

Amelioration of endothelial dysfunction by sodium glucose co-transporter 2 inhibitors: pieces of the puzzle explaining their cardiovascular protection

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Abstract

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Abstract (150 words)

Sodium glucose co-transporter 2 inhibitors (SGLT-2i's) significantly improve cardiovascular outcome in both diabetic and non-diabetic patients. Preclinical studies suggest that SGLT-2i's directly affect endothelial function in a glucose-independent manner. The effects of SGLT-2i's include reduction of oxidative stress and inflammatory reaction in endothelial cells. Furthermore, SGLT2i's have been shown to restore endothelial-related vasodilation and to regulate angiogenesis. The favorable cardiovascular effects of SGLT-2i's might be mediated via multiple pathways: 1) by inhibition of the overactive sodium-hydrogen exchanger; 2) by reduction of nicotinamide adenine dinucleotide phosphate oxidases expression; 3) by alleviation of mitochondrial injury; 4) by the suppression of inflammatory-related signaling pathways (e.g. by affecting nuclear factor kappa beta); 5) by modulation of glycolysis, as well as 6) by restoring impaired nitric oxide bioavailability. This review focuses on the most recent progress and existing gaps in preclinical investigations concerning the direct effects of SGLT-2i's on endothelial dysfunction and their underlying mechanisms.

Key words: sodium glucose co-transporter 2 inhibitors (SGLT-2i's); endothelial cell (EC) dysfunction; sodium-hydrogen exchanger (NHE); reactive oxygen species (ROS), inflammation

Abbreviations

| Abbreviations | Terms |
|------------------|---|
| AGEs | Advanced glycation end products |
| Akt | Protein kinase B |
| AMPK | Adenosine monophosphate-activated protein kinase |
| Ang II | Angiotensin II |
| Ca ²⁺ | Calcium |
| CANA | Canagliflozin |
| CM | Cardiomyocyte |
| CMEC | Cardiac microvascular endothelial cell |
| DAPA | Dapagliflozin |
| DM | Diabetes mellitus |
| EC | Endothelial cell |
| EMPA | Empagliflozin |
| eNOS | Endothelial nitric oxide synthase |
| ERK | Extracellular signal regulated kinase |
| GADPH | Glyceraldehydes-3-phosphate dehydrogenase |
| GCX | Glycocalyx |
| HAEC | Human aortic endothelial cell |
| HCAEC | Human coronary artery endothelial cell |
| HF | Heart failure |
| HFpEF | Heart failure with preserved ejection fraction |
| HIF-1 α | Hypoxia-induced factor-1 α |
| HKII | Hexokinase II |
| HUVEC | Human umbilical vein endothelial cell |
| ICAM-1 | Intracellular adhesion molecule 1 |
| IL | Interleukin |
| JNK | c-Jun N terminal kinase |
| MAPK | Mitogen-activated protein kinase |
| mtROS | Mitochondrial reactive oxidative species |
| NF- κ B | Nuclear factor kappa B |
| NHE | Sodium-hydrogen exchanger |
| NLRP3 | NOD-like receptor pyrin domain containing 3 |
| NO | Nitric oxide |
| NOX | Nicotinamide adenine dinucleotide phosphate oxidase |

| Abbreviations | Terms |
|---------------|--|
| PI3k | Phosphatidylinositol 3 kinase |
| PKC | Protein kinase C |
| PON | Ponatinib |
| RAGE | Receptor for advanced glycation end products |
| ROS | Reactive oxidative species |
| SFK | Src Family kinase |
| SGLT-1/2 | Sodium glucose co-transporter 1/2 |
| SGLT-2i's | Sodium glucose co-transporter 2 inhibitors |
| SMC | Smooth muscle cell |
| TNF- α | Tumor necrosis factor- α |
| VCAM-1 | Vascular cell adhesion molecule 1 |
| VE-cadherin | Vascular endothelial cadherin |
| VEGF | Vascular endothelial growth factor |

Introduction

Patients with diabetes mellitus (DM) more frequently suffer from heart failure (HF), in particular heart failure with preserved ejection fraction (HFpEF), than individuals without DM (Seferovic *et al.* , 2018). Sodium glucose co-transporter 2 inhibitors (SGLT-2i's), a novel class of glucose-lowering drugs, significantly reduce the risk of cardiovascular death and hospitalisation in patients with existing HF, regardless of the presence of DM (Zinman *et al.* , 2015; Neale *et al.* , 2017; Wiviott *et al.* , 2019; Packer *et al.* , 2020). Treatment with empagliflozin (EMPA) also reduces the combined outcome of worsening HF, rehospitalization of HF and death for HF in patients with acute HF (Damman *et al.* , 2020). Recently, the EMPEROR-Preserved phase III trial has established EMPA as the first potential therapy capable to improve cardiovascular outcome in patients suffering from HFpEF (Anker *et al.* , 2021). Until today, the exact mechanisms underlying these “off-target” effects of SGLT-2i's remain largely unknown. Previous studies have highlighted the direct cardiac effects of SGLT-2i's (Packer, 2020; Kleinbongard *et al.* , 2020), which are mediated by alleviation of oxidative stress, inflammation, apoptosis, and calcium (Ca²⁺) overload of cardiomyocytes (CMs) (Uthman *et al.* , 2018; Trum *et al.* , 2021).

The PROMIS-HFpEF trial has prospectively demonstrated a high prevalence of coronary microvascular disorder and systemic endothelial dysfunction in patients with HF (Shah *et al.* , 2018). Endothelial cells (ECs) form a monolayer over the inner surface of vessels (Kruger-Genge *et al.* , 2019). In the adult human heart, ECs account for 12.2% of total cells within the arterial tissues and 7.8% within the ventricular regions (Litvinukova *et al.* , 2020). Physiologically, ECs serve to maintain cardiovascular function by ensuring the production of endothelium-derived vasoactive factors, preventing monocyte adhesion and platelet aggregation, regulating the proliferation of smooth muscle cells (SMCs) as well as the contraction and relaxation of CMs (Monteiro *et al.* , 2019). In patients with diabetes, hyperglycemia impairs endothelial function and ultimately causes the development of macro- and micro-vascular complications (Shi *et al.* , 2017). Thus, ECs might serve as a novel target to improve cardiac function of patients with HF.

Previous studies have shown that SGLT-2i's directly ameliorate endothelial dysfunction in both euglycaemic and hyperglycaemic conditions (Alshnbari *et al.* , 2020; Durante *et al.* , 2021; Salvatore *et al.* , 2021) and that EMPA mitigates endothelial and cardiac dysfunction in patients with HFpEF via reducing inflammatory-oxidative pathways (Kolijn *et al.* , 2020). Our current manuscript focuses on the potential role of improved endothelial function as an indispensable contributor to the enhanced cardiac function in patients receiving SGLT-2i's. We will review the current data and most recent progress in preclinical investigations concerning the direct effects of SGLT-2i's on endothelial dysfunction, with the aim to improve the understanding of their compelling cardiovascular effect on patients with HF.

1 SGLT-2i's inhibit reactive oxygen species (ROS) production in ECs

1.1 Mechanisms behind increased ROS production in ECs

At moderate concentrations, ROS play an important role in maintaining the proliferation and survival of ECs, but excessive ROS have detrimental effects on the vascular system (Forstermann *et al.* , 2017). In patients with DM, hyperglycaemia accelerates protein glycation and forms advanced glycation end products (AGEs) (Kay *et al.* , 2016). These AGEs bind with cell surface receptors for AGEs (RAGE) to activate downstream signaling pathways, such as extracellular signal-regulated kinase (ERK), and subsequently increase ROS production (Yuan *et al.* , 2019). Excessive ROS also trigger nuclear poly ADP-ribose polymerase, which inhibits activity of glyceraldehydes-3-phosphate dehydrogenase (GADPH) and activates the polyol pathway of glucose (Giri *et al.* , 2018). Activated polyol pathway produces ROS via depleting nicotinamide adenine dinucleotide phosphate and glutathione, as well increasing the oxidation of nicotinamide adenine dinucleotide hydrogen during the conversion of sorbitol to fructose. Inhibition of GADPH also accelerates the generation of diacylglycerol, subsequently activating protein kinase C (PKC) and stimulating nicotinamide adenine dinucleotide phosphate oxidases (NOXs) to generate additional ROS (Yuan *et al.* , 2019). Mitochondria are the central regulators for aerobic energy generation, and ROS are the essential by-product during this process. Diabetes and hyperglycemia disrupt the mitochondrial respiratory chain and alter mitochondrial ultrastructure (e.g. mitochondrial fission and fusion), thereby increasing the ROS production within mitochondria (Forrester *et al.* , 2018; Brownlee, 2001). Hyperglycemia upregulates both abundance and activity of the sodium-hydrogen exchanger (NHE) within ECs (Klug *et al.* , 2021). Activated NHE promotes the influx of sodium influx and enhances intracellular calcium (Ca^{2+}) via triggering sodium-calcium exchanger (Baartscheer *et al.* , 2017). Increased intracellular sodium (Na^+) triggers the sodium-calcium exchanger (NCX) and enhances calcium (Ca^{2+}) influx into the cytosol. The increased cytosolic Ca^{2+} then stimulates the PKC-NOXs pathway, which further increases ROS production (Rastogi *et al.* , 2016). Recently, Uthman et al have directly proven the causal link between NHE activity and oxidative stress in ECs: tumor necrosis factor- α (TNF- α) enhanced NHE activity and intracellular sodium (Na^+), as well ROS production, and the increased ROS generation was mitigated by cariporide, a potent inhibitor for NHE. The crucial role of NHE/ Na^+ -axis in inflammatory related oxidative stress was further supported by the fact that sodium pump inhibitor ouabain increased intracellular Na^+ and ROS production in human ECs (Uthman *et al.* , 2022).

Excessive ROS increase vascular tone and undermine cardiac inotropic function, contributing to cardiomyopathy (Ritchie *et al.* , 2020). Oxidative stress causes endothelial nitric oxide synthase (eNOS) uncoupling and impairs NO production, the key vasodilator. ROS produced by NOXs also oxidize the sarcoendoplasmic reticulum calcium transport ATPase and limit the sensitivity of SMC to NO (Griendling *et al.* , 2021). Besides, ROS induce vascular stiffness via upregulating the expression of vasoactive factors like vascular endothelial growth factor (VEGF) and extracellular proteins like matrix metalloproteinases (Griendling *et al.* , 2021). Vascular remodeling elevates blood pressure and increases the intensity of cyclic stretch caused by vasoconstriction-dilation circles (Ohishi, 2018). Enhanced stretch might exacerbate oxidative stress via upregulating expression of NOXs in ECs, further increasing ROS production within in ECs (Li *et al.* , 2021). Besides, oscillatory shear stress created by disturbed blood flow also induces oxidative stress in ECs via activating NOXs (Siu *et al.* , 2016).

Depletion of nitric oxide (NO) is a crucial mediator for ROS related cardiac dysfunction (Shah *et al.* , 2021). EC-derived NO triggers soluble guanylate cyclase (cGMP) and protein kinase G (PKG) of adjacent CMs and leads to the phosphorylation of troponin I and reduction of myofilament Ca^{2+} sensitivity, thus enhancing myocardial relaxation in both isolated CMs and whole hearts (Królet *et al.* , 2021; Feil *et al.*). Activation of the NO/cGMP/PKG signaling pathway also maintains phosphorylation of titin within CMs and prevents the development of cardiac hypertrophy (Shah *et al.* , 2021). Moreover, oxidative stress leads to increased secretion of pro-inflammatory cytokines and chemokines from ECs, as well as upregulated expression of adhesion molecules and enhanced monocyte-endothelial attachment (Yuan *et al.* , 2019). Intensified ROS production activates src family kinase (SFK) to phosphorylate vascular endothelial-cadherin (VE-cadherin), leading to VE-cadherin internalisation and adherens junction disruption. Activated SFK also promotes the transformation from G actin to F actin to generate stress fibres under the cellular membrane, increasing the

intracellular tension (Zhang *et al.* , 2017). ROS are also involved in the endothelial dysfunction induced by stretch and oscillatory shear stress through intracellular cascades, such as mitogen-activated protein kinase p38 (p38 MAPK), ERK, c-Jun N-terminal kinase (JNK) and nuclear factor kappa B (NF- κ B) (Lehoux, 2006). The pivotal role of EC-derived ROS in development of cardiovascular disease is summarised in Figure 1.

1.2 Anti-oxidative effect of SGLT-2i's

Mounting evidence reveals a class effect of ROS inhibition for SGLT-2i's. *In vivo* studies showed that ipragliflozin decreased urinary 8-hydroxy-2'-deoxyguanosine (a marker for DNA oxidative injury) in hyperglycaemic mice (Salim *et al.* , 2016), and dapagliflozin (DAPA) attenuated elevated vascular ROS generation in aortic atherosclerotic tissues of diabetic mice (Leng *et al.* , 2016). However, it might be considered that these observed ROS inhibitory effects of SGLT-2i's were mediated by the decreased blood glucose in mice with diabetes.

Live cell imaging suggested that SGLT-2i's directly inhibited inflammation-stimulated ROS production within human ECs from both venous and arterial vessels (Uthman *et al.* , 2019). Two other studies showed that EMPA reduced ROS production within cardiac microvascular endothelial cells (CMECs) exposed to pro-inflammatory cytokines and uremic acid (Juni *et al.* , 2019; Juni *et al.* , 2021). By preventing ROS accumulation within ECs, EMPA restored NO bioavailability in co-cultured CMs, indicating that the ROS inhibitory capacity of SGLT-2i's contributes to the improvement of contraction and relaxation of adjacent CMs through an endothelial-NO pathway (Juni *et al.* , 2019; Juni *et al.* , 2021). A more recent study reported a novel ROS inhibitory effect of SGLT-2i's in human coronary artery endothelial cells (HCAECs) undergoing enhanced cyclic stretch, suggesting that SGLT-2i's might also alleviate oxidative stress caused by mechanical forces (Li *et al.* , 2021). This study firstly showed that SGLT-2i's prevented the loss of VE-cadherin and alleviated barrier dysfunction in HCAECs undergoing enhanced stretch, which was mediated by their ROS inhibitory effect (Li *et al.* , 2021).

1.3 Potential mechanisms underlying ROS inhibition by SGLT-2i's

Several factors contribute to the anti-oxidative effect of SGLT-2i's. SGLT-2i's revert upregulation of NOXs and inhibit oxidative stress in the macro- and micro-vascular system (Ganbaatar *et al.* , 2020; Kuno *et al.* , 2020). In diabetic mice, EMPA reduced NOX2 expression at messenger RNA (mRNA) levels in aortic endothelium (Ganbaatar *et al.* , 2020). Correspondingly, EMPA also suppressed the increase of NOX2 and NOX4 in renal tissue of rats with acute kidney injury (Kuno *et al.* , 2020). An *in vitro* study showed that EMPA exerted a similar inhibitory capacity like GKT136901, a specific inhibitor for NOX1 and NOX4, on ROS generation in HCAECs undergoing enhanced stretch. Combination of EMPA and GKT136901 did not further reduce the stretch-induced ROS production, suggesting that the anti-oxidative effect of EMPA is mediated via NOXs (Li *et al.* , 2021). Furthermore, EMPA prevented hyperglycaemia-induced mitochondrial disruption, thereby attenuating the overproduction of cytosolic ROS and mitochondrial ROS (mtROS) in ECs isolated from mice and humans (Juni *et al.* , 2021; Zhou *et al.* , 2018). This mechanism is further supported by the fact that induction of mitochondrial fission abrogated the inhibitory effects of EMPA on mtROS in mice CMECs (Zhou *et al.* , 2018).

Another potential mechanism that might explain the antioxidant effects of SGLT2i's is the direct inhibition of the sodium-hydrogen exchanger (NHE) by SGLT2i's, firstly discovered in CMs (Baartscheer *et al.* , 2017; Uthman *et al.* , 2018). A recent study from our laboratory showed that 10 μ M cariporide blocked the increase of oxidative stress in HCAECs undergoing enhanced cyclic stretch, and this effect of cariporide on ROS production was not further enhanced when combined with EMPA. These data indirectly suggest the involvement of NHE in the anti-oxidative capacity of EMPA in ECs (Li *et al.* , 2021). For the first time, Uthman et al directly proved that EMPA inhibited ROS production in ECs via NHE inhibition: EMPA treatment lowered the NHE activity and Na⁺ concentration in human ECs triggered by TNF- α (measured with SNARF-AM and SBFI-AM fluorescence probes respectively), and also mitigated the increased ROS production. The combination of EMPA and cariporide did not demonstrate additional ROS reduction in

cells, showing that EMPA reduced TNF- α induced ROS production via NHE inhibition (Uthman *et al.* , 2022).

Yet, there is still an ongoing discussion regarding the role of NHE in the inhibitory effect on ROS of SGLT-2i's. In support of our finding, Cappetta *et al.* previously reported NHE inhibition by DAPA in “non-stimulated” human umbilical vein endothelial cells (HUVECs) (Cappetta *et al.* , 2020). In contrast, using cardiac microvascular ECs exposed to uremic serum, Juni *et al.* recently observed a stronger ROS inhibitory capacity of 1 μ M EMPA when compared to 10 μ M cariporide (63% vs 38%), indicating that part of the anti-oxidative effect of EMPA might be unrelated to NHE inhibition (Juni *et al.* , 2021). Chung *et al.* reported a neutral effect of EMPA (1-30 μ M) on NHE activity within isolated rat CMs (Chung *et al.* , 2020), which is in contrast to the studies of Baartscheer *et al.*, Uthman *et al.* and Zuurbier *et al.* showing that 1 μ M EMPA inhibited the NHE activity in both isolated cardiac myocytes and isolated intact hearts of different rodents (mice and rabbits) (Baartscheer *et al.* , 2017; Uthman *et al.* , 2018; Zuurbier *et al.* , 2021). Diversities in the employed methodology might explain the differences between these studies.

The involvement of sodium glucose co-transporter 1/2 (SGLT-1/2) in the anti-oxidative effect of SGLT-2i's has been recently investigated. Recent studies showed that high glucose and angiotensin II (Ang II) increased the expression of SGLT-1 and -2 in porcine ECs, and that EMPA showed an inhibitory effect on the induced SGLT-1/2 expression (Khemais-Benkhiat *et al.* , 2020; Park *et al.* , 2021). At 24 h, sotagliflozin (a dual inhibitor for SGLT-1 and -2) and empagliflozin abolished the Ang II-induced ROS production. Reduction of extracellular glucose and Na⁺ concentrations significantly inhibited the pro-oxidant reaction to Ang II, indicating the crucial role of SGLT-1 and -2 in a glucose and sodium dependent ROS production (Park *et al.* , 2021). Intriguingly, the sustained oxidative stress triggered by Ang II could also be alleviated by inhibition of NHE, NCX and NOXs, further supporting the functional link between NHE/Na⁺/Ca²⁺ pathway and ROS production by NOXs within ECs (Park *et al.* , 2021). However, expression of SGLT-2 in ECs is still a matter of debate, especially in the case of human cells. Mancini *et al.* showed the absence of SGLT-2 at mRNA level in HUVECs (Mancini *et al.* , 2018), corresponding with the most recent study of Juni *et al.* using human CMECs (Juni *et al.* , 2021). In contrast, using Western blot, Behnammanesh *et al.* detected the presence of SGLT-2 in human ECs (Behnammanesh *et al.* , 2019). Uthman *et al.* also reported a potential existence of SGLT-2 in human ECs at protein level with a commercially available antibody (Uthman *et al.* , 2019). But this signaling for SGLT-2 protein persisted after the target gene being silenced at mRNA level, and the qPCR revealed no existence of SGLT-2 (Uthman *et al.* , 2019).

2 SGLT-2i's inhibit the inflammatory reaction of ECs upon different stimuli

2.1 Increased vascular inflammation in ECs

During hyperglycaemia, binding of AGEs to RAGE elevates adhesion molecule expression, production of cytokines and growth factors via the activation of ERK, JNK, and phosphoinositol 3-kinase (PI3k) pathways. AGEs directly stimulate monocytes to produce inflammatory mediators like interleukins (ILs) and TNF- α , and thereby further enhance local inflammation of the vascular wall (Jin *et al.* , 2018). Moreover, excessive production of ROS induces vascular inflammation in ECs via upregulating intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Daiber *et al.* , 2020). The oxidative stress triggers cytokine secretion mainly in an “inflammasome-dependent” manner (Bai *et al.* , 2020). Briefly, ROS activate NF- κ B and upregulate the expression level of NOD-like receptor containing pyrin domain 3 (NLRP3) inflammasome as well as pro-IL-1 β . Then, NLRP3 inflammasome is activated through assembling of NLRP3, caspase-1 and apoptosis-associated speck-like protein containing a CARD (caspase activation and recruitment domain), forming a complex for the final production of active caspase-1, IL-1 β and IL-18. This process is also accelerated by ROS (Ferrucci *et al.* , 2018; Toldo *et al.* , 2021). Intriguingly, pro-inflammatory mediators released during inflammation like TNF- α can induce ROS production, thus promoting the “vicious circle” of oxidative stress and inflammation (Yuan *et al.* , 2019).

2.2 Anti-inflammatory capacity of SGLT-2i's

A recent clinical trial revealed that 24-week treatment with EMPA significantly reduced serum ICAM-1 level

and prevented leukocyte-endothelium interactions in patients with DM (Canet *et al.* , 2021). EMPA inhibited macrophage accumulation, as well the expression of monocyte chemoattractant protein-1 and ICAM-1 in the aortic arch of diabetic mice (Ganbaatar *et al.* , 2020) and DAPA attenuated the high-salt diet-induced upregulation of VCAM-1 in euglycaemic rats and lowered NF- κ B expression within rat ECs (Cappetta *et al.* , 2020). Another study showed that EMPA limited Ang II-induced abdominal aortic aneurysm in ApoE knockout mice, partially through inhibiting activation of p38 MAPK and NF- κ B in aortas, as well reducing macrophage infiltration within lesions (Ortega *et al.* , 2019).

Consistently, *in vitro* studies revealed that DAPA attenuated the increased ICAM-1 and VCAM-1 secretion of HUVECs exposed to hyperglycaemia or TNF- α for 24 h (Gaspari *et al.* , 2018) and that EMPA inhibited the TNF- α triggered leukocyte adhesion towards human ECs cultured under flow (Cooper *et al.* , 2019). However, the anti-inflammatory effect in Cooper's study was achieved by very with high concentration of EMPA (50 μ M) (Cooper *et al.* , 2019). Uthman *et al.* reported a neutral effect of EMPA (1 μ M) on TNF- α -induced adhesion molecules expression in static human ECs (Uthman *et al.* , 2019). In the latter study, ICAM-1 and VCAM-1 were measured 4 h after TNF- α stimulation with flow cytometry, instead of using the supernatant of cell culture as in the study of Gaspari *et al.* (Uthman *et al.* , 2019; Gaspari *et al.* , 2018). The dosage of SGLT-2i's also differed in these two study (1 μ M vs. 1-5 nM, respectively). Yet, lower doses of DAPA (1 and 10 nM) did not inhibit expression of adhesion molecules either in the study of Uthman *et al.* (Uthman *et al.* , 2019). Additionally, static cells are not directly comparable to cells cultured under flow. Static cells develop a thinner glycocalyx (GCX) layer than dynamically activated ECs, and the latter model is considered more physiologically relevant because *in situ* endothelial cells are constantly exposed to mechanical forces generated by blood flow (Chistiakov *et al.* , 2017; Haymet *et al.* , 2021).

Uthman *et al.* recently reported a compound-specific effect of canagliflozin (CANA) in inhibiting IL-6 secretion by HCAECs exposed to lipopolysaccharide (Uthman *et al.* , 2020). In this study, only CANA activated adenosine monophosphate-activated protein kinase (AMPK), while EMPA or DAPA did not alter AMPK phosphorylation in HCAECs (Uthman *et al.* , 2020). The different impact on AMPK might partially explain the diverse effects of the three SGLT-2i's on vascular inflammation. Moreover, CANA-induced IL-6 reduction was significantly diminished after glycolytic hexokinase II (HKII) knockdown, revealing a novel anti-inflammatory mechanism of CANA via inhibiting glycolysis (Uthman *et al.* , 2020).

3 SGLT-2i's restore impaired NO bioavailability

3.1 Mechanisms of reduced NO bioavailability in ECs

Patients with HF show an increased vascular tone in both macro- and micro- vessels, mainly because of blunted NO bioavailability (Forstermann *et al.* , 2017). Physiologically, NO production in ECs is initiated by phosphorylation of eNOS via the PI3K/protein kinase B (Akt) pathway. Hyperglycaemia compromises NO generation by inhibiting the expression of eNOS as well as suppressing the phosphorylation of the active site of eNOS (e.g. Ser1177) (Meza *et al.* , 2019; Forstermann *et al.* , 2017). In addition, excessive ROS in ECs consumes NO, which can be prevented by anti-oxidative agents (Meza *et al.* , 2019). Next to the above mentioned mechanisms, mechanical forces play a crucial role in modulating NO production within ECs. When exposed to unidirectional high shear stress (12-15 dyne/cm²), the GCX transduces mechanical stimulation to intracellular compartments and triggers diverse downstream pathways like PI3K/Akt/eNOS, thus promoting the release of NO (Chistiakov *et al.* , 2017). Increased blood glucose levels degrade endothelial GCX of diabetic mice (Zuurbier *et al.* , 2005), suggesting a potential interaction between hyperglycaemia and GCX in eNOS-dependent NO production.

3.2 Restoration of NO by SGLT-2i's

In vivo studies showed that SGLT-2i's restored the endothelium-dependent vasodilation in both hyperglycaemic and euglycaemic animals (Salim *et al.* , 2016; Sayour *et al.* , 2019). Additionally, *in vitro* studies showed that EMPA and DAPA reverted the loss of NO in human ECs exposed to TNF- α (Uthman *et al.* , 2019; Juni *et al.* , 2021). However, phosphorylation of eNOS at Ser1177 in isolated ECs was not affected by EMPA, the rescued NO bioavailability in isolated human ECs was most likely mediated by the ROS

inhibitory capacity of SGLT-2i's (Uthman *et al.* , 2019; Juniet *et al.* , 2021). In contrast, EMPA promoted eNOS phosphorylation via activating AMPK in ECs isolated from mice (Zhou *et al.* , 2018), which could be partially explained by species difference. Moreover, the studies of Uthman and Juni were performed within static ECs (Uthman *et al.* , 2019; Juni *et al.* , 2021). A further improvement in cellular studies could be the incorporation of dynamically activated ECs.

4 SGLT-2i's revert disturbed angiogenesis of ECs

4.1 Pathological modulation of angiogenesis

Angiogenesis is defined as the formation of new capillaries from established vessels. Physiologically, this process is limited to the embryonic and postnatal stage and rarely found within healthy adults, apart from female reproductive organs (Eelen *et al.* , 2020). Pathological alterations in angiogenesis are highly diverse in different organs/tissues. For example, excessive angiogenesis occurs in diabetic retinopathy/nephropathy, while the angiogenetic capacity is significantly impaired in cardiac and cerebral tissue of patients with DM (Okonkwo *et al.* , 2017). Growth factors like VEGFs play a key role in promoting vascular growth and remodeling, and they can be dramatically upregulated by hyperglycaemia, ROS and pro-inflammatory mediators like TNF- α , but mostly by the lack of oxygen in respective organs. Local hypoxic conditions activate hypoxia-induced factor (HIF)-1 α and increase the expression of VEGFs (Apte *et al.* , 2019). Conditional knockout of HIF-1 α attenuated VEGF overproduction and prevented the development of diabetic retinopathy within mice, suggesting a crucial role of the HIF-1 α /VEGFs pathway in pathological angiogenesis (Lin *et al.* , 2011). Vasodilators like NO are temporarily increased during pregnancy and may promote placental angiogenesis, but whether this effect persists in adult tissue is unclear (Umapathy *et al.* , 2020).

4.2 The effect of SGLT-2i's on angiogenesis

The effect of SGLT-2i's on angiogenesis is still controversial. Previously, Zhou *et al.* demonstrated that EMPA promoted angiogenesis of CMECs in diabetic mice, thus improving myocardial microcirculatory perfusion and cardiac function (Zhou *et al.* , 2018). This study showed that EMPA preserved angiogenetic capacity of isolated CMECs through stabilization of F-actin. The effect of EMPA was nearly abolished when EMPA was combined with FCCP, a mitochondrial fission activator to promote mtROS generation (Zhou *et al.* , 2018). Excessive ROS arrested the cell cycle transition from G0/G1 to S and interrupted CMECs proliferation, which was re-established by EMPA and mdiv1 (an inhibitor for mitochondrial fission) (Zhou *et al.* , 2018). Taken together, prevention of mitochondrial fission and subsequent oxidative stress by EMPA was involved in its pro-angiogenetic effects on CMECs. Correspondingly, a recent study from Nikolaou *et al.* showed that chronic administration (6 weeks) of EMPA reduced myocardial infarct size after ischemia-reperfusion in non-diabetic mice, and this cardioprotective effect of EMPA could be partially explained by the improved CMECs survival (Nikolaou *et al.* , 2021). An *in vitro* study with human ECs exposed to hypoxia/reoxygenation stress also proved that EMPA increased cellular viability via activating signal transducer and activator of transcription 3 pathway (Nikolaou *et al.* , 2021). Using human aortic endothelial cells (HAECs), another study revealed that the autophagy is also involved in the pro-angiogenic effect of EMPA. The anti-leukemia agent ponatinib (PON) induces vasculotoxicity via mitochondrial damage, while the autophagy-mediated removal of injured mitochondria represents a cardiovascular protective mechanism against the toxic insult of PON. HAECs exposed to PON showed decreases in autophagy marker expression (LC3-I/II), tube formation as well cell viability, which were reverted by EMPA (Madonna *et al.* , 2021).

In contrast, Behnammanesh *et al.* reported a robust anti-proliferative and anti-migration effect of CANA in HUVECs and HAECs (Behnammanesh *et al.* , 2019). In clinically relevant dosages, CANA (5 μ M and 10 μ M) inhibited the proliferation of HUVECs by reducing the expression of cyclin A, as well as by reducing phosphorylation of the retinoblastoma protein, while EMPA and DAPA barely influenced the proliferative capacity of ECs in their physiological dosage (1-2 μ M) (Behnammanesh *et al.* , 2019; Devineni *et al.* , 2016; Tomlinson *et al.* , 2017). The anti-proliferative effect of CANA could also be beneficial: CANA suppressed the increased proliferation and tubular formation of HUVECs during co-culture with Huh7 and HepG2 (hepatocyte-derived carcinoma cell lines), as well the enhanced production of angiogenic cytokines

(e.g. IL-8), thus inhibiting the growth of liver cancer (Kaji *et al.* , 2018). More recently, another study showed that CANA had a dosage-dependent inhibitory effect on the VEGF-A expression and angiogenesis of HUVECs co-cultured with HepG2 cell line (Luo *et al.* , 2021).

Several factors might explain the observed opposite effects of CANA and EMPA on angiogenesis. Firstly, these studies were performed with ECs under different “stress” conditions. Cells in the EMPA studies were activated with pathological stimuli (diabetes, hypoxia/reoxygenation and PON) that impaired their cellular viability and angiogenesis capacity (Zhou *et al.* , 2018; Nikolaou *et al.* , 2021; Madonna *et al.* , 2021), while the anti-angiogenic effect of CANA was reported in “non-stimulated” cells or ECs co-cultured with cancer cells (Behnammanesh *et al.* , 2019; Kaji *et al.* , 2018; Luo *et al.* , 2021). The potential involvement of SGLT-1 is also of importance, considering that anti-proliferating effect seems to be compound-specific for CANA (Behnammanesh *et al.* , 2019). Compared with EMPA and DAPA, CANA is relatively less selective for SGLT-2 over SGLT-1, and the latter is highly expressed in human ECs (Ohgaki *et al.* , 2016). However, in a HepG2 and ECs co-culture model, CANA reduced glucose uptake and growth of liver cancer cells via inhibiting SGLT-2 rather than SGLT-1 (Kaji *et al.* , 2018). Moreover, the effect of SGLT-2i’s on ECs proliferation and migration might vary among different organs/tissues, and more research is required to explain these existing differences.

5 Summary and Perspective

In summary, SGLT-2i’s demonstrate prominent cardiovascular protective effects in patients with existing HF, with and without diabetes (Anker *et al.* , 2021; Packer *et al.* , 2020). Whereas earlier studies focused on the direct impact of SGLT-2i’s on CMs, recent work has also emphasised ECs as a promising therapeutic target of SGLT-2i’s (Durante *et al.* , 2021; Salvatore *et al.* , 2021). Preclinical studies show that SGLT-2i’s exert favorable effects against endothelial dysfunction, including inhibiting oxidative stress and inflammatory reactions, restoring NO bioavailability, and modulating angiogenesis (The major findings of discussed literatures are listed in Table 1). This property might contribute to the cardiovascular benefits observed in patients receiving SGLT-2i’s (Figure 2).

However, there are lots of controversies concerning the mechanisms underlying these observed endothelial effects. Species differences can only partly explain these discrepancies, and *in vivo* studies using rats and mice are vulnerable to the influence of the animals biokinetic parameters, making it difficult to extrapolate the results to humans (Saeidnia *et al.* , 2015). Moreover, the relatively limited studies in human ECs are of high diversity in origin (micro- or macro- circulation) and developmental stage (adult or postnatal) of the cells (Alshnbari *et al.* , 2020). Further studies with CMECs are required, considering the high prevalence and clinical relevance of coronary microvascular dysfunction in patients with HF (Shah *et al.* , 2018). Remarkably, most of these *in vitro* studies are performed in static cells.

Taken together, given that endothelial dysfunction is a crucial promoter for the development of heart failure and that SGLT-2i’s exert promising protective effects on ECs, SGLT-2i’s have a great potential in the treatment of cardiovascular disease. Investigations regarding the direct endothelial effect of SGLT-2i’s are needed for a better understanding of the mechanisms underlying these cardiovascular effects.

6 Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY <http://www.guidetopharmacology.org> and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander *et al.* , 2021; Alexander *et al.* , 2021; Alexander *et al.* , 2021).

7 Author Contributions

Original draft preparation: XL, BP and NCW; revision and editing: NCW, BP, CJZ, MWH, JH and XL; conceptualization: NCW, BP, CJZ, MWH and JH; literature search and analysis: XL and NCW; figure/table preparation: XL.

Table 1 Major findings of the direct endothelial effects of SGLT-2i's

| References | Cells/Tissues | Drug (Dosage) | Stimulant (Dosage) | Major findings |
|----------------------------------|--|---|---|---|
| <i>Behnammanesh et al., 2019</i> | HUVECs HAECs Mice aortic ECs | CANA (0-50 μ M)/ 1-3 d | ... | Angiogenesis — Cell proliferation — Cyclin A and retinoblastoma phosphorylation — NHE activity — |
| <i>Cappetta et al., 2020</i> | HUVECs | DAPA (1 μ M) | ... | VCAM-1 — NF- κ B — |
| <i>Cooper et al., 2019</i> | Rat vasculature HAECs | DAPA (0.1 mg/kg/d)/ 6 w EMPA (50 μ M)/24 h | High-salt (8% NaCl)/ 11 w TNF- α (10 μ g/ml)/ 24 h Wall shear stress (10 dyne/cm ²)/24 h | Leukocyte- endothelium adhesion — Glycocalyx integrity — |
| <i>Ganbaatar et al., 2020</i> | Abdominal aorta of Apo E ^{-/-} mice | EMPA (20 mg/kg/d)/ 8-12 w | STZ (75 mg/kg) | NOX2 — Macrophage accumulation — Monocyte chemoattractant protein-1 — ICAM-1— |
| <i>Gaspari et al., 2018</i> | HUVECs | DAPA (1 nM-1 mM)/ 24 h | TNF- α (10 ng/ml)/ 24 h Hyperglycaemia (10-30 mM)/24 h High-fat diet (22% fat+0.15% cholesterol)/ 20 w | ICAM-1 — VCAM-1 — NF- κ B — |
| <i>Juni et al., 2019</i> | Aortic sections of Apo E ^{-/-} mice Human CMECs | DAPA (1 mg/kg/d)/ 4 w EMPA (1 μ M)/6 h | TNF- α (10 ng/ml)/6 h | ICAM-1 — Cytoplasmatic ROS — Mitochondrial ROS — NO bioavailability — |
| <i>Juni et al., 2021</i> | Human CMECs | EMPA (1 μ M)/6 h | Uremic serum (15%)/ 6 h | Cytoplasmatic ROS — Mitochondrial ROS — Mitochondrial fragmentation — NO bioavailability — |

| References | Cells/Tissues | Drug (Dosage) | Stimulant (Dosage) | Major findings |
|--------------------------------------|--|---|--|--|
| <i>Kaji et al., 2018</i> | HUVECs | CANA (10 μ M)/12 h | Co-culture with Huh7 and HepG2/24 h | HUVEC proliferation — Tubular formation — IL-8 — Angiogenin — Metalloproteinase-1 — |
| <i>Khemais-Benkhiat et al., 2020</i> | Porcine coronary artery ECs | EMPA (0.1-100 nM)/ 30 min-96 h | High glucose (25 mmol/L)/48 or 96 h | Senescence associated-beta-galactosidase — p-21 & p-16 protein — ROS — NOX — eNOS & NO — Glucose uptake — VCAM-1 — |
| | Porcine coronary segment | EMPA (100 nM)/ 30 min | High glucose (25 mmol/L)/24 h | SGLT-1 & 2 — eNOS — VCAM-1 — |
| <i>Leng et al., 2016</i> | Aortic sections of Apo E ^{-/-} mice | DAPA (1.0 mg/kg/d)/ 12 w | STZ (130 mg/kg) +high-fat diet (15% fat+0.15% cholesterol)/4 w | ROS — NLRP3 inflammasome — |
| <i>Li et al., 2021</i> | HCAECs | EMPA (1 μ M)/24 h DAPA (1 μ M)/24 h CANA (3 μ M)/24 h | Cyclic stretch (1 Hz, 10%)/24 h | ROS — Cell permeability — VE-cadherin — |
| <i>Luo et al., 2021</i> | HUVECs | CANA (10 & 20 μ M)/ 24 h | Co-culture with HepG2/24 h | Angiogenesis — VEGF-A protein — |
| <i>Madonna et al., 2021</i> | HAECs | EMPA (100 & 300 nM)/0-48 h DAPA (100 nM)/ 0-48 h | PON (1.7 nM)/0-48 h | Tube formation — Cell viability — Autophagy — Senescence — |
| <i>Mancini et al., 2018</i> | HUVECs HAECs | CANA (10 μ M)/ 6 & 24 h | IL-1 β (10 ng/ml)/ 6 & 24 h | IL-6 — Monocyte chemoattractant protein-1 — Monocyte adhesion — AMPK activity — |
| <i>Nikolaou et al., 2021</i> | Mice CMECs | EMPA (10 mg/kg/d)/ 6 w | Ischaemia (30 min)/ Reperfusion (2 h) | VEGF — Superoxidedimutase 2 — Cell survival — |
| | Human CMECs | EMPA (500 nM)/24 h | Hypoxia (3 h)/ Reoxygenation (1 h) | Cell viability — ROS — |

| References | Cells/Tissues | Drug (Dosage) | Stimulant (Dosage) | Major findings |
|----------------------------|---|--|--|---|
| <i>Park et al., 2021</i> | Porcine coronary artery ECs | EMPA or sotagliflozin (100 nM)/30 min-24 h | Ang II (100 nM) | ROS — SGLT-1 & 2 — Senescence associated-beta-galactosidase — eNOS & NO — |
| | Porcine coronary artery ECs | EMPA or sotagliflozin (100 nM)/48 h | Microparticles from patients with coronary artery disease (10 nM)/48 h | SGLT-1 & 2 — eNOS & NO — VCAM-1 — |
| <i>Ortega et al., 2019</i> | Aortic aneurysm sections of Apo E ^{-/-} mice | EMPA (3 mg/kg/d)/ 28 d | Ang II (1000 ng/kg/d)/ 28 d | Macrophage infiltration — p38 MAPK — NF- κ B — VEGF — |
| | HAECs | EMPA (3 μ M)/24 h | Ang II (1 μ M)/24 h | Leukocyte-endothelium interactions — ICAM-1 — VCAM-1 — |
| <i>Salim et al., 2016</i> | Mice abdominal aorta | Ipragliflozin (3 mg/kg/d)/3 w | STZ (150mg/lkg) | Akt phosphorylation — eNOS phosphorylation — Monocyte chemoattractant protein-1 — ICAM-1 — VCAM-1 — |
| <i>Uthman et al., 2019</i> | HCAECs HUVECs | EMPA (1 μ M)/4-24 h DAPA (1 μ M)/4-24 h | TNF- α (10 ng/ml)/ 4-24 h | ROS — NO bioavailability — |
| <i>Uthman et al., 2020</i> | HCAECs | CANA (10 μ M)/16 h | Lipopolysaccharide (1 μ g/ml)/16 h | IL-6 — ERK1/2 phosphorylation — AMPK phosphorylation — HK II — |
| <i>Uthman et al., 2022</i> | HCAECs HUVECs | EMPA (1 μ M)/6 h | TNF- α (10 ng/ml)/6 h | NHE activity — Intracellular Na ⁺ — ROS — |

| References | Cells/Tissues | Drug (Dosage) | Stimulant (Dosage) | Major findings |
|--------------------------|---------------|-------------------------|--------------------|--|
| <i>Zhou et al., 2018</i> | Mice CMECs | EMPA (10 mg/kg/d)/ 20 w | STZ (50mg/kg)/5 d | Angiogenesis — Migration — Wound healing — Cytoplasmatic ROS — Mitochondrial ROS — Mitochondrial fission and fusion — Senescence — |

Abbreviations: Protein kinase B (Akt); Adenosine monophosphate-activated protein kinase (AMPK); Angiotensin II (Ang II); Canagliflozin (CANA); Cardiac microvascular endothelial cell (CMEC); Dapagliflozin (DAPA); Empagliflozin (EMPA); Endothelial nitric oxide synthase (eNOS); Extracellular signal regulated kinase (ERK); Human aortic endothelial cell (HAEC); Human coronary artery endothelial cell (HCAEC); Hexokinase II (HK II); Human umbilical vein endothelial cell (HUVEC); Intracellular adhesion molecule 1 (ICAM-1); Interleukin (IL); Mitogen-activated protein kinase (MAPK); Nuclear factor kappa B (NF- κ B); Sodium-hydrogen exchanger (NHE); NOD-like receptor pyrin domain containing 3 (NLRP3); Nitric oxide (NO); Nicotinamide adenine dinucleotide phosphate oxidase (NOX); Ponatinib (PON); Reactive oxygen species (ROS); Sodium glucose co-transporter (SGLT); Streptozotocin (STZ); Tumor necrosis factor- α (TNF- α); Vascular cell adhesion molecule 1 (VCAM-1); Vascular endothelial cadherin (VE-cadherin); Vascular endothelial growth factor (VEGF)

Figure legends

Figure 1 :

The pivotal role of ROS in endothelial dysfunction and CM hypertrophy :

In patients with diabetes, hyperglycaemia along with inflammatory reaction, enhanced cyclic stretch and oscillatory shear stress, increases production of reactive oxygen species in endothelial cells (ECs). ROS trigger vascular inflammation via activating multiple downstream pathways, including extracellular signal-regulating kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK), as well by increasing the expression of nuclear factor kappa B (NF- κ B). Excessive ROS induce uncoupling of endothelial nitric oxide synthase (eNOS) and loss of nitric oxide (NO) bioavailability of endothelial cells. The latter results in the inactivation of protein kinase G-cyclic guanosine monophosphate (PKG-cGMP) and hypo-phosphorylation of titin in adjacent cardiomyocyte (CM), therefore promoting CM hypertrophy. Increased ROS production promotes the formation of F-actin stress fibre and vascular endothelial (VE)-cadherin internalisation via activating src family kinase (SFK), leading to the disruption of adherens junction (Created with Biorender.com).

Figure 2

Direct endothelial protection by SGLT-2i's :

Sodium-glucose co-transporter 2 inhibitors (SGLT-2i's) have favourable effects on endothelial cells (ECs), including (1) inhibition of reactive oxidative species (ROS) production, (2) prevention of the inflammatory reaction, (3) restoration of nitric oxide (NO) bioavailability, and (4) modulation of angiogenesis. Anti-oxidative effects of SGLT-2i's might be mediated via inhibition of nicotinamide adenine dinucleotide phosphate oxidases (NOXs), sodium-hydrogen exchanger (NHE), and mitochondrial fission. The anti-inflammatory effect of SGLT-2i's is hallmarked with downregulation of vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1), reduction in cytokine secretion, and prevention of monocyte-endothelium

adhesion. SGLT-2i's might restore NO bioavailability of endothelial cells via improving phosphorylation of endothelial nitric oxide synthase (eNOS) and scavenging ROS (Created with Biorender.com).

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