MicroRNA-154 Targets the Wnt/β-Catenin Signaling Pathway Following Injury to Human Vascular Endothelial Cells by Hydrogen Peroxide

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Background:
Endothelial cells are involved in vascular homeostasis, and endothelial cell dysfunction is involved in the pathogenesis of cardiovascular disease. This study aimed to investigate the effects of microRNA-154 in human umbilical vein endothelial cells (HUVECs) following injury induced by hydrogen peroxide (H₂O₂).

Material/Methods:
Cell viability and apoptosis of HUVECs treated with H₂O₂ were measured. The expression of microRNA-154 was detected by quantitative real-time polymerase chain reaction (qRT-PCR). Cell survival, caspase-3 activity, and the apoptosis rate were evaluated in H₂O₂-treated HUVECs cells after the upregulation and down-regulation of microRNA-154 expression. The interaction between microRNA-154 and Dickkopf WNT signaling pathway inhibitor 2 (DKK2) was predicted by bioinformatics analysis and was verified by luciferase reporter gene assay and Western blot. The effects of DKK2 short-interfering RNA (siRNA) on antioxidant injury in HUVECs cells were determined.

Results:
The survival rate of HUVECs exposed to H₂O₂ was significantly reduced and the apoptosis rate was significantly increased, and H₂O₂ significantly inhibited the expression of microRNA-154 in a dose-dependent manner. Overexpression of microRNA-154 increased cell survival, reduced the activity of caspase-3, and reduced cell apoptosis. Inhibition of microRNA-154 expression decreased cell survival, increased the activity of caspase-3, and promoted cell apoptosis. Luciferase reporter gene assay and Western blot showed that microRNA-154 interacted with the Wnt pathway molecule DKK2 in HUVECs. Also, DKK2 siRNA resulted in a similar protective effect on H₂O₂-treated HUVECs as overexpression of microRNA-154.

Conclusions:
Oxidative injury in HUVECs was regulated by microRNA-154 targeting the Wnt/β-catenin signaling pathway.

MeSH Keywords:
Cardiovascular Diseases • Cardiovascular System • Endothelial Cells • MicroRNAs

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Background

The common cardiovascular diseases that seriously affect human health include hypertension, coronary artery disease, and chronic heart failure, which may result from dysfunction of normal endothelial cell homeostasis [1]. Endothelial cell dysfunction has been shown to be the basis of the pathogenesis of cardiovascular disease [2]. Oxidative stress, endothelial dysfunction, and cell injury can be caused by hydrogen peroxide ($H_2O_2$) produced by blood vessels and inflammatory cells [3,4], which results in the occurrence and development of cardiovascular and cerebrovascular diseases, including hypertension and atherosclerosis. Therefore, it is important that studies should be undertaken on the molecular mechanism of oxidative stress-induced endothelial dysfunction and cell injury to develop treatments for cardiovascular disease.

MicroRNAs (or miRNAs) are conservative non-coding RNAs with a length of only 22 nucleotides (nt). MicroRNAs can bind to target sites in the three prime untranslated region (3’-UTR) of target RNA, which degrades or inhibits the expression of target genes post-transcription [5–7]. Also, microRNAs in endothelial cells have been shown to be involved in the development of diseases of the cardiovascular system [8]. MicroRNA-154 has been shown to be one of the most abundant microRNAs in human endothelial cells and is involved in endothelial cell migration and cell survival [9–12].

Target sites of microRNA-154 have been reported in the DKK2, SFRP4, and WIF-1 genes. Importantly, the DKK2 gene encodes for Dickkopf WNT signaling pathway inhibitor 2 (DKK2). MicroRNA-154 may activate the Wnt pathway by regulating the activity of the DKK2, SFRP4, and WIF-1 genes. However, it is unclear whether microRNA-154 is involved in endothelial dysfunction and cell injury induced by oxidative stress. Therefore, this study aimed to investigate the effects of microRNA-154 in human umbilical vein endothelial cells (HUVECs) following injury induced by $H_2O_2$.

Material and Methods

Reagents and equipment

Hydrogen peroxide (China National Medicines Co. Ltd., Shanghai, China), the cytotoxicity and caspase-3 activity detection kit (Biyuntian Institute of Biotechnology, Nantong, China), the electrochemiluminescence (ECL) immunoblotting substrate kit (Merck Millipore, Burlington, MA, USA), antibody to DKK2 (Abcam, Cambridge, MA, USA), microplate reader (Bio-Rad, Hercules, CA, USA), 1640 medium and fetal bovine serum (FBS) (Gibco, Thermofisher Scientific, Waltham, MA, USA), and trypsin, the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) cell apoptosis detection kit (Nanjing KeyGen Biotech Co. Ltd., Nanjing, China) were obtained from the respective companies. The other reagents were of analytical grade purity. A 680 full-automatic microplate reader (Bio-Rad, Hercules, CA, USA), an AE31EF-INV inverted microscope (Motic Microscopy, Tokyo, Japan), a CO$_2$ cell incubator (Sanyo Co., Tokyo, Japan), and a C6 flow cytometer (Beckman Coulter, Brea, CA, USA) were used for the assays.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were cultured with 5% fetal bovine serum (FBS) and endothelial cell growth supplement (ECGS) culture medium. The cells were cultured in an incubator with 5% $CO_2$ at 37°C. Cells were digested with EDTA trypsin and subcultured in cell culture flasks. Follow-up assays were performed when the cells were in the logarithmic growth phase.

Lentivirus cell transfection

Recombinant lentiviral particles expressing DKK2 short-interfering RNA (siRNA) or control RNA, and recombinant lentiviral particles expressing microRNA-154 mimic or inhibitor and control microRNA were obtained from GenePharma Company, Shanghai, China. Following the manufacturer’s instructions, HUVECs were obtained as a single cell suspension by adding trypsin when the HUVECs had reached the logarithmic growth phase. HUVECs were then inoculated into a 24-well cell culture plate and cultured in a complete medium and transfection with lentiviruses particles was performed for 48 h. The cells were treated with 8 μg/mL polybrene (a cationic polymer) to improve the transfection. The siRNA and polybrene did not affect the cell viability, and siRNA had no targeting effect and had no effect on the cell adhesion, shape, and viability of HUVECs during the transfection and treatment period.

MTS cell proliferation assay

A 100 μL inoculum of cells in the logarithmic growth phase ($1\times10^4$ cells/mL) was added to each well of a 96-well plate, followed by 100 μL $H_2O_2$ at a concentration of 0, 10, 50, 100, or 200 μmol/L in serum-free medium. After 72 hours of treatment, the medium was extracted by adding MTS reagent, according to the manufacturer’s instructions. Finally, the absorbance at 490 nm was measured using the microplate reader, and the cell proliferation rate was calculated.

Caspase-3 activity

Caspase-3 activity was evaluated by a caspase-3 activity assay kit. The cells were lysed and the total cell protein was extracted.
The total cell protein was measured by a protein kit and incubated overnight with equivalent acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA) at 37°C for determination of caspase-3. The pNA release was evaluated by measuring the absorbance at 405 nm by enzyme-linked immunosorbent assay (ELISA). Caspase-3 activity was calculated as the average measured absorbance/average control absorbance ×100%.

Detection of apoptosis by flow cytometry

The cells were treated, as above, and digested with trypsin to form a single cell suspension. The cells were washed twice with precooled phosphate-buffered saline (PBS) and centrifuged at 1000 rpm for 5 min. The cells were then fixed with 1 mL of cold 70% ethanol, and stained with PI. The apoptotic rate was measured by flow cytometry.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA of the HUVECs was extracted by TRIzol reagent. Reverse transcription was performed using a PrimeScript RT-PCR kit and Promega RT-PCR kit. The reaction conditions were initial denaturation at 94°C for 4 min, denaturation at 94°C for 40 s, annealing at 52°C for 40 s, and extension at 72°C for 40 s, with a total of 40 cycles. The qRT-PCR was performed on an ABI 7500 amplifier using SYBR Premix Ex Taq (Takara, Minato-ku, Tokyo, Japan). The PCR results were analyzed by the ΔΔCt method. The PCR primers were designed and synthesized by Shanghai Sangon Biotech Company, with U6 as an internal reference.

Bioinformatics analysis and the luciferase reporter gene detection

Bioinformatic analysis was performed using DIANA Tools (Athena Innovation, Greece). According to the predicted sequence of binding sites between DKK2 and miR-154, DKK2 was found to be mutated at the presumed binding site. Endothelial cells were cultured with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and co-transfected with pmir-GLO, pmir-GLO-DKK2-wt, or pmir-GLO-DK2-mut, and mir-154 mimic or microRNA-NC. After 48 hours of preparation and transfection, the relative luciferase activity of endothelial cells was measured using the E1910 Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western blot

The expression of Dickkopf WNT signaling pathway inhibitor 2 (DKK2) protein was detected by Western blot after protein isolation from different cell groups. The cell protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was sealed with 5% skimmed milk powder and incubated with anti-DKK2 monoclonal antibody. The GAPDH monoclonal antibody was used as an internal reference. The proteins were visualized by chemiluminescence. The protein bands were quantified and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The difference in the expression of microRNAs and proteins between the two groups was compared using a non-paired t-test (two-tailed). The data were expressed as the mean ± standard deviation (SD). The data for cell growth and apoptosis were analyzed by two-way analysis of variance (ANOVA). All statistical data were analyzed by GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA) and SPSS version 18.0 software (IBM, Chicago, IL, USA). A P-value <0.05 was considered to be statistically significant.

Results

Down-regulation of microRNA-154 expression in human umbilical vein endothelial cells (HUVECs) treated with hydrogen peroxide (H₂O₂)

The effect of microRNA-154 in endothelial cell injury induced by H₂O₂ was evaluated by an in vitro cell system using different concentrations of H₂O₂. The endothelial cell injury was induced by H₂O₂ in a concentration-dependent way, as shown by the findings of the MTT assay (Figure 1A), which supported that the model of endothelial cell injury induced by H₂O₂ was successfully established. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine the expression levels of microRNA-154 and showed that with the increasing concentrations of H₂O₂, the expression of microRNA-154 was down-regulated (Figure 1B). This finding supported that expression of microRNA-154 might be related to endothelial cell injury induced by H₂O₂.

Regulation of microRNA-154

MicroRNA-154 was underexpressed or overexpressed by regulating the genes in endothelial cells (Figure 2A–2D). Endothelial cell injury was reduced by microRNA-154 overexpression and increased by microRNA-154 down-regulation. The results indicated that endothelial cell injury could be regulated by microRNA-154.

The role of microRNA-154 in caspase-3 activity

Changes in caspase-3 activity, an important index of apoptosis, were also detected. The increase of caspase-3 activity induced
Figure 1. Down-regulation of microRNA-154 expression during endothelial cell injury induced by hydrogen peroxide. (A) The MTT assay was used to detect the effects of different hydrogen peroxide concentrations (0–200 µM) on endothelial cell survival. When the concentration of hydrogen peroxide was 50–200 µM, the endothelial cell activity decreased significantly. (B) Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the changes in the level of microRNA-154. The expression of microRNA-154 was found to be down-regulated in a dose-dependent manner during endothelial cell injury induced by hydrogen peroxide. * P<0.05 represents a significant difference compared with the group treated with 0 µM hydrogen peroxide.

Figure 2. Effects of microRNA-154 regulation on endothelial cell injury induced by hydrogen peroxide. (A) Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of microRNA-154 in endothelial cells infected with the microRNA-154 mimic and mimic control. (B) Overexpression of microRNA-154 could alleviate the endothelial cell injury induced by H₂O₂. (C) qRT-PCR was used to detect the expression of microRNA-154 in endothelial cells infected with the microRNA-154 inhibitor and the mimic inhibitor. (D) Down-regulation of microRNA-154 expression increased the endothelial cell injury induced by hydrogen peroxide. * P<0.05 represents a significant difference compared with the control group. * P<0.05 represents a significant difference compared with the hydrogen peroxide group.
by H$_2$O$_2$ was limited by overexpression of microRNA-154, whereas inhibition of microRNA-154 led to an increase of caspase-3 activity (Figure 3A, 3B). These findings indicated that microRNA-154 was involved in a change in caspase-3 activity induced by H$_2$O$_2$ in endothelial cells.

**Figure 3.** Involvement of microRNA-154 in the changes in caspase-3 activity induced by hydrogen peroxide in endothelial cells. (A) Overexpression of microRNA-154 reduced caspase-3 activity induced by hydrogen peroxide in endothelial cells. (B) Inhibition of microRNA-154 could increase the caspase-3 activity induced by hydrogen peroxide in endothelial cells. * P<0.05 represents a significant difference compared with the control group. * P<0.05 represents a significant difference compared with the hydrogen peroxide group.

**Figure 4.** (A, B) Effects of microRNA-154 overexpression on endothelial cell apoptosis induced by hydrogen peroxide. * P<0.05 represents a significant difference compared with the control group. * P<0.05 represents a significant difference compared with the hydrogen peroxide-treated group.

Overexpression of microRNA-154 and endothelial cell apoptosis

Cell apoptosis was detected by flow cytometry to study the effect of microRNA-154 in endothelial cell injury induced by H$_2$O$_2$. The cell apoptosis was reduced by overexpression of microRNA-154, which suggested that the cell apoptosis was inhibited by upregulation of microRNA-154 (Figure 4A, 4B).
Figure 5. Effects of inhibition of microRNA-154 expression on endothelial cell apoptosis induced by hydrogen peroxide. * P<0.05 represents a significant difference compared with the control group. # P<0.05 represents a significant difference compared with the hydrogen peroxide-treated group.

Figure 6. Involvement of microRNA-154 and Dickkopf WNT signaling pathway inhibitor 2 (DKK2) in endothelial cell apoptosis induced by hydrogen peroxide. (A) Bioinformatics analysis predicted an interaction between Dickkopf WNT signaling pathway inhibitor 2 (DKK2) and microRNA-154. (B) Luciferase reporter gene analysis confirmed the interaction between DKK2 and microRNA-154. (C) The expression of DKK2 protein was detected during the overexpression and inhibition of microRNA-154. * P<0.05 represents a significant difference compared with the vehicle control group. # P<0.05 represents a significant difference compared with the hydrogen peroxide group.
Inhibition of microRNA-154 and endothelial cell apoptosis

Cell apoptosis induced by H₂O₂ was detected by flow cytometry and was increased by microRNA-154 inhibition, which suggested that the cell apoptosis was promoted by down-regulation of microRNA-154 (Figure 5A, 5B).

Effects of microRNA-154 and DKK2 on endothelial cell apoptosis

MicroRNAs are known to interact with target genes to produce phenotypic effects. The interaction between microRNA-154 and DKK2 was predicted from bioinformatics database analysis (Figure 6A). In the reporter gene analysis, the luciferase activity decreased significantly after the mutant DKK2 3’UTR plasmid was transferred into the endothelial cells with overexpressed microRNA-154 (Figure 6B). Also, Western blot showed that overexpression of microRNA-154 down-regulated the protein expression of DKK2, indicating a relationship between DKK2 and microRNA-154 (Figure 6C).

The role of DKK2 in endothelial cell apoptosis

To study the mechanism of DKK2 in the endothelial cell apoptosis induced by H₂O₂ and mediated by microRNA-154, the endothelial cells were transfected with DKK2 short-interfering RNA (siRNA) to down-regulate the level of DKK2. Endothelial cell apoptosis induced by H₂O₂ was reduced by DKK2 siRNA, and the increase in caspase-3 activity induced by H₂O₂ was limited by the overexpression of DKK2 siRNA (Figure 7A–7C). These results indicated that the expression level of microRNA-154 was regulated by DKK2 siRNA in endothelial cell apoptosis induced by H₂O₂.

Discussion

In this study, the effects of microRNA-154 on cell viability and apoptosis of human umbilical vein endothelial cells (HUVECs)
following injury induced by hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) were studied, and the expression of microRNA-154 was determined by quantitative real-time polymerase chain reaction (qRT-PCR). Cell survival and caspase-3 activity of HUVECs treated with H\textsubscript{2}O\textsubscript{2} were evaluated with upregulation and down-regulation of microRNA-154. The relationship between microRNA-154 and Dickkopf WNT signaling pathway inhibitor 2 (DKK2) protein and DKK2 gene were predicted by bioinformatic analysis, and verified by luciferase reporter gene assay and Western blot. The effect of DKK2 short-interfering RNA (siRNA) on H\textsubscript{2}O\textsubscript{2} injury in HUVECs was determined, and the role of microRNA-154 in oxidative stress induced by H\textsubscript{2}O\textsubscript{2} in HUVECs was studied. The findings from this study showed that both the survival rate of HUVECs and the apoptosis rate were significantly increased. Also, the expression of microRNA-154 was inhibited by H\textsubscript{2}O\textsubscript{2} in a dose-dependent way. The survival rate of HUVECs exposed to H\textsubscript{2}O\textsubscript{2} was increased and the activity of caspase-3 was decreased by overexpression of microRNA-154, and the survival rate of HUVECs exposed to H\textsubscript{2}O\textsubscript{2} was decreased and the activity of caspase-3 was increased by microRNA-154 interference. The expression of DKK2 was inhibited by microRNA-154 in the luciferase reporter gene assay and Western blot, and DDK2 siRNA had similar protective effects on H\textsubscript{2}O\textsubscript{2}-treated HUVECs with overexpression of microRNA-154.

Endothelial cell growth, differentiation, migration, and adhesion have been previously shown to be regulated by Wnt/β-catenin signaling pathway, with the mechanism classified as following a normative or non-normative pathway [13]. As the key signaling molecule in the normative pathway, β-catenin has been reported to be activated by its stability and nuclear translocation [13].

DKK2 is a Wnt antagonist [14]. DKK2 can specifically bind to the Wnt receptor and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) to prevent their combination, with the downstream signal transduction inhibited by the Wnt-Frizzled complex [15,16]. The relationship between microRNAs and the Wnt/β-catenin signaling pathway has previously been established, and the potential value of their regulation in diagnosis, treatment, and prognosis of human diseases has been studied. Zhou et al. [17] reported that microRNA-154 activated Wnt/β-catenin signaling pathway, including phosphatase, the tumor suppressor gene Wnt inhibitor 1 (WIF1), tension protein homologs, and deletion of PTEN and Wnt5a on chromosome 10. It has been previously reported that the pathological changes associated with microRNA-154 activation in malignancy could accelerate epithelial-mesenchymal transformation (EMT) and promote tumor metastasis. Also, Milosevic et al. [18] reported that pulmonary fibrosis was promoted by microRNA-154 activation of the Wnt signaling transduction pathway. Budak et al. [19] found that the expression of some microRNAs similar to microRNAs-154 resulted in cardiac hypertrophy and heart failure. Therefore, microRNAs and the Wnt signal transduction pathway, including microRNA-154, may have a role in cardiovascular disease.

The relationship between microRNA-154 and the Wnt pathway has been previously analyzed using miRanda [20] and RNAhybrid software [21], and the potential target sites of microRNA-154 have been found in the 3’UTR of the DKK2 gene. DKK2 is a member of the antagonist protein family in the Wnt signaling pathway that includes five extracellular molecules, including WIF1, secreted frizzled-related proteins (sFRP), Wise, Cerberus, and DKK2 [22]. Yanagida et al. [23] reported that the Wnt signaling pathway was inhibited by DKK2 and that the expression of the antifibrosis gene SMAD7 and the fibrosis promoting genes ASMA and COLI1 were induced by DKK2, with the activation of hematopoietic stem cells (HSCs) and inhibition of hepatic fibrosis.

The findings of the present study showed that the expression of β-catenin in endothelial cells was increased by microRNA-154, whereas the expression of β-catenin was reduced by DKK2, confirmed by transfection with microRNA-154 inhibitors and DKK2 short-interfering RNA (siRNA), respectively. Also, overexpression of DKK2 and miR-154 in the same cells resulted in no difference in Wnt signaling pathways, which confirmed that the Wnt pathway was activated by microRNA-154 having an inhibited effect on DKK2, which supported the findings and the proposed mechanism reported by Milosevic et al. in human lung fibroblasts [18]. In this previously reported study, Wnt pathway inhibitors (DKK2, DIXDC1, and PPP2CA) were significantly decreased, whereas Wnt receptor activators (FZD4/5/6, LRP, and KREMEN1) were significantly increased following microRNA-154 transfection into fibroblasts [18]. Activation of the Wnt signaling pathway and the proliferation and migration of lung fibroblasts were significantly promoted, induced by TGF-β1, which was inhibited following microRNA-154 inhibitor transfection into fibroblasts [18]. In the study findings reported by Bernardo et al. [24], inhibition of microRNA-154 in a mouse model of hypertrophic cardiomyopathy could treat cardiac dysfunction, reduce cardiac remodeling and fibrosis, and reduce adverse cardiac events. The results from these previous studies indicated that microRNA-154 could activate Wnt signaling pathways to promote fibrosis, increase cell proliferation and migration, and inhibit cell apoptosis, while cell apoptosis was promoted by microRNA-154 inhibitors and DKK2 overexpression. Xian et al. [25] reported that cell migration and differentiation were promoted by the activation of the Wnt signaling pathway, and Yanagida et al. [23] reported that the expression of DKK2 in HSC was decreased in the absence of Septin 4 (SEPT4), which promoted the inhibition of DKK2 in the classical Wnt pathway. Therefore, the findings from these previous studies are consistent with the results of the present study.
with the reported effect of microRNA-154 on targeting DKK2 expression and upregulating β-catenin expression to activate the classical Wnt pathway in HUVECs.

Conclusions

In cultured human umbilical vein endothelial cells (HUVECs), oxidative injury was regulated by microRNA-154 targeting the Wnt/β-catenin signaling pathway.

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Conflict of interest

None.