SGLT2 independent effects of the SGLT2 inhibitor empagliflozin

Focus on the heart

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Chapter 01

General introduction

Introduction

Heart failure

The escalating prevalence of heart failure (HF) poses a global health challenge, impacting over 64 million individuals[1]. HF, characterized by dyspnea, compromised ventricular function, and exertional limitations, results in significant morbidity and mortality. The one-year mortality rate for chronic HF is 7.2% and one-year hospitalization rate is 31.9% , escalating to 17.4% and 43.9% for acute cases[2]. HF is classified by ejection fraction (EF) of left ventricle, distinguishing between HF with reduced EF (HFrEF), mildly reduced EF (HFmrEF) and preserved EF with EF≤ 40%, 40%<EF<50% and EF≥50% respectively[1,3]. Guideline-directed therapy for HFrEF focuses on the utilization of diuretics to alleviate symptom and implementing beta-blocker, angiotensin receptor blocker/neprilysin inhibitor or mineralocorticoid receptor antagonist, aimed at reducing hospitalizations, all-cause mortality, and cardiovascular mortality[2,4]. Despite advancements, a plateau in survival improvement prompts exploration of novel interventions[2]. However, HFpEF and HFmrEF present diagnostic challenges, and standard medications show limited effectiveness[1]. Clinical trials with sacubitril-valsartan/candesartan and spironolactone report modest effects on cardiovascular outcomes[5]. Recent breakthroughs highlight sodium glucose cotransporter 2 inhibitors (SGLT2i) as incremental contributors, reducing HF development and progression. The EMPEROR-Reduced trial demonstrates empagliflozin's (EMPA) efficacy in improving renal outcomes and reducing cardiovascular death in HFrEF, irrespective of diabetes status[6]. Similarly, the EMPEROR-Preserved trial shows that EMPA reduces cardiovascular risks in patients with HFpEF and HFmrEF, irrespective of diabetes. As a consequence, SGLT2i have now become part of the first-line therapy in HF[5].

Sodium glucose cotransporter 2

Sodium glucose cotransporter 2 (SGLT2) exhibits a distinctive distribution characterized by a high-capacity and low-affinity cotransporter mainly localized along the early segments of the proximal renal tubule[7,8]. Approximately 90% of filtered glucose is reabsorbed by SGLT2 situated in the S1 segments of the proximal tubule , thereby precipitating unique renal effects[7,9]. The inhibition of SGLT2 contributes to increased glucose excretion, resulting in reduced plasma glucose, which underscores the rationale for classifying SGLT2i as ‘antidiabetic drugs’[7]. Furthermore, these pharmacological agents induce natriuresis and osmotic diuresis together with glycosuria, thereby exhibiting characteristics similar to diuretics with a ’hybrid’ mechanism[7]. Additionally, SGLT2i lead to an elevation in sodium concentration at the macula densa, facilitating enhanced sodium influx into the cell and subsequent elevation of its osmolarity. Consequently, there is an amplification in the conversion of ATP to adenosine, which can result in afferent arterioles to constrict through tubule glomerular feedback together with a simultaneous decrease in the glomerular filtration rate. This cascade is significant as it attenuates the hyperfiltration of glomerular, the

pressure of intraglomerular, and consequent proteinuria, thereby decelerating the progression of nephropathy[10].

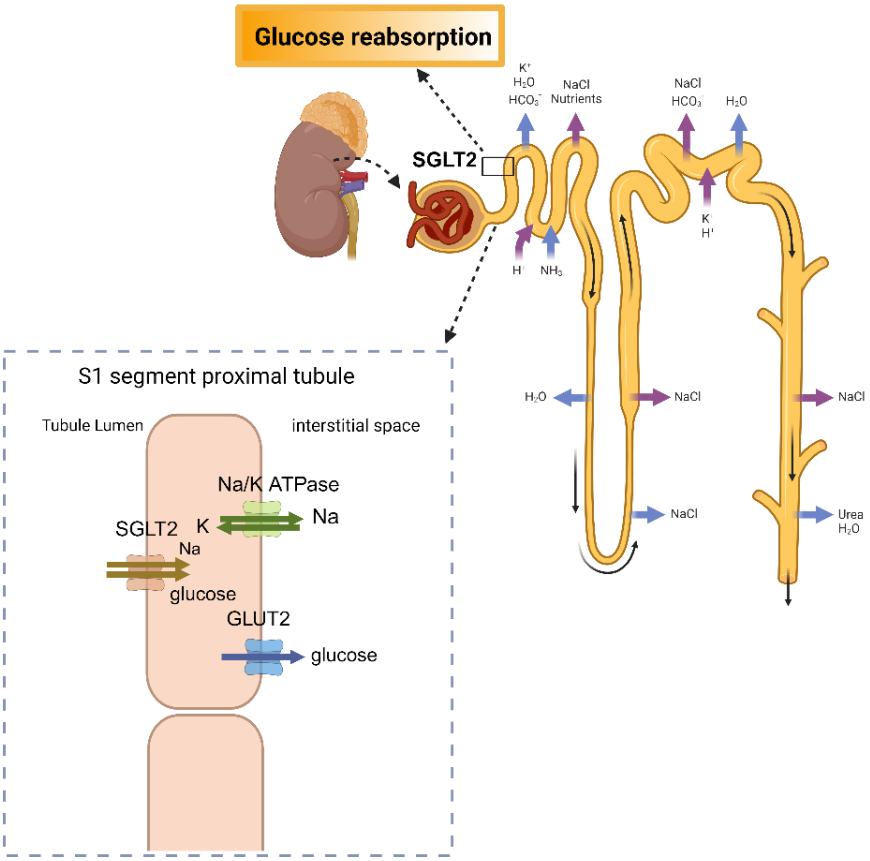

Figure 1. Glucose reabsorption in proximal tubule in kidney. Around 90% glucose is reabsorpted by SGLT2 from glomerular into epithelial cells in segment 1 of the proximal tubule. Then glucose is subsequently transported into the interstitial fluid through the glucose transporter 2 (GLUT2)[8]. (Created with BioRender.com)

Although initially developed for type 2 diabetes, SGLT2i exhibit substantial benefits in reducing cardiovascular disease risk, particularly in HF. Revised guidelines advocate their incorporation alongside optimal medical therapy for HF[11,12]. While their primary mechanism involves supressing sodium and glucose reabsorption back into blood in the kidney, the prompt reduction in cardiovascular and renal adverse events post-initiation and their persistence imply mechanisms beyond glycemic control, because improvements in glycemic control alone would take years to manifest measurable cardiovascular beneficial effects[8,11]. Therefore, the scientific interest in

investigating the underlying actions of EMPA independently of diabetes has grown over the last years[8,11]. The main questions are to what extent the targeted protein, SGLT2, is actually needed for SGLT2i to provide cardiovascular protection, and which other off-target mechanisms may contribute to the observed cardiovascular protection. This thesis addresses these questions.

Ischemia/reperfusion injury

In this thesis the focus is mainly on understanding SGLT2i's effects on the healthy and diseased myocardium in *ex vivo* and *in vivo* conditions. However, the direct impact of SGLT2i on the myocardium remains questionable, given that SGLT2 is not existed in myocardium from human and rodent[13,14]. In the realm of translational research, SGLT2i holds promise as a valuable extension to the arsenal for secondary prevention of acute myocardial injury, as EMPA has been found that it may help mitigate myocardial cell death resulting from I ischemia/reperfusion (I/R) injury[15]. In our own laboratory we were one of the first demonstrating that EMPA affected the isolated ischemic mouse heart directly by delaying the development of ischemic contracture[16]. Besides, Lu et al. demonstrate that the contractility of hypoxic cardiomyocyte and the recovery of heart after ischemia in an *ex vivo* heart perfusion set-up are improved and *in vivo* myocardial infarct size is reduced, with EMPA treatment. These off-target effects on heart disease could be due to the activation of AMP-activated protein kinase (AMPK)[17]. Additionally, acute dapagliflozin (DAPA) administration during cardiac I/R injury reduces cardiac infarct size and increases left ventricle (LV) function in non-diabetic male Wistar rats[18]. Furthermore, Yu et al. demonstrate that a high-dose DAPA pretreatment significantly reduces infarct size and decreases the serum levels of creatine kinase-myoglobin binding (CK-MB), troponin I, and lactate dehydrogenase (LDH) in non-diabetic mice myocardial I/R model[19]. Nikolaoue et al. have provided evidence that in non-diabetic mice, chronic administration of EMPA and DAPA reduce infarct size, while cardioprotection by the SGLT2i is not correlated to SGLT2 inhibition in the kidney. The cardiac protection by SGLT2i is dependent on the phosphoinositide 3-kinase (PI3K) and signal transducer and activator of transcription 3 (STAT3), and is associated with increased caveolin-3 (Cav-3) expression and fibroblast growth factor 2 (FGF2)[15,20]. Collectively, this body of evidence demonstrates that SGLT2i can protect against I/R injury, independent of SGLT2 inhibition.

Ion Regulation

The sodium hydrogen exchanger 1 (NHE1) has emerged as the first identified off-target cardiac mechanism of SGLT2i[21]. Studies have demonstrated that NHE1 activity is inhibited by SGLT2i in isolated cardiomyocytes from mice and rabbits [22]. Furthermore, Trum et al. have provided evidence of EMPA's ability to inhibit NHE1 in both diseased human cardiomyocytes and healthy mouse cardiomyocytes, with a level of inhibition comparable to that of the specific NHE1 inhibitor cariporide[23]. Additionally,

EMPA has been shown to mitigate angiotensin II-induced hypertrophy by suppressing NHE1 expression[24]. Lin et al. have demonstrated that DAPA exerts direct protection against saturated fatty acid (FA)-triggered cardiomyocyte injury through an NHE1-dependent mechanism, and confirmed that DAPA can directly bind NHE1 protein in cardiomyocytes[25]. Moreover, Kim et al. observed a suppression of NHE1 expression levels and activities by SGLT2i in lipopolysaccharide (LPS)-treated macrophages[26]. In the context of vascular dysfunction, EMPA has shown efficacy in improving inflammation and stretch-induced barrier dysfunction of human coronary artery endothelial cells (HCAECs), mediated by NHE1 inhibition[27,28]. Despite consistent documentation of NHE1 inhibition by SGLT2i across various cell types[25,28-33], conflicting results have been reported in one study[34]. Studies suggest that enzymes utilized in cell isolation may compromise channel proteins which are embedded in the cell membrane. Specifically, serine proteases such as protease XIV and XXIV have been identified as contributors to the degradation of the hERG1 potassium channel and current in both cardiomyocytes and HEK cells[35]. Hence, the careful selection of isolation techniques is crucial, as it significantly impacts the integrity and functionality of membrane proteins, including those targeted by pharmaceutical agents such as SGLT2i. Overall, the modulation of NHE1 activity merges as a promising therapeutic target for SGLT2i in addressing cardiovascular disorders, emphasizing the need for further research to clarify the underlying mechanisms and clinical implications of their off-target effects on cardiac function and the cause of conflicting results need to be explored.

Mechanically, inhibition of the NHE1 by SGLT2i can result in a reduction in intracellular Na^+ and Ca^{2+} levels. The decrease of intracellular Na^+ leads to a secondary rise in intracellular Ca^{2+} via the sodium calcium exchanger (NCX). Studies provide evidence that reductions in intracellular Na^+ and intracellular Ca^{2+} mitigate signaling cascades that eventually cause mitochondrial homeostasis to be less dysregulated, including enhanced energetics, reduced ROS production and enhanced NO presence, ultimately resulting in improvement of cardiac hypertrophy and remodeling[22,36,37]. Kim et al. also have observed that DAPA or EMPA suppress the expression levels of NCX, consequently downregulating PI3K/Protein kinase B (AKT)/rapamycin complex 1 (mTORC1) signaling and glycolysis in LPS-treated macrophages[26]. Besides, Wijnker et al. show that SGLT2i alters sodium calcium exchange current in mutant human induced pluripotent stem cell-derived cardiomyocytes[38]. In addition, a decrease in intracellular Ca^{2+} concentrations can decrease the activity of Ca^{2+}/calmodulin-dependent kinase II (CaMKII), and a decrease in ROS production. It has been demonstrated that EMPA is able to reduce the activity of CaMKII and sequentially decrease sarcoplasmic reticulum Ca^{2+} leak in ventricular myocytes from human and murine[39]. Lastly, it has been demonstrated that 1 μM EMPA reverts HFpEF-induced upregulation od late sodium current [40]. The downstream effects of NHE1 inhibition on intracellular ion homeostasis and cellular signaling provide a compelling rationale for the therapeutic use and effects of SGLT2i in cardiovascular disease management.

Vascular regulation

The endothelium serves as a continuous barrier, intimately engaging with nearly every bodily system to regulate vascular development, homeostasis, and pathogenesis. Its tendency to exhibit endothelial dysfunction, often an early hallmark of various cardiovascular diseases (CVD), marks it as a harbinger of future cardiovascular outcomes[41]. Synthesized by nitric oxide synthase (NOS), nitric oxide (NO) plays pivotal roles in the modulation of coronary blood flow together with myocardial contractility, especially in ischemic hearts[42]. NO can diffuse across vascular smooth muscle cell (VSMC) membranes to initiate the activation of the enzyme soluble guanylate cyclase (sGC), which facilitates the formation of cyclic guanosine monophosphate (cGMP). This cyclic nucleotide serves as the key mediator in triggering the activation of protein kinase G (PKG), which in turn leads to the phosphorylation of multiple cellular targets, thereby diminishing intracellular Ca^{2+} concentrations. Consequently, the principal outcome of activation of this cascade is the induction of vascular relaxation or vasodilation[43]. Besides, NO plays an important role in preserving vascular health. NO can regulate vascular tone to modulate blood pressure and blood flow and exert anti-inflammatory and antithrombotic effects via inhibiting platelet aggregation, leukocyte adhesion, and infiltration within the vessel wall. Additionally, NO inhibits VSMC proliferation and migration, as well as oxidative phosphorylation[44]. EMPA and DAPA have demonstrated the ability to restore NO bioavailability in TNFα-induced endothelial cells[45]. This evidence suggests that NO could serve as one of the end-effectors of SGLT2i's cardioprotective effects.

Antioxidant effect

Advancements in cardiovascular research have underscored oxidative stress as a critical pathophysiological pathway implicated in the development and progression of HF, marked by a dysregulation in the equilibrium between reactive oxygen species (ROS) production and the endogenous antioxidant defense system[46]. Uthman et al. have demonstrated that EMPA and DAPA effectively mitigate ROS generation, thereby restoring NO bioavailability[45]. Additionally, Li et al. showcase that the protective effects of DAPA and CANA against cyclic stretch-triggered increased permeability in human endothelial cells are likely through ROS inhibition[27]. Besides, Jhuo et al. reveal that EMPA significantly reduces mitochondrial ROS production and levels of the associated antioxidant marker, superoxide dismutase 1 (SOD1), in high-fat diet (HFD)-triggered metabolic obesity and disorder[47]. Acute intravenous administration of canagliflozin (CANA) following ischemia onset shields against myocardial I/R injury by reducing the pre-oxidant marker, 4-hydroxynonenal (4-HNE)[48,49]. In addition, phlorizin treatment mitigates another oxidative stress marker, 3-nitrotyrosine (3-NT) in Streptozotocin-induced diabetic kidney and EMPA significantly reduces some pathological oxidative parameters, such as H_2O_2, glutathione and lipid peroxide in cardiomyocyte cytosol and mitochondria[50,51]. Furthermore, in HFpEF, EMPA has been shown to attenuate oxidative stress and

thereby enhances NO/sGC/cGMP/PKGIα pathway, which causes pathological cardiomyocyte stiffness to reduce[51]. These findings collectively shed light on promising strategies to alleviate oxidative stress-related complications in cardiovascular and metabolic disorders by therapeutic application of SGLT2i.

Cardiac energy metabolism

SGLT2i have shown effects on metabolism[52]. Alterations in cardiac energy metabolism play a crucial role in the severity of HF, highlighting the modulation of cardiac metabolism as a promising clinical target[53]. Cardiac metabolism relies on the integration of the production and consumption pathways of ATP, with ATP serving as a pivotal energy provider and a primary energy source in the purinergic signaling pathway, garnering a lot of attention in recent research. Although no changes in cardiac PCr/ATP ratio were detected in HF patients treated with EMPA[54], cardiac ATP was increased in a preclinical mouse model treated with SGLT2i[55]. The SGLT2i increase blood ketone bodies (KB), suggesting potential improvements in abnormal skeletal muscle metabolism in HF, including enhanced fatty acid (FA) metabolism, glycolysis suppression, and increased utilization of KB for mitochondrial energy production, thus explaining their effectiveness and potential in HF management[56]. Studies by Levin et al. showed DAPA capacity to enhance glucose oxidation in all organs[57]. Additionally, CANA can inhibit glycolytic metabolism, exerting dose-dependent anti-proliferative effects in Huh7 and HepG2 cells[58]. In a non-diabetic in vivo model, EMPA is able to redirect myocardial fuel utilization from glucose toward free FA, KB, and branched-chain amino acids, thus enhancing myocardial energetics, improving LV systolic function, and mitigating LV remodeling in a non-diabetic porcine model[59]. However, it is possible that these results were a result of prolonged fasting in this porcine model, thereby increasing SGLT2i's effects on plasma KB levels and overestimating these metabolic effects when compared to non-fasted conditions. For example, no effects on KB were observed with SGLT2i in another porcine model[60]. In addition, both SGLT2-expressing or non-expressing cells exhibit consistent gene expression changes in metabolic genes following DAPA treatment[61]. Therefore, investigating the change of metabolism caused by SGLT2i becomes very important in

understanding the mechanisms of SGLT2i-mediated cardioprotection, especially for direct effect of SGLT2i on heart.

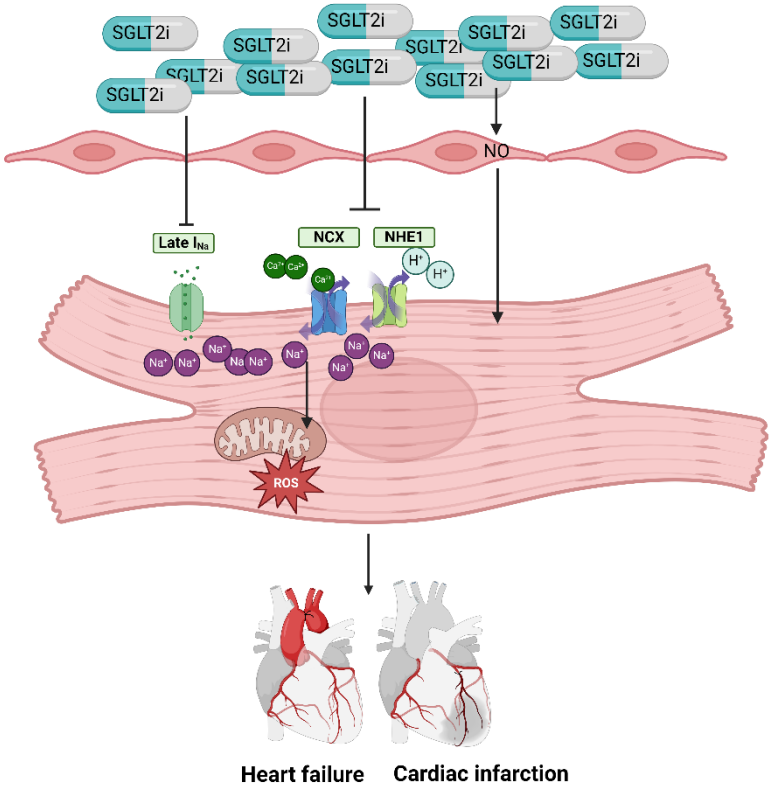

Figure 2 the direct effects of SGLT2i in cardiomyocytes and endothelial cells. In heart, SGLT2i can inhibit NHE1 on plasma membrane to change ion homeostasis in cytoplasm. Consequently, the oxidative stress in mitochondria is inhibited to mitigate HF and myocardial infarction. In endothelial cells, SGLT2i is able to increase NO production to mitigate heart dysfunction. (Created with BioRender.com)

Aims of this thesis

The general aims of this thesis are to answer the questions: 1) to what extent are SGLT2i empagliflozin protective effects mediated through inhibition of the SGLT2 protein, and 2) which cellular mechanism(s) within the heart are affected by EMPA, and may contribute to its cardioprotective effects. We firstly review the progressions

and limitations *in in vitro*, *ex vivo* and preclinical studies exploring the direct effects of SGLT2i's on cardiomyocytes, endothelial cells, fibrobasts, smooth muscle cells, isolated hearts and platelets, to provide more insight into potential underlying cellular mechanism of these drugs by direct cardiac effects (**Chapter 2**). Next, we have examined direct cardiac effects of EMPA on the metabolism of type 2 diabetic *ex vivo* murine hearts, and explore the role of NHE1 Inhibition (**chapter 3**). Then, we use these diabetic hearts to first explore direct cardiac effects of EMPA on the serine/threonine kinome, and explore discovered kinases in a rat cardiomyoblast hypertrophic model (**chapter 4**). Subsequently, in **chapter 5** we examine whether the use of a specific enzyme to isolate cardiomyocytes can explain divergent results obtained from different laboartories. Then we focus on the question whether SGLT2 protein is needed to obtain the cardioprotective effects of SGLT2 inhibition, and which other cellular mechanisms are affected by EMPA. To this end, we generate a global SGLT2 knock-out mice by Crisper/Cas9 technique and study whether EMPA-mediated cardioprotection against *in vivo* cardiac infarction is dependent on SGLT2 (**Chapter 6**). Furthermore, we use transverse aortic constriction (TAC) surgery plus Deoxycorticosterone Acetate (DOCA) pellet implantation to establish a combined HFmrEF and HFrEF mice model for exploring whether cardioprotection by EMPA on HF is dependent on SGLT2 and/or on the NHE1/NCX/oxidative stress/NO pathway (**Chapter 7**). Finally, we employ our TAC/DOCA model of HF to examine the role of empagliflozin and SGLT2 on mechanics, metabolomics and fluxomics of the isolated Langendorff-perfused mouse heart (chapter 8). In chapter 8 we provide a general discussion of the major obtained results, discuss the pros- and cons of our research projects, and make suggestions for future research.

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