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 mitochondrial 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 proapoptotic 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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Received 14 February 2013 and accepted 1 August 2013.

DOI: 10.2337/db13-0251

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db13-0251/-/DC1. © 2013 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See http://creativecommons.org/licenses/by-nc-nd/3.0/ for details.
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 mitochondrial 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).

Mitochondrial morphology. Mitochondria were stained with 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 mM glucose at 37°C with 5% CO₂ in the incubator and then incubated with 75 nmol/L MitoTracker Red CMXRos 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 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 mM 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 mM 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 stimulated with 33.3 mM 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 (30 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 mM glucose and rhein (1 µg/mL) for 3 days. After the treatment, cells and islets were incubated with 20 µmol/L JC-1 (Invitrogen) by incubation with 5.5 or 33.3 mM glucose 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/m, and subgroups). At the beginning, db/db and db/m 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 for each subgroup per week. Blood samples were collected. For TUNEL assay, the apoptotic pancreatic β-cells were detected using an in situ apoptosis detection kit (Roche) on paraffin-embedded 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 SDSPAGE and transferred to polyvinylidene fluoride using standard procedures. The blot was incubated with anti-Drp1 antibody (Abcam), p(16)-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 lyase 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 12,000g for 10 min at 4°C for p(16)-Drp1, Drp1, and Drp1 expression or islets immunoblotting. Some isolated islets from db/db or 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 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
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. 2A, 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. 2B), 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 2A 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. 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 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. 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 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. 3B). 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 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 kidney proximal tubular (HK2) cells. As shown in Fig. 4A and
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. 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 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 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 through modulating mitochondria structure and function.


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. 5A, 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. 5B). 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. 6A) and islets (Supplementary Fig. 6B) 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. 5C). As expected, the analysis of mitochondria fragmentation (Fig. 5D), glucose-stimulated insulin secretion (GSIS) (Fig. 5E and F), and caspase 3 activation (Fig. 5H 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 down-regulation 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. 5F and G) and caspase 3 activation (Fig. 5H and I), the results are in agreement that rhein protects pancreatic β-cells from HG-induced dysfunction mainly through suppressing mitochondrial 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. 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.

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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. 6A) and mouse islets (Fig. 6B) 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. 6C) or islets (Fig. 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

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 Mito-Tracker 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.
and H₂O₂, a bona fide antioxidant and a pro-oxidant, respectively, strongly reduced and enhanced ROS levels in NIT-1 cells (Fig. 6A) and islets (Fig. 6B), as well as the expression of Drp1 (Fig. 6C 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 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 expression 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.

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 is more prevalent, 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 expression was markedly decreased in rhein-treated db/db mice compared with db/db mice treated with vehicle control.

**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.

**TABLE 1.** Summary of cell apoptosis and Drp1 expression in HG-treated NIT-1 cells after Drp1 upregulation and downregulation.

**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 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 concentrations, can induce apoptosis in certain cancer cells through mitochondrial-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 mitochondrial 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 expression was markedly decreased in rhein-treated db/db mice compared with db/db mice treated with vehicle control.
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. 10A), and ROS level in primary β-cells was affected by HG but not Drp1-siRNA (Supplementary Fig. 10B).

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.

ACKNOWLEDGMENTS
This work was supported by grants from the National Basic Research Program of China (2012CB517603), the National Natural Science Foundation of China (30980003, 30800004, 30900046, 30871019, 31071223, 31000323, 90608010, and 90819035), and the Natural Science Foundation of Jiangsu Province (BK201113).

No potential conflicts of interest relevant to this article were reported.

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.

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