European Review for Medical and Pharmacological Sciences 2017; 21: 1091-1098

microRNA-199a-5p mediates high glucose-induced reactive oxygen species production and apoptosis in INS-1 pancreatic β-cells by targeting SIRT1

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Abstract. – OBJECTIVE: Hyperglycemia-induced pancreatic β-cell loss is a pathologic hallmark of type 2 diabetes mellitus (T2DM). This study was conducted to clarify the function of microRNA (miR)-199a-5p in high glucose-elicited β-cell toxicity and associated molecular mechanisms.

MATERIALS AND METHODS: INS-1 rat pancreatic β-cells were cultured under normal (11 mM) or high (30 mM) glucose for 16-72 h and examined for miR-199a-5p expression. Gain and loss-of-function studies were performed to determine the role of miR-199a-5p in high glucose-induced apoptosis and reactive oxygen species (ROS) production. Additionally, the involvement of SIRT1 in the action of miR-199a-5p was checked.

RESULTS: High glucose caused a significant upregulation of miR-199a-5p in INS-1 cells compared to cells under normal glucose conditions. Pre-transfection with anti-miR-199a-5p inhibitors prevented the reduction in cell viability and inhibited ROS generation in INS-1 cells after high glucose treatment. In contrast, overexpression of miR-199a-5p significantly reduced cell viability and promoted apoptosis and ROS formation in INS-1 cells, which was coupled with a downregulation of SIRT1. Knockdown of SIRT1 led to apoptotic death in INS-1 cells. Moreover, enforced expression of SIRT1 blocked miR-199a-5p-induced ROS generation and attenuated high glucose-mediated apoptosis in INS-1 cells.

CONCLUSIONS: miR-199a-5p is upregulated in pancreatic β-cells in response to high glucose and promotes apoptosis and ROS generation by targeting SIRT1. The miR-199a-5p/SIRT1 axis may represent a promising target for the treatment of T2DM.

Key Words:
Apoptosis, Hyperglycemia, miR-199a-5p, Oxidative stress, SIRT1.

Introduction

Pancreatic β-cell dysfunction and loss is a pathologic hallmark of type 2 diabetes mellitus (T2DM)[1,2]. Chronic exposure to hyperglycemia is an important cause of the deterioration of pancreatic β-cells[3]. High-glucose-induced β-cell toxicity has been casually linked to the production of excessive reactive oxygen species (ROS)[4]. Several antioxidant agents were reported to confer protection against glucotoxicity in pancreatic β-cells[5,6]. In this respect, inhibition of ROS generation may represent a promising strategy to treat T2DM.

microRNAs (miRNAs) are a class of endogenous, small non-coding RNAs that play important roles in the pathogenesis of T2DM[7]. In murine models, β-cell-specific overexpression of miR-200 causes β-cell death and lethal T2DM, whereas depletion of mIR-200 prevents β-cell apoptosis and alleviates T2DM[8]. It has been shown that both miR-101a and miR-30b are implicated in inflammatory cytokine-induced dysfunction of β-cells[9]. Another study[10] demonstrated that miR-19a-3p can augment cell proliferation and insulin secretion and reduce apoptosis in pancreatic β-cells. miR-199a-5p has a wide range of biological activities, including anticancer[11], osteogenic[12], and anti-inflammation[13]. Arsenic-transformed bronchial epithelial cells showed downregulation of miR-199a-5p via alteration of ROS formation[14], suggesting a link between miR-199a-5p and oxidative stress. Several miRNA expression-profiling studies[7] have reported that miR-199a-5p is deregulated in animal models of T2DM. However, the role of miR-199a-5p in the pathogenesis of T2DM is still unclear. In this work, we used an INS-1 rat pancreatic β-cell

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model and investigated the expression and function of miR-199a-5p in response to high glucose. The target genes mediating the activity of miR-199a-5p were identified and functionally characterized.

Materials and Methods

Cell Culture and Treatment

INS-1 rat pancreatic β-cells were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). They were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), streptomycin (100 μg/mL), and penicillin (100 units/mL). For high glucose treatment, INS-1 cells were exposed to 30 mM of glucose for 16-72 h. Cells cultured in fresh RPMI-1640 medium containing 11 mM glucose were used as a control.

Quantification of Mature miR-199a-5p Expression

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized using a specific stem-loop primer. Quantitative real-time PCR (qRT-PCR) was carried out on the Applied Biosystems StepOne Plus Detection System (Applied Biosystems, Foster City, CA, USA). The Taqman microRNA assay kit (Applied Biosystems) was used to detect mature miR-199a-5p expression. The results were normalized to the level of U6.

Plasmids and Oligonucleotides

A fragment containing miR-199a-5p precursor was yielded by PCR amplification of genomic DNA and cloned into the pSUPER expression vector. Full-length human cDNA of SIRT1 lacking the 3'-untranslated region (3'-UTR) was purchased from OriGene (Rockville, MD, USA) and cloned into pcDNA3.1(+) expression vector. Locked nucleic acid (LNA)-modified anti-miR-199a-5p inhibitor and negative control oligonucleotides were purchased from Exiqon (Vedbaek, Denmark). Small interfering RNA (siRNA) targeting *SIRT1* gene and negative control siRNA were provided by GenPharm (Shanghai, China).

Cell Transfection

INS-1 cells were seeded at a density of 5×10^3 cells/well in 96-well plates or 2×10^5 cells/well in 24-well plates. Cells were individually transfected with miR-199a-5p-expressing plasmid (1 μg), *SIRT1* siRNA (50 nM), and corresponding controls using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). In some experiments, cells were pre-transfected with anti-miR-199a-5p inhibitors (40 nM) or pcDNA3.1/SIRT1 plasmid (1 μg) 24 h before high glucose treatment. To reverse the effect of miR-199a-5p, cells were co-transfected with miR-199a-5p-expressing plasmid (0.2 μg) and pcDNA3.1/SIRT1 plasmid (1 μg) 24 h before ROS measurement.

MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done to determine cell viability. In brief, 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells. After incubation for 4 h at 37°C, dimethyl sulfoxide (DMSO) was used to dissolve the formazan. Absorbance was recorded at a wavelength of 570 nm.

Apoptosis Detection by Flow Cytometry

Cell apoptosis was detected using the Annexin-V/propidium iodide (PI) Apoptosis Detection Kit (Becton Dickinson Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. After staining with FITC-conjugated annexin-V and PI, apoptotic cells were analyzed by a flow cytometer using the CellQuest software (Becton Dickinson Biosciences, San Jose, CA, USA).

Western Blot Analysis

Cells were lysed in ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% NP-40, and 0.5% sodium deoxycholate) supplemented with proteinase inhibitors (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using the Bradford assay. Protein samples (40 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated at 4°C overnight with primary antibodies (1: 400 dilution) against cleaved caspase-3 and -9, Bcl-2, SIRT1, and β-actin (Cell Signaling Technology, Beverly, MA, USA). After washing three times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA). Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

Measurement of ROS Production

Measurement of ROS formation was performed as described previously[15]. A permeable probe 2'-7'-dichlorodihydrofluoresce in diacetate (DCFH-DA) was used in this assay, which can be oxidized to generate the fluorescent product dichlorofluorescein (DCF). Cells were incubated with 20 μM of DCFH-DA for 15 min at 37°C in the dark. After washing, cells were re-suspended in phosphate buffered saline and analyzed by flow cytometry.

Statistical Analysis

Data are expressed as means ± standard deviation (SD). Statistical differences were determined using the Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey's test. $p < 0.05$ were considered statistically significant.

Results

miR-199a-5p Mediates High Glucose-Induced Toxicity in INS-1 Cells

qRT-PCR analysis revealed that high glucose-treated INS-1 cells displayed an induction of miR-199a-5p expression, compared to control cells under normal glucose conditions ($p < 0.05$; Figure 1A). The levels of miR-199a-5p were elevated by 1.8- and 3.2-fold after exposure to high glucose for 16 and 72 h, respectively. To determine the role of miR-199a-5p in high glucose-induced toxicity, INS-1 cells were transfected with anti-miR-199a-5p inhibitors before exposure to high glucose. MTT assay showed that high glucose treatment for 72 h led to a 54% decline in the viability of INS-1 cells ($p < 0.05$; Figure 1B). Pre-transfection with anti-miR-199a-5p inhibitors significantly prevented the cytotoxic effect of high glucose on INS-1 cells. Next, we asked whether overexpression of miR-199a-5p could affect the viability of INS-1 cells. Notably, miR-199a-5p-overexpressing INS-1 cells had a 42% lower viability than empty vector-transfected cells ($p < 0.05$; Figure 1C). These results suggest that miR-199a-5p is involved in high glucose-induced toxicity in INS-1 cells.

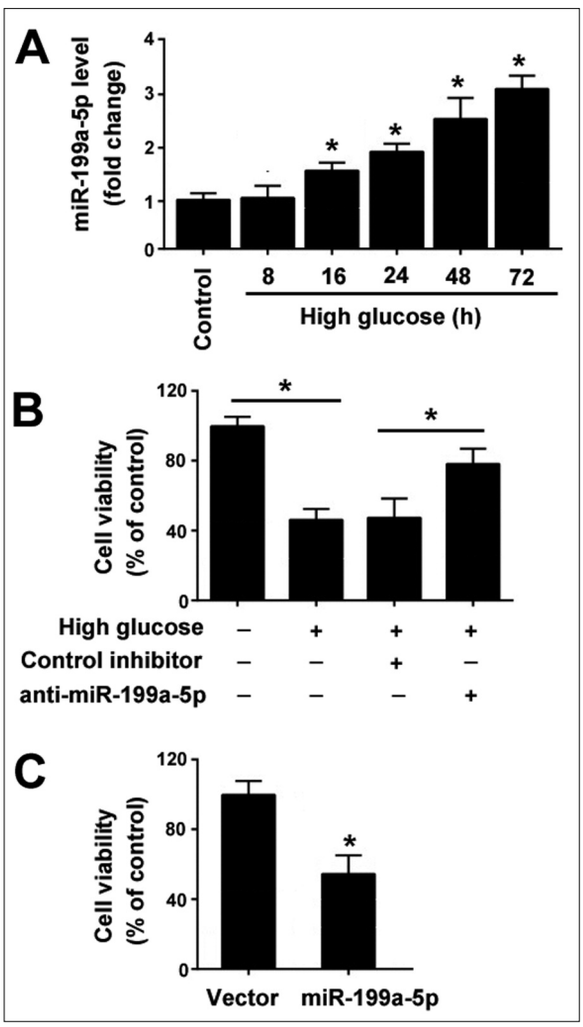

Figure 1. miR-199a-5p mediates high glucose-induced toxicity in INS-1 cells. ***A,*** Measurement of miR-199a-5p levels in high glucose-treated INS-1 cells by qRT-PCR analysis. *$p < 0.05$ vs. control cells under normal glucose conditions. ***B,*** MTT assays were done to measure the viability of INS-1 cells pre-transfected with control or anti-miR-199a-5p inhibitors before high glucose treatment for 72 h. *$p < 0.05$. ***C,*** Measurement of the viability of INS-1 cells transfected with vector or miR-199a-5p-expressing plasmid by MTT assays. *$p < 0.05$ vs. vector-transfected cells.

Enforced Expression of miR-199a-5p Triggers Apoptosis in INS-1 Cells

Next, we investigated the effect of miR-199a-5p overexpression on the apoptosis of INS-1 cells. Flow cytometric analysis showed that miR-199a-5p-overexpressing INS-1 cells had a significantly greater percentage of apoptotic cells than empty vector-transfected cells (21.2 ± 1.9% *vs.* 4.8 ± 0.8%, $p < 0.05$; Figure 2A). Western blot analysis confirmed that miR-199a-5p overexpression led to an enhancement of caspase-3 and caspase-9 cleavage, as well as decease in Bcl-2 expression (Figure 2B).

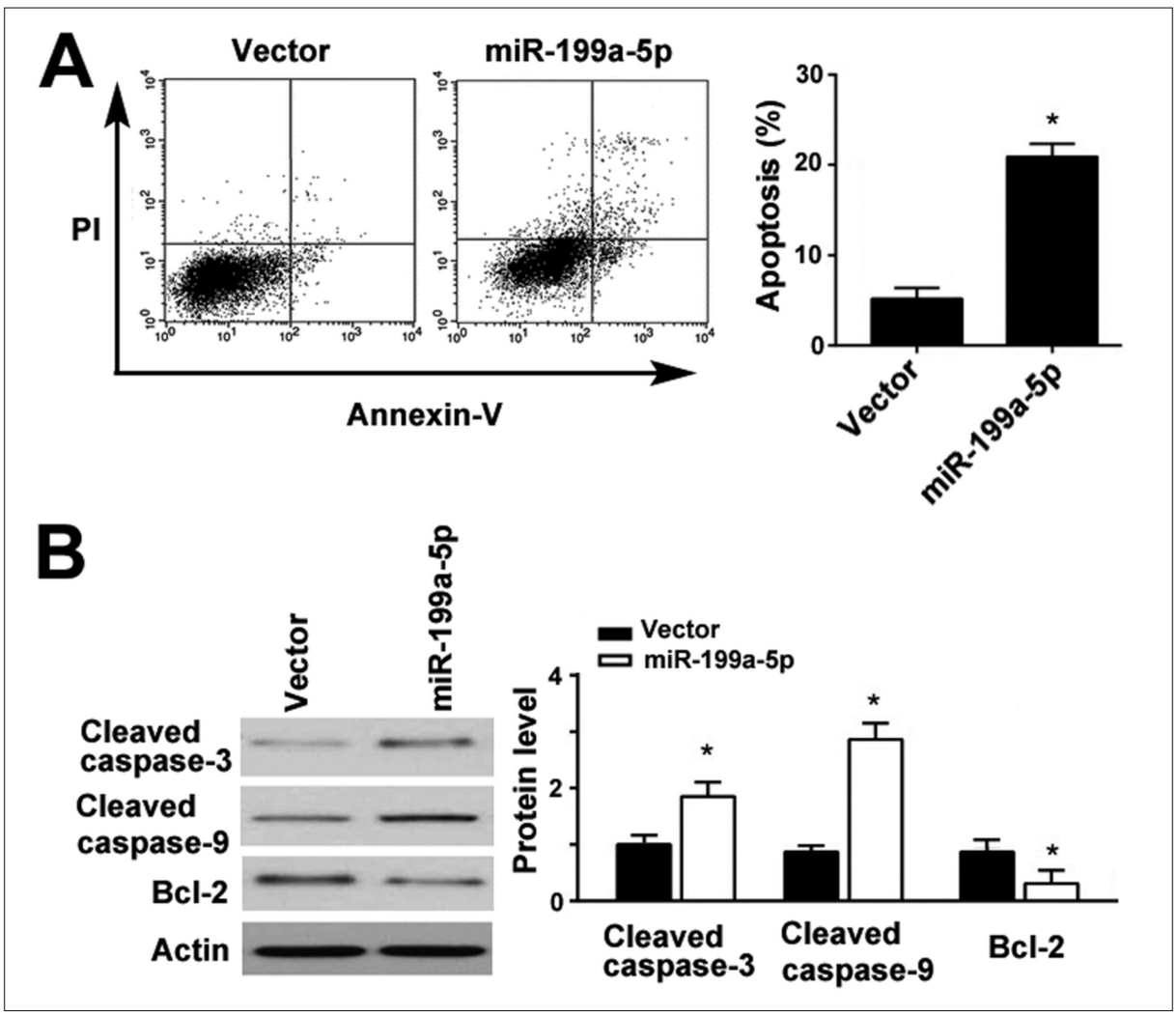

Figure 2. Enforced expression of miR-199a-5p triggers apoptosis in INS-1 cells. ***A,*** Flow cytometric analysis of apoptosis in INS-1 cells tranfected with vector or miR-199a-5p-expressing plasmid. *Left*, representative flow cytometry dot plots after Annexin-V/PI staining. *Right*, quantification of apoptosis from three independent experiments. ***B,*** Western blot analysis of indicated proteins in INS-1 cells tranfected with vector or miR-199a-5p-expressing plasmid. Bar graphs show densitometric analysis of protein intensities from three independent experiments. *$p < 0.05$ vs. vector-transfected cells.

miR-199a-5p Promotes ROS Production by Targeting SIRT1

Excessive ROS is an inducer of apoptosis in pancreatic β-cells in response to high glucose[16,17]. We next investigated the role of miR-199a-5p in ROS production. As shown in Figure 3A, there was 8.3-fold increase in ROS levels in miR-199a-5p-overexpressing INS-1 cells, compared to vector-transfected cells ($p < 0.05$). Delivery of anti-miR-199a-5p inhibitors was noted to impair ROS generation in INS-1 cells after high glucose exposure (Figure 3B). These results suggest that miR-199a-5p contributes to high glucose-induced ROS formation.

Next, we attempted to identify the target genes involved in the induction of ROS by miR-199a-5p. It has been reported[18] that SIRT1 is a direct target of miR-199a-5p in the hippocampus. Since SIRT1 shows antioxidant activity in various biological settings[19,20], we hypothesized that miR-199a-5p may promote ROS generation in INS-1 cells via targeting of SIRT1. In line with this hypothesis, we showed that enforced expression of miR-199a-5p significantly suppressed the endogenous expression of SIRT1 in INS-1 cells (Figure 4A). Most importantly, overexpression of SIRT1 (Figure 4B) significantly impaired miR-199a-5p-induced ROS formation (Figure 4C).

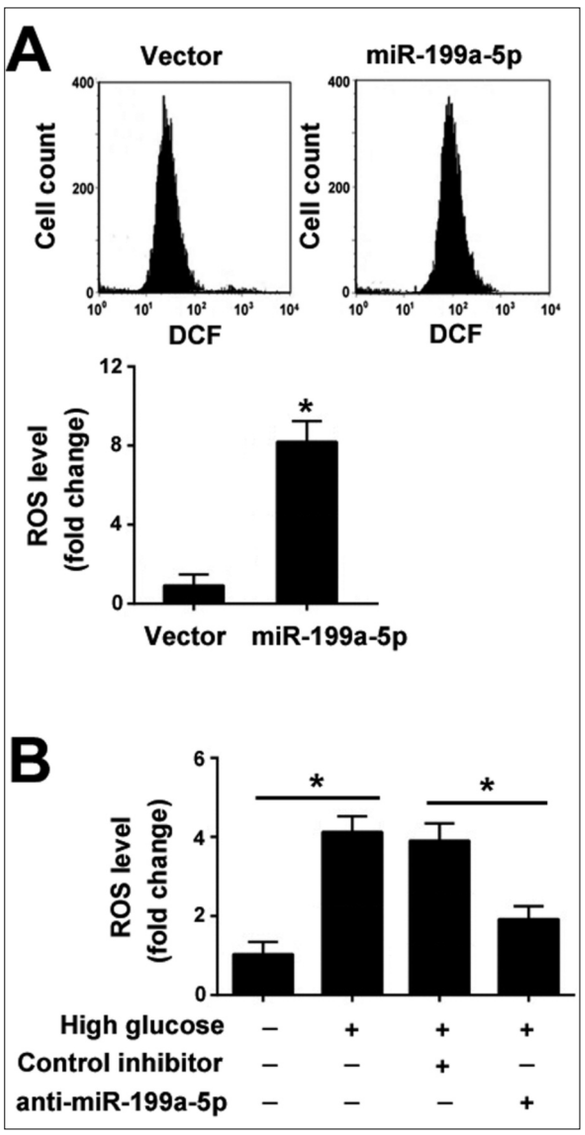

Figure 3. miR-199a-5p contributes to high glucose-induced ROS formation. ***A,*** Measurement of ROS levels in INS-1 cells tranfected with vector or miR-199a-5p-expressing plasmid. Top, representative flow cytometry histograms showing ROS levels determined by DCF fluorescence. Bottom, quantitative data from three independent experiments. *$p < 0.05$ *vs.* vector-transfected cells. ***B,*** Measurement of ROS production in INS-1 cells pre-transfected with control or anti-miR-199a-5p inhibitors before high glucose treatment for 72 h. *$p < 0.05$.

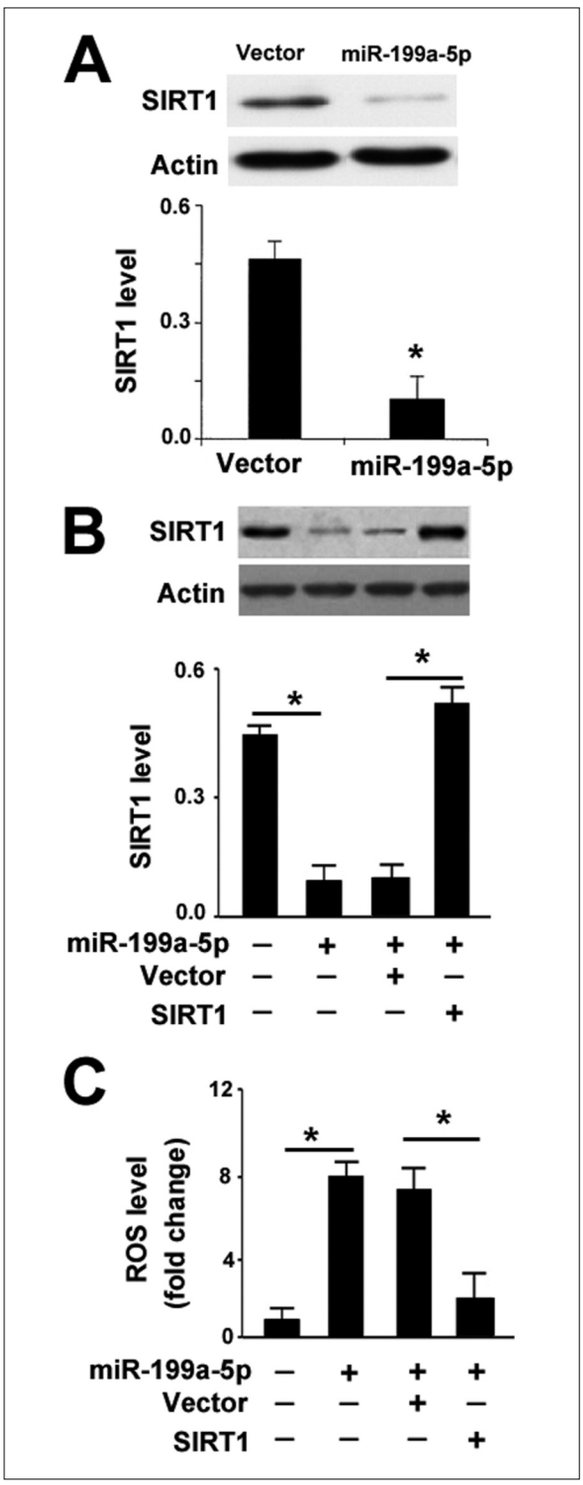

Figure 4. miR-199a-5p promotes ROS production by targeting SIRT1. ***A,*** Western blot analysis of SIRT1 protein levels in INS-1 cells tranfected with vector or miR-199a-5p-expressing plasmid. *$p < 0.05$ vs. vector-transfected cells. ***B,*** Western blot analysis of SIRT1 protein levels in INS-1 cells transfected with indicated constructs. *$p < 0.05$. ***C,*** Measurement of ROS production in INS-1 cells transfected with indicated constructs. *$p < 0.05$.

SIRT1 Downregulation Contributes to High Glucose-Induced Apoptosis in INS-1 Cells

Finally, we clarified the roles of SIRT1 in high glucose-induced an apoptotic response. Similar to high glucose treatment, silencing of SIRT1 (Figure 5A) was found to cause significant apoptosis in INS-1 cells (Figure 5B). In contrast,

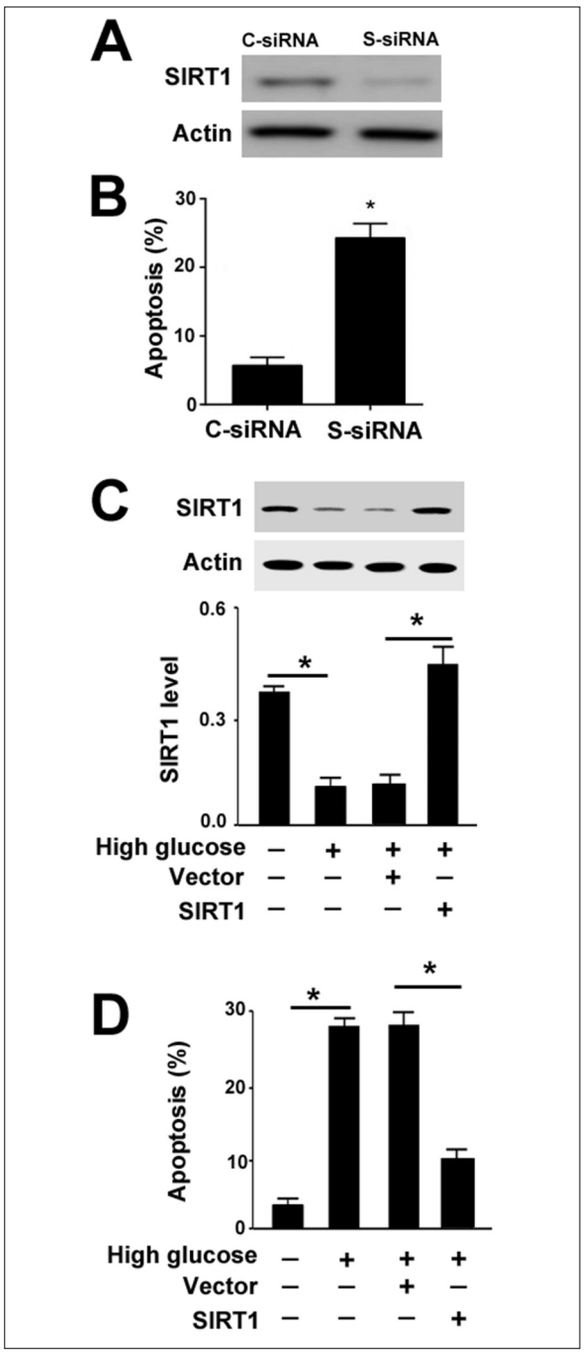

Figure 5. SIRT1 downregulation contributes to high glucose-induced apoptosis in INS-1 cells. ***A,*** Western blot analysis of SIRT1 protein levels in INS-1 cells transfected with control siRNA (C-siRNA) or SIRT1-targeting siRNA (S-siRNA). ***B,*** Apoptosis detection in INS-1 cells transfected with C-siRNA or S-siRNA by flow cytometry after Annexin-V/PI staining. *$p < 0.05$ *vs*. C-siRNA-transfected cells. ***C,*** Western blot analysis of SIRT1 protein levels in INS-1 cells pre-transfected with vector or SIRT1-expressing plasmid before high glucose treatment. ***D,*** Flow cytometric analysis of apoptosis in INS-1 cells treated as in ***(C)***. Bar graphs represent quantitative data from three independent experiments. *$p < 0.05$.

overexpression of SIRT1 (Figure 5C) decreased the percentage of apoptosis from 28.9 ± 2.3% to 10.2 ± 1.9% in high glucose-treated INS-1 cells ($p < 0.05$, Figure 5D), suggesting an involvement of SIRT1 in the survival of β-cells.

Discussion

In this work, we showed that miR-199a-5p was upregulated in INS-1 cells after exposure to high glucose. Functional studies revealed that inhibition of miR-199a-5p attenuated high glucose-induced toxicity. In contrast, overexpression of miR-199a-5p significantly reduced the viability of INS-1 cells. These data indicate that miR-199a-5p functions in pancreatic β-cells to mediate the cytotoxicity induced by high glucose. Consistent with our findings, high glucose-induced upregulation of miR-199a-5p has also been noted in rat mesangial cells, where miR-199a-5p modulates high glucose-elicited fibrotic and inflammatory responses[21].

Our data further demonstrated that miR-199a-5p overexpression significantly induced apoptosis in INS-1 cells. At the molecular level, there was an enhancement of caspase-3 and caspase-9 cleavage in miR-199a-5p-overexpressing cells. The caspase-9/caspase-3 cascade can be activated by cytochrome c released from the mitochondria[22]. Bcl-2 acts as an anti-apoptotic protein through blocking mitochondrial cytochrome c efflux[23]. Notably, we found that miR-199a-5p overexpression led to a downregulation of Bcl-2 in INS-1 cells. Therefore, miR-199a-5p may activate the mitochondria-dependent apoptotic pathway in INS-1 cells. Accompanying induction of apoptosis, ectopic expression of miR-199a-5p augmented the production of ROS in INS-1 cells. It has been suggested that high glucose exposure triggers apoptotic response in pancreatic β-cells by facilitating ROS formation[16,17]. Notably, we found that depletion of miR-199a-5p impaired the production of ROS in high glucose-exposed INS-1 cells. These results suggest that induction of ROS formation may account for miR-199a-5p-mediated apoptosis in pancreatic β-cells. SIRT1 has been identified to be a target gene of miR-199a-5p in the hippocampus[18]. Consistently, we showed that miR-199a-5p also had the ability to downregulate SIRT1 expression in INS-1 cells. Most importantly, overexpression of SIRT1 reversed the effect of miR-199a-5p on ROS formation. Taken together, we provide evidence that miR-199a-5p promotes ROS generation

in INS-1 cells likely through downregulating SIRT1.

Given the important role of SIRT1 in the regulation of ROS production[19,20], we tested the hypothesis that SIRT1 is involved in glucotoxicity in pancreatic β-cells. Interestingly, knockdown of SIRT1 significantly promoted apoptosis in INS-1 cells, which phenocopied the effect of miR-199a-5p overexpression on INS-1 cells. Moreover, overexpression of SIRT1 reduced apoptotic response in INS-1 cells after treatment with high glucose. Altogether, these results suggest that the miR-199a-5p/SIRT1 axis regulates the survival of β-cells in response to high glucose. In agreement with our observations, previous reports showed that overexpression of SIRT1 confers protection against cytokine toxicity[24] and hexosamine-induced apoptosis in pancreatic β-cells[25].

However, it should be noted that miR-199a-5p can regulate multiple target genes other than SIRT1[26,27]. It has been reported that FZD6 is negatively regulated by miR-199a-5p in human colorectal cancer cells[26]. Another study[27] demonstrated that miR-199a-5p could target GLUT4 to repress glucose uptake in rat L6 myoblast cells. Therefore, it is possible that miR-199a-5p may simultaneously coordinate some target genes including SIRT1, consequently contributing to high glucose-induced β-cell toxicity.

Conclusions

We provide evidence that miR-199a-5p is induced in pancreatic β-cells upon high glucose exposure and facilitates ROS generation and apoptotic death, largely through the negative regulation of SIRT1 expression. Knockdown of miR-199a-5p or overexpression of SIRT1 confers protection against high glucose-induced apoptosis in pancreatic β-cells. The miR-199a-5p/SIRT1 axis may constitute a promising target for prevention of β-cell loss in the treatment of T2DM.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 81400804).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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