Overexpression of microRNA-155 alleviates palmitate-induced vascular endothelial cell injury in human umbilical vein endothelial cells by negatively regulating the Wnt signaling pathway

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Abstract. The present study aimed to investigate the effect of microRNA155 (miR-155) on palmitate-induced vascular endothelial cell injury in human umbilical vein endothelial cells (HUVECs) via the regulation of the Wnt signaling pathway. HUVECs were treated with 0.1 mM palmitate. After transfection with mimic, antagonim or the Wnt pathway inhibitor XAV939, HUVECs were divided into six treatment groups: Control, palmitate, mimic + palmitate, mimic + palmitate + XAV939, antagonim + palmitate, antagonim + palmitate + XAV939. miR-155 expression was detected using reverse transcription-quantitative PCR. The expression levels of the Wnt signaling pathway-related factors β-catenin and Cyclin D, and the inflammatory factors interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), were detected using western blot analysis. MTT and Transwell assays were used to detect the proliferation and migration of cells, respectively. Apoptosis and reactive oxygen species (ROS) levels were determined using flow cytometry. The localization of β-catenin in cells was determined by immunofluorescence. Palmitate reduced the expression level of miR-155 in HUVECs. In palmitate-induced HUVECs, overexpression of miR-155 promoted cell proliferation, reduced the levels of apoptosis, downregulated the levels of inflammatory factors and reduced ROS levels. Inhibition of the Wnt signaling pathway enhanced the anti-endothelial cell injury effect caused by the overexpression of miR-155 in palmitate-induced HUVECs, thereby promoting proliferation, reducing apoptosis, downregulating the levels of inflammatory factors and reducing ROS levels. In summary, overexpression of miR-155 inhibited palmitate-induced apoptosis, ROS production and levels of inflammatory factors, and promoted the proliferation of HUVECs by negatively regulating the Wnt signaling pathway. This present study provides a theoretical basis for the prevention and treatment of cardiovascular diseases associated with endothelial cell injury.

Introduction

Endothelial cell injury and dysfunction are important events in the pathogenesis of cardiovascular disease (1). Palmitate, a 16-carbon saturated fatty acid, is synthesized by fatty acid synthase (2). Saturated free fatty acids (FFAs), such as palmitate, can induce cardiomyocyte apoptosis, which is related to cardiac dysfunction in obesity and diabetes (3). FFAs can promote the expression of pro-inflammatory cytokines, lipid metabolites and cellular stress as well as causing endothelial dysfunction, resulting in atherosclerosis (4-6). Additionally, previous studies reported that palmitate, a common circulating saturated FFA in plasma, can induce apoptosis in vascular endothelial cells by producing intracellular reactive oxygen species (ROS) or by decreasing the expression of anti-apoptotic molecules (7,8). However, the molecular mechanism of palmitate-induced endothelial cell injury is unclear.

MicroRNAs (miRNAs/miRs), endogenous -22 nucleotide RNAs, play important regulatory roles by targeting mRNAs for cleavage or translational repression. miRNAs play important roles in many biological processes, including proliferation, differentiation, apoptosis, signal transduction and organ development (9,10). miR-155, an oncogenic miRNA, is expressed at high levels in various types of cancer and is often associated with a poor prognosis (11). A previous study demonstrated that miR-155 was induced by TNF-α in human endothelial cells and elevated miR-155 expression is beneficial in vascular endothelial cells (12). The Wnt/β-catenin signaling pathway, a canonical Wnt pathway, is important for developmental and physiological
palmitate-induced vascular endothelial cell injury. Whether miR-155 regulates the Wnt signaling pathway in endothelial cell injury in response to palmitate, and to examine whether miR-155 regulates the Wnt signaling pathway in palmitate-induced vascular endothelial cell injury.

Materials and methods

Cell culture. Primary human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell Research Laboratories, Inc., and cultured in endothelial cell medium produced by ScienCell Research Laboratories, Inc. Cells at a density of 1x10^5 were cultured in a T25 culture flask containing 5 ml culture medium in an incubator at 37°C and 5% CO2 for 24 h. After 24 h, the culture medium was changed. The cells were sub-cultured at a ratio of 1:3 or plated for the experiment when cell confluence reached 80-90%. Cells between the 3rd and 10th generation were used for experiments.

Cell transfection and grouping. HUVECs were cultured routinely and the cell growth was observed using an inverted microscope. When confluence reached 60-70%, the cell density was adjusted to 3x10^5 cells/ml. Cells were seeded into 24-well plates with 500 µl medium/well and cultured in a CO2 incubator for 6-8 h. HUVECs were transfected with mimic (miR-155 mimic, 5'-uua auG cua auc GuG aua GGG Guc ccu auc -3'; Santa Cruz Biotechnology, Inc.) or antagomir (miR-155 antagomir, 5'-acc ccu auc acG cgG aAG CTa aTc GaT aGG-3'; Santa Cruz Biotechnology, Inc.) using lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was synthesized using the ReverTra Ace® qPCR RT kit (Toyobo Co., Ltd.), the reaction conditions were as follows: 42°C for 2 min, 95°C for 5 sec and 37°C for 15 min. Then, RT-qPCR was carried out using SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.) on an Applied Biosystems 7500 Sequence Detection System. The thermocycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing at 60°C for 20 sec. U6 was used as internal reference. Relative fold changes in expression were calculated with the 2^−ΔΔCT method (17) using data from three independent experiments. The reverse primer for miR-155 was 5'-TTAAATGCTAAATCGATAGG-3' and the forward primer was 5'-GTGCAAGGGTCCAGGTT-3'. The reverse primer for U6 was 5'-CTCGCTTCCGACA-3' and the forward primer was 5'-ACGCTTACGAATTGTTGGTG-3'.

Western blot analysis. HUVECs were washed twice with PBS, lysed in RIPA buffer [1 mM EDTA, 150 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4], and heated at 98°C for 10 min. The protein concentration was measured using the bicinchoninic acid method. Equal amounts of protein (20 µg protein/well) were separated using a 9% SDS/polyacrylamide gel and transferred onto a PVDF (EMD Millipore) membrane at 100 V for 2 h at 4°C. The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then incubated with primary antibodies, including anti-β-catenin (cat. no. 8480S; 1:1,000; Cell Signaling Technology), anti-Cyclin D (cat. no. ab16663; 1:100; Abcam), anti-IL-6 (cat. no. sc-57135; 1:200; Santa Cruz Biotechnology, Inc.) and anti-TNF-α (cat. no. sc-52746; 1:500; Santa Cruz Biotechnology, Inc.), anti-GAPDH (cat. no. ab181602; 1:10,000; Abcam), overnight at 4°C. After washing with TBS-Tween 20 (0.05%), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody anti-Immunglobulin G (1:2,000; cat. no. A7539; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Finally, the proteins were visualized using an ECL detection system (EMD Millipore) and quantified by densitometry using Quantity One software (version v4.6.6; Bio-Rad Laboratories, Inc.). GAPDH was used for the internal control.

MTT colorimetric method. When the confluence of the HUVECs reached ~80%, the cells were washed twice with PBS and trypsinized to form a single cell suspension. Cells were then counted with a hemocytometer. The cells were plated into 96-well plates at a density of 3-6x10^4 cells/well (200 µl/well) and cultured in a 37°C and 5% CO2 incubator for 24-72 h. A total of six identical wells were plated for each experiment. MTT solution was added to each well and incubated at 37°C for a further 4 h. The culture medium was then discarded and 150 µl DMSO was added to each well for 10 min to dissolve the formazan crystals. The optical density values at 570 nm of each well were determined using an enzyme-labeled instrument (Bio-Rad Laboratories, Inc.) after 24, 48 and 72 h of culture. The experiment was repeated three times. Transwell assay. HUVECs were cultured in human endothelial serum-free basal medium (cat. no. 1111044; Gibco;
Thermo Fisher Scientific, Inc.) containing 0.5% FBS for 24 h, and then digested with 0.25% trypsin. The cell concentration was adjusted to 2.0x10^4 cells/well. The HUVECs were incubated in the upper chamber of the Transwell chamber (Corning, Inc.) and 500 µl DMEM medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) was added into the lower chamber, followed by incubation at 37°C for 24 h. The chamber was then removed and the culture medium discarded. Cells that did not cross the membrane inside the chamber were gently removed with a cotton swab. Cells that had passed through the membrane were washed three times with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, washed three times with PBS and stained with 0.1% crystal violet for 15 min at room temperature. A total of five visual fields (magnification, x200) were randomly selected to count the number of cells passing through the membrane and images were captured using an optical microscope.

**Immunofluorescence.** HUVECs from each treatment group were smeared using centrifugation at 1,000 x g for 5 min at 20°C onto slides coated with 25 µg/ml polylysine (Sigma-Aldrich; Merck KGaA) solution, fixed with 4% paraformaldehyde at room temperature for 20 min, washed with distilled water for 5 min, soaked in PBS for 5 min, dripped with 3% H₂O₂ in deionized water and incubated at room temperature for 30 min. After that, the samples were washed three times with PBS, each for 5 min, permeabilized with 0.3% Triton X-100 for 30 min, washed three times with PBS, each for 5 min, and blocked with 1% BSA for 10 min. All these steps were performed at room temperature. The samples were then incubated with rabbit anti-human β-catenin monoclonal antibody (1:100; cat. no. ab32572; Abcam) and incubated overnight at 4°C. The slides were washed three times with PBS for 5 min each time at room temperature. The samples were then incubated with FITC-labeled goat anti-rabbit secondary antibody (1:200; cat. no. ab6717; Abcam) at room temperature for 30 min. The slides were washed three times with PBS for 5 min each time at room temperature. The nuclei were stained using DAPI for 5 min at 37°C and washed three times with PBS for 5 min each time. Images were captured using a laser confocal (Axioskop 2 Plus; Carl Zeiss AG; magnification, x200).

**Apoptosis detection by flow cytometry.** HUVECs from each treatment group were plated into 6-well plates and divided into groups, and the assay was conducted according to the manufacturer's protocol of the apoptosis detection kit (Nanjing Keygen Biotech Co., Ltd.). Briefly, cells were digested with 0.25% trypsin (without EDTA), collected by centrifugation at 1,000 x g for 5 min at 20°C, washed with PBS three times and added to 500 µl pre-cooled 1X binding buffer. This was then mixed with 5 µl Annexin-V-FITC and 2.5 µl propidium iodide. The cells were detected using flow cytometry (BD FACSArial I; BD Biosciences). The percentage of apoptotic cells in each quadrant was calculated using FlowJo software (version 7.2.2; FlowJo LLC).

**Determination of ROS level.** The levels of ROS were determined using the 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) probe (Honeywell Fluka; Thermo Fisher Scientific, Inc.). H2DCF-DA can be oxidized to 2',7'-dichlorodihydrofluorescein, generating a fluorescent signal. HUVECs were plated into 6-well plates. After treatments according to the aforementioned procedure, 10 µM H2DCF-DA was added to each well. After incubation for 30 min, the levels of ROS were determined using flow cytometry and analyzed using FlowJo software (version 7.2.2; FlowJo LLC) to calculate the percentage of positive cells.

**Statistical analysis.** SPSS 19.0 software (IBM Corp.) was used for data analysis. All experiments were repeated in triplicate. Data are presented as the mean ± SD. One-way ANOVA was used for comparisons among multiple groups (the homogeneity of variance was tested before analysis), and the least significant difference post hoc test was used for the pairwise comparison of multiple groups/means. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Effect of palmitate on mir-155 expression in HUVECs.** HUVECs were treated with palmitate to model vascular endothelial cell injury and changes in the expression level of mir-155 were detected by RT-qPCR. As shown in Fig. 1, the expression of miR-155 in palmitate-induced HUVECs was significantly downregulated compared to control cells, suggesting that palmitate could reduce the expression of miR-155. In order to study the effects of the overexpression and inhibition of mir-155 on vascular endothelial cell injury, HUVECs were treated with palmitate following transfection with miR-155 mimic or mir-155 antagonist. The RT-qPCR results showed that compared to the palmitate group, the expression of miR-155 in HUVECs was significantly increased after transfection of the cells with the mir-155 mimic. Transfection of cells with the mir-155 antagonist significantly inhibited the expression of miR-155, indicating that the transfections had been successful.
Figure 2. Expression of Wnt signaling pathway-related factors in palmitate-induced human umbilical vein endothelial cells. (A) Western blot analysis was used to detect the expression of the Wnt signaling pathway-related factors β-catenin and Cyclin D. (B) The localization of β-catenin in cells was determined by immunofluorescence. The data are presented as the mean ± SD from three independent experiments. *P<0.05 vs. the control group, #P<0.05 vs. the palmitate group.

Figure 3. Effect of miR-155 on the survival and migration of palmitate-induced human umbilical vein endothelial cells through the regulation of the Wnt signaling pathway. (A) The MTT assay was used to detect the survival of cells in each treatment group at 24, 48 and 72 h. (B) The Transwell assay was used to determine cell migration ability. The Wnt signaling pathway was inhibited using XAV939. The data are presented as the mean ± SD from three independent experiments. *P<0.05 vs. the control group, †P<0.05 vs. the palmitate group, &P<0.05 vs. the mimic + palmitate group, ▲P<0.05 vs. the antagomir + palmitate group.
Expression of Wnt signaling pathway-related factors in palmitate-induced HUVECs. In order to investigate the role of the Wnt signaling pathway in palmitate-induced vascular endothelial cell injury, the expression of β-catenin and Cyclin D were analyzed by western blotting. As shown in Fig. 2A, the expression of β-catenin and Cyclin D in HUVECs increased significantly after palmitate treatment. The expression of β-catenin and Cyclin D in palmitate-induced HUVECs was significantly decreased following transfection with the miR-155 mimic. After transfection with the miR-155 antagonist, the protein expression of β-catenin and Cyclin D increased significantly. The immunofluorescence results (Fig. 2B) indicated that the expression of β-catenin in the nucleus of HUVECs was increased after treatment with palmitate. The expression of β-catenin in the nucleus of HUVECs transfected with the miR-155 mimic decreased. The expression of β-catenin in the nucleus of HUVECs transfected with miR-155 antagonist increased. These results indicated that miR-155 regulates the expression of the Wnt signaling pathway-related factors β-catenin and Cyclin D in palmitate-induced HUVECs.

miR-155 affects palmitate-induced cytotoxicity and migration in HUVECs by regulating the Wnt signaling pathway. In this present study, it was found that miR-155 regulates the Wnt signaling pathway in palmitate-induced HUVECs. To investigate the molecular mechanism underlying the regulation of palmitate-induced vascular endothelial cell injury by miR-155, the effect of miR-155 on the Wnt signaling pathway in palmitate-induced vascular endothelial cell injury was examined further. The Wnt signaling pathway in HUVECs transfected with miR-155 mimic or miR-155 antagonist was inhibited. MTT assays showed that the number of HUVECs was lower following treatment with palmitate compared with control HUVECs. Compared to the palmitate group, the number of HUVECs increased in the palmitate + mimic group; while the number of HUVECs in the antagonist + palmitate group decreased. In HUVECs transfected with miR-155 mimic or miR-155 antagonist, the number of HUVECs was increased by inhibiting the Wnt signaling pathway (Fig. 3A). The migration ability of HUVECs was determined using a Transwell test. Compared to the control group, the migration ability of HUVECs treated with palmitate was significantly decreased. The migration of HUVECs after transfection with miR-155 mimic increased and migration decreased after transfection with the miR-155 antagonist. The migration ability of HUVECs was increased...
miR-155 affects palmitate-induced apoptosis in HUVECs by regulating the Wnt signaling pathway. The level of apoptosis in HUVECs was determined by flow cytometry. The results showed that compared to the control group, the levels of apoptosis in palmitate-induced HUVECs were significantly higher. miR-155 overexpression significantly reduced palmitate-induced apoptosis in HUVECs, while inhibition of the Wnt signaling pathway further reduced the level of apoptosis. Inhibition of miR-155 increased the level of palmitate-induced apoptosis in HUVECs; inhibition of the Wnt signaling pathway decreased the degree of apoptosis (Fig. 4). These results demonstrated that miR-155 inhibits palmitate-induced apoptosis in HUVECs by negatively regulating the Wnt signaling pathway.

miR-155 affects the production of ROS in palmitate-induced HUVECs by regulating the Wnt signaling pathway. This study examined the level of ROS in palmitate-induced vascular endothelial cell injury in HUVECs, and the effect of miR-155. The results showed that compared to the control group, the palmitate group showed a higher level of ROS production (P<0.05). miR-155 overexpression significantly reduced the production of ROS and inhibition of the Wnt signaling pathway further inhibited the production of ROS. Inhibition of miR-155 increased the production of ROS, but increased ROS levels were again inhibited by suppression of the Wnt signaling pathway (Fig. 5). These results suggested that miR-155 negatively regulates the Wnt signaling pathway and reduces the level of ROS produced by palmitate-induced vascular endothelial cell injury in HUVECs.

miR-155 regulates the levels of inflammatory factors in palmitate-induced vascular endothelial cell injury in HUVECs by regulating the Wnt signaling pathway. In order to further investigate the effect of palmitate on the inflammation level of injured vascular endothelial cells, levels of the inflammatory factors IL-6 and TNF-α were determined by western blot analysis. As shown in Fig. 6, the levels of IL-6 and TNF-α in palmitate treated HUVECs were significantly higher than in control cells. Transfection with the miR-155 mimic reduced palmitate-induced inflammation while transfection with the miR-155 antagonir increased palmitate-induced inflammation. Compared to the mimic + palmitate group, the levels of IL-6 and TNF-α in the mimic + palmitate + XAV939 group decreased significantly. Compared to the antagonir + palmitate group, the levels of IL-6 and TNF-α in the antagonir + palmitate + XAV939 group decreased. These results indicated that the Wnt signaling pathway can promote palmitate-induced vascular endothelial cell inflammation, and this process is regulated by miR-155.

Discussion

Endothelial cells play an important role in vascular biology by regulating vasodilation and constriction by autocrine, paracrine and hormonal-like mechanisms, as well as molecules such as nitric oxide, prostacyclin, endothelin and thromboxane (18). Therefore, maintaining the integrity and functional activity of endothelial cells is a potential target in the prevention and treatment of cardiovascular disease. In this present study, the role of miR-155 in palmitate-induced vascular endothelial cell injury in HUVECs was investigated. Overexpression of miR-155 alleviated palmitate-induced vascular endothelial cell injury in HUVECs by negatively regulating the Wnt signaling pathway.

To investigate the role of miR-155 in palmitate-induced vascular endothelial cell injury in HUVECs, the effect of palmitate on miR-155 expression was examined. In the present study, palmitate inhibited miR-155 expression. In a previous study, the exposure of rat aortic endothelial cells to palmitate
for 24 h resulted in an increased level of apoptosis (19). It has been reported that miR-155 plays an important role in the regulation of endothelial cell apoptosis, vascular smooth muscle migration, lipid metabolism and inflammatory reaction, and thus affects the occurrence of atherosclerosis via physiological and pathological processes (20).

The possible molecular mechanism by which miR-155 regulates vascular endothelial cell injury induced by palmitate through the Wnt signaling pathway was investigated. miR-155 promoted the survival and migration of HUVECs cells and reduced the level of apoptosis through the negative regulation of the Wnt signaling pathway. With the expression of Wnt signaling pathway-related factors assessed, the findings of the present study indicated that there were increased levels of β-catenin and Cyclin D expression following treatment with palmitate, which could be reversed by the transfection of the miR-155 mimic. Palmitoylation and other lipid modifications, including myristoylation, are important regulatory switches in many signal transduction pathways, including the Wnt/β-catenin signaling pathway (21,22). Consistent with the present study, a previous report found that the upregulation of fatty acid synthase could lead to the accumulation and activation of membranous and cytoplasmic β-catenin (23). As a cancer-related miRNA, the ability of miR-155 to regulate components of the Wnt/β-catenin signaling pathway indicates that mutations in Adenomatous Polyposis Coli, which regulates miRNA expression, may also modulate the Wnt/β-catenin signaling pathway (24). Furthermore, ectopic expression of miR-155 was reported to induce HUVEC network formation, proliferation, invasion and migration (25), which is consistent with the results from the present study.

In the present study, it was demonstrated that ROS production and the inflammatory reaction were increased in palmitate-stimulated HUVECs. ROS are highly reactive molecules that have the potential to impair DNA, proteins and fatty acids, and it has been reported that upregulated ROS production can reduce endothelial function, not only in patients with cardiovascular disease, but also in animal models (26). The present study is consistent with a previous report that found palmitate could increase the production of ROS (27). Saturated fatty acids, such as palmitate, can activate inflammatory pathways in primary microvascular endothelial cells and induce an endoplasmic reticulum stress response in macrophages (28,29). Furthermore, the present study found that miR-155 reduced the production of ROS and the inflammatory reaction induced by palmitate in HUVECs by negatively regulating the Wnt signaling pathway. TNF-α is a multifunctional pro-inflammatory cytokine, with multiple functions in the innate immune response, including macrophage activation, inflammatory reactions and apoptosis (30). It has been reported that TNF-α induces the production of ROS in endothelial cells, resulting in endothelial dysfunction (31,32). miR-155 plays an important role in modulating inflammation and tumorigenesis, overexpression of miR-155 was found to result in the nuclear accumulation of β-catenin and a concomitant increase in cyclin D1 (33).

In conclusion, the present study identified miR-155 as an important and novel feedback regulator of palmitate-induced vascular endothelial cell injury in HUVECs through the negative regulation of the Wnt signaling pathway. The results of the present study suggested that modulating miR-155 levels could be used as a therapeutic intervention for vascular endothelial cell injury induced by palmitate.

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Availability of data and materials

The datasets analyzed in the present study are available from the corresponding author on reasonable request.

Authors’ contributions

QL, YZ and WR conceived and designed the present study., YW, XY and GW performed the experiments. JD and MD analyzed and interpreted the data. YZ, WR and YW drafted and revised the paper. All the authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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