MiR-32-5p/AIDA Mediates OxLDL-Induced Endothelial Injury and Inflammation

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Summary

The role of endothelial injury and inflammation in atherosclerosis has been well established. miRNAs have been found to be key regulators in the development of atherosclerosis. Here we investigated whether miR-32-5p and its predicted target gene axin interactor, dorsalization associated (AIDA) are involved in endothelial injury and inflammation. Human umbilical vein endothelial cells (HUVECs) were treated with oxidized low-density lipoprotein (oxLDL) to induce endothelial injury and inflammation. AIDA was predicted to be a target gene of miR-32-5p using TargetScan software. Cell viability, migration, and angiogenesis were evaluated using Cell Counting Kit-8, wound-healing, and tube formation assays, respectively. The expression of inflammatory factors was detected using quantitative PCR, enzyme-linked immunosorbent assay, and western blot. We found that miR-32-5p expression was significantly decreased, whereas AIDA expression was significantly increased in oxLDL-treated HUVECs and the increased AIDA expression was reversed by the up-regulation of miR-32-5p. Moreover, both miR-32-5p mimic and knockdown of AIDA enhanced cell viability, promoted cell migration and angiogenesis and suppressed the expression of inflammatory factors including IL-1β, IL-6, TNF-α, ICAM-1, and VCAM-1 in oxLDL-induced HUVECs. Furthermore, miR-32-5p was verified to directly target AIDA using dual-luciferase reporter assay. Overall, these findings suggest that miR-32-5p/AIDA signal plays an important role in oxLDL-induced endothelial injury and inflammation. This study provides new insights into novel molecular mechanisms of endothelial dysfunction and atherosclerosis.

Key words: Coronary artery disease, Atherosclerosis, Endothelial dysfunction, miRNA

Coronary artery disease (CAD) is a common cardiovascular disease that seriously threatens human health and life globally. It has been estimated that 17.8 million of deaths occurred worldwide in 2017 due to cardiovascular disease, more than half of which were caused by CAD.1 Although the mortality of CAD has begun to decline in some developed countries in recent years owing to the success in prevention and treatment, CAD is still a global public health problem that needs to be solved urgently.2 Atherosclerosis, a chronic progressive inflammatory process, is the pathological basis of CAD. The development of atherosclerosis is complex and has been well established to be closely associated with endothelial injury and inflammation.3,4 Endothelial injury occurs when endothelial cells are exposed to harmful stimuli (hyperglycemia and hyperlipidemia), which facilitates lipid infiltration and inflammatory activation, thus promoting the development of atherosclerosis.5 Previous studies have identified that various bioactive molecules, including transcription factors, miRNAs and IncRNAs, play critical roles in the regulation of endothelial injury and inflammation.6,7 Among these molecules, miRNAs have attracted much attention and been recognized to be potential biomarkers and therapeutic targets for atherosclerosis.8 miRNAs are small noncoding RNA molecules that posttranscriptionally affect mRNA stability and translation by binding to the 3’ untranslated region (UTR) of their target genes.9 Accumulating evidence indicates that miRNAs are key regulators in the occurrence and progression of cardiovascular disease.10 Recently, we reported that miR-32-5p levels were elevated in the serum-derived exosomes from patients with stable CAD.10 Of interest, serum miR-32-5p expression has been found to be associated with the

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biomarker expression of endothelial injury and inflammatory response, suggesting that miR-32-5p may be involved in endothelial injury and inflammation. However, it has not been clear whether miR-32-5p participates in endothelial injury and inflammation and the underlying mechanism.

Using the online software TargetScan, we found that axin interactor, dorsalization associated (AIDA) is a putative target gene of miR-32-5p. The AIDA gene, located in the 1q41 region, contains 10 exons and is widely expressed in heart, brain, fat, and other tissues. AIDA protein was first reported in a study of embryonic development in zebrafish and found to affect dorsalization by Axin interaction. Recent studies have provided valuable clues that AIDA may be involved in the development of CAD. First, a report revealed that AIDA prevented obesity by regulating the activity of fat synthesis enzymes and intestinal fat absorption. Accordingly, AIDA may have effects on the development of CAD because obesity is an independent risk factor for CAD. Moreover, AIDA was identified as a candidate gene of CAD and found to be up-regulated in TNF-α-treated endothelial cells. However, the role of AIDA in the pathogenesis of CAD and the regulation of endothelial function has not been reported.

In this study, we investigated the role of miR-32-5p and its predicted target gene AIDA in endothelial injury and inflammation and whether miR-32-5p targets AIDA, providing new insights into novel molecular mechanisms of endothelial dysfunction and broadening the understanding of the pathogenesis of atherosclerosis.

Methods

Culture, treatment, and transfection of endothelial cells: Human umbilical vein endothelial cells (HUVECs) from the Cell Bank of the Chinese Academy of Sciences were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (Gibco, USA) in a humidified atmosphere containing 5% CO₂ at 37°C. Cells from passages 4-10 were used. To induce endothelial injury and inflammation, HUVECs were treated with oxidized low-density lipoprotein (oxLDL, Union-Biol, China) for 24 hours. When HUVECs reached 60%-70% confluence, the cells grew to confluence, scratch wounds were made using sterile pipette tips (200 μL). After being washed twice with PBS, the scratched cells were cultured in serum-free medium. Later, the cells were photographed at 0 and 24 hours on marked positions. The scratch area of each photograph was quantified using ImageJ software, and the cell migration rate was calculated using the formula (scratch area at 0 hours − scratch area at 24 hours)/scratch area at 0 hours.

Tube formation assay: The ability of HUVEC tube formation was assessed using Matrigel (Corning, USA). After being thawed overnight at 4°C, Matrigel was added into a precooled 48-well plate at 150 μL per well and incubated at 37°C for 30 minutes. HUVECs (5 × 10⁵ cells per well) were added into the wells coated with Matrigel and coincubated for 6 hours under the cell culture conditions. After the coincubation, capillary-like tubes were photographed randomly. The number of nodes and total length of the capillary-like tubes used to evaluate tube formation ability were measured using ImageJ software.

Enzyme-linked immunosorbent assay (ELISA): The concentrations of IL-1β, IL-6, and TNF-α in the culture supernatant of HUVECs were measured using commercial ELISA kits (LiankeBio, China) according to the manufacturer’s guidelines. Optical density was read at 450 nm using a microplate reader (Thermo Fisher Scientific, USA). The assay for each sample was repeated twice, and the average of the duplicate values was used for statistical analysis.

Western blot: HUVECs were homogenized in RIPA lysis buffer supplemented with 1% protease inhibitor cocktail (Bimake, USA) at 4°C. Protein concentration was determined using the BCA method. Total protein with equal amounts were loaded into 10% polyacrylamide gels, transferred to PVDF membranes (Millipore, USA) and blocked with 5% BSA in TBST at 37°C for 2 hours. The membranes were incubated with appropriate primary antibodies.
overnight at 4°C: anti-ICAM-1 (1:2000, #ab53013, Abcam, USA), anti-VCAM-1 (1:1000, #ab134047, Abcam, USA), anti-AIDA (1:1000, #ab234419, Abcam, USA), and β-actin (1:1000, #4970s, Cell Signaling Technology, USA). Subsequently, the membranes were incubated with HRP-conjugated secondary antibody (1:2000, #A9169, Sigma-Aldrich, Germany) for 1.5 hours at 37°C. The protein bands were visualized by chemiluminescence using the ECL kit (Millipore, USA). The grayscale analysis of the protein bands was performed using ImageJ software. β-Actin was used as a reference to normalize the expression levels of target proteins.

**Luciferase reporter assay:** The putative binding site of miR-32-5p and AIDA 3′UTR was predicted using Target-Scan (www.targetscan.org). A fragment of AIDA 3′UTR containing the putative miR-32-5p binding site was amplified by PCR using the forward primer 5′-CCTGGGCCAA TTGTAATAGAACT-3′ and the reverse primer 5′-AACCC AAAGCTTGACGTTAAGT-3′. The amplified fragment was cloned into psiCHECK™-2 vector (Promega, USA) downstream of the Renilla luciferase gene. The mutation of the putative miR-32-5p binding site was generated using a fast mutagenesis system (TransGen Biotech, China) with the forward primer 5′-CTTTTCAATTAATGTTCCA TTAATAGTCT-3′ and the reverse primer 5′-GGACAAAT TAATGAGAAATACCATGTT-3′. The recombinant psiCHECK™-2 vectors containing wild-type and mutant 3′UTR of AIDA (wt-AIDA and Mut-AIDA) were verified by sequencing. Afterward, the verified vectors were cotransfected into HUVECs with miR-32-5p mimic or NC mimic using Lipofectamine 2000 (Invitrogen, USA). After cotransfection for 48 hours, the luciferase activity of the cell lysate was measured using a dual-luciferase reporter assay system (Promega, USA) and normalized to firefly activity.

**Statistical analysis:** Statistical analysis was conducted using GraphPad Prism 8.0 statistical software. Each experiment was repeated at least three times. Student’s t-test and one-way ANOVA followed by Bonferroni’s correction were applied to compare the difference between two groups or multiple groups. P < 0.05 was considered statistically significant.

**Results**

**Treatment of HUVECs with oxLDL induces endothelial injury and inflammation:** To induce endothelial injury and inflammation, we first investigated the effects of oxLDL at different concentrations on the cell viability of HUVECs. CCK-8 assay showed that cell viability was first increased and then decreased significantly with the increase of oxLDL concentration and the turning point was at the concentration of 80 μg/mL (Figure 1A). Moreover, oxLDL at the concentration of 80 μg/mL significantly suppressed the migration and angiogenesis of HUVECs while promoting the expression of inflammatory factors including IL-1β, IL-6, TNF-α, ICAM-1, and VCAM-1 in HUVECs (Figure 1B-I). On the basis of these results, we chose to treat HUVECs with oxLDL at the concentration of 80 μg/mL to induce endothelial injury and inflammation in subsequent experiments.

oxLDL treatment decreases the expression of miR-32-5p in HUVECs: To explore whether oxLDL treatment affects the expression of miR-32-5p in HUVECs, we detected the expression of miR-32-5p in HUVECs treated with oxLDL at different concentrations for 24 hours and at the concentration of 80 μg/mL for different time points using qPCR assay. As shown in Figure 2, oxLDL reduced miR-32-5p expression in HUVECs in a dose-dependent manner, and the expression levels of miR-32-5p in HUVECs treated with oxLDL (80 μg/mL) for 12 and 24 hours were comparable.

**Overexpression of miR-32-5p in HUVECs attenuates oxLDL-induced endothelial injury and inflammation:** To determine the role of miR-32-5p in oxLDL-induced endothelial injury and inflammation, miR-32-5p expression in HUVECs was up-regulated by the transfection with miR-32-5p mimic. We found that overexpression of miR-32-5p significantly increased cell viability and the abilities of cell migration and angiogenesis (Figure 3A-E) but decreased the mRNA and protein expression levels of inflammatory factors including IL-1β, IL-6, TNF-α, ICAM-1, and VCAM-1 in HUVECs treated with oxLDL (Figure 3F-I). These data suggest that the up-regulation of miR-32-5p in HUVECs could attenuate oxLDL-induced endothelial injury inflammation.

**miR-32-5p inhibits oxLDL-induced increase in the expression of AIDA:** As AIDA was predicted to be a target gene of miR-32-5p using bioinformatics analysis, we next investigated whether the expression of AIDA is altered in oxLDL-treated HUVECs and whether it is regulated by miR-32-5p. Cells were treated with miR-32-5p mimic in the presence of oxLDL. Notably, both the mRNA and protein expressions of AIDA were significantly increased in HUVECs treated with oxLDL (Figure 4A, B), which was reversed by miR-32-5p mimic (Figure 4C, D).

**Knockdown of AIDA ameliorates oxLDL-induced endothelial injury and inflammation:** To investigate whether AIDA plays a role in endothelial injury and inflammation induced by oxLDL, AIDA expression in HUVECs was down-regulated by the transfection with siAIDA. Efficient knockdown of miR-32-5p in HUVECs using siAIDA was confirmed by western blot (Figure 5A). CCK-8, wound-healing, and tube formation assays demonstrated that knockdown of AIDA significantly increased cell viability and promoted the migration and angiogenesis of HUVECs induced by oxLDL (Figure 5B-F). Results of qPCR, ELISA, and western blot showed that knockdown of AIDA significantly decreased the mRNA expression of IL-1β, TNF-α, ICAM-1, and VCAM-1 and the protein expression of IL-6, IL-1β, ICAM-1, and VCAM-1 in oxLDL-treated HUVECs, whereas the mRNA expression of IL-6 and protein expression of TNF-α were not affected (Figure 5G-J). These results indicate that knockdown of AIDA ameliorates oxLDL-induced endothelial injury and inflammation.

**miR-32-5p directly targets AIDA in HUVECs:** Because AIDA was predicted to be a target gene of miR-32-5p, we further investigated the interaction of miR-32-5p with AIDA using dual-luciferase reporter assay. The putative binding site of miR-32-5p and AIDA was predicted using TargetScan software (Figure 6A). Dual-luciferase reporter
Figure 1. Treatment with oxLDL induces endothelial injury and inflammation in HUVECs. A: Cell viability of HUVECs treated with oxLDL at different concentrations (0, 20, 40, 80, and 160 μg/mL) for 24 hours was evaluated using CCK-8 assay (n = 6). B-I: HUVECs were treated with oxLDL at a concentration of 80 μg/mL for 24 hours. B: Representative images of the scratched HUVECs at 0 and 24 hours after the scratch. C: Comparison of the cell migration rates (n = 3). D: Representative images of the capillary-like tubes. E: Comparisons of the number of nodes and total length of the tubes (n = 3). F-I: The expression levels of inflammatory factors were detected using qPCR, ELISA, and western blot (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group. oxLDL indicates oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; CCK-8, Cell Counting Kit-8; and ELISA, enzyme-linked immunosorbent assay.
The expression of miR-32-5p is decreased in oxLDL-treated HUVECs. The expression of miR-32-5p in HUVECs treated with oxLDL was detected using qPCR analysis (n = 3). **P < 0.01, ***P < 0.001. oxLDL indicates oxidized low-density lipoprotein; and HUVECs, human umbilical vein endothelial cells.

Discussion

In the current study, we found that miR-32-5p and its target gene AIDA play significant roles in oxLDL-induced endothelial injury and inflammation. Treatment with oxLDL decreased miR-32-5p expression but increased AIDA expression. Additionally, the increased expression of AIDA was reversed by miR-32-5p mimic. Up-regulation of miR-32-5p by both mimic and knockdown of AIDA by siRNA suppressed the mRNA and protein expressions of AIDA in HUVECs (Figure 6C-E), further confirming that miR-32-5p targets AIDA in HUVECs.

miRNAs play crucial roles in the occurrence and development of cardiovascular disease including CAD. miR-32-5p has been reported to be dysregulated in patients with CAD and involved in the pathogenesis of cardiovascular disease. For instance, a recent study illustrated that the serum miR-32-5p expression was increased in patients with AMI and associated with the expression of biomarkers for myocardial damage, endothelial injury, and inflammation, indicating that miR-32-5p may be involved in the regulation of these pathological processes. However, this study has not explored the role of miR-32-5p in the aforementioned processes. In the present study, we found that miR-32-5p expression was significantly decreased in oxLDL-treated HUVECs and the up-regulation
Figure 3. Overexpression of miR-32-5p attenuates oxLDL-induced endothelial injury. The expression of miR-32-5p in HUVECs was increased by the transfection of miR-32-5p mimic before treatment with oxLDL for 24 hours. A: Cell viability was measured using CCK-8 assay (n = 6). B: Representative images of the scratched HUVECs at 0 and 24 hours after the scratch. C: Comparison of the cell migration rates (n = 3). D: Representative images of the capillary-like tubes. E: Comparisons of the number of nodes and total length of the tubes (n = 3). F-I: The expression levels of inflammatory factors were detected using qPCR, ELISA, and western blot (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001. oxLDL indicates oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; CCK-8, Cell Counting Kit-8; and ELISA, enzyme-linked immunosorbent assay.
miR-32-5p inhibits oxLDL-induced increase in the expression of AIDA. The mRNA and protein levels of AIDA were detected using qPCR and western blot (n = 3). The mRNA (A) and protein expression (B) of AIDA was increased in oxLDL-treated HUVECs. Overexpression of miR-32-5p decreased the mRNA (C) and protein expression (D) of AIDA in oxLDL-treated HUVECs. *P < 0.05, **P < 0.001. oxLDL indicates oxidized low-density lipoprotein; AIDA, axin interactor, dorsalization associated; and HUVECs, human umbilical vein endothelial cells.

miR-32-5p alleviated oxLDL-induced endothelial injury and inflammation. Our findings implied that miR-32-5p might play a critical role in protecting endothelial function in the development of atherosclerosis. Additionally, previous studies have demonstrated that miR-32-5p plays vital roles in the pathophysiological processes of cardiovascular disease including calcification of vascular smooth muscle cells (VSMCs) and phenotypic alteration of cardiac fibroblast. However, the existing studies on the roles of miR-32-5p in cardiovascular disease are still in the preliminary stage of exploration, and more research is needed to further elucidate its roles and mechanisms in cardiovascular disease.

Additionally, miR-32-5p has been widely studied in various cancers and inflammatory disease. Previous studies demonstrated that miR-32-5p could regulate the proliferation, migration, invasion, apoptosis and angiogenesis of tumor cells by suppressing PTEN and activating the PI3K/Akt pathway. Notably, it is well established that PTEN and PI3K/Akt pathways also play important roles in the development of atherosclerosis through regulating endothelial function, VSMC proliferation and migration, and inflammation. Therefore, we speculated that miR-32-5p may regulate endothelial injury and inflammation by affecting PTEN expression and PI3K/Akt activity, which remains to be further investigated. Moreover, several studies have shown that miR-32-5p affects the expression of inflammatory factors. In macrophages infected with Mycobacterium tuberculosis, miR-32-5p could inhibit the expression of inflammatory factors IL-1β, TNF-α, and IL-6, which was in line with our findings in HUVECs induced by oxLDL. However,
Knockdown of AIDA ameliorates oxLDL-induced endothelial injury and inflammation. The expression of AIDA in HUVECs was down-regulated by the transfection with siAIDA before treatment with oxLDL for 24 hours. A: Knockdown of AIDA in HUVECs was verified using western blot (n = 3). B: Cell viability was measured using CCK-8 assay (n = 6). C: Comparison of the cell migration rates (n = 3). D: Representative images of the scratched HUVECs at 0 and 24 hours after the scratch. E: Representative images of the capillary-like tubes. F: Comparisons of the number of nodes and total length of the tubes (n = 3). G-J: The expression levels of inflammatory factors were detected using qPCR, ELISA, and western blot (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001. AIDA indicates axin interactor, dorsalization associated; oxLDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; CCK-8, Cell Counting Kit-8; and ELISA, enzyme-linked immunosorbent assay.
Figure 6. miR-32-5p directly targets AIDA in HUVECs. A: The putative binding site of miR-32-5p and AIDA mRNA 3’UTR was predicted using TargetScan software. B: Dual-luciferase activity assay was performed (n = 3). C: The mRNA expression of AIDA was detected using qPCR (n = 3). D, E: The protein expression of AIDA was assessed using western blot (n = 3). AIDA indicates axin interactor, dorsalization associated; HUVECs, human umbilical vein endothelial cells; UTR, untranslated region; Wt-AIDA, wild-type AIDA 3’UTR; and Mut-AIDA, mutant AIDA 3’UTR. *P < 0.05, **P < 0.01, ***P < 0.001.

in intestinal epithelial cells infected with Helicobacter pylori and spinal microglial cells treated with lipopolysaccharide, miR-32-5p could up-regulate TNF-α and IL-6 expressions.26,29) The inconsistent effects of miR-32-5p on the expression of inflammatory factors in the aforementioned studies may be explained by the notion that the same miRNA may function differently in different disease models.

To explore the underlying mechanism of miR-32-5p in endothelial injury and inflammation induced by oxLDL, we first predicted the targets of miR-32-5p using TargetScan software and then obtained the information about the biological functions of the predicted targets by reviewing previous literature. As a result, one of the targets, AIDA, has been recently reported as a novel candidate gene for CAD.15) However, little is known about the role of AIDA in the pathogenesis of CAD. We next investigated whether AIDA is involved in endothelial injury and inflammation. Contrary to miR-32-5p, AIDA expression was significantly increased in HUVECs induced by oxLDL. Moreover, AIDA knockdown could attenuate endothelial injury and down-regulate inflammatory factors induced by oxLDL, which was the same as miR-32-5p overexpression. However, AIDA knockdown significantly decreased the expression levels of the secreted IL-6 protein and TNF-α mRNA but did not affect IL-6 mRNA or
the secreted TNF-α protein. The possible reason lies in the regulation of inflammatory factors by AIDA at the posttranscriptional level. Finally, we verified that miR-32-5p directly targeted AIDA in HUVECs. These results suggest that miR-32-5p probably ameliorates oxLDL-induced endothelial injury and inflammation by targeting AIDA. As a newly identified protein, AIDA is rarely reported. A previous study showed that AIDA expression was increased in human coronary artery endothelial cells treated with TNF-α, suggesting AIDA may be involved in the inflammatory activation of endothelial cells. The current study further demonstrated that AIDA aggravated oxLDL-induced endothelial injury and inflammation, which provides new insights into the role of AIDA in the regulation of endothelial function. A study of embryonic development in zebrafish showed that AIDA inhibited the dorsalizing activity of zebrafish by interfering with the activity of Axin-mediated c-Jun N-terminal kinase (JNK), which is a multifunctional kinase activated in response to stress stimuli and involved in many pathophysiological processes. In endothelial cells treated with various stimuli such as cytokines, high glucose, and oxLDL, JNK has been found to be activated and involved in the processes of inflammatory responses, cell apoptosis, and angiogenesis. Although it remains to be further studied, the available evidence leads us to hypothesize that AIDA regulates oxLDL-induced endothelial injury and inflammation through its inhibitory effect on JNK.

However, some limitations of this study need to be mentioned. For example, animal experiments have not been conducted because of the limited time. Also, the downstream mechanism of AIDA in endothelial injury and inflammation has not been investigated. Meanwhile, clinical correlation between AIDA and atherosclerosis should be explored in further studies.

In summary, our findings suggest miR-32-5p and its target gene AIDA are important regulators in oxLDL-induced endothelial injury and inflammation. This study provides new insights into novel molecular mechanisms of endothelial function and helps to develop novel therapeutic targets for atherosclerosis.

Disclosure

Conflicts of interest: None.

References


