Rhein Protects Pancreatic β-Cells From Dynamin-Related Protein-1–Mediated Mitochondrial Fission and Cell Apoptosis Under Hyperglycemia

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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 (FBG) 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. 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. ***Diabetes*** **62:3927–3935, 2013**

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is an anthraquinone compound isolated from rhubarb that 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). Using mouse nonalcoholic fatty liver disease as an animal model associated with obesity, insulin resistance, and inflammatory disorders, Sheng et al. (5) reported that rhein could ameliorate fatty liver disease in diet-induced obese mice via negative energy balance, hepatic lipogenous regulation, and immunomodulation. Recent antihyperglycemic study by Chatterjee et al. (6) suggests that rhein, as well as other natural inhibitors such as aloins and capparisine, may be a foundation for a better antidiabetic 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 levels of glucose can also amplify lipotoxicity (10). The thiazolidinedione peroxisome proliferator–activated receptor-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), optic atrophy protein 1 (Opa1) (13), prohibitin (14), and mitofusin (15), collectively control the dynamic balance of mitochondria fission and fusion processes 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 Arnoult (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 (HG), 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.

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In the current study, we used *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). NIT-1 cells were cultured in F-12 Ham's medium containing 10% 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). Mito Tracker red reactive oxygen species (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 high-performance liquid chromatography assay showed the purity of rhein (batch number 0403A) was over 98%. H_2O_2 solution (30%) and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, MO). Various lentiviruses, including those expressing Drp1, GFP, Drp1 small interfering RNA (siRNA), and scramble control, were obtained from Invitrogen.

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

Distribution of rhein in NIT-1 cells. NIT-1 cells were cultured in F-12 Ham's medium containing 75 nmol/L 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 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 mmol/L glucose at 37°C with 5% CO_2 in the incubator and then incubated with 75 nmol/L 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 mmol/L glucose or a small piece of pancreas (1-mm^3 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 levels, mitochondrial membrane potential, and insulin secretion. After incubation for 3 days in the presence or absence of 1 μg/mL rhein in F-12 Ham's medium containing 5.5 or 33.3 mmol/L glucose at 37°C, NIT-1 or groups of five islets with a similar mass were incubated for 1 h in medium with 5.5 or 33.3 mmol/L glucose at 37°C. ATP was extracted in 0.1% trichloroacetic acid and neutralized in 0.1 mol/L Tris acetate. ATP levels were determined using an ATP bioluminescence assay kit (Beyotime, China, catalog number S0026) according to the manufacturer's protocol. To measure mitochondrial membrane potential, NIT-1 or groups of five islets were loaded with JC-1 (Invitrogen) by incubation with 5.5 or 33.3 mmol/L glucose at 37°C for 1 h. Fluorescence was measured in a Flexstation II plate reader (Molecular Device, Union City, CA) first at lengths of excitation and emission 530/580 nm ("red") and then at 485/530 nm ("green"). The ratio of red to green reflects the $\Delta\psi m$. Secretion tests were performed on NIT-1 or batches of five isolated islets straight out of being cultured in 200 μL of F-12 Ham's medium (1% FBS) with 5.5 or 33.3 mmol/L glucose at 37°C in 5% CO_2, 95% air, in 96-well plates for 1 h. For insulin secretion, 10 μL of medium was collected from each culture. 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, and 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 mmol/L 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 antimouse cytochrome c (1:100; Abcam), followed by treatment with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (1:50; Dako). Cells were then counterstained with 100 nmol/L 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 mmol/L glucose in the presence or absence of 1 μg/mL rhein. The cells were washed once in PBS and incubated with 5 μL (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 ROS production. Intracellular ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe, 2′,7′-dichlorofluorescin diacetate, as described previously (22). NIT-1 cells or groups of five islets were stimulated with 33.3 mmol/L of glucose and rhein (1 μg/mL) for 3 days. After the treatment, cells and islets were incubated with 20 μmol/L 2′,7′-dichlorofluorescin diacetate at 37°C. The NIT-1 cells were measured by flow cytometry with the emission at 525 nm (FACS Aria). More than 10,000 cells were acquired for each sample, and the content of ROS was assessed by mean fluorescence intensity. 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 U.S. National Institutes 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. There were two groups of mice, each containing three 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 (FBG) 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, six mice per group and a minimum of three 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 (1:1,500, 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 radioimmunoprecipitation assay 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 polyvinylidene fluoride 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 previously (23). Some of the isolated islets were lysed in radioimmunoprecipitation assay 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% CO_2 in F-12 Ham's medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine, and 1% FBS.

Statistical analysis. The data shown are presented as the mean ± SE of three or more independent experiments. Differences are considered

statistically significant at $P < 0.05$, assessed using the Student t test (for paired samples) or the ANOVA test (for more than two groups).

RESULTS

Rhein decreased the 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 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 with that of *db/db* mice without rhein treatment (Supplementary Fig. 1), administration of rhein effectively decreased the FBG and improved glucose intolerance in *db/db* mice (Fig. 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 Supplementary Fig. 2*A*, when 70 mg/kg rhein was used, rhein had a half-life of ~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, feces, and bile acids was less than 25% of total amount of rhein administered, suggesting that rhein is mainly metabolized or reincorporated within the animal body. The tissue distribution study by high-performance liquid chromatography–mass spectrometry clearly showed that rhein was accumulated in pancreas, as well as various rat organs (Supplementary Fig. 2*B*), 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 Figure 2*A* and *B*, 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 with 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 (Fig. 2*C*). 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 with *db/db* mice without rhein treatment, mice treated with rhein showed a significantly higher insulin secretion at either basal level or induced by HG (Fig. 2*D*).

The protective effect of rhein on pancreatic β-cells was further tested using cultured NIT-1 cells. As shown in Figure 3*A*, the viability of NIT-1 cells significantly decreased under HG. 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 (Fig. 3*B*). The level of activated caspase 3 (cleaved form) was also elevated under high glucose conditions. 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 Figure 3*D*, 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 kidney proximal tubular (HK2) cells. As shown in Fig. 4*A* and

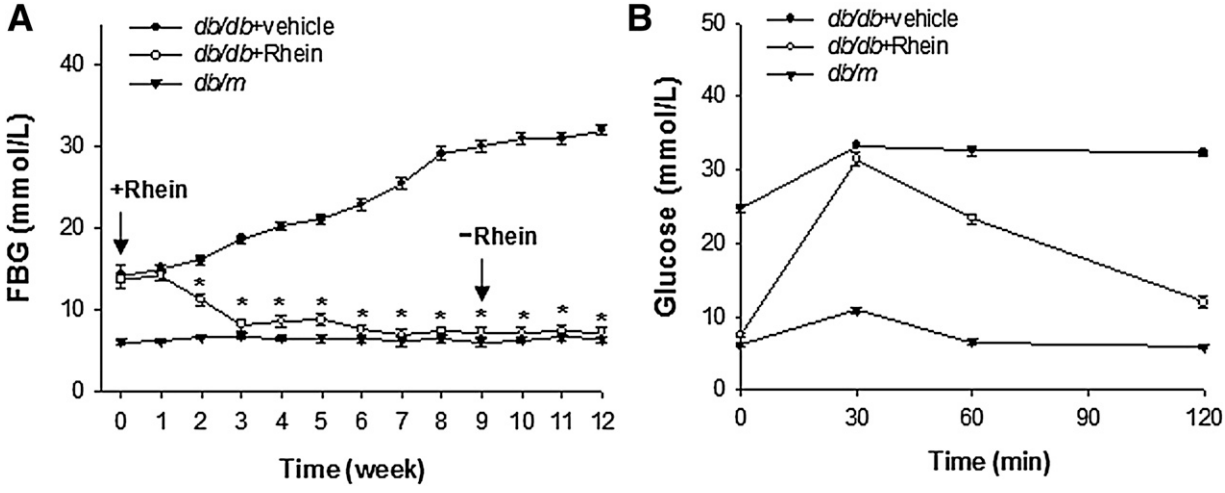

FIG. 1. Effects of rhein on FBG (*A*) and glucose intolerance (*B*) in *db/db* mice. Mice were orally treated with rhein (120 mg/kg) or vehicle for 8 weeks. FBG was measured weekly. After the last administration, half of them were used for oral glucose intolerance experiments and the others given a 1-month 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 with mice treated with vehicle control. 20 × 13 mm (300 × 300 DPI).

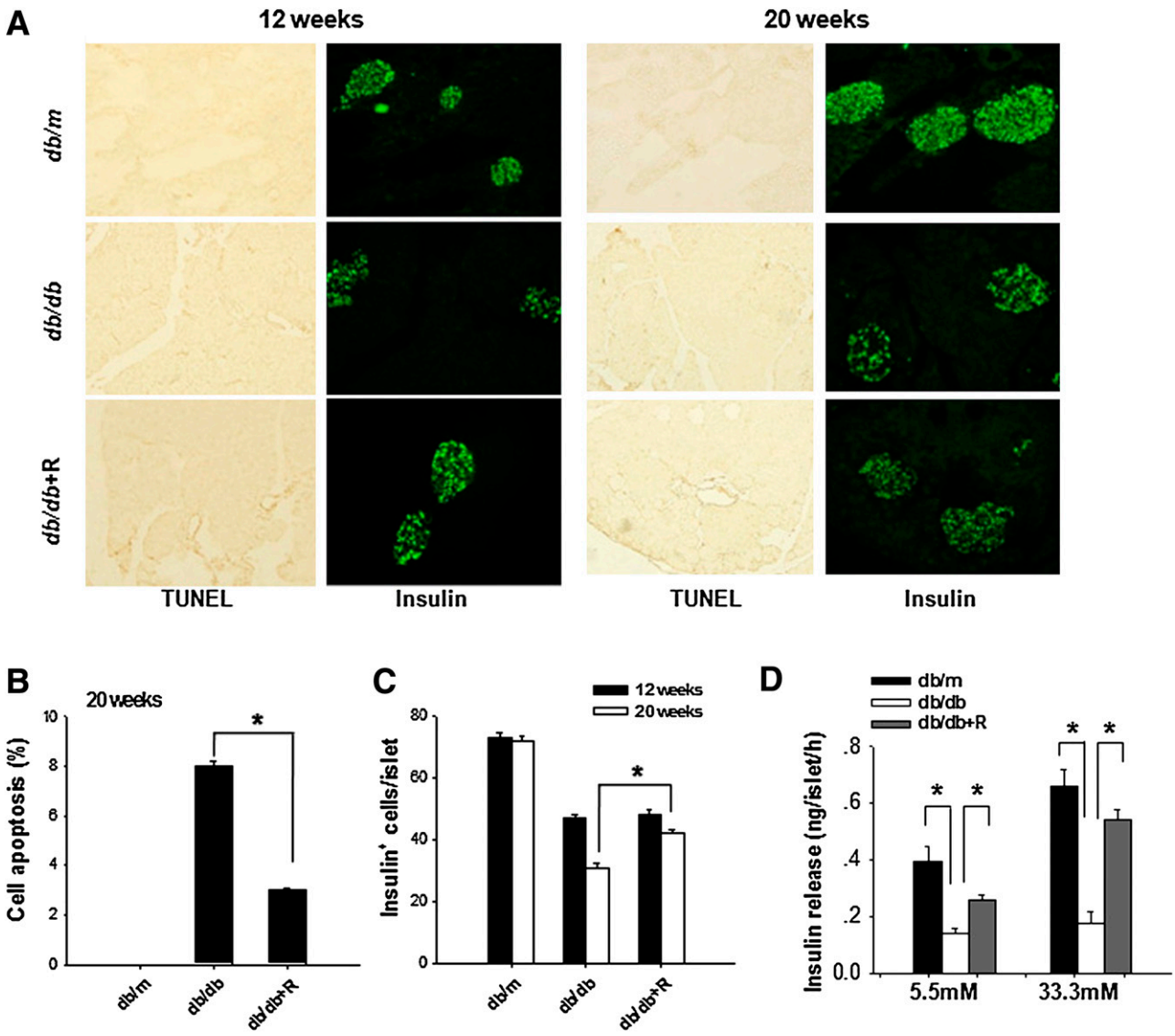

FIG. 2. Protective effect of rhein on pancreatic β-cell apoptosis and function. *A*: Representative images of β-cell apoptosis in pancreatic sections examined by TUNEL assay (brown color). Pancreatic tissues were collected from *db/db* mice at 12 and 20 weeks of age. To identify the β-cells, we stained 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 five islets with a similar mass were hand picked under microscope and cultured in 200 μL F-12 Ham's medium (1% FBS) with 5.5 or 33.3 mmol/L glucose for 1 h. After incubation, 10 μL of medium was collected from each culture condition for insulin content assay. *$P < 0.05$. 54 × 48 mm (300 × 300 DPI). R, rhein.

Supplementary Fig. 3, rhein was largely localized in the cytoplasm of NIT-1 and HK2 cells, particularly at mitochondria as shown by the colocalization of rhein with mitochondrial fluorescent probe, MitoTracker Red CMXRos. Specific localization of rhein in mitochondria suggests that a protective role of rhein in the pancreatic β-cell or renal HK2 cell is likely through modulating mitochondria structure and function. Indeed, compared with mitochondria in control cells, which display 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 (Supplementary Fig. 4) and ATP production (Supplementary Fig. 5) in NIT-1 cells. To further analyze the possible mechanism underlying the protective effect of rhein on mitochondria, we examined β-cell mitochondria at the ultrastructural level (Fig. 4*B*). 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 a long shape and filamentous structure. Compared with 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 4*C*, 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

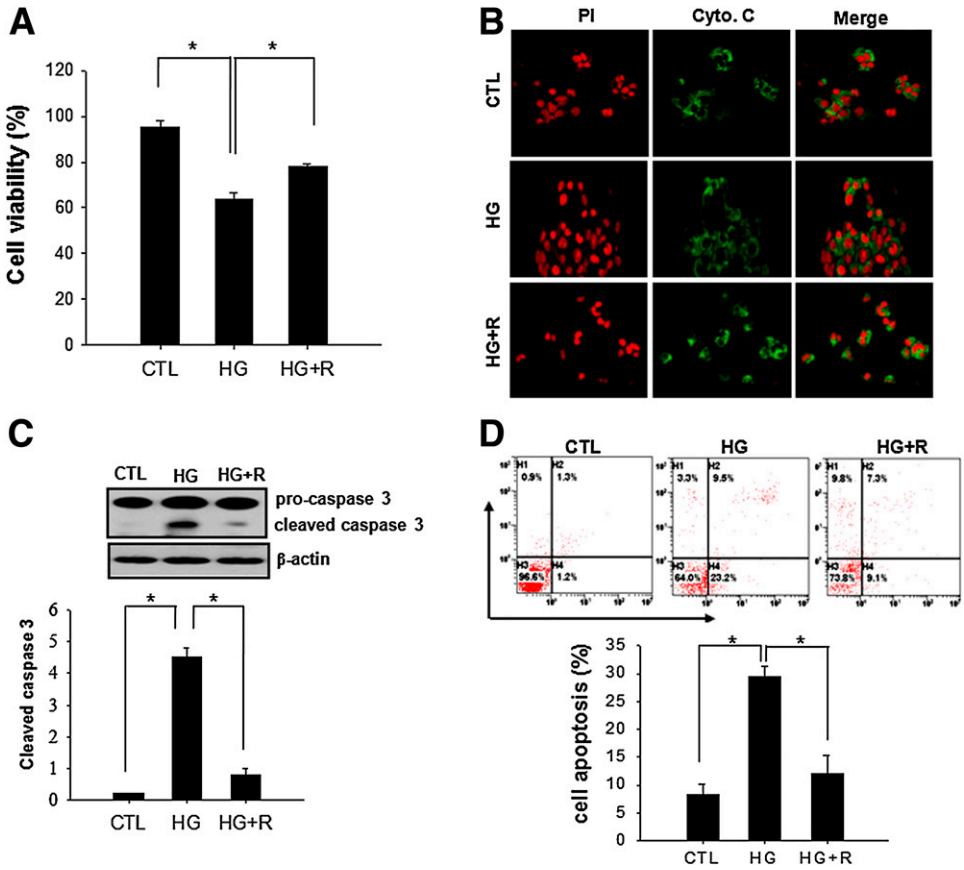

FIG. 3. Effect of rhein on HG-induced NIT-1 cell apoptosis. The cells were cultured in medium containing 5.5 or 33.3 mmol/L glucose in the presence or absence of 1 μg/mL rhein for 3 days at 37°C. *A*: Cell viability determined by MTT assay. *B*: The release of cytochrome c. Green and red fluorescence corresponds 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. *$P < 0.05$. 57 × 50 mm (300 × 300 DPI). CTL, control; Cyto, cytochrome; R, rhein.

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 Fig. 5*A*, compared with 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 (Fig. 5*B*). We further detected the level of phosphorylated Drp1 in NIT-1 cells and isolated islets. The results showed that the level of active Drp1 in NIT-1 cells (Supplementary Fig. 6*A*) and islets (Supplementary Fig. 6*B*) 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, 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 (15,19). We thus detected the level of Opa1 in β-cells treated with or without HG and rhein, and the result showed that NIT-1 cell Opa1 level was not affected by HG and rhein (Supplementary Fig. 7). 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) (Fig. 5*C*). As expected, the analysis of mitochondria fragmentation (Fig. 5*D*), glucose-stimulated insulin secretion (GSIS) (Fig. 5*E*), cell apoptosis (Fig. 5*F* and *G*), and caspase 3 activation (Fig. 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 (Fig. 5*F* and *G*) and caspase 3 activation (Fig. 5*H* and *I*), the results are in agreement 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 (Supplementary Fig. 8).

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

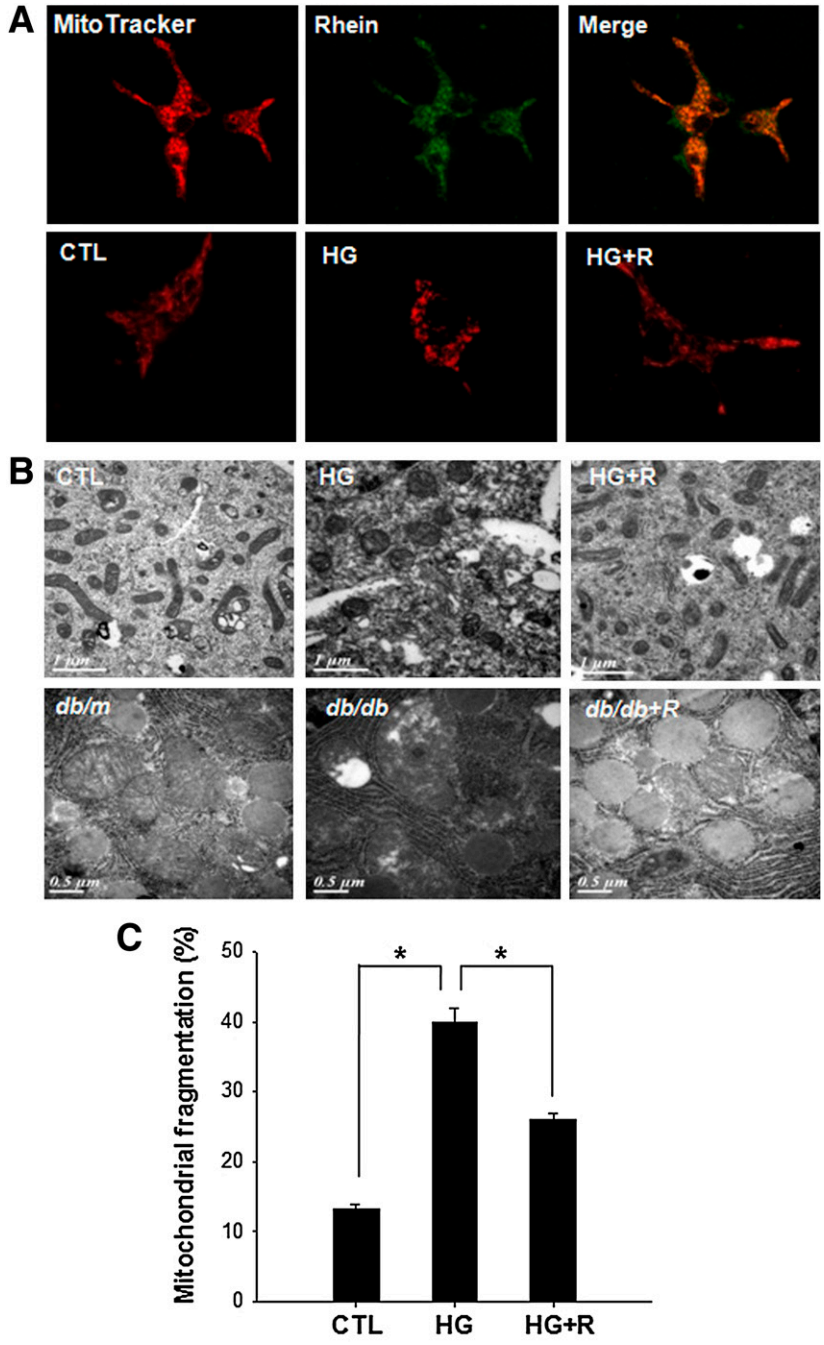

FIG. 4. Rhein inhibits high glucose or hyperglycemia-induced mitochondria fission. *A*: The distribution of rhein in NIT-1 cells and the changes in mitochondrial morphology induced by HG (33.3 mmol/L). Note that rhein was largely colocalized with mitochondrial fluorescent probe MitoTracker Red CMXRos at mitochondria, and rhein treatment prevented NIT-1 cells from HG-induced mitochondrial fragmentation and preserved the filamentous shape of mitochondria (original magnification ×1,000). *B*: Representative electron microscope images showing that HG (33.3 mmol/L) 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 with 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). *P < 0.05. 79 × 111 mm (300 × 300 DPI). CTL, control; R, rhein.

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 mmol/L glucose. As shown in Figure 6, the intracellular ROS levels were significantly increased in HG group compared with the low concentration glucose control group. Interestingly, rhein almost completely abolished the induction of ROS in NIT-1 cells (Fig. 6*A*) and mouse islets (Fig. 6*B*) by HG. Correlating with the ROS level, Drp1 expression was also increased in the HG group compared with the control group, and HG-induced Drp1 expression in either NIT-1 cells (Fig. 6*C*) or islets (Fig. 6*D*) 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

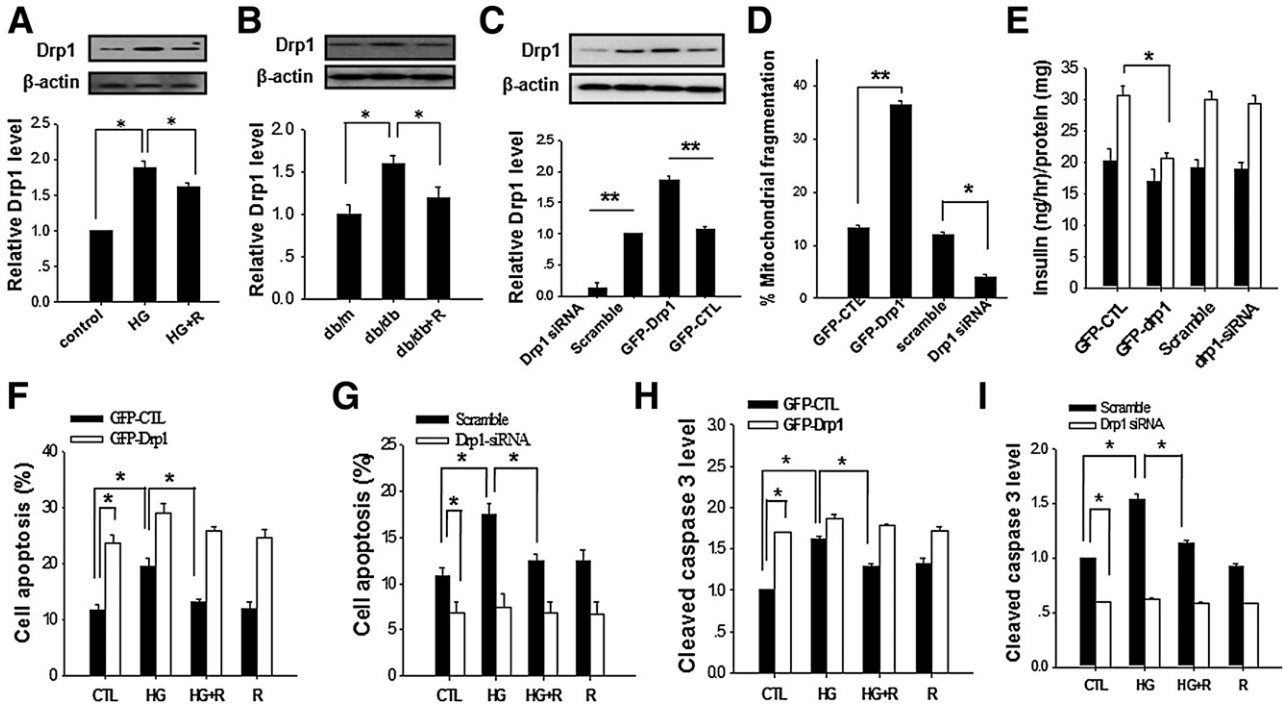

FIG. 5. Rhein inhibits HG-induced Drp1 expression in pancreatic β-cells and Drp-1-mediated cell apoptosis. *A*: Rhein inhibited Drp1 induction in NIT-1 cells by HG. *B*: Drp1 level in primary isolated islet β-cells was markedly decreased in rhein-treated *db/db* mice compared with *db/db* mice treated 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). *P < 0.05; **P < 0.01. 25 × 15 mm (300 × 300 DPI). CTL, control; hr, hour; R, rhein.

and H_2O_2, a bona fide antioxidant and a pro-oxidant, respectively, strongly reduced and enhanced ROS levels in NIT-1 cells (Fig. 6*A*) and islets (Fig. 6*B*), as well as the expression of Drp1 (Fig. 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 showed 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 a protective role of rhein remains unclear. In the current 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 and 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 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 GSIS, glucose metabolism generates ATP in mitochondria and increases the ATP/ADP ratio in β-cells. The elevation of the ATP/ADP ratio results in the closure of K_{ATP} channels, depolarizing the β-cells, increasing Ca^{2+} 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 concentrations, can induce apoptosis in certain cancer cells through mitochondrion-dependent pathways (37,38). Taking advantage of the autofluorescence property of rhein, we observed the cellular localization of rhein and found that rhein was largely colocalized 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, the 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 (12,42), Drp1 level in cultured NIT-1 cells was significantly increased under HG, compared with controls with normal glucose levels, whereas rhein treatment strongly abolished the elevation of Drp1 level induced by HG. A similar inhibitory effect of rhein on Drp1 was also observed in *db/db* mice, in which Drp1

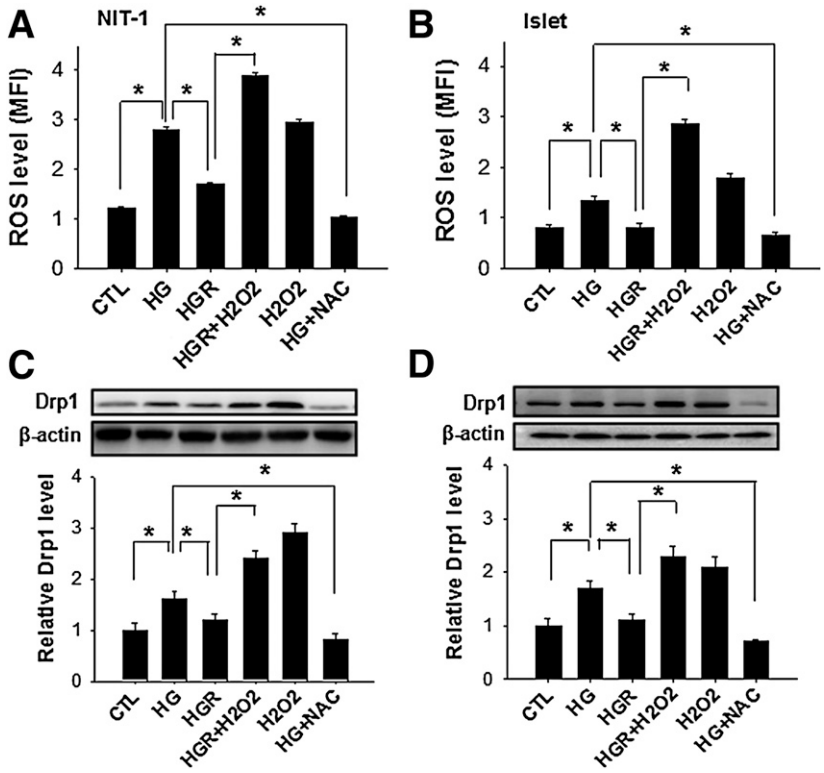

FIG. 6. Rhein suppressed high glucose–induced Drp1 expression via decreasing cellular ROS production. ROS generation was examined in the presence of rhein or antioxidants NAC (0.4 mmol/L) or pro-oxidants H_2O_2 (1 μmol/L). Cellular ROS level was determined by flow cytometry. *A*: NIT-1 cells were cultured for 30 min with NAC or H_2O_2 before stimulation with HG for 3 days. *B*: Primary pancreatic β-cell from *db/m* were cultured for 30 min with NAC or H_2O_2 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. *$P < 0.05$. 26 × 20 mm (300 × 300 DPI). CTL, control; MFI, mean fluorescence intensity.

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 upregulated 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 Supplementary Figure 9, 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 Supplementary Fig. 10, primary β-cells treated with HG + Drp1-siRNA + rhein displayed a decreased apoptosis compared with cells treated with HG + Drp1-siRNA (Supplementary Fig. 10*A*), and ROS level in primary β-cells was affected by HG but not Drp1-siRNA (Supplementary Fig. 10*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 ROS production (47,48). In the current study, we demonstrated that rhein could decrease the ROS production induced by HG 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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J.L., Z.C., Y.Z., M.Z., X.Z., and Y.F. researched and analyzed data. S.S., K.Z., and Z.L. contributed to discussion and reviewed and edited the article. K.Z. and Z.L. designed

experiments. J.L. and K.Z. wrote the manuscript. K.Z. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

REFERENCES

1. Huang Q, Lu G, Shen HM, Chung MC, Ong CN. Anti-cancer properties of anthraquinones from rhubarb. Med Res Rev 2007;27:609–630
2. Zheng JM, Zhu JM, Li LS, Liu ZH. Rhein reverses the diabetic phenotype of mesangial cells over-expressing the glucose transporter (GLUT1) by inhibiting the hexosamine pathway. Br J Pharmacol 2008;153:1456–1464
3. Jia ZH, Liu ZH, Zheng JM, Zeng CH, Li LS. Combined therapy of rhein and benazepril on the treatment of diabetic nephropathy in db/db mice. Exp Clin Endocrinol Diabetes 2007;115:571–576
4. Du H, Shao J, Gu P, Lu B, Ye X, Liu Z. Improvement of glucose tolerance by rhein with restored early-phase insulin secretion in db/db mice. J Endocrinol Invest 2012;35:607–612
5. Sheng X, Wang M, Lu M, Xi B, Sheng H, Zang YQ. Rhein ameliorates fatty liver disease through negative energy balance, hepatic lipogenic regulation, and immunomodulation in diet-induced obese mice. Am J Physiol Endocrinol Metab 2011;300:E886–E893
6. Chatterjee S, Narasimhaiah AL, Kundu S, Anand S. Anti hyperglycaemic study of natural inhibitors for Insulin receptor. Bioinformation 2012;8:1195–1201
7. Weyer C, Bogardus C, Mott DM, Pratley RE. The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. J Clin Invest 1999;104:787–794
8. Robertson RP, Harmon J, Tran PO, Poitout V. Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. Diabetes 2004;53(Suppl. 1):S119–S124
9. Kaiser N, Leibowitz G, Nesher R. Glucotoxicity and beta-cell failure in type 2 diabetes mellitus. J Pediatr Endocrinol Metab 2003;16:5–22
10. Poitout V, Robertson RP. Glucolipotoxicity: fuel excess and beta-cell dysfunction. Endocr Rev 2008;29:351–366
11. Defronzo RA. Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. Diabetes 2009;58:773–795
12. Men X, Wang H, Li M, et al. Dynamin-related protein 1 mediates high glucose induced pancreatic beta cell apoptosis. Int J Biochem Cell Biol 2009;41:879–890
13. Zhang Z, Wakabayashi N, Wakabayashi J, et al. The dynamin-related GTPase Opa1 is required for glucose-stimulated ATP production in pancreatic beta cells. Mol Biol Cell 2011;22:2235–2245
14. Lee JH, Nguyen KH, Mishra S, Nyomba BL. Prohibitin is expressed in pancreatic beta-cells and protects against oxidative and proapoptotic effects of ethanol. FEBS J 2010;277:488–500
15. Leboucher GP, Tsai YC, Yang M, et al. Stress-induced phosphorylation and proteasomal degradation of mitofusin 2 facilitates mitochondrial fragmentation and apoptosis. Mol Cell 2012;47:547–557
16. Leinninger GM, Backus C, Sastry AM, Yi YB, Wang CW, Feldman EL. Mitochondria in DRG neurons undergo hyperglycemic mediated injury through Bim, Bax and the fission protein Drp1. Neurobiol Dis 2006;23:11–22
17. Peng L, Men X, Zhang W, et al. Dynamin-related protein 1 is implicated in endoplasmic reticulum stress-induced pancreatic β-cell apoptosis. Int J Mol Med 2011;28:161–169
18. Estaquier J, Arnoult D. Inhibiting Drp1-mediated mitochondrial fission selectively prevents the release of cytochrome c during apoptosis. Cell Death Differ 2007;14:1086–1094
19. Kushnareva YE, Gerencser AA, Bossy B, et al. Loss of OPA1 disturbs cellular calcium homeostasis and sensitizes for excitotoxicity. Cell Death Differ 2013;20:353–365
20. Fang F, Wang JB, Zhao YL, et al. A comparative study on the tissue distributions of rhubarb anthraquinones in normal and CCl4-injured rats orally administered rhubarb extract. J Ethnopharmacol 2011;137:1492–1497
21. Dimmer KS, Scorrano L. (De)constructing mitochondria: what for? Physiology (Bethesda) 2006;21:233–241
22. Chen J, Chen S, Chen Y, et al. Circulating endothelial progenitor cells and cellular membrane microparticles in db/db diabetic mouse: possible implications in cerebral ischemic damage. Am J Physiol Endocrinol Metab 2011;301:E62–E71
23. Demozay D, Tsunekawa S, Briaud I, Shah R, Rhodes CJ. Specific glucose-induced control of insulin receptor substrate-2 expression is mediated via Ca2+-dependent calcineurin/NFAT signaling in primary pancreatic islet β-cells. Diabetes 2011;60:2892–2902
24. Trudeau K, Molina AJ, Guo W, Roy S. High glucose disrupts mitochondrial morphology in retinal endothelial cells: implications for diabetic retinopathy. Am J Pathol 2010;177:447–455
25. Li Y, Wu H, Khardori R, Song YH, Lu YW, Geng YJ. Insulin-like growth factor-1 receptor activation prevents high glucose-induced mitochondrial dysfunction, cytochrome-c release and apoptosis. Biochem Biophys Res Commun 2009;384:259–264
26. Molina AJ, Wikstrom JD, Stiles L, et al. Mitochondrial networking protects beta-cells from nutrient-induced apoptosis. Diabetes 2009;58:2303–2315
27. Frank S, Gaume B, Bergmann-Leitner ES, et al. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell 2001;1:515–525
28. Tanaka A, Youle RJ. A chemical inhibitor of DRP1 uncouples mitochondrial fission and apoptosis. Mol Cell 2008;29:409–410
29. Peng L, Men X, Zhang W, et al. Involvement of dynamin-related protein 1 in free fatty acid-induced INS-1-derived cell apoptosis. PLoS ONE 2012;7:e49258
30. Cai Y, Martens GA, Hinke SA, Heimberg H, Pipeleers D, Van de Casteele M. Increased oxygen radical formation and mitochondrial dysfunction mediate beta cell apoptosis under conditions of AMP-activated protein kinase stimulation. Free Radic Biol Med 2007;42:64–78
31. Nicholls DG, Budd SL. Mitochondria and neuronal survival. Physiol Rev 2000;80:315–360
32. Maedler K, Sergeev P, Ris F, et al. Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. J Clin Invest 2002;110:851–860
33. Pang Z, Wu N, Zhang X, et al. GPR40 is partially required for insulin secretion following activation of beta3-adrenergic receptors. Mol Cell Endocrinol 2010;325:18–25
34. Goehring I, Sauter NS, Catchpole G, et al. Identification of an intracellular metabolic signature impairing beta cell function in the rat beta cell line INS-1E and human islets. Diabetologia 2011;54:2584–2594
35. Maechler P, Wollheim CB. Mitochondrial function in normal and diabetic beta-cells. Nature 2001;414:807–812
36. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. Science 2005;307:384–387
37. Heo SK, Yun HJ, Park WH, Park SD. Rhein inhibits TNF-alpha-induced human aortic smooth muscle cell proliferation via mitochondrial-dependent apoptosis. J Vasc Res 2009;46:375–386
38. Lai WW, Yang JS, Lai KC, et al. Rhein induced apoptosis through the endoplasmic reticulum stress, caspase- and mitochondria-dependent pathways in SCC-4 human tongue squamous cancer cells. In Vivo 2009;23:309–316
39. Chan DC. Mitochondrial fusion and fission in mammals. Annu Rev Cell Dev Biol 2006;22:79–99
40. Yoon Y, Galloway CA, Jhun BS, Yu T. Mitochondrial dynamics in diabetes. Antioxid Redox Signal 2011;14:439–457
41. Zhang B, Alysandratos KD, Angelidou A, et al. Human mast cell degranulation and preformed TNF secretion require mitochondrial translocation to exocytosis sites: relevance to atopic dermatitis. J Allergy Clin Immunol 2011;127:1522–1531 e1528
42. Wang W, Wang Y, Long J, et al. Mitochondrial fission triggered by hyperglycemia is mediated by ROCK1 activation in podocytes and endothelial cells. Cell Metab 2012;15:186–200
43. Goyal G, Fell B, Sarin A, Youle RJ, Sriram V. Role of mitochondrial remodeling in programmed cell death in Drosophila melanogaster. Dev Cell 2007;12:807–816
44. Kaneto H, Kajimoto Y, Miyagawa J, et al. Beneficial effects of antioxidants in diabetes: possible protection of pancreatic beta-cells against glucose toxicity. Diabetes 1999;48:2398–2406
45. Kang D, Hamasaki N. Alterations of mitochondrial DNA in common diseases and disease states: aging, neurodegeneration, heart failure, diabetes, and cancer. Curr Med Chem 2005;12:429–441
46. Hodgin JB, Nair V, Zhang H, et al. Identification of cross-species shared transcriptional networks of diabetic nephropathy in human and mouse glomeruli. Diabetes 2013;62:299–308
47. Wu YB, Shi LL, Wu YJ, Xu WH, Wang L, Ren MS. Protective effect of gliclazide on diabetic peripheral neuropathy through Drp-1 mediated-oxidative stress and apoptosis. Neurosci Lett 2012;523:45–49
48. Shenouda SM, Widlansky ME, Chen K, et al. Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus. Circulation 2011;124:444–453