Original Article

miR-146a regulates inflammatory cytokines and reverses high-glucose- and high-insulin-induced insulin resistance in 3T3-L1 adipocytes by targeting Traf6 through the NF-κB signaling pathway

Shu-Ping Liao, Wei-Qing Wu, Jun Zeng, Yan Wu

Department of Endocrinology Management, The Second Medical College of Jinan University, Shenzhen People's Hospital, Shenzhen 518020, Guangdong, China

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Abstract: Insulin resistance (IR) is a common feature of type 2 diabetes mellitus. Therefore, the molecular mechanism of IR in adipocytes is becoming a focal point of diabetes research. Emerging evidence suggests that miR-146a might be associated with diabetes and its related complications. However, the role of miR-146a in the development of IR remains unclear. In this study, we found that the expression of miR-146a was significantly inhibited in IR-3T3-L1 adipocytes compared with that in normal 3T3-L1 adipocytes, while that of Traf6 was significantly promoted. The overexpression of miR-146a significantly inhibited the expression of Traf6, silenced the NF-κB signaling pathway, reduced the secretion of inflammatory cytokines, and increased glucose uptake in IR-3T3-L1 adipocytes. Traf6 overexpression had no effect on the expression of miR-146a, but partially alleviated the effect of miR-146a in adipocytes transfected with miR-146a mimic. In conclusion, we demonstrated that miR-146a silenced the NF-κB signaling pathway, decreased the secretion of inflammatory cytokines, and improved glucose uptake in IR-3T3-L1 adipocytes by targeting Traf6. The findings provide information regarding a potential new therapeutic strategy to control IR in adipocytes.

Keywords: miR-146a, inflammatory cytokines, insulin resistance, adipocytes, Traf6, NF-κB

Introduction

Type 2 diabetes mellitus (T2DM), which leads to a high concentration of glucose in the blood, has harmful long-term effects on several body organs, especially the kidneys, eyes, heart, and nerves, and represents a major global public health problem [1]. Insulin resistance (IR) is a common feature of T2DM [2]. Recent research has suggested that dysfunctional adipocytes represent a key link between obesity and IR [3]. Therefore, the molecular mechanism of IR in adipocytes is becoming a focal point of research on T2DM. MicroRNAs (miRNAs or miRs) are a novel class of highly-conserved small non-coding RNAs that degrade their target mRNA sequences by binding to the mRNA’s 3’ untranslated region [4]. Evidence from recent studies has suggested that miRNAs regulate insulin secretion [5], sugar and lipid metabolism [6], and pancreatic β-cell development [7]. Thus, certain miRNAs might have applications in the prevention and treatment of T2DM and its related complications. It has been demonstrated that some miRNAs such as miR-21 and miR-320 regulate the development of IR [8, 9].

Several studies have provided evidence suggesting that miR-146a was associated with T2DM and its related complications by regulating the expression of interleukin-1 receptor-associated kinase-1 (Iрак1) and tumor necrosis factor receptor associated factor 6 (Traf6), and reduced the expression of Il-6, TNF-α, and IL-1β [10-12]. However, the role of miR-146a in the development of IR remains unclear. In this study, we investigated the expression and effect of miR-146a in 3T3-L1 adipocytes with induced IR (IR-3T3-L1 adipocytes). The results showed that the expression of miR-146a was...
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significantly inhibited in IR-3T3-L1 adipocytes as compared with that in normal 3T3-L1 adipocytes. The overexpression of miR-146a significantly inhibited the activity of the NF-κB signaling pathway, reduced the secretion of inflammatory cytokines, and increased glucose uptake by inhibiting the expression of Traf6. In conclusion, we demonstrate that miR-146a improved glucose uptake in IR-3T3-L1 adipocytes by targeting Traf6. The findings provide information regarding a potential new therapeutic strategy to control IR.

Materials and methods

Cell culture and establishment of an IR model

3T3-L1 pre-adipocytes (ATCC, Manassas, VA, USA) were cultured and induced to differentiate into mature adipocytes, as described previously [13]. Briefly, 3T3-L1 pre-adipocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco®, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco®) in a 5% CO₂ atmosphere at 37°C. For the induction of differentiation into mature adipocytes, 3T3-L1 pre-adipocytes were collected and cultured in differentiation media supplemented with 0.5 mmol/L 3-isobutyl-1-methylxantine, 10 mg/mL insulin, and 1 μmol/L dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). At 2 days post-culture, the cells were cultured in DMEM media supplemented with 10% FBS and 10 mg/mL insulin, and replenished every other day with fresh media and insulin until day 9, when the cells were fully differentiated into adipocytes. To establish an IR model, 3T3-L1 adipocytes were cultured for 24 h at 37°C in DMEM supplemented with 10% FBS, 1 μmol/L insulin, and 0, 5, or 25 mmol/L glucose. As a control, 3T3-L1 adipocytes were preincubated for 24 h at 37°C in DMEM supplemented with 10% FBS, 1 mmol/L insulin, and 0, 5, or 25 mmol/L glucose.

2 Deoxyglucose transport assay

2-Deoxyglucose (2DG) transport was analyzed using a 2DG uptake measurement kit (Cosmo Bio, Tokyo, Japan), according to the manufacturer’s instructions. Adipocytes in six-well plates were incubated in serum-free medium for 6 h and washed three times with Krebs-Ringer phosphate buffer (pH 7.4). Insulin was added to a final concentration of 1 μM and the cells were incubated at 37°C for 30 min at 37°C. 2DG was then added to a final concentration of 1 mM and the cells were incubated at 37°C for 20 min. The medium was then removed and the cells were gently washed three times with cooled phosphate-buffered saline containing 200 μM phloretin. Next, 3 mL 10 mM Tris-HCl buffer (pH 8.0) was added to each well and the adipocytes were disrupted using a microtip sonicator. The cell lysates were collected into fresh sterile plastic tubes and heat treatment was applied at 80°C for 15 min. The lysates were then centrifuged at 4°C and 15,000 × g for 20 min and each supernatant was transferred into a new tube. An aliquot of each supernatant was diluted ≥ 1:4 with 1 × sample diluent buffer and the optical density of each well at 420 nm was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

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

Total RNA was extracted from 3T3-L1 adipocytes, using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). For the quantification of miR-146a, total RNA was reverse transcribed to cDNA using an miRcute miRNA first-strand cDNA synthesis kit (TIANGEN Biotech, Beijing, China) and miR-146a expression was measured using an miRcute miRNA qPCR detection kit (SYBR® Green, TIANGEN). U6 served as the internal reference. For the quantification of Traf6, total RNA was reverse transcribed into cDNA using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio, Ōtsu, Japan). qPCR was performed using Power SYBR® Green PCR master mix (Applied Biosystems, Foster City, CA, USA). β-actin was used as an internal control. qPCR was performed on a 7500 real-time PCR system (Applied Biosystems). Gene expression was measured in triplicate, quantified using the 2ΔΔCT method, and normalized to an internal control. For miR-146a, the forward and reverse primers were 5‘-GGCGTTAGAACTGAATTCCA-3’ and 5‘-GTTACAGGTCCAGAATGCT-3’, respectively. For U6, the forward and reverse primers were 5‘-CTCGCTTCGGCAGAGGA-3’ and 5‘-AACGCTTCAGAATTGCG-3’, respectively. For Traf6, the forward and reverse primers were 5‘-TACTTACAGAAGACAGATCG-3’ and 5‘-AAAGTACTGAATGTCATGG-3’, respectively. For β-actin, the forward and reverse primers were 5‘-CTCCTTCGGGAGCGACACAGCATG-3’ and 5‘-CTGCATAGTCTCAGGTGTTG-3’, respectively.
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were 5′-TGCCACCTCCAGAGATGT-3′ and 5′-AGCTCACGACGAGCACGTTA-3′, respectively.

miR-146a mimic construction, Traf6-pcDNA3.0 construction and transfection

A miR-146a mimic (mimic, 5′-UGAGAACUGAAUUCAGGGGU-3′) and a negative control (NC, 5′-UUCCUCCAGUGUGACGUTT-3′) were synthesized by RiboBio (Guangzhou, Guangdong, China). The full coding sequence of Traf6 (NM_145803) was synthesized by GENEWIZ (Suzhou, Jiangsu, China) and inserted into the pcDNA3.0 vector (Traf6-pcDNA3.0). For transient transfection, IR-3T3-L1 adipocytes were plated at 50% confluence and transfected with 200 nM miR-146a mimic or NC, using Lipofectamine® RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s protocol. IR-3T3-L1 adipocytes were then transfected with miR-146a mimic and plated at 50% confluence and transfected with 200 nM Traf6-pcDNA3.0 or pcDNA3.0 using Lipofectamine® 3000 transfection reagent (Invitrogen). Cells were harvested 24 or 48 h after transfection for further analysis.

Western blotting

3T3-L1 adipocytes were washed twice with ice-cold phosphate-buffered saline and lysed in ice-cold radioimmunoprecipitation assay buffer containing 1 mmol/L phenylmethanesulfonyl fluoride and a cocktail of protease inhibitors (1:100 dilution; Beyotime, Nantong, China). Cell lysates were centrifuged at 4°C for 15 min at 13,000 × g. The protein content of each supernatant was quantified using a bicinchoninic acid protein assay kit (Beyotime). Samples containing 30 μg total protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Pall Corporation, Port Washington, NY, USA). The membranes were blocked at 37°C for 1 h in a buffer containing 0.1% Tween® 20 and 5% milk. For the detection of TRAF6, the membranes were incubated with antibodies against TRAF6 (1:1000; Abcam, Cambridge, UK) and then washed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000 dilution) for 1 h at room temperature. Finally, the membranes were visualized by the enhanced chemiluminescence method and a densitometric analysis was performed using Image Pro-Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). GAPDH served as a reference protein.

Inflammatory cytokine analysis

At 48 h post-treatment, the concentrations of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α secreted by the IR-3T3-L1 adipocytes were analyzed using a Bio-Plex Human cytokine group I 23-plex assay (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer’s instructions.

Statistical analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used to perform all statistical analyses. Continuous variables are presented as the mean ± standard deviation. The significance of differences among multiple groups was analyzed by one-way analysis of variance (ANOVA) followed by a post-hoc least significant difference (LSD) test. The significance of differences between two groups was analyzed by the unpaired t test. P values <0.05 were considered statistically significant.

Results

miR-146a expression was inhibited in IR-3T3-L1 adipocytes

To evaluate whether the IR-3T3-L1 adipocyte model was successfully established, insulin sensitivity of the cells was measured by the 2DG transport assay (Figure 1A). Under the same glucose concentration, addition of 1 μmol/L insulin significantly increased glucose transport of 3T3-L1 adipocytes compared with addition of 1 nmol/L insulin (P<0.05). In the presence of 1 μmol/L insulin, addition of 25 mmol/L glucose led to a significant decrease in the rate of glucose transport in 3T3-L1 adipocytes compared with the addition of 0 or 5 mmol/L glucose (P<0.05). The results indicated that high concentrations of glucose (25 mmol/L) and insulin (1 μmol/L) resulted in the development of IR in the 3T3-L1 adipocytes and suggested that the IR-3T3-L1 adipocyte model was successfully established. The expression of miR-146a showed a trend similar to that of glucose transport (Figure 1B). Under the same glucose concentration, addition of 1 μmol/L insulin led to a significant increase in
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**Figure 1.** 2-Deoxyglucose (2DG) transport (A) and miR-146a expression (B) in 3T3-L1 adipocytes were analyzed using a 2DG uptake measurement kit and by quantitative real-time polymerase chain reaction (qRT-PCR), respectively.

**Figure 2.** miR-146a expression (A) and 2DG transport (B) were analyzed by qRT-PCR and using 2-DG uptake measurement kit, respectively, at 48 h post-transfection in IR-3T3-L1 adipocytes that had been transfected with an miR-146a mimic.

**Figure 3.** Effects of miR-146a on the NF-κB signaling pathway and the secretion of inflammatory cytokines in IR-3T3-L1 adipocytes. (A) miR-146a overexpression significantly reduced secretion of IL-1β, IL-6, and TNF-α in IR-3T3-L1 adipocytes. (B) miR-146a overexpression significantly inhibited expression of NF-κB p65 and p-IκBα, while it promoted expression of IκBα in IR-3T3-L1 adipocytes.
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The expression of miR-146a as compared with the addition of 1 nmol/L insulin ($P<0.05$). In the presence of 1 μmol/L insulin, addition of 25 mmol/L glucose led to a significant decrease in the expression of miR-146a as compared with the addition of 0 or 5 mmol/L glucose ($P<0.05$). The results suggest that expression of miR-146a was inhibited in IR-3T3-L1 adipocytes as compared with that in 3T3-L1 adipocytes.

Transfection of miR-146a mimic into IR-3T3-L1 adipocytes upregulated miR-146a expression and increased glucose uptake

To assess the biological function of miR-146a in IR-3T3-L1 adipocytes, the miR-146a mimic or NC was transiently transfected into IR-3T3-L1 adipocytes. miR-146a expression and glucose uptake were significantly increased in IR-3T3-L1 adipocytes transfected with the miR-146a mimic compared with in those cells transfected with the NC at 48 h post-transfection ($P<0.05$, Figure 2A and 2B).

miR-146a overexpression inhibited the secretion of inflammatory cytokines by IR-3T3-L1 adipocytes

We assessed the effects of miR-146a on the activity of the NF-κB signaling pathway and the secretion of inflammatory cytokines. We found that miR-146a overexpression in IR-3T3-L1 adipocytes significantly reduced the secretion of IL-1β, IL-6, and TNF-α (Figure 3A), inhibited the expression of NF-κB p65 and p-IκBα, and promoted the expression of IκBα, silencing the NF-κB signaling pathway (Figure 3B).
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miR-146a regulate Traf6 expression in IR-3T3-L1 adipocytes

To demonstrate the association between Traf6 and miR-146a in IR-3T3-L1 adipocytes, the expression of Traf6 was detected in IR-3T3-L1 adipocytes treated with 1 μmol/L insulin plus 0, 5, or 25 mmol/L glucose. The results are shown in Figure 4A and 4B. In the presence of 1 μmol/L insulin, the addition of 25 mmol/L glucose led to a significant increase in the expression of Traf6 as compared with the addition of 0 or 5 mmol/L glucose (P<0.05). The results suggest that the expression of Traf6 was promoted in IR-3T3-L1 adipocytes. Additionally, the cells transfected with the miR-146a mimic exhibited a significant reduction in their expression of Traf6 (Figure 4C and 4D). These data indicated that Traf6 is a potential target of miR-146a in IR-3T3-L1 adipocytes.

Overexpression of Traf6 partially reversed the effect of miR-146a on glucose uptake in IR-3T3-L1 adipocytes transfected with a miR-146a mimic

To overexpress Traf6, the Traf6-pcDNA3.0 vector was constructed and transfected into IR-3T3-L1 adipocytes that had also been transfected with a miR-146a mimic. The cells were then harvested for qRT-PCR and Western blot analysis. The results showed that the transfection of the cells with the Traf6-pcDNA3.0 vector significantly promoted their expression of Traf6.
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(Figure 5A and 5B) but had no effect on their expression of miR-146a (Figure 5C) as compared with the transfection of NC-pcDNA3.0 into miR-146a mimic-transfected IR-3T3-L1 adipocytes. The results also showed that the transfection of the Traf6-pcDNA3.0 vector into miR-146a mimic-transfected IR-3T3-L1 adipocytes significantly inhibited glucose uptake as compared with the transfection of NC-pcDNA3.0 into the same cells (Figure 5D).

Overexpression of Traf6 partially reversed the effect of miR-146a on the NF-κB signaling pathway and inflammatory cytokines in IR-3T3-L1 adipocytes transfected with a miR-146a mimic

Finally, we assessed the effects of miR-146a and Traf6 on the activity of the NF-κB signaling pathway and the secretion of inflammatory cytokines. We found that Traf6 overexpression promoted the secretion of IL-1β, IL-6, and TNF-α, enhanced the expression of NF-κB p65 and p-IκBα, and inhibited the expression of IκBα, silencing the NF-κB signaling pathway in IR-3T3-L1 adipocytes transfected with a miR-146a mimic (Figure 6A and 6B). Overexpression of Traf6 partially reversed the effect of miR-146a on NF-κB signaling pathway and inflammatory cytokines in IR-3T3-L1 adipocytes.

Discussion

IR is defined as a reduction in the responsiveness of cells or tissues to insulin. IR mainly occurs in adipose tissue, liver, and skeletal muscle, and the cells comprising those tissues abundantly express insulin receptors on their outer membranes. IR mainly manifests as a decrease in glucose uptake by muscle and adipose tissue alongside a reduction in glucose output by the liver. In the present study, we investigated the role of miR-146a in the development of IR in 3T3-L1 adipocytes. This study showed that miR-146a expression was downregulated and Traf6 expression was upregulated in IR-3T3-L1 adipocytes as compared with that in 3T3-L1 adipocytes. Additionally, overexpression of miR-146a significantly promoted glucose uptake in IR-3T3-L1 adipocytes, while overexpression of Traf6 had no effect on the expression of miR-146a but significantly inhibited glucose uptake in miR-146a mimic-transfected IR-3T3-L1 adipocytes. These results showed that Traf6 overexpression partially alleviated the effect of miR-146a on glucose uptake, suggesting that miR-146a promotes glucose uptake by regulating Traf6. Previous studies have reported that miR-146a can regulate disease progression by targeting Traf6 [14, 15], which is concordant with the results of the present study.

Inflammatory factors such as C-reactive protein, TNF-α, and IL-6 have been associated with the development of T2DM [16-18]. Inflammatory factors play important roles in the diagnosis, prevention, and treatment of T2DM [19, 20] and the concept of regulating such factors has provided new ideas for the development of new drugs for treating T2DM. Previous studies have...
found that decreased expression of miR-146a promotes the expression of inflammatory factors, contributing to disease progression in chronic obstructive pulmonary disease [21] and lupus nephritis [22]. In this study, we found that miR-146a overexpression significantly reduced the secretion of IL-1β, IL-6, and TNF-α by IR-3T3-L1 adipocytes, while upregulation of Traf6 expression partially promoted their secretion in miR-146a mimic-transfected IR-3T3-L1 adipocytes. These results showed that Traf6 overexpression partially alleviated the inhibitory effect of miR-146a on the secretion of IL-1β, IL-6, and TNF-α by regulating Traf6 in lupus nephritis [22].

NF-κB is involved in inflammation, apoptosis, and the immune response [23, 24]. Silencing of NF-κB has been reported to protect adipocytes from inflammation and IR-induced injury, and it would be helpful to understand the regulatory mechanisms of NF-κB signaling in IR-3T3-L1 adipocytes to elucidate the pathogenesis of insulin resistance and provide potential new therapeutic targets [25, 26]. In this study, we found that miR-146a overexpression significantly inhibited expression of NF-κB p65 and p-IκBα, while it promoted expression of IκBα in IR-3T3-L1 adipocytes. Experimental overexpression of Traf6 partially promoted expression of NF-κB p65 and p-IκBα, while it inhibited expression of IκBα in miR-146a mimic-transfected IR-3T3-L1 adipocytes. These results showed that Traf6 overexpression partially alleviated the inhibitory effect of miR-146a on the NF-κB signaling pathway, suggesting that miR-146a silenced the NF-κB signaling pathway by regulating Traf6, which corresponds with the results of previous studies in ischemia/reperfusion injury [27] and osteoarthritis-derived chondrocytes [28].

In conclusion, this study demonstrates that miR-146a overexpression can silence the NF-κB signaling pathway, inhibit secretion of inflammatory factors, and promote glucose uptake by targeting Traf6. Based on the results of the present study, miR-146a could represent a potential new therapeutic target for controlling IR.

Disclosure of conflict of interest

None.

Address correspondence to: Yan Wu, Department of Endocrinology Management, the Second Medical College of Jinan University, Shenzhen People’s Hospital, 1017 Dongmen Bei Road, Luohu District, Shenzhen 518020, Guangdong, China. Tel: +86-13802230166; E-mail: wyan_2017@126.com

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