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MiR17 improves insulin sensitivity through inhibiting expression of ASK1 and anti-inflammation of macrophages

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ABSTRACT

Objectives: MicroRNAs (miRNAs) are involved in the pathological progression of various disease including type 2 diabetes (T2D). Chronic inflammation in adipose tissue is a cause of insulin resistance and T2D. MiR-17 palys an anti-inflammatory role in many biological processes. We hypothesized that miR-17 suppressed inflammatory macrophage that is related to insulin resistance in patients with T2D.

Methods: Macrophage migration and secretion of inflammatory cytokines including TNF-α, IL-6 and IL-1β were detected through transwell migration assay and enzyme-linked immunosorbent assay, respectively. Insulin-stimulated glucose uptake was tested by the radioactivity of tritium-labeled glucose in 3T3-L1 adipocytes. Dual luciferase reporter gene assay was employed to evaluate the interaction between miR-17 and 3′UTR of ASK1.

Results: Our results showed that miR-17 inhibited macrophage infiltration and secretion of TNF-α, IL-6 and IL-1β. Moreover, insulin-stimulated glucose uptake of 3T3-L1 was suppressed by treatment with LPS-induced macrophage conditioned media (CM), whereas the opposite effect was showed after treatment with the CM of macrophages transfected with miR-17. Furthermore, we found that miR-17 directly prevented expression of ASK1 by binding to its 3′UTR.

Conclusion: miR-17 improved inflammation-induced insulin resistance by suppressing ASK1 expression in macrophages. These results indicated that miR-17 had an anti-diabetic acitivity by its anti-inflammation effect on macrophage.

1. Introduction

Type 2 diabetes (T2D) is characterized by chronic inflammation [1]. Adipose tissue macrophages (ATMs) promote chronic inflammatory responses by the release of inflammatory cytokines [2,3], which plays an important role in development of T2D. Macrophages are commonly polarized, which include M1 and M2 phenotypes [4]. Usually, local microenvironments are key regulators of transition between M1 and M2 phenotypes [5,6]. Activation of M1 macrophages secretes inflammatory cytokines and promotes pro-inflammatory responses. Alternatively, activation of M2 macrophages plays an anti-inflammatory role. The transition of macrophage polarization regulates the development of T2D. It has been reported that chronic inflammation was caused by an imbalanced M1/M2 ratio in adipose tissue of T2D patients [1].

Obesity-related insulin resistance causes and promotes diabetic pathophysiology [7]. Activation of M1 macrophages is associated with insulin resistance in obesity individuals [1,8,9]. At the beginning of T2D, macrophages infiltrated into adipose tissue and induced inflammatory response [10]. M1 macrophages secrete inflammatory cytokines including Interleukin-6 (IL-6), IL-1β and tumor necrosis factor (TNF)-α disturb insulin signaling, which then lead to insulin resistance [4,10]. Conversely, M2 macrophages promote insulin-dependent glucose uptake by producing anti-inflammatory cytokines [11].

MicroRNAs (miRNAs) are small noncoding RNAs (~19–25 nucleotides) that completely or partially paired with 3′untranslated region (3′UTR) of their targeting mRNA, and then lead to post-transcriptional gene repression by promoting mRNA degradation and/or inhibiting translation [12]. miRNAs were involved in various physiological and pathological process including obesity, insulin resistance, inflammation, diabetes and metabolic syndrome [13–15]. It has been reported that dysregulation of miRNAs in peripheral blood mononuclear cells (PBMC) regulates the development of diabetes [16,17]. MiR-17–92 is located in the C13orf25 (chromosome 13 open reading frame 25) that also was named locus of MIR17HG (miR-17–92 cluster host gene). The

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miR-17–92 cluster is transcribed from the same promoter. As a polycistronic primary transcript, it encodes six miRNAs including miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92 [18]. MiR-17–92 was found to be implicated in inflammatory response [18,19]. It has been reported that Inhibition of miR-17-5p inhibits the activation of macrophage in type 2 diabetes mellitus patients [20]. Moreover, miR-17-5p alleviates the suppressive function of myeloid-derived suppressor cells by directly blocking expression of STAT3 [21] that is a major regulatory pathway of macrophage activation. ASK1 also regulates activation of macrophage [22]. Although miR-17 downregulation activated ASK1 pathway and led to neural stem cell apoptosis [23], the connection between miR-17 and ASK1 in diabetic inflammation and macropahge activation is still unknown. In the current study, we discuss the possible effects of miR-17 in macrophages-associated inflammation that might lead to insulin resistance and T2D. Our data suggested that miR-17 might be a key linker between obesity-associated inflammation and insulin resistance, which provide new the biological mechanism of inflammation mediated T2D.

2. Material and methods

2.1. Animals

Twelve-week-old C57BL/6 mice were obtained Beijing Vital River (Beijing, China). Twelve-week-old ob/ob mice were obtained from Model Animal Research Center of Nanjing University. Fasting glucose levels were measured after starvation for 12 h. Fasting blood glucose levels > 250 mg/dl were considered diabetic mice that were selected for further study. Mouse Peritoneal macrophages were isolated and cultured as described previously [24]. All animal studies were approved by the Institutional Animal Care and Use Committee of Tianjin Nankai Hospital.

2.2. Cell culture

T3-L1 adipocytes cells (3T3-L1s) and mouse macrophage cell line (RAW264.7 macrophages) were attained from Shanghai Institute of Chinese Academy of Sciences (China). 3T3-L1s were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific Inc., USA) containing 10% calf serum (CS, Gibco, Thermo Fisher Scientific Inc., USA) under conditions of 5% CO_2, 37 °C in a humidity incubator. RAW264.7 cells were grown in DMEM (Gibco, Thermo Fisher Scientific Inc., USA) containing 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific Inc., USA) at 37°C in a humidity incubator within 5% CO_2. All culture media were supplemented with penicillin (100 U/mL, Sigma-Aldrich) and streptomycin (100 μg/mL, Sigma-Aldrich).

2.3. Plasmid construction and transfection

ShRNAs of ASK1 were purchased from Sigma Aldrich (St. Louis, Missouri, USA). RAW264.7 macrophages were seeded into six-well plates. At 70–80% confluence, cells were transfected with pre-miRNAs of miR-17 (miR-17, 100 nM) or NC–pre-miRNAs (miR-NC, 100 nM) or miR-17 inhibitor (anti–miR-17, 150 nM) or inhibitor NC (anti-NC, 150 nM) using Lipofectamine RNAiMAX transfection reagent (Life Technologies) for 48 h and then incubated with LPS (200 ng/mL) for 24 h. MiR-17, miR-NC, anti–miR-17 and anti-NC were obtained from Life Technologies, Inc. (Gaithersburg, MD, UAS). For ASK1 overexpression, ASK1 cDNA without 3′-UTR were cloned into pCDNA3 (Addgene, USA). An empty vector (Vector) was used as a negative control. Plasmids of shRNA or pCDNA3-ASK1 were transfected using Lipofectamine2000 (Invitrogen, USA) according to the manufacturer's description. All constructions were confirmed by plasmid DNA sequencing.

2.4. Dual luciferase assays

A wild-type 3′-UTR fragment of ASK1 cDNA (WT) was amplified by PCR and cloned into pmirGLO dual-luciferase vector (Promega, Madison, WI, USA). The mutant variant of ASK1 3′-UTR (MUT) was generated based on WT by mutating eight nucleotides that potentially bind to miR-17. RAW264.7 macrophages were seeded into a 24-well plate at 2×10^4 per well. At 80–90% confluence, cells were transiently co-transfected with vectors (WT or Mut) and pre-miRNAs (100 nM) of miR-17 or NC–pre-miRNAs. After transfection for 48h, luciferase activity was assayed using Dual-Luciferase® Reporter 1000 Assay System (Promega, Madison, USA). Each experiment was repeated in triplicate.

2.5. RNA extraction and real-time PCR (qPCR)

Total RNAs were extracted from cells and tissues using Trizol (Invitrogen), and then were reverse-transcribed into cDNA by SuperScript First Strand cDNA Synthesis Kit (Life Technologies) under the manufacturer's instructions. Expression of miR-17 was quantified using TaqMan microRNA Assays with U6snRNA as control (Life Technologies, Inc. Gaithersburg, MD, UAS). Expression of ASK1 mRNA was quantified by qPCR using 2× TransStar™ Green qPCR superMix (Beijing TransGen Biotech Co., China), and U6snRNA (Life Technologies, Inc. Gaithersburg, MD, UAS) was used as control. Quantification of relative expression was done using the ΔΔCt method. The primer of mouse ASK1 [25] was forward 5′- CCCTGGAGACCCTG CATTT -3′ and reverse 5′- CATCTCCACCACAGCAATATCTG-3′.

2.6. Protein extraction and western blot

Total proteins were extracted using a RIPA lysis buffer supplemented with complete protease inhibitor (Beyotime Biotech Inc., China). Protein concentrations were quantified using the BCA Protein Assay kit (Applygen, Beijing, China). 40 μg of proteins were loaded on SDS-page gel and electrophoresed, and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat milk for 1 h. Specific proteins were detected using primary antibodies: rabbit anti-ASK1 (1:500, Santa Cruz, USA) and rabbit anti-β-actin (1:1000, Santa Cruz, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies, immunoreactive bands were visualized using enhanced chemiluminescence detection reagent (Thermo Scientific, USA). The images were captured by a ChemoDoc XRS detection system (Bio-Rad, Milan, Italy), and analyzed by the ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA). β-Actin is used as a loading control.

2.7. Transwell assay

Transwell assay were used to detect macrophage migration. After transfection, 5×10^4 cells were seeded into the upper chamber (BD Bioscience, USA) with 100 μL serum-free medium, while the lower chambers were filled with 800μL serum-free media containing different concentrations of LPS 0, 200 ng/mL). After incubation for 6 h, the cells inside the upper chamber were removed by cotton swabs. Migrated cells in outside were fixed in 4% paraformaldehyde and stained with crystal violet, and counted (six random 100 × fields per well). Three independent experiments were performed.

2.8. Macrophages conditioned media (CM) preparation and inflammatory factors detection

CM was prepared as described previously [26]. After transfection, 70–80% confluence of cells in 10 cm dish were starved with serum-free media with or without LPS (200 ng/mL) for 24 h. Then the cells were incubated in serum-free medium for another 24 h. The cell-cultured conditioned media were collected, and were defined as macrophages