Rhein protects pancreatic β-cells from dynamin-related protein-1-mediated mitochondrial fission and cell apoptosis under hyperglycemia

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ABSTRACT

Rhein, an anthraquinone compound isolated from rhubarb, has been shown to improve glucose metabolism disorders in diabetic mice. The mechanism underlying the protective effect of Rhein, however, remains unknown. Here, we demonstrate that Rhein can protect the pancreatic β-cells against hyperglycemia-induced cell apoptosis through stabilizing mitochondrial morphology. Oral administration of Rhein for 8 or 16 weeks in db/db mice significantly reduced fasting blood glucose level and improved glucose tolerance. Cell apoptosis assay using both pancreatic sections and cultured pancreatic β-cells indicated that Rhein strongly inhibited β-cell apoptosis. Morphological study showed that Rhein was mainly localized at β-cell mitochondria and Rhein could preserve mitochondrial ultrastructure by abolishing hyperglycemia-induced mitochondrial fission protein dynamin-related protein 1 (Drp1) expression. Western blot and functional analysis confirmed that Rhein protected the pancreatic β-cells against hyperglycemia-induced apoptosis via suppressing mitochondrial Drp1 level. Finally, mechanistic study further suggested that decreased Drp1 level by Rhein might be due to its effect on reducing cellular reactive oxygen species (ROS). Taken together, our study demonstrates for the first time that Rhein can serve as a novel therapeutic agent for hyperglycemia treatment, and Rhein protects pancreatic β-cells from apoptosis by blocking the hyperglycemia-induced Drp1 expression.
INTRODUCTION

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is an anthraquinone compound isolated from rhubarb which has been used for more than 2,000 years in China to treat constipation, gastrointestinal hemorrhage, and ulcers (1). In our previous work, we found that Rhein could improve glucose metabolism disorders in diabetic mice, and its effect on reducing blood glucose level was even stronger than rosiglitazone and benazepril (2; 3). Moreover, Rhein also inhibited apoptosis of islet cells and protected islet function (4). Employing mouse nonalcoholic fatty liver disease as an animal model associated with obesity, insulin resistance and inflammatory disorders, Sheng and co-workers (5) reported that Rhein could ameliorate fatty liver disease in diet-induced obese mice via negative energy balance, hepatic lipogenic regulation, and immunomodulation. Recent anti-hyperglycemic study by Chatterjee et al. (6) suggests that Rhein, as well as other natural inhibitors such as Aloins and Capparisine, may lay a foundation for a better anti-diabetic therapy. However, the mechanism underlying these protective effects of Rhein remains unclear.

Increasing evidence suggests that β-cell failure is the mainstay of the pathogenesis of type 2 diabetes (7). Although the precise mechanisms underlying the β-cell dysfunction in type 2 diabetes are not fully understood, hyperglycemia has been shown as a major factor to cause the β-cell apoptosis. Once hyperglycemia develops, the pancreatic β-cell is exposed to increased metabolic flux and associated cellular stress, leading to impairment of β-cell function and survival, a process called glucotoxicity (8; 9). In type 2 diabetes, hyperglycemia is commonly associated with deregulation of lipid metabolism and elevation of free fatty acids, which also contribute to β-cell dysfunction (8; 10). Moreover, high level of glucose can also amplify lipotoxicity (10). The thiazolidinedione PPAR-γ activator drugs, rosiglitazone and pioglitazone, have been widely used to suppress insulin resistance in type 2 diabetic patients (11). Although Rhein shows a similar or even better effect on reducing mouse blood glucose level than rosiglitazone, the underlying mechanism remains unclear. It has been known that mitochondrial fission and fusion modulators, dynamin-related protein 1 (Drp1) (12), Opal (13), Prohibitin (14) and Mitofusin (15) collectively controls the dynamic balance of mitochondria fission and fusion process, and consequent mitochondria functions. Previous studies have demonstrated that Drp1 plays an important role in promoting hyperglycemia-induced apoptosis of β-cells and neurons (12; 16; 17). Drp1 expression was increased drastically in islet β-cells under hyperglycemia...
conditions. Estaquier and co-workers (18) further demonstrated that inhibiting Drp1-mediated mitochondrial fission could selectively prevent the release of cytochrome c, a mediator of apoptosis, from mitochondria. In contrast to the mitochondria fission modulators, which are upregulated or activated by stress factors such as high concentration of glucose, mitochondria fusion modulators are generally reduced when cells are challenged with pro-apoptotic insults. Recent studies by Kushnareva et al. (19) and Leboucher et al. (15) showed that stress-induced loss of Opa1 and mitofusin can facilitate mitochondrial fragmentation and cell apoptosis. However, it remains to be determined whether Rhein executes its protective role in pancreatic β-cells through regulating the expression or activation of these mitochondria fission/fusion modulators.

In the present study, we employed db/db mice and a pancreatic β-cell line (NIT-1) to study the protective effect of Rhein. Our results showed that Rhein largely localized at mitochondria in the β-cells and that it strongly protected pancreatic β-cells from hyperglycemia-induced apoptosis through suppressing Drp1 activation and Drp1-mediated mitochondria fission.
RESEARCH DESIGN AND METHODS

Cells, antibodies and reagents.

A mouse pancreatic β-cell line (NIT-1) was obtained from American Type Cell Culture Collection (Rockville, MD, USA). NIT-1 cells were cultured in F-12 Ham's medium containing 10% fetal bovine serum (FBS). All antibodies were obtained from Cell Signaling (Beverly, MA) or Abcam (Cambridge, MA), and a mouse-insulin ELISA kit was purchased from Millipore (Billerica, MA, USA). Mito Tracker red ROS was purchased from Invitrogen (Carlsbad, CA). Rhein, purified by alkali extraction and acid precipitation described previously (20), was a gift from Dr. Guangji Wang (China Pharmacology University, Nanjing, China). Reversed-phase HPLC assay showed the purity of Rhein (batch number 0403A) was over 98%. H$_2$O$_2$ solution (30%), N-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, MO). Various lentiviruses, including those expressing Drp1, GFP, Drp1 siRNA, and scramble control, were obtained from Invitrogen (Carlsbad, CA).

Lentiviral infection

NIT-1 cells or primary islet cells isolated from db/m were seeded in 6-well tissue culture plates. Lentivirus stock was added to the cells together with polybrene (a final concentration of 8µg/ml), and incubated for 6 h before switched to F-12 Ham's medium. More than 90% of the cells were infected as shown by GFP expression after 72 h.

The distribution of Rhein in NIT-1 cells

NIT-1 cells were cultured in F-12 Ham's medium containing 75 nM mitochondrial fluorescent probe MitoTracker RED CMXRos for 30 min at 37°C. The cells were washed three times with PBS, followed by incubation with 1 µg/ml of Rhein for 5 min and PBS wash for three times. The cells were viewed under LSM 510 laser scanning confocal microscope (Carl Zeiss, Germany).

Mitochondrial Morphology

The cells were treated with 1 µg/ml Rhein for 3 days in F-12 Ham's medium containing 5.5 or 33.3 mM glucose at 37°C with 5% CO$_2$ in the incubator, and then incubated with 75 nM MitoTracker Red for 20 min.
Cells labeled with mitochondrial fluorescent probe MitoTracker RED CMXRos were analyzed by confocal microscopy. Fragmented mitochondria were shortened, punctate, and sometimes rounded, whereas filamentous mitochondria showed a thread-like tubular structure (21). In most cases, the mitochondria within one cell were either filamentous or fragmented. In rare cases that mitochondria displayed mixed morphologies, we classified it based on the morphology of majority (> 70%) of mitochondria.

Electron microscopy
Pancreatic cells treated with or without 1 µg/ml Rhein for 3 days in medium containing 5.5 or 33.3 mM glucose or a small piece of pancreas (1-mm³ cubes) were fixed in 2.5% glutaraldehyde solution. The samples were then processed following standard protocol, including dehydration, embedding and sectioning, and then examined and photographed under a Hitachi 7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Measurement of ATP evels, mitochondrial membrane potential and insulin secretion
After incubation for 3 days in the presence or absence of 1µg/ml Rhein in F-12 Ham's medium containing 5.5 or 33.3 mM glucose at 37°C, NIT-1 or groups of 5 islets with a similar mass were incubated for 1 h in medium with 5.5 mM or 33.3 mM glucose at 37°C. ATP was extracted in 0.1% trichloroacetic acid and neutralized in 0.1 M Tris acetate. ATP levels were determined using an ATP Bioluminescence Assay kit (Beyotime, China, Cat.#. S0026) according to the manufacturer’s protocol. To measure mitochondrial membrane potential, NIT-1 or groups of 5 islets were loaded with JC-1 (Invitrogen, Carlsbad, CA) by incubation with 5.5 or 33.3 mM glucose at 37°C for1 h. Fluorescence was measured in a Flexstation II plate reader (Molecular Device, Union City, CA) with first at lengths of excitation and emission 530 nm/580 nm (“red”) and then at 485 nm/530 nm (“green”). The ratio of red to green reflects the ∆ψm. NIT-1 or batches of 5 isolated islets were performed secretion tests straight out of the cultured in 200 µl of F-12 Ham's medium (1% FBS) with 5.5 or 33.3 mM glucose at 37°C in 5% CO₂, 95% air, in 96-well plates for 1 h. 10 µl of medium was collected from each culture for insulin secretion. The remaining medium was aspirated, cells or islets were washed twice with PBS, and 20 µl of ice-cold 0.1% Triton X-100 was added. Cell lysates were collected and assayed for protein, islet lysates were assayed for insulin content.
Cell viability assay

The cells were cultured with F-12 Ham's medium containing 5.5 or 33.3 mM glucose in the presence or absence of 1 µg/ml Rhein for 72 h. At the end of treatment, 20 µl of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (5 mg/ml in PBS) was added to each well. The plates were then incubated at 37°C for 4 h in the dark. The medium was removed and MTT reduction product dissolved in Dimethyl Sulfoxide was added. The absorbance at 490 nm was measured using a microplate reader.

Cytochrome C assay

NIT-1 cells were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100. For cytochrome C staining, cells were stained with rabbit anti-mouse cytochrome C (1:100; Abcam), followed by treatment with FITC-conjugated swine anti-rabbit IgG (1:50; Dako). Cells were then counterstained with 100 nM propidium iodide (PI) and then observed and imaged by confocal microscopy (LSM 510; Carl Zeiss, Germany).

Cell apoptosis assay

For Annexin V-FITC labeling, NIT-1 cells or primary islet cells were cultured for 72 h in medium containing 5.5 or 33.3 mM glucose in the presence or absence of 1 µg/ml Rhein. The cells were washed once in PBS and incubated with 5µl (20 µg/ml) Annexin V-FITC according to the manufacturer’s instruction. The cells were incubated with PI (50 µg/ml) for 5 min on ice and then analyzed by flow cytometry (FACS Aria, Becton Dickinson, San Jose, CA). This assay discriminates between intact cells, early apoptotic cells, and late apoptotic or necrotic cells.

Determination of reactive oxygen species (ROS) production

Intracellular ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), as described (22). NIT-1 cells or groups of 5 islets were stimulated with 33.3 mM of glucose and Rhein (1 µg/mL) for 3 days. After the treatment, cells and islets were incubated with 20 uM of DCFH-DA at 37°C. The NIT-1 cells were measured by flow cytometry with the emission at 525 nm (FACS Aria, Becton Dickinson, San Jose, CA). More than 10,000 cells were acquired for
each sample and the content of ROS was assessed by mean fluorescence intensity (MFI). Green fluorescence derived from islet ROS generation was monitored using a plate-reader fluorometer.

**Animal experiments**

Animal maintenance and experimental procedures were carried out in accordance with the US National Institute of Health Guidelines for Use of Experimental Animals and approved by the Animal Care Committee of Nanjing University (Nanjing, China). Male db/db diabetic mice in C57BL6 background and their nondiabetic littermate control db/m mice (4 weeks old) were obtained from the Jackson Laboratory. Mice were housed in a room at a constant temperature of 22 ± 2°C with 12-h light/dark cycles. Two groups of mice each containing 3 subgroups (db/db, db/db + Rhein, and db/m, n = 6 in each subgroup). The db/db mice in the first group were treated with Rhein (120 mg/kg) or vehicle by oral administration for 8 weeks and then killed by exsanguination under anesthesia after blood samples were collected. Body weight and fasting blood glucose levels were measured every week. For oral glucose tolerance test, mice with or without Rhein treatment received an oral glucose challenge (1 g/kg). Blood samples were collected from the tail vein at 0, 30, 60, and 120 min. Blood glucose levels were determined using the blood glucose meter from Johnson (Milpitas, CA). In the second group, db/db mice were treated with Rhein (120 mg/kg) or vehicle in the same way, and subjected to islet TUNEL assay and insulin-positive cell analysis at 12 and 20 weeks of age, respectively. The distal portion of each pancreas was fixed overnight in 10% formalin. Serial paraffin sections were cut at a thickness of 5 µm. For data analysis, 6 mice per group and minimum of 3 sections per mouse were blindly examined. For TUNEL Assay, the apoptotic pancreatic β-cells were detected using In Situ Apoptosis Detection Kit (Roche) on paraffin-imbedded pancreatic sections. To determine the total number of insulin-positive cells per section, sections were immunostained with mouse anti-insulin antibody (1:1500, Abcam). The insulin-positive cells were counted for a total of 20 islets in each subgroup.

**Western blotting**

NIT-1 cells or primary islet cells were lysed in RIPA buffer at 4°C and then centrifuged at 12,000 g for 10 min at 4 °C. The lysates were boiled for 5 min in sample buffer, separated by SDS-PAGE and transferred to PVDF using standard procedures. The blot was incubated with anti-Drp1 antibody (Abcam), p(616)-drp1
(Cell Signal Technologies) and anti-caspase 3 (Cell Signal Technologies) followed by horseradish peroxidase–conjugated secondary antibodies (Abcam). The detection of signal was performed with ECL Western blotting detection system (Rockford, IL).

**Pancreatic islet isolation, culture, and lysate preparation**

Islets were isolated from male C57BL6 db/db or db/m mice by collagenase digestion and Histopaque density gradient centrifugation as described (23). Some the isolated islets were lysed in RIPA buffer at 4°C and then centrifuged at 12,000 g for 10 min at 4°C for p(616)-Drp1, Drp1 and Opa1 expression with immunoblotting. Some isolated islets from db/m mice were dissociated into single cells by trypsin for ROS and apoptosis assay. The rest were used for insulin secretion assay after cultured overnight at 37°C in 5% CO₂ in F-12 Ham's medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 1% FBS.

**Statistical analysis**

The data shown are presented as the mean ± standard error of three or more independent experiments. Differences are considered statistically significant at \( p<0.05 \), assessed using the Student’s \( t \) test (for paired samples) or the ANOVA test (for more than two groups).
RESULTS

Rhein decreased the fasting blood glucose (FBG) and improved glucose intolerance in db/db mice

To test whether Rhein can improve mouse glucose intolerance, we administered db/db mice with or without 120 mg/kg Rhein orally for 8 weeks, and their wild-type (WT) littermates db/m as control. Six mice for each group were used. The data clearly showed that, although the body weight of db/db mice treated with Rhein was slightly decreased compared to that of db/db mice without Rhein treatment (Supplemental Figure S1), administration of Rhein effectively decreased the fasting blood glucose (FBG) and improved glucose intolerance in db/db mice (Figure 1). We also observed convalescent phase FBG in db/db mice for 4 weeks after stopping Rhein treatment, and to our surprise, we found that the FBG was maintained at a normal level.

We next performed experiments to determine the pharmacokinetics and tissue distribution of Rhein in animals. As shown in Supplemental Figure S2A, when 70 mg/kg Rhein was used, Rhein had a half-life of about 4.30 ± 1.55 h in rat plasma. The measurement of the plasma protein binding rate of Rhein implicated that Rhein had a high binding rate (~ 93.46 ± 1.10%) with plasma protein, which may allow animals to maintain a considerable basal concentration of Rhein in the plasma. In addition, the metabolism analysis on Rhein showed that, after 60 h, the total elimination of Rhein through rat urine, faeces and bile acids was less than 25% of total amount of Rhein administered, suggesting that Rhein is mainly metabolized or re-incorporated within the animal body. The tissue distribution study by HPLC-MS clearly showed that Rhein was accumulated in pancreas, as well as various rat organs (Supplemental Figure S2B), indicating that Rhein is rapidly delivered to and accumulated in various animal organelles.

Rhein inhibits islet β-cell apoptosis induced by hyperglycemia

To determine whether Rhein can benefit long-term cell survival, we monitored pancreatic β-cell apoptosis by TUNEL assay. As shown in Figures 2A and 2B, pancreatic β-cell apoptosis in db/db mice at 20 weeks was significantly higher than that in db/db mice at 12 weeks. However, compared to the vehicle control, Rhein treatment strongly reduced islet β-cell apoptosis in db/db mice. In agreement with this, by staining the consecutive sections with anti-insulin antibody, we found that Rhein treatment also significantly preserved the loss of insulin-positive β-cells in db/db mice (Figure 2C). To confirm that islets isolated from Rhein-treated
*db/db* mice at 20 weeks still maintain insulin secretory function, we isolated and primarily cultured islets from *db/db* mice treated with or without Rhein for insulin assay. Compared to *db/db* mice without Rhein treatment, mice treated with Rhein showed a significantly higher insulin secretion at either basal level or induced by high concentration of glucose (HG) (Figure 2D).

The protective effect of Rhein on pancreatic β-cells was further tested using cultured NIT-1 cells. As shown in Figure 3A, the viability of NIT-1 cells significantly decreased under high concentration of glucose. However, the decrease of cell viability was prevented by Rhein treatment. We next examined the release of cytochrome C in the presence of high glucose. Previous studies have shown that, under stress such as high glucose, cytochrome C can be released from mitochondria into the cytosol, and the release of cytochrome C to cytosol can result in activation of caspase cascade, leading to cell apoptosis (24; 25). In agreement with this, we found the significant amount of relocation of cytochrome C from mitochondria to cytoplasm in NIT-1 cells (Figure 3B). The level of activated caspase 3 (cleaved form) was also elevated under high glucose condition. In contrast, the HG-induced cytochrome C relocation from mitochondria to cytoplasm and activation of caspase 3 were both strongly abolished by Rhein treatment. To quantify the protective effects of Rhein on pancreatic β-cells, we assessed the cell apoptosis by analyzing Annexin V/PI staining using flow cytometry. As shown in Figures 3D, HG induced nearly 30% apoptosis in NIT-1 cells, whereas Rhein treatment strongly suppressed HG-induced cell apoptosis.

**Rhein blocks mitochondrial fission in β-cells via suppressing Drp1 expression**

Taking advantage of autofluorescence of Rhein, we determined the localization of Rhein in pancreatic β cell and human renal HK2 cells. As shown in Figure 4A and Supplemental Figure S3, Rhein was largely localized in the cytoplasm of NIT-1 and HK2 cells, particularly at mitochondria as shown by the co-localization of Rhein with mitochondrial fluorescent probe, MitoTracker RED CMXRos. Specific localization of Rhein in mitochondria suggests that protective role of Rhein in pancreatic β cell or renal HK2 cell is likely through modulating mitochondria structure and function. Indeed, compared to mitochondria in control cells, which displaying a long, filamentous shape in the cytoplasm, NIT-1 cells and HK2 cells treated with HG showed a mitochondrial network broken down into small punctate organelles. The mitochondrial fragmentation induced
by high glucose, however, could be prevented by Rhein treatment. In support of this, we also observed the protective effect of Rhein on HG-induced disruption of mitochondrial membrane potential (Supplemental Figure S4) and ATP production (Supplemental Figure S5) in NIT-1 cells. To further analyze the possible mechanism underlying the protective effect of Rhein on mitochondria, we examined β-cell mitochondria at ultra-structural level (Figure 4B). We found that HG treatment induced marked deformation of mitochondria with dilation of the cristae in NIT-1 cells, whereas Rhein treatment largely reduced the number of mitochondria with aberrant cristae, and preserved the mitochondria with long shape and filamentous structure. Compared to pancreatic β-cells from db/m mice, the ones from db/db mice frequently showed swollen or vesicular mitochondria. In contrast, these swollen or vesicular mitochondria were seldom observed in db/db mice treated with Rhein. The percentage of cells with fragmented mitochondria was determined by cell counting. As shown in Figure 4C, Rhein treatment strongly prevented the hyperglycemia-induced mitochondria fragmentation in pancreatic β-cells. The dramatic morphological alteration of β-cell mitochondria in the presence or absence of Rhein suggests that the protective role of Rhein in pancreatic β-cells is likely due to its modulation of mitochondria fission. Mitochondrial fission has been previously shown to play a role in cell apoptosis (26).

Given that Drp1 is a key regulator of mitochondrial fission (27) and also responsible for cytochrome C release and caspase activation (18; 28; 29), we next determined whether Drp1 expression in pancreatic β-cell mitochondria was affected by high glucose and Rhein. As shown in Figure 5A, compared to low concentration of glucose control, HG strongly increased the β-cell Drp1 expression in NIT-1 cells, whereas this HG-induced Drp1 expression was largely abolished by Rhein treatment. Rhein treatment also inhibited the Drp1 expression in primary isolated islets from db/db mice (Figure 5B). We further detected the level of phosphorylated Drp1 (pDrp1) in NIT-1 cells and isolated islets. The results showed that the level of active Drp1 in NIT-1 cells (Supplemental Figure S6A) and islets (Supplemental Figure S6B) was also elevated by HG and the HG-induced upregulation of active Drp1 was blocked by Rhein. Mitochondria morphology is collectively modulated by several key molecules. Besides Drp1 which facilitates mitochondria fission, optic atrophy protein 1 (Opa1) (13) and mitofusin (15) promote mitochondria fusion. Previous studies had shown that loss of Opa1 and mitofusin resulted in cell mitochondrial fragmentation and sensitization to apoptotic insults (15;
We thus detected the level of Opa1 in β-cell treated with or without HG and Rhein, and the result showed that NIT-1 cell Opa1 level was not affected by HG and Rhein (Supplemental Figure S7). To test whether mitochondrial fragmentation, caspase 3 activation and β-cell apoptosis could be enhanced by directly increasing Drp1 expression level but reduced by decreasing Drp1 level, we either elevated Drp1 expression level in β-cells by infecting the cells with Drp1-expressing lentivirus, or decreased its expression level with lentivirus expressing Drp1-specific siRNA (with empty lentivirus and lentivirus expressing scramble oligonucleotide serving as controls, respectively) (Figure 5C). As expected, the analysis of mitochondria fragmentation (Figure 5D), glucose-stimulated insulin secretion (GSIS) (Figure 5E), cell apoptosis (Figure 5, F and G), and caspase 3 activation (Figure 5, H and I) confirmed the role of Drp1 in promoting mitochondrial fragmentation and caspase 3-dependent cell apoptosis in β-cells and reducing β-cell GSIS. Interestingly, since Drp1 upregulation or downregulation by infection with lentivirus expressing Drp1-GFP or Drp1 siRNA also largely abolished or enhanced the protective effect of Rhein on HG-induced cell apoptosis (Figure 5, F and G) and caspase 3 activation (Figure 5, H and I), the results are in agreement with that Rhein protects pancreatic β-cells from HG-induced dysfunction mainly through suppressing mitochondria Drp1 expression. Drp1 overexpression-induced cell apoptosis was also observed in primary pancreatic β-cells (Supplemental Figure S8).

**Rhein protected from high glucose-induced ROS production in β-cells.**

Cellular ROS have been widely shown to play a critical role in mediating mitochondrial dysfunction and cell apoptosis (30; 31). To investigate the effect of Rhein on hyperglycemia-induced ROS generation, NIT-1 cells and isolated rat islets were treated with or without 1 μg/ml Rhein in medium containing 5.5 or 33.3 mM glucose. As shown in Figure 6, the intracellular ROS levels were significantly increased in HG group compared with low concentration glucose control group. Interestingly, Rhein almost completely abolished the induction of ROS in NIT-1 cells (Figure 6A) and mouse islets (Figure 6B) by HG. Correlating with the ROS level, Drp1 expression was also increased in HG group compared with control group, and HG-induced Drp1 expression in either NIT-1 cells (Figure 6C) or islets (Figure 6D) was blocked by Rhein. HG-induced ROS level and Drp1 expression was also blocked by NADPH oxidase inhibitors, apocynin and diphenyl iodonium (data not shown). As expected, NAC and H$_2$O$_2$, a bona fide antioxidant and pro-oxidant, respectively, strongly
reduced and enhanced ROS levels in NIT-1 cells (Figure 6A) and islets (Figure 6B), as well as the expression of Drp1 (Figure 6, C and D). The inhibition of HG-induced Drp1 expression in pancreatic β-cells and isolated islets by Rhein and ROS inhibitors suggests that Drp1 induction under hyperglycemia may be, at least partially, dependent upon the ROS production.
DISCUSSION

Our previous study has shown that Rhein can improve glucose tolerance in db/db mice by restoring early phase insulin secretion and inhibiting apoptosis of islet cells (4). However, the underlying mechanism of protective role of Rhein remains unclear. In the present study, we demonstrate that Rhein is mainly localized at mitochondria in pancreatic β-cells and can prevent hyperglycemia-induced β-cell apoptosis through suppressing ROS production thereby Drp1 expression.

During progression of type 2 diabetes, glucotoxicity is an important factor that contributes to advancing pancreatic β-cell failure and development of diabetes (32). In db/db mice, we found that Rhein could effectively decreased FBG and FBG remained at a normal level after stopping Rhein for 4 weeks, indicating that Rhein has a prolonged effect on prevention of relapse after withdrawal. It is generally believed that mitochondria play a major role in insulin secretion in pancreatic β-cells. During the course of glucose-stimulated insulin secretion, glucose metabolism generates ATP in mitochondria and increases ATP/ADP ratio in β-cells. The elevation of ATP/ADP ratio results in the closure of KATP channels, depolarizing the β-cells, increasing Ca2+ influx and ultimately stimulating insulin secretion (33; 34). Mitochondrial failure in β-cells has emerged as an important step in the pathogenesis of type 2 diabetes (35; 36). In support of the concept that Rhein modulates mitochondria structure and function, studies have shown that Rhein, at higher concentration, can induce apoptosis in certain cancer cells through mitochondrion-dependent pathways (37; 38). Taking advantage of autofluorescence property of Rhein, we observed the cellular localization of Rhein and found that Rhein was largely co-localized with mitochondrial fluorescent probe in NIT-1 cells, implicating a role of Rhein in mitochondria.

Mitochondria are a class of dynamic organelles, constantly undergoing fission and fusion (26; 39; 40). The shape of mitochondria is thus controlled by fission and fusion events. Under normal conditions, fusion process prevails and as a result, mitochondria assume a long or filamentous morphology. However, when a cell is under stress, the dynamic balance will shift to fission, leading to punctate fragments of mitochondria. Mitochondrial fission requires the activation of a Drp1, a GTPase that causes scission of mitochondrial outer membrane and fission of mitochondrial tubules into fragments (27; 41). In agreement with previous findings
Drp1 level in cultured NIT-1 cells was significantly increased under high glucose, compared to controls with normal glucose levels; whereas Rhein treatment strongly abolished the elevation of Drp1 level induced by high glucose. A similar inhibitory effect of Rhein on Drp1 was also observed in db/db mice, in which Drp1 expression in mouse islet was markedly decreased by Rhein treatment. Furthermore, we found that the number of fragmented mitochondria in NIT-1 cells under HG was significantly decreased by the treatment with Rhein. Drp1 is also responsible for cytochrome c release and caspase activation (18; 28; 29). Whether Drp1 could induce islet β-cell apoptosis, however, is unknown. By infecting NIT-1 cells with lentivirus expressing GFP-Drp1 or Drp1 siRNA to upregulate or downregulate Drp1 expression level, we found that downregulation of Drp1 expression decreased mitochondrial fission and cell apoptosis, whereas up-regulation expression of Drp1 increased mitochondrial fission and cell apoptosis. The elegant work by Goyal et al. (43) suggests that Drp1 knockdown delays but does not prevent cell apoptosis. We thus performed a longer time cell apoptosis assay using primary β cells. As shown in Supplemental Figure S9, HG-induced β cell apoptosis, albeit to less degree, was observed in the cells treated with Drp1 siRNA at 72 h incubation. However, this delayed apoptosis of primary β cells was further reduced by Rhein. The result suggests that Rhein can also protect pancreatic β cells from HG-induced apoptosis through a mechanism independent of Drp1. Our further study also confirmed that Rhein prevents mouse primary β cells from HG-induced apoptosis through its antioxidant activity. As shown in Supplemental Figure S10, primary β cells treated with HG+Drp1-siRNA+Rhein displayed a decreased apoptosis compared to cells treated with HG+Drp1-siRNA (A), and ROS level in primary β cells was affected by HG but not Drp1-siRNA (B).

It has been reported that excess cellular ROS can cause the perturbations in mitochondrial function and play a role in the pathogenesis of diabetes complications (44; 45). The generation of ROS in response to the high concentrations of glucose may also cause mitochondrial dysfunction and trigger β-cells apoptosis (46). In support of this, altered mitochondrial dynamics was found to be associated with increased mitochondrial reactive oxygen species production (47; 48). In the present study, we demonstrated that Rhein could decrease the ROS production induced by high concentration of glucose, and that Drp1 expression was positively linked to the cellular ROS level, suggesting that Rhein might protect mitochondrial function through depleting cellular ROS. In conclusion, our study presents the first evidence that Rhein prevents hyperglycemia-induced pancreatic β-cell apoptosis by blocking ROS-Drp1-mitochondrial fission-apoptosis pathway.
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JL, ZC, YZ, XZ, YF and MZ researched and analyzed data. ZL, SS and KZ contributed to discussion and reviewed and edited the article. ZL and KZ designed experiments. JL and KZ wrote the manuscript.

KZ is the person who take full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.
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Figure 1. Effects of Rhein on fasting blood glucose (A) and glucose intolerance (B) in db/db mice. Mice were orally treated with Rhein (120 mg/kg) or vehicle for 8 weeks. Fasting blood glucose (FBG) was measured weekly. After the last administration, half of them were used for oral glucose intolerance experiment, and the others given a 1-month recovery period in which FBG was also determined weekly. Mice treated with or without Rhein received an oral glucose challenge (1 g/kg). Blood samples were collected from the tail vein at 0, 30, 60, and 120 min. Blood glucose levels were determined using the blood glucose meter. Data are presented as mean ± SD (n=6) (*, p < 0.05, compared to mice treated with vehicle control).
Figure 2. Protective effect of Rhein on pancreatic β-cell apoptosis and function. A) Representative images of β-cell apoptosis in pancreatic sections examined by TUNEL assay (brown color). Pancreatic tissues were collected from db/db mice at 12 and 20 weeks of age. To identify the β-cells, we stained the consecutive pancreatic sections with anti-insulin antibody (green color). B) Quantitative analysis of β-cell apoptosis in TUNEL assay. C) Analysis of the number of insulin-positive cells per islet. D) After primary islet isolation at 20 weeks, batches of 5 islets with a similar mass were hand-picked under microscope and cultured in 200μl F-12 Ham’s medium (1% FBS) with 5.5 or 33.3 mM glucose for 1 h. After incubation, 10 μl of medium was collected from each culture condition for insulin content assay. R, Rhein. *, p < 0.05.
Figure 3. Effect of Rhein on high concentration of glucose (HG)-induced NIT-1 cell apoptosis. The cells were cultured in medium containing 5.5 mmol/l or 33.3 mmol/l glucose in the presence or absence of 1 µg/ml Rhein for 3 days at 37°C. A) Cell viability determined by MTT assay. B) The release of cytochrome C. Green and red fluorescence correspond to cytochrome C and mitochondria, respectively. C) Level of cleaved caspase 3 in NIT-1 cells. D) Apoptosis was assessed by flow cytometry after Annexin V/PI staining. The data were presented as the mean ± SD of three independent experiments. R, Rhein. *, p < 0.05.
Figure 4. Rhein inhibits high glucose or hyperglycemia-induced mitochondria fission. A) The distribution of Rhein in NIT-1 cells and the changes in mitochondrial morphology induced by HG (33.3 mM). Note that Rhein was largely co-localized with mitochondrial fluorescent probe MitoTracker RED CMXRos at mitochondria, and Rhein treatment prevented NIT-1 cells from HG-induced mitochondrial fragmentation and preserved the filamentous shape of mitochondria (magnification, ×1000). B) Representative EM images showing that, HG (33.3 mM) treatment induced marked deformation of the mitochondria with dilation of the cristae in NIT-1 cells, while with Rhein treatment the long and filamentous shape of mitochondria was preserved; Compared to islet cells from db/m mice, swollen or vesicular mitochondria were frequently observed in islet cells from db/db mice, but rarely in the islet cells from Rhein-treated db/db mice. C) Quantitative analysis of effects of Rhein on mitochondrial fragmentation in NIT-1 cells. Data were presented as mean ± SD, n = 3 (≥ 100 cells were counted in three independent experiments). R, Rhein. *, p < 0.05.
Figure 5. Rhein inhibits high concentration glucose (HG)-induced Drp1 expression in pancreatic β-cells and Drp1-mediated cell apoptosis. A) Rhein inhibited Drp1 induction in NIT-1 cells by HG. B) Drp1 level in primary isolated islet β-cells was markedly decreased in Rhein-treated db/db mice compared to db/db mice treated with vehicle control. C) Downregulation and upregulation of Drp1 in NIT-1 cells by infections with lentiviruses expressing Drp1 siRNA and Drp1, respectively. D) Effect of Drp1 upregulation on mitochondrial fragmentation in NIT-1 cells. E) Effect of Drp1 downregulation and upregulation on NIT-1 cell GSIS. F) Effect of Drp1 downregulation on HG-induced NIT-1 cell apoptosis in the presence or absence of Rhein. G) Effect of Drp1 upregulation on HG-induced NIT-1 cell apoptosis in the presence or absence of Rhein. H) Effect of Drp1 downregulation on HG-induced NIT-1 cell caspase 3 activation in the presence or absence of Rhein. I) Effect of Drp1 upregulation on HG-induced NIT-1 cell caspase 3 activation in the presence or absence of Rhein.

Data were presented as mean ± SD (n=3). R, Rhein. *, p < 0.05. **, p < 0.01.

25x15mm (300 x 300 DPI)
Figure 6. Rhein suppressed high glucose-induced Drp1 expression via decreasing cellular ROS production. ROS generation was examined in the presence of Rhein or antioxidants NAC (0.4 mM) or pro-oxidants H2O2 (1µM). Cellular ROS level was determined by flow cytometry. A) NIT-1 cells were cultured for 30 min with NAC or H2O2 before stimulation with HG for 3 days. B) Primary pancreatic β cell from db/m were cultured for 30 min with NAC or H2O2 before stimulation with HG for 3 days. C) NIT-1 cell Drp1 levels determined by western blotting. D) Primary pancreatic β cell Drp1 levels determined by western blotting. Note that both Rhein and antioxidants significantly inhibited the HG-induced Drp1 expression. Results represent the mean ± SD from three independent experiments. *, p<0.05.
Supplemental Figure S1. Effect of Rhein on body weight of db/db mice. Note that the body weight of db/db mice treated with Rhein was only slightly decreased compared to that without Rhein treatment.
Supplemental Figure S2. The concentration–time curve of Rhein in rat plasma (A) and rat pancreas tissue distribution of Rhein (B). The plasma concentrations of rhein were measured by high performance liquid chromatography with postcolumn fluorescence derivatization (HPLC-FLD). In this experiment, the concentration of Rhein was 70mg/kg bodyweight. For A, total of 6 rats (named as 1, 2, 3, 4, 5 and 6) were used. For B, rat tissues were obtained at 1 h with or without Rhein treatment and then homogenated on ice. Purified Rhein was used as a standard control.
Supplemental Figure S3. The distribution of Rhein in human renal HK2 cells and the changes of mitochondrial morphology induced by HG (33.3 mM). Note that Rhein was largely co-localized with mitochondrial fluorescent probe MitoTracker RED CMXRos at mitochondria, and Rhein treatment protected HK2 cells from HG-induced mitochondrial fragmentation and preserved the filamentous shape of mitochondria (magnification, ×400).
Supplemental Figure S4. The effect of Rhein on mitochondrial membrane potential in NIT-1 cells (A and B) and isolated islets (C). Note that, compared with the control group, high concentration of glucose (HG) decreases the intensity of JC-1 aggregates (red) but increases the intensity of JC-1 monomers (green), whereas Rhein (R) treatment reverses the alteration of JC-1 aggregates and JC-1 monomers induced by HG. *, $P<0.05$. 
Supplemental Figure S5. The effect of Rhein on ATP production in NIT-1 cells (A) and primary islet β cells (B). Note that high concentration of glucose (HG) inhibits ATP production comparing to control group, whereas Rhein (R) restores the ATP production. *, $P < 0.05$. 
Supplemental Figure S6. Rhein treatment suppresses the active Drp1 (p-Drp1) levels induced by HG in both NIT-1 cells (A) and primary islet β cells (B). * P < 0.05.
Supplemental Figure S7. The level of Opa1 in isolated mouse islets detected by western blot. Note that Opa1 level in mouse pancreatic β cells is not affected by HG or HG plus Rhein.
Supplemental Figure S8. Forced expression of Drp1 in primary β cells results in cell apoptosis, which is not reversed by Rhein.* $P < 0.05$.
Supplemental Figure S9. Knockdown of Drp1 level via Drp1 siRNA transfection delays HG-induced primary β cell apoptosis. Note that HG-induced apoptosis of primary β cells transfected with Drp1 siRNA, which occurs at 72 h incubation, is further inhibited by Rhein. *, $P < 0.05$. 
Supplemental Figure S10. Rhein prevents mouse primary β cells apoptosis though its antioxidant activity. Note that primary β cells treated with HG+Drp1-siRNA+Rhein have a reduced apoptosis compared to cells treated with HG+Drp1-siRNA (A), and that ROS level in primary β cells is affected by HG but not Drp1-siRNA (B). *, P < 0.05.