

# Glucagon-Like Peptide I Attenuates Lipotoxicity-Induced Islet Dysfunction in ApoE<sup>-/-</sup> Mice

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**Aim:** Glucagon-like peptide-1 (GLP1) is known to decrease glucagon release and may be beneficial for the reduction of elevated blood glucose. However, its role and mechanism of action in diabetes remain elusive. This study aimed to examine the function of GLP1 and analyze the mechanism of effect that GLP1 exerts on inducible nitric oxide synthase (iNOS) in diabetic mice.

**Methods:** A diabetes model was established in ApoE<sup>-/-</sup> mice fed a high-fat diet and treated with GLP1 and/or lentivirus-expressing PARP1. PARP1, iNOS, and inflammatory factors in islets were detected by Western blot and ELISA. Islet  $\alpha$  cells and  $\beta$  cells and CD8<sup>+</sup> T lymphocytes were detected by immunostaining. Islet-cell apoptosis was detected by TUNEL.

**Results:** GLP1 inhibited the expression of PARP1 and iNOS in islets, alleviated decrease in  $\beta$  cells, and suppressed cell apoptosis induced by the high-fat diet. Moreover, GLP1 recovered the decline in insulin sensitivity and glucose tolerance in ApoE<sup>-/-</sup> mice fed the high-fat diet, and the effects of GLP1 were related to the inhibition of COX2 and NF $\kappa$ B expression.

**Conclusion:** GLP1 significantly alleviated the decrease in  $\beta$ -cell numbers, suppressed  $\beta$ -cell apoptosis induced by the high-fat diet, inhibited the expression of iNOS, and alleviated inflammatory islet injury via inhibiting the COX2-NF $\kappa$ B pathway.

**Keywords :** GLP1, islet function, PARP1

## Introduction

Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance and impaired insulin response to glucose.<sup>1-3</sup> Impaired function of islet  $\beta$  cells is one of the major causes of DM.<sup>4</sup> Studies have shown that high glucose, high lipids, and inflammatory factors contribute to the apoptosis of islet  $\beta$  cells in T2DM patients, mainly due to the imbalance of the reactive oxygen species (ROS)- and reactive nitrogen species (RNS)-production and -clearance system.<sup>5,6</sup> Nitric oxide (NO) is an oxygen-free species synthesized by the action of NO synthetase (NOS).<sup>7,8</sup> It is generally believed that NO produced by constitutive NOS (cNOS) mainly plays a physiological regulatory role, while the NO produced by inducible NOS (iNOS) causes cell damage and plays a cytotoxic role. In addition, NO can directly activate Fas and induce apoptosis through p53.<sup>2,9</sup>

PARP1 is a member of the PARP family, which are nuclear proteins and serve as DNA-break sensors.<sup>10</sup> PARP1 can be activated via binding to DNA-strand breaks and facilitates damage repair through poly(ADP-ribosyl)ation of target proteins, such as histones, transcription factors, and PARP1 itself.<sup>11</sup> Moreover, PARP1 can

regulate the expressions of inflammatory factors, including iNOS, ICAM1, and VCAM1.<sup>12</sup> Accumulated evidence shows that exposure of islets to high lipids induces PARP1 expression, concomitant with reduced insulin response to glucose.<sup>13,14</sup>

Nutrient ingestion stimulates the secretion of gut hormones, including GLP1 and gastric inhibitory polypeptide, to amplify glucose-stimulated insulin release. GLP1 is known to decrease glucagon release and may be beneficial in the reduction of elevated blood glucose.<sup>15–17</sup> We recently showed that GLP1 can restore impairment of glucose-stimulated insulin release in the islets of lipid-infused rats, which may be mediated by increased cyclic AMP levels and the suppression of both neuronal cNOS and iNOS.<sup>13</sup> The exposure of islets to high glucose in healthy animals resulted in enhanced production of iNOS-derived NO in a short time.<sup>14,15</sup> However, the molecular mechanism by which GLP1 exerts its effects remains unclear. In the current research, our purpose was to investigate how the abnormal expression of iNOS in insulin and glucagon cells of ApoE<sup>−/−</sup> mice and GLP1 works on insulin secretion, and to analyze the mechanism that GLP1 exerts on iNOS in ApoE<sup>−/−</sup> mice, so as to find a new treatment target for T2DM.

## Methods

### Reagents

Monoclonal antibodies for PARP1, glucagon, insulin, and iNOS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Cell Signaling Technology (Beverly, MA, USA). Primary antibodies of nitrotyrosine were purchased from Cayman Biochemicals (Ann Arbor, MI, USA). Primary antibodies for COX2 and NFκB p65 were purchased from Abcam. Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Recombinant human GLP1 (rhGLP1; 7–36) was obtained from Huayi Bio-Lab (Shanghai). PARP1 lentivirus or control lentivirus was obtained from Genechem (Shanghai). All other drugs and chemicals were purchased from Merck (Darmstadt, Germany). Radioimmunoassay kits for insulin and glucagon were obtained from Diagnostika (Falkenberg, Sweden) and Euro Diagnostica (Malmö, Sweden), respectively.

### Animals

Animal experiments were approved by the Experimental Animal Welfare and Ethics Committee, Shandong

University (approval DWLL-2015-006, 30 July, 2015), and conducted in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Laboratory animals underwent all operations under anesthesia, and every effort was made to minimize pain and death. ApoE<sup>−/−</sup> mice were from Jackson Laboratories (Bar Harbor, ME, USA) and housed in a pathogen-free animal-care facility with free access to water and food. Mice were divided into six treatment groups: normal diet (n=15), high-fat diet (n=15), high-fat diet treated with GLP1 (n=15), high-fat diet infected with PARP1 lentivirus (n=15), high-fat diet infected with control lentivirus (n=15), and high-fat diet infected with PARP1 lentivirus and treated with rhGLP1 group (n=15). The normal diet-group were fed a normal diet for 32 weeks and subcutaneously injected with saline. The remaining mice were fed high-fat diet for 32 weeks. Diabetic mice in the high-fat diet treated with GLP1 group were then subcutaneously injected with 80 µg/kg rhGLP1 for 8 weeks (rhGLP1 was diluted with 10 mL sterile saline). Diabetic mice in the high-fat diet with PARP1 lentivirus-infected group and high-fat diet infected with control-lentivirus group were subcutaneously injected with 5 µL PARP1 lentivirus or control lentivirus (10<sup>9</sup> TU/mL) for 8 days. After PARP1-lentivirus infection, mice serving as the high-fat diet infected with PARP1 lentivirus and treated with rhGLP1 were then subcutaneously injected with 80 µg/kg rhGLP1 for 8 weeks. Blood samples were collected by angular venipuncture in mice fasted overnight to measure total cholesterol (TC), triglycerides (TGs), low-density-lipoprotein cholesterol (LDL-C), and high-density-lipoprotein cholesterol (HDL-C).

### Histology and Immunostaining

Pancreata were dissected from mice and fixed in 10% methanol, deparaffinized, rehydrated, and washed in PBS, then stained for 5 minutes with hematoxylin and 2 minutes with eosin. For immunostaining, sections were stained overnight at 4°C with antinitrotyrosine primary antibodies (1:100), then stained by appropriate secondary antibodies at 37°C for 30 minutes. Then, coverslips were sealed using Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). Images were acquired by laser scanning confocal microscopy (LSM710; Carl Zeiss, Germany) and analyzed using Image-Pro Plus 6.0.

### TUNEL Assays

The number of apoptotic cells was evaluated with a TUNEL-assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. In brief, sections were

washed with xylene twice, then dipped in ethanol (100%, 95%, 90%, 80%, and 70%) for 3 minutes in each concentration and then incubated in a TUNEL reaction mixture in the dark for 1 hour at 37°C. Labeled samples were visualized under fluorescence microscopy (Olympus, Tokyo, Japan) and analyzed with Zen 2011 software (Carl Zeiss).

## ELISAs

Pancreatic tissue was collected and concentrations of IL1 $\beta$ , (ELH-IL1 $\beta$ ; RayBiotech), MCP-2, (ELH-MCP2; RayBiotech), IL6 (ELH-IL6; RayBiotech, and TGF $\beta$  measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. In addition, plasma samples were collected and CRP levels measured using the ELISA kits according to the manufacturer's instructions.

## Western Blot Analysis

Protein was extracted from the pancreata and equal amounts of protein separated with 10% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were blocked in 5% nonfat milk powder for 2 hours at room temperature and washed three times in TBS-T. Then, membranes were probed with primary antibodies overnight at 4°C. Primary antibodies included anti- $\beta$ -actin (1:1,000), anti-glucagon (1:2,000), anti-insulin (1:2,000), anti-PARP1 (1:500), anti-iNOS (1:250), COX2 (1:500), and NF $\kappa$ B p65 (1:1,000). After being washed in TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 hours at room temperature. Signals were detected by enhanced chemiluminescence (Millipore) and analyzed using Image-Pro Plus 6.0.

## Determination of Glucose-Tolerance, Blood-Glucose, and Blood-Insulin Levels

After 10 hours' fasting, venous blood glucose in mice was measured with a blood-glucose meter from the tail. Blood-insulin levels were measured using ELISA. Glucose solution (2 mg glucose/g body weight) and insulin (0.5 U/kg body weight) was injected intraperitoneally into the mice and intraperitoneal glucose-tolerance testing (IPGTT) and intraperitoneal insulin-tolerance testing (IPITT) were performed.

## Statistical Analysis

Data are expressed as means  $\pm$  SEM, and were analyzed with Graph-Pad Prism 5.0 software (GraphPad Software,

San Diego, CA, USA). Statistical analyses were performed using one-way ANOVA. Differences with  $p < 0.05$  were considered statistically significant.

## Results

### High-Fat Diet Aggravates Islet Injury

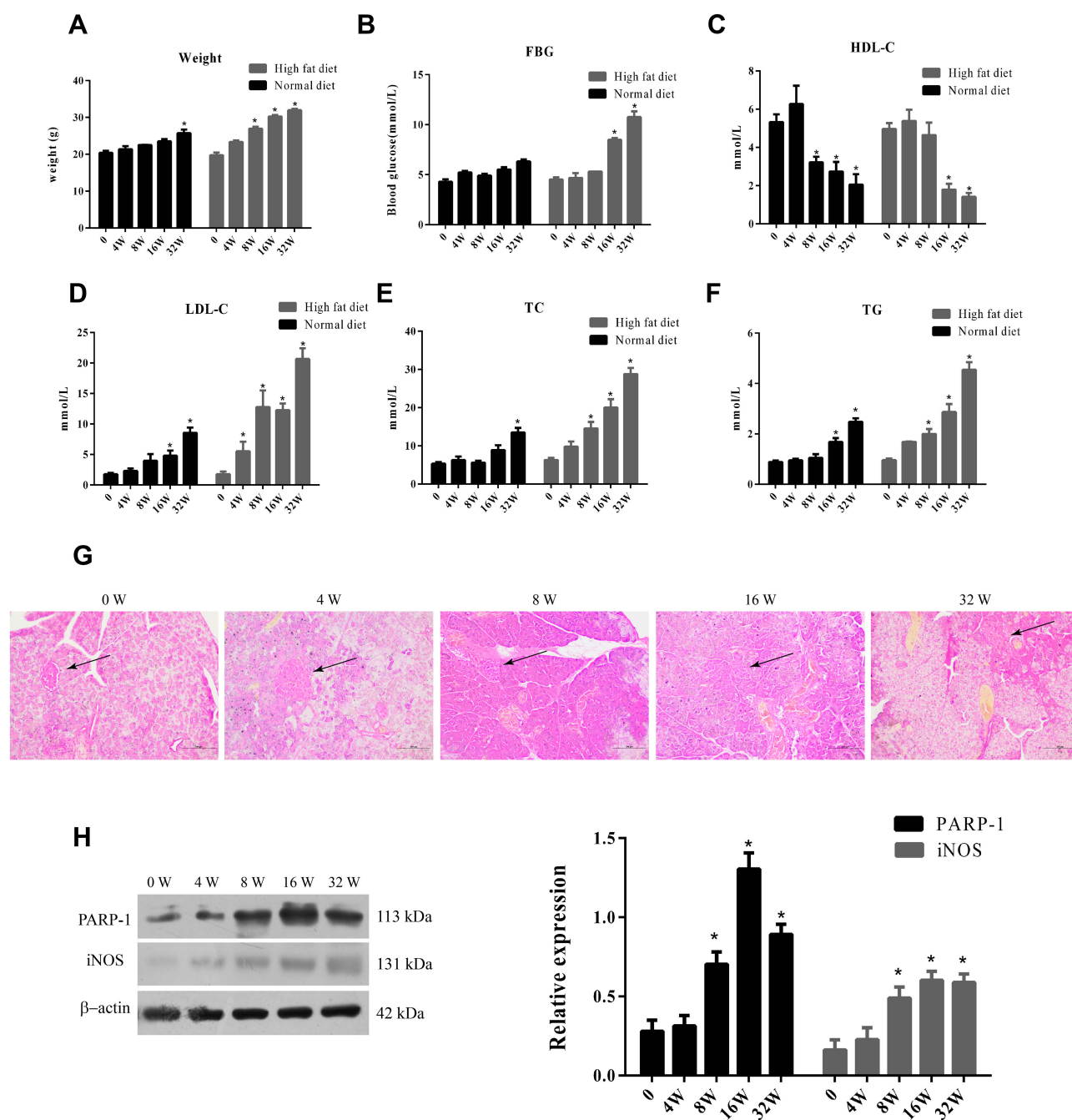
First, we measured fasting blood sugar, weight, HDL-C, LDL-C, TC, and TGs in diabetic mice. The mice fed a high-fat diet showed significant increases in body weight compared to the normal-diet group at 8, 16, and 32 weeks (Figure 1A) and significant increases in fasting blood-sugar levels compared to the normal-diet group at 16 and 32 weeks ( $p < 0.05$ ) (Figure 1B). Moreover, the mice fed the high-fat diet had significantly higher TC, LDL-C, and TG levels, but significantly lower HDL-C levels than the control group at 16 and 32 weeks ( $p < 0.05$ , Figure 1C–F).

Next, we examined histology of the islets in ApoE $^{-/-}$  mice fed the high-fat diet. H&E staining showed that the morphology of the islets was round or oval in normal mice. In contrast, with increased duration of the high-fat diet, the islets became small, atrophic, and distorted, and had increased inflammatory injury (Figure 1G). Furthermore, we analyzed PARP1 and iNOS expression in islets of ApoE $^{-/-}$  mice fed the high-fat diet. PARP1 and iNOS protein had levels increased significantly at 8, 16, and 32 weeks compared to 0 and 4 weeks ( $p < 0.05$ , Figure 1H).

### GLP1 Recovers Declines in Insulin Sensitivity and Glucose Tolerance in ApoE $^{-/-}$ Mice Fed High-Fat Diet

Next, we investigated blood-glucose tolerance and insulin sensitivity in each group of mice. IPITT values increased significantly in the high-fat diet group compared to the normal-diet group, but GLP1 decreased IPITT values in the high-fat diet group and high-fat diet group with PARP1 treatment (Figure 2A). Similarly, IPGTT values increased significantly in the high-fat diet group compared to the normal-diet group, but GLP1 decreased IPGTT values in the high-fat diet group and high-fat diet group with PARP1 treatment (Figure 2B). Consistently, PARP1 and iNOS expression increased significantly in the high-fat diet group compared to the normal-diet group, but GLP1 decreased PARP1 and iNOS expression in the high-fat diet group and high-fat diet group with PARP1 treatment (Figure 2C).

Since iNOS can cause tissue damage via NO radicals, we detected 3-nitrotyrosine (3-NT), a marker for RNS in the damaged pancreas.<sup>18,19</sup> Immunohistochemistry staining



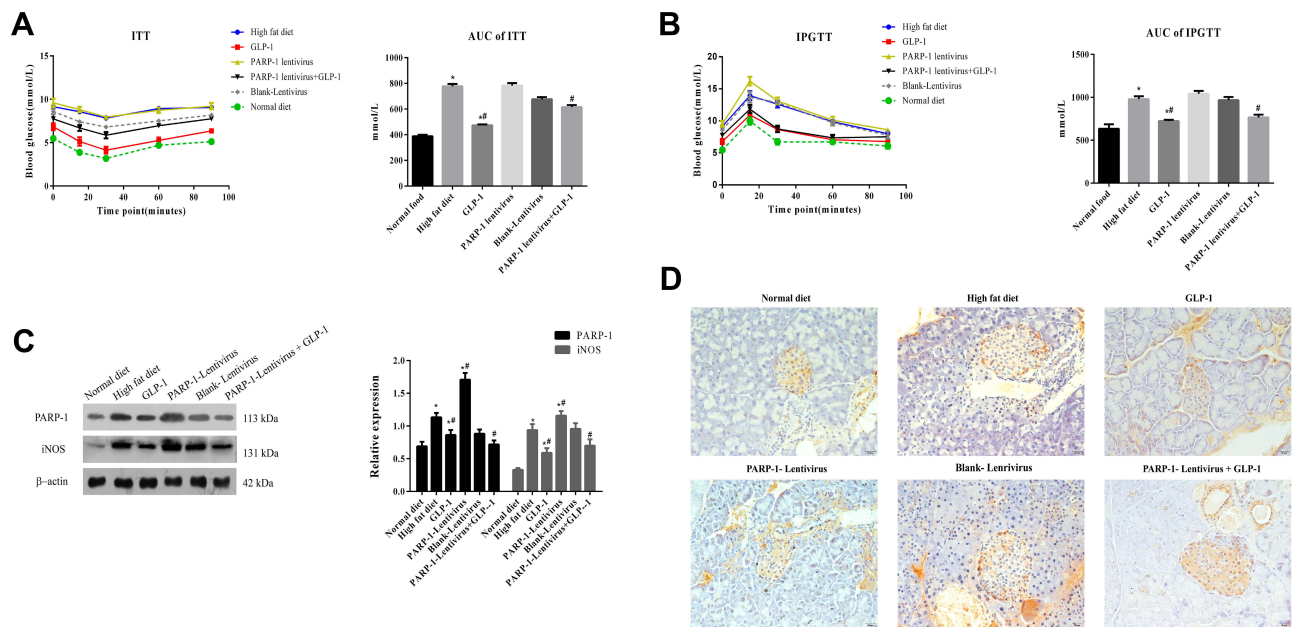
**Figure 1** Weight, glucose and lipid indexes in high fat diet fed ApoE<sup>-/-</sup> mice.

**Notes:** (A) Body weight of mice in normal diet group and high fat diet group. (B) FBG of mice in normal diet group and high fat diet group. (C) HDL-C of mice in normal diet group and high fat diet group. (D) LDC-C of mice in normal diet group and high fat diet group. (E) TC of mice in normal diet group and high fat diet group. (F) TG of mice in normal diet group and high fat diet group. (G) HE staining of islets (†) of mice in normal diet group and high fat diet group. Scale Bar = 100  $\mu$ m. (H) Western blot analysis of PARP-1 and iNOS expression in islets of mice in normal diet group and high fat diet group. Data were analyzed using a one-way ANOVA followed by Dunnett's test, or by Kruskal-Wallis test followed by Steel's test. \*,  $p < 0.05$  compared with NF + vehicle.

showed that 3-NT levels increased significantly in the high-fat diet group compared to the normal-diet group, but GLP1 decreased 3-NT levels in the high-fat diet group and high-fat diet group with PARP1 treatment (Figure 2D). Taken

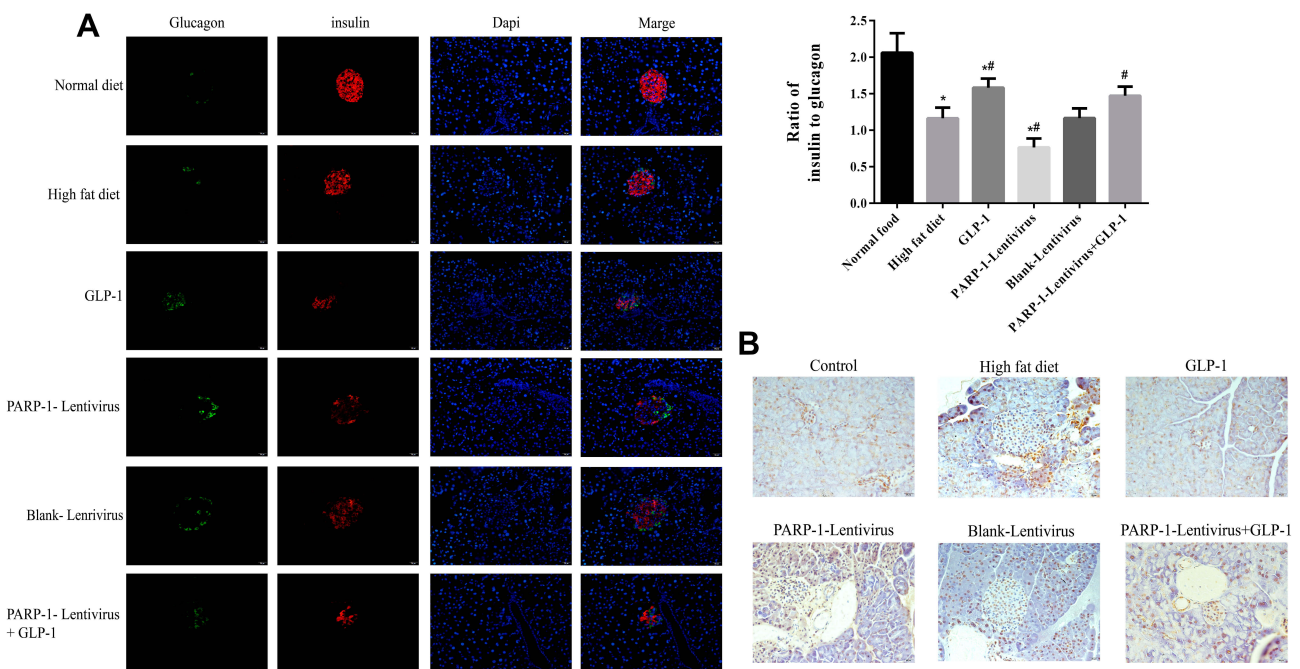
together, these data indicate that GLP1 may alleviate high-fat diet-induced declines in insulin sensitivity and glucose tolerance, and this is related to increased expression of iNOS and increased production of 3-NT in the islets.





**Figure 2** GLP-1 recovers the decline of insulin sensitivity and glucose tolerance and reduces PARP-1, iNOS and 3-NT levels in islets.

**Notes:** (A) Insulin tolerance test (ITT) value and area under curve (AUC) of mice in ApoE<sup>-/-</sup> mice fed with high fat diet and treated with GLP-1 or/and lentivirus expressing PARP-1. (B) Intraperitoneal glucose tolerance test (IPGTT) value and area under curve (AUC) of mice in ApoE<sup>-/-</sup> mice fed with high fat diet and treated with GLP-1 or/and lentivirus expressing PARP-1. (C) Western blot analysis of PARP-1 and iNOS expression in islets in ApoE<sup>-/-</sup> mice fed with high fat diet and treated with GLP-1 or/and lentivirus expressing PARP-1. (D) Immunohistochemical staining of 3-NT in islets in ApoE<sup>-/-</sup> mice fed with high fat diet and treated with GLP-1 or/and lentivirus expressing PARP-1. Brown particles label 3-NT-positive areas. Scale Bar = 20  $\mu$ m. Data were analyzed using a one-way ANOVA followed by Dunnett's test, or by Kruskal-Wallis test followed by Steel's test. \*,  $p < 0.05$  compared with NF + vehicle. ##,  $p < 0.05$ , compared with high fat diet group. #,  $p < 0.05$ , compared with PARP-1 lentivirus group.



**Figure 3** GLP-1 inhibits pancreas cell apoptosis.

**Notes:** (A) Immunofluorescence analysis (40 $\times$ ) of representative images of pancreatic sections with anti-insulin (red) and anti-glucagon (green) antibodies. Results are representative of 5–6 sections of the whole pancreas of each mouse. GLP-1 induced a marked suppression of glucagon and improved insulin expression in ApoE<sup>-/-</sup> mice islets. (B) TUNEL assay of cell apoptosis in pancreas. The islet cell apoptosis was aggregated after 16w high fat diet compared to the control group, while GLP-1 reduced islet cell apoptosis. Results are representative of 5–6 pancreas slides of each mouse. \*,  $p < 0.05$  compared with NF + vehicle. ##,  $p < 0.05$ , compared with high fat diet group. #,  $p < 0.05$ , compared with PARP-1 lentivirus group.

## GLP1 Reduces Islet-Cell Apoptosis

Islet  $\beta$  cells and  $\alpha$  cells were identified by insulin antibody and glucagon antibody, respectively. Immunostaining showed that the high-fat diet significantly decreased the number of  $\beta$  cells, but increased the number of  $\alpha$  cells compared to the control group ( $1.163 \pm 0.145$  vs  $2.062 \pm 0.266$ ,  $p < 0.05$ ). GLP1 inhibited glucagon and improved insulin secretion in islets of high-fat diet-fed mice ( $1.583 \pm 0.125$  vs  $1.163 \pm 0.145$ ,  $p < 0.05$ ). In high-fat diet-fed mice infected with PARP1 lentivirus, the number of  $\beta$  cells had further decreased and the number of  $\alpha$  cells further increased compared to high-fat diet-fed mice ( $0.764 \pm 0.121$  vs  $1.163 \pm 0.145$ ,  $p < 0.05$ ), while GLP1 reversed these results ( $1.470 \pm 0.104$  vs  $0.764 \pm 0.121$ ,  $p < 0.05$ ) (Figure 3A). TUNEL assays showed that islet-cell apoptosis was aggregated in mice fed the high-fat diet for 16 weeks compared to the control group, while GLP1 reduced islet-cell apoptosis. Moreover, PARP1 lentiviral infection further increased islet-cell apoptosis compared to the high-fat diet group, while GLP1 inhibited the further increase of apoptosis (Figure 3B).

## GLP1 Inhibits Islet Inflammation

H&E staining showed that the islets in the high-fat diet group were irregular and had inflammation, while GLP1 alleviated islet injury. When high-fat diet-fed mice were infected with the PARP1 lentivirus, inflammation in islets was more severe than the high-fat diet group, and GLP1 reversed the inflammation (Figure 4A). To confirm pancreatic inflammation, CD8<sup>+</sup> T lymphocytes were stained and mononuclear cell infiltration into islet was observed, as previously described. Glucagon was stained to outline a residual islet.<sup>20</sup> We found large numbers of CD8<sup>+</sup> T lymphocytes in islets of the high-fat diet group, while GLP1 decreased the number of CD8<sup>+</sup> T lymphocytes. Infection with PARP1 lentivirus increased the number of CD8<sup>+</sup> T lymphocytes infiltrating the islets, and GLP1 reversed the increase in CD8<sup>+</sup> T lymphocytes due to PARP1 overexpression (Figure 4B).

Furthermore, we detected concentrations of inflammatory factors in the islets. Compared with the control group, the high-fat diet increased concentrations of IL1 $\beta$ , IL6, TNF $\alpha$ , and MIP2, while GLP1 treatment significantly decreased their concentrations. Infection with PARP1 lentivirus further increased concentrations of inflammatory factors compared with the high-fat diet group, and GLP1 inhibited the increase due to PARP1 overexpression (Figure 4C). To explore the

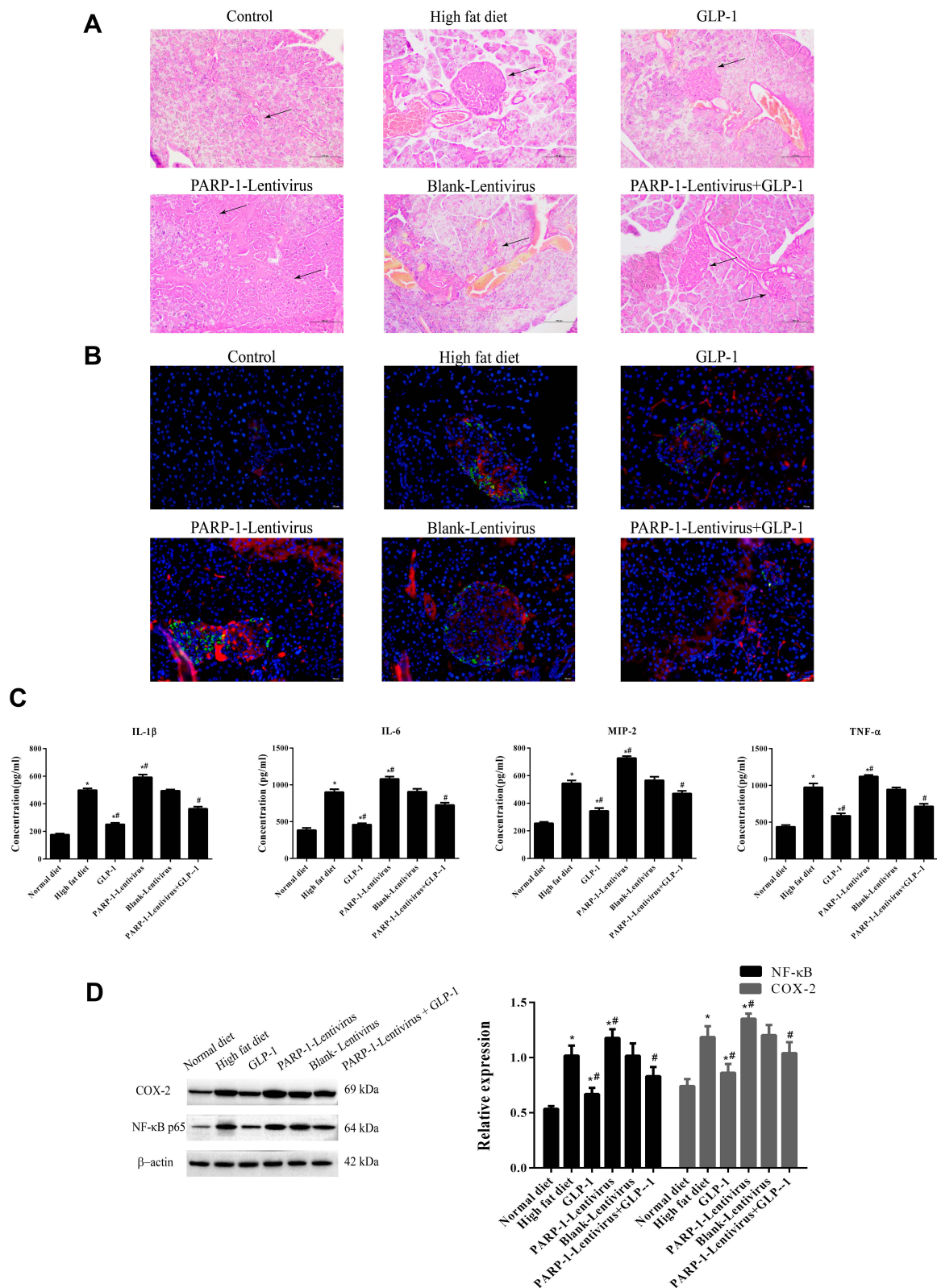
mechanism by which GLP1 inhibits inflammation, we detected expression of COX2 and NF $\kappa$ B. We found that expression of COX2 and NF $\kappa$ B p65 had significantly increased in the islets of the high-fat diet group compared to the control group, but decreased after GLP1 was applied. Infection with PARP1 lentivirus further increased the expression of COX2 and NF $\kappa$ B p65 compared with the high-fat diet group, and GLP1 inhibited increased expression of COX2 and NF $\kappa$ B p65 due to PARP1 overexpression (Figure 4D). Collectively, these data indicate that GLP1 protects the islets by inhibiting inflammation.

## Discussion

The prevalent hypothesis is that damage to islet function and insulin resistance are the main pathological aspects of T2DM. Recent studies have shown that GLP1 improves glycemic control and reduces islet damage in patients with T2DM.<sup>21–23</sup> However, the molecular mechanism by which GLP1 reverses fat-induced islet injury remains poorly understood. In the current study, we found that GLP1 inactivated PARP1 and suppressed islet damage via inhibiting COX2–NF $\kappa$ B pathway-mediated inflammation.

A distinctive feature of T2DM is the dysfunction of insulin-secreting pancreatic  $\beta$  cells. Under hyperglycemic conditions, islet  $\beta$  cells will initially enhance the secretion of insulin in a compensatory way, but after a long period they will develop decompensation and eventually fail, leading to loss of secretion function and DM.<sup>24–26</sup> Therefore, increasing the number of islet  $\beta$  cells is an important aspect in effective treatment of T2DM. In the current study, we found that GLP1 alleviated decreases in  $\beta$  cells by inhibiting cell apoptosis induced by the high-fat diet. Consequently, GLP1 recovered the decline in insulin sensitivity and glucose tolerance. Our results are consistent with a previous study reporting that GLP1 protected  $\beta$ -cell viability against streptozocin-induced toxicity, inhibited weight gain, and relieved symptoms of polydipsia.<sup>27</sup>

Oxidized LDL is a harmful cholesterol produced when LDL-C interacts with free radicals generated by macrophages. Uptake of oxidized LDL by islet  $\beta$  cells leads to oxidative damage, ROS production, and PARP1 activation.<sup>28–31</sup> PARP1 is known to activate iNOS expression and enhance NO production, and is involved in a variety of diseases, including chronic obstructive pulmonary disease, atherosclerotic lesions, and DM.<sup>32,33</sup> We found that the application of GLP1 suppressed the expression of PARP1 and iNOS and inhibited RNS generation



**Figure 4** GLP-1 inhibits inflammatory injury in islets.

**Notes:** (A) HE staining (20 $\times$ ) of islets ( $\uparrow$ ) in ApoE $^{-/-}$  mice fed with high fat diet and treated with GLP-1 or/and lentivirus expressing PARP-1. Scale Bar = 100  $\mu$ m. (B) CD8 $^{+}$ T lymphocytes were stained as Red to observe mononuclear cell infiltration into the islets. Glucagon was stained as Green to outline a residual islet. Results are representative of 5–6 pancreas slides of each mouse. (C) ELISA assay of inflammatory factors in the islets. (D) Western blot analysis of COX-2 and NF- $\kappa$ B levels in the islets. Data were analyzed using a one-way ANOVA followed by Dunnett's test, or by Kruskal-Wallis test followed by Steel's test. \*,  $p < 0.05$  compared with NF + vehicle. \*#,  $p < 0.05$ , compared with high fat diet group. #,  $p < 0.05$ , compared with PARP-1 lentivirus group.



mediated by PARP1. Moreover, the severity of mononuclear cell infiltration was alleviated and the expression of inflammatory factors decreased after GLP1 had been applied, suggesting that GLP1 protected the islets by inhibiting inflammation.

COX2 functions as an inducible enzyme with low or undetectable levels in most tissue types, and its expression can be markedly increased by a number of inflammatory, mitogenic, and physical stimuli.<sup>34–36</sup> In the primary stage of inflammation, NFκB is activated and upregulates the expression of COX2 and iNOS, thereby promoting NO production. iNOS also enhances the activation of NFκB, which further promotes inflammation effects.<sup>37</sup> In this study, we found that GLP1 suppressed the expression of COX2 and NFκB induced by a high-fat diet. These results indicate that GLP1 attenuates islet-inflammation damage by inhibiting COX2 and NFκB pathways. In conclusion, we found that GLP1 significantly alleviated decreased β-cell numbers and suppressed β-cell apoptosis induced by a high-fat diet, inhibited the expression of iNOS, and alleviated inflammatory-islet injury via inhibiting the COX2–NFκB pathway.

## Author Contributions

All authors made substantial contributions to conception and design, acquisition of or analysis and interpretation of data, took part in drafting the article or revising it critically for important intellectual content, gave final approval to the version to be published, and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare that they have no conflicts of interest.

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