RESISTIN INHIBITS GLUCOSE-STIMULATED INSULIN SECRETION THROUGH MIR-494 BY TARGET ON STXBP5

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Abstract

Aims. Resistin has been reported to impair the pancreatic beta cells and associated with insulin resistance. MicroRNAs (miRNAs) are short, endogenously produced non-coding ribonucleotides that bind mRNAs and function mainly as negative regulators in mammals. MiRNAs have been implicated in many diseases, including insulin resistance and diabetes. A considerable body of evidence has indicated an important function for miRNAs in insulin secretion. The current study was designed to investigate the effects of miR-494 in the reductions in insulin secretion attributable to resistin.

Methods. Insulin secretion was determined by ELISA, and expressions of genes were identified using quantitative RT-PCR (qRT-PCR) or Western blot analysis.

Results. Insulin secretion was significantly reduced by resistin. Overexpression of miR-494 inhibited insulin secretion both in diet culture and high glucose medium in MIN6 cell lines. MiR-494 down-regulated the protein level of STXBP5 by pairing with sites in the 3'UTR.

Conclusion. miR-494 is involved in the insulin secretion regulated by resistin via its effects on STXBP5 in MIN6 cells.

Key words: resistin, insulin secretion, miR-494, STXBP5.

INTRODUCTION

Diabetes is a metabolic syndrome characterized by hyperglycemia company with lipid and protein metabolism disorders. It is induced by insufficient insulin secretion or insulin resistance (1, 2). Type 2 diabetes mellitus (T2DM) is increasingly common worldwide (3) and correlated with age (4), obesity (5, 6), diet (7), and other factors, and has a strong heritable character (8). Insulin is the critical factor in the development of diabetes, and a complete understanding of the

pathophysiological cause of diabetes can provide novel methods for successful prevention and treatment.

Resistin is considered the link between obesity and both insulin resistance and diabetes because of its role in the development of insulin resistance (9). It is an adipocyte-derived cytokine first described in 2001 (10). Resistin is also expressed in islets and up-regulated in insulin resistance (11). The number of human pancreatic islet cells expressing resistin was significantly higher in patients of T2DM (12). Increasing amounts of evidence suggest that resistin may impair the function of pancreatic β -cells and so affect insulin secretion (13, 14).

MiRNAs are a negative factor, and they regulate the expression of target genes through pairing with the seed sites in the 3' untranslated region (3'-UTR) of mRNA (15). Increasing amounts of evidence suggest that miRNAs may play an important role in many physiological and pathological processes, and dysfunction of miRNAs and their target genes can lead to a variety of disorders, particularly insulin resistance and metabolic syndrome (16–19). Identification of glucose-regulated miRNAs from pancreatic β -cells has indicated that 61 glucose-regulated miRNAs from a total of 108 miRNAs detectable in MIN6 cells and miR-494 were up-regulated in the presence of high concentrations of glucose (20). In a previous study, we found miR-494 to be down-regulated in the liver treated with resistin (21). These data suggests that miR-494 correlate with the function of pancreatic β -cells. In this study, we aimed to investigate the effect of miR-494 on resistin-induced inhibition of insulin secretion.

MATERIALS AND METHODS

Materials

TRIzol reagent was purchased from TaKaRa (Dalian, China), Lipofectamine™ 2000 was from

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Invitrogen (Carlsbad, CA, U.S.). Recombinant mouse resistins were purchased from PeproTech, Inc. (Rochy Hill, NJ, U.S.). MiRNA mimics and inhibitors were purchased from GenePharma (Shanghai, China). A miRcute miRNA isolation kit was obtained from Tiangen Biotech Co. Ltd. (Beijing, China). STXBP5 antibody was purchased from PeproTech, Inc. (Rochy Hill, NJ, U.S.) and β -actin was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.).

Cell culture and treatment

MIN6 cells were maintained in DMEM medium (HyClone, Thermo Scientific, U.S.) supplemented with 20% fetal bovine serum (FBS) (Gibco, Rockville, MD, U.S.) containing 100 U/mL penicillin and 100 μ g/mL streptomycin. The cells were grown at 37°C in a humidified chamber containing 5% CO₂.

RNA isolation

Total RNA was extracted from MIN6 cells using a Tiangen miRcute miRNA isolation kit in accordance with the manufacturer's instructions. RNA quality was assessed using UV-vis spectrophotometry (SMA4000). RNA was generally of high quality (average 260/280 ratio of 2.02, and an average 260/230 ratio of 1.79).

Real-time PCR (qRT-PCR)

Total RNA extracted from MIN6 cells was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega) and gene-specific stem-loop primers or oligo (dT18). The sequences of the primers used are listed in Table 1. Real-time PCR was performed using SYBR Green PCR Mix (ABI, CA, U.S.) with a CFX96TM Real-time PCR System (Bio-Rad). PCR cycles were as follows: initial denaturation

at 95°C for 5 min followed by 35 cycles of 95°C for 15 s and 60°C for 30 s. The average threshold (Ct) was determined for each gene and normalized to 36B4 mRNA or snRu6 miRNA as internal normalization controls. Fold changes in relative expression were calculated using the 2- $\Delta\Delta$ Ct method.

Transfection

Except where otherwise indicated, all experiments were performed in triplicates, in three independent experiments. All cells were grown and maintained in DMEM supplemented with 20% FBS. The miRNA mimics and inhibitors were transfected into cells in serum-free medium; after 6 h, media were replaced with DMEM with 20% FBS. Cells and culture medium were harvested 24 h after transfection to isolate RNA or 48 h to extract protein.

Enzyme-linked immunosorbent assay (ELISA)

The insulin secreted to culture medium was measured by ELISA using test kits (Huamei Biotech, China) following manual instruction. Collected medium samples were centrifuged at 1,500 g for 10 min. Supernatants were transferred into 96-well plates, along with blank control and standard samples. After incubation at 37°C for 30 min, supernatants were discarded and rinsed 5 times with washing buffer. Enzyme-linked reagents were added into each well except the blank control for further incubation. Reagents A and B were then added, mixed, and incubated in the dark at 37°C for 15 min. Color development was stopped by addition of quenching buffer. A microplate reader was used to measure optical density (OD) values of each well at 450 nm. A standard curve was first plotted using serially diluted standard samples. Sample concentration was then deduced from the regression function.

Western blotting

Cells were harvested and lysed in RIPA lysis buffer (Beyotime, P0013C) with or without phosSTOP phosphatase inhibitor cocktail tablets (Roche Applied Science). Protein concentrations were determined using a BCA kit (Beyotime). Protein samples were separated by electrophoresis on 10% or 12% SDS-polyacrylamide gels and then transferred to PVDF membranes (Bio-Rad). Membranes were blocked with 5% non-fat dry milk in TBS-Tween. Target proteins were detected using specific polyclonal antibodies; β -actin served as an internal control.

Table 1. Primers for real-time RT-PCR

Gene name	Sequences (5'-3')
Mus Stxbp5	F: CGGTGCACAATCTCTCGAT R: CACCTTCGATCCCACCAG
Mus Vamp2	F: TGCACCTCCTCCAAACCTTA R: AGCTCCGACAACCTCTGGTC
Mus Stxbp1	F: ATATCAACAAGCGCCGAGAG R: ATGGGGTGATGAGGTACACG
Mus Stx1a	F: AAGAAGGCCGTCAAGTACCA R: CCAGAATCACACAGCAAATGA
Mus Snap25	F: GGCTCCTCCACTCTTGCTAC R: CAGCAAGTCAGTGGTGCTTC
Mus 36B4	F: TCGCTTTCTGGAGGGTGTC R: CAGATGGATCAGCCAGGAAGG

Luciferase assays

Cells were seeded into 24 well plates and allowed to grow overnight. Cells were then incubated in serum-free DMEM for 6 h before transfection. Recombinant vectors or empty vector (pGL3-Basic) were co-transfected into the cells using Lipofectamine™ 2000 (Invitrogen) in accordance with the manufacturer's protocol. pGL3-Basic vector, containing the firefly luciferase reporter was used for normalization. After 24 h, firefly activity and Renilla luciferase were measured separately using the Dual Luciferase Assay. Three independent experiments were performed in triplicate.

Statistical analysis

Data are presented as means $\pm$ standard deviation (SD). Statistical analyses were performed using unpaired two-tailed t-test (for two groups) and analysis of variance (ANOVA; for multiple groups). P-values <0.05 were considered statistically significant.

RESULTS

Resistin Inhibits Glucose-stimulated Insulin Secretion in MIN6 Cells

To confirm that resistin decreases the secretion of insulin, islet β -cells MIN6 were pretreated with various concentrations of recombinant resistin for 24

hours and cultured in high-glucose culture medium (25 mM glucose). The insulin concentration in the culture medium was measured. Results showed that, under high-glucose conditions, insulin secretion by MIN6 cells was inversely dependent on the concentration of resistin. The concentration of insulin decreased gradually as that of resistin increased. The concentration of insulin declined significantly when resistin reached a concentration of 50 ng/mL and 100 ng/mL (Fig. 1A). For these reasons, 50 ng/mL resistin was chosen for the following experiment.

To further confirm that resistin significantly impaired the glucose-induced secretion of insulin from MIN6 cells, insulin secretion was assessed in the presence of resistin under low-glucose (5.5 mM) and high-glucose (25 mM) condition. The results showed that resistin significantly inhibited glucose-induced insulin secretion in MIN6 cells under both sets of conditions (Fig. 1B).

MiR-494 Overexpression Inhibits Insulin Secretion

To ascertain whether miR-494 is correlated with insulin secretion regulated by resistin, the expression of miR-494 was detected in MIN6 cells after resistin treatment. Results showed miR-494 to be significantly upregulated by resistin in MIN6 cells and that miR-494 inhibitor reversed the effect of resistin (Fig. 2A).

Figure 1. Resistin Inhibits Glucose-stimulated Insulin Secretion in MIN6 Cells. The insulin concentration in the culture medium which treated with or without resistin was measured, and insulin secretion was assessed in the presence of resistin under low-glucose (5.5 mM) and high-glucose (25 mM) condition respectively. (A) MIN6 cells were treated with resistin at doses of 25, 50, and 100 ng/mL for 24 h. Cellular insulin secretion was measured by ELISA assay. (B) MIN6 cells were treated with 50 ng/mL resistin and incubated with either 5.5 mM or 25 mM glucose medium for 24 h. Insulin secretion was measured by ELISA assay. Data are here presented as means $\pm$ SD of percentage of controls. (*, $P<0.05$, significant; **, $P<0.01$, very significant; ns, not significant).

Next, the role of miR-494 in glucose-induced insulin secretion was studied. miR-494 mimics were transfected into MIN6 cells at final concentrations of 100 nM, 150 nM, and 200 nM, and MIN6 cells were subsequently cultured in low-glucose and high-glucose medium. Insulin content was measured 24 hours later. Results showed insulin secretion to be dependent on miR-494 concentration in both low-glucose and high-glucose culture medium. As the concentration of miR-494 increased, cellular insulin secretion was decreased. Insulin secretion was significantly reduced in the presence of 100 nM miR-494 (Fig. 2B).

In order to determine whether resistin regulates insulin secretion through miR-494, cells transfected with miR-494 inhibitor were treated with resistin and glucose-stimulated insulin secretion was measured. Results showed that miR-494 inhibitor reversed the reduction of glucose-stimulated insulin secretion by resistin in MIN6 cells effectively (Fig. 2D). This suggested that miR-494 was involved in the insulin secretion process that had been suppressed by resistin in islet β cells.

The effect of miR-494 on exocytosis of insulin was studied to identify the regulatory mechanism by which miR-494 affects glucose-stimulated insulin secretion in islet β -cells. The SNARE complex is involved in the regulation of the fusion of insulin particles with the plasma membrane. First, miR-494 was over-expressed and inhibited in two separate sets of MIN6 cells. Then both sets were treated with resistin. Gene expression of SNARE complex components, Vamp2, Snap25, Stx1a (syntaxin-1a), and Stxbp1, Stxbp5 was examined. The results showed that over-expression of miR-494 or resistin down-regulated Vamp2, Stx1a, and Stxbp1 and that the miR-494 inhibitor reversed down-regulation of these genes (Fig. 2E, F). This suggested that miR-494 may inhibit assembly of the SNARE complex.

MiR-494 Inhibit Insulin Secretion Through Target on STXBP5

MiRNA is incorporated into the RNA-induced silencing complex (RISC) (22). It is partially complementary to the sequences in the 3' UTR of its target mRNA, to which it binds, inducing mRNA degradation and so suppressing protein translation and expression of the target gene (15). Previous studies have shown that miR-494 over-expression significantly inhibits glucose-induced insulin secretion in MIN6 cells. *In silico* tools were used to find target genes of miR-494 to further elucidate the molecular mechanism

by which miR-494 regulates insulin secretion in MIN6 cells. A conservative miR-494 binding site at 3' UTR of Stxbp5 was found (Fig. 3A).

Two dual luciferase reporter plasmids were constructed to validate the interaction between miR-494 and 3' UTR on Stxbp5, one of which contained Stxbp5 mRNA 3' UTR and the other Stxbp mRNA 3' UTR with mutations on the miR-494 binding site. The results showed that, compared to empty vector, miR-494 inhibited the luciferase activity of Stxbp5 3' UTR reporter plasmid, while the luciferase activity of mutant plasmids remained unchanged (Fig. 3B). This suggested that miR-494 bound directly to the 3' UTR region of Stxbp5 mRNA.

The effect of miR-494 on Stxbp5 gene expression was examined at the cellular level to confirm that Stxbp5 is the target gene for miR-494. Previous data have shown that over-expression of miR-494 had no impact on Stxbp5 mRNA level (Fig. 2E). microRNA regulates gene expression post-transcriptionally, mostly at translational level. In this way, the Stxbp5 protein level was determined and results showed that over-expression of miR-494 significantly suppressed Stxbp5 protein level (Fig. 3C). The data suggested that miR-494 inhibited Stxbp5 expression by binding directly to the 3' UTR of Stxbp5.

Results have shown that Stxbp5 is enriched on insulin particles and that it is required for exocytosis of insulin in islet β cells. The result of the present study indicated that miR-494 can bind to 3' UTR of Stxbp5 mRNA and inhibit Stxbp5 expression at the translational level. In order to determine whether miR-494 can inhibit insulin secretion in islet β cells through Stxbp5, miR-494 was co-transfected with Stxbp5 expression plasmid to MIN6 cells. Over-expression of Stxbp5 was found to reverse the inhibition of glucose-stimulated insulin secretion (Fig. 3D). This confirmed that miR-494 inhibited glucose-stimulated insulin secretion via inhibiting Stxbp5 expression in islet β cells.

DISCUSSION

Resistin was discovered in human and mouse pancreases in 2003, and it was found to be up-regulated in a mouse model of insulin resistance mouse (23). Mice that over-express resistin secrete less insulin. *In vitro* treatment of islet β -cells with resistin induces insulin resistance and impairs glucose-induced insulin secretion. Studies have shown that, in islet β cells, resistin impairs glucose-induced insulin

Figure 2. MiR-494 Overexpression Inhibits Insulin Secretion. The expression of miR-494 was detected in MIN6 cells after resistin treatment and the role of miR-494 in glucose-induced insulin secretion was studied. (A) The expression of miR-494 was examined by real-time PCR. (B) MIN6 cells were transfected with NC, 100 nM, 150 nM, or 200 nM miR-494 mimics, respectively, then incubated with either 5.5 mM or 25 mM glucose medium for 24 h. Insulin secretion was measured by ELISA assay. (C) The expression of miR-494 was examined using real-time PCR. (D) MIN6 cells were treated with recombinant resistin (50 ng/mL) for 24 h, then transfected with miR-494 inhibitor, cells were incubated with either 5.5 mM or 25 mM glucose medium for 24 h. Insulin secretion was measured by ELISA assay. (E) MIN6 cells were transfected with miR-494 mimics or miR-494 inhibitor for 24 h. The expression of genes involved in insulin secretion was examined by real-time PCR. (F) MIN6 cells were treated with recombinant resistin for 24 h, then transfected with miR-494 inhibitor. The expression of genes involved in insulin secretion was examined using real-time PCR. Data are here presented as means $\pm$ SD of percentage of controls. (*, $P < 0.05$, significant; **, $P < 0.01$, very significant; ns, not significant).

Figure 3. MiR-494 Inhibit Insulin Secretion Through Target on STXBP5. The target genes of miR-494 were predicted and analyzed, and also the target gene was verified. (A) The result of bioinformatics prediction shows that miR-494 can bind to the 5253–5259 bp sites of the *Stxbp5* mRNA 3'UTR region. (B) miR-494 and *Stxbp5* 3'UTR report vector or binding site mutant vector (*Stxbp5* -3'UTR-M) were co-transfected into NIH-3T3 cells and luciferase activity was assessed after 24 h according to the instructions included with the Dual Luciferase Reporter Assay System. (C) Effect on *Stxbp5* protein level of miR-494 by Western blot. (D) miR-494 and *Stxbp5* were co-transfected into MIN6 cells, which were incubated with either 5.5 mM or 25 mM glucose medium for 24 h. Insulin secretion was measured by ELISA assay. Data are here presented as means $\pm$ SD of percentage of controls. (*, $P < 0.05$, significant; **, $P < 0.01$, very significant; ns, not significant).

secretion at least partially via reduction of insulin-induced phosphorylation of Akt and induction of SOCS-3 expression (13). Other studies have concluded that resistin down-regulates insulin receptor mRNA and protein levels in islet β -cells (14). These studies collectively suggest that resistin has a role in regulation of islet β -cell functions. The current study shows that resistin can significantly inhibit insulin secretion in MIN6 cells under both high-glucose and low-glucose culture conditions.

An earlier study by the present team showed resistin to be down-regulated in liver tissue arrays from resistin-treated mice (21). Other reports have shown that miR-494 is up-regulated in the myocardial microvascular endothelial cells of diabetic GK rats (24). This indicates that miR-194 may play a role in the initiation and development of diabetes. The essential distinction between type I and type II diabetes involves insulin sensitivity, so it was here hypothesized that miR-494 may be correlated with insulin secretion as

regulated by resistin. To test this hypothesis, miR-494 was over-expressed in MIN6 cells, and results showed that miR-494 could also inhibit glucose-stimulated insulin secretion. Inhibition of miR-494 in the presence of resistin rescued glucose-stimulated insulin secretion. miR-494 was also up-regulated by resistin. These data suggested that resistin inhibited glucose-stimulated insulin secretion by up-regulating the expression of miR-494 in MIN6 cells.

To determine the specifics of the mechanism by which miR-494 affects the resistin-induced reduction of glucose-stimulated insulin secretion in islet β -cells, real-time RT-PCR was used to screen for marker genes associated with insulin exocytosis. Results showed that, when miR-494 was over-expressed, Vamp2, Stx1a (Syntaxin-1a), and Stxbp1 mRNA levels were significantly decreased. This was consistent with the results observed after resistin stimulation. Vamp2 and syntaxin-1 are components of SNARE complex, which suggests that miR-494 might indirectly regulate the assembly of the SNARE protein complex. However, miR-494 showed no effect on Stxbp5 transcriptional level. *In silico* analysis, dual luciferase reporter assay, and Western blotting assay demonstrated that miR-494 inhibited Stxbp5 translation by binding directly to the 3' UTR of its mRNA.

Studies have shown that Stxbp5 specifically binds to syntaxin-1 (25, 26). In islet β -cells, knock-down of Stxbp5 decreases insulin exocytosis, which suggests that Stxbp5 is required for insulin exocytosis (27, 28). In the current study, miR-494 and Stxbp5 expression plasmids were co-transfected in MIN6 cells, and results showed that over-expression of Stxbp5 partially rescued miR-494-mediated, glucose-stimulated insulin secretion in MIN6 cells. This suggests that miR-494 inhibited glucose-stimulated insulin secretion in islet β -cells and did so at least in part by inhibiting Stxbp5 protein expression. Over-expression of Stxbp5 did not promote insulin exocytosis, as had been expected (Fig. 3D). The current study and previous works all suggest that Stxbp5 plays a critical role in glucose-stimulated insulin secretion. However, the details of the underlying mechanism remain to be determined and merit further investigation.

In conclusion, resistin suppresses glucose-stimulated insulin secretion by up-regulating miR-494 and abrogating Stxbp5 expression in the post-translational level by targeting the 3'UTR in the corresponding mRNA in MIN6 cells.

Conflict of interest

There are no potential conflicts of interest relevant to this article to report.

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Informed consent

All co-authors have seen and agree with the contents of the manuscript and there are no financial interests to report.

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