Empagliflozin Protects Cardiac Mitochondrial Fatty Acid Metabolism in a Mouse Model of Diet-Induced Lipid Overload

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Abstract

Purpose Sodium-glucose cotransporter 2 (SGLT2) inhibitors prevent heart failure and decrease cardiovascular mortality in patients with type 2 diabetes. Heart failure is associated with detrimental changes in energy metabolism, and the preservation of cardiac mitochondrial function is crucial for the failing heart. However, to date, there are no data to support the hypothesis that treatment with a SGLT2 inhibitor might alter mitochondrial bioenergetics in diabetic failing hearts. Thus, the aim of this study was to investigate the protective effects of empagliflozin on mitochondrial fatty acid metabolism.

Methods Mitochondrial dysfunction was induced by 18 weeks of high-fat diet (HFD)-induced lipid overload. Empagliflozin was administered at a dose of 10 mg/kg in a chow for 18 weeks. Palmitate metabolism in vivo, cardiac mitochondrial functionality and biochemical parameters were measured.

Results In HFD-fed mice, palmitate uptake was 1.7, 2.3, and 1.9 times lower in the heart, liver, and kidneys, respectively, compared with that of the normal chow control group. Treatment with empagliflozin increased palmitate uptake and decreased the accumulation of metabolites of incomplete fatty acid oxidation in cardiac tissues, but not other tissues, compared with those of the HFD control group. Moreover, empagliflozin treatment resulted in fully restored fatty acid oxidation pathway-dependent respiration in permeabilized cardiac fibers. Treatment with empagliflozin did not affect the biochemical parameters related to hyperglycemia or hyperlipidemia.

Conclusion Empagliflozin treatment preserves mitochondrial fatty acid oxidation in the heart under conditions of chronic lipid overload.

Keywords Empagliflozin · Fatty acid oxidation · Mitochondria · Heart

Introduction

Sodium-glucose cotransporter 2 (SGLT2) inhibitors are the newest class of antihyperglycemic agents that have been shown to prevent heart failure and decrease cardiovascular mortality in patients with type 2 diabetes [1]. Importantly, these studies have suggested that the observed cardioprotective effects of SGLT2 inhibitor therapy cannot be fully explained by their antidiabetic action. Various hypotheses on the cardioprotective mechanisms of SGLT2 inhibitor action have been proposed (reviewed in [2]): diuretic action, decreased blood pressure, anti-inflammatory effects, mediation through the renin-angiotensin-aldosterone system, restoration of mitochondrial calcium handling, and enhanced production of ketone bodies (“thrifty substrate” hypothesis). Growing number of findings indicate that the mechanism of SGLT2 inhibitor action involves also modulation of mitochondrial dynamics (biogenesis, fission, and fusion) (reviewed in [3]). However, the current knowledge about the potential impact of SGLT2 inhibitors on cardiac mitochondrial bioenergetics is limited.

In a recent study, the SGLT2 inhibitor empagliflozin was shown to improve the cardiac energy pool in diabetic hearts by increasing glucose and fatty acid oxidation but not ketone
oxidation [4]. Moreover, it has been shown that in nondiabetic hearts after myocardial infarction, empagliflozin treatment restores glucose and fatty acid metabolism-related gene expression, and these changes are associated with stimulated mitochondrial biogenesis and reduction of mitochondrial DNA damage [5]. In addition, treatment with empagliflozin normalized the size and number of mitochondria in cardiomyocytes in a rat model of type 2 diabetes [6]. Overall, the available data indicate that treatment with empagliflozin could improve mitochondrial function and metabolic flexibility in the diabetic heart. The conclusions about improved mitochondrial function after treatment with SGLT2 inhibitors came from previous studies that mainly investigated processes of ionic homoeostasis and mitochondrial dynamics [6–10]. However, to date, there are no data demonstrating that treatment with empagliflozin or other SGLT2 inhibitors would alter mitochondrial bioenergetics in diabetic hearts. Because disturbances in mitochondrial energy metabolism play a major role in the pathogenesis of diabetes-induced heart failure [11], the aim of the present study was to evaluate for the first time the cardiac mitochondrial functionality after treatment with the SGLT2 inhibitor empagliflozin, with a focus on fatty acid oxidation, in a model of high-fat diet-induced insulin resistance.

Materials and Methods

Animals and Treatment

Twenty-four male C57bl/6N mice (7 weeks old, Envigo, Netherlands) were housed under standard conditions (21–23 °C, relative humidity 50% ± 10%, 12 h shifted light-dark cycle) with unlimited access to food and water. The experimental procedures were carried out in accordance with the guidelines of the European Community (2010/63/EU) and local laws and policies and were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia. These studies are reported in accordance with the ARRIVE guidelines. Mice were adapted to local conditions for 2 weeks before the start of treatment and were housed in individually ventilated cages with softwood bedding. The control group (n = 8 with an average body weight of 29.0 ± 0.9 g) received a standard diet (R70 diet, Lantmännen, Sweden) for 18 weeks. For induction of insulin resistance, the remaining animals were randomly divided into 2 groups (n = 8 per group, with an average body weight of 29.0 ± 0.6 g) that received a high-fat diet (HFD) (21% fat and 0.15% cholesterol, Western RD, Special Diets Services, UK) or HFD with empagliflozin (dose 10 mg/kg in the chow) for 18 weeks. The dose of empagliflozin of 10 mg/kg was chosen based on the data from previously published pre-clinical studies [4, 7, 10]. At the end of the treatment, the animals were then fasted overnight and sacrificed by decapitation, and samples of plasma and tissues were collected and immediately used for the assessment of mitochondrial functionality or stored at −80 °C until analysis.

Measurements of Palmitate Uptake and Oxidation In vivo

After 18 weeks of treatment, for analysis of the palmitate uptake in vivo, mice were fasted overnight, and 1 μCi of [9,10-3H]palmitate (specific activity, 60 Ci/mmol) per mouse was administered subcutaneously. After 10 min, the mice were sacrificed by decapitation, and tissue homogenates (1:5, w/v in water) were prepared. For determination of the accumulated metabolite ratio, the samples were treated as previously described [12]. The detailed description of method is included in the Supplementary Material.

Mitochondrial Functionality in Permeabilized Cardiac Fibers

The mitochondrial functionality was determined in permeabilized cardiac fibers. Mitochondrial respiration measurement was performed at 37 °C using Oxygraph-2 k (O2k; Oroboros Instruments, Innsbruck, Austria) with MiR05 (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM MgCl2, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, pH 7.1, 0.1% BSA essentially free of fatty acids). ADP was added to a concentration of 5 mM to initiate oxidative phosphorylation-dependent respiration (OXPHOS state) with endogenous substrates. Afterwards, palmitoylcarnitine, carnitine, and malate (10 μM, 700 μM, and 0.5 mM, respectively) were added to measure fatty acid oxidation-dependent mitochondrial respiration (F(N)-pathway). Pyruvate (5 mM, complex I substrate, N-pathway) was then added to reconstitute FN-pathway-linked respiration. Glutamate (10 mM) was added as an additional substrate for the N-pathway. Succinate (10 mM, complex II substrate, S-pathway) was added to reconstitute convergent FOS-linked respiration. Titration with the uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) at 0.5-μM stepwise increases was performed to determine the electron transfer system (ETS) capacity. Then, rotenone (0.5 μM, inhibitor of complex I) and antimycin A (2.5 μM, inhibitor of complex III) were added to determine the S-linked ET capacity and residual oxygen consumption (ROX), respectively. The respiratory rate was corrected for ROX. For determination of the contribution of each substrate to the respiration rate, the flux control factor was calculated as follows:

\[
1 - \frac{\text{Resp. rate before the addition of substrate}}{\text{Resp. rate after the addition of substrate}}
\]
For determination of mitochondrial mass in the heart, citrate synthase activity in tissue homogenate was measured spectrophotometrically.

**Determination of Biochemical Measures**

Plasma insulin concentrations were determined with a RIA kit (Millipore, Billerica, USA). Glucose and triglycerides in plasma samples were measured by Instrumentation Laboratory (Milan, Italy) enzymatic kits. Plasma fatty acids and β-hydroxybutyrate were measured by Wako (Neuss, Germany) and Biosystems S.A (Barcelona, Spain) enzymatic kits. In addition, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated according to the formula:

\[
\frac{\text{fasting insulin (mU/L) \times fasting plasma glucose (mg/dL)}}{22.5}
\]

To perform glucose tolerance test, mice were fasted overnight. Then, a glucose solution at dose 0.5 g/kg of body weight was administrated intraperitoneally, and blood glucose concentration was measured at 0 (fasting), 15, 30, 60, and 240 min using Accu-Chek Instant blood glucose meter and strips.

**Statistical Methods**

Data are presented as the mean ± SD. Statistically significant differences in the mean values were evaluated using a one-way ANOVA with Tukey’s post-test. The differences were considered significant when \( P < 0.05 \). The data were analyzed using GraphPad Prism software (GraphPad, Inc., La Jolla, USA).

**Results**

**Fatty Acid Metabolism**

To determine the effect of empagliflozin treatment on fatty acid metabolism, we evaluated \(^3\text{H}\)-palmitate uptake and oxidation in the tissues in vivo. As shown in Fig. 1a, in HFD-fed mice, the palmitate uptake in the heart, liver, and kidneys was 1.7, 2.3, and 1.9 times lower, respectively, than that in the normal chow control group. Compared with the HFD control group, the empagliflozin-treated group showed significantly increased palmitate uptake by 35% in the heart but not in other tissues. Moreover, the palmitate uptake was comparable in empagliflozin-treated and normal chow control group hearts. To characterize fatty acid oxidation, we calculated the percentage of acid-soluble \(^3\text{H}\)-palmitate metabolites of total \(^3\text{H}\)-palmitate taken up into tissues. In HFD-fed mouse hearts, the percentage of acid-soluble metabolites was 1.8 times higher than that in normal chow group hearts, indicating incomplete fatty acid oxidation in the cardiac mitochondria (Fig. 1b). Empagliflozin treatment significantly reduced the percentage of acid-soluble \(^3\text{H}\)-palmitate metabolites in cardiac tissues compared with that in the HFD-treated group (Fig. 1b). These results indicate that empagliflozin specifically affects heart energy metabolism and, by preserving fatty acid oxidation, protects against HFD-induced disturbances in cardiac metabolism.

**Mitochondrial Functionality**

To further investigate the observed effects of HFD and empagliflozin on palmitate uptake and oxidation, we assessed mitochondrial functionality in cardiac tissues from all experimental groups. The cardiac F(N)-pathway-dependent respiration rate (using palmitoylcarnitine as a substrate) was significantly decreased by 59% in the HFD control group compared with the normal chow group (Fig. 2a). Empagliflozin

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**Fig. 1** Empagliflozin (Empa) treatment-induced effects on the fasted state \(^3\text{H}\)-palmitate uptake (a) and percentage of acid-soluble \(^3\text{H}\)-palmitate metabolites (b). Each value represents the mean ± SD of 6–7 (a) or 4–6 (b) animals. *Significantly different from the normal chow control group, #Significantly different from the HFD control group (ANOVA followed by Tukey’s test, \( P < 0.05 \))
treatment fully restored F(N)-pathway-dependent respiration in permeabilized cardiac fibers to the level of the normal chow group. Although pyruvate metabolism was upregulated in the HFD control level, as indicated by the flux control factor (Fig. 2b), the FN-pathway-linked respiration rate in the OXPHOS state was decreased compared with that of the normal chow group. Moreover, HFD induced a significant impairment in glutamate and succinate metabolism (Fig. 2b) and decreased FN- and FNS-pathway-linked respiration rates in the OXPHOS and ET states (Fig. 2a). The analysis of flux control factors showed that empagliflozin only had a tendency to restore the S-pathway, but not other pathways (Fig. 2b), indicating that restored respiration rate observed in empagliflozin-treated hearts (Fig. 2a) is related to preserved F-pathway. The measurements of cardiac citrate synthase activity, as a marker of mitochondrial mass, demonstrated that there was no significant difference between the groups (data not shown). Overall, these results show that empagliflozin treatment normalizes mitochondrial function in the heart, mainly due to an increase in fatty acid oxidation.

**Biochemical Parameters**

The entire data set on biochemical parameters, glucose tolerance, and body weight gain are included in the Supplementary Material. Lipid overload induced by HFD diet for 18 weeks resulted in a significant increase in fasting plasma glucose, insulin, and β-hydroxybutyrate concentrations by 30% (7.0 ± 1.6 vs 10.0 ± 1.6 mM), threefold (3.0 ± 1.9 vs 10.4 ± 3.8 ng/ml), and 1.8 times (0.37 ± 0.23 vs 0.66 ± 0.28 mM), respectively, when compared with normal chow control. Moreover, in HFD-fed control group, glucose tolerance was significantly impaired as well as HOMA-IR was significantly higher when compared with normal chow control. In HFD-fed control group, mice triglyceride concentration was increased by 42% (0.38 ± 0.21 vs 0.54 ± 0.09 mM), while the concentration of nonesterified fatty acids was not affected (1.10 ± 0.32 vs 1.01 ± 0.24 mM). Treatment with empagliflozin reduced a HFD-induced increase in β-hydroxybutyrate concentration (Table S1) and slightly decreased insulin concentration (8.2 ± 4.8 ng/ml). The glucose tolerance and HOMA-IR values after treatment with empagliflozin were comparable with HFD-fed control group values (Supplementary Fig. S1, Table S1).
Table S1). Moreover, empagliflozin had no effect on body weight gain and hyperglycemia- and hyperlipidemia-related measures compared with HFD-fed control group (Supplementary Fig. S1, Table S1).

**Discussion**

The most distinct effect of SGLT2 inhibitors observed in clinical studies is a delay in the progression of heart failure [13]; however, the mechanism of this effect is still unclear. It has been proposed that SGLT2 inhibitors enhance cardiac efficiency via improving ATP generation from ketone body oxidation [14]. However, a recent study by Verma et al. [4] showed that the beneficial effects of empagliflozin on diabetic heart function are most likely due to the normalization of glucose and fatty acid oxidation. In a murine obesity model, increased fatty acid availability in combination with impaired fatty acid oxidation ultimately leads to contractile dysfunction [15]. Consistent with these findings, we demonstrate that treatment with empagliflozin preserves fatty acid oxidation in lipid overload-damaged heart mitochondria. Moreover, further analysis of mitochondrial functionality demonstrated that the preserved fatty acid metabolism is associated with overall improvement of cardiac mitochondrial oxidative phosphorylation capacity. These data indicate that treatment with empagliflozin protects cardiac mitochondria and preserves fatty acid utilization instead of stimulating oxidation of ketone bodies in the heart.

Several investigators suggested that SGLT2 inhibitors augment AMPK phosphorylation [16–18]. However, previous study addressing this issue in cardiac tissues shows that empagliflozin treatment does not affect AMPK pathway [7]. Moreover, a recent study in skeletal muscles showed that empagliflozin-treatment preserves fatty acid oxidation without affecting AMPK pathway [19]. Importantly, AMPK pathway regulates fatty acid metabolism by affecting CPT1 activity, while in our study, we investigated the effects of empagliflozin on fatty acid oxidation downstream of CPT2 using palmitoylcarnitine as a substrate; and metabolism of palmitoylcarnitine is not regulated by CPT1 activity. Thus, previously reported effect of SGLT2 inhibitors on AMPK phosphorylation cannot explain the improvement of mitochondrial fatty acid oxidation observed in our study.

The recently demonstrated inhibition of cardiac sodium hydrogen exchanger (NHE) by empagliflozin has been suggested as an additional mechanism of action [20]. By inhibiting NHE activity, empagliflozin reduces cardiac cytosolic Na⁺ and Ca²⁺ concentrations and subsequently enhances mitochondrial Ca²⁺ concentration [8]. This effect has been previously linked to normalization of glucose metabolism in diabetic heart [4] and possible stimulation of Krebs cycle dehydrogenases. Since changes in mitochondrial Ca²⁺ regulate the fatty acid oxidation rate [21], we can speculate that the observed increase in mitochondrial fatty acid oxidation in this study might also be related to the previously observed increase in mitochondrial Ca²⁺ concentration.

Fatty acid metabolism in mitochondria is the main energy source of the heart, and the inability to generate energy from fatty acids has long been considered the primary mechanism linking mitochondrial dysfunction with cardiac dysfunction. In failing hearts, reduced fatty acid oxidation in mitochondria is initially compensated by an increase in glucose oxidation [22]. Further progression of mitochondrial dysfunction results in disturbances related to glucose oxidation that lead to increased glycolysis, which is uncoupled from oxidation. Our results indicate that treatment with empagliflozin protects mitochondria against dietary lipid overload-induced damage and helps to maintain fatty acid metabolism. In addition, the benefit of sustained fatty acid oxidation likely extends beyond the energy supply. Thus, given the important role of mitochondrial fatty acid metabolism in cardiac energy production, empagliflozin treatment-induced effects on mitochondrial metabolism could be a critical mechanism for the prevention of heart failure development.

Several limitations of the present study should be mentioned. First, HFD-induced lipid overload is a model to induce cardiac metabolic disturbances, but it is not a typical model of cardiac dysfunction; therefore, the cardiac mechanic function was not monitored. Thus, we cannot directly link empagliflozin-induced protection of mitochondrial fatty acid oxidation with better preserved cardiac function in our experimental model. Nevertheless, previous studies have confirmed that lipid overload, as a result of impaired fatty acid oxidation, is closely linked to cardiac dysfunction [15, 23], and stimulation of fatty acid metabolism is beneficial for the improvement of function in insulin-resistant heart (reviewed in [11]). Since facilitated fatty acid oxidation is beneficial for preservation of failing heart function, further studies need to be conducted to investigate the effect of empagliflozin treatment on energy metabolism in experimental models of heart failure. Second, in our study, we used only carnitine palmitoyltransferase 2 (CPT2)-dependent substrate, palmitoylcarnitine, to study the effect of empagliflozin treatment on mitochondrial fatty acid oxidation.

In conclusion, the present study demonstrates that treatment with empagliflozin protects against high-fat diet-induced dysfunction of cardiac mitochondria. The direct beneficial effect of empagliflozin treatment in mitochondria is due to the increased efficiency of fatty acid oxidation and the subsequent improvement of the fatty acid intermediate clearance and overall mitochondrial bioenergetics in the heart (Fig. 2c). Since treatment with empagliflozin did not affect hyperinsulinemia, hyperglycemia, or hyperlipidemia, we can conclude that empagliflozin-induced protection of mitochondrial metabolic functions in the heart is not associated with a
general improvement of metabolic status of animals and the observed effects are heart specific. Overall, these findings provide a basis for a novel mechanism of empagliflozin action.

**Availability of Data and Materials** The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Author Contributions** M.M.-K., M.D., and E.L. designed the research. M.M.-K., S.K., M.V., K.V., H.C., and J.K. conducted experiments. M.M.-K., M.D., and E.L. analyzed and interpreted the data. M.M.-K. wrote the manuscript. The study was supervised by M.M.-K., M.D., and E.L. All authors read and approved the final manuscript.

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**Compliance with Ethical Standards**

**Conflict of Interests** The authors declare that they have no conflict of interest.

**Ethics Approval and Consent to Participate** The experimental procedures involving animals were performed in accordance with the guidelines of the European Community and local laws and policies, and all of the procedures were approved by the Food and Veterinary Service, Riga, Latvia.

**Abbreviations** CCCP, Carbonyl cyanide m-chlorophenyl hydrazine; ETS, Electron transfer system; F-pathway, Fatty acid oxidation-dependent pathway; HDF, High-fat diet; N-pathway, NADH-dependent pathway; NHE, Sodium hydrogen exchanger; OXPHOS, Oxidative phosphorylation; ROX, Residual oxygen consumption; SGLT2, Sodium-glucose cotransporter 2; S-pathway, Succinate-pathway

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