RETRACTED ARTICLE: Notoginsenoside R1 alleviates TNF-α-induced pancreatic β-cell Min6 apoptosis and dysfunction through up-regulation of miR-29a

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disorder that seriously harms human health. Notoginsenoside R1 (NGR1) can be used in various diseases. We explored consequences of NGR1 on tumour necrosis factor (TNF)-α-stimulated Min6 and rat primary islets β cells. The results were that TNF-α significantly cut down cell activity, raised cell apoptosis and iNOS expression and decreased insulin secretion in Min6 and rat primary islets β cells. NGR1 alleviated TNF-α-treated cell dysfunctions. In addition, miR-29a was positively regulated by NGR1 in TNF-α-treated Min6 and rat primary islets β cells. miR-29a knockdown damaged protection roles of NGR1 through cutting down cell activity and insulin secretion, raising apoptosis and iNOS in TNF-α-treated Min6 and rat primary islets β cells. The phosphorylation of Wnt3a, β-catenin and the rate of p/t-AKT/PI3K was all increased, while p/t-GSK3β was decreased by the administration with NGR1. In conclusion, NGR1 alleviated TNF-α-stimulated Min6 and rat primary islets β cells apoptosis and worn roles via positively regulating miR-29a. This process might be through actuation of Wnt/β-catenin and PI3K/AKT/GSK3β signal ways.

Introduction

Diabetes mellitus (DM) is a hyperglycaemia caused by insufficient insulin secretion and low insulin consequents or defects in both [1]. Insulin is a class of proteins (hormones) that are synthesized by pancreatic β cells and can lower blood sugar levels and mainly respond to glucose stimulation [2]. Several pathogenic progressions are the cause of diabetes, and DM can be mainly classified into two categories: type 1 DM (T1DM) is caused by insufficient insulin production, which is due to the destruction of autoimmune in pancreatic β cells [3]; and type 2 DM (T2DM) is an irregular release of insulin to keep normal blood glucose levels [4]. Recently, many researchers have conducted molecular mechanism of β cells failure and insulin resistance. With the increasing prevalence of DM, much attention has been drawn to this disease.

Using traditional herbal medicines to treat DM has been existed in Asian for centuries [5]. Despite advanced technology and researches, more and more investigation of herbal medicine was performed for novel treatments. Notoginsenoside R1 (NGR1) is a phytoestrogen, purified from Panax notoginseng and can be used in various diseases, such as cardiac dysfunction [6], ischemia-reperfusion injury [7] and Alzheimer disease [8]. Importantly, studies have proved that NGR1 has potential effects of antidiabetes. For example, NGR1 ameliorated podocyte injury in rats with diabetic nephropathy through actuating phosphatidylinositol 3'-kinase (PI3K)/protein kinase B (AKT) signal ways [9]. Recent studies showed that NGR1 provided a novel treatment for diabetic kidney disease [10], and NGR1 could ameliorate diabetic encephalopathy [11]. However, seldom studies about the effects of NGR1 on DM were performed till now.

MicroRNAs (miRNAs), a class of very conservative noncoding RNAs, play regulated roles in gene expressing via post-transcriptional consequences [12]. A previous report showed that 40 miRNAs were notably out of tune in T2DM [13]. miR-375 is altered in the serum of children with newly diagnosed T1DM [14]. Among these identified miRNAs, miR-29 family is one of the most abundant microRNAs of pancreas. Importantly, miR-29a has been found to be a main conditioner in secreting insulin in vivo, and under unfolded protein stimulation, extracellular machinery causes β cells to respond notably to diabetes [15]. In addition, miR-29a, which was belonging to diabetes-related serum miRNA [16], was down-regulated by high glucose [17]. Moreover, miR-29a leads to influence in insulin action in HepG2 cells [18]. Taken together, we hypothesized that miR-29a also be possibly related to the progression of NGR1 on DM model in regulating insulin secretion and other biological activities.

β cell living and damaged roles are the main causes of diabetes processes. In our study, we used tumour necrosis factor (TNF)-α to treat a murine insulinoma cell line Min6 and rat primary islets β cells to simulate cell DM injury and explored the functions of NGR1 and the underlying
mechanisms. Our study points to the administration of herbal medicine used in the treatment of DM.

Materials and methods

Cell

Min6 cells were gained from American Type Culture Collection (ATCC, Manassas, USA) and maintained in 25 mM glucose Dulbecco’s-modified Eagle medium (DMEM) (Hyclone, Logan, UT, USA), supplemented with 10% foetal bovine serum (FBS, Life Science, UT, USA), 100 U/mL penicillin, 50 mmol/L β-mercaptoethanol and 0.1 mg/mL streptomycin at 37°C with 5% CO2. The cultured cells were treated with NGR1 (dissolved in dimethyl sulphoxide (DMSO)) in DMEM medium for 24 h. TNF-α solution was readied through preheated medium and through a 2.2-mm sterile filter. Cells were pretreated with or without NGR1 and then treated by TNF-α for 12 h. All the chemical substance was from Sigma-Aldrich (St Louis, MO, USA).

Rat primary islets β cells

Islets were separated through digesting pancreas of collagenase in adult male Wistar rats (Shandong University Laboratory Animal Center, Shandong, China). β cells were detached from non-β cells through autofluorescence from a FACSVantage (Becton Dickinson, Oxford, UK). Islet scattered cells, selected β cells and selected non-β cells were incubated in DMEM including 11 mM glucose, 0.05 mg/mL gentamicin, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% FBS (Life Science, UT, USA). Cells were cultured on 804 G ECM-coated plates and were adhered and spread in 24 h before starting assays.

Cell counting kit-8 (CCK-8) assay

After the above treatment, cell activity was tested through CCK-8 (Yeasen, Shanghai, China) experiment. First, inoculate the cell suspension in a 96-well plate (5 x 10⁴ cells/well) and place the plate in an incubator for pre-incubation (37°C, 5%
CO₂). Then, 10 µL CCK-8 solution was replenished to every well, and the plate was kept in an incubator for 1–4 h. Finally, the absorbance at 450 nm was tested with a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis assay

Cell apoptosis was tested through Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) testing kit (Beijing Biosea Biotechnology, Beijing, China). First, cells were gathered in centrifuge tubes, and the number of cells per sample was 1 × 10⁶ cells/mL. The supernatant was discarded by centrifugation at 111.8 g. Then, cells were cleaned twice with cold PBS, and a suspension of 1 × 10⁶ cells/mL was made with 1 × Binding Buffer, gently mixed and kept for 15 min at room temperature (20 °C–25 °C) under shading. Finally, flow cytometry (Beckman Coulter, USA) analysis was performed that excitation wavelength was measured at 488 nm. FITC fluorescence was tested with a passband filter with a wavelength of 515, and 560 nm for PI. Results judgments were that normal cells (FITC –/PI –), necrotic cells (FITC +/PI +) and apoptotic cells (FITC +/PI –).

Glucose-stimulated insulin secretion assay (GSIS)

For insulin secreting assay, Min6 and main islets β cells at the denseness of 5 × 10⁵ cells/well were planted in 24-well plates and pretreated with or without NGR1, and then incubated with TNF-α. After treatment, the cells were incubated for 1 h in glucose-free KRB buffer (115 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 20 mmol/L NaHCO₃, 16 mmol/L HEPES, 2.56 mmol/L CaCl₂ and 0.2% BSA) and then handle cells in KRB buffer for 1 h with low (3.3 mmol/L) or high (16.7 mmol/L) consistencies of glucose. Supernatants were gathered, and insulin concentrations were determined through radioimmunoassay.

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

Overall RNA was got through Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) referring to

![Figure 2. Notoginsenoside R1 (NGR1) reduced TNF-α-caused Min 6 damage. (A,B) Cell activity was tested via CCK-8. (C,D) Cell apoptosis was inhibited by NGR1. (E) Inducible nitric oxide synthase (iNOS) expression was decreased by NGR1. (F) Insulin secretion was increased by NGR1. *p < .05, **p < .01, ***p < .001.](image-url)
producer’s directions. MiR-29a expression was tested through Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA). U6 was the inside comparison. Use $2^{\Delta\Delta C_t}$ way to compute results.

**miRNAs transfection**

MiR-29a inhibitor and its NC were compounded (Life Technologies Corporation, MD, USA) and transfected into cells. Cultured cells in 24-well plates were transfected in vitro with pri-miR-29a plasmids, miR-29a inhibitor and their corresponding comparison through Lipofectamine 2000 transfection reagent (Invitrogen). Seventy-two hours was the most suitable time of following assays. Keep the stable transfected cells for 4-week inculture medium including 0.5 mg/mL G418 (Sigma-Aldrich, St Louis, USA) to get G418-resistant cells.

**Western blot**

First, extraction of experimental protein was done through RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) and protease inhibitors (Roche, Basel, Switzerland). Second, protein quantification was done through BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Then, protein samples was isolated through SDS-PAGE and then transferred to the polyvinylidene difluoride (PVDF) membrane. Fully immerse the membrane in the no protein blocking solution (Sangon Biotech, Shanghai, China). Primary antibodies included the following: anti-pro-Caspase-3 antibody (ab32499), anti-cleaved Caspase-3 (ab49822), anti-B-cell lymphoma 2 (Bcl-2) antibody (ab692), anti-Bax antibody (ab32503), anti-inducible nitric oxide synthase (iNOS) antibody (ab178945), anti-β-actin antibody (ab8227), all from Abcam (Cambridge, UK); anti-Wnt3a antibody (2721), anti-β-catenin antibody (8480), anti-t-PI3K antibody (4249), anti-p-PI3K antibody (4228), anti-t-AKT antibody (4691), anti-p-AKT antibody (9614), anti-t-glycogen synthase kinase 3β (GSK3β) antibody (5676), anti-p-GSK3β antibody (9327), from Cell Signaling Technology (Beverly, MA, USA). Next, the membrane was cultured with these primary antibodies for 10 min in room and kept at 4 °C all the night. After rinsing, incubate the membrane with secondary antibodies for 1 h in room. Finally, after rinsing six times with TBST, add 200 µL Immobilon Western
pancreatic \(\beta\)-cells apoptosis against immunological and inflammatory stimulation [19]. We found that iNOS was positively regulated by TNF-\(\alpha\) \((p < .001, \text{Figure 1(E)}\)), demonstrating that TNF-\(\alpha\) successfully caused Min6 cell injury.

**NGR1 alleviated TNF-\(\alpha\)-caused damage in Min6 cells**

Cell viability under different concentrations of NGR1 was detected for choosing the proper treatment concentration. As shown in Figure 2(A), cell activity was not notably influenced by various consistencies of NGR1 (0, 1, 5, 10, 50 and 100 \(\mu\)M). However, higher concentrations of NGR1 10 \(\mu\)M \((p < .05), 50 \mu\)M \((p < .01)\) and 100 \(\mu\)M \((p < .01)\) showed increasing cell viability under TNF-\(\alpha\) treatment (Figure 2(B)). NGR1 at 50 \(\mu\)M was selected in future assays. Simultaneously, apoptosis was significantly decreased by NGR1 \((p < .01, \text{Figure 2(C)}\) in addition, cleaved Caspase-3 and Bax were negatively regulated, whereas Bcl-2 was positively regulated by NGR1 under TNF-\(\alpha\) treatment (Figure 2(D)). iNOS expression was down-regulated by NGR1 under TNF-\(\alpha\) treatment \((p < .01, \text{Figure 2(E)}\). Furthermore, insulin secretion was up-regulated by NGR1 under TNF-\(\alpha\) treatment \((p < .05, \text{Figure 2(F)}\). We got that NGR1 alleviated Min6 cell injury induced by TNF-\(\alpha\).

**NGR1 alleviated TNF-\(\alpha\)-caused damage in rat primary islets \(\beta\)-cells**

To validate whether the effects of NGR1 on Min6 cells were also applied in the other cells, especially the clinical specimens, similar experiments were performed in rat primary islets \(\beta\)-cells. Interestingly, we got that cell activity was also suppressed through TNF-\(\alpha\) in a dose-relying ways \((p < .01, p < .01, \text{Figure 3(A)}\). Furthermore, TNF-\(\alpha\) also induced apoptosis \((p < .001, \text{Figure 3(B)}\). Moreover, Western blot revealed that cleaved-Caspase-3 and Bax were apparently increased, whereas Bcl-2 was down-regulated by TNF-\(\alpha\) (Figure 3(C)).

Then what happened when NGR1 was administrated? Similar as in Min6 cells, NGR1 supplement inhibited apoptosis \((p < .01, \text{Figure 3(D)}\), altered apoptotic protein accumulated levels (Figure 3(E)) and down-regulated the expression of iNOS (Figure 3(F)). This information above indicated that NGR1 could also promote cell growth in rat primary islets \(\beta\)-cells. In addition, the insulin secretion was increased which suggested that NGR1 alleviated rat primary islets \(\beta\)-cells injury to some extent.

**NGR1 positively regulated miR-29a in both Min 6 and rat primary islets \(\beta\)-cells**

miR-29a was closely related to the concentration of glucose [17]. Here, miR-29a was negatively regulated by TNF-\(\alpha\) \((p < .05\) or \(p < .01)\), while positively regulated after supplying of NGR1 \((p < .01\) or \(p < .001)\) (Figure 4(A,B)). Our data demonstrated that miR-29a might be related to the protective functions of NGR1 in TNF-\(\alpha\)-induced Min6 and primary islets \(\beta\)-cell injury.
NGR1 alleviated TNF-α-induced damage via positively regulating miR-29a in Min6 cells

Here, we tested the roles of miR-29a. Significantly inhibition of miR-29a in a time-dependent manner after miR-29a inhibitor transfection suggested great transfection effectiveness (p < .05 or p < .01, Figure 5(A)). Interestingly, miR-29a inhibitor transfection inhibited protection roles of NGR1 through cutting down cell activity (p < .01, Figure 5(B)), increasing apoptosis (p < .01, Figure 5(C,D)) compared with NC. Additionally, iNOS (p < .01, Figure 5(E)) was positively regulated and insulin secretion was cut down (p < .01, Figure 5(F)) after miR-29a inhibitor transfection contrasted with NC. Our data suggested that NGR1 attenuated TNF-α-caused Min6 cell damage via positively regulating miR-29a.

NGR1 alleviated TNF-α-caused damage through positively regulating miR-29a in primary islets β cell

Similarly, we determined the influence of miR-29a down-regulation on TNF-α-stimulated primary islets β cells to
detect whether miR-29a was also worked in other cell type. We transfected miR-29a inhibitor into primary islets β cells and high transfection was also obtained (Figure 6(A)). Interestingly, the effects of miR-29a down-regulation demonstrated similar effects on cell viability (Figure 6(B)), apoptosis (Figure 6(C)), apoptotic factors (Figure 6(D)), iNOS (Figure 6(E)) as well as insulin secretion (Figure 6(F)). This series assays were done to confirm the consequents of NGR1 in regulating TNF-α-induced cell injury not only in Min 6 but also in primary islets β cells.

NGR1 activated Wnt/β-catenin and pi3K/AKT/GSK3β signal ways by positively regulating miR-29a

The previous study shed a light on the relation between miR-29a and PI3K in regulating insulin, and the results showed that miR-29a was important in negatively regulating insulin signalling through PI3K [15]. We investigated the roles of miR-29a and PI3K/AKT/GSK3β signal ways. Wnt3a and β-catenin and the rate of p/t-PI3K/AKT were all down-regulated (all p < .05), while p/t-GSK3β was up-regulated by TNF-α (p < .01,
Figure 7(A,B)), while administration with NGR1 reversed these results (Figure 7(A,B)). In addition, miR-29a inhibitor transfection caused converse consequents compared with the group, which was NGR1 administration and transfection with NC in TNF-α-treated Min6 cells (Figure 7(A,B)). These results demonstrated that NGR1 activated Wnt/β-catenin and PI3K/AKT/GSK3β signal ways by positively regulating miR-29a.

**Discussion**

We studied the functions of NGR1 on TNF-α-treated Min6 cells and rat primary islets β cells is to mimic effects on DM. Results demonstrated that NGR1 significantly increased cell viability and insulin secretion, while decreased apoptosis and iNOS expression in TNF-α-treated Min6 cells and rat primary islets β cells through up-regulation of miR-29a. Further studies revealed that NGR1 activated Wnt/β-catenin and PI3K/AKT/GSK3β signal ways via up-modulating miR-29a.

DM is a main hazard element of cardiovascular illness and stroke [3]. Insulin is central in the process of this illness [20]. Proinflammatory cytokine TNF-α is essential in destruction of β cells [21]. In our study, TNF-α-treated Min6 cells led to the cut down of cell activity, increase of apoptosis, up-regulation of iNOS and decrease of insulin secretion, suggesting cell injury model was successfully established in vitro.

To investigate the functions of NGR1 on the TNF-α-treated Min6 cell injury, various factors were measured. Cell activity was raised, whereas apoptosis was cut down after NGR1 administration, indicating that NGR1 could promote Min6 cell growth. Consistently, NGR1 treatment ameliorates the diabetes-induced apoptosis of podocytes [9].

Regulation of iNOS has latent function in insulin resistivity, diabetes and heart failure [22]. Importantly, iNOS expression was closely correlated with diabetes [23] because iNOS over-expression of DM caused β cell death [24]. In our study, administration with NGR1 notably cuts down iNOS expression, indicating the protective consequent of NGR1 on TNF-α-treated Min6 cells. This is consistent with the previous report that NGR1 could attenuate iNOS expression [6].

DM could promote glucose irritate the emancipation of enough number of insulin from β cells to keep regular blood glucose [25]. Hence, novel cure methods to elevate β cells or insulin secretion are believed to be beneficial [26]. In our study, NGR1 significantly increased insulin secretion under high glucose concentration, which indicated that NGR1 might enact protective effects in TNF-α-treated Min6 cells for
improving β cells insulin secretion ability. NGR1 is often found worked with miRNAs to carry out its functions. For example, NGR1 treatment caused a notable cut down of miR-21, miR-26a and miR-126 and raised miR-20a expression in mice [27]. Meanwhile, emerging studies have shown that miRNAs play diverse roles in DM [28]. Here, we explored the probable consequents of miR-29a in TNF-α-treated Min6 cells, according to close relationship of miR-29a with T1DM [29] and T2DM [13]. However, the roles of miR-29a in different cells revealed different functions. For example, miR-29a suppressed cell proliferation and caused cell cycle arrest in human gastric cancer [30]. Of contrast, miR-29a down-regulation contributed to cell apoptosis in large-cell lymphomas [31], and inhibited prostate cancer cell growth and invasion [32]. The current study revealed miR-29a down-regulation cuts down cell activity and caused apoptosis in TNF-α-treated Min6 cells, which was similar to the past research [31,32]. Further studies proved that the functions of NGR1 in TNF-α-treated Min6 cells were via positively regulating miR-29a.

Wnt/β-catenin and PI3K/AKT/GSK3β were reported to be closely related to DM [33,34]. Wnt/β-catenin signalling is an important regulator for insulin secretion and might be involved in the pathogenesis of T2DM [35]. In our study, NGR1 activated Wnt/β-catenin and PI3K/AKT/GSK3β ways through positively regulating miR-29a in TNF-α-treated Min6 cells. Various reports supported our results. For example, NGR1 treatment induced activation of the PI3K/AKT signalling in rats [9]. miR-29a activated Wnt/β-catenin signalling way in pancreatic cancer cells [36].

In conclusion, NGR1 promoted cell growth, alleviated the expression of iNOS and increased insulin secretion in TNF-α-treated Min6 cells via positively regulating miR-29a. Our results might offer a foundation for the further researches and suggested NGR1 as a novel target for DM in the further.

Disclosure statement
No potential conflict of interest was reported by the authors.

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