Original Research Article

MiR-10b alleviates high glucose-induced human retinal endothelial cell injury by regulating TIAM1 signaling

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Abstract

Purpose: To investigate the effects of microRNA (miR)-10b on high glucose (HG)-induced human retinal endothelial cell (HREC) injury and the mechanisms involved.

Methods: Levels of miR-10b were measured in HRECs using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) after the addition of glucose (5.5 and 30 mM). Cell viability was measured using Cell Counting Kit-8 assay, while levels of reactive oxygen species (ROS) were determined using fluorimetry. An enzyme-linked immunosorbent assay (ELISA) was used to measure cellular apoptosis. Luciferase reporter assay was used to validate the miR-10b-binding sites of target genes. The levels of T-cell lymphoma invasion and metastasis (TIAM1) and NADPH oxidase-2 (NOX2) were determined using qRT-PCR. Ras-related C3 botulinum toxin substrate 1 (Rac1) activation was evaluated using a pull-down assay. The protein levels of TIAM1 and Rac1 were assayed by western blotting.

Results: After HG stimulation, miR-10b expression was downregulated. Viability of HRECs decreased, whereas ROS production increased. However, the overexpression of miR-10b inhibited apoptosis and ROS production in HG-treated HRECs (p < 0.05), while luciferase reporter analysis revealed a possible binding site for miR-10b to target the 3'-untranslated region (UTR) of TIAM1. In addition, the overexpression of miR-10b distinctly reduced the expression levels of TIAM1 and NOX2, but decreased the activation of Rac1 in HG-treated HRECs (p < 0.05); these inhibitory effects of miR-10b were significantly reversed after TIAM1 application.

Conclusion: MiR-10b alleviates HG-induced HREC injury by regulating TIAM1 signaling. MiR-10b therapy is a potential therapeutic strategy for patients suffering from diabetic retinopathy.

Keywords: MicroRNA-10b, Human retinal endothelial cells, High glucose, TIAM1-Rac1 axis

INTRODUCTION

Diabetic retinopathy (DR), a microvascular complication of diabetes, is the main cause of blindness in adults worldwide [1]. Its morbidity ranges between 17.6 and 33.2% [2,3]. Unfortunately, with the increasing prevalence of diabetes in Asian countries, especially China and India, the prevalence of DR has been inaccurately estimated [4]. It is believed that the...
duration and severity of hyperglycemia leads to the development of DR [5]. Thus, it is important to minimize damaging effects caused by hyperglycemia.

Short non-protein-coding RNAs, microRNAs (miRNAs or miRs), are highly expressed in many species [6]. Most mature miRNAs precisely regulate target genes by binding to the 3'-untranslated region (UTR) of their mRNAs [7]. Compelling evidence has indicated that miRNAs play vital roles in many biological processes, including tumorigenesis, apoptosis, proliferation, and cell differentiation [8-10]. For example, miRNA-1273g-3p and miRNA-451a have been shown to play vital roles in the regulation of DR; thus, they may serve as new targets for DR treatment [11,12]. The downregulation of miR-10b has been observed in cervical cancer, and miR-10b overexpression has been found to repress cellular proliferation, migration, and invasion, and induce apoptosis by targeting T-cell lymphoma invasion and metastasis (TIAM1) [6]. However, the effects of miR-10b on high glucose (HG)-induced injury of human retinal endothelial cells (HRECs) and the signaling mechanisms involved have not yet been investigated. Therefore, HRECs were selected to investigate the effects of miR-10b on HG-treated HRECs.

**EXPERIMENTAL**

**Cell culture and treatment**

HRECs were purchased from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 0.1% gelatin in Dulbecco’s modified Eagle's medium (DMEM)/F12 medium (1:1) containing 5 mM glucose (MSKCC Media Facility, New York, NY, USA), and supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 5% ECGS, 1% penicillin/streptomycin, and 1 × insulin-transferrin-selenium at 37 °C in 5% CO₂ environment.

HRECs were maintained in 6-well plates containing 5.5 mM glucose [normal glucose (NG)] for 24 h and then incubated in HG (30 mM) for 48 h.

**Cell transfection with microRNA mimics**

HRECs were transfected with miRNA mimics (has-miR-10b) using oligofectamine (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s instructions. Forty-eight hours before harvest, 30 nM has-miR-10b was added to induce the overexpression of miR-10b. The negative control group was treated with an equal concentration (30 nM) of a mimic negative control (Invitrogen). The NG and HG groups were not treated with the mimic. The overexpression of miRNA was verified using quantitative polymerase chain reaction (PCR).

**Evaluation of cell viability**

HRECs were maintained in 6-well plates overnight until they reached 100% confluence. The Cell Counting Kit-8 (Sigma, St. Louis, MO, USA) was utilized to determine cell viability according to the manufacturer’s instructions. Cell viability was determined at 450 nm using a microplate reader (Nippon-InterMed, Tokyo, Japan).

**Determination of levels of reactive oxygen species (ROS)**

ROS levels were determined using fluorimetry: 2'7'-Dichlorodihydrofluorescein diacetate (10 μM; Thermo Fisher, Waltham, MA, USA) was added and incubated with HRECs for 1 h. Following the addition of HG, the stained cells were observed under a fluorescence microscope (KEYENCE Japan, Osaka, Japan). After counterstaining with DAPI, images were obtained using fluorescence microscopy, and ROS production was quantified in 10 high-power fields.

**Assessment of cellular apoptosis**

HRECs (1 × 10⁵ cells/well) were seeded into a 6-well plate in DMEM/F12 containing NG. After 24 h, cells were washed twice with cold phosphate-buffered saline and then treated with HG for 48 h. An enzyme-linked immunosorbent assay (ELISA) was used to quantify cellular apoptosis (Cell Death Detection ELISA kit, Roche Applied Science, Branford, CT, USA), according to the manufacturer’s instructions. The absorbance was measured at 405 nm.

**Quantitative PCR**

Total cellular RNA was isolated using the Trizol reagent (Invitrogen), and miRNA from HRECs was isolated using the miRcute miRNA Isolation kit (Tiangen Biotech, Beijing, China). Complementary DNA (cDNA) from total RNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Lithuania). For the detection of mature miR-10b, RNA was reverse-transcribed using the miRcute miRNA first strand cDNA synthesis kit (Invitrogen). The mRNA levels of TIAM1 and NOX2 were determined using the QuantiTect...
SYBR Green PCR kit (Qiagen, Germany). The miRcute miRNA qPCR detection kit (Tiangen Biotech, Beijing, China) was used for the quantitative analysis of miR-10b. The relative expression levels of the target genes were quantified using the ΔΔCt method [13], with either U6 small nuclear RNA (U6 snRNA) or β-actin as an endogenous reference gene for quantification and normalization. Primer pairs used for amplification are listed in Table 1.

Table 1: Primer pairs used for quantitative PCR amplification

| Gene       | Primer pair                        |
|------------|------------------------------------|
| miR-10b    | 5'-GGATACCCCTGTAGAACCAGA-3' (forward) |
|            | 5'-GGGAACTGGGCTGTGAATGA-3' (forward) |
| TIAM1      | 5'-TCCCTGCTGTGCTCCTC-3' (forward)  |
|            | 5'-CTTCCTTGGTGTTGCTCCT-3' (reverse) |
| NOX2       | 5'-GGGAACTGGGCTGTGAATGA-3' (forward) |
|            | 5'-CCAGTGCTGACCCAAGAAGT-3' (reverse) |
| U6 small   | CTCGCTTCGGCAGCA                     |
| nuclear    | RNA                                |
| β-actin    | 5'-AGCCTCGCCTTGGGCATCGG-3' (forward) |
|            | 5'-TCTCTTGGGCTGCAGCTCGG-3' (reverse) |

Luciferase reporter assay

TargetScan (http://www.targetscan.org/) was used to predict the miR-10b-binding sites of target genes. Both wild-type (WT) and mutant (Mut) 3'-UTRs of TIAM1 were amplified by PCR, including the putative miR-10b-binding site, and cloned into the GV272 vector (JiKai Gene Medical Technology Co., Ltd, Shanghai, China). Then, 293T cells (1 × 10^5 cells/well) were cultured in 24-well plates and co-transfected with 0.1 μg firefly luciferase reporter, 0.02 μg renin luciferase (normalized), and 0.4 μg miRNA expression plasmid for 48 h. The cells were then lysed, and a dual luciferase reporter analysis system (Promega, Inc., Madison, WI, USA) was used for the luciferase activity assay. The firefly luciferase value was normalized to that of Renilla, and the ratio between them was calculated.

Determination of Rac1 activation

Ras-related C3 botulinum toxin substrate 1 (Rac1) activity assays were performed, as previously described [14]. A glutathione S-transferase-Rac/Cdc42-binding domain of Pak fusion protein was produced in bacteria and purified by binding to glutathione-coated Sepharose beads. A pull-down assay kit (Cytoskeleton, Denver, CO, USA) was utilized to determine the activation of Rac1. Activated Rac1 levels were quantified using western blotting.

Western blot analysis

HRECs were lysed on ice (Beyotime, Shanghai, China), and whole-protein lysates were separated by electrophoresis on a 12% SDS-polyacrylamide gel. The separated proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad, California, USA) and blocked in Tris-buffered saline/Tween 20 containing 5% skim milk for 2 h, followed by incubation with anti-TIAM1 (1:800, Santa Cruz, CA, USA) and anti-Rac1 (1:1,000, Transduction Laboratories, Lexington, KY, USA) antibodies overnight. Then, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz, California, USA) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence.

Statistical analysis

SPSS statistical software (version 17.0, SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Data are expressed as the mean ± SD. One-way analysis of variance was used for comparisons between multiple groups, followed by Tukey’s multiple comparison test. The level of statistical significance was set at p < 0.05.

RESULTS

HG induces the downregulation of miR-10b in HRECs

The viability of HRECs following HG treatment was visibly reduced compared with HREC viability after NG treatment (Figure 1 A, p < 0.01). Stimulation with HG significantly elevated ROS levels compared with stimulation with NG (Figure 1 B, p < 0.01). In Figure 1 C, the results show that HG induced more apoptosis of HRECs than NG (Figure 1 C, p < 0.01), whereas HG distinctly downregulated miR-10b levels (Figure 1 D, p < 0.01). HG induced the downregulation of miR-10b in HRECs.

Overexpression of miR-10b inhibits apoptosis and ROS production in HG-treated HRECs

Figure 2 A shows that the relative level of miR-10b was significantly increased compared with the control and miR-NC groups (p < 0.01). However, the level of miR-10b was distinctly decreased after HG treatment compared with...
that after NG treatment ($p < 0.01$, Figure 2 B). HG treatment repressed cell viability, whereas overexpression of miR-10b visibly increased cell viability (Figure 2 C, $p < 0.01$). HG treatment increased ROS production and cell apoptosis, but overexpression of miR-10b significantly suppressed ROS production and cell apoptosis (Figure 2 D and F, $p < 0.01$). Overexpression of miR-10b repressed apoptosis and ROS production in HG-treated HRECs.

**TIAM1 is a target of miR-10b in HRECs**

The TargetScan results revealed that TIAM1 had a putative 3′-UTR-binding site for miR-10b (Figure 3 A). A luciferase reporter experiment was performed to verify the target gene for miR-10b. The results revealed that the expression of miR-10b remarkably reduced the activity of the luciferase reporter gene for the WT gene ($p < 0.01$) compared miR-NC expression (Figure 3 B). Figure 3 C showed that miR-10b was upregulated in the miR-10b-overexpressing group compared with the miR-NC group ($< 0.01$), but the results showed a visible reduction in miR-10b after treatment with an miR-10b inhibitor compared with an miR-NC inhibitor ($p < 0.01$). In addition, the overexpression of miR-10b significantly reduced TIAM1 mRNA and protein levels compared with the miR-NC group, whereas the miR-10b inhibitor significantly elevated TIAM1 levels (Figure 3 D and E, $p < 0.01$). TIAM1 was a direct target of miR-10b in HRECs.
MiR-10b regulates NOX2 activation via the TIAM1-Rac1 axis in HG-treated HRECs

Figure 4 A and B shows that HG stimulation induced higher levels of TIAM1 and NOX2 than after NG treatment (< 0.01), but miR-10b overexpression inhibited TIAM1 and NOX2 after HG treatment compared with HG treatment of the miR-NC group (p < 0.01). After TIAM1 treatment, the inhibitory effects of miR-10b on TIAM1 and NOX2 were significantly reversed (p < 0.01). Figure 4 C shows that HG induced the activation of Rac1 compared with NG, whereas miR-10b overexpression suppressed the activation of Rac1. TIAM1 treatment also reversed the inhibitory effects of miR-10b on the activation of Rac1. MicroRNA-10b modulated NOX2 activation via the TIAM1-Rac1 axis in HG-treated HRECs.

DISCUSSION

The incidence of diabetes is increasing worldwide, and it is associated with a high risk of blindness [15]. DR, a serious complication of diabetes, is primarily caused by hyperglycemia, [5], severely affecting a patient’s quality of life. Therefore, it is critical to alleviate hyperglycemia for patients with DR.

In the current study, miR-10b was downregulated after HG stimulation, which decreased the viability of HRECs and increased ROS production. However, the overexpression of miR-10b inhibited apoptosis and ROS production in HG-treated HRECs. In addition, the overexpression of miR-10b significantly reduced TIAM1 and inhibited NOX2 via the TIAM1-Rac1 axis in HG-treated HRECs.

MiRNAs are key post-transcriptional regulatory factors that play pivotal roles in biological processes, but their functions appear to be inconsistent. A previous study showed that in esophageal squamous cell carcinoma, serum miR-10b levels were elevated, whereas levels of miR-29c and miR-205 were distinctly decreased [16]. In contrast, miR-543 was downregulated during osteogenic differentiation; its overexpression repressed osteogenic differentiation via directly modulating morphogenetic protein-2 [17]. Yu et al [6] found that miR-10b was decreased in cases of cervical cancer, whereas miR-10b overexpression suppressed the proliferation, migration, and invasion of cancer cells, and induced their apoptosis by targeting TIAM1. In the current study, miR-10b levels were reduced after HG stimulation, and miR-10b overexpression suppressed apoptosis and ROS production in HG-treated HRECs.

To elucidate the mechanism by which miR-10b inhibits apoptosis and ROS production, a luciferase reporter assay was performed. The results indicated miR-10b binding of the 3'-UTR region of TIAM1, indicating that TIAM1 is a target of miR-10b. In addition, miR-10b overexpression significantly reduced TIAM1 levels.

TIAM1, a guanine nucleotide exchange factor for Rac, is reportedly involved in many important cellular processes. It has been found to be overexpressed in certain malignant neoplasms and could thus be considered a novel supplementary biomarker for such cancers [18]. The activation of NOX2 and p38 MAP kinase, which is mediated by the TIAM1-Rac1 axis, was shown to contribute to mitochondrial dysfunction and the development of DR [19]. Further, in the early stages of diabetes, the activation of the TIAM1-Rac1-NOX2 axis led to increased intracellular ROS levels, resulting in mitochondrial damage and the progression of DR [20]. Moriarty [21] found that miR-10b inhibited TIAM1 in breast cancer cells by interacting with the 3'-UTR of TIAM1. Importantly, miR-10b suppressed breast cancer cell invasion and migration by inactivating Rac-stimulated TIAM1. In the current study, HG treatment increased the levels of TIAM1 and NOX2, but the overexpression of miR-10b significantly
suppressed TIAM1 and NOX2 by modulating the TIAM1-Rac1 axis.

**CONCLUSION**

The findings of this study show that MiR-10b alleviates HG-induced injury in HRECs by targeting TIAM1-Rac1 axis, suggesting the importance of the regulatory roles of miR-10b in this process. The results imply that miR-10b might be a therapeutic strategy for patients suffering from DR. Although this is the first report of the effects of miR-10b on HG-induced injury of HRECs, the regulation of TIAM1 signaling requires further study.

**DECLARATIONS**

**Conflict of interest**

No conflict of interest is associated with this work.

**Contribution of authors**

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims related to the content of this article are borne by the authors. Yaohua Chen designed the study, supervised the data collection, and analyzed the data; Yanqing Zhu interpreted the data and prepared the manuscript for publication; Sheng Zhao supervised the data collection, analyzed the data, and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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