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ORIGINAL ARTICLE

Effects of caloric restriction on SIRT1 expression and apoptosis of islet beta cells in type 2 diabetic rats

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Abstract Increasing evidence suggests that a restricted caloric intake extends the life span of mammals, and SIRT1 may play a key role in this process. To study the effects of caloric restriction on SIRT1 expression and apoptosis of islet beta cells in type 2 diabetic rats, we first induced a model of type 2 diabetes in rats with a low-dose of streptozotocin. Then, the rats were fed with a normal diet, high-fat diet or 60% caloric restriction, respectively. As a result, the apoptosis ratio of islet beta cells in diabetic rats was dramatically increased compared to the control group, and mRNA and protein expression of SIRT1 in islet beta cells were much lower than those of the control group. After caloric restriction for 1 month, the blood glucose and serum insulin of rats decreased. The mRNA and protein expression of SIRT1 in islet beta cells significantly increased; however, the apoptosis ratio of islet beta cells decreased remarkably. These data show that caloric restriction notably improves the sensitivity to insulin and significantly increases mRNA and protein expression of SIRT1 while decreasing the apoptosis ratio of islet beta cells in diabetic rats. Therefore, SIRT1 may play an important role in the apoptosis of islet beta cells of type 2 diabetes.

Keywords Caloric restriction · SIRT1 · Apoptosis · Islet beta cells · Rats

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Introduction

Diabetes mellitus is a polygenetic disease characterized by hyperglycemia because of absolute or relative insulin deficiency. Possible mechanisms responsible for diabetes include beta cell death and impaired function, which contributes to insulin deficiency. An increase in beta cell apoptosis is an important factor contributing to beta cells loss and the onset of type 2 diabetes [1, 2]. Islet beta cells are very sensitive to various pro-apoptotic stimuli. There is in vitro evidence that glucotoxicity and lipotoxicity exert synergistic effects to impair the secretory function of beta cells and to promote apoptosis in type 2 diabetes. However, the molecular mechanisms responsible for abnormal beta cell apoptosis have not been elucidated. A growing body of evidence suggests that restricted caloric intake extends the life span of a number of organisms including yeast, worms, flies and even mammals, and reduces the incidence of age-related diseases such as cancer, cardiovascular disease and diabetes in animal models [3]. SIRT1, a homolog of the yeast protein silent information regulator 2 (Sir2), which encodes an NAD^+ (nicotinamide adenine dinucleotide)-dependent histone deacetylase may play a key role in the regulation of cell apoptosis, cell cycle, genetic transcription and other cell processes to extend life span [4]. Some studies had suggested that pancreatic beta cell-specific

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SIRT1 over-expression improved glucose tolerance as a result of an enhanced glucose-stimulated secretion of insulin [5, 6], but the effects of caloric restriction (CR) on SIRT1 expression and the apoptosis of islet beta cells requires further investigation. In this study, we induced chronic hyperglycemia in rats with low-dose streptozotocin (STZ), and fed rats with a normal diet, a high-fat diet or CR, respectively. Finally, we investigated the effects of CR on SIRT1 expression and the apoptosis of islet beta cells.

Materials and methods

Animals and diets

Normal male Wistar rats weighing 140–180 g were obtained from the Experimental and Animal Centre of Tongji Medical College affiliated to Huazhong University of Science Technology. Normal chow was purchased from an Experimental Animal Centre of Hubei province of China. To make the high-fat diet, 20% lard stearin (wt/wt), 10% sucrose and 0.1% bile salt were added to normal chow (Table 1). The rats received either the normal diet with 18.94% of energy derived from fat, 31.67% from protein and 49.39% from carbohydrates or the high-fat diet with 50.55% of energy derived from fat, 15.72% from protein and 33.73% from carbohydrates. The rats were individually housed in special cages on a 12 h day/night cycle and provided ad libitum access to food and water, except when specified by experimental protocol. Animals were fasted for 12 h before any study. All protocols for animal experimentation and maintenance were approved by the Animal Ethics Committee in our hospital and carried out in accordance with the Institutional Guidelines of China.

Table 1 Composition of experimental diets

Ingredient (%)	Normal diet	High-fat diet
Indian corn	40	28
Wheat bran	33	23
Soybean	10	7
Fish flour	12.5	8.7
Yolk flour	0.5	0.4
Milk powder	1.5	1
Shells flour	1	0.7
Calcium phosphate	1	0.7
Mulvital	0.035	0.024
Minor element	0.15	0.1
Sodium chloride	0.35	0.24
Lard stearin	–	20
Sucrose	–	10
Choline bitartrate	–	0.1
Total (%)	100.0	100.0

Biochemical reagents

Glucose assay kits were purchased from Beihua Kangtai Clinic Reagent Company Limited (Beijing, China). Insulin assay kits were obtained from Jiuding Bioengineering Company Limited (Tianjin, China). The RNA extract reagent Trizol was purchased from Invitrogen (CA, USA). MMLV retroviridase was purchased from Toyobo Company (Osaka, Japan). Taq enzyme was the product of Tiangen Biotechnology Company Limited (Beijing, China). RNA primers were synthesized by Shanghai Invitrogen Biotechnology Company Limited (Shanghai, China). Rabbit anti-rat SIRT1 (H-300) polyclonal antibody was from Santa Cruz Biotechnology, Inc. (CA, USA). Mouse anti-rat β-actin monoclonal antibody and secondary antibody horseradish peroxidase-conjugated goat immunoglobulin G (IgG) for immunochemistry were purchased from Boster Company (Wuhan, China). The streptozotocin and type V collagenase were purchased from Sigma Company (MO, USA). Enhanced chemiluminescence kit was purchased from Pierce Company (IL, USA). Prestained Marker, SDS–polyacrylamide gel preparation kit, lysis buffer for Western blots, BCA protein assay kits and Hoechst apoptosis dyeing kit were supplied by Beyotime Biotechnology Company (Jiangsu, China). Other biochemical reagents were purchased from Dingguo Biotechnology Company Limited (Beijing, China).

Experimental protocol

Upon arrival, 36 male Wistar rats were fed with normal chow and allowed to acclimatize for 1 week. After acclimatization, the fasting plasma glucose (FPG) and random plasma glucose (RPG) of rats were measured. In this study, RPG was defined as plasma glucose not on an empty stomach. Then, the rats were randomly divided into two groups. Animals in the normal control (NC, $n = 7$) and type 2 diabetic model group (DM, $n = 29$) were fed ad libitum with normal chow. The rats in DM group were induced by low-dose streptozotocin (35 mg/kg) intraperitoneal injection, but the NC group only received an intraperitoneal injection with citrate buffer solution. The RPG of all rats was measured after 1 week. The standard for type 2 diabetes induction is an RPG $\geq$ 11.1 mmol/l [7], and the rats that did not attain this glucose standard were rejected. Then, the diabetic rats were randomly divided into two groups: group DNC fed with normal chow (DNC, $n = 6$) and group DHF0 fed with high-fat diet (DHF0, $n = 16$). After 2 mo of this feeding protocol, the rats in group DHF0 were further randomly divided into continuous high-fat feeding group (DHF, $n = 8$) and CR group

(DCR, $n = 6$). The rats in group DCR were fed with normal chow with 60% CR for 1 mo. The method of CR was performed as previously described [8]. Briefly, the rats receiving CR were housed singly in cages and fed ad libitum with normal chow for a few days. Food intake was measured each day in all rats, and the average daily food intake of each rat was obtained. The dietary caloric restriction was accomplished by 10% reduction in the average daily food intake every 5 days until a 40% reduction was achieved, and then rats were fed with 60% CR for 1 month. At the end of the experiment, all rats were anesthetized with an injection of an intraperitoneal dose (60 mg/kg body weight) of 1% sodium pentobarbital. Blood samples were collected from a heart puncture. The islets of the pancreas were isolated by type V collagenase digestion. Some pancreatic tissue was fixed in 4% buffered paraformaldehyde solution and then cut into pathological sections.

General characteristics observation

The food intake and body weight (BW) of rats in each group were observed weekly. Animals were fasted for 12 h before any study, and then blood samples were obtained by heart puncture. Some biochemical parameters such as FPG, RPG and fasting serum insulin (FINS) were tested. The plasma glucose was measured by the oxidation enzyme method and serum insulin by radioimmunoassay.

Islet isolation

A modified method by vibration digestion after pancreatic ductal injection of collagenase solution was used [9]. Briefly, animals were anesthetized with pentobarbital (60 mg/kg body weight) by intraperitoneal injection. For the exposure of the whole pancreas, the abdominal wall was opened via a midline incision. The common bile duct was first ligated at its entrance to the liver to prevent collagenase solution retrogradely entering the liver, and then the pancreatic duct proximal to the duodenum was exposed. Then, cannulation of the pancreatic duct was performed using a polyethylene catheter, which was connected to a syringe filled with 5 ml of precooled Dulbecco's phosphate-buffered saline (DPBS) solution. This solution additionally contained 8 mg collagenase V per 10 ml DPBS without Ca^{++} and Mg^{++}. The aorta was transected immediately to minimize interstitial hemorrhage in the pancreas before the duct was retrogradely injected with DPBS solution. Then, 3–5 ml DPBS was injected into pancreas duct until the pancreas was distended completely. Finally, the whole pancreas was excised quickly and rinsed in cooled DPBS, and unnecessary adipose tissue and anadesma were removed. Then, the pancreas was put into a Petri dish containing another 5 ml of 37°C DPBS solution with collagenase V. The Petri dish with the pancreatic tissue in the DPBS solution was immediately placed in a water bath at 37°C vibrating gently for 20–30 min to further allow tissue digestion. Then, the Petri dish was taken out and put on ice to stop the digestion process. Tissue suspensions were then washed two to three times at 10-min intervals and passed through a stainless steel filter net (800 μm pores) in 5 ml of DPBS solution. For islet cell assessment, 1 ml cell suspension was incubated with 10 μl dithizone for about 10 min and tested under microscope. A great quantity of comparatively large cells that were dyed scarlet was identified as islet cells. Finally, the islets were handpicked carefully with a pipette under a stereomicroscope and transferred to Petri dishes for the next step.

Detection of apoptotic islet beta cells

The apoptotic islet beta cells dyed with a Hoechst apoptosis dyeing kit were detected by fluorescence microscope. Briefly, formalin-fixed and paraffin-embedded pancreatic tissues were processed to sections using standard techniques. The sections were deparaffinized and hydrated routinely, and then 0.5 ml Hoechst staining solution was added after washing twice with 0.9% sodium chloride. After 5 min of dyeing, the staining solution was discarded. Then, washing twice with 0.9% sodium chloride, an anti-fluorescence quench mounting solution was added and a coverslip was added. Finally, the sections were observed under a fluorescence microscope. The cellular nucleus under the fluorescence microscope was blue, but the nucleus of apoptotic cells displayed as thickly dyed blue or shivers. The apoptosis of islet beta cells under a high-power lens was observed, and then the average apoptosis ratio of each group was calculated.

SIRT1 RNA expression using reverse transcription PCR (RT–PCR)

Total RNA was isolated from islets using modifications of an acid guanidinium thiocyanate-phenol–chloroform extraction method [10]. A minimum of 150 islets were extracted using Trizol Reagent according to the manufacturer's directions. The RNA pellet was resuspended in 15 μl DEPC-treated H_2O at 65°C for 5 min and stored at −70°C. The quality and concentration of the RNA were assessed using the OD 260/280 ratio, and only samples with ratios above 1.5 were used in the experiments. For PCR analysis, total RNA was reverse transcribed using the Moloney murine leukemia virus (MMLV) retroviridase. The 2 μl of resulting cDNAs was amplified by PCR carried out on a 25 μl reaction mixture. Primers used to amplify

SIRT1 cDNA were 5′-CCT GAC TTC AGA TCA AGA GAC GGT A-3′ and 5′-CTG ATT AAA AAT GTC TCC ACG AAC AG-3′ (444-base pair fragment). As an internal standard, β-actin cDNA was amplified using primers, 5′-CTG GCA CCA CAC CTT CTA CA-3′ and 5′-AGT ACT TGC GCT CAG GAG GA-3′ (758-base pair fragment). PCR amplification consisted of a 5-min hot start at 95°C, followed by 32 cycles (SIRT1) at 54°C and 26 cycles (β-actin) at 58°C for 45 s, and 72°C for 5 min. The products were electrophoresed on a 1% agarose gel, and analyzed by the MGIAS-1000 gel analytical system (Bio-Rad Co, München, Germany). Levels of SIRT1 mRNA were expressed as the ratio of the signal intensity relative to that for β-actin.

SIRT1 protein expression by Western blot analysis

Samples of pancreatic islets homogenate were analyzed by Western blot as described previously [11]. Rabbit anti-rat SIRT1 (H-300) polyclonal antibody and mouse anti-rat β-actin monoclonal antibody were used to detect respective proteins. A minimum of 300 islets were handpicked with a pipette under a stereomicroscope. The islets were rapidly homogenized at 4°C in 0.4 ml lysis buffer. Homogenates were centrifuged at 12,000*g* for 20 min at 4°C to yield supernatants. The protein concentrations were determined using a BCA protein assay kit. Equivalent amounts of proteins from homogenates were run in sodium dodecyl sulfate–polyacrylamide gel electrophoresis using an 8% acrylamide resolving gel. Separated proteins were transferred to nitrocellulose membranes. Membranes were blocked in TBS-T (1 × TBS, 0.1% Tween-20) containing 5% milk for 1 h at room temperature followed by incubation with SIRT1 antibody (1:200) or β-actin antibody (1:400) at 4°C overnight. Then, membranes were probed with their respective antibody labeled with horseradish peroxidase (1:4,000) for 2 h at room temperature and visualized using the enhanced chemoluminescence (ECL) reagent. After membranes were exposed to Kodak films for 5–10 min, the resulting images were quantitated by densitometry. Intensity of bands was analyzed with Gel-Pro® analyzer 4.0 software. The results were expressed as a ratio of SIRT1 to β-actin densitometry.

Statistical analysis

All results were expressed as the mean ± SD and analyzed using one-way *ANOVA*. Student–Newman–Keuls multiple-range test was then used to compare results between two groups. $P < 0.05$ was considered statistically significant. All statistical analyzes were performed with the use of SAS 8.1 software.

Results

General characteristics of rats during experiments

The rats in DM model group were induced by a low-dose streptozotocin (35 mg/kg) intraperitoneal injection. Most rats displayed obvious symptoms such as polydipsia, polyuria and polyphagia. According to the standard of RPG ≥ 11.1 mmol/l, seven rats did not attain the standard. The achievement ratio of the model for type 2 diabetes was 76%. After 2 weeks of streptozotocin treatment, the velocity of BW increase in diabetic model rats was slower than normal controls. At the end of experiment, BW of DHF group rats began to decrease because of severe diabetes. However, CR could reduce BW of rats sharply (Fig. 1). In all diabetic rats, the significant characteristics was that RPG increased significantly, but FPG was normal (<6.0 mmol/l) at the beginning. After 2 months feeding according to groups, the rats in DHF0 group fed with high-fat diet displayed more severe polydipsia and polyuria. The FPG and RPG of rats were increased, and FINS decreased significantly. However, the RPG of rats in the DNC group fed with normal chow was increased notably, but FPG always remained in the normal range. Subsequently, after 1 month of CR, the plasma glucose and serum insulin of rats in the DCR group (the number changed due to the unexpected death of two rats because of heart puncture) were all decreased sharply. Figure 2 shows the development changes of FPG in each group. General characteristics of rats are summarized in Table 2.

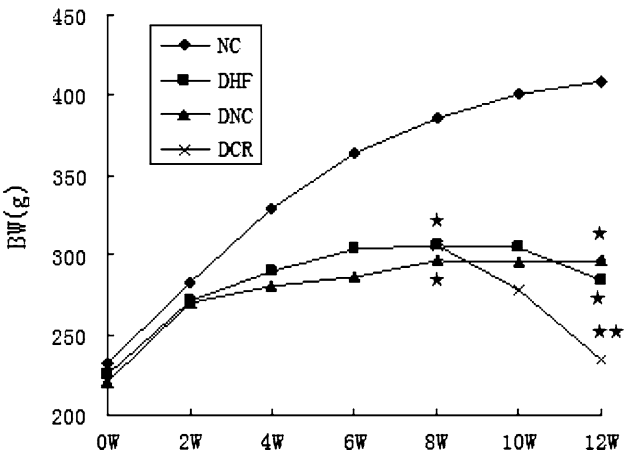

Fig. 1 Changes in body weight of each group rats in different phases. *NC* normal control, *DNC* type 2 diabetic model fed with normal chow, *DHF* type 2 diabetic model fed with high-fat diet continuously, *DCR* type 2 diabetic model fed with normal chow with 60% calorie restriction, *BW* body weight. $^{\star}P < 0.01$ (versus group NC); $^{\star\star}P < 0.01$ (versus group DNC)

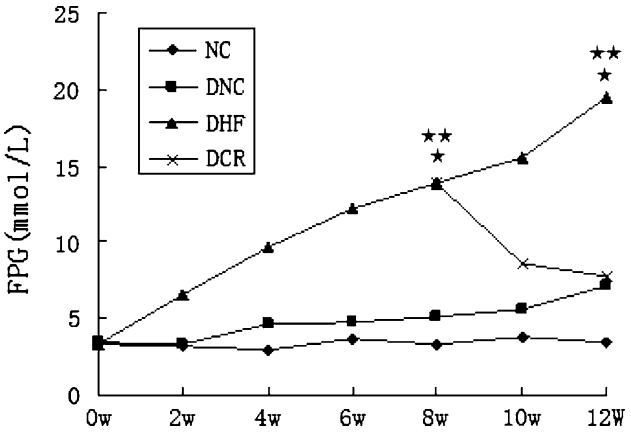

Fig. 2 Changes in fasting plasma glucose of each group rats in different phases. *NC* normal control, *DNC* type 2 diabetic model fed with normal chow, *DHF* type 2 diabetic model fed with high-fat diet continuously, *DCR* type 2 diabetic model fed with normal chow with 60% calorie restriction, *FPG* fasting plasma glucose. $^{\star}P < 0.01$ (versus group NC); $^{\star\star}P < 0.01$ (versus group DNC)

Apoptosis of islet beta cells in each group

Apoptosis of islet beta cells in each group is shown in Fig. 3. In the fluorescence photomicrographs, those cells in the islets with thickly dyed blue nuclei were apoptotic beta cells. As a result, compared with rats in the control NC group, the apoptosis ratio of islet beta cells of rats in the DNC and DHF groups was elevated significantly ($P < 0.01$), but the apoptosis ratio of the DHF group was higher than that of DNC group ($P < 0.01$). Interestingly, CR (DCR group) notably reduced the apoptosis ratio of islet beta cells.

SIRT1 mRNA and protein expression of islet beta cells

As shown in Fig. 4, compared with the control NC group, the type 2 diabetic rats fed with normal chow or the high-fat diet displayed an obviously reduced mRNA expression of SIRT1 in islet beta cells, but the mRNA expression of SIRT1 in the high–fat-fed rats was prominently lower than the normal chow fed rats. However, after 1 month CR, SIRT1 mRNA expression of rats was increased significantly compared with that of the DNC ($P < 0.05$) group or the DHF group ($P < 0.01$). In the corresponding time period, we also measured the protein expression of SIRT1 in each group of rats by Western blot. Consequently, the SIRT1 protein expression of beta cells in the DNC and DHF group was also decreased. After 1 month CR, the SIRT1 protein expression significantly increased in the same manner as the mRNA expression. SIRT1 protein expression is shown in Fig. 5.

Discussion

Glucolipotoxicity is a dominant feature of type 2 diabetes [12]. In the development of type 2 diabetes, apoptosis of islet beta cells plays a key role. CR is a basic measure of type 2 diabetes treatment. However, the study provides in vivo evidence concerning the effects of glucolipotoxicity and strict CR (It is impossible for humans to maintain a 60% CR). However, data on the apoptosis of beta cells have been insufficient so far. High-fat feeding is a reliable method to induce lipotoxicity in rats [13]. To study the effects of glucolipotoxicity on the apoptosis of islet beta cells, we first used low-dose streptozotocin to induce

Table 2 Comparison of tested parameters of rats in each group at different phases

Group	*n*	BW (g)	FPG (mmol/l)	RPG (mmol/l)	FINS (mU/l)
After 2-month feeding					
NC	7	385 ± 46	3.3 ± 0.43	6.5 ± 0.64	14.30 ± 1.94
DNC	6	297 ± 21^{b}	5.2 ± 1.12	16.3 ± 3.77^{b}	9.28 ± 1.02^{b}
DHF_0	16	305 ± 21^{b}	14.2 ± 3.84^{ba}	18.3 ± 2.16^{b}	6.75 ± 0.69^{ba}
After 3-month feeding					
NC	7	408 ± 44	4.4 ± 0.57	6.5 ± 0.76	13.09 ± 1.18
DNC	6	297 ± 21^{b}	7.2 ± 1.99	16.3 ± 2.16^{b}	10.52 ± 2.14^{b}
DHF	8	284 ± 19^{b}	19.5 ± 3.86^{ba}	22.0 ± 3.10^{ba}	6.59 ± 0.83^{ba}
DCR	6	235 ± 22^{ba}	7.8 ± 2.28	8.6 ± 2.32^{a}	4.83 ± 0.63^{ba}

NC normal control, *DNC* type 2 diabetic model fed with normal chow, DHF_0 type 2 diabetic model fed with high-fat diet. After 2-month feeding, the rats in group DHF_0 were further randomly divided into group DHF and DCR. *DHF* type 2 diabetic model fed with high-fat diet continuously, *DCR* type 2 diabetic model fed with normal chow with 60% calorie restriction. *BW* body weight, *FPG* fasting plasma glucose, *RPG* random plasma glucose, *FINS* fasting serum insulin. All results were expressed as the mean ± SD and analyzed using one-way *ANOVA*. Student–Newman–Keuls multiple-range test was used to compare results between two groups. Versus group NC, ^{b} $P < 0.01$; versus group DNC, ^{a} $P < 0.01$

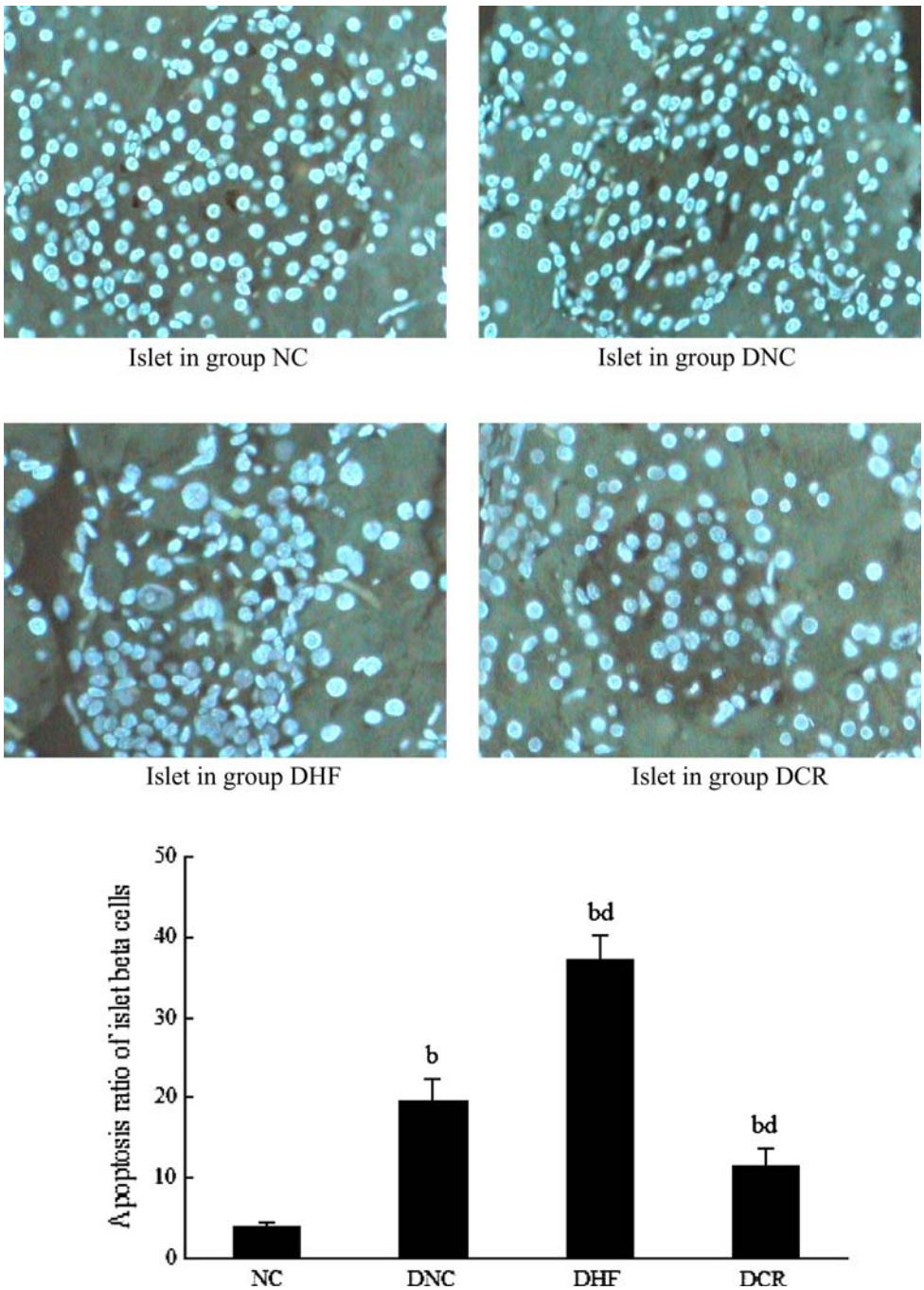

Fig. 3 Fluorescence photograph of islet beta cells apoptosis and comparison of apoptosis ratio of islet beta cells in each group. *NC* normal control, *DNC* type 2 diabetic model fed with normal chow, *DHF* type 2 diabetic model fed with high-fat diet continuously, *DCR* type 2 diabetic model fed with normal chow with 60% calorie restriction. All results were expressed as the mean ± SD and analyzed using one-way *ANOVA*. Student–Newman–Keuls multiple-range test was used to compare results between two groups. Each value represents the mean ± SD of three independent experiments. versus group NC, $^{b}P < 0.01$; versus group DNC, $^{d}P < 0.01$

insulin deficiency and chronic hyperglycemia in rats. Then, we simulated lipotoxicity by high-fat feeding. By observing effects of CR on the apoptosis of islet beta cells, we investigated the molecular mechanisms by which glucolipotoxicity influenced the apoptosis of islet beta cells. Previous studies have suggested that an injection of 24–100 mg/kg streptozotocin produced a dose-dependent hyperglycemia, and a 50 mg/kg or lower dose of streptozotocin intraperitoneally injected into rats induced a type 2 diabetic animal model [8, 14]. Our results showed that Wistar rats treated with low-dose streptozotocin (35 mg/kg) displayed significantly high RPG and normal FPG, which suggested that the partly impaired islet beta cells could maintain a normal FPG, but were not tolerant of supernumerary glucose load. Therefore, rats we treated with low-dose streptozotocin were mildly type 2 diabetic animals at the beginning.

On the basis of the type 2 diabetic model, we fed the rats with normal chow or high-fat diet for 3 months. Consequently, the rats in the DNC group fed with normal chow displayed significantly increased RPG and decreased FINS, but there was no obvious difference in the FPG when

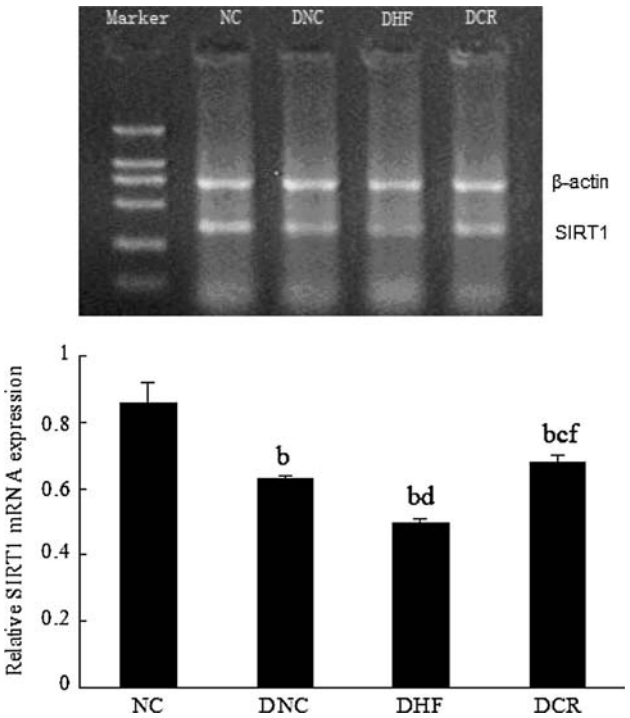

Fig. 4 Expression of SIRT1 mRNA in islet beta cells of rats. *NC* normal control, *DNC* type 2 diabetic model fed with normal chow, *DHF* type 2 diabetic model fed with high-fat diet continuously, *DCR* type 2 diabetic model fed with normal chow with 60% calorie restriction. All results were expressed as the mean ± SD and analyzed using one-way *ANOVA*. Student–Newman–Keuls multiple-range test was used to compare results between two groups. Each value represents the mean ± SD of four independent experiments. Versus group NC, $^{b}P < 0.01$; versus group DNC, $^{c}P < 0.05$, $^{d}P < 0.01$; versus group DHF, $^{f}P < 0.01$

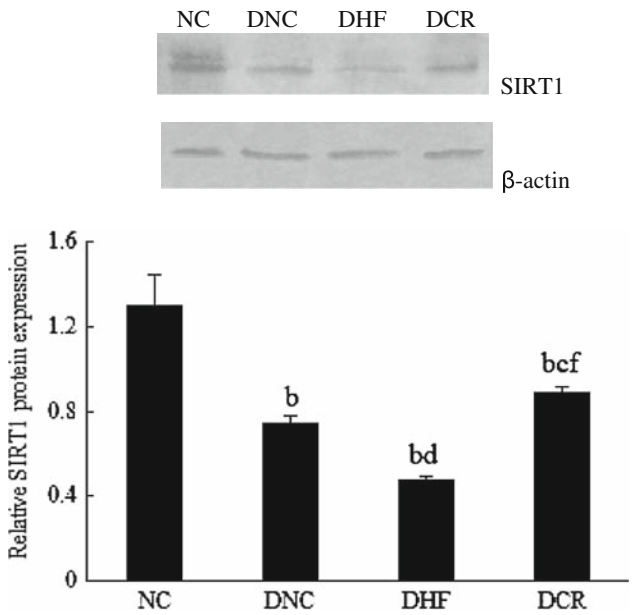

Fig. 5 Expression of SIRT1 protein in islet beta cells of rats. *NC* normal control, *DNC* type 2 diabetic model fed with normal chow, *DHF* type 2 diabetic model fed with high-fat diet continuously, *DCR* type 2 diabetic model fed with normal chow with 60% calorie restriction. All results were expressed as the mean ± SD and analyzed using one-way *ANOVA*. Student–Newman–Keuls multiple-range test was used to compare results between two groups. Each value represents the mean ± SD of four independent experiments. Versus group NC, $^{b}P < 0.01$; versus group DNC, $^{c}P < 0.05$, $^{d}P < 0.01$; versus group DHF, $^{f}P < 0.01$

compared with that of the control group NC. These results suggest that the islet beta cells were further impaired by chronic hyperglycemia in the DNC group, but the function of islet beta cells could still keep the FPG in the normal range overall. However, the FPG and RPG of rats fed high-fat chow in the DHF group were much higher than that of the NC and DNC groups. In addition, FINS was further reduced, which suggested that the islet beta cells underwent a major injury, and the function of islet beta cells could not maintain a normal FPG. These results showed that lipotoxicity induced by high-fat feeding notably promoted the impairment of islet beta cells and further reduced insulin secretion when chronic hyperglycemia was present. By quantifying the apoptosis of islet beta cells, we found that the ratio of apoptosis of islet beta cells in DNC and DHF rats was raised significantly when compared with that of normal controls. Furthermore, the ratio of islet beta cell apoptosis in the DHF group was much higher than that of the DNC group. This finding suggests that the hyperlipidemia and hyperglycemia can cooperate to promote the apoptosis of islet beta cells, and high-fat feeding further promoted apoptosis in the background of chronic hyperglycemia.

Much research has clearly shown that short-term CR can dramatically increase the reactivity of rat skeletal muscle to insulin. Along with prolonged CR, the rats display an overt hypometabolism status. Especially, the core body temperature, plasma glucose and serum insulin of rats were all reduced significantly [15–17]. These were also confirmed in our study. In this study, the weight of rats in group DCR that underwent strict CR for 1 month was sharply decreased compared with the DHF group. Though serum insulin was reduced significantly, the RPG and FPG were lower than that of the DHF group, which suggested that the sensitivity to insulin notably increased because of CR. Meanwhile, the ratio of apoptosis of islet beta cells in the DCR group was reduced remarkably when compared with the DHF group, which showed that strict CR can ameliorate apoptosis of islet beta cells induced by glucolipotoxicity. However, as a limitation of this study, maybe immunohistochemistry of islets should be done to directly elucidate the effects of glucolipotoxicity and CR on synthesis and secretion of insulin in type 2 diabetic rats.

In mammals, CR delays the onset of numerous age-associated diseases including cancer, atherosclerosis and diabetes and can greatly increase life span. The molecular

mechanisms may be associated with the reduced apoptosis of cells [18]. Research on *Saccharomyces cerevisiae* showed that the small molecular protein encoded by Sir2 gene, which plays a key role in CR effects may mediate the silence of chromatin, telomere, rDNA and the repairing of DNA, which accordingly maintains the stability of the genome and increases life span [19, 20]. Therefore, the Sir2 gene has been known as the longevity gene. The mammalian SIRT1 gene is the homolog of yeast sir2. Moreover, SIRT1 has a remarkably varied spectrum of nuclear substrates that include TAF_I68 (Tata box-binding protein-associated factor I of 68 kDa), p53, FOXO (Forkhead box class O transcription factors), p300/CBP, myoD (myoblast determination protein), Ku70 (thyroid autoantigen of 70 kDa or Ku antigen), etc. SIRT1 is a protein deacetylase able to deacetylate these substrates both in vitro and in vivo [21–24]. From physiological functions of these substrates, we presumed that it is possible that SIRT1 may regulate cell apoptosis, cell cycle control, genetic transcription and other cell processes. In type 2 diabetes, mitochondria malfunction is dramatic. The mitochondria pathway is a major signal transduction pathway of the apoptosis of islet beta cells. In this pathway, abnormal changes of pro-apoptosis proteins (Bax, Bak, Bid, etc.) and anti-apoptosis proteins (Bcl-2, Bcl-xl, etc.) and p53 may be the keys of cellular apoptosis [25–27]. Previous research has demonstrated that in some cells such as brain cells, kidney cells, fat cells and liver cells, SIRT1 down-regulates several pro-apoptotic factors such as p53, FOXO and Bax, which potentializes the ability of CR animals to resist stress [24]. However, these effects in islet beta cells need to be elucidated, especially in vivo. Our study has primarily demonstrated that CR has a great effect on SIRT1 expression and the apoptosis of islet beta cells, and that the changes of SIRT1 expression may be involved in the islet beta cell apoptosis process. In this study, chronic hyperglycemia significantly promoted the apoptosis of islet beta cells. Meanwhile, the mRNA and the protein expression of SIRT1 of islet beta cells were reduced notably. Then, the high-fat feeding further aggravated the apoptosis of beta cells when coupled with chronic hyperglycemia, and the mRNA and protein expression of SIRT1 in islet beta cells were further reduced. In our study, the mRNA and protein expression of SIRT1 in islet beta cells of rats that had undergone strict CR for 1 month were increased significantly, and the apoptosis rate of islet beta cells was reduced significantly. Recently, some studies [5, 6, 28] suggested that SIRT1 is only expressed in islets, but not in the exocrine pancreas, which indicates that SIRT1 may be involved in the special physiological function of islets. SIRT1 exists in the nucleus and endochylema of islet beta cells. The SIRT1 binding promoter region of uncoupling protein 2 (UCP2) directly represses the expression of the UCP2 gene and regulates glucose-stimulated insulin secretion (GSIS). Increased SIRT1 expression significantly promotes GSIS. According to the physiological functions of SIRT1 substrates, the special effects of SIRT1 in islet beta cells and our experimental results, it is reasonable to believe that SIRT1 expression is not only involved in regulating beta cell function to secrete insulin, but also is associated with the apoptosis of islet beta cells. Recently, Lee JH et al. [29] also reported an additional effect of SIRT1: through inhibition of NF-κB by deacetylating p65, it could protect β-cells from cytokine toxicity. Their results suggest that SIRT1 may have diverse roles in addition to regulating insulin secretion in pancreatic β-cells and that it constitutes a novel mechanism for treating type 1 diabetes. In our study, we used type 2 diabetic rat model to study the effects of SIRT1 on apoptosis of islet beta cells. In some extent, type 2 diabetes is also a kind of inflammatory disease. The histology of islets from patients with type 2 diabetes displays an inflammatory process characterized by the presence of cytokines, apoptotic cells, immune cell infiltration, amyloid deposits and eventually fibrosis [30, 31]. Therefore, our results about SIRT1 on apoptosis of islet beta cells of type 2 diabetes are essentially consistent with that of Lee JH et al. study on type 1 diabetes. Currently, small molecule activators of SIRT1 have been used as therapeutics for the treatment of type 2 diabetes [32]. However, the causal relationship between SIRT1 expression and the apoptosis of islet beta cells, and the precise molecular mechanisms to regulate the apoptosis of islet beta cells need to be further elucidated.

In summary, we successfully induced type 2 diabetic rat model by low-dose STZ. Chronic hyperglycemia (glucotoxicity) significantly promoted the apoptosis of islet beta cells. Lipotoxicity further increased the apoptosis of beta cells coupled with the background of chronic hyperglycemia. Glucotoxicity and lipotoxicity cooperated to promote the apoptosis of beta cells. Intriguingly, CR notably improved the sensitivity to insulin and had a great effect on SIRT1 expression and the apoptosis of islet beta cells. CR significantly increased the expression of SIRT1 in islet beta cells and decreased the apoptosis ratio of islet beta cells remarkably in CR animals. SIRT1 may play an important role in the apoptosis of islet beta cells in type 2 diabetes.

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