single-stranded RNA molecules of 19–25 nucleotides in length that serve as important post-transcriptional gene regulators [10]. Subsequently, they bind with RNA-induced silencing complex (RISC) and base-pair with tar-
miRNA-134 regulates AT1R in VSMCs

get mRNAs to inhibit translation or promote mRNAs degradation. The key features of miRNAs regulate cell proliferation and differentiation of various cell types. A growing number of studies have demonstrated that the pathogenic change in various tissues has been linked to miRNAs [11-14]. Interestingly, miRNAs are expressed and play many important biological functions in the cardiovascular system, such as miRNA-181b, miRNA-599 and miRNA-322 which regulate VSMCs proliferation and differentiation [15-17]. However, the underlying molecular mechanisms of miRNAs in Ang II-mediated VSMC dysfunction by targeting AT1R remain to be determined.

In this study, we hypothesize that a post-translational mechanism might exist for AT1R signaling, which could be regulated by miRNAs in Ang II-mediated VSMCs dysfunction. By using PicTar, TargetScan, and miRBase database and microarray assay, we found that miRNA-134 was a regulator of AT1R through the predicted binding sites in its 3'-UTR. Therefore, the aim of the present study was to explore the effects of miRNA-134 on the Ang II-induced VSMCs dysfunction and also to investigate the role of the AT1R signaling in this process.

Materials and methods

Cell culture

The human vascular smooth muscle cells (VSMCs) were obtained from the Cell Resource Center, Shanghai Institutes for Biological Sciences (SIBS, China), and maintained in RPMI-1640 (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) at 37°C in a humidified incubator (Thermo, USA), 5% CO2, 95% air atmosphere.

Cell viability detection by MTT

VSMCs proliferation was monitored by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation/Viability Assay kit (R&D SYSTEMS) in accordance to the guidelines.

Nitric oxide quantification

VSMCs were plated and treated in 96-well plates and were stimulated with Ang II or transfected with miRNA-134. Twelve hours later centrifuged to obtain the supernatant, and the level of nitric oxide was measured by nitrite production using the Griess reagent (Invitrogen, USA) at 540 nm using an ELISA reader (BioTek, USA) according to the manufacturer’s instructions.

Detection of Ca2+ concentrations

VSMCs were plated and treated in 12-well plates and were incubated with Ang II or transfected with miRNA-134 to detect changes in Ca2+ levels. Cells were harvested and washed twice, and re-suspension in Indo 1/AM (3 μg/ml) at 37°C for 30 min and analyzed by flow cytometry.

Determination of the mitochondrial membrane potential

The mitochondrial membrane potential was assessed using a fluorometric probe, DiOC6 (Molecular Probes). Briefly, cells were plated in 6-well culture dishes. After reaching confluence, cells were treated with Ang II or transfected with miRNA-134. After incubation, cells were stained with DiOC6 (40 nM) for 15 min at 37°C. Cells were collected, washed twice in PBS, and analyzed by FACScan flow cytometry.

Luciferase reporter gene activity assay

The 3'-UTR of AT1R gene containing the predicted target sites for miRNA-134 was obtained by PCR amplification. The fragment was inserted into the multiple cloning sites in the pMIR-REPORT luciferase microRNA expression reporter vector (Ambion, Austin, USA). VSMVs were co-transfected with 100 ng of luciferase reporters containing AT1R 3'-UTR and miRNA-134 mimics by Lipofectamine 2000 (Invitrogen, CA, USA). We harvested the cell lysates after 12 hours transfection and measured the luciferase activity with a dual luciferase reporter assay kit according to manufacturer’s instruction.

Transfection of miRNA-134 mimics and inhibitor

The FAM modified 2′-OMe-oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography (GenePharma, Shanghai, China). The 2′-OMe-miRNA134 mimics were composed of RNA duplexes with the following sequence: 5′-UG-UGACUGGUACAGAGGGG-3′. The sequenc-
miRNA-134 regulates AT1R in VSMCs

Real time-polymerase chain reaction

RNA extraction was performed according to the TRIzol manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 4 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen) with oligo dT (15) primers (Fermentas) as described by the manufacturer. miRNA-134 level was quantified by the mirVana qRT-PCR miRNA detection kit (Ambion, Austin, USA) in conjunction with real-time PCR with SYBR Green. After circle reaction, the threshold cycle (Ct) was determined and relative miRNA-134 level was calculated based on the Ct values and normalized to U6 level in each sample. PCR with the following primers: ACE, Forward 5’-CCCATCTGCTAGGGAACATGT-3’ and Reverse 5’-GGTTGCTCCCTGCTTTATCA-3’; AT1R, Forward 5’-CCATCACCAGATCAAGTGCA-3’ and Reverse 5’-TGGGGCAGTCATCTTGAATTCT-3’; AT2R, Forward 5’-CAGTTGACGTGATGCACAGG-3’ and Reverse 5’-CGGTTGAAGTCGTGGAGCCC-3”; GAPDH, Forward 5’-GCACCGTCAAGCTGAGAAC-3’ and Reverse 5’-TGGTGAAGACGCCAGTGGA-3’.

Western blotting

VSMCs were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 30 μg of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with primary antibodies: ACE, AT1R and AT2R (Santa Cruz Biotechnology, CA, USA). After three washes with TBST, The membranes were next incubated with the appropriate HRP (horseradish peroxidase)-conjugated antibody visualized with chemiluminescence (Thermo, USA).

Statistical analysis

The data from these experiments were reported as mean ± standard errors of mean (SEM) for each group. All statistical analyses were performed by using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey’s multiple comparison test as a post test to compare the group means if overall P < 0.05. Differences with P value of < 0.05 were considered statistically significant.

Results

Identification of Ang II-regulated miRNAs in human VSMCs

To identify differentially expressed miRNAs in response to Ang II in cultured VSMCs, we performed microarray assay with small RNA libraries generated using total RNA extracted from control group or Ang II-stimulated (10 nM) VSMCs for 12 hours. We found that miRNA-134 and miRNA-185 was significantly lowly expressed in Ang II-treated group as compared to control group (Figure 1A). Among the Ang II-induced miRNAs, we concluded that miRNA-134 and miRNA-185 might be closely related to VSMCs dysfunction in the present of Ang II. miRNA-134 and miRNA-185 have been reported to be expressed as a highly conserved cluster and can modulate diverse functions in different cell systems [18, 19]. Therefore, we further investigated the functional roles of miRNA-134 and miR-185 in VSMCs when they were exposed to Ang II. Time course and concentration dependent experiments showed that Ang II markedly suppressed miRNA-134 and miRNA-185 expression in VSMCs (Figure 1B and 1C). Subsequently, pretreatment with AT1R blocker Losartan (10 μM) for 12 hours significantly reversed Ang II-induced down-regulated miRNA-134 and miRNA-185 in VSMCs (Figure 1D). However, miRNA-134 in response to Ang II-treated in VSMCs was more sensitive to miRNA-185, and we finally focused on miRNA-134 in our study.

Ang II-stimulated RAS components in human VSMCs

As a key active peptide in RAS, Ang II has been shown to act on VSMCs dysfunction [20]. However, the RAS components expression that
miRNA-134 regulates AT1R in VSMCs

induced by Ang II in VSMCs were not fully understood. In the present study, the mRNA and protein expression of RAS components, such as angiotensin-converting enzyme (ACE), AT1R and AT2R, were measured by real-time PCR and western blotting respectively. The results showed that the mRNA and protein expression of ACE and AT1R were significantly increased in a time dependent manner when the VSMCs were exposed to Ang II at concentration of 10 nM (Figure 2A and 2B). In contrast to that the mRNA and protein expression of AT2R were significantly decreased when the VSMCs were exposed to Ang II after 6 hours (Figure 2C). These results demonstrated that RAS signaling might be involved in VSMCs dysfunction in vitro.

miRNA-134 targets AT1R

Because each miRNA target prediction algorithm (PicTar, TargetScan and miRBase) has its own advantages, targets theoretically predicted by multiple bioinformatics software and having conserved seed sequences are more likely to be true targets. Therefore, we used these bioinformatics tools and selected the targets that were predicted by at least two databases and conserved across rat, mouse and human. We found the potential miRNA-134 binding sites within the 3'-UTR of AT1R in human sapiens (Figure 3A). To verify if AT1R is a direct target of miR-134, we cloned the 3'-UTR of the wild-type or mutant-type AT1R gene and co-
miRNA-134 regulates AT1R in VSMCs

transfected it along with miRNA-134 or NC oligonucleotides into VSMCs. Luciferase assays were performed 24 hours post-transfection. Results showed significantly reduced luciferase activity in miRNA-134-transfected cells compared with NC cells. In contrast, co-transfection miRNA-134 into AT1R mutant-type 3'-UTR cells, the luciferase activity did not show significant difference compared with NC group (Figure 3B). Next, we observed that transfection of VSMCs with miR-134 mimic oligonucleotides or miR-134 inhibitors significantly downregulated or upregulated, respectively, both AT1R mRNA (Figure 3C) and protein (Figure 3D) levels.

Overexpressed miRNA-134 protects against Ang II-induced VSMCs dysfunction

To investigate the potential roles of miRNA-134 on Ang II-induced the proliferation of VSMCs, we first examined the effect of miRNA-134 on cell survival by MTT assay. The results indicated that treatment of VSMCs with Ang II induced cell proliferation in a time-dependent manner, and the number of VSMCs was markedly increased in Ang II-treated group. However, co-incubated with Ang II and miRNA-134 mimics in VSMCs, the cell viability was significantly decreased as compared to Ang II single treatment group. Moreover, miRNA-134 mimics single treatment could also inhibit the cell proliferation compared with control group (Figure 4A). To assess Ang II-induced secretory dysfunction of VSMCs and the modulation effect of miRNA-134, we measured the levels of NO and p-eNOS in VSMCs. As shown in Figure 4B and 4C, the levels of NO was significantly increased, and p-eNOS was significantly decreased when the cells were exposed to Ang II at concentration of 10 nM. However, VSMCs transfected with miRNA-134 for 12 hours reversed the increased level of NO or decreased level of p-eNOS in VSMCs (Figure 4B and 4C). These
miRNA-134 regulates AT1R in VSMCs

Results suggested that miRNA-134 could modulate the Ang II-induced secretory dysfunction of VSMCs. Furthermore, Ang II-induced mitochondrial dysfunction and the potential protective effects of miRNA-134 was investigated. As shown in Figure 4D and 4E, treatment of VSMCs with Ang II induced the loss of the mitochondrial membrane potential and increase of Ca\(^{2+}\) releases as compared to control group. Ang II combination with miRNA-134 transfection could significantly improve mitochondrial membrane potential and suppress Ca\(^{2+}\) releases in VSMCs (Figure 4D and 4E).

Overexpressed miRNA-134 Inhibits RAS Components in Human VSMCs

To determine whether RAS components were involved in miRNA-134 inhibition of VSMC proliferation, we used real-time PCR and western blotting to examine RAS components mRNA and protein expression in miRNA-134 overexpressed VSMCs. The mRNA and protein expression of ACE and AT1R were significantly decreased in miRNA-134 overexpressed VSMCs as compared to control group. Moreover, overexpressed miRNA-134 could reverse Ang II-induced increase of ACE and AT1R in VSMCs (Figure 5A and 5B). In contrast to that, the mRNA and protein expression of AT2R were significantly increased in miRNA-134 overexpressed VSMCs as compared to control group. As expected, overexpressed miRNA-134 could reverse Ang II-induced decrease of AT2R in VSMCs (Figure 5C). These results demonstrated that miRNA-134 played a protective effect on Ang II-induced VSMCs dysfunction in vitro, and the underlying mechanism was mediated, at least partially, through the inhibition of AT1R signaling.

Discussion

It has been postulated that Ang II plays a crucial role in cardiovascular remodeling, as well as in the pathogenesis of atherosclerosis,
Figure 4. MTT assays were performed on VSMCs transfected with miRNA-134 mimics or inhibitor for 0, 6, 12 and 24 hours (A). VSMCs were transfected with miRNA-134 mimics or inhibitor for 12 hours, and the NO concentration was detected by ELISA assay (B). The protein expression of p-eNOS and eNOS was measured by western blotting (C).
miRNA-134 regulates AT1R in VSMCs

VSMCs were transfected with miRNA-134 mimics or inhibitor for 12 hours, the mitochondrial membrane potential (D) and the release of Ca$^{2+}$ (E) were examined by flow cytometry. Values were expressed as mean ± SEM, n = 3 in each group. **P < 0.01, ***P < 0.001.

![Graphs showing mRNA and protein expression of ACE, AT1R, and AT2R](image)

Figure 5. VSMCs were transfected with miRNA-134 mimics or inhibitor for 12 hours, mRNA and protein expression of ACE (A), AT1R (B) and AT2R (C) were analyzed by real-time PCR or western blotting respectively. Values were expressed as mean ± SEM, n = 3 in each group. *P < 0.05, **P < 0.01, ***P < 0.001.

miRNA-134, located on 14q32.31, has been reported to be dysregulated in several malignant tumors and acts as a tumor suppressor in breast cancer [18], non-small cell lung cancer (NSCLC) [24], ovarian cancer [25] and osteosarcoma [26]. Recent studies have shown that miRNA-134 plays a role in vascular biology and are involved in biochemical and molecular pathways dysregulated during vascular injury and may regulate lipid accumulation and proinflammatory cytokine secretion [27, 28]. Therefore, we analyzed the functional relevance of miR-134 in Ang II-induced VSMC dysfunction. Our studies suggested that treatment of VSMCs with Ang II induced cell proliferation in a time-
miRNA-134 regulates AT1R in VSMCs

dependent manner, and the number of VSMCs was markedly increased in Ang II-treated group. We established that directly transfecting miRNA-134 into VSMCs could improve Ang II-induced cell proliferation, secretory and mitochondrial dysfunction. Collectively, these studies indicated that miRNA-134 was downregulated in many pathophysiological processes, and our study extended these observations by showing that overexpressed miRNA-134 plays an important role in Ang II-induced VSMCs dysfunction.

Previous studies indicate that miRNA-134 by targeting angiopoietin-like 4 in THP-1 macrophages regulates lipid accumulation and inflammatory response [28], modulates the proliferation of human cardiomyocyte progenitor cells by targeting Meis2 [29] and regulates ischemia/reperfusion injury-induced neuronal cell death by regulating CREB signaling [30]. In this study, we found that miRNA-134, as a multifunctional signal miRNA, could target AT1R via the potential miRNA-134 binding sites within the 3' UTR of AT1R in VSMCs. To our knowledge, the biological effects of Ang II are mediated by AT1R and AT2R, and Ang II-induced VSMC proliferation is mediated mainly by AT1R, rather than AT2R [4, 22]. Other studies in macrophages have shown that AT1R antagonists inhibit the effect of Ang II, but AT2R antagonists the opposite has happened [31]. In the present study, overexpressed miRNA-134 could reverse Ang II-induced increase of ACE and AT1R and decrease of AT2R in VSMCs. These results demonstrated that miRNA-134 played a protective effect on Ang II-induced VSMCs dysfunction in vitro, and the underlying mechanism was mediated, at least partially, through the inhibition of AT1R signaling.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhi-Liang Li, The Department of Cardiology, Zhujiang Hospital of Southern Medical University, Guangzhou 510280, China. Tel: (+86) 13809774642; E-mail: xinlu_088@163.com

References

[15] Li TJ, Chen YL, Gua CJ, Xue SJ, Ma SM and Li XD. MicroRNA 181b promotes vascular smooth
miRNA-134 regulates AT1R in VSMCs


[22] Diep QN, Li JS and Schiffrin EL. In vivo study of AT(1) and AT(2) angiotensin receptors in apoptosis in rat blood vessels. Hypertension 1999; 34: 617-624.


