Original Paper

The Sodium-Glucose Co-Transporter 2 Inhibitor, Empagliflozin, Protects against Diabetic Cardiomyopathy by Inhibition of the Endoplasmic Reticulum Stress Pathway

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Key Words

Diabetic cardiomyopathy • Empagliflozin • Endoplasmic reticulum stress

Abstract

Background/Aims: This study aimed to determine whether or not the sodium-glucose co-transporter 2 inhibitor, empagliflozin (EMPA), can protect against diabetic cardiomyopathy (DCM) and to elucidate the related mechanism. **Methods:** Rats were divided into the following four groups: a non-diabetic group; diabetic cardiomyopathy rats without EMPA treatment; and diabetic cardiomyopathy rats with EMPA treatment (low- and high-dose EMPA). Hemodynamic measurements were performed to evaluate left ventricular systolic and diastolic function. The histopathology of the heart was examined with hematoxylin-eosin staining. Expression of glucose-regulated protein (GRP)78, enhancer-binding protein homologous protein (CHOP), and caspase-12 was detected by Western blot, and the mRNA levels of XBP1, ATF4, and TRAF2 were analysed by real-time PCR. **Results:** EMPA significantly decreased the blood glucose level when compared with vehicle. EMPA strongly improved cardiac function based on hemodynamic and histopathologic analyses. Moreover, EMPA can significantly down-regulate the expression of GRP78, CHOP, and caspase-12 ($P < 0.01$). Additionally, the mRNA levels of XBP1, ATF4, and TRAF2 were markedly decreased by administration of EMPA ($P < 0.01$). **Conclusion:** EMPA protects against DCM by inactivating the endoplasmic reticulum stress pathway.

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Introduction

Diabetes mellitus (DM), a serious and complex metabolic disorder, currently affects over 350 million people worldwide, and type 2 DM (T2DM) accounts for 90–95% of all diagnosed diabetes in adults [1, 2]. Studies have demonstrated that DM is associated with certain cardiovascular diseases such as diabetic cardiomyopathy (DCM) [1, 3]. DCM is a disease

process which affects the myocardium in diabetic patients, and its prevalence reaches to 60% in T2DM [4]. It includes a wide range of structural abnormalities such as cardiomyocyte hypertrophy and apoptosis, ventricular dilation, and prominent interstitial fibrosis [5, 6], which eventually leads to diastolic or systolic dysfunction [7, 8].

Empagliflozin (EMPA), a new anti-diabetic drug, is a potent competitive Sodium-Glucose Co-transporter (SGLT) inhibitor with high selectivity to SGLT-2 over other SGLT isoforms [9]. EMPA can effectively reduce the blood glucose and haemoglobin level, increase insulin sensitivity and urinary glucose, and improve glucose tolerance without making hypoglycaemia [10, 11]. Furthermore, recent clinical trial, known as Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients-Removing Excess Glucose (EMPA-REG OUTCOME), has indicated that EMPA could reduce mortality in patients with T2DM at high cardiovascular risk [12]. However, relatively little information is available about whether EMPA improves cardiac function in DCM and the possible associated mechanism has not been defined.

In DCM, there are multiple pathophysiologies which seem related to different mechanisms, including impaired calcium homeostasis, myocardial insulin resistance, increased lipid uptake, glucose toxicity, activation of the renin-angiotensin system, and increased oxidative stress [13]. Recently, the pathological role of endoplasmic reticulum (ER) stress is increasingly recognized in DCM. The ER plays an important role in protein folding and maturation, and lipid synthesis, where several situations including hypoxia, elevated protein synthesis, hyperglycemia, and exposure to free radicals, can induce the pathological accumulation of unfolded proteins, a condition known as ER stress (ERS) [14]. ERS is known to be involved in a number of complexes homeostatic signaling pathways among which the unfolded protein response (UPR) is most commonly recognized [15]. UPR can activate glucose-regulated protein 78 (GRP78) which plays an important role in exporting the accumulated proteins out of ER or degrading protein with the help of cytoplasmic proteasomes. Moderate ERS can restore the homeostasis, while severe ERS can result in persistent or excessive protein misfolding leading to consequences such as apoptosis. It was noted that the transcriptional induction of CHOP, activation of JNK and caspase-12-dependent pathways was important in the processes of apoptosis [16]. Drugs such as valsartan and liraglutide could relieve the ERS-associated apoptosis, preventing against the development of DCM [17, 18]. Nevertheless, it remains unclear whether EMPA could improve the DCM condition via ERS special pathway.

Therefore, in the present study, we investigated whether EMPA improves DCM in the streptozotocin (STZ)-induced diabetic rats. Our results imply that EMPA improves cardiac function through the inhibition of ERS induced cardiomyocyte apoptosis in the rats with DCM.

Materials and Methods

Animals and grouping

Sixty adult male Wistar rats (200 ± 20 g) were purchased from the Experimental Animals Ministry of China Medical University (Shenyang, China). The rats were kept under a 12 h light/dark cycle at room temperature ($20-22^{\circ}\text{C}$) and humidity (50%-60%). Rats were separated into high-fat diet rats ($n = 36$) and control rats ($n = 12$). The former rats were fed a high-fat diet for 8 weeks, then given a single intraperitoneal (i.p.) injection of STZ (35 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) dissolved in citrate buffer at pH 4.5. The control rats were fed regular chow and injected with the same dose of citrate buffer. Seventy-two hours following the STZ injection, blood samples were collected from the tail vein to measure the blood glucose level. Rats with fasting blood glucose levels > 16.7 mmol/l were considered successful DM models and were used for further investigation [19]. The blood glucose level of control rats was within the normal range. All of the remaining rats were used in the experiments and divided into 4 groups: non-DCM group (CON, $n = 12$); non-EMPA, but with vehicle group (DCM, 0.5% hydroxyethylcellulose/d p. o., daily, $n = 12$); high dose of EMPA (high-EMPA, 30 mg/kg/d *per os* [p.o.] daily, $n = 12$); and low dose of EMPA (low-EMPA, 10 mg/kg/d

p.o. daily, n = 12). The rats were treated for 8 weeks. All rats were anesthetized with chloral hydrate and sacrificed following weeks of treatment, and heart tissues were obtained for the following experiments. The study was approved by Ethics Committee of China Medical University (Shenyang, China).

Determination of blood glucose level

Following a 12-h fast, all of the blood from the rat tail peripheral capillary was extracted to detect the fasting blood glucose level using the Roche Accu-CHEK active blood glucose meter (Roche, Mannheim, Germany).

Hemodynamic measurements

Rats were anesthetized with an i.p. injection of 10% chloral hydrate (3.5 ml/kg), and a 2F catheter connected to a polygraph system (BL-420; Thai Union, Chengdu, China) was introduced into the left ventricle via the right carotid artery to obtain the following measurements: left ventricular systolic pressure (LVSP); left ventricular end-diastolic pressure (LVEDP); maximal ascending rate of left ventricular pressure (+dp/dt); and maximal descending rate of left ventricular pressure (−dp/dt).

TUNEL assay

Cardiomyocyte apoptosis was detected with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) *In Situ* Cell Death Detection Kit (Wanleibio, Shenyang, China) according to the manufacturer's instructions. The apoptotic index was calculated as the percentage of TUNEL-positive cells. Ten representative fields were evaluated for each group and the average value was calculated.

Hematoxylin-eosin (HE) staining

The pathologic specimens were fixed for 24 h with 10% neutral formalin using the conventional method, dehydrated through an ethanol gradient, embedded in paraffin, and stained with HE. The remaining section of the sample was stored at −80°C prior to use in Western blotting and a real-time polymerase chain reaction (PCR).

Western blotting

After extraction of myocardial proteins, equal amounts of the protein preparations were separated by 15% SDS-PAGE. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) for 90 min at 80 V. The membrane was blocked with 5% non-fat milk in Tris-buffered saline Tween (TBST) for 1 h at room temperature, then incubated with a primary antibody against Grp78 (1:500; Wanleibio), caspase-12 (1:500; Abcam, Shanghai, China), CHOP (1:500; Wanleibio), and β-actin (1: 1000; Wanleibio) at 4°C overnight. After incubation with 1:4000 horseradish peroxidase (HRP)-conjugated secondary antibodies (Wanleibio), the blots were developed using enhanced chemiluminescence (Wanleibio).

Quantitative reverse transcription real-time PCR (qRT-PCR)

Total RNA was extracted using a one-step method according to the instructions for the TRIzol® reagent (BioTeke, Beijing, China). The expression of TRAF2, ATF4, and XBP1 mRNA was analysed using real-time PCR with a 2 × Power Taq PCR Master Mix (BioTeke). To determine the RNA concentration, a 1-μl RNA sample was obtained and 79 μl of DEPC water was added to detect OD₂₆₀ and OD₂₈₀ values using an ultraviolet spectrophotometer. A ratio between 1.8 and 2.0 suggested that the RNA purity of the sample was sufficient. cDNA was then synthesized using reverse transcription in accordance with the kit instructions (BioTeke). The ATF4, TRAF2, XBP1, and β-actin primers are shown in Table 1. The relative expression of genes was calculated using the 2^{−ΔΔCT} method.

Table 1. ATF4, TRAF2, XBP1, and β-actin primers

	Forward	Reverse
ATF4	ATGAGCCCTGAGTCCTACCT	GCTGTCTTGT'TTTGCTCCAT
TRAF2	AACCTGTGATGGCTGTGGC	CTTGGGACTCCAGGAACGA
XBP1	CCTTCTCCCTTCAGCGACAT	CAGTGGTGGGTGGCTTTAGACA
β-actin	GGAGATTACTGCCCTGGCTCCTAGC	GGCCGGACTCATCGTACTCCTGCTT

Statistical analysis

All data are presented as the mean $\pm$ SD. Statistical analyses were performed with SPSS 22.0 software, using a Student t-test or analysis of variance (ANOVA) with *post hoc* analysis, as appropriate. A P value < 0.05 was considered statistically significant.

Results

EMPA has an effect on blood glucose level

The blood glucose level was determined 8 weeks after EMPA treatment. The blood glucose level was increased in DCM rats when compared with the low- and high-EMPA groups, and CON rats ($P < 0.01$). Following long-term treatment with EMPA, the blood glucose level in the high-EMPA group was significantly lower than the low-EMPA group (Fig. 1).

EMPA improves cardiac function in DCM rats

As shown in Figure 2, the LVSP in DCM rats was clearly reduced in comparison with CON rats ($P < 0.01$); however, after administration of EMPA, the LVSP in both EMPA groups was significantly increased compared to DCM rats (Fig. 2a). In contrast, the LVEDP in DCM rats was significantly increased compared to CON rats ($P < 0.01$). Compared with DCM rats, the LVEDP was significantly decreased when treated with low- or high-dose EMPA (Fig. 2b); however, there was no significant difference in the LVSP and LVEDP between the low- and high-dose EMPA groups (Fig. 2a-b). In DCM rats, both the $+dp/dt$ and $-dp/dt$ were decreased compared to CON rats ($P < 0.01$). After administration of EMPA for 8 weeks, the $+dp/dt$ and $-dp/dt$ were increased compared with DCM rats (Fig. 2c-d); however, there was no statistical difference in the $\pm dp/dt$ between the low- and high-dose EMPA groups (Fig. 2c-d).

Fig. 1. Effect of EMPA on the blood glucose level. Blood glucose levels in different groups of rats after different treatments for 8 weeks. Data are shown as the mean $\pm$ SD. * $P < 0.01$ vs. CON group rats; # $P < 0.01$ vs. DCM group rats; \$ $P < 0.01$ vs. low-EMPA group rats.

Fig. 2. EMPA improves cardiac function in DCM rats. (a) LVSP, (b) LVEDP, (c) $+dp/dt$, and (d) $-dp/dt$ in different groups of rats after different treatments for 8 weeks. Data are shown as the mean $\pm$ SD. * $P < 0.01$ vs. CON group rats; # $P < 0.01$ vs. DCM group rats.

Fig. 3. HE staining of cardiomyocytes. HE staining of myocardium in (a) DCM, (b) low-EMPA, (c) high-EMPA, and (d) CON group rats. 200 x: Original magnification $\times 200$; 400 x: Original magnification $\times 400$.

Fig. 4. EMPA inhibits cardiac myocyte apoptosis in DCM rats. Representative observations of TUNEL assay of myocardium cells in (a) DCM group, (b) low-EMPA group, (c) high-EMPA group, and (d) CON group. Original magnification $\times 400$. (e) Number of TUNEL-positive myocardium cells in the four groups. Data are shown as the mean $\pm$ SD. * $P < 0.01$ vs. CON group rats; # $P < 0.01$ vs. DCM group rats; \$ $P < 0.01$ vs. low-EMPA group rats.

EMPA alleviates histopathologic changes in the myocardium of DCM rats

HE staining was performed to clarify the effect of EMPA on the histopathologic changes in the myocardium. The cardiomyocytes were clearly striated and regularly arrayed in the CON rats (Fig. 3d), while disordered cell arrays and focal necrosis were characteristics of the DCM rats (Fig. 3a), but were improved following treatment with low- or high-dose EMPA (Fig. 3b-c). Additionally, the histopathologic changes in the high-EMPA group improved to a greater degree than the low-EMPA group.

EMPA inhibits cardiomyocyte apoptosis in DCM rats

The TUNEL assay was performed to understand the effect of EMPA on cardiomyocyte apoptosis in DCM rats. Cardiomyocytes of rats with DNA fragmentation were labeled brown by TUNEL, whereas cardiomyocytes without DNA fragmentation were counterstained blue by hematoxylin (Fig. 4a-d). The number of TUNEL-positive myocardium cells was low in the control rats (Fig. 4d). A number of cardiomyocytes were TUNEL-positive in the DCM group (Fig. 4a); however, the number of apoptotic myocardium cells was significantly decreased after treatment with EMPA for 8 weeks (Fig. 4e).

EMPA decreases ERS-induced cardiomyocyte apoptosis by down-regulating the expression of CHOP and GRP78, and inactivating caspase-12

GRP78 is an important molecular chaperone localized in the ER, and is usually regarded as a marker reflecting activation of ERS. GRP78 expression was abundant in the myocardium of DCM rats, suggesting the presence of myocardial ERS; however, GRP78 expression in

Fig. 5. Immunoblot analysis for proteins of ERS special pathway. Western blot analysis of (a) GRP78, (c) CHOP, and (d) caspase-12 expression in the myocardium of DCM and CON rats. Western blot was performed using each relevant antibody. β -actin is shown as a loading control. Quantitative analysis of (b) GRP78, (d) CHOP, (f) procaspase-12, and (g) cleaved caspase-12 for Western blot. Data are shown as the mean $\pm$ SD. * P < 0.01 vs. CON group rats; # P < 0.01 vs. DCM group rats; \$ P < 0.01 vs. low-EMPA group rats.

DCM rats treated with EMPA was markedly lower than the DCM group (Fig. 5a-b). We next examined the expression of CHOP because of the essential role in ERS-induced cardiomyocyte apoptosis in diabetes. The expression of CHOP had a similar trend with the incidence of cardiomyocyte apoptosis in DCM rats. Interestingly, administration of low- or high-dose EMPA attenuated the expression of CHOP (Fig. 5c-d). To further elucidate the ERS process, we noted caspase-12 activation in the different groups. Caspase-12 activity was markedly increased in the DCM group when compared with the CON group (P < 0.01). When DM rats were treated with EMPA, both the low- and high-EMPA groups demonstrated a reduced level of caspase-12 activation (Fig. 5e-g).

EMPA blocks mRNA transcription of some ERS-associated factors

Because ATF4, TRAF2, and XBP1 are related to ERS, we further analysed the mRNA levels using RT-qPCR. As shown in Figure 6, the levels of ATF4, TRAF2, and XBP1 mRNA were significantly higher in the myocardium of DCM rats than CON rats (P < 0.01); however, EMPA treatment decreased the mRNA levels, and the levels of ATF4, TRAF2, and XBP1 mRNA in the high-dose EMPA group was lower than the low-dose EMPA group (Fig. 6).

Discussion

Numerous studies have demonstrated that DM is closely related to cardiomyopathy [20, 21]. Epidemiologic investigations have also indicated that there is an increase in the

Fig. 6. EMPA blocks mRNA transcription of ER stress-associated factors. Quantitative analysis of the level of AEG-1 mRNA and level of mRNA expression of (a) ATF4, (b) TRAF2, and (c) XBP1 in rat myocardium of each group. Data are shown as the mean $\pm$ SD. *P < 0.01 vs. CON group rats; #P < 0.01 vs. DCM group rats; \$P < 0.01 vs. low-EMPA group rats.

incidence of cardiac dysfunction and heart failure in diabetic patients. DCM is characterized by diastolic and systolic dysfunction, and hyperglycemia. In the current study, a STZ-induced DCM model was successfully established, and the blood glucose level and cardiomyocyte function were detected. This is the most widely used and accepted way for establishing a diabetic model, consistent with that used in previous studies [19, 22]. The combination of STZ and a high-fat diet is particularly suitable for exploration of the pathophysiology of DCM [23]. In the current study, we determined whether or not EMPA could improve cardiac function and alleviate cardiomyocyte apoptosis in DCM rats. Additionally, the potential mechanism associated with this process was also discussed.

EMPA, an SGLT2 inhibitor, is a new anti-diabetic drug and offers a novel approach for the treatment of T2DM by enhancing urinary glucose excretion. In the present study, the blood glucose level was significantly reduced by EMPA, which is consistent with the results of others [24]. Moreover, we showed that DCM rats treated with EMPA (low- or high-dose) had improved cardiac function by analysing the LVSP, LEDVP, and $\pm dp/dt$. Additionally, the level of cardiomyocyte apoptosis was also alleviated by EMPA.

Recent studies have shown that reactive oxygen species (ROS) are associated with cardiac function in DCM during exercise or treatment with exogenous hydrogen sulfide, which can suppress high glucose-induced cardiomyocyte apoptosis [25-27]. ERS is one of the mechanisms contributing to ROS-mediated cell apoptosis [28]. A previous study suggested that hyperglycemia-associated ERS is important in the DCM [29], and EMPA can reduce the blood glucose level [10, 11]. Thus, we further carried out experiments to determine whether or not EMPA achieves cardioprotection through an ERS-associated mechanism in DCM. GRP78, a molecular chaperone in the ER, plays a critical role in recognizing the abnormal proteins. In the current study, GRP78 is involved in ERS in DCM rats and EMPA can down-regulate the expression of GRP78. GRP78 also serves as a main modulator for UPR via binding to three transmembrane proteins of ER (protein-kinase-RNA-like ER kinase [PERK], inositol-requiring 1 [IRE1], and activating transcription factor 6 [ATF6]) [30]. Once activated, IRE1 can induce splicing of X-box-binding protein mRNA. The spliced protein functions as a transcription factor, which in turn has an effect on the ERS gene, GRP78 [31, 32]. Our results showed that the level of XBP1 mRNA is increased in DCM rats and decreased after administration of EMPA. This result parallels the over-expression of GRP78.

CHOP is the downstream protein of the apoptotic pathway, which plays a vital role in ERS-induced apoptosis. CHOP can be activated by the over-transcription of ATF4, TRAF2, and XBP1 [33, 34]. Our results indicated that CHOP is significantly overexpressed in DCM rats compared to CON rats. The up-expression of CHOP in DCM rats can be significantly inhibited by low- or high-dose EMPA. Moreover, the high-dose EMPA group exhibited a better

inhibitory effect than the low-dose EMPA group. Once CHOP is triggered, CHOP contributes to the increased expression of pro-apoptotic factors, such as death receptor 5 (DR5) [35], tribbles-related protein 3 (Trb3) [36], and Bim, and CHOP can suppress Bcl-2 expression [37].

Caspase-12-mediated apoptosis is a specific apoptosis pathway of ER, which is independent of mitochondria or death receptor activation. Once caspase-12 is activated, CHOP can directly trigger the caspase protein in the cytosol, including caspase-3 and -9 [38]. In the current study, caspase-12 also was shown to take part in ERS in DCM rats. Pro-caspase-12 was significantly decreased and cleaved caspase-12 (activated caspase-12) was up-regulated in DCM rats compared with CON rats. Activated caspase-12 could be blocked by the administration of EMPA, which showed a similar dose-dependent relationship as the expression of CHOP. The activation of TRAF2 is considered to be a biomarker of ERS, which can activate caspase-12 [39]. TRAF2-JNK is another pathway involved in ER-associated apoptosis [40]. The up-regulated level of TRAF2 mRNA might induce the activation of caspase-12 and the JNK apoptotic pathway. In the current study, EMPA inhibited ERS via down-regulation of TRAF2 directly or indirectly, which was in parallel with changes in GRP78, CHOP, and cleaved caspase-12. Furthermore, the increased induction of those markers in DCM rats were also in parallel with the deterioration in cardiac function and aggravation of cardiomyocyte apoptosis. Thus, our results imply that the protective function of EMPA may be induced by inactivation of the apoptotic pathway of ERS via the expression of CHOP, caspase-12, and the JNK pathway in DCM rats.

Liu and colleagues [41] indicated that the glucagon-like peptide-1 (GLP1) analog, liraglutide, could improve cardiac function significantly and decrease the expression of GRP78, CHOP, and caspase-12, and the transcription of ATF4, TRAF2, and XBP1 [41]. It is known that GLP1 can be up-regulated by EMPA with the doses administered in the current study. Therefore, it is likely that EMPA might improve cardiac function in DCM due to ERS because of the increased level of GLP1. Additionally, Ferrannini et al. [42] concluded that EMPA achieves cardioprotection through mild, but persistent hyperketonemia, which prevails during treatment with SGLT2 inhibitors and can improve the transduction of oxygen consumption into work efficiency in the endangered myocardium. Thus, EMPA may improve function of the myocardium in DCM via several mechanisms, and future studies are needed to demonstrate the hypothesis.

In conclusion, the present study suggests not only that EMPA is capable of down-regulating a high glucose level in DCM rats, but also can alleviate cardiomyocyte apoptosis by inhibiting the three ERS-induced apoptotic pathways, which in turn improve heart function. Therefore, EMPA may represent a novel therapeutic method for DCM.

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Disclosure Statement

No conflict of interest associated with this work.

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