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Knockdown of Long Noncoding RNA (lncRNA) AK094457 Relieved Angiotensin II Induced Vascular Endothelial Cell Injury

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Hypertension could induce many serious diseases, including damage to vascular endothelial cells. As a noncoding RNA, long noncoding RNA (lncRNA) has received much attention in scientific research and has a regulating efficacy on many critical life activities in human body. The level of lncRNA AK094457 is thought to be elevated in hypertensive rats. However, there is no research indicating the relationship between the level of lncRNA AK094457 and vascular endothelial injury.

Material/Methods: In our study, we used lentiviral to knockdown lncRNA AK094457, and the human umbilical vein endothelial cells (HUVECs) were stimulated by the Ang II to imitate the vascular endothelial cell damage caused by hypertension. The Cell Counting Kit-8 assays were used to detect the cells viability. Western blotting was performed to detect the endothelial nitric oxide synthase (eNOS), p-eNOS and endothelin-1 (ET-1). After that the production of the NO was monitored. At last, the reactive oxygen species (ROS) levels and apoptosis rates were detected in this study.

Results: According to the results, we found that knockdown lncRNA AK094457 could alleviate the decrease of vascular endothelial cell viability induced by angiotensin II (Ang II). The knockdown of lncRNA AK094457 also relieved the downregulation of eNOS and p-eNOS, and the decreasing of NO release. At the same time, the knockdown of lncRNA inhibited the levels of Ang II-induced proinflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-1, and IL-6) and cell adhesion molecules (vascular cell adhesion molecule 1 [VCAM-1], intercellular adhesion molecule 1 [ICAM-1], and monocyte chemoattractant protein-1 [MCP-1]). The levels of ROS and apoptosis rates also decreased after the knockdown of lncRNA AK094457.

Conclusions: All these results indicated that lncRNA AK094457 could promote Ang II-induced vascular endothelial cell injury. On the contrary, knockdown of lncRNA AK094457 could alleviate this damage.

MeSH Keywords: Hypertension • RNA, Long Noncoding • Vascular System Injuries

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Background

Hypertension is a type of disease characterized by elevated blood pressure in the systemic arteries [1–3]. Studies have shown that the incidence of essential hypertension rising continuously in China and the proportion of young patients has also increased, which has caused widespread concern [4]. High blood pressure is one of the causes of many cardiovascular diseases such as the atherosclerosis and congestive heart failure [5]. Furthermore, hypertension can also induce damage to vascular endothelial cells, vascular injury mainly induces pathological processes such as endothelial cell injury, smooth muscle cell proliferation and vascular remodeling which threat to the safety of the people lives [6,7]. On the other hand, researches have pointed out that endothelial nitric oxide synthase (eNOS) phosphorylation at 1177 serine plays a crucial role in the production of nitric oxide (NO) in endothelial cells, and under normal conditions, NO produced by eNOS can play the role in promoting normal blood flow and vasodilation, maintaining anti-inflammatory and anti-thrombotic vascular endothelium [8]. Consequently, NO also plays a crucial role in the pathophysiology of hypertension and may impact the damage of vascular endothelial cells which was induced by the hypertension.

Long noncoding RNA (lncRNA) is one kind of the RNA which consists of more than 200 nucleotides. Although it does not have the coding function, it can participate in many vital physiological processes in the body [9,10]. Some studies reported that the lncRNA could affect the development of the cardiovascular diseases such as the hypertension and atherosclerosis [11,12]. There are also some lncRNAs participated in the regulation of the vascular smooth muscle cells function [11,13]. Studies have shown that the expression of lncRNA AK094457 in rats with essential hypertension is higher than that of normal rats, and the levels decreased after the treatment with notoginsenoside R1 [14]. However, the role of lncRNA AK094457 in hypertension-induced vascular endothelial injury and its specific molecular mechanism have not been reported.

Therefore, we used the angiotensin II (Ang II) to stimulate the human umbilical vein endothelial cells (HUVECs) to simulate the hypertension-induced vascular endothelial cell injury model in our study. Then we detect the expression of the lncRNA AK094457 in these cells. After that, we used the sh-RNA to stably knockdown the lncRNA AK094457 in HUVECs. At the same time, we used the Ang II to stimulate the HUVECs and observe the proliferation of these cells. Furthermore, the damage of the vascular endothelial cells induced the changing of expression of e-NOS and p-eNOS was detected by the Western-blotting. Next the 3 vascular endothelial related factors molecules, VCAM-1 (vascular cell adhesion molecule 1), ICAM-1 (intercellular adhesion molecule 1) and the MCP-1 (monocyte chemoattractant protein-1) were detected by the enzyme-linked immunosorbent assay (ELISA). At last, the levels of the inflammation factors and the oxidative stress were tested. The detection of these indicators fully reveals the role of lncRNA AK094457 in Ang II-induced vascular endothelial injury.

Material and Methods

Cell culture and treatment

The HUVECs were purchased from the American Type Culture Collection (Manassas, VI, USA). The HUVECs was cultured in the endothelial medium 2 containing 10% fetal bovine serum (FBS; Gibco, BV000) and antibiotics (penicillin 100 IU/mL and streptomycin 100 μg/mL, Invitrogen, California, USA). The cells were placed in the cell incubator which was 37°C and has the humid atmosphere with 5% CO_2. The Ang II was diluted with the medium and added into the cell culture plate for 24 hours.

Cell viability assays

The cells viability assay was performed with the Cell Counting Kit-8 assays (CCK-8) assay. The cells were planted into 3 divided 96-well plates. After 12 hours, CCK-8 (Dojindo, Japan) was diluted with medium and added to a 96-well plate. Then the absorbance was observed with the microplate reader. The other 2 plates were detected at 24 hours and 48 hours respectively.

Western blotting

The protein was extracted from the cells with the RIPA (Beyotime, P0013C). The protein concentration was determined using the bicinchoninic acid (BCA) method, and then the protein was incubated at 99°C for 10 minutes to denature the protein. After that the samples were separated by the 10% sodium dodecyl sulfate (SDS) gels (Beyotime, P0012A). Then the proteins were transferred to the PVDF (polyvinylidene fluoride) membranes (Millipore, USA). After that the targeted proteins were incubated with the primary anti-body p-eNOS (Abcam, ab76199), eNOS (Abcam, ab184154), endothelin-1 (ET-1) (Abcam, ab2786), Bax (CST, #14796), Bcl-2 (CST, #15071), cleaved caspase-3 (CST, #9664), caspase-3 (CST, #9662), α1-AMPK (Abcam, ab32047), Nox-2 (Abcam, ab80508), and GAPDH (CST, #5174) at 4°C overnight. In the second day, the membranes were washed by the PBST (phosphate-buffered saline Tris) for 3 times. After that, the membranes were incubated with the second anti-body rabbit IgG (CST, #14708) and anti-mouse IgG (CST, #7076) for 2 hours at the room temperature. Then the membranes were washed by the PBST again. Then the membranes were exposed in the machine.

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Real-time polymerase chain reaction (RT-PCR)

The total mRNA was extracted from the HUVECs which was stimulated by the Ang II by the TRIzol (Thermo Fisher, USA) method. The reverse transcription kit (Takara, Japan) was used for the cDNA synthesis and the cDNA is then used for the amplification of the cDNA. And the process was according to the manufacturer's instructions. The GAPDH was used as the loading control. The forward primer of the eNOS was: 5'-GACCAGAAACTGTC TCACCTG-3 the reverse primer of the eNOS was: 5'-CGAACATCGAACGTCTCA CA-3. The forward primer of the GAPDH was: 5- GGAGCGAGATCCCTCCAAAAT-3 the reverse primer of the GAPDH was: 5-GGCTGTTGTCATACTTCTCATGG-3. The mRNA expression level of eNOS was normalized to GAPDH, and the results were analyzed using the method of $2^{-\Delta\Delta Ct}$.

Construction of lentivirus vectors and cells transfection

To establish the HUVECs that stably knockdown the lncRNA AK094457, we constructed the lentiviral vectors and transfected the cells. The lentiviral particles were obtained from the Genechem Shanghai. The sequence of sh-RNA1 was 5'-GCATGCTTTCGTACCTCAATG-3' and the sequence of sh-RNA2 was 5'-GCGTTTGTTTAGTTCACTTGT-3'. These lentivirus and corresponding negative control were used for the transfection of the HUVECs. The polybrene was applied to improve the transfection efficacy. All the operations were followed the manufacturer's instructions. After that the medium containing the puromycin (Sigma, USA) was used for the culturing of the cells which was transfected with the lentivirus. Next the qPCR was performed for the detecting of the knockdown efficiency of the lentivirus vectors.

Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) were performed to detect the levels of the VCAM, ICAM-1, MCP-1, tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 in the culture medium of the HUVECs. The supernatant was collected during the process of cell culture. After that, the supernatant was centrifuged and transferred into clean tubes for the detection. ICAM-1 human ELISA kit (Abcam, ab174445), VCAM-1 human ELISA kit (Sigma Aldrich, RAB-0505), MCP-1 human ELISA kit (Sigma Aldrich, RAB0054), TNF-α human ELISA kit (Abcam, ab181421), IL-1 human ELISA kit (Abcam, ab214025), and IL-6 human ELISA kit (Abcam, ab178013) were used for the ELISA assays. The operation of these assays was followed the instructions.

Nitric oxide (NO) detection

The nitric oxide (NO) was measured by the total NO assay kit (Beyotime, S0021). NO is unstable and could be quickly transformed to nitrates and nitrites in cells. After that, the nitrate reductase converts the nitrates into nitrites. Therefore, the Griess reagent method was used to the detect the nitric oxide indirectly.

Reactive oxygen species (ROS) staining

The dichlorofluorescein diacetate (DCFH-DA) was used to detect the level of reactive oxygen species (ROS) in the HUVECs. First the DCFH-DA (Beyotime, S0033) which was diluted with the serum-free medium was added after the removing of the cell culture medium. After that, the cells were incubated in the cell incubator for 30 minutes. Then the cells were washed by the phosphate-buffered saline (PBS). At the last, the laser confocal microscope (Olympus, Japan) was used to observe the immunofluorescence.

Apoptosis

The apoptosis rates of HUVECs were detected with the apoptosis assay kit (Beyotime, C1062L). The cells were washed with the PBS for 3 times, and then the Annexin-V and propidium iodide (PI) were added. After that these cells were incubated in the cell incubator for 40 minutes. After that the apoptosis rates of these cells were measured with the flow cytometry.

Statistical analysis

The analyses were performed with the Graphpad Prism 6.0 (Graphpad Software Inc., USA). The Student's *t*-test was used for the comparison of the experiment and control groups. There is significant difference between of the different groups when the value of *P* is less than 0.05. The data presented as the mean±standard deviation (SD). All the experiments in this paper were repeated 3 times.

Results

The knockdown of the lncRNA AK094457 relieved the Ang II-induced vascular endothelial injury

The Ang II was used to stimulate HUVECs to mimic vascular endothelial injury induced by hypertension. Then we detected the level of lncRNA AK094457 in the HUVECs after the stimulation of the Ang II. The results (Figure 1A) showed that the expression of the lncRNA AK094457 in the stimulation groups were higher than the control group. After that the HUVECs were transfected with 2 different sh-RNA sequences to knockdown the expression of the lncRNA AK094457. Then the levels of the lncRNA AK094457 were validate by qPCR. As shown in the Figure 1B, the sh-AK094457-1 has the expected function but the sh-AK094457-2 did not work. Therefore,

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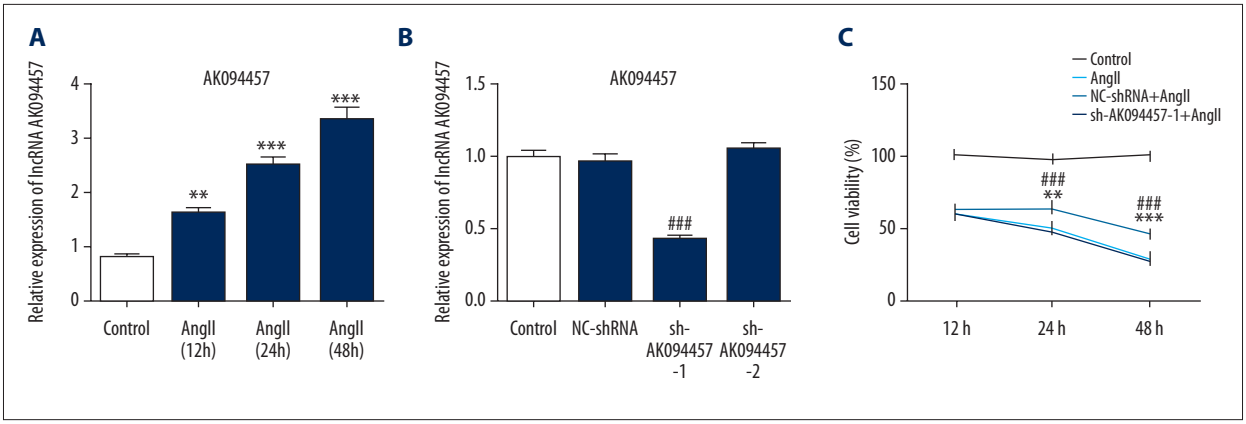

Figure 1. The knockdown of the lncRNA AK094457 enhanced the cell viability of HUVECs. (**A**) The expression of lncRNA AK094457 after the stimulation of Ang II. (**B**) The validation of the HUVECs which was stably knockdown the lncRNA AK094457. (**C**) The cell viability of the HUVECs was detected by the CCK-8 assays. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. HUVECs – human umbilical vein endothelial cells; Ang II – angiotensin II; lncRNA – long noncoding RNA; CCK-8 – Cell Counting Kit-8.

the sh-AK094457-1 group was used for the next experiment. The CCK-8 assay was performed to test the cell viability of HUVECs. The results (Figure 1C) showed that the cells viability of the knockdown group was upregulated after the stimulation of the Ang II.

The expression of eNOS and p-eNOS was upregulated in the knockdown group

Because the vascular endothelial injury was usually induced by the hypertension. Therefore, the expression of the eNOS and p-eNOS were detected by the qPCR and western-blotting. The results (Figure 2A) were showed that the protein level of the p-eNOS was downregulated in the Ang II group. On the other side, the mRNA level of the eNOS was also downregulated after the stimulation of Ang II (Figure 2B). However, the level of p-eNOS and eNOS was rescued after the knockdown of the lncRNA AK094457. The function of the eNOS was associated with the production of the NO. Therefore, we next detected the NO during the culture of these cells, and we found that the release of NO was inhibited when these cells were stimulated by the Ang II. Nevertheless, the amount of NO released was higher than the Ang II and negative control groups after the knockdown of the lncRNA AK094457 (Figure 2C). The endothelin-1 (ET-1) is a kind of vasoconstriction and maintain vascular tension in normal physiological conditions, but the level of ET-1 was elevated in different kinds of cardiovascular diseases [15,16]. In our results (Figure 2D) we found that the expression of the ET-1 was upregulated after the stimulation of the Ang II, but the level of the ET-1 was partially inhibited when the expression of lncRNA AK094457 was decreased.

The knockdown of lncRNA AK094457 suppressed the Ang II induced upregulation of the proinflammatory factors and vascular cell adhesion molecules

The higher levels of vascular cell adhesion molecules (VCAM, ICAM-1, and MCP-1) and proinflammatory factors (TNF-α, IL-1, and IL-6) was harmful for the health of vascular endothelial cells. In our study, the ELISA assays were performed to detect the level of vascular endothelial cytokines and inflammatory factors. These ELISA assays were performed to study the levels of the VCAM, ICAM-1, MCP-1, TNF-α, IL-1, and IL-6 in the HUVECs. The results (Figure 3A–3F) showed that the levels and of VCAM, ICAM-1 MCP-1, TNF-α, IL-1, and IL-6 were facilitated after the stimulation of the Ang II. However, the variation tendency was reversed after the knockdown of the lncRNA AK094457.

Knockdown of lncRNA AK094457 alleviate the Ang II induced rising of ROS level and apoptosis rates of HUVECs

Oxidative damage caused by high levels of reactive oxygen species (ROS) and apoptosis are also important indicators of cell damage, so we next examined the level of intracellular ROS and the proportion of apoptosis cells. As shown in the Figure 4A, the staining of Ang II stimulation group and negative control group is significantly lighter than that of control group. But after the knockdown of lncRNA AK094457, the ROS level of HUVECs was downregulated. Similarly, the apoptosis rates of the Ang II group and negative control group were higher than the control group. However, the knockdown of lncRNA AK094457 significantly relieved the apoptosis of the HUVECs (Figure 4B). Then the levels of associated apoptosis genes were detected, and the result (Figure 4C) was consistent with the previous results. The levels of the Bax and cleaved caspase-3 were promoted in the Ang II group and negative

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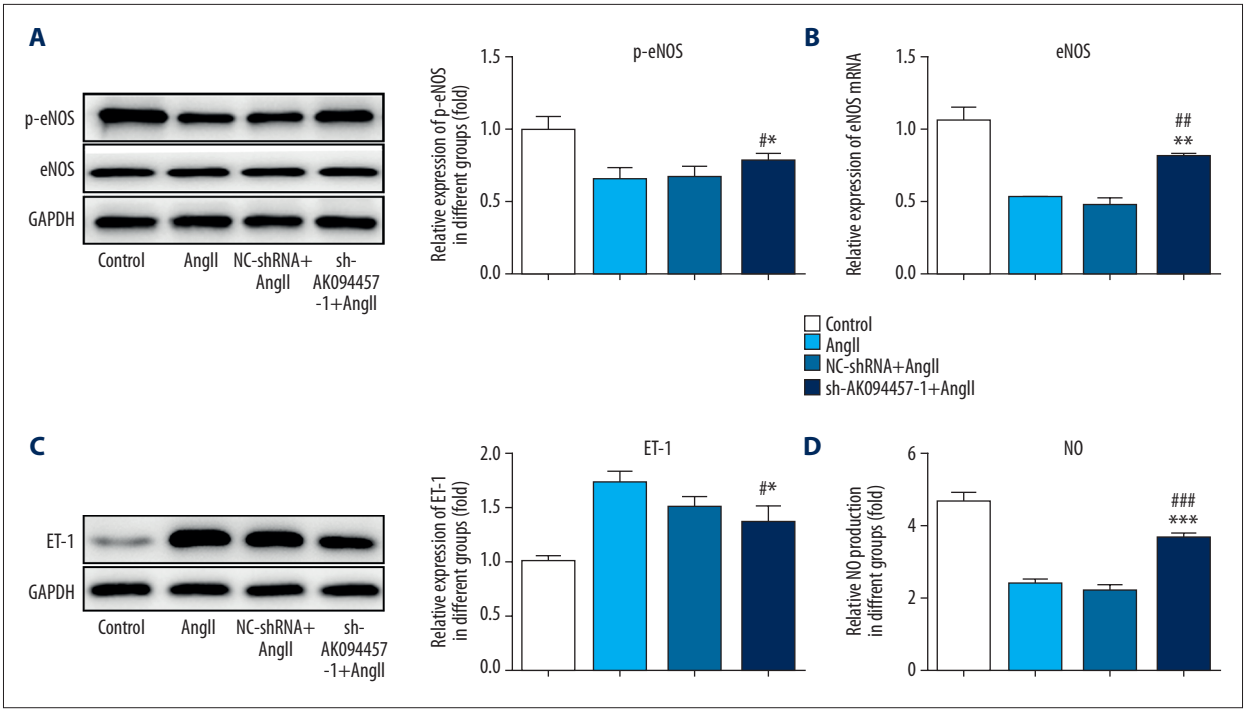

Figure 2. The knockdown of lncRNA AK094457 alleviated the decreasing of the NO production. (**A**) Western-blotting to detect the expression of p-eNOS and eNOS. (**B**) q-PCR was performed to detect the expression of eNOS. (**C**) The NO production *in vitro*. (**D**) The expression of ET-1 in different groups was detected by the western-blotting. * *P*<0.05, ** *P*<0.01, *** *P*<0.001. lncRNA – long noncoding; NO – nitric oxide; eNOS – endothelial nitric oxide synthase; q-PCR – quantitative polymerase chain reaction; ET-1 – endothelin-1.

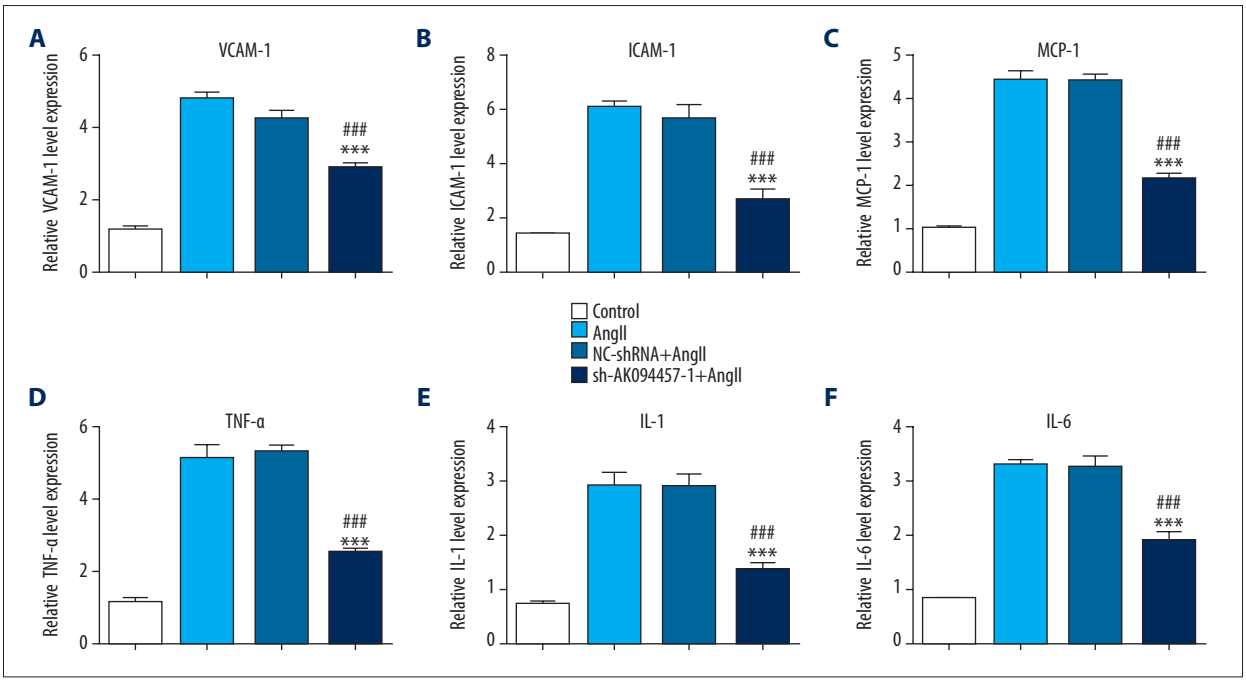

Figure 3. (**A–F**) The knockdown of lncRNA AK094457 relieved the upregulation of the endothelial adhesion molecules and inflammation factors. (**A–F**) The expression of the VCAM-1, ICAM-1, MCP-1, TNF-α, IL-1 and IL-6 were detected by the ELISA assays. * *P*<0.05, ** *P*<0.01, *** *P*<0.001. lncRNA – long noncoding; VCAM-1 – vascular cell adhesion molecule 1; ICAM-1 – intercellular adhesion molecule 1; MCP-1 – monocyte chemoattractant protein-1; TNF – tumor necrosis factor; IL – interleukin (IL).

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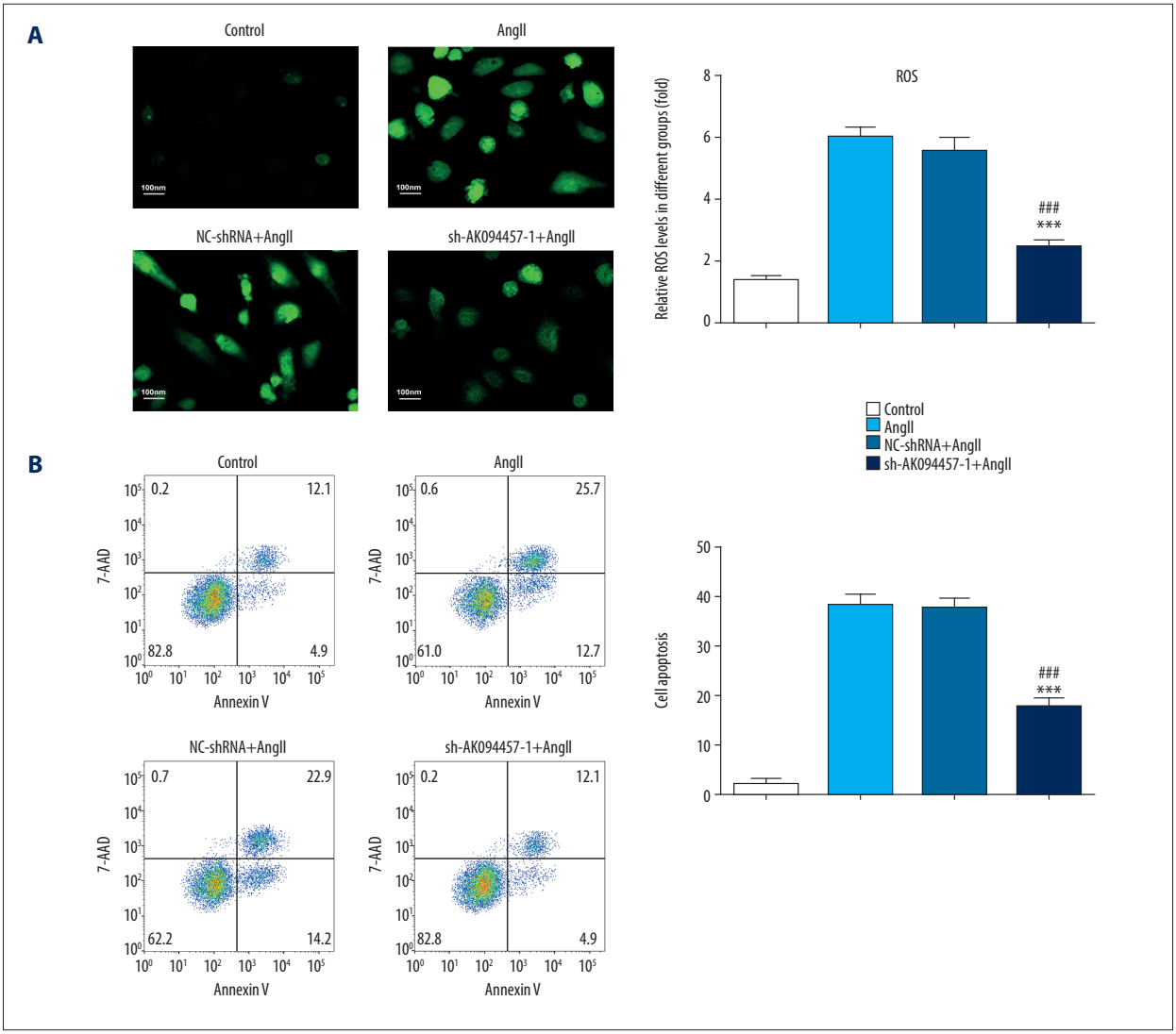
control group, but the expression of these proteins was rescued after the knockdown of the lncRNA AK094457. On the other side, the trend of expression of Bcl-2 was complete opposite with these proteins. AMP-activated protein kinase (AMPK) is a critical regulator of the vascular inflammation, furthermore the α1AMPK was considered as an anti-inflammatory protein [17,18]. Therefore, we detected the expression of the α1AMPK in HUVECs. According to the results (Figure 5A–5C), we found that the level of α1AMPK was downregulated in the Ang II group and negative control group. But, the expression of α1AMPK has recovered after the knockdown of the lncRNA AK094457. Next, the level of NADPH oxidative enzymes Nox-2 was also researched and the results showed that the expression of Nox-2 was enhanced when the HUVECs was stimulated with the Ang II. When the lncRNA AK094457 was knocked down, the level of Nox-2 was partially recovered.

Discussion

Hypertension which is a basic circulatory disease has been widely concerned in recent years. Furthermore, the vascular endothelial injury caused by hypertension is a crucial factor in many cardiovascular diseases. However, the understanding of the molecular mechanisms of hypertension-induced vascular endothelial injury is limited. Therefore, the research of new mechanisms of vascular endothelial injury induced by hypertension, the search for therapeutic targets is of great significance for the treatment of hypertension and vascular endothelial injury.

On the other side, there are some reports revealed that the lncRNA plays a critical role in different physiological processes in human body [19–21]. And the level of lncRNA AK094457 was higher in the hypertensive rats, however, the expression of the lncRNA AK094457 was suppressed after these rats were



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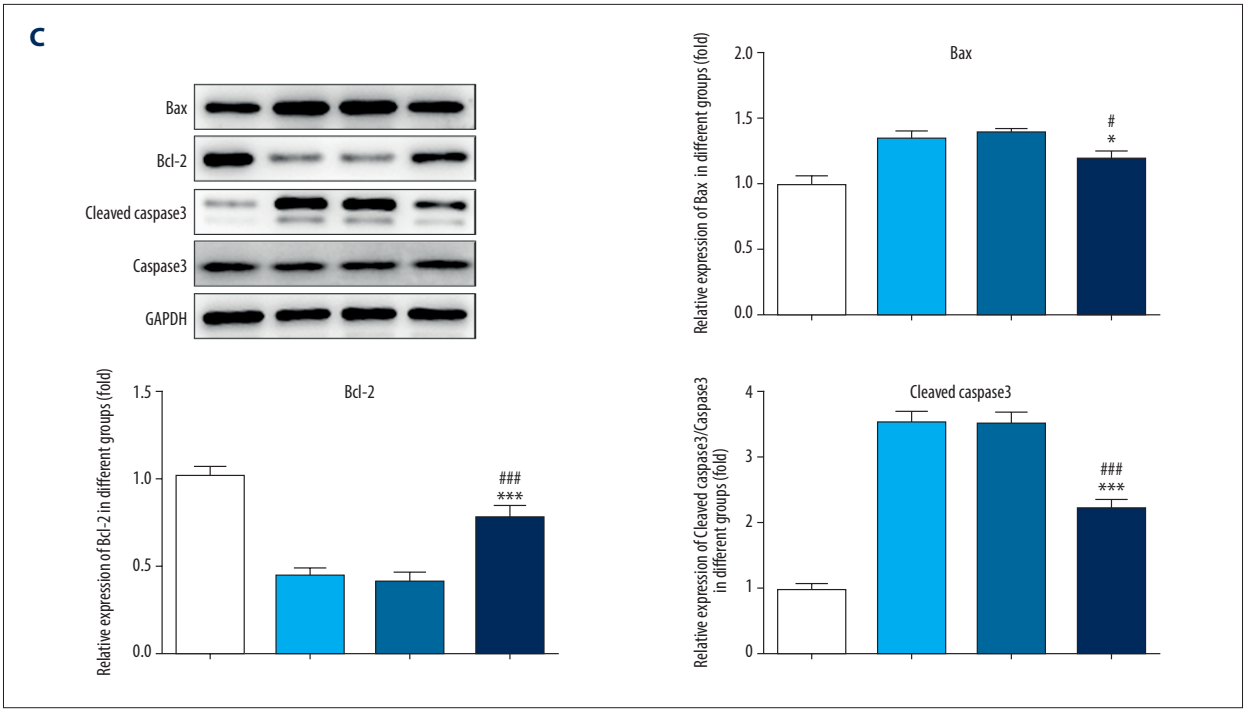

Figure 4. Knockdown of lncRNA AK094457 relieved the increasing apoptosis rates and ROS levels in HUVECs. (**A**) The ROS staining was used to detect the ROS levels in different groups. (**B**) The apoptosis rates of HUVECs were detected by the flow cytometry. (**C**) The apoptosis relative proteins(Bax, Bcl-2, caspase-3 and cleaved caspase-3) were detected with the western blotting. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. lncRNA – long noncoding; ROS – reactive oxygen species; HUVECs – human umbilical vein endothelial cells.

treated with the notoginsenoside R1 [14]. It indicated that the lncRNA AK094457 may play a vital role in the process of the development of hypertension [22]. There are already some studies revealed that the relationship between the lncRNA and the vascular endothelial cells injury. For instance, the lncRNA Meg3 was consider as a protective element during the injury process of the vascular endothelial cells [23]. However, the lncRNA ZEB1-AS1 enhanced the oxidative low-density lipoprotein induced damage of the vascular endothelial cells [24]. The lncRNA NEAT1 has shown that could suppressed the oxidative stress induced injury of vascular endothelial cells by activating the miR181d-5p/CDKN3 [25]. In our study, we designed the experiments to explore the efficacy of lncRNA AK094457 on the Ang II induced vascular endothelial cells injury. According to the results, we found that the level of lncRNA AK094457 was upregulated and the cell viability was decreased after the stimulation of Ang II. After the knockdown of lncRNA AK094457, the cell viability was partially recovered. These results indicated that the combination of lncRNA AK094457 and Ang II could enhance the damage effect on the vascular endothelial cells.

It is well known that NO can maintain normal contraction and relaxation of blood vessels, and NO is mainly synthesized by eNOS. In this study, the expression of the eNOS and p-eNOS were downregulated in the Ang II group. And after the knockdown of the lncRNA AK094457, the levels of eNOS and p-eNOS were rescued. The releasing of the NO showed the same tendency. It indicated that the Ang II could induce the disorder of the releasing of the NO and the expression of the eNOS and p-eNOS which could lead to the injury of the vascular endothelial cells. On the other side, the abnormal expression of ET-1 is thought to be involved in the development and progression of various cardiovascular diseases [26]. The study revealed that the level of ET-1 was promoted after the stimulation of Ang II [27]. Similarly, the expression of the ET-1 was increased in our study, but the level of ET-1 decreased a little after the knockdown of the lncRNA AK094457. The results implied that the knockdown of lncRNA AK094457 inhibited the level of ET-1 and protected the vascular endothelial cells.

The vascular cell adhesion molecule 1 (VCAM-1) is present on the surface of endothelial cells and plays an important role in atherosclerosis [28]. The intercellular adhesion molecule 1 (ICAM-1) is also an endothelial cell adhesion molecule which was increased in atherosclerotic tissues [29]. Similarly, in our research the levels of VCAM-1, ICAM-1, and MCP-1 were upregulated after the stimulation of the Ang II. The knockdown of the lncRNA AK094457 partially inhibited the increasing expression of these proteins. And these results revealed that the lncRNA AK094457 strengthen the damage of Ang II on

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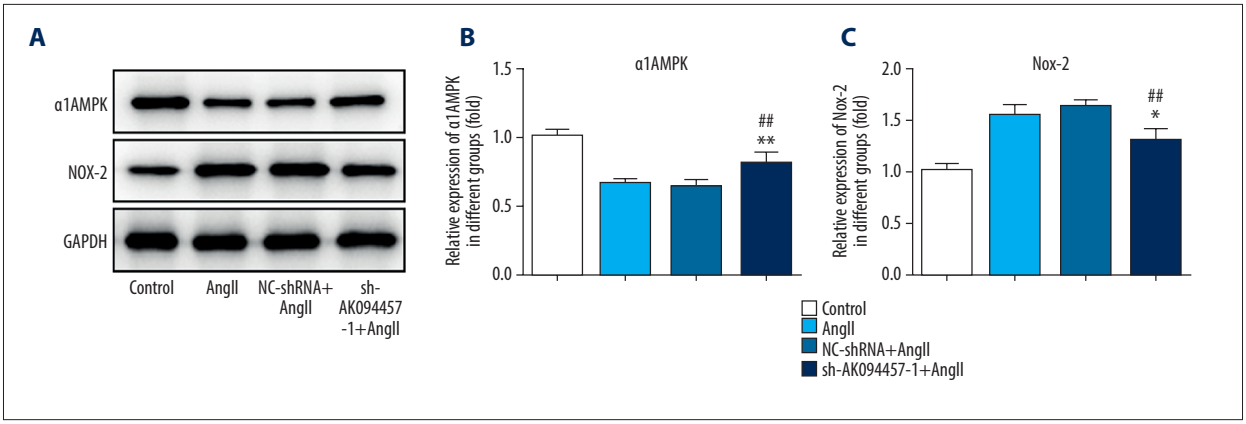

Figure 5. Knockdown of lncRNA AK094457 rescued the expression of α1AMPK and repressed the level of Nox-2. (**A**) The levels of the α1AMPK and Nox-2 were detected with the western blotting. (**B, C**) The quantitative of the α1AMPK and Nox-2 proteins were performed with the ImageJ. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. lncRNA – long noncoding; ROS – reactive oxygen species; HUVECs – human umbilical vein endothelial cells.

these cells. Furthermore, the inflammation of the vascular endothelial cells was another form of the damage. Furthermore, vascular endothelial cells are susceptible to inflammatory responses induced by ischemia-reperfusion [30,31]. Other studies have also pointed out that vascular endothelial inflammation plays a crucial role in all stages of atherosclerosis, and low-density lipoprotein enhances the occurrence and development of atherosclerosis by inducing the vascular endothelial inflammation [32–34]. In our study, after the knockdown of lncRNA AK094457 the expression of the TNF-α, IL-1, and IL-6 was inhibited compared to the Ang II group and negative control group. Therefore, the lncRNA could protect the vascular endothelial cells from the inflammatory damage.

The rising ROS levels and the apoptosis rates are also the important indicators for the vascular endothelial cells injury. Many studies shown that higher levels of ROS and apoptosis rates could induce dysfunction of the vascular endothelial cells, meanwhile, ROS and apoptosis is also an important factor during the process of vascular endothelial cell damage [35,36]. In our study, the levels of the ROS and apoptosis rates was induced by the Ang II but downregulated in the knockdown of the lncRNA AK094457. Furthermore, studies have shown that the decreasing of the expression of α1AMPK *in vivo* leads to the increasing in the expression of Nox-2, and the changing of these proteins leads to endothelial dysfunction and vascular endothelial inflammation [37]. Our results indicated that the knockdown of the lncRNA AK094457 could enhance the expression of α1AMPK and repress the level of the Nox-2.

Above all, the detection of these indicators proved that the stimulation of Ang II could induce the injury of the vascular endothelial cells and the lncRNA AK094458 could enhance this process. After the knockdown of lncRNA AK094457 the damage of these cells was relieved. Therefore, the lncRNA AK094457 might be a new target for the treatment of hypertension induced vascular endothelial cells injury.

Conclusions

In our study, we revealed that the lncRNA AK094457 enhanced the Ang II induced vascular endothelial cells injury. The knockdown of the lncRNA AK094457 could relieve the Ang II induced decrease of the cell viability. At the same time, the knockdown of lncRNA AK094457 could also maintain the production of NO and partially inhibited the levels of vascular adhesion molecules to ensure the performance of normal ability of the vascular endothelium. Furthermore, knockdown of the lncRNA AK094457 suppressed the Ang II induced elevating of ROS levels and higher apoptosis rates of HUVECs. All these indicators imply that the knockdown of the lncRNA AK094457 alleviate the Ang II induced damage of the vascular endothelial cells. And lncRNA AK094457 may provide a new therapeutic target for hypertension-induced vascular endothelial injury.

Conflicts of interest

None.

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