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Anti-inflammatory effects of grape seed procyanidin B2 on diabetic pancreas

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Abstract

Prevalence of type 2 diabetes mellitus (T2DM) has increased considerably in the recent years, highlights the importance of developing new therapeutic strategies. Insulin-resistance and gradual dysfunction of pancreatic islets is the mainstay in the progression of T2DM. Therefore, preserving the function of pancreas may lead to new prospective approaches. Our previous studies suggested that grape seed procyanidin B2 (GSPB2), a natural polyphenol product, exhibited protective effects on diabetic vasculopathy. However, effects of GSPB2 on diabetic pancreas remain unknown. In this study, we provided strong evidence that GSPB2 exerted protective effects on diabetic pancreas. GSPB2 attenuated the elevated body weights, food intake and advanced glycation end-products (AGEs) levels in db/db mice (p < 0.05), though had no significant effects on glucose levels. The increased islet sizes, insulin levels, as well as HOMA-IR, were also improved by GSPB2 treatment in db/db mice (p < 0.05). Milk fat globule epidermal growth factor-8 (MFG-E8), an estimated target of GSPB2 in our previous studies, was upregulated in pancreatic tissues whereas GSPB2 treatment down-regulated its protein level (p < 0.05). Since MFG-E8 is highly involved in inflammation, we further investigate pro-inflammatory cytokines interleukin-1β (IL-1β) and NLRP3 levels. We found that protein levels of IL-1β and NLRP3 increased in diabetic pancreas while GSPB2 treatment notably attenuated these alterations (p < 0.05). In conclusion, our results suggest that inflammation is involved in the damage of diabetic pancreas and GSPB2 provides protective effects at least in part through anti-inflammation.

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Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by hyperglycemia and insulin-resistance. Traditional anti-diabetic therapy mainly focuses on glycemic control. However, the dysfunction of pancreatic islets, which gradually lose the ability to secrete sufficient insulin against insulin-resistance, is the mainstay in the pathogenesis of T2DM, determining the progression and prognosis of diabetic patients.\(^1\)-\(^3\) Although molecular mechanisms underlying dysfunction of pancreas and insulin-resistance are not fully understood, accumulated evidence shows that inflammation is implicated. Release of pro-inflammatory cytokines was increased at both local and systemic levels in the development of \(\beta\)-cells dysfunction and insulin-resistance.\(^4\),\(^6\) Therefore, development of new treatment against inflammation may have great benefits on diabetic therapy.

Plant polyphenols, especially flavonoids, are of great potential as anti-diabetic agents for their ability to protect against early-stage diabetes and the development of complications.\(^7\) Flavonoids-rich extracts from cocoa show beneficial effects in strengthening anti-oxidant defenses of pancreatic \(\beta\)-cells in vitro and delaying the loss of functional \(\beta\)-cell mass in vivo.\(^8\),\(^9\) An extract of French maritime pine bark, pycnogenol, whose major constituents are bioflavonoids, was reported to possess anti-inflammatory and pancreatic protective actions.\(^10\) Procyanidins, the main group of flavonoids, are widely distributed in fruits and vegetables. Tamarind seed extracts with procyanidin content exerted protective effects in pancreatic islets in STZ-induced diabetic rats.\(^11\),\(^12\)

Procyanidins extracted from grape seeds are attracting much attention. In some diet-induced insulin resistant models, grape seed procyanidin exerted favorable effects in modulation of glucose homeostasis and insulin-resistance.\(^13\),\(^14\) Procyanidins treatment in rats modulated the miRNA and proteome profile in pancreatic islets.\(^15\),\(^16\) However, in genetic model of diabetes, such as db/db mice, results of grape seed procyanidin on pancreas were controversial. Even in cultured \(\beta\) cells under hyperglycemic conditions, grape seed procyanidin showed pro-apoptotic effects.\(^17\) Grape seed procyanidin B2 (GSPB2) is a dimeric form of grape seed procyanidin extracts, one effective components among others. Our previous studies demonstrated that GSPB2 could prevent diabetic arterial damage, as well as diabetic nephropathy at an early stage in db/db mice.\(^18\),\(^19\) However, whether GSPB2 has protective effects on pancreas at the early stage of diabetes remains unknown.

In the present study, we aimed to investigate the effects of GSPB2 on pancreatic changes in db/db mice, a rodent model for T2DM. Moreover, our previous studies showed that milk fat globule epidermal growth factor-8 (MFG-E8) was a potential target of GSPB2.\(^18\),\(^19\) MFG-E8, also known as lactadherin, is a membrane-associated glycoprotein which involves inflammatory response.\(^20\) These findings prompt us to investigate whether MFG-E8 and inflammation are involved in diabetic pancreatic changes.

Animals and Methods

Animals

Male C57BLKS/J db/db and db/m mice aged 7 weeks were obtained from Model Animal Research Center of Nanjing University (Nanjing, China). They were housed in a temperature- and humidity-controlled room with free access to standard chow and water under a 12/12 h light/dark cycle. After acclimation for one week, the diabetic db/db mice were randomly divided into two groups: one
group was treated with GSPB2 (purity > 95%, 30 mg/kg body weight per day, diluted in normal saline solution) (provided by Jianfeng Inc, Tianjin, China) by intragastric administration every morning at 8 a.m. for 10 weeks (DMT, n=8); the other group was treated with the same amount of normal saline solution (DM, n=8). The non-diabetic db/m mice were selected as control group (CC, n=8). At the end of the 18th week, mice were fasted overnight and anesthetized using sodium pentobarbital. Fasting blood was collected from ophthalmic veins. Then all mice were perfused with ice-cold normal saline. Pancreases were dissected out. The serum and tissues were stored at -80 °C for further analysis. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the animal ethics committee of Shandong University.

Measurement of body weights, food intake, fasting blood glucose (FBG), advanced glycation end products (AGEs), serum insulin and Homeostasis Model Assessment of Insulin Resistance (HOMA-IR)

All mice were weighed every week. Food intake was monitored three times a week. FBG and serum insulin were detected using DVI-1650 Automatic Biochemistry and Analysis Instrument (Bayer, Germany). AGEs-specific fluorescence detection was estimated by measuring emission at 440 nm on excitation at 370 nm using a fluorescence spectrophotometer (HITACHI F-2500, Japan). HOMA-IR was calculated using the formula: HOMA-IR = blood glucose (mmol/L) × serum insulin (mIU/L) / 22.5.\(^{21}\)

Data was expressed as fold increase over the control group.

Pancreas morphology and islet size analysis

Pancreas was fixed in 4% paraformaldehyde for 48 h and dehydrated in graded ethanol solutions, followed by embedding in paraffin. The embedded specimens were sectioned at a thickness of 5 \(\mu\)m. Then hematoxylin–eosin (HE) staining was conducted. The morphological changes of pancreas were examined under light microscope. For quantitative analysis, at least 6 sections from each pancreas (4 pancreas from each group) were used. Areas of pancreas and islets were measured by Image J. Islet/pancreas area was determined by the sum of all islet areas from one section divided by the area of the whole section and averaged for each pancreas. Data was expressed as fold increase over the control group. Similar analysis was used in previous studies.\(^{22, 23}\)

Western blot:

Equal amount of protein samples from pancreas were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and then transferred onto a poly(vinylidene difluoride) membrane. After blocking (TBST containing 5% non-fat milk) for 1 h at room temperature, the immunoblots were probed with primary antibodies overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies. The primary antibodies were goat anti-MFG-E8 (1:100, AF2805, R&D systems, Minneapolis, USA), rabbit anti-IL-1\(\beta\) (1:1000, ab9722, Abcam, Cambridge, UK) and goat anti-NLRP3 (1:100, sc-34411, Santa Cruz, CA, USA). The secondary antibodies were rabbit anti-goat and goat anti-rabbit secondary antibodies from ZSGB-BIO (Beijing, China)(1:3000). Bands were visualized by ECL detection system and analyzed using Image J. GAPDH was used as loading control.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software. Experiment data was expressed as mean ± standard deviation. Significant differences among groups were evaluated using one-way analysis of variance followed by Tukey’s HSD test. \(P < 0.05\) was considered statistically significant.
**Results**

Effects of GSPB2 on body weight, FBG and AGEs

During the experimental period, db/db mice without any treatment showed obvious obesity compared with normal mice. As shown in Fig 1A, body weights of db/db mice were consistently higher than that of db/m mice ($p < 0.05$). However, from the second week after GSPB2 treatment, the increase of body weight in db/db mice was significantly attenuated ($p < 0.05$). Meantime, food intake of db/db mice was significantly increased, compared with db/m mice ($p < 0.05$). GSPB2 treatment effectively inhibited the increasing food intake after the second week of treatment ($p < 0.05$) (Fig 1B). FBG and AGEs were also estimated. At the end of the experiment, serum levels of FBG and AGEs in db/db mice were notably higher than that in db/m mice. After GSPB2 treatment, levels of AGEs in db/db mice were significantly reduced ($p < 0.05$). However, levels of FBG were not significantly affected by GSPB2, compared with db/db mice (Fig 1C and D).

Effects of GSPB2 on islet size, insulin levels and HOMA-IR

At the early stage of diabetes, a compensatory increase of insulin secretion was induced by peripheral insulin-resistance, accompanied by the increased $\beta$-cell mass. But consistent increase of insulin secretion leads to $\beta$-cell exhaustion.$^{24, 25}$ Therefore, preventing $\beta$-cells from over-secretion of insulin might be of great benefits. Our histologic results showed that islet size of db/db mice was strikingly larger than that of db/m mice ($p < 0.05$). After GSPB2 treatment, the enlargement of islet was significantly ameliorated ($p < 0.05$) (Fig 2A and B). Serum level of insulin was also elevated in db/db mice, whereas GSPB2 treatment decreased these elevations ($p < 0.05$) (Fig 2C). HOMA-IR, representing insulin resistance, increased largely in db/db mice, compared with normal mice. However, GSPB2 treatment notably reduced HOMA-IR of db/db mice ($p < 0.05$) (Fig 2D).

Effects of GSPB2 on the protein expression of MFG-E8

In order to explore the mechanisms underlying the protective effects of GSPB2 on diabetic pancreas, we evaluated the protein expression of MFG-E8 in pancreas. As shown in Fig 3, MFG-E8 was expressed in normal pancreas. In db/db mice, protein level of MFG-E8 was largely increased, compared with CC group ($p < 0.05$). After treatment with GSPB2, the increased protein level of MFG-E8 was significantly attenuated in DMT group, compared with DM group ($p < 0.05$).

Effects of GSPB2 on protein levels of interleukin 1$\beta$ (IL-1$\beta$) and NLRP3

Since MFG-E8 is largely involved in inflammatory response, we further evaluated the protein levels of IL-1$\beta$ and NLRP3 in pancreas. As shown in Fig 4, protein level of IL-1$\beta$ was notably increased in db/db mice, compared with control mice. By GSPB2 treatment, the increased protein level of IL-1$\beta$ was significantly suppressed, compared with db/db mice ($p < 0.05$). Meanwhile, NLRP3 was remarkably up-regulated in pancreas of db/db mice, while GSPB2 treatment suppressed the up-regulation of NLRP3 ($p < 0.05$).

**Discussion**

Pancreas plays an important role in secreting hormones to maintain the homeostasis of blood glucose.
Progressive dysfunction of pancreatic islets and \( \beta \)-cells is the key point among the multiple pathogenic mechanisms of T2DM.\(^{26}\) Study shows that loss of pancreatic function begins to occur several years before the onset of hyperglycemia.\(^{27}\) Therefore, early intervention to preserve the function of pancreas is crucial for delaying the progression of diabetes. However, current therapies for diabetes are limited to supplement of insulin and improvement of tissue response to insulin.\(^{28, 29}\) Development of new therapies targeting on protection of pancreatic function thus becomes high priorities.

Grape seed procyanidin extract is a natural complex of polyphenol polymers. It was reported that procyanidin B2 exerted greater effects than other dimers, in regarding to some biological potential, for example anti-tumor-promoting potential.\(^{30}\) However, one report demonstrated that procyanidin B2 alone did not exhibit growth-inhibitory effects on human prostate carcinoma LNCaP cells.\(^{31}\) View on procyanidin B2 seems controversial. In our opinion, each component from grape seed procyanidins may have different biological activities depending on their different structures and targeting models. Our previous studies demonstrated that GSPB2 prevented diabetic arterial damage, as well as diabetic nephropathy at an early stage in db/db mice.\(^{18, 19}\) The present study aimed to find whether GSPB2 has protective effects on pancreas, since no direct related data was available, as far as we know. Db/db mice, a commonly used model of T2DM, have been observed to develop similar diabetic symptoms as humans, including obesity, hyperglycemia and insulin-resistance.\(^{32}\) Thus, our present study used db/db mice to investigate the effects of GSPB2 on diabetic pancreatic changes. Since 30mg GSPB2/kg bw treatment significantly changed the proteome profile of kidney and aorta in db/db mice and exerted protective effects in our previous studies, we chose the same dose in this study.\(^{18, 19}\)

Obesity is a significant symptom of db/db mice, which is due to the severely increased food intake and reduced energy expenditure.\(^{33}\) Previous studies reveal the effects of procyanidins against obesity, where they prevent weight gain and adipose tissue mass increase. One possible mechanism is that adipocyte is a target of procyanidins. Procyanidins mobilize lipid stores by modulating intracellular signaling cascades.\(^{34}\) Moreover, procyanidins limit adipocyte formation by altering the gene expression profile during in vitro adipocyte differentiation.\(^{35}\) Being consistent with these data, our results showed that GSPB2 treatment significantly inhibited the gain of body weight, indicating the anti-obesity effect of GSPB2 in db/db mice. On the other hand, our study showed that food intake of db/db mice was higher than db/m mice, while GSPB2 treatment significantly decreased the food intake of db/db mice. This effect can be explained by the opinion that procyanidins inhibit the activity of pancreatic lipase, which is the most important enzyme for dietary triacylglycerols digestion.\(^{36}\)

Advanced glycation end products (AGEs) are resulting from hyperglycemia and accumulating in the plasma and tissue of diabetic patients.\(^{37}\) They play a critical role in endothelial dysfunction and diabetic vasculopathy.\(^{38, 39}\) It has been reported that AGEs could induce apoptosis of pancreatic islet endothelial cells via a RAGE/NF-\( \kappa \)B/COX-2/PGE\(_2\) signaling pathway.\(^{40}\) In another model of cultured rat pancreatic islets, AGEs exerted pro-apoptotic effects after \( 72 \) h of exposure.\(^{41}\) AGEs also injure pancreatic \( \beta \) cells through mitochondrial pathway, as well as JNK and p38 MAPK signaling pathways.\(^{42}\) Therefore, preventing the production of AGEs is of great importance in maintaining the normal function of pancreas. Our results showed that AGEs levels were much higher in db/db mice, whereas GSPB2 treatment notably decreased the levels of AGEs, indicating the pancreatic protective effects of GSPB2. In our hypothesis, these effects were at least in part through decreasing AGEs level thus inhibiting the pro-apoptotic effects and oxidative injury of AGEs on pancreatic islets. However, more evidence is needed to verify our hypothesis.

In our results, GSPB2 failed to improve hyperglycemia. However, there are opposite results showing
that grape seed procyanidins improved glucose homeostasis in streptozotocin and high-fat-induced diabetic animal models, \(^{43, 44}\) though no reports provide data of GSPB2 on \(db/db\) mice. In our opinion, there may be two possible explanations of our results: First, a compensatory phase exists at the early stage of diabetes, during which insulin secretion increase, leading to hyperinsulinemia. Recently, there is view that hyperinsulinemia is often both a result and a driver of insulin-resistance, in that rats and humans treated with escalating doses of insulin show insulin resistance.\(^{45}\) Thus the hyperinsulinemia could further aggravate insulin-resistance. GSPB2 treatment was not enough or too late to reverse the hyperglycemia. Second, \(db/db\) mice is a genetic model of diabetes, which manifest T2DM-like characteristics due to the congenital deficiency of leptin receptor. GSPB2 was not sufficient to counteract the genetic background of \(db/db\) mice in aspect of hyperglycemia. In another leptin receptor deficient models, Zucker fatty rats, GSPE treatment did not improve glucose homeostasis either, though proteins involved in insulin synthesis and secretion were modulated.\(^{16}\) Our histological results showed that pancreatic islet in \(db/db\) mice were much larger than normal ones, indicating they were undergoing the compensatory period. GSPB2 treatment significantly prevented the over-enlargement of pancreatic islet in \(db/db\) mice. What’s more, our results showed that hyperinsulinemia and insulin-resistance occurred in \(db/db\) mice, which were largely improved by GSPB2 treatment. These results support our first explanation of GSPB2’s effects on glucose homeostasis. However, the second explanation is still of great possibility which needs to be further studied.

MFG-E8 was first identified to be responsible for phagocytic clearance of apoptotic cells.\(^{46}\) Our previous clinical studies on diabetic patients showed that MFG-E8 was correlated with poor blood glucose control.\(^{47}\) Moreover, significant up-regulation of MFG-E8 expression was found in chronic pancreatitis patients.\(^{48}\) These findings imply that MFG-E8 is implicated in glucose homeostasis and pancreatic inflammation. Our study showed that protein level of MFG-E8 was increased in \(db/db\) mice, compared with control ones. After GSPB2 treatment, the increased protein level of MFG-E8 was reduced. The increased level of MFG-E8, as well as the hyperglycemia, was in accordance with the previous findings that MFG-E8 was involved in glucose homeostasis, though involvement of inflammation still needs to be studied. In a MFG-E8 deficient transgenic mouse model, MFG-E8 promoted proliferation in premalignant angiogenic islets and might enhance cell survival.\(^{49}\) The underlying mechanism may involve increased Akt phosphorylation.\(^{50}\) Our results showed up-regulated MFG-E8 in pancreas, as well as increased pancreatic islets. Together these findings may imply that MFG-E8 in some extent promotes islets increase, but further studies are needed to verify. Targeting MFG-E8 might be one way for GSPB2 to exert protective effects on diabetic pancreas. However, we still cannot exclude the possibility that decreased MFG-E8 is the outcome of reduced diabetes severity after GSPB2 treatment. To solve this question, transgenic mice with MFG-E8 knocking-down or exogenous recombinant are needed in our future project.

Apart from the inflammatory implication of MFG-E8, grape seed procyanidin exerts anti-local and anti-systemic inflammatory effects via modulating cytokines in diet-induced obesity rats.\(^{51, 52}\) These findings prompt us to find more evidence of inflammatory involvement in diabetic pancreas. Our present study showed that both IL-1\(\beta\) and NLRP3 were up regulated in pancreas of \(db/db\) mice, compared with control mice. Pro-inflammatory cytokine IL-1\(\beta\) has emerged as a primary therapeutic target for inflammatory conditions. Exposure of islets to glucose induces release of IL-1\(\beta\).\(^{53}\) The mechanism includes that high-concentration glucose activates the thioredoxin-interacting protein, which binds to NLRP3, member of the Nod-like receptor family, resulting the processing of the inactive IL-1\(\beta\) precursor to mature active IL-1\(\beta\).\(^{54, 55}\) Once released, IL-1\(\beta\) can amplify its signals by self-activation, leading to a
vicious cycle of inflammation. Local inflammation and increased IL-1β levels play a key role in the progression of β-cell failure. Thus inhibiting the local levels of IL-1β and NLRP3 at an early stage of diabetes is of great importance to restore the function of islets. In our study, GSPB2 treatment significantly decreased the levels of IL-1β and NLRP3 in db/db mice, suggesting the protective effects of GSPB2 on diabetic pancreas may at least in part due to the anti-inflammatory effects.

Conclusions
Our data demonstrate that inflammation is involved in the damage of diabetic pancreas and natural product GSPB2 exhibits protective effects on diabetic pancreas. The protective effects of GSPB2 are at least in part through inhibiting inflammation, in which regulating MFG-E8, IL-1β and NLRP3 are potentially involved. Our research may provide new avenues for anti-diabetic therapy.

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References


Fig. 1  Effects of GSPB2 on body weight, food intake, FBG and AGEs in db/db mice. A, changes of body weight during the experimental period. B, changes of food intake during the experimental period. C and D, measurement of FBG and AGEs levels after 10 weeks of GSPB2 treatment. *P < 0.05 versus CC group; # P < 0.05 versus DM group. CC, control db/m mice; DM, untreated db/db mice; DMT, db/db mice treated with GSPB2; FBG, fasting blood glucose; AGEs, advanced glycation end products.
Fig. 2 Effects of GSPB2 on islet size and insulin levels in db/db mice. A, representative hematoxylin-eosin (HE) staining pictures of pancreatic islets (200×). B, quantitation of islet/pancreas area. C, measurement of serum insulin levels. D, quantitation of HOMA-IR. *P < 0.05 versus CC group; # P < 0.05 versus DM group.
Fig. 3  Effects of GSPB2 on the protein expression of MFG-E8 in pancreas of db/db mice. A, representative western blots of MFG-E8. GAPDH was used as loading control. B, quantitative analysis of MFG-E8/GAPDH ratio (n=4, means ±SD). *P < 0.05 versus CC group; # P < 0.05 versus DM group.
Fig. 4 Effects of GSPB2 on protein levels of IL-1β and NLRP3 in pancreas of db/db mice. A and B, representative western blots of IL-1β and NLRP3. GAPDH was used as loading control. C and D, quantitative analysis of IL-1β/GAPDH ratio and NLRP3/GAPDH ratio (n=4, means ±SD). *P < 0.05 versus CC group; # P < 0.05 versus DM group.