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Original Article

miR-155 suppresses palmitic acid-induced vascular smooth muscle cell dysfunction by targeting angiotensin II type 1 receptor

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Abstract: miR-155 is a multifunctional post-translational modulator that participates in a variety of diseases. However, the relationship between palmitic acid (PA), miR-155 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 PA-treated VSMCs. First, we found that PA could accelerate the mitochondrial dysfunction and DNA damage and induce VSMCs apoptosis in vitro. Next, abnormally expressed AT1R and miR-155 were simultaneously induced by PA in VSMCs. Intriguingly, bioinformatic analysis showed the potential miR-155 binding sites within the 3'-UTR of AT1R in human VSMCs. Luciferase assays verified significantly reduced luciferase activity in miR-155-transfected wild-type VSMCs compared with NC cells. In addition, miR-155 could inhibit PA-induced apoptosis and reverse PA-induced increase of AT1R in VSMCs. In conclusion, the results suggested that overexpressed miR-155 inhibited PA-induced VSMCs dysfunction, and the underlying mechanism was mediated, at least partially, through the suppression of AT1R expression.

Keywords: miR-155, vascular smooth muscle cell, atherosclerosis, AT1R

Introduction

Palmitic acid (PA) is the most common saturated free fatty acid and is known to induce vascular smooth muscle cells (VSMCs) dysfunction that can lead to atherosclerosis (AS), hypertension and vascular restenosis [1]. In particular, PA can induce apoptosis, oxidative stress and inflammatory cytokines, such as C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) and interleukin-6 (IL-6), all cytokines that aggravate the vascular damage and cellular infiltration [2-4]. Moreover, a variety of cytokines and growth factors activate their corresponding receptors in VSMCs, which can activate cytoplasmic signal transduction pathways and induce dysfunction of VSMCs and finally lead to the progression of various cardiovascular diseases [5, 6].

miRNAs are endogenous non-coding RNAs, a class of small non-coding (18-25 nucleotides), and regulate the translation of messenger RNAs (mRNAs) by binding to the target mRNAs 3'-untranslated regions (3'-UTR), which is an important post-transcriptional gene down-regulation mechanisms in cell growth and development [7, 8]. Recent studies have demonstrated that the pathogenic change in various of tissues has been linked to miRNAs, and abnormal miRNAs expression via various cell signaling pathways regulate cancer development [9, 10]. A number of miRNAs have been identified as markedly up- or down-regulated in atherosclerosis development and progression [11-13]. miR-155 is a typical multi-functional miRNA with distinct expression profiles and has a crucial role in various physiological and pathological processes, such as hematopoietic lineage differentiation, immunity, inflammation, and cancer [14, 15]. miR-155 attenuates the effect of exogenous Ang II-induced ERK1/2 activation to reduce HUVEC damage and apoptosis [16]. Moreover, miR-155 inhibits angiotensin II-induced VSMCs proliferation [2]. In the atherosclerotic plaque samples, the expression levels

of miR-155 are significantly upregulated compared with those in the normal control samples, and miR-155 can modulate the cell proliferation by targeting endothelial nitric oxide synthase in VSMCs [17]. However, the underlying molecular mechanisms of miR-155 in PA-induced VSMCs dysfunction via targeting angiotensin II type 1 receptor (AT1R) remains unknown. The present study was undertaken to determine the effects of miR-155 on the PA-induced VSMCs dysfunction and also to investigate the role of the AT1R signaling in this process.

Materials and methods

Cell culture

The vascular smooth muscle cells (VSMCs) were maintained in RPMI-1640 (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) at 37°C in a humidified incubator (Thermo, USA), 5% CO_2, 95% air atmosphere. The medium was replenished every day.

Cell viability detection by CCK8

VSMCs (5.0×10^3/well) were plated and treated in 96-well plates (three wells per group) with various concentrations of palmitic acid (Sigma, USA) for 24 h, 48 h or 72 h respectively. 10 μL of CCK8 (Beyotime, China) was added to the cells, and the OD value of the cells was measured at 450 nm using an ELISA reader (BioTek, USA) according to the manufacturer's instructions.

Nitric oxide quantification

VSMCs (1.0×10^5/well) were plated and treated in 96-well plates (three wells per group) and were stimulated with palmitic acid or miR-155 mimics in the presence or absence. Forty-eight hours later centrifugate 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 Ca^{2+} concentrations

VSMCs were plated and treated in 12-well plates and were incubated with PA to detect changes in Ca^{2+} 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 (Becton Dickinson, USA).

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 PA. 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 (Becton Dickinson, USA).

The comet assay

Briefly, fully frosted slides were precoated on each end with 100 mL of 0.8% agarose in phosphate-buffered saline (pH = 7.4) and covered with a 22 mm × 22 mm glass coverslip and left at room temperature for 20 minutes. Then, 30 mL of the cell culture was mixed with 70 mL of 1% low-melting point agarose in phosphate-buffered saline and maintained at 42°C on a dry-bath incubator. The mixture was immediately spread onto each end of a precoated slide and covered with a fresh glass coverslip, and the comets were captured with an Olympus microscope equipped with a CCD camera connected to the fluorescent microscope.

Caspase-3 activity and cell apoptosis assay

VSMCs lysates were prepared and incubated with anti-caspase 3. Immunocomplexes were incubated with peptide substrate in assay buffer for 2 h at 37°C. Release of p-nitroaniline was measured at 405 nm using an ELISA reader (MD SpectraMax M5, USA) according to the manufacturer's instructions. Results signify percent change in activity compared to untreated control.

Cell apoptosis was measured using an Annexin-V and Propidium Iodide (PI) Apoptosis Detection Kit (Beyotime, China) by a flow cytometer (Becton Dickinson, USA) according to the guidelines. Quantitative assessment of apoptotic cells was assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling (TUNEL) method,

which examines DNA-strand breaks during apoptosis by using BD ApoAlertTM DNA Fragmentation Assay Kit. Cells were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% sodiumcitrate. After being washed, the cells were incubated with the reaction mixture for 60 min at 37°C. Cells were immediately analyzed using FACScan and the Cellquest program (Becton Dickinson, USA).

Transfection of miR-155 mimics and inhibitor

The FAM modified 2'-OMe-oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography (Gene Pharma, Shanghai, China). The 2'-OMe-miR-155 mimics were composed of RNA duplexes with the following sequence: 5'-UUAAUGCUAAUCGUGAUAGGGGU-3'. The sequences of 2'-OMe-miR-155 inhibitor and 2'-Ome-scramble oligonucleotides were as follows: 5'-ACCCCUAUCACGAUUAGCAUUAA-3'; and 5'-CGUAGGCUAGUUGACGUAGGCGA-3'. Cells were transfected using Lipofectamine2000 (Invitrogen, CA, USA) at a final concentration of 50 nM or 100 nM. At 24 h post-transfection, the culture medium was changed. After 12 hours, cells were harvested for analysis.

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. miR-155 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 miR-155 level was calculated based on the Ct values and normalized to U6 level in each sample. PCR with the following primers: AGT, Forward 5'-CGAGTGGGAGAGGTTCTCAA-3' and Reverse 5'-CTCGTAGATGGCGAACAGGA-3'; ACE, Forward 5'-CCCATCTGCTAGGGAACATGT-3' and Reverse 5'-GGTGTCCCTGCTTTATCA-3'; AT1R, Forward 5'-CCATCACCAGATCAAGTGCA-3' and Reverse 5'-TGGGGCAGTCATCTTGAATTCT-3'; GAPDH, Forward 5'-GCACCGTCAAGCTGAGAAC-3' and Reverse 5'-TGGTGAAGACGCCAGTGGA-3'.

Western blotting

VSMCs were 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: AT1R, iNOS, caspase3 and caspase9 (Santa Cruz Biotechnoogy, 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 6.0 (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

PA induces VSMCs dysfunction

To determine whether PA regulated the proliferation of VSMCs, we used the CCK-8 assay to monitor cell viability. The results indicated that the treatment of VSMCs with PA inhibited cell proliferation in a time- and doe-dependent manner, and the number of VSMCs was markedly decreased 16.1%, 27.3% and 36.4 exposure to PA for 72 h at the concentration of 0.1 mM, 0.5 mM and 1 mM respectively (**Figure 1A**). Next, we investigated whether PA induces cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used for the detection of PS externalization, a hallmark of early phase of apoptosis. Consistent with the CCK8 assay, the results showed that the proportion of the apoptosis cells had gained in the present of PA with a dose-dependent manner (**Figure 1B**). Mitochondrial dysfunction and DNA damage are involved in VSMCs proliferation inhibition and apoptosis [18, 19]. To fur-

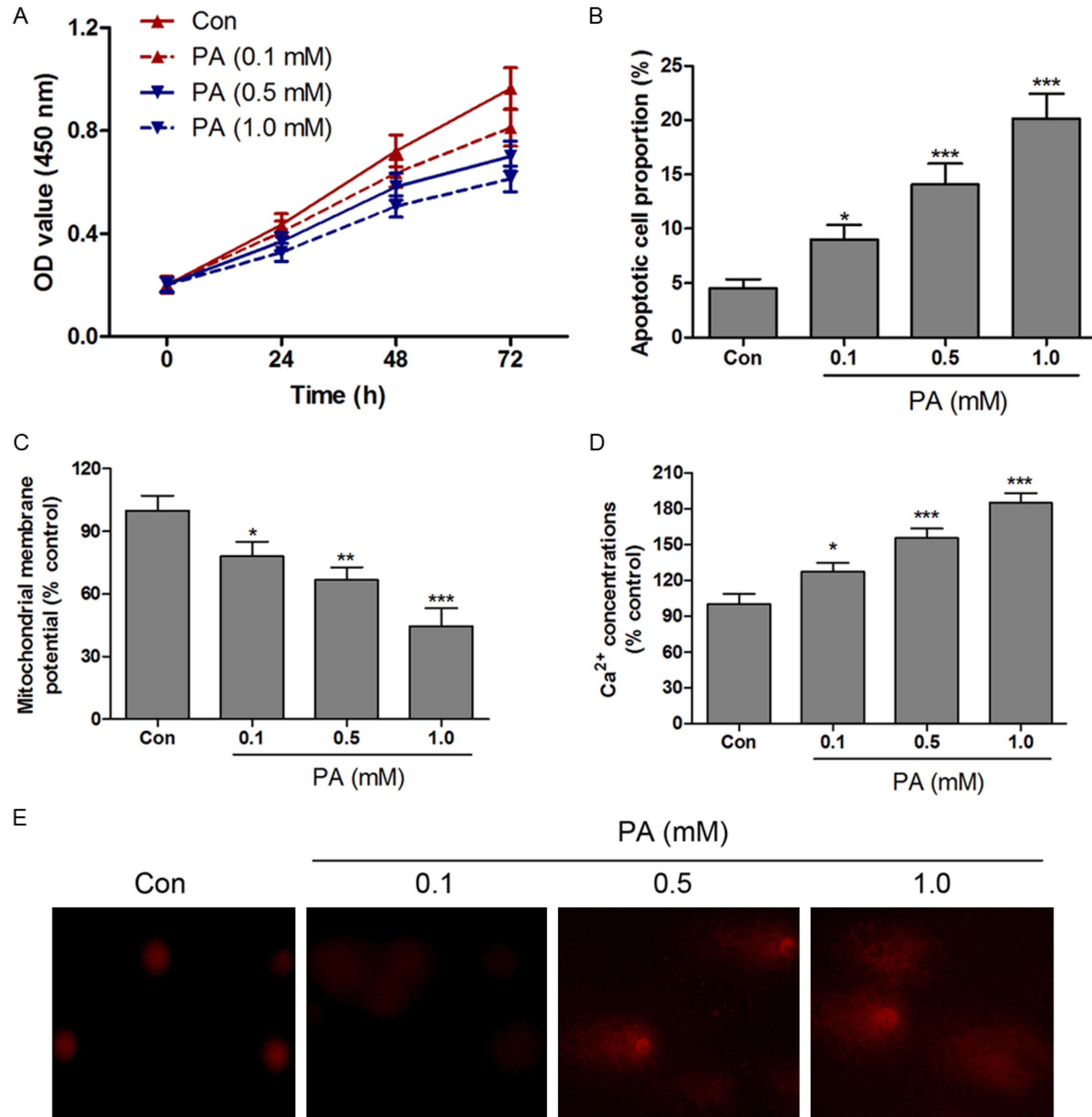

Figure 1. VSMCs were incubated with palmitic acid, and the cell viability was examined by CCK8 assay (A). VSMCs were incubated with palmitic acid for 48 h, the percentage of apoptotic cells was analyzed by flow cytometric analysis of annexin V/PI double staining (B). VSMCs were incubated with palmitic acid for 48 h, the mitochondrial membrane potential (C) and the release of Ca^{2+} (D) were examined by flow cytometry. VSMCs were incubated with palmitic acid for 48 h, and the cell DNA damage was measured by the comet assay (E). Values were expressed as mean ± SD, n = 3 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group.

ther explore whether PA-induced cell apoptosis was mediated through mitochondrial dysfunction and DNA damage. We determined the mitochondrial membrane potential with the mitochondria-sensitive dye, DiOC6, using flow cytometry. As shown in **Figure 1C**, the loss of the mitochondrial membrane potential in a dose-dependent manner was observed when the VSMCs was exposed to PA for 48 h. Moreover, we assessed the effect of PA on the mobilization of Ca^{2+}. When VSMCs were treated with PA, Ca^{2+} levels were significantly increased in the present of PA as compared with the control group (**Figure 1D**). The results demonstrated that PA promoted the secretory dysfunction of Ca^{2+} in VSMCs. Intriguingly, DNA damage had been found in VSMCs with PA treatment, and the tail length in the PA-treated group was

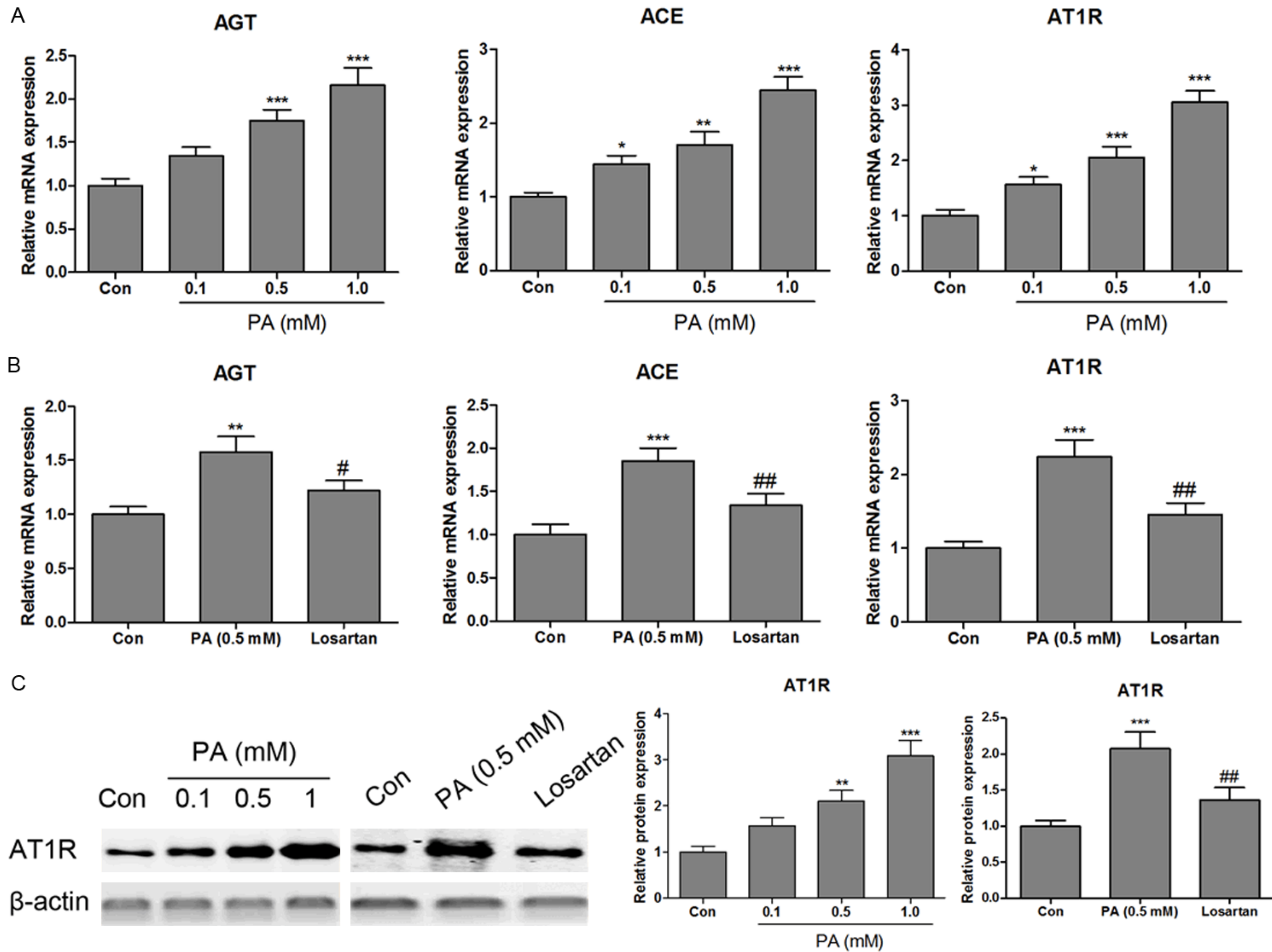
A
AGT
ACE
AT1R
Relative mRNA expression
Con
0.1
0.5
1.0
PA (mM)
B
AGT
ACE
AT1R
Relative mRNA expression
Con
PA (0.5 mM)
Losartan
C
PA (mM)
Con
0.1
0.5
1
Con
PA (0.5 mM)
Losartan
AT1R
β-actin
AT1R
Relative protein expression
Con
0.1
0.5
1.0
PA (mM)
AT1R
Relative protein expression
Con
PA (0.5 mM)
Losartan

Figure 2. VSMCs were incubated with palmitic acid, and mRNA expression of AGT, ACE and AT1R were analyzed by real-time PCR (A). VSMCs were incubated with palmitic acid (0.5 mM) or Losartan (100 μM), and mRNA expression of AGT, ACE and AT1R were analyzed by real-time PCR (B). VSMCs were incubated with palmitic acid or Losartan, and protein expression of AT1R was analyzed by western blotting (C). Values were expressed as mean ± SD, n = 3 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus PA-treated group.

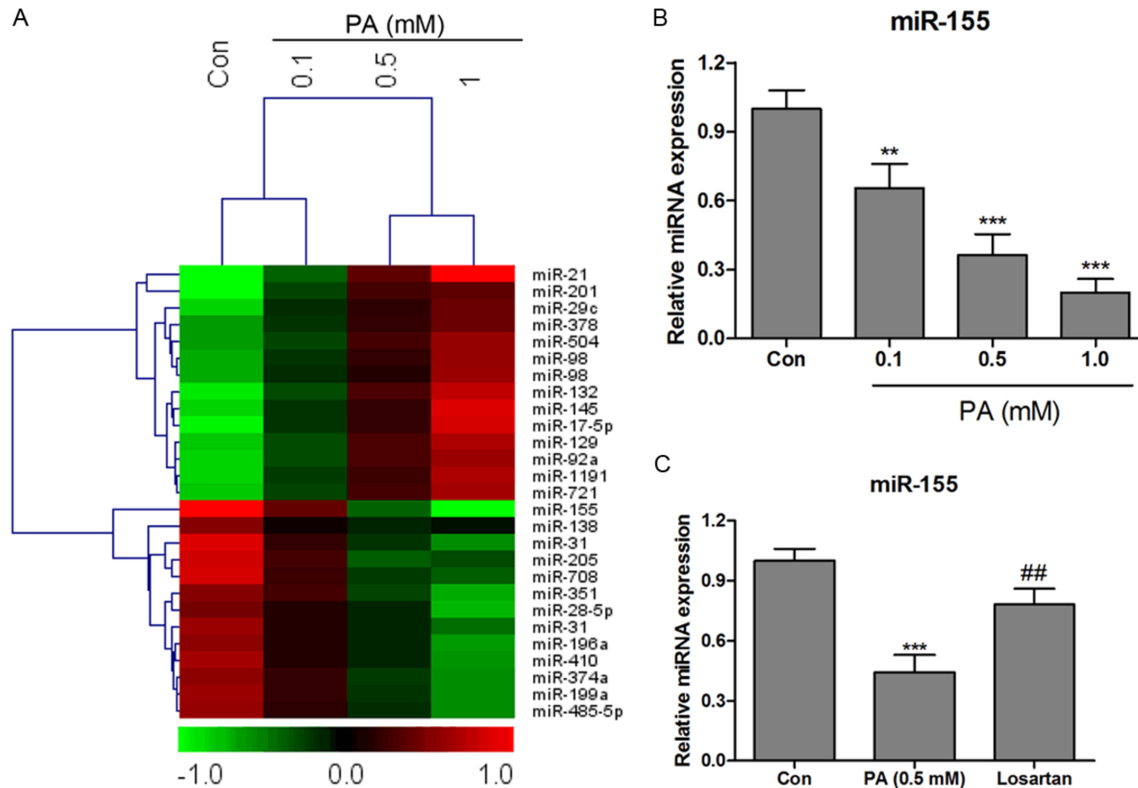

Figure 3. Unsupervised hierarchical clustering of differentially expressed miRNAs (> 2-fold) in VSMC treated with PA for 24 h. The figure is drawn by MeV software (version 4.2.6). Correlation similarity matrix and average linkage algorithms are used in the cluster analysis. Each row represents an individual miRNA, and each column represents different treatment. The dendrogram at the left side and the top displays similarity of expression among miRNAs and samples individually. The color legend at the bottom represents the level of miRNA expression, with red indicating high expression levels and green indicating low expression levels (A). miR-155 was analyzed by real-time PCR in control or PA-treated group at the different concentration for 24 hours (B). miR-155 was analyzed by real-time PCR in control, PA-treated (0.5 mM) and losartan-treated (100 μM) group for 24 hours (C). Values were expressed as mean ± SD, n = 3 in each group. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group. $^{\#\#}P < 0.01$ versus PA-treated group.

markedly longer than in the control group (**Figure 1E**). These results suggested that PA could accelerate the mitochondrial dysfunction and DNA damage in VSMCs.

PA upregulates RAS components expression in VSMCs

In the present study, the mRNA expression of RAS components, such as angiotensinogen (AGT), angiotensin-converting enzyme (ACE) and AT1R were measured by real-time PCR when the VSMCs were exposed to PA for 48 h. The results showed that the mRNA expression of AGT, ACE and AT1R was significantly increased in a dose-dependent manner (**Figure 2A**). However, losartan treatment could reverse PA-induced upregulated AGT, ACE and AT1R mRNA expression in VSMCs (**Figure 2B**). These results suggested that RAS signaling might be involved in PA-induced VSMCs dysfunction in vitro. Furthermore, the protein expression of AT1R was measured by western blotting when the VSMCs were exposed to PA for 48 h. As shown in **Figure 2C**, AT1R protein expression was significantly increased in PA-treated groups

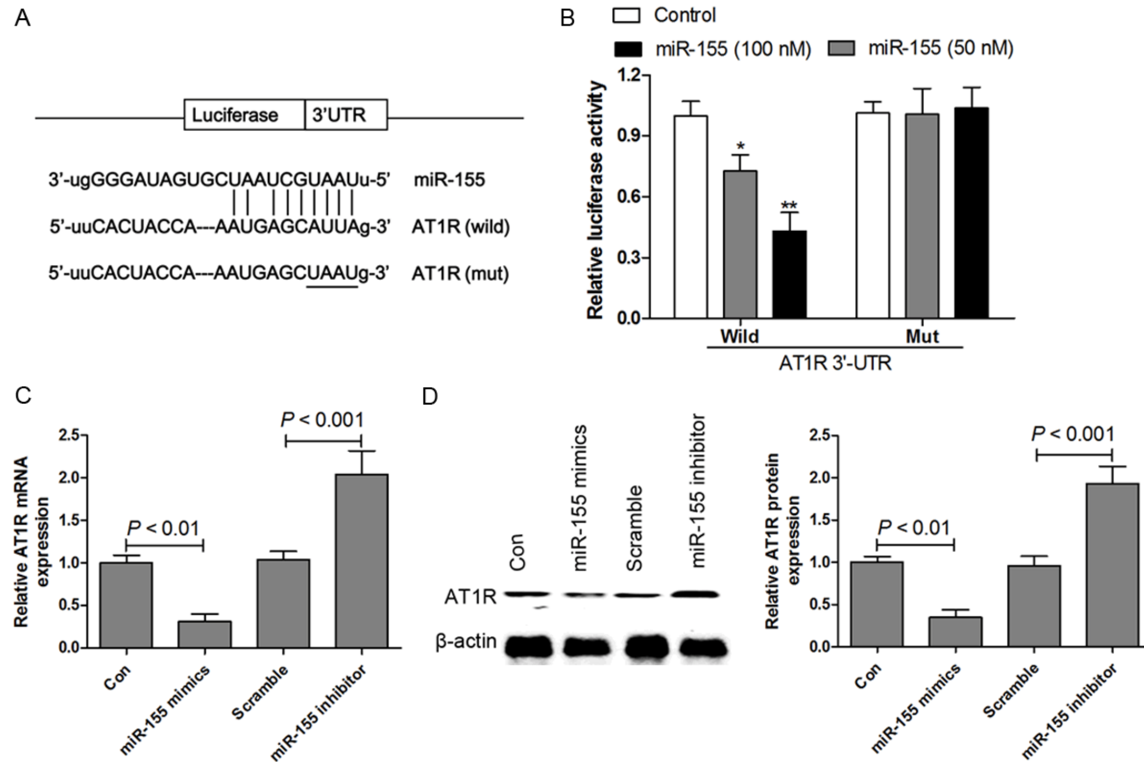

Figure 4. Schematic representation of the putative miR-155 binding site in the AT1R 3'UTR as in Targetscan (A) and luciferase activity assay (B). mRNA (C) and protein expression of AT1R (D) were analyzed by real-time PCR or western blotting, respectively, in VSMCs with miR-155 mimics or g miR-155 inhibitor. Values were expressed as mean ± SEM, n = 3 in each group. *$P < 0.05$, **$P < 0.01$ versus control group.

as compared to control group. However, losartan treatment could reverse PA-induced upregulated AT1R protein expression in VSMCs.

PA regulates miR-155 expression in VSMCs

Previous studies show that various miRNAs play key roles in regulating VSMCs differentiation, proliferation and apoptosis [20, 21], but the involvement of miRNAs in PA-mediated effects in VSMC is still largely unknown. To identify differentially expressed miRNAs in response to PA in cultured VSMCs, we performed microarray assay with small RNA libraries generated using total RNA extracted from control group or PA-stimulated (0.1, 0.5 or 1 mM) VSMCs for 24 hours. We found that miR-155 was significantly lowly expressed in PA-treated groups as compared to control group (**Figure 3A**). Among the PA-induced miRNAs, we concluded that miR-155 might be closely related to VSMCs dysfunction in the present of PA. miR-155 has been reported to inhibit angiotensin II-induced VSMCs proliferation and regulate AT1R expression and phenotypic differentiation in vascular adventitial fibroblasts [2, 15, 16]. Therefore, we further investigated the functional roles of miR-155 in VSMCs when they were exposed to PA. The does-dependent experiments showed that PA markedly suppressed miR-155 expression in VSMCs (**Figure 3B**). However, losartan treatment could reverse PA-induced downregulated miR-155 levels in VSMCs (**Figure 3C**).

miR-155 interferes with ATR1 expression

According to the miRBase Target database (http://www.mirbase.org), we found the potential miRNA-155 binding sites within the 3'-UTR of AT1R in human sapiens (**Figure 4A**). To verify if AT1R is a direct target of miR-155, we cloned the the 3'-UTR of the wild-type or mutant-type AT1R gene and co-transfected it along with miR-155 or NC oligonucleotides into VSMCs. Luciferase assays were performed 24 hours post-transfection. Results showed significantly reduced luciferase activity in miR-155-trans-

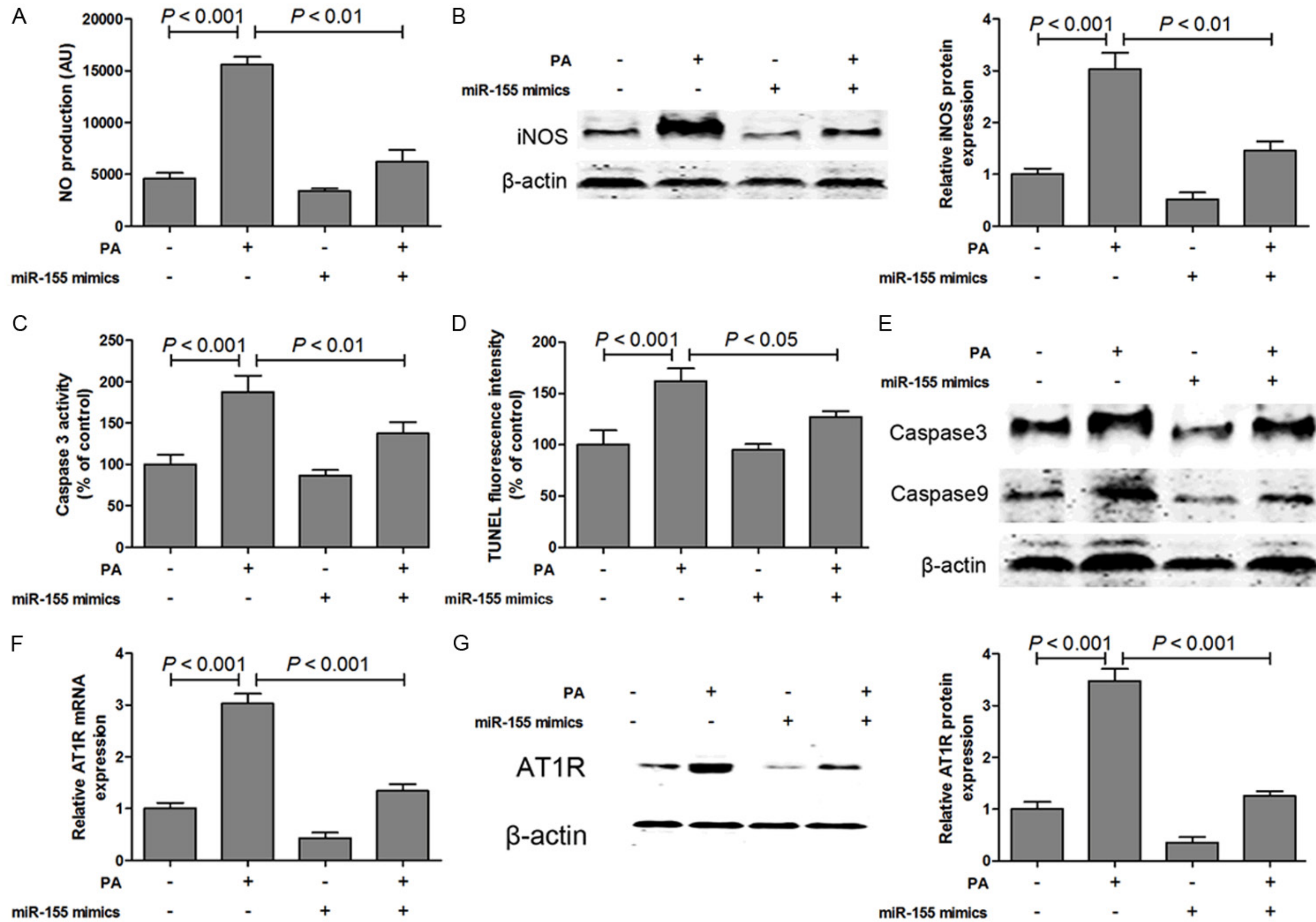

Figure 5. VSMCs were transfected with miR-155 mimics or inhibitor in the present of PA for 48 hours, and the NO concentration was detected by ELISA assay (A). The protein expression of iNOS was measured by western blotting (B). VSMCs were transfected with miR-155 mimics or inhibitor in the present of PA for 48 hours, and the caspase3 concentration was detected by ELISA assay (C), Triphosphate nick-end labeling (TUNEL) staining (D) were measured by flow cytometry, caspase3 and

caspase9 protein expression were measured by western blotting (E). VSMCs were transfected with miR-155 mimics or inhibitor in the present of PA for 48 hours, mRNA (F) and protein (G) expression of AT1R was analyzed by real-time PCR and western blotting respectively.

fected cells compared with NC cells. In contrast, co-transfection miRNA-155 into AT1R mutant-type 3'-UTR cells, the luciferase activity did not show significant difference compared with NC group (**Figure 4B**). Next, we observed that transfection of VSMCs with miR-155 mimic oligonucleotides or miR-155 inhibitors significantly downregulated or upregulated, respectively, both AT1R mRNA (**Figure 4C**) and protein (**Figure 4D**) levels.

Overexpressed miR-155 inhibits PA-induced VSMCs dysfunction via targeting AT1R

To assess PA-induced secretory dysfunction of VSMCs and the modulation effect of miRNA-155, we measured the levels of NO and iNOS in VSMCs. As expected, the levels of NO and iNOS were significantly increased when the VSMCs were exposed to PA at the concentration of 0.5 mM. However, VSMCs transfected with miR-155 for 48 hours reversed the increased level of NO and iNOS in VSMCs (**Figure 5A** and **5B**). Moreover, we examined whether overexpressed miR-155 inhibited PA-induced VSMCs apoptosis. Caspase-3 activity assay and triphosphate nick-end labeling (TUNEL) staining were measured after VSMCs exposure to PA for 48 h. The results indicated that the VSMCs with PA treatment showed significant increased caspase-3 activity. In the contrast to that VSMCs transfected with miR-155 mimics suppressed PA-induced increased caspase-3 activity (**Figure 5C**). TUNEL staining showed that PA-treated induced cell apoptosis, and miR-155 mimics suppressed PA-induced apoptosis in VSMCs (**Figure 5D**). Western blotting confirmed that caspase3 and caspase9 protein expression were significantly upregulated in PA-treated group as compared to control group. However, overexpressed miR-155 reversed PA-induced upregulated caspase3 and caspase9 protein expression in VSMCs (**Figure 5E**). To determine whether AT1R was involved in miR-155 inhibition PA-induced VSMCs dysfunction, we used real-time PCR and western blotting to examine AT1R mRNA and protein expression in PA-treated or miR-155-overexpressed VSMCs. The mRNA and protein expression of AT1R were significantly increased in PA-treated group as compared to control group. However, overexpressed miR-155 could reverse PA-induced increase of AT1R in VSMCs (**Figure 5F** and **5G**). These results suggested that overexpressed miR-155 inhibited PA-induced VSMCs dysfunction, and the underlying mechanism was mediated, at least partially, through the suppression of AT1R expression.

Discussion

Our data suggest that miR-155 is involved in PA-induced VSMCs dysfunction. Previous studies suggest that the exogenous overexpression of miR-155 significantly enhances cell proliferation by inhibiting apoptosis in human aortic SMCs (HASMCs), and it also promoted the migratory ability of the cells [17]. Moreover, miR-155 has been shown to be highly expressed in HUVECs and VSMCs, and AT1R has been shown to be a target of miR-155. By targeting AT1R, miR-155 decreases HUVECs and VSMCs migration in response to Ang II [2, 22]. Subsequently, miR-155 is being postulated and proposed in the etiology of AS [2]. Although AS development appears to be the result of multiple factors, a particularly important risk factor in the pathogenesis of AS is saturated free fatty acid, which contributes to VSMCs dysfunction [1, 23]. As a major component of dietary saturated fat and 20% of the total serum free fatty acids, PA has cytokine-like properties and induces inflammatory response and oxidant [24]. During the development of AS, PA promotes VSMC migration, proliferation, apoptosis and phenotype translation, as well as the secretion and expression of many kinds of pro-inflammatory cytokines, growth factors and extracellular matrix proteins [1]. Therefore, PA and VSMCs have important pathophysiological roles in the formation and development of AS. However, the underlying molecular mechanisms of miR-155 in PA-induced VSMC dysfunction by targeting AT1R remains to be determined.

miRNAs function as critical regulators of protein expression by binding to the 3'-UTRs of target mRNAs result in inhibition of protein translation in mammalian cells [25, 26]. In this study,

we found the potential miRNA-155 binding sites within the 3'-UTR of AT1R in human sapiens. The following a dual luciferase reporter assay manifested that miR-155 regulated luciferase expression by interacting with the 3'-UTR of AT1R in VSMCs. Moreover, our studies indicated that AT1R expression was simultaneously suppressed by overexpressed miR-155 at the transcriptional and translational level. In human primary lung fibroblasts, transfection of miR-155 reduces endogenous expression of hAT1R compared with non-transfected cells [27]. In the vascular adventitial fibroblasts, AT1R expression was reduced by miR-155 at the translational, not transcriptional level [17]. These results suggest that miR-155-mediated translational repression of AT1R can play a vital role in cardiovascular diseases.

miR-155 is found within the BIC gene on Chromosome 16 in mouse and Chromosome 21 in human [28]. miR-155 is a multifunctional signal miRNA, which has been reported to play a critical role in various physiological and pathological processes such as immunity, inflammation, cancer and cardiovascular disease [2]. In our work, we investigated the functional roles of miR-155 in VSMCs when they were exposed to PA. The does-dependent experiments showed that PA markedly suppressed miR-155 expression in VSMCs, and losartan treatment could reverse PA-induced downregulated miR-155 levels in VSMCs. Moreover, PA induced cell apoptosis was mediated through mitochondrial dysfunction and DNA damage, which was confirmed by the loss of the mitochondrial membrane potential, the promotion of the Ca^{2+} secretion and the tail length in VSMCs. Interestingly, overexpressed miR-155 reversed PA-induced dysfunction and apoptosis in VSMCs. Meanwhile, we hypothesized that a post-translational mechanism might exist for dysfunction and apoptosis in VSMCs, which could be regulated by miRNAs in the present of PA. By using PicTar, TargetScan, and miRBase database and microarray assay, we found that miR-155 was a regulator of AT1R through the predicted binding sites in its 3'-UTR. In vitro experiment indicated that the mRNA and protein expression of AT1R were significantly increased in PA-treated group as compared to control group, and overexpressed miR-155 could reverse PA-induced increase of AT1R in VSMCs. Therefore, we have reason to believe that overexpressed miR-155 inhibits PA-induced VSMCs dysfunction, and the underlying mechanism is mediated, at least partially, through the suppression of AT1R expression.

Disclosure of conflict of interest

None.

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