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Research Article

Empagliflozin prevents cardiomyopathy via sGC-cGMP-PKG pathway in type 2 diabetes mice

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Cardiovascular complications contribute to the major mortality and morbidity in type 2 diabetes. Diabetic cardiomyopathy (DCM) is increasingly recognized as an important cause of heart failure. EMPA-REG OUTCOME trial has reported that empagliflozin, the sodium-glucose cotransporter 2 inhibitor, exerts cardiovascular benefits on diabetic population. However, the mechanism by which empagliflozin alleviates DCM still remains unclear. In the current study, we investigated the cardiac protective effects of empagliflozin on spontaneous type 2 diabetic db/db mice and its potential mechanism. Eight weeks of empagliflozin treatment (10 mg/kg/day) decreased body weight and blood glucose level, and increased urinary glucose excretion (UGE) in diabetic mice. Echocardiography revealed that both systolic and diastolic functions of db/db mice were also obviously improved by empagliflozin. Furthermore, empagliflozin-treated diabetic mice presented with amelioration of cardiac hypertrophy and fibrosis. In addition, diabetic hearts exhibited the deterioration of oxidative stress, apoptosis and pyroptosis, while these effects were significantly counteracted after empagliflozin treatment. Moreover, empagliflozin rescued diabetes-induced suppression of sGC (soluble guanylate cyclase enzyme)-cGMP (cyclic guanosine monophosphate)-PKG (cGMP-dependent protein kinase) pathway. However, when sGC-β expression of hearts was inhibited by transvascular delivery of small interfering RNA, cardiac dysfunction was aggravated and the advantages of empagliflozin were reversed through inhibiting sGC-cGMP-PKG pathway. Collectively, these findings indicate that empagliflozin improves cardiac function involving the inhibition of oxidative stress-induced injury via sGC-cGMP-PKG pathway and may be a promising therapeutic option for DCM.

Introduction

According to the latest survey by International Diabetes Federation, in 2017 there are 451 million adults with diabetes worldwide and it is predicted to rise to 693 million by 2045 [1]. Cardiovascular complications such as diabetic cardiomyopathy (DCM) are the increasing cause of morbidity and mortality in patients with diabetes [2]. Diabetes-induced myocardial injury is defined as DCM and more than half of diabetic patients suffer from DCM, therefore the incidence and prevalence of DCM is rising continuously [3]. DCM is characterized by early diastolic dysfunction and systolic dysfunction at a later stage, which is accompanied by cardiac hypertrophy. Multiple molecular mechanisms have been proposed to result in the development of DCM including myocardial inflammation, fibrosis, cardiomyocyte oxidative stress, apoptosis, autophagy, mitochondrial dysfunction and so on [4]. In addition, the NOD-like receptor 3 (NLRP3) inflammasome-induced pyroptosis, a novel programmed cell death process, is increasingly recognized in cardiovascular disease including DCM [5]. Caspase1 is activated by ligands of NLRP3 inflammasome, which further triggers the cleavage of pro-inflammatory cytokinesis such as interleukin-1β, 18 (IL-1β,

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IL-18) and gasdermin D (GSDMD). Cleaved GSDMD causes the formation of membrane pore which is required and sufficient for the secretion of pro-inflammatory cytokinesis [6–8]. Although above key pathophysiological features have been indicated in the progression of DCM, the exact mechanism is still unknown.

Nitric oxide is synthesized in endothelial cells by the catalytic effect of endothelial nitric oxide synthase (eNOS) and then activates its intracellular receptor the soluble guanylate cyclase enzyme (sGC) causing the release of the second messenger cyclic guanosine monophosphate (cGMP). cGMP further activates the cGMP-dependent protein kinase (PKG) to regulate lots of physiological functions [9]. sGC-cGMP-PKG signaling was found to exert as a common mediator of cardioprotection [10] and this pathway participated in regulating the systolic and diastolic dysfunctions under diabetic conditions [11]. Increasing evidence also supports a pivotal role for sGC-cGMP-PKG pathway in myocardial fibrotic remodeling, hypertrophy, oxidative stress and apoptosis in diabetic heart [11–13].

Sodium-glucose cotransporter 2 (SGLT2) inhibitors are a new class of new glucose-lowering agents through inhibiting renal proximal tubular glucose reabsorption and increasing urinary glucose excretion (UGE) [14]. Empagliflozin is a potent and competitive inhibitor of SGLT2 with the highest selectivity compared with other SGLT2 inhibitors [15]. The well-known EMPA-REG OUTCOME trial reported that both the risk of major cardiovascular events and the incidence of heart failure hospitalization have been substantially reduced in type 2 diabetes mellitus (T2DM) patients after treating with empagliflozin [16]. The surprising benefits of empagliflozin on cardiovascular outcomes involved the simultaneous modulation of pleiotropic molecular and biochemical pathways beyond its glucose-lowering effect [17]. Recent studies described the beneficial effects of empagliflozin on diastolic dysfunction in ob/ob and db/db mice [18,19], myocardial microvascular injury in streptozotocin-induced diabetic mice [20], atherosclerosis in ApoE$^{-/-}$ mice [21], cardiac injury in prediabetic rats [22] or myocardial infarction in animals fed with Western diet [23]. Other studies have also proved that empagliflozin protects against DCM by blocking endoplasmic reticulum stress and ameliorating cardiac interstitial fibrosis and oxidative stress [24,25]. However, little information is available about the action of empagliflozin on cardiac function in DCM via sGC-cGMP-PKG pathway.

In the present study, we investigated the effects of empagliflozin on cardiac dysfunction and myocardial injury in type 2 diabetes mice. Moreover, *in vivo*, we explored the functional interplay between sGC-cGMP-PKG pathway and heart injury in mediating empagliflozin's cardioprotective actions.

Materials and methods

Animals and experimental protocol

Seven-week-old male diabetic db/db mice were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). Meanwhile, nondiabetic littermate db/m mice were obtained as the normal control. The present study was approved by the Ethical Committee of Tianjin Medical University (Tianjin, China). All experimental mice were maintained on a 12:12 h light–dark cycle at 22 ± 2°C under specific pathogen-free conditions in the animal facility at Tianjin Medical University (Tianjin, China). After adaptive feeding for 1 week, db/db mice with similar blood glucose level and body weight were randomly assigned to db/db group and db/db+Empa group. Mice in db/db+Empa group were given empagliflozin (Boehringer-Ingelheimat, Germany) a dose of 10 mg/kg/day for 8 weeks through intragastric administration, while db/m and db/db mice were gavaged with equal amounts of saline. General status of the mice was monitored once every other week. Mice were placed in individual metabolic cage to calculate 24 h food and water intake and obtain 24 h urine samples. UGE was determined by an automatic biochemistry analyzer (Roche, Germany). At the end of treatment, overnight-fasted mice were killed by exsanguination under anesthesia with inhaled 5% isoflurane in room air. After collecting blood samples, heart weight was measured and heart tissues were removed for further experiments.

sGC-β knockdown *in vivo*

As described previously [26,27], models of genes knockdown were successfully obtained by intravenous injections of siRNA *in vivo*. Six male db/db mice received weekly intravenous injections of negative control siRNA or sGC-β-siRNA (si-NC or si-sGC-β) for eight consecutive weeks, respectively. Briefly, 50μg synthetic control or sGC-β siRNA using the transfection reagent *in vivo*-jetPEI (Polyplus Transfection, France) following the manufacturer's protocol was rapidly injected via tail vein into mice. All mice were daily treated with 10 mg/kg empagliflozin for 8 weeks. Cholesterol-modified siRNA was synthesized by GenePharma (Suzhou, China). The target sequences used in the present study were as follows: sGC-β sense 5′-GCCUGUAUCUGAGUGACAUTT-3′; sGC-β antisense 5′-AUGUCACUCAGAUACAGGCTT-3′; negative control sense 5′-UUCUCCGAACGUGUCACGUTT-3′; negative control antisense 5′-ACGUGACACGUUCGGAGAATT-3′.

Table 1 Effects of empagliflozin treatment on cardiac function in db/db mice

	db/m	db/db	db/db +Empa
Heart rate (beats/min)	585 ± 35	568 ± 49	593 ± 43
Left ventricular mass (mg)	89.2 ± 9.3	127.6 ± 25.4[1]	104.8 ± 14.6[4]
EF (%)	71.30 ± 4.86	52.22 ± 7.39[1]	60.63 ± 4.95[4]
FS (%)	40.25 ± 3.39	28.15 ± 4.53[1]	33.42 ± 2.30[4]
IVRT (ms)	15.2 ± 2.2	21.1 ± 3.0[1]	16.4 ± 2.5[3]
DT (ms)	25.8 ± 3.3	32.3 ± 3.9[2]	27.5 ± 2.7[4]
E/A	1.57 ± 0.22	1.18 ± 0.16[1]	1.44 ± 0.18[3]

Data are presented as mean ± SD, *n*=8 per group. Abbreviations: DT, deceleration time; E/A, velocity of early mitral flow to velocity of late mitral flow ratio; EF, ejection fraction; FS, fraction shortening; IVRT, isovolumetric relaxation time.
[1]$P<0.001$
[2]$P<0.01$ represents significant differences between db/m and db/db mice.
[3]$P<0.01$
[4]$P<0.05$ represents significant differences between db/db and db/db+Empa.

Echocardiography

After the 8-week empagliflozin treatment or siRNA transfection, cardiac function of experimental animals was assessed by echocardiography. Mice were anesthetized with isoflurane and underwent echocardiography by one echocardiographer. Echocardiography was conducted using an echocardiogram machine equipped with a 24-MHz linear transducer (Visual Sonics, Canada). Left ventricular systolic function was measured directly in M-mode, and diastolic function was obtained using pulsed-wave Doppler from apical four chamber view.

Histology

Heart tissues were fixed with formalin, embedded with paraffin and cut into 4-μm thick sections. The sections were stained with HE and Masson staining kits (Leagene Biotechnology, China) according to the manufacturer's protocols. For immunohistochemistry staining, after the deparaffinization, rehydration and heat-induced antigen retrieval, heart sections were blocked with 3% H_2O_2. Subsequently, the sections were incubated with the following primary antibodies at 4°C overnight: anti-sGC-β (Proteintech, China). Finally, the sections were visualized with a DAB kit and counterstained with Hematoxylin followed by incubation with HRP-conjugated secondary antibody.

Western blot analysis

Heart proteins were separated by SDS/PAGE gels, and then transferred to PVDF membranes. After being blocked with 5% defatted milk, membranes were incubated with the following primary antibodies overnight at 4°C: transforming growth factor-β (TGF-β) (Proteintech, China), Collagen I (Proteintech, China), Collagen III (Proteintech, China), superoxide dismutase 2 (SOD2) (Abcam, U.S.A.), NADPH oxidase (NOX) 2 (NOX2) (Abcam, U.S.A.), NOX4 (Proteintech, U.S.A.), Bcl2 (CST, U.S.A.), Bax (CST, U.S.A.), cleaved Caspase3 (CST, U.S.A.), NLRP3 (Abclonal, China), cleaved Caspase1 (CST, U.S.A.), IL-1β (Abclonal, China), GSDMD (Abcam, U.S.A.), p-eNOS^{Ser1179} (Abcam, U.S.A.), t-eNOS (Abcam, U.S.A.), sGC-β (Proteintech, China), PKG1α (Proteintech, China), phosphorylated vasodilator-stimulated phosphoprotein (p-VASP^{Ser239}) (CST, U.S.A.), total VASP (t-VASP) (CST, U.S.A.) and β-actin (Sungene Biotech, China). After washing, the HRP–conjugated secondary antibody (Sungene Biotech, China) was incubated for 1 h at room temperature. Finally, protein bands were visualized with an ECL kit (Advansta, U.S.A.). β-actin was used as a loading control. Quantification of each band was analyzed with ImageJ software.

Real-time PCR

Total RNA was reverse-transcribed to cDNA using a reverse transcription system kit (Thermo Scientific, U.S.A.). The primers were obtained from AuGCT Biotechnology (Beijing, China), and their sequences were listed in Supplementary Table S1. PCR was performed with a SYBR Green PCR reagent kit (Sangon Biotech, China). CFX Manager system (Bio-Rad, U.S.A.) was used to monitor gene expression and analyze data. The relative RNA levels were normalized to β-actin.

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Reactive oxygen species detection

To detect reactive oxygen species (ROS) production in myocardium, the frozen heart sections were incubated with 5 μmol/ml ROS Fluorescent Probe-DHE (US Everbright, China) for 1 h at 37°C in the dark. Fluorescence images were obtained using a fluorescence microscope (Olympus, Japan). The fluorescence intensity of the heart sections was determined in ten arbitrarily selected regions using Image-Pro Plus software.

TUNEL assay

A TUNEL assay kit (KeyGEN BioTECH, China) was used in order to examine cardiomyocyte apoptosis according to the manufacturer's instructions. Briefly, the slides were incubated with freshly diluted proteinase K for 20 min at 37°C to enhance the permeability. After the enzymatic reaction, sections were incubated with Streptavidin-FITC for 30 min in the dark. Finally, the sections were stained with DAPI for 10 min and images were obtained with fluorescence microscope (Olympus, Japan). TUNEL-positive cell nuclei were calculated in ten randomly selected fields of each slide.

cGMP measurement

The cGMP ELISA kit (Enzo Life Sciences, U.S.A.) was used to determine the level of cGMP in serum and myocardium according to the manufacturer's protocol. The activity of myocardial cGMP level was normalized to total protein concentration and results were presented as pmol per milligram of protein.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. All data were expressed as mean ± SD. Unpaired Student's *t* test was used for comparisons between two groups and one-way ANOVA and Tukey's test was used for comparisons among multiple groups. P-value <0.05 was considered statistically significant.

Results

Effects of empagliflozin on body weight, blood glucose, food and water intake, urine volume and UGE of mice

Compared with the db/m mice, db/db mice showed significantly heavier body weight, higher blood glucose levels, more urine volume and UGE accompanied by excessive food and water intake (Figure 1). Significant reduction in body weight of db/db+Empa mice was observed after 6 weeks of the treatment (Figure 1A). Empagliflozin effectively decreased blood glucose levels of db/db mice throughout 8 weeks of the treatment (Figure 1B). Meanwhile, water intake, urine volume and UGE of mice were markedly increased in the db/db+Empa group relative to the db/db group (Figure 1D–F). There was no significant difference in food intake between db/db and db/db+Empa groups during the treatment (Figure 1C).

Empagliflozin improved cardiac function in db/db mice

The db/db mice exhibited a significant decrease in left ventricular systolic parameters, including ejection fraction (EF) and fraction shortening (FS) compared with the db/m group, while empagliflozin-treated mice showed a remarkable improvement in the FS and EF (Table 1). On Doppler flow analysis, the increase in deceleration time (DT) and isovolumetric relaxation time (IVRT) and decrease in velocity of early mitral flow to velocity of late mitral flow ratio (E/A) indicated that left ventricular diastolic function was markedly impaired in db/db mice. In contrast, empagliflozin treatment significantly reversed these diastolic function parameters (Table 1). We did not observe significant differences in heart rate among the three groups of mice (Table 1). Interestingly, diabetic mice showed a marked decrease in blood glucose after receiving insulin injection, but no improvements were observed in their heart function (Supplementary Table S2). In summary, empagliflozin itself improved diabetes-induced cardiac diastolic and systolic functions in db/db mice.

Empagliflozin alleviated cardiac hypertrophy in diabetic mice

The heart of db/db mice was obviously larger, heart weight to tibia length ratio (HW/TL) was higher and left ventricular mass was heavier than those in db/m mice (Figure 2A,B and Table 1). Moreover, HE staining showed that cardiomyocytes were clearly striated and regularly arrayed in db/m mice while myocardial fibers were disordered and cross-sectional areas in cardiomyocytes were increased in db/db mice (Figure 2C). However, empagliflozin treatment decreased heart size, HW/TL and left ventricular mass, as well as mitigated diabetes-induced histopathologic

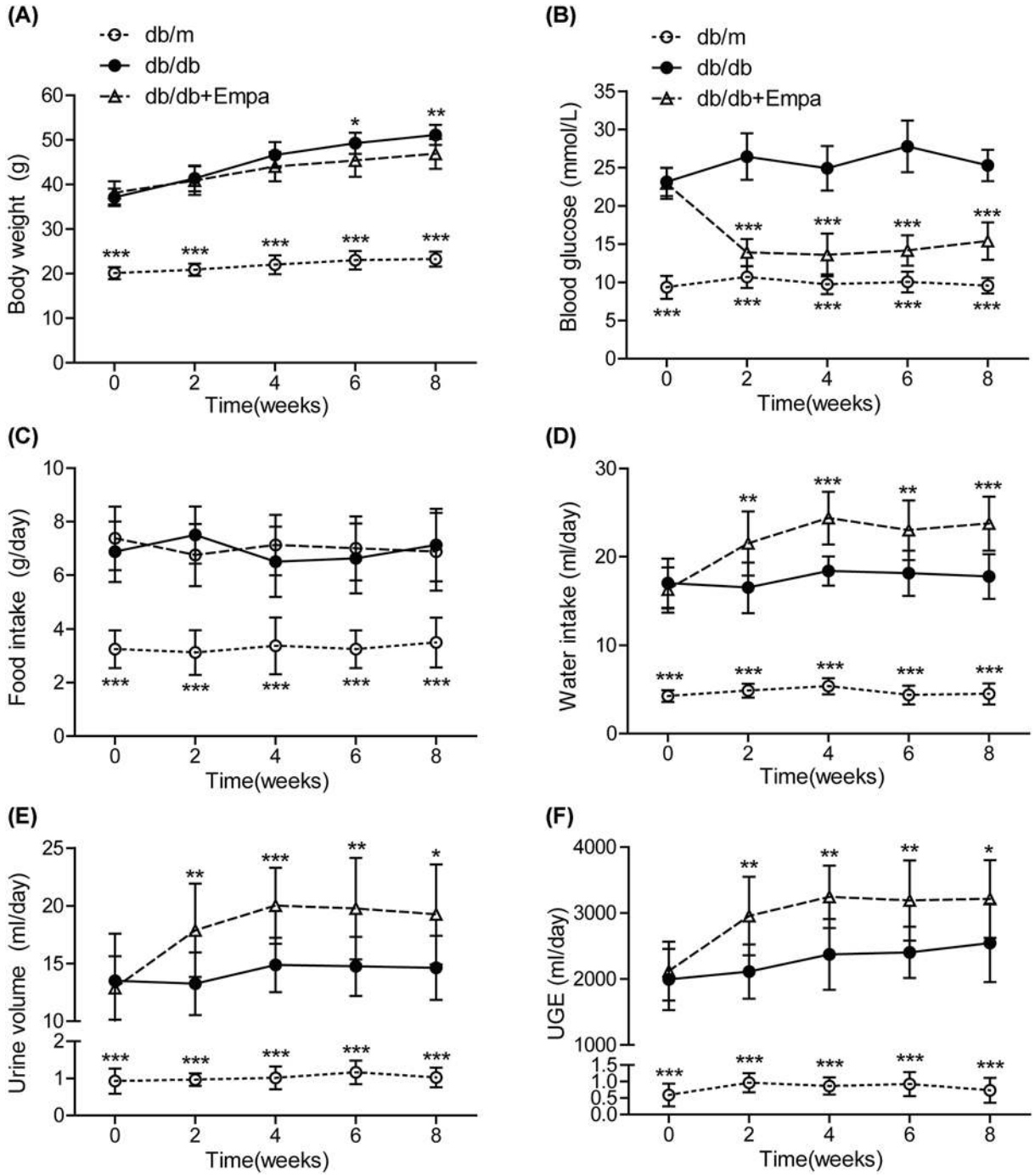

Figure 1. Effects of empagliflozin treatment on basic parameters in different groups

(**A–F**) Body weight, blood glucose, food intake, water intake, urine volume and UGE of mice after different treatments for 8 weeks. Data are presented as mean ± SD, n=8 per group. ***P<0.001, **P<0.01 and *P<0.05 *vs.* the db/db group. Abbreviation: UGE, urinary glucose excretion.

changes in hearts (Figure 2A–C). To further verify these cardiac changes, the expression of hypertrophy markers was detected by RT-PCR analysis. The transcription levels of molecular markers of cardiac hypertrophy, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and myosin heavy chain-β (β-MHC) were markedly elevated in db/db mice, but empagliflozin decreased diabetes-associated expression of ANP, BNP and β-MHC (Figure

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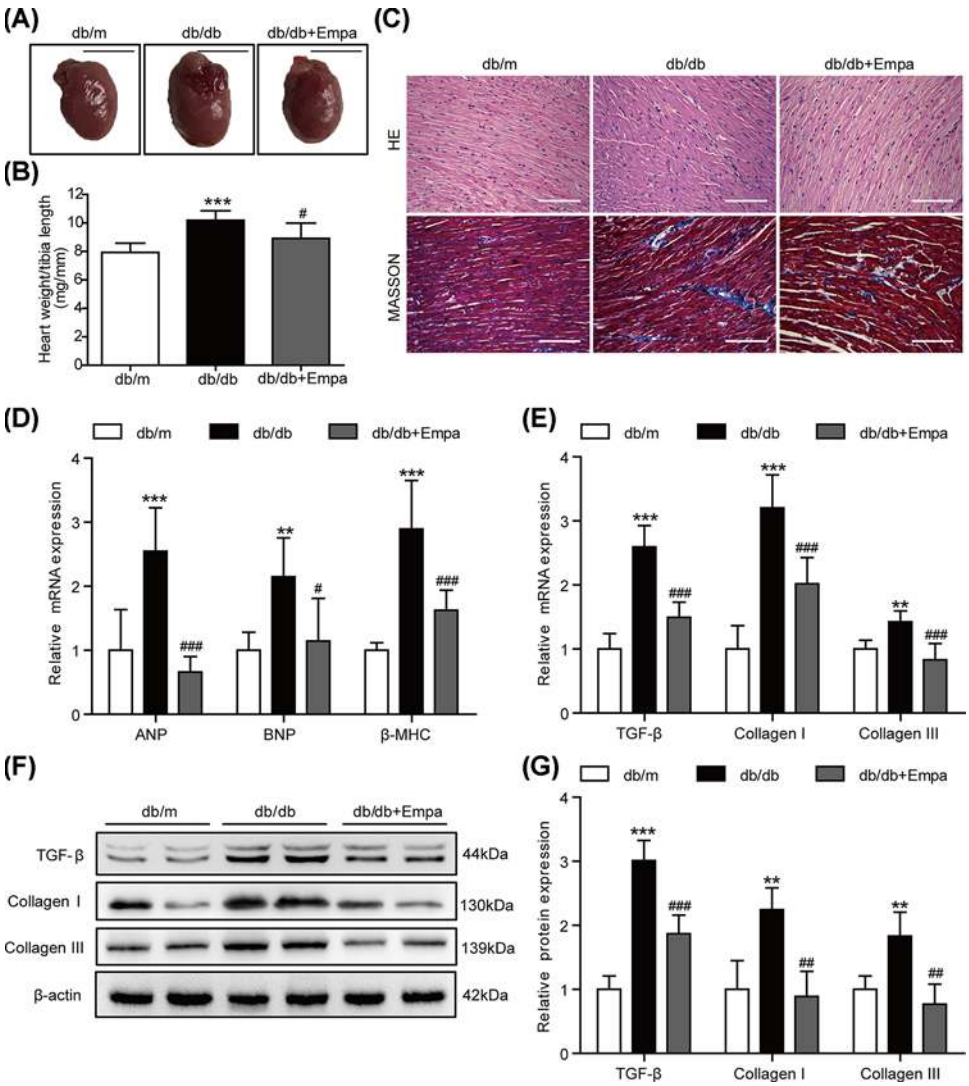

Figure 2. Effects of empagliflozin treatment on cardiac hypertrophy and fibrosis in diabetes

(**A**) Representative images of whole hearts from mice in different groups. The scale bar represents 5 mm. (**B**) The ratio of heart weight to tibia length of the mice was shown, n=6 per group. (**C**) Representative images of HE and Masson stained heart sections. Magnification ×400. The scale bar represents 100 μm. (**D**,**E**) mRNA expression of ANP, BNP, β-MHC, TGF-β, collagen I and collagen III was measured by quantitative RT-PCR, n=6 per group. (**F**) Protein expression of TGF-β, collagen I and collagen III in the hearts of mice was determined by western blot. (**G**) Quantitative analysis of TGF-β, collagen I and collagen III for Western blot, n=4 per group. Data are presented as mean ± SD. $^{***}P<0.001$ and $^{**}P<0.01$ represent significant differences between db/m and db/db mice; $^{###}P<0.001$, $^{##}P<0.01$ and $^{#}P<0.05$ represent significant differences between db/db and db/db+Empa.

2D). To sum up, these results demonstrated that empagliflozin treatment inhibited cardiac hypertrophy in diabetic mice.

Empagliflozin reduced diabetes-induced fibrosis in hearts

The accumulation of collagen fibers presented by Masson staining of cardiac sections were increased in db/db mice relative to the db/m group, while the signs of cardiac fibrosis were significantly alleviated after 8-week treatment with empagliflozin (Figure 2C). Since collagen fibers in myocardium mainly consisted of collagen I and collagen III [28], we further detected the mRNA and protein expression of these genes. Diabetes resulted in evident increases in collagen I and collagen III mRNA and protein levels in diabetic db/db mice, but empagliflozin attenuated diabetes-induced gene transcription of collagen fibers (Figure 2E–G). In addition, the pro-fibrotic factor, TGF-β expression was significantly

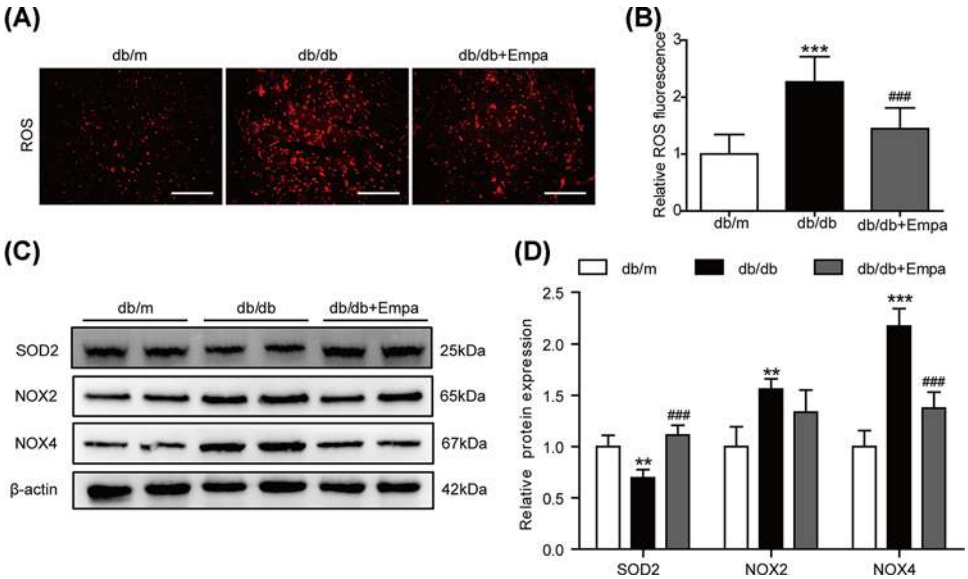

Figure 3. Effects of empagliflozin treatment on oxidative stress in diabetic hearts

(**A**) Representative images of Dihydroethidium (DHE) staining of the heart sections. Magnification ×400. The scale bar represents 100 μm. (**B**) Quantitative analysis of ROS productions for DHE staining, n=10 per group. (**C**) Protein expression of SOD2, NOX2 and NOX4 in the hearts of mice was determined by Western blot. (**D**) Quantitative analysis of SOD2, NOX2 and NOX4 for Western blot, n=4 per group. Data are presented as mean ± SD. $^{***}P<0.001$ and $^{**}P<0.01$ represent significant differences between db/m and db/db mice; $^{\#\#\#}P<0.001$ represents significant differences between db/db and db/db+Empa.

elevated at the mRNA and protein levels shown by qPCR and Western blot analyses; however, empagliflozin relieved the up-regulation of the pro-fibrotic gene (Figure 2E–G). Therefore, severe cardiac fibrosis of diabetic myocardium in db/db mice was significantly ameliorated by empagliflozin treatment.

Empagliflozin attenuated cardiac oxidative stress in diabetic mice

Next, we assessed the redox status in the mouse hearts. According to the results of DHE staining, we found that ROS expression was obviously increased in the myocardium of db/db mice compared with the db/m group, while empagliflozin markedly reduced ROS levels (Figure 3A,B). NOX is the major source of ROS in diabetic hearts [29], so we further made a thorough inquiry of whether empagliflozin could affect the activity of NOX. As expected, we observed an apparent up-regulation of NOX2 and NOX4 in diabetic hearts in comparison with the db/m group, and empagliflozin treatment reversed the expression of NOX4, but not significantly altered NOX2 expression (Figure 3C,D). In addition, ROS generation is highly related to mitochondrial functions, we examined the changes in proteins associated with mitochondrial fission and fusion. Empagliflozin reduced mRNA expression of fission-involved factors fission and dynamin-related protein 1, and increased fusion-involved factors mitofusin 1 and mitofusin 2 (Supplementary Figure S2). Furthermore, antioxidant SOD2 expression was visibly decreased relative to the db/m group; when diabetic mice were treated with empagliflozin, the SOD2 levels were significantly higher than those in animals without treatment (Figure 3C,D). Thus, empagliflozin treatment protected diabetic db/db hearts against oxidative stress.

Empagliflozin ameliorated cardiomyocyte apoptosis in db/db mice

To further evaluate the cardiac apoptosis levels, we performed TUNEL staining and analyzed the expression of anti-apoptotic Bcl2, pro-apoptotic Bax and cleaved Caspase3. As shown by Western blot analysis, diabetic hearts displayed significantly up-regulated percentage of TUNEL positive nuclei compared with the db/m group (Figure 4A,B). Meanwhile, cardiomyocyte apoptosis was enhanced as evidenced by decreased Bcl2/Bax expression and increased levels of cleaved Caspase3 (Figure 4C,D). Intriguingly, empagliflozin administration decreased percentage of TUNEL positive nuclei, cleaved Caspase3 expression as well as increased Bcl2/Bax expression (Figure 4A–D). These changes convincingly manifested that empagliflozin exerted beneficial effects on cardiomyocyte apoptosis.

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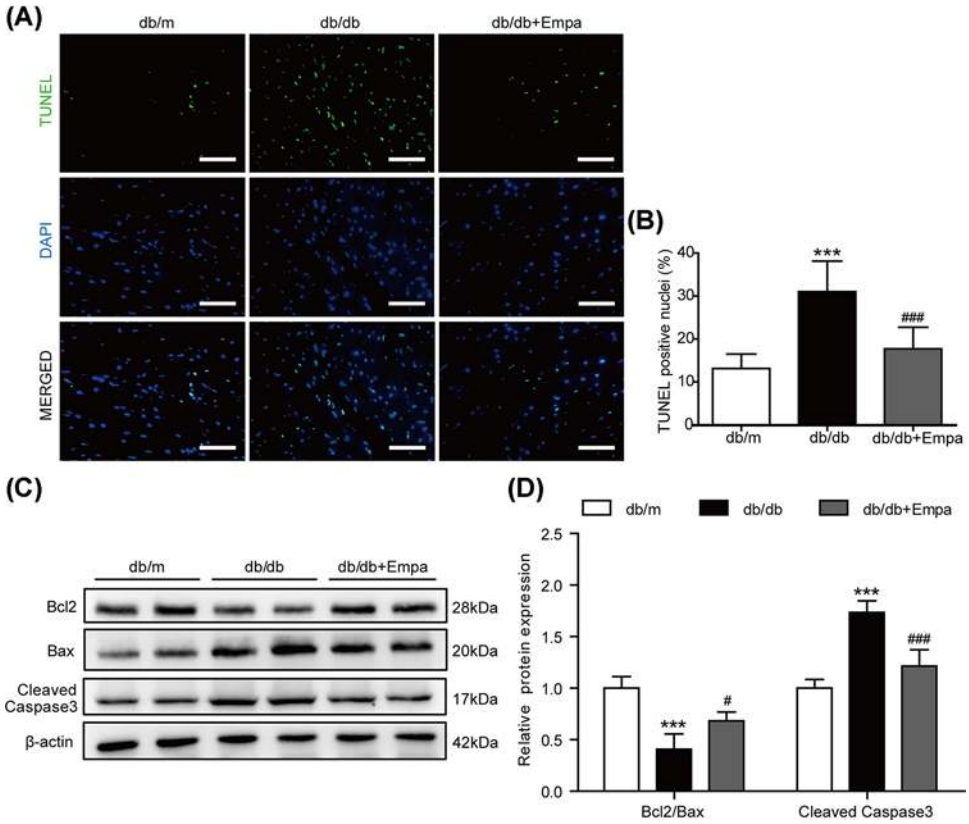

Figure 4. Effects of empagliflozin treatment on myocardial apoptosis of db/db mice

(**A**) Representative images of TUNEL staining of the heart sections. Magnification ×400. The scale bar represents 50 μm. (**B**) Percent of TUNEL positive nuclei in each group, n=10 per group. (**C**) Protein expression of Bcl2, Bax and cleaved Caspase3 in the hearts of mice was determined by Western blot. (**D**) Quantitative analysis of Bcl2/Bax and cleaved Caspase3 for Western blot, n=4 per group. Data are presented as mean ± SD. $^{***}P<0.001$ represents significant differences between db/m and db/db mice; $^{###}P<0.001$ and $^{\#}P<0.05$ represent significant differences between db/db and db/db+Empa.

Empagliflozin relieved NLRP3-induced pyroptosis in diabetic hearts

We further investigated the effects of empagliflozin on diabetes-induced NLRP3 inflammasome activation and NLRP3-induced pyroptosis. In comparison with the db/m group, myocardial NLRP3, cleaved Caspase1, IL-1β and cleaved GSDMD expression levels were significantly augmented but full-length GSDMD was reduced in the hearts of db/db mice (Figure 5A–C); however, activation of NLRP3 and Caspase1 as well as production of IL-1β and cleaved GSDMD were evidently decreased while full-length GSDMD was increased in hearts from db/db mice treated with empagliflozin (Figure 5A–C). Therefore, we first confirmed that empagliflozin alleviated the activation of NLRP3 inflammasome and subsequent cardiomyocyte pyroptosis in diabetic heart.

sGC-cGMP-PKG pathway in diabetes condition was altered by empagliflozin

To further explore the potential molecular mechanism regarding protective action of empagliflozin *in vivo*, the activity of sGC-cGMP-PKG pathway were examined. According to Western blot analysis or immunohistochemistry results, p-eNOS/t-eNOS and sGC-β expression presented a significant decrease in diabetic heart (Figure 6A,D,E). Meanwhile, we also observed that myocardial cGMP levels were down-regulated in db/db mice compared with the db/m group although no notable changes were found in serum cGMP levels (Figure 6B,C). Furthermore, cardiac PKG1α expression in db/db mice was lower than that in db/m mice (Figure 6D–F). Since Ser^{239}-phosphorylation of VASP is often used to assess PKG activation [13], we next determined the p-VASP/VASP ratio and observed that diabetic heart displayed a decrease in Ser^{239}-phosphorylation (Figure 6D–F). Interestingly, application of empagliflozin effectively up-regulated the activation of sGC-cGMP-PKG pathway as indicated by markedly increased

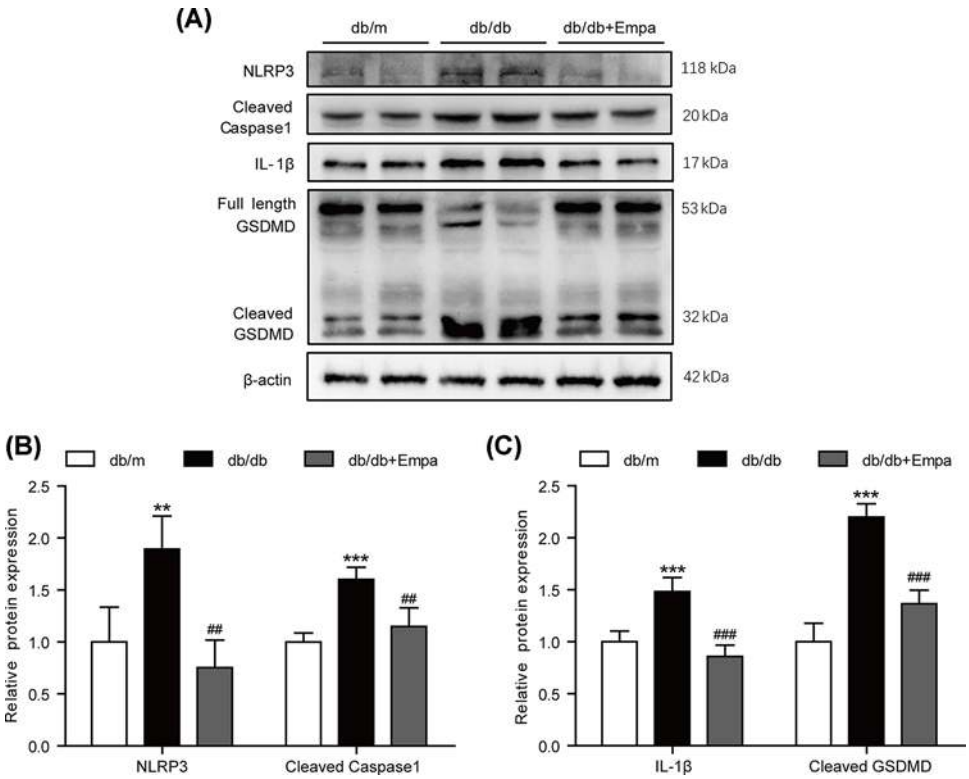

Figure 5. Effects of empagliflozin treatment on cardiac pyroptosis in diabetic myocardium

(**A**) Protein expression of NLRP3, cleaved Caspase1, IL-1β, full-length GSDMD and cleaved GSDMD in the hearts of mice was determined by Western blot. (**B**,**C**) Quantitative analysis of NLRP3, cleaved Caspase1, IL-1β and cleaved GSDMD for Western blot. Data are presented as mean ± SD, n=4 per group. $^{***}P<0.001$ and $^{**}P<0.01$ represent significant differences between db/m and db/db mice; $^{###}P<0.001$ and $^{##}P<0.01$ represent significant differences between db/db and db/db+Empa.

p-eNOS/t-eNOS, sGC-β, PKG1α expression, p-VASP/VASP ratio as well as cGMP levels (Figure 6A–F). In the present study, we found that the SGLT2 expression in kidney was over 100-times higher than that in heart (Supplementary Figure S1). These results illustrated that sGC-cGMP-PKG pathway played a vital role in the cardioprotective effects of empagliflozin on diabetic myocardium, while these effects might be not directly regulated by SGLT2 inhibition.

Cardioprotective effects of empagliflozin were dulled by sGC-β knockdown

To ascertain the exact regulation of empagliflozin in diabetic heart injury by sGC-cGMP-PKG signaling, we used an opposite strategy to inactivate this pathway through down-regulating sGC-β expression *in vivo*. As shown in Supplementary Figure S2, transfection of sGC-β siRNA substantially inhibited the mRNA and protein expression of sGC-β compared with the mice injected with negative control siRNA and no significant differences in p-eNOS/t-eNOS expression were observed after sGC-β transfection (Supplementary Figure S3A,D,E). In addition, si-sGC-β injection caused no marked changes in body weight and blood glucose compared with the si-NC group while HW/TL of mice was slightly increased (Figure 7A–C). Notably, knockdown of sGC-β suppressed the activity of sGC-cGMP-PKG signaling, characterized by reduced PKG1α expression, p-VASP/VASP ratio and cGMP levels both in myocardial tissue and serum (Supplementary Figure S3B–D,F). According to the echocardiographic analysis, systolic parameters EF and FS were markedly decreased in the db/db+Empa+si-sGC-β group relative to the negative control; in addition, diastolic parameters DT and IVRT were significantly increased as well as E/A ratio was decreased (Figure 7D–H). Therefore, these data suggested that sGC-β inhibition significantly damaged the left ventricular systolic and diastolic function which empagliflozin improved in db/db mice and sGC-cGMP-PKG signaling was indispensable for the advantage of empagliflozin on cardiac protection in diabetic condition.

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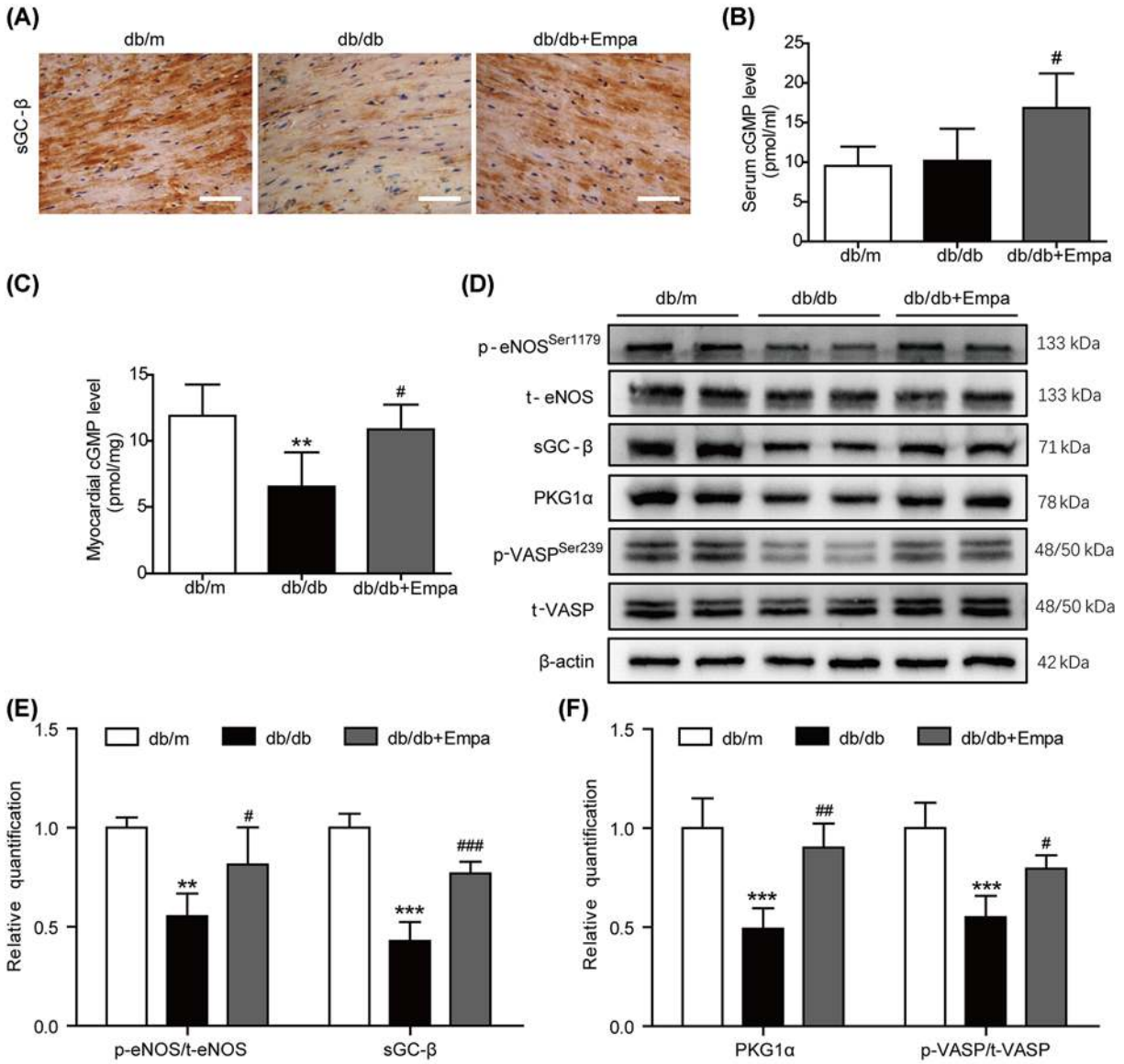

Figure 6. Effects of empagliflozin treatment on the activity of sGC-cGMP-PKG pathway in the hearts

(**A**) Representative immunohistochemistry staining of sGC-β of the heart sections. Magnification ×400. The scale bar represents 50 μm. (**B**,**C**) Representative levels of cGMP in serum and myocardium were evaluated by ELISA, n=6 per group. (**D**) Protein expression of p-eNOS, t-eNOS, sGC-β, PKG1α, p-VASP and t-VASP in the hearts of mice was determined by Western blot. (**E**,**F**) Quantitative analysis of p-eNOS/t-eNOS, sGC-β, PKG1α and p-VASP/t-VASP for Western blot, n=4 per group. Data are presented as mean ± SD. ***P<0.001 and **P<0.01 represent significant differences between db/m and db/db mice; ###P<0.001, ##P<0.01 and #P<0.05 represent significant differences between db/db and db/db+Empa.

Discussion

T2DM is closely associated with a substantially increased cardiomyopathy risk [30]. DCM was characterized by the impairment of diastolic and systolic myocardial performance, independent of coronary artery disease and hypertension [31]. First, the body weight, blood glucose and cardiac function of mice were detected, and these results indicated that we had successfully established DCM model. In the current study, we investigated whether empagliflozin could improve cardiac function and alleviate some typical lesions of DCM in db/db mice. Moreover, the potential mechanism underlying the protective role of empagliflozin was also discussed.

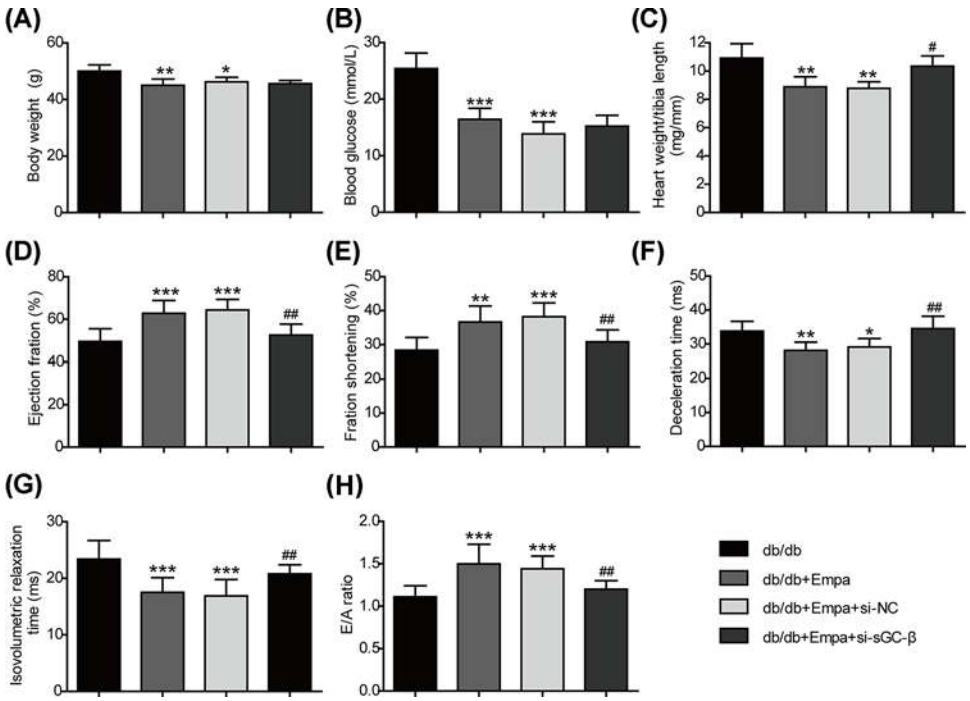

Figure 7. General and echocardiographic features of mice after si-sGC-β transfection at 8 weeks

(**A**–**C**) Body weight, blood glucose and the ratio of heart weight to tibia length of mice after transfection. (**D**–**H**) Measurement of EF, FS, DT, IVRT and E/A ratio after 8 weeks of transfection. Data are presented as mean ± SD, n=8 per group. $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$ represent significant differences between db/db and db/db+Empa mice; $^{\#\#}P<0.01$ and $^{\#}P<0.05$ represent significant differences between db/db+Empa+si-NC and db/db+Empa+si-sGC-β.

Empagliflozin, an SGLT2 inhibitor, is a new antidiabetic agent that promotes the glucose excretion from the urine. Recently, a large number of clinical trials have reported that empagliflozin application improved cardiovascular outcomes in diabetic patients [16,32,33]. Numerous experimental studies on diabetic animals show that empagliflozin predominantly delayed the progression of cardiomyopathy [18,24,25]. In the present study, water intake, urine volume and UGE were significantly enhanced, and the blood glucose and body weight were decreased by empagliflozin, which is consistent with the results of other studies [18,22,23,34]. Additionally, although early stage of DCM was manifested by diastolic dysfunction, we observed that empagliflozin treatment improved both diastolic and systolic functions by analyzing the data on echocardiography in db/db mice. Previous study found that only diastolic function was improved in ob/ob mice treated with empagliflozin for 6 weeks but systolic function was not altered [35]. We speculated that different animal model and therapeutic course may lead to this inconsistent result. Notably, the difference in mortality between the empagliflozin group and the placebo group occurred 12 weeks after the start of the EMPA-REG OUTCOME trial, and it is clear that such early benefits are unlikely to be achieved by short-term anti-hyperglycemic effect [16]. Furthermore, the CVD-REAL study suggested that treatment with SGLT2 inhibitors versus other glucose-lowering drugs was associated with a lower risk of hospitalization for heart failure and cardiovascular death [32]. In addition, the treatment period in our study is 8 weeks, and insulin injection could not improve heart function in spite of the glucose-lowering effect. Therefore, the unexpected cardiovascular benefits might be explained by the role of empagliflozin itself.

Cardiac hypertrophy is a feature of lots of cardiomyopathies, which can contribute to ventricular dysfunction in heart failure [36,37]. Animal studies discovered that drug therapy through inhibiting myocardial hypertrophy ameliorates DCM [11,38]. The changes in HW/TL, size and structure of cardiomyocytes and molecular markers of hypertrophy were compromised by empagliflozin, confirming that the advantageous result of empagliflozin in cardiac hypertrophy, which was also aligned with other studies [18]. Myocardial fibrosis is another pathological characteristic of DCM, which can aggravate ventricular stiffness and reduce cardiac compliance, and finally leads to cardiac dysfunction [39]. Fibrotic remodeling occurred as a result of excess accumulation of extracellular matrix (ECM) proteins in hearts, primarily type I and III collagen [28], which are mediated by TGF-β, a well-known pro-fibrotic factor [39].

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Fortunately, empagliflozin reversed the ECM deposition and up-regulation of fibrotic changes. These data suggested that empagliflozin might be effective in preventing the diabetic hypertrophy and fibrosis of hearts.

Based on the above results, we further probed the feasible mechanism underlying the protective role of empagliflozin. The pivotal contribution of hyperglycemia-associated oxidative stress to DCM has been well elucidated [40,41]. Andreadou et al. [23] observed that empagliflozin prevented myocardial infarction via the activation of STAT3 antioxidant and anti-inflammatory properties. In addition, our group found that empagliflozin significantly ameliorated myocardial oxidative stress injury and cardiac fibrosis diabetic KK-Ay mice [42]. We further determined whether empagliflozin could suppress oxidative stress in diabetic db/db mice. As shown in our study, diabetes led to accelerating oxidative stress, while empagliflozin treatment saved hearts from oxidative damage. On one hand, one paper published in *Nature Immunology* has confirmed the precise link between oxidative stress and NLRP3 inflammasome activation [43]. Oxidative stress was also reported to mediate NLRP3-induced pyroptosis in the process of biochemical and structural alterations during DCM [6]. In our study, enhanced NLRP3 inflammasome activation and myocardial pyroptosis were found in diabetic hearts. Moreover, several experiments have explored the role of SGLT2 inhibitors in pyroptosis. Although empagliflozin treatment protects against the diet-induced NLRP3 inflammasome activation of kidney and liver in mice fed high fat-high sugar diet for 4 months, this effect was not observed in heart [34]. In another study, however, dapagliflozin reduces the activation of the NLRP3 inflammasome and attenuates the development of DCM in T2DM mice model [44]. In this study, empagliflozin reduced NLRP3 inflammasome activation and pyroptosis level in hearts of db/db mice. We consider that the anti-pyroptotic effect of empagliflozin on cardiomyocytes is possibly owing to the reduced oxidative damage, which is similar to previous study [6]. On the other hand, accumulating evidence demonstrates that apoptosis activated by oxidative stress is involved in heart diseases [45,46]. Evidence for oxidative stress-mediated apoptosis in the development of DCM was found *in vivo* and *in vitro* [47]. In our study, excessive oxidative stress in diabetes was accompanied by elevated level of apoptosis. Not surprisingly, empagliflozin also alleviated the level of cardiomyocyte apoptosis in diabetic mice, which is also concordant with data from recent publications emphasizing the anti-apoptotic role of EMPA in hearts [24]. Empagliflozin rescues diabetic myocardial microvascular injury via the inhibition of mitochondrial fission [20]. In addition, empagliflozin normalizes the size and number of mitochondria via suppression of ROS after myocardial infarction [48]. We also found that empagliflozin changed the mitochondrial dynamics in diabetic hearts. Therefore, inhibition of ROS generation by empagliflozin may improve mitochondrial functions.

Next, we further probed the potential signaling pathway underlying the effects of empagliflozin on cardiomyocytes. It is widely recognized that sGC-cGMP-PKG pathway serves as a common mediator of cardioprotection and the impairment of this signaling and low cGMP levels are conspicuous traits arising in various cardiovascular diseases [10,13,49]. Moreover, accumulating evidence supports that sGC-cGMP-PKG signaling pathway is inactivated in DCM [11]. Likewise, we observed that sGC-cGMP-PKG pathway was remarkably down-regulated in the diabetic hearts. The observed inconsistency between impairment of myocardial cGMP level and unchanged serum cGMP level in db/db mice may be a compensatory reaction that intracellular cGMP was released from other tissues to serum under diabetic conditions [11]. A recent study has suggested that empagliflozin diminished the inhibition of sGC-cGMP-PKG pathway in diabetic aorta [50]. Not surprisingly, we discovered a significant effect of empagliflozin on diabetes-induced inactivation of the sGC-cGMP-PKG pathway in hearts. Additionally, other studies have demonstrated that enhanced activation of sGC-cGMP-PKG pathway potently alleviates oxidative damage [13,51], which suggests that empagliflozin might reduce diabetes-associated oxidative stress via sGC-cGMP-PKG pathway. Subsequently, utilizing siRNA knockdown animal models, we observed that sGC-β depletion weaken the effects of empagliflozin on diabetic hearts. It is obvious to find that when sGC-cGMP-PKG signaling was inactivated in db/db mice receiving empagliflozin treatment, their heart function was similar to the status of db/db mice without treatment. Therefore, it is reasonable to conclude that sGC-cGMP-PKG signaling is likely to be the mechanistic actions of empagliflozin toward DCM. Previous review provides supportive evidence that cGMP signaling decreases cardiac hypertrophy but promotes apoptosis, although the downstream mechanisms have not been fully determined [52]. In our study, empagliflozin enhanced sGC-cGMP-PKG signaling in cardiomyocytes of diabetic mice, meanwhile, we observed an inhibition of cardiac apoptosis. Moreover, very recent studies also show that PKG knockdown could induce aggravated endoplasmic reticulum stress, oxidative stress, and cellular apoptosis in myocardial ischemia-reperfusion injury [53,54]. Thus, increasing activation of sGC-cGMP-PKG signaling may suppress apoptosis by down-regulating oxidative injury.

SGLT2 displays a ubiquitous distribution pattern with highest expression levels in kidney, particularly in renal tubular epithelium [55]. Given that SGLT2 is little expressed in heart and empagliflozin is a selective SGLT2 inhibitor [19,55], how empagliflozin activates the sGC-cGMP-PKG signaling in diabetic cardiomyocytes will be investigated in future studies. However, several studies have proved that empagliflozin can directly influence the metabolism and

function of cardiomyocytes. In isolated cardiomyocytes, empagliflozin decreased cytoplasmic Na^+ and Ca^{2+} and enhanced mitochondrial Ca^{2+} through direct inhibition of the Na^+/H^+ exchanger independent of SGLT2 activity [56]. Another *in vitro* study concluded that empagliflozin ameliorated high glucose-induced cardiac dysfunction via the down-regulation of SGLT1 and SGLT2 expression in cardiomyocytes [57]. Two similar studies have also revealed that empagliflozin may increase cardiac energy production in isolated diabetic hearts and prevent worsening cardiac function in isolated hearts from mice with heart failure [58,59]. These all suggest that the benefit of empagliflozin may be due to a direct and sustained cardiac effect. Taken together, our results indicate that DCM is related to oxidative stress-induced pyroptosis and apoptosis, and empagliflozin prevents the diabetes-associated oxidative stress, pyroptosis, and apoptosis and attenuates the accelerated development of cardiac hypertrophy and fibrosis. More importantly, we first identify that empagliflozin achieves cardioprotection at least in part through activating sGC-cGMP-PKG pathway under diabetic conditions.

Clinical perspectives

- EMPA-REG OUTCOME trial has reported that empagliflozin exerts cardiovascular benefits on diabetic population. However, the mechanism by which empagliflozin alleviates DCM still remains unclear.
- We found that empagliflozin improved cardiac function and extenuated cardiomyopathy involving the inhibition of oxidative injury via sGC-cGMP-PKG pathway in type 2 diabetes mice.
- Our data provide strong evidence for the clinical priority of the empagliflozin in patients with diabetes and cardiovascular disease.

Author Contribution

Liming Chen and Bei Sun designed the experiment. Mei Xue, Yunpeng Chang, and Ying Cheng performed the experiment and wrote the manuscript. Yunhong Lu and Xiangyang Liu interpreted the results and analyzed the data. Linxin Xu, Xiaoyu Li revised the manuscript. Ting Li and Xiaochen Yu assisted in conducting the experiment. During the revision, Yue Wang performed the supplementary experiment and revised the article. All authors approved the final version for publication.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; β-MHC, myosin heavy chain-β; cGMP, cyclic guanosine monophosphate; DCM, diabetic cardiomyopathy; DT, deceleration time; eNOS, endothelial NO synthase; EF, ejection fraction; E/A, velocity of early mitral flow to velocity of late mitral flow ratio; FS, fraction shortening; GSDMD, gasdermin D; HW/TL, heart weight to tibia length ratio; IL-1β, interleukin-1β; IVRT, isovolumetric relaxation time; NLRP3, NOD-like receptor 3; NOX, NADPH oxidase; PKG, cGMP-dependent protein kinase; ROS, reactive oxygen species; sGC, soluble guanylate cyclase enzyme; SOD2, superoxide dismutase 2; T2DM, type 2 diabetes mellitus; TGF-β, transforming growth factor-β; UGE, urinary glucose excretion; VASP, vasodilator-stimulated phosphoprotein.

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