Protective effects of calorie restriction on insulin resistance and islets function in STZ-induced type 2 diabetes rats

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Research

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Abstract

Background Caloric restriction (CR) has become increasingly attractive in the treatment of type 2 diabetes mellitus (T2DM) because of the increasingly common high-calorie diet and sedentary lifestyle. This study aimed to evaluate the role of CR in T2DM treatment and further explore its potential molecular mechanisms.

Methods Sixty male Sprague-Dawley rats were used in this study. The diabetes model was induced by 8 weeks of high-fat diet (HFD) followed by a single dose of streptozotocin injection (30 mg/kg). Subsequently, the diabetic rats were fed HFD at 28 g/day (diabetic control) or 20 g/day (30% CR regimen) for 20 weeks. Meanwhile, normal rats fed a free standard chow diet served as the vehicle control. Body mass, plasma glucose levels, and lipid profiles were monitored. After diabetes-related functional tests were performed, the rats were sacrificed at 10 and 20 weeks, and glucose uptake in fresh muscle was determined. In addition, western blotting and immunofluorescence were used to detect alterations in AKT/AS160/GLUT4 signaling.

Results We found that 30% CR significantly attenuated hyperglycemia and dyslipidemia, leading to alleviation of glucolipotoxicity and thus protection of islet function. Insulin resistance was also markedly ameliorated, as indicated by notably improved insulin tolerance and homeostatic model assessment for insulin resistance (HOMA-IR). However, the improvement in glucose uptake in skeletal muscle was not significant. The upregulation of AKT/AS160/GLUT4 signaling in muscle induced by 30% CR also attenuated gradually over time. Interestingly, the consecutive decrease in AKT/AS160/GLUT4 signaling in white adipose tissue was significantly reversed by 30% CR.

Conclusion CR (30%) could protect islet function from hyperglycemia and dyslipidemia, and improve insulin resistance. The mechanism by which these effects occurred is likely related to the upregulation of AKT/AS160/GLUT4 signaling.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a kind of metabolic disorder whose occurrence and progression are closely related to individual lifestyles apart from other risks like genetic factors^[1]. According to the prediction of the International Diabetes Federation, the number of diabetes were at least 463 million in 2019, and it will climb to 700 million people by 2045^[2]. Caloric restriction (CR), as the only approved scientific method that can slow aging, has been frequently proved to improve insulin resistance (IR) and islets dis-function—two key pathological links of T2DM. Moreover, recent clinical study^[3] on the remission of T2DM by very low-calorie diet has created a precedent that T2DM may be cured by intensive lifestyle intervention. However, as the pathogenesis of T2DM is not clear, and the mechanism underlying CR is complicated and uncovered incompletely, thus the potential molecular mechanism of CR in T2DM treatment remains to be studied further despite that mechanisms in the integral level such as improving obesity, reducing ectopic lipid deposition, etc. has been repeatedly confirmed.

Insulin signal pathway is key to normal insulin action. Generally, insulin binds to the insulin receptor (INSR), and then recruits insulin receptor substrate (IRS) family, the best-described class of INSR scaffolds, leading to its tyrosine-phosphorylation. Tyrosine-phosphorylated IRS proteins recruit and activate phosphoinositide-3-kinase (PI3K), further induce the phosphorylation of protein kinase B (AKT)^[4]. In skeletal muscle or white adipose tissue, activated AKT phosphorylates AKT substrate of 160 kDa (AS160) to block the inactivation of small Rab GTPase protein switches that control vesicle trafficking, thus facilitate the translocation of glucose transporter 4 (GLUT4), further mediating glucose uptake in these tissues to maintain glucose homeostasis^[4]. Actually, IR can be defined to some extent as a decrease in insulin-induced glucose uptake, especially in skeletal muscle, as most of insulin's other effects are retained or only slightly affected in insulin resistant individuals, therefore the decrease of insulin-induced glucose uptake is a key manifestation of IR, and this effect is mainly mediated by GLUT4^[4]. So, normal transduction of AKT/AS160/GLUT4 signaling in skeletal muscle or white adipose tissues is critical to guarantee well-balanced glucose uptake in response to insulin action to maintain normal insulin sensitivity. It was reported that the phosphorylation level of AKT in patients with insulin resistance decreased by up to 50% when compared with healthy controls^[5–7]. Muscle and adipose tissue-specific GLUT4 knockout could induce global insulin resistance and hyperinsulinemia^[8, 9]. Furthermore, mutations of AS160 which regulate GLUT4 translocation in adipocyte and muscle cells also led to insulin resistance and increased risk of progression to T2DM in human^[10].

In terms of the aforementioned information, AKT/AS160/GLUT4 signaling may play an important role in the onset of insulin resistance and T2DM, while the positive effects of CR on AKT and its phosphorylation have been observed in many studies^[11–13], and there were also several preliminary studies reported CR's potential role in regulating GLUT4^[14, 15]. So, we speculated that the mechanism behind CR's metabolic effects on T2DM disease may related to its regulation on AKT/AS160/GLUT4 signaling. T2DM model rats were induced by high-fat feeding combined with a single low dose of streptozotocin injection, and then kept on 30% CR regimen to test our hypotheses.

2. Methods And Materials

2.1 Materials

Gansulin-R was purchased from Tonghua Dongbao pharmaceutical Co. (Beijing, China). Streptozotocin and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Rat insulin ELISA assay was purchased Merckodia (Merckodia AB, Uppsala, Sweden). The primary and secondary antibodies used in our experiments are summarized in table. (Supplementary Table 1).

2.2 Experimental procedures

2.2.1 Animals

A total of 60 weaned and specific-pathogen-free SD rats (male, 6–8 weeks, 180–190 g) were obtained from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). The animal experiments were performed according to internationally followed ethical standards and approved by the research ethics committee of School of Public Health, Sun Yat-Sen University (NO. 2019-001). Rats were kept on a 12 h light/dark cycle in a temperature-controlled room maintained at $(24 \pm 1) ^\circ\text{C}$ with a relative humidity of $(50 \pm 5) \%$ and were maintained on a standard chow diet. Rats were habituated to the conditions for 1 week before modeling.

2.2.2 Induction of diabetes

With reference to the method widely used in an abundance of literatures^[16–18], rats were fed on a high-fat diet for 8 weeks and then received a single low dose of streptozotocin (STZ, 30 mg/kg) injection to induce T2DM model. Specifically, rats were randomly allocated to the blank control group (N = 20) and the model group (N = 40) according to body mass. Rats in the blank control group had a free normal diet (ND, Guangdong Medical Laboratory Animal Center, Foshan, China; contains 58% carbohydrates, 18% protein, 4.5% fats, and 4% essential vitamins and trace elements, total calorie is 3.45 kcal/g). Rats in the model group were kept on a high-fat diet (HFD, Guangdong Medical Laboratory Animal Center, Foshan, China; contains 53.6% normal chow, 15% sucrose, 10% lard, 20% protein, 1.2% cholesterol, 0.2% sodium cholate, total calorie is 4 kcal/g). After 8 weeks of feeding, rats in the model group were intraperitoneally injected with 1%STZ dissolved in 0.1M citric acid buffer (PH = 4.2–4.5) at a single dose of 30 mg/kg, while the blank control rats received same amount of 0.1M citric acid buffer injection. It's important to note that the whole procedure of preparation and injection of STZ solution needs to avoid light and the dissolved STZ solution should be used as soon as possible (within 15 min). After STZ administration, model rats continued to eat *ad libitum* with the high-fat diet in order to attain relatively stable blood glucose level, then 14 days after STZ injection, random blood glucose (RBG) was measured by portable glucometer (Beijing Yicheng biotechnology co. LTD, China) through tail vein, and rats whose RBG were higher than or equal to 16.7 mmol/L were considered as diabetic^[19] and were further used in the following experiment.

2.2.3 CR procedure

During high-fat diet feeding, the total food intake of rats in each cage was recorded to calculate average daily food intake per rat which turned out to be 28.54 ± 4.78 g/day. Thus, 30% CR (20 g/day) was applied in the following experiment. Diabetes model was successfully induced in a total of 29 rats after STZ administration, then according to blood glucose level, these diabetic rats were randomly assigned to the model control group eating *ad libitum* with high-fat diet (HFD + AL + STZ, MCT group, n = 15) or the CR intervention group feeding on high-fat diet but with 30% CR regimen as we stated above (HFD + CR + STZ, CR group, n = 14). In the meanwhile, rats in the blank control group continued to have a free normal diet (ND + AL, BCT group, n = 20). The intervention period lasted for 20 weeks. Water intake in all rats was free during the whole experiment. The whole process of this experiment was indicated in **Fig. 1**.

Body mass and water intake were recorded every week, while RBG and fasting blood glucose (FBG) were measured every 2 or 4 weeks respectively. To assess insulin resistance, insulin tolerance test (ITT) was conducted at baseline before modeling, after modeling, at 10 and 20 weeks post-treatment. In addition, FBG and fasting insulin (FINS) were also detected at the above indicated time points through tail vein after a 12 h overnight fasting, and insulin level was measured by ELISA operating according to the manufacturer's instructions. Then, homeostasis model assessment of insulin resistance (HOMA-IR) was calculated with the following equation: $\text{HOMA-IR} = \text{FBG} \times \text{FINS} / 22.5$. Intraperitoneal glucose tolerance test (IPGTT) and glucose stimulated insulin secretion (GSIS) were accomplished after modeling (before intervention), 10 and 20 weeks after intervention to evaluate glucose tolerance and islets secretion.

At the time point of 10 weeks and 20 weeks after intervention, 6 to 10 rats in each group were anesthetized by 2% pentobarbital sodium (60 mg/kg) after an overnight fasting (12 h), and then blood, liver, pancreas, skeletal muscle and white adipose tissue (WAT, specifically epididymal fat pad) were collected rapidly. Blood samples were centrifuged at 4°C, 3500 rpm for 15 min to collect the serum (stored at -80°C till use). Lipid profile was measured in the central laboratory of the First Affiliated hospital of Sun Yat-sen University.

Figure 1. Flow chart of animal study design

2.2.4 IPGTT

Rats were intraperitoneally injected with 50% glucose solution (2 g/kg) after overnight fasting for 12 h. Blood glucose was measured before injection (0 min) and 15 min, 30 min, 60 min, 90 min and 120 min after injection through tail vein with portable glucometer. Six rats from each group were randomly chosen for use.

2.2.5 ITT

Rats were intraperitoneally injected with Gansulin-R (0.5 IU/kg) after fasting for 6 h. Blood glucose was measured before injection (0 min) and 15 min, 30 min, 45 min, 60 min and 90 min after injection through tail vein with portable glucometer. Six rats from each group were randomly chosen for use.

2.2.6 GSIS

Rats were intraperitoneally injected with 50% glucose solution (2 g/kg) after 12 hours of fasting at night. Blood samples were collected from the tail vein before injection (0 min) and 15 min, 30 min and 60 min after injection. The blood samples were centrifuged at 3500 rpm, 4 °C for 15 minutes. The separated serum was collected and the insulin level was detected by ELISA. Six rats from each group were randomly chosen for use.

2.2.7 Glucose uptake in isolated soleus

At the end of the experiment, glucose uptake in isolated soleus was measured in 4 rats from each group. The effect of CR in glucose uptake was detected with reference to the method described in previous study with slightly modifications^[20–22]. Generally, the isolated fresh soleus was immediately rinsed with Krebs-

Ringer buffer, removed the muscle fascia, tendons and attached connective tissue as far as possible. Subsequently, the muscle tissue was placed into the brain slice mold (Reward, China) to cut it into thin slices of similar size with a blade, then divided into three parts (roughly the same weight) and put into 2 ml ep tubes separately. The muscle tissue was then incubated with 1.5 ml oxygenated glucose-free Krebs-Hensleit buffer (KRH), containing 0.1% bovine serum albumin (BSA) and 10 mU/ml insulin (Gansulin-R) at 37 °C, 300 rpm (shaking) for 60 min to deplete intercellular glucose. After that, the liquid was removed, 1.5 ml oxygenated KRH (containing 11.1 mM glucose, 0.1%BSA, and 40 mM mannitol) with 0, 10 or 20 mU/ml insulin was added in. In the meantime, another tube contains equal volume of oxygenated KRH but no muscle tissue served as blank control. All the four tubes were incubated at 37 °C, 300 rpm (shaking) for 30 min. A 1 mL aliquot was collected from each incubation tube before and after the incubation, and the glucose concentration was determined. Glucose uptake of muscle was calculated as the amount of glucose (mg) taken up per gram of muscle tissue using the following formula: Muscle glucose uptake = $(GC_b - GC_a) / \text{muscle tissue weight}$, where GC_b and GC_a are glucose concentrations before and after incubation, respectively.

2.2.8 Histological assessment

Liver and pancreas tissues from 3 rats in each group were randomly selected for histological evaluation. After fixation in 10% formalin overnight at 4 °C, one portion of liver and pancreas tissue from each rat were embedded in paraffin blocks, sliced at 10 µm thickness, and stained with hematoxylin and eosin (H&E) for histological studies. Besides, another portion of them were fixed in 4% paraformaldehyde overnight at 4 °C and embedded in OCT for frozen section, sliced at 10 µm thickness, then stained with Oil Red O and counterstained with hematoxylin to determine hepatic and pancreatic lipid accumulation. Photomicrographs were taken with a digital camera (Nikon Eclipse Ci-L, Japan).

2.2.9 Immunohistochemistry

Pancreas tissues from 3 rats in each group were randomly selected for insulin immunohistochemistry. After fixation in 10% formalin overnight at 4 °C, pancreas tissue samples were embedded in paraffin blocks, sliced at 10 µm thickness. Antigen retrieval was carried out in EDTA buffer (pH 9.0) by heating to 99 °C for 15 min. Subsequently, deparaffinized sections were incubated with 3% H₂O₂ for 25 min at room temperature to block endogenous peroxidase activity and then rinsed three times with phosphate-buffered saline (PBS). After blocking with 3% BSA for 30 min at room temperature, each section was incubated with anti-insulin antibody (1:600, Servicebio, China) in a wet box at 4 °C overnight. Thereafter, the sections were rinsed three times with PBS, and then incubated with horseradish peroxidase-conjugated secondary antibody (1:200, Servicebio, China) for 50 min at room temperature. After washing with PBS for three times, the sections were stained with a 3,3'-diaminobenzidine solution and then counterstained with haematoxylin for 3 min. Photomicrographs were taken with a digital camera (Nikon Eclipse Ci-L, Japan).

2.2.10 Immunofluorescence

Skeletal muscle from 3 rats in each group were randomly selected for GLUT4 immunofluorescence experiment. Briefly, after fixation in 4% paraformaldehyde overnight at 4 °C, followed equilibrated muscle tissues in 20% sucrose overnight until the tissues settle to bottom at 4 °C, then transferred them into 30% sucrose overnight till they settle to bottom at 4 °C, and next they were embedded in OCT for frozen section, sliced at 10 µm thickness. Subsequently, frozen sections were rinsed gently with 4 °C PBS for three times, fixed with 4 °C 4% PFA for 30 min at room temperature and then washed three times with PBS again. After incubation with 0.2% Triton-X100 in PBS for 20 min, sections were blocked with 3%BSA for 30 min at room temperature and then incubated overnight with rabbit anti-GLUT4 antibody (1:250, Abcam, ab654) at 4 °C in a wet box. After washing with Tris-buffered saline containing 0.1% Tween 20, the sections were co-incubated with donkey secondary antibody conjugated with Alexa Fluor® 568 (1:400, Invitrogen, A-11011) for 3 h at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1 µg/ml, Sigma-Aldrich, USA). All photomicrographs were taken with an inverted fluorescence microscope (Nikon Eclipse Ti-E, Japan).

2.2.11 Western blotting

Total protein extracts of skeletal muscle and epididymal fat pad tissues were obtained by homogenizing and lysing with RIPA buffer (Beyotime, China), and then centrifuging at 14000 g for 5 min at 4 °C. The protein concentration was measured with a BCA assay kit (Beyotime, China). An equal amount of protein (20 µg) was loaded in each lane. Proteins were separated using sodium dodecylsulfate-polyacrylamide (SDS-PAGE) gel electrophoresis and electrically transferred to a polyvinylidene difluoride membrane (Millipore). After blocking the membrane with 5% skim milk, target proteins were immunodetected using specific antibodies. After incubation with the secondary antibody, bands were visualized using an ECL plus kit (ThermoFisher, USA) and exposed to autoradiographic films according to the manufacturer's instruction. The intensities of bands were performed using Image J 1.52q software.

2.3 Statistical analysis

Statistical analysis was performed using SPSS 23.0 software. Data were presented as mean ± S.D., and statistical significance was determined using one-way analysis of variance (ANOVA) followed by multiple comparisons with Tukey's test. A *p*-value < 0.05 was considered as statistically significant difference.

3. Results

A total of 40 rats received STZ injection after 8 weeks of HFD feeding, among which 29 attained targeted RBG levels (≥ 16.7 mmol/L) and were used in the following experiment. Five rats died within 3 days of STZ administration because of intolerance to STZ toxicity. Furthermore, RBG concentrations of another 6 rats fluctuated in the range of 5.5–9.8 mmol/L, and were therefore excluded from the following experiment. During the entire intervention period, there were no death in the blank control group (ND + AL). However, two rats in the MCT group (HFD + AL + STZ) died within 6 weeks, probably due to higher blood glucose levels (fluctuated in 22–27 mmol/L) and lower body weight, which influenced their

acquisition of food and water. One rat in the CR group (HFD + CR + STZ) died accidentally from excessive anesthesia with isopentane inhalation when drawing blood from the tail vein in the GSIS experiment.

3.1 Effects of CR on body mass and glucolipotoxicity

Body weight at each time point in the CR group was lower than that in the MCT group, but the difference was not statistically significant (Fig. 2A and B, $p > 0.05$). However, when compared with the baseline, a significant decrease was observed in the CR group (Fig. 2A and B, $p < 0.05$) but not in the MCT group (Fig. 2A and B, $p > 0.05$). Meanwhile, water intake in the MCT group increased gradually, while it declined to the blank control level after 20 weeks of CR intervention (Fig. 2C and D, $p > 0.05$).

During the intervention period, RBG and FBG levels increased significantly in the MCT group and decreased gradually in the CR group. Ultimately, hyperglycemia was ameliorated by 20 weeks of CR intervention. (Fig. 2E and F, $p < 0.05$). Meanwhile, improvement in lipid profile including free fatty acids (FFAs, Fig. 2K, $p < 0.05$) was also detected after CR intervention (Supplementary Fig. 1). These results demonstrated that CR intervention provide relief from glucolipotoxicity. Accordingly, we found that glucose tolerance in the CR group at 20 weeks was significantly improved when compared to that at baseline or in the MCT group. (Fig. 2I and J, $p < 0.05$). The area under the curve (AUC) of the IPGTT also showed a similar variation tendency (Fig. 2J, $p < 0.05$).

3.2 Effects of CR on insulin resistance and islets function

After 20 weeks of intervention, insulin sensitivity was notably improved in the CR group compared with that in the MCT group, as indicated by the ITT results. (Fig. 3A, $p < 0.05$). Interestingly, homeostatic model assessment for insulin resistance (HOMA-IR) values in the CR and MCT groups both exhibited a significant decreasing tendency, and were significantly lower than their baseline values (Fig. 3D, $p < 0.05$). Glucose-stimulated insulin secretion levels in the CR and MCT groups were both much lower than those in the BCT group (Fig. 3B and C, $p < 0.05$). Nevertheless, GSIS gradually improved in the CR group and progressively deteriorated in the MCT group over time (Fig. 3B and C). Insulin levels and AUC of GSIS in the CR group at 20 weeks were significantly higher than those in the baseline and the MCT group, although they were still markedly lower than those in the BCT group (Fig. 3B and C, $p < 0.05$).

3.3 Effects of CR on glucose uptake in skeletal muscle

The glucose uptake rate of muscle in the BCT group increased proportionally with the elevation of insulin concentration applied for stimulation, while this effect was distinctly impaired in both the CR and MCT groups (Fig. 3E). Although the glucose uptake rate in the CR group at each insulin concentration was slightly higher than that in the MCT group, the difference was not statistically significant (Fig. 3E, $p > 0.05$).

3.4 Effects of CR on AKT/AS160/GLUT4 signaling

In skeletal muscle, CR intervention could exert protective effects on the greatly reduced expression of AKT, p-AKT, AS160, and GLUT4 proteins induced by T2DM pathology and HFD feeding. However, the protective effects of CR wore off over time. The expression of the aforementioned proteins and the average fluorescence intensity of GLUT4 decreased to the same levels as the MCT group at 20 weeks (Fig. 4A, B and C; Fig. 5, $p > 0.05$). In contrast, in white adipose tissue, the protein expression of AKT, p-AKT, AS160, and GLUT4 in the MCT group decreased sharply over time, and were markedly lower than those in the CR groups at 20 weeks (Fig. 4D, E, and F, $p < 0.05$).

4. Discussion

In a diabetic rat model induced by HFD feeding combined with a single low-dose injection of STZ, we demonstrated that 30% CR could protect islet function against glucolipotoxicity during the progression of T2DM by markedly relieving hyperglycemia and dyslipidemia. CR also improved insulin resistance significantly, with probable underlying molecular mechanisms related to the upregulation of AKT/AS160/GLUT4 signaling.

CR has attracted increasing attention in the treatment of metabolic diseases, as overeating and obesity are increasingly common in modern society. Therefore, weight loss has always been considered as the main effect of CR and the source of its other accompanying metabolic benefits. However, some studies^[22, 23] have indicated that weight loss may not be the only key function of CR in improving glucose metabolism. In our study, hyperglycemia in MCT rats deteriorated over time, resulting in aggravated osmotic diuresis, massive loss of glucose, and subsequent continuous weight loss. In contrast, diabetic rats receiving CR intervention experienced great relief from hyperglycemia, protecting them from continuous weight loss. However, insufficient calorie intake might offset the effect of weight gain resulting from hyperglycemia relief, and may eventually lead to a slight (but not significant) decline in body mass when compared with MCT rats.

Glucotoxicity refers to long-term chronic hyperglycemia leading to downregulated insulin gene expression and chronic irreversibly decreased insulin synthesis. Meanwhile, lipotoxicity refers to the toxic effect of high concentrations of fatty acids in circulation on β cells^[24]. In our study, hyperglycemia was constantly aggravated in the MCT group over time, indicating more and more severe glucotoxicity. Accordingly, the results from GSIS also revealed almost exhausted insulin secretion. Fortunately, 30% CR markedly reversed the deterioration of hyperglycemia, leading to relief from glucotoxicity and thus protection of islet function. The role of CR in improving the lipid profile has been widely demonstrated in previous studies^[25, 26], as well as in this study. High FFAs level is an independent risk factor for T2DM^[27, 28]. In vitro studies have shown that FFAs can damage glucose-stimulated insulin secretion in β cells and primary islets, and increase β -cell apoptosis and necrosis^[29, 30]. Our data demonstrated that sharply increased FFAs level induced by HFD feeding and T2DM pathology could be reversed by 30% CR. The alleviation of lipotoxicity seemed to exert positive effects on β cells as GSIS was eventually improved after CR intervention in our study.

The insulin signaling pathway is crucial for normal insulin action. Defects in the transduction or phosphorylation of the amongst signaling molecules such as PI3K, AKT, and GLUT4 may be relate to the onset of IR. Our data indicated that AKT/AS160/GLUT4 signaling in skeletal muscle was consecutively downregulated with the progression of diabetes and HFD feeding, while CR intervention seemed to exert limited protective effects. Although the expression of AKT, p-AKT, AS160, and GLUT4 proteins was restored to some extent by 30% CR at 10 weeks, their expression declined to the levels observed in MCT rats at 20 weeks. In accordance with this, the improvement in glucose uptake induced by 30% CR was not significant. These results probably suggest that the overall IR improvement induced by 30% CR may not be due to the alleviation of IR in muscle. This needs to be further confirmed in the future studies. An in vitro study^[31] found no significant change in basal glucose uptake rate of skeletal muscle tissue treated with medium containing glucose and insulin equivalent to the CR level in vivo when compared with the control group. Mechanism analysis detected no notable changes in p-AKT, AS160, GLUT1, and GLUT4, which was similar to our results. In visceral white adipose tissue, the protein expression levels of AKT, p-AKT, AS160 and GLUT4 in the MCT group decreased progressively over time, which was consistent with the downregulation of insulin signaling in IR individuals reported by many studies. However, these effects could be significantly reversed by 30% CR intervention, suggesting the potential of 30% CR in delaying impairment of insulin signaling in white adipose tissue during the process of T2DM.

In addition, it was suggested^[32] that the remaining β cells may proliferate after the destruction of STZ, resulting in spontaneous recovery from hyperglycemia. Hence, diabetic rats were fed HFD to maintain model stability in our experiment. However, overt glucolipotoxicity induced by consecutive HFD feeding accelerated islet failure, especially in the MCT group. Then, the decrease in FINS was much greater than the increase in FBG; therefore, the HOMA-IR value of the MCT group calculated with the formula also exhibited a decreasing tendency, contrary to the aggravated IR reflected by the ITT curve.

Admittedly, the implementation of CR with HFD due to the instability of the STZ-induced diabetic model may have greatly weakened the protective effects of CR. This is one of the limitations of our research. In addition, we did not include included more rats or other diabetic animal models, such as transgenic models (*db/db*, *ob/ob*), to verify the results repeatedly. In the future, transgenic animal models and larger sample sizes should be employed to better interpret the benefits of CR. The protective effects of CR under the condition of blocked AKT/AS160/GLUT4 signaling have not been explored yet, and further studies are warranted to consolidate our findings.

5. Conclusions

In conclusion, our study demonstrated that during the progression of T2DM, 30% CR could exert protective effects on islet function through significant alleviation of glucolipotoxicity, reflected by markedly improved hyperglycemia and dyslipidemia, and ameliorate insulin resistance. The underlying molecular mechanism are likely related to the upregulation of AKT/AS160/GLUT4 signaling in white adipose tissue. Further studies associated with of AKT/AS160/GLUT4 signaling need to be conducted to enhance the credibility of our results.

List Of Abbreviations

T2DM, type 2 diabetes mellitus; CR, Calorie restriction; HFD, high-fat diet; IR, insulin resistance; INSR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphoinositide-3-kinase; AKT, protein kinase B; AS160, AKT substrate of 160kDa; GLUT4, glucose transporter 4; STZ, streptozotocin; RBG, random blood glucose; FBG, fasting blood glucose; ITT, insulin tolerance test; FINS, fasting insulin; HOMA-IR, homeostasis model assessment of insulin resistance; IPGTT, intraperitoneal glucose tolerance test; GSIS, glucose-stimulated insulin secretion; WAT, white adipose tissue; KRH, Krebs-Henseleit buffer; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FFAs, free fatty acids; TG, triglyceride, TC, total cholesterol, HDL-c, high-density lipoprotein cholesterol, LDL-c, low-density lipoprotein cholesterol.

Declarations

Ethics approval and consent to participate

The animal experiments were performed according to internationally followed ethical standards and approved by the research ethics committee of the School of Public Health, Sun Yat-Sen University (NO. 2019–001).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JQ, LZ, and YJH conceived and designed the experiments. LZ, YJH, JPS, TYZ, and TLL performed the study. LZ, YJH, and BK drafted the manuscript. XFS, HL, GPZ, and ZYY analyzed the data. JQ, BK, and XFS revised the manuscript critically for important intellectual content, clarity, and grammar. All authors read and approved the final manuscript.

References

- 1 Simpson RW, Shaw JE, Zimmet PZ The prevention of type 2 diabetes–lifestyle change or pharmacotherapy? A challenge for the 21st century. *Diabetes Res Clin Pract*, 2003, 59(3): 165-80.
- 2 International Diabetes Federation Diabetes Atlas 9th edition, IDF, Brussels. Available at: https://diabetesatlas.org/upload/resources/2019/IDF_Atlas_9th_Edition_2019.pdf
- 3 Lean MEJ, Leslie WS, Barnes AC, *et al*. Primary care-led weight management for remission of type 2 diabetes (DiRECT): an open-label, cluster-randomised trial. *Lancet*, 2018, 391(10120): 541-51.
- 4 Petersen MC, Shulman GI Mechanisms of Insulin Action and Insulin Resistance. *Physiological Reviews*, 2018, 98(4): 2133-223.
- 5 Storgaard H, Song XM, Jensen CB, *et al*. Insulin signal transduction in skeletal muscle from glucose-intolerant relatives of type 2 diabetic patients [corrected]. *Diabetes*, 2001, 50(12): 2770-8.
- 6 Pratipanawatr W, Pratipanawatr T, Cusi K, *et al*. Skeletal muscle insulin resistance in normoglycemic subjects with a strong family history of type 2 diabetes is associated with decreased insulin-stimulated insulin receptor substrate-1 tyrosine phosphorylation. *Diabetes*, 2001, 50(11): 2572-8.
- 7 Tonks KT, Ng Y, Miller S, *et al*. Impaired Akt phosphorylation in insulin-resistant human muscle is accompanied by selective and heterogeneous downstream defects. *Diabetologia*, 2013, 56(4): 875-85.
- 8 Abel ED, Peroni O, Kim JK, *et al*. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature*, 2001, 409(6821): 729-33.
- 9 Zisman A, Peroni OD, Abel ED, *et al*. Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat Med*, 2000, 6(8): 924-8.
- 10 Moltke I, Grarup N, Jorgensen ME, *et al*. A common Greenlandic TBC1D4 variant confers muscle insulin resistance and type 2 diabetes. *Nature*, 2014, 512(7513): 190-3.
- 11 Li T, Chen K, Liu G, *et al*. Calorie restriction prevents the development of insulin resistance and impaired lipid metabolism in gestational diabetes offspring. *Pediatr Res*, 2017, 81(4): 663-71.
- 12 Prasannarong M, Vichaiwong K, Saengsirisuwan V Calorie restriction prevents the development of insulin resistance and impaired insulin signaling in skeletal muscle of ovariectomized rats. *Biochim Biophys Acta*, 2012, 1822(6): 1051-61.

- 13 Wang H, Sharma N, Arias E, *et al.* Insulin Signaling and Glucose Uptake in the Soleus Muscle of 30-Month-Old Rats After Calorie Restriction With or Without Acute Exercise. *The journals of gerontology Series A, Biological sciences and medical sciences*, 2015, 71(
- 14 Wheatley KE, Nogueira LM, Perkins SN, *et al.* Differential effects of calorie restriction and exercise on the adipose transcriptome in diet-induced obese mice. *J Obes*, 2011, 2011(265417).
- 15 Park SY, Choi GH, Choi HI, *et al.* Calorie restriction improves whole-body glucose disposal and insulin resistance in association with the increased adipocyte-specific GLUT4 expression in Otsuka Long-Evans Tokushima Fatty rats. *Archives of Biochemistry and Biophysics*, 2005, 436(2): 276-84.
- 16 Xu W, Lu Z, Wang X, *et al.* *Gynura divaricata* exerts hypoglycemic effects by regulating the PI3K/AKT signaling pathway and fatty acid metabolism signaling pathway. *Nutr Diabetes*, 2020, 10(1): 31.
- 17 Reed MJ, Meszaros K, Entes LJ, *et al.* A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat. *Metabolism*, 2000, 49(11): 1390-4.
- 18 Yao H, Gu J, Shan Y, *et al.* Type 2 diabetes mellitus decreases systemic exposure of clopidogrel active metabolite through upregulation of P-glycoprotein in rats. *Biochem Pharmacol*, 2020, 180(114142).
- 19 Nambirajan G, Karunanidhi K, Ganesan A, *et al.* Evaluation of antidiabetic activity of bud and flower of Avaram Senna (*Cassia auriculata* L.) In high fat diet and streptozotocin induced diabetic rats. *Biomed Pharmacother*, 2018, 108(1495-506).
- 20 Koshinaka K, Sano A, Howlett KF, *et al.* Effect of high-intensity intermittent swimming on postexercise insulin sensitivity in rat epitrochlearis muscle. *Metabolism-Clinical and Experimental*, 2008, 57(6): 749-56.
- 21 Chukwuma CI, Islam MS Effects of xylitol on carbohydrate digesting enzymes activity, intestinal glucose absorption and muscle glucose uptake: a multi-mode study. *Food & Function*, 2015, 6(3): 955-62.
- 22 Ajiboye BO, Oyinloye BE, Agboinghale PE, *et al.* *Cnidioscolus aconitifolius* (Mill.) I. M. Johnst leaf extract prevents oxidative hepatic injury and improves muscle glucose uptake ex vivo. *Journal of Food Biochemistry*, 2019, 43(12):
- 23 Baskota A, Li S, Dhakal N, *et al.* Bariatric Surgery for Type 2 Diabetes Mellitus in Patients with BMI <30 kg/m²: A Systematic Review and Meta-Analysis. *PLoS One*, 2015, 10(7): e0132335.
- 24 Perry RJ, Peng L, Cline GW, *et al.* Mechanisms by which a Very-Low-Calorie Diet Reverses Hyperglycemia in a Rat Model of Type 2 Diabetes. *Cell Metabolism*, 2018, 27(1): 210+.
- 25 van Raalte DH, Diamant M Glucolipotoxicity and beta cells in type 2 diabetes mellitus: Target for durable therapy? *Diabetes Research and Clinical Practice*, 2011, 93(S37-S46).

- 26 Holowko J, Michalczyk MM, Zajac A, *et al.* Six Weeks of Calorie Restriction Improves Body Composition and Lipid Profile in Obese and Overweight Former Athletes. *Nutrients*, 2019, 11(7):
- 27 Kraus WE, Bhapkar M, Huffman KM, *et al.* 2 years of calorie restriction and cardiometabolic risk (CALERIE): exploratory outcomes of a multicentre, phase 2, randomised controlled trial. *Lancet Diabetes & Endocrinology*, 2019, 7(9): 673-83.
- 28 Cnop M, Welsh N, Jonas JC, *et al.* Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes - Many differences, few similarities. *Diabetes*, 2005, 54(S97-S107).
- 29 Paolisso G, Tataranni PA, Foley JE, *et al.* A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM. *Diabetologia*, 1995, 38(10): 1213-7.
- 30 Poitout V, Robertson RP Minireview: Secondary beta-cell failure in type 2 diabetes - A convergence of glucotoxicity and lipotoxicity. *Endocrinology*, 2002, 143(2): 339-42.
- 31 Lupi R, Dotta F, Marselli L, *et al.* Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets - Evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes*, 2002, 51(5): 1437-42.
- 32 Su EN, Alder VA, Yu DY, *et al.* Continued progression of retinopathy despite spontaneous recovery to normoglycemia in a long-term study of streptozotocin-induced diabetes in rats. *Graefes Archive for Clinical and Experimental Ophthalmology*, 2000, 238(2): 163-73.
- 33 Arias EB, Cartee GD In vitro simulation of calorie restriction-induced decline in glucose and insulin leads to increased insulin-stimulated glucose transport in rat skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*, 2007, 293(6): E1782-E8.

Figures

Figure 1

Flow chart of animal study design

Figure 2

Effects of CR on metabolic indices The diabetic Sprague-Dawley rats model was induced by feeding HFD for 8 weeks, followed by a single STZ injection (30 mg/kg). The rats were either provided HFD *ad libitum* (HFD + AL + STZ group) or fed HFD with a 30% restriction regimen (HFD + CR + STZ group). Rats given free access to normal chow diet served as a blank control (ND + AL group). All interventions lasted for 20

weeks. (A) and (B) body mass; (C) and (D) water intake; (E) and (F) RBG; (G) and (H) FBG; (I) IPGTT; (J) AUC of IPGTT; (K) FFAs. Data were presented as the mean $\pm$ standard deviation (SD). For A–H, $n=10-20$ for the ND + AL group and $n=6-14$ for the other two groups. Six rats from each group were randomly chosen for the IPGTT experiment and lipid profile detection. * $p < 0.05$ between the two groups; CR, calorie restriction; HFD, high-fat diet; STZ, streptozotocin; RBG, random blood glucose; FBG, fasting blood glucose; IPGTT, intraperitoneal glucose tolerance test; AUC, area under curve; FFAs, free fat acids; ND + AL, normal diet provided ad libitum (BCT group); HFD + AL + STZ, HFD provided ad libitum and STZ injection (MCT group); HFD + CR + STZ, HFD with 30% CR and STZ injection (CR group).

Figure 3

Effects of CR on insulin resistance and islets function The diabetic Sprague-Dawley rats model was induced by feeding HFD for 8 weeks, followed by a single STZ injection (30 mg/kg). The rats were either provided HFD ab libitum (HFD + AL + STZ group) or fed HFD with a 30% restriction regimen (HFD + CR + STZ group). Rats given free access to normal chow diet served as a blank control (ND + AL group). All interventions lasted for 20 weeks. (A) ITT ($n=6$); (B) GSIS ($n=6$); (C) AUC of GSIS; (D) HOMA-IR ($n=6-10$); (E) Glucose uptake in muscle ($n=4$); Data were presented as the mean $\pm$ standard deviation (SD). * $p < 0.05$ between the two groups; CR, calorie restriction; HFD, high-fat diet; STZ, streptozotocin; ITT, insulin tolerance test; GSIS, glucose simulate insulin secretion; AUC, area under curve; HOMA-IR, homeostasis model assessment of insulin resistance; IOD, integrated optical density; ND + AL, normal diet provided ad libitum (BCT group); HFD + AL + STZ, HFD provided ad libitum and STZ injection (MCT group); HFD + CR + STZ, HFD with 30% CR and STZ injection (CR group).

Figure 4

Effects of CR on AKT/AS160/GLUT4 signaling The diabetic Sprague-Dawley rats model was induced by feeding HFD for 8 weeks, followed by a single STZ injection (30 mg/kg). The rats were either provided HFD ab libitum (HFD + AL + STZ group) or fed HFD with a 30% restriction regimen (HFD + CR + STZ group). Rats given free access to normal chow diet served as a blank control (ND + AL group). All interventions lasted for 20 weeks. Effects of CR on the expressions of AKT, p-AKT, AS160, and GLUT4 were determined by western blotting. (A) Expressions of AKT, p-AKT, AS160 and GLUT4 in skeletal muscle tissue after 10 and 20 weeks of intervention. (B) and (C) Densitometric measurements of band intensity were performed using ImageJ 1.52q. (D) Expressions of AKT, p-AKT, AS160 and GLUT4 in white adipose tissue after 10 and 20 weeks of intervention. (E) and (F) Densitometric measurements of band intensity were performed using ImageJ 1.52q. Data were from at least three independent experiments and presented as the mean $\pm$ standard deviation (SD). * $p < 0.05$ between the two groups; CR, calorie restriction; HFD, high-fat diet; STZ, streptozotocin; ND + AL, normal diet provided ad libitum (BCT group); HFD + AL + STZ, HFD provided ad libitum and STZ injection (MCT group); HFD + CR + STZ, HFD with 30% CR and STZ injection (CR group).

Figure 5

GLUT4 immunofluorescence in skeletal muscle The diabetic Sprague-Dawley rats model was induced by feeding HFD for 8 weeks, followed by a single STZ injection (30 mg/kg). The rats were either provided HFD ab libitum (HFD + AL + STZ group) or fed HFD with a 30% restriction regimen (HFD + CR + STZ group). Rats given free access to normal chow diet served as a blank control (ND + AL group). All interventions lasted for 20 weeks. (A) Representative images of GLUT4 immunofluorescence in skeletal muscle (100×). (B) Densitometric measurements of the average fluorescence were performed by ImageJ 1.52q. Data were from at least three independent experiments and presented as the mean ± standard deviation (SD). * $p < 0.05$ between the two groups; CR, calorie restriction; HFD, high-fat diet; STZ, streptozotocin; ND + AL, normal diet provided ad libitum (BCT group); HFD + AL + STZ, HFD provided ad libitum and STZ injection (MCT group); HFD + CR + STZ, HFD with 30% CR and STZ injection (CR group).

Supplementary Files

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